

THE BIOLOGICAL AND MOLECULAR ANALYSIS OF A TICK-ENCODED
SERINE PROTEASE INHIBITOR (S6) AND ITS ROLE IN THE FEEDING CYCLE
OF THE LONE STAR TICK, *AMBLYOMMA AMERICANUM* (L) (ACARI:
IXODIDAE)

A Thesis

by

KATELYN COX CHALAIRE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Entomology

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Approved by:

Chair of Committee,	Albert Mulenga
Committee Members,	Patricia Pietrantonio
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ABSTRACT

The Biological and Molecular Analysis of a Tick-Encoded Serine
Protease Inhibitor (S6) and its Role in the Feeding Cycle of the Lone
Star Tick, *Amblyomma americanum* (L) (Acari: Ixodidae.) (August 2010)

Katelyn Cox Chalaire, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Albert Mulenga

Serine protease inhibitors (serpins) are a large superfamily of proteins that regulate critical proteolytic pathways by inhibiting serine proteases. Tick-encoded serpins are thought to play a vital role in the feeding process. To determine the relationship of *Amblyomma americanum* serpin 6 (S6) to tick feeding regulation, this study attempted to define the biological significance of this molecule through transcription and protein expression profiles, biochemical characterization of recombinant s6 (rS6), and the effects of *in vivo* post-transcriptional gene silencing on blood meal acquisition and fecundity.

Transcriptional analysis revealed that S6 mRNA is ubiquitously expressed in unfed and partially fed ticks through the initial 5 days of the feeding period. S6 mRNA abundance in dissected tick organs showed a 3.7, 3.4, and 1.7- fold upregulation from 24 h to 96 h in the salivary gland (SG), midgut (MG) and the carcass (CA) remnant after removal of SG, MG respectively before downregulating at 120 h. Native S6 protein is downregulated in response to tick feeding, with correlation between transcription and protein expression profiles only consistent from the unfed to 48 h. Similarly, S6 protein

expression in dissected female tick tissues is reduced as feeding progresses, with S6 being identified in SG, MG, ovary (OV), and CA from 24 h until 72 h. Biochemical characterization of S6 was not achieved, as rS6 did not form an irreversible complex when incubated with chymotrypsin or trypsin. Although complete silencing of S6 and S6/S17 mRNA was achieved, post-transcriptional gene knockdown had no effect on tick feeding efficiency or fecundity. These findings have been discussed in regards to the development of a vaccine against *A. americanum* and necessary future studies have been suggested for further characterization and assessment of biological significance.

DEDICATION

This work is dedicated to my mother, Kelly Chalaire, and my sister, Amanda “Pookey” Chalaire. It is my honor and privilege to call you family. This thesis is also dedicated to John Weimar. I love you.

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I would like to thank my committee chair, Dr. Mulenga, for all of the time and patience he provided me in the pursuit of my master's degree. Working in the Mulenga Lab has been a life-changing experience that has given me a solid foundation in research. I must also thank my amazing committee members, Dr. Pietrantonio and Dr. Snowden, for their guidance and support throughout the course of this project.

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CHAPTER I

INTRODUCTION

Ticks and Tick-borne Diseases

Ticks are obligate blood sucking arthropods that parasitize a wide range of vertebrate hosts and can be found in all terrestrial regions of the world (Sonenshine, 1993). The proposed origin of ticks varies between the Silurian period around 443 MYA (Oliver, 1989) to the more recent late Cretaceous period at approximately 120 MYA (Klompen et al., 1996). The oldest tick fossil, found in New Jersey amber, was dated around 94-90 MYA by Klompen and Grimaldi (2001). Ticks, making up the suborder Ixodida, are the most primitive members of the class Arachnida (Sonenshine et al., 2002). There are well over 800 species distributed among three tick families, 650 of which belong to the 13 genera of Ixodidae, while Argasidae contains about 172 species distributed among 5 genera, and Nuttalliellidae has one species (Sonenshine et al., 2006). The two tick families of principal importance are argasids and ixodids, respectively referred to as soft ticks and hard ticks. Hard ticks hunt their blood meal source while soft ticks are nest-dwelling, therefore argasids do not pose the same public health threat as ixodids (Balashov, 2006).

Although direct tick feeding can cause damage to their hosts, they are mostly

known for their role as vectors of disease-causing pathogens. Ticks surpass any other known arthropods in terms of the diversity of disease agents which they can transmit to both humans and animals, and are only outranked by mosquitoes in terms of their medical importance (Sonenshine, 1993). Ticks and tick-borne diseases (TBD) are one of the biggest concerns in global agriculture, particularly in regards to the cattle industry. Tick control and treatment of TBDs is estimated at 13 to 18 US dollars per animal, which translates to billions of dollars in global expenditures annually (de Castro, 1997). Important livestock TBDs include east coast fever (*Theileria parva*) vectored by *R. appendiculatus*, cattle tick fever (*Babesia bigemina* and *B. bovis*) vectored by *Rhipicephalus* (formerly, *Boophilus*) *microplus* and *R. annulatus* ticks, and tropical theileriosis (*T. annulata*) vectored by *Hyalomma anatolicum* (Sonenshine et al., 2002; Uilenberg et al., 2004; Gratz, 2006). Other livestock TBDs include bovine ehrlichiosis (*Anaplasma bovis*) and anaplasmosis that affect cattle (*A. marginale*) and sheep (*A. ovis*), which are transmitted by various genera of ticks (Sonenshine et al., 2002; Uilenberg et al., 2004; Gratz, 2006). In companion animals some of the commonly encountered TBDs include canine monocytotropic ehrlichiosis (*Ehrlichia canis*) and canine granulocytotropic ehrlichiosis (*E. ewingii*) (Sonenshine et al., 2002; Neer and Harrus, 2006), babesiosis caused by the *Babesia canis* and *B. gibsoni* protozoan parasites. Dogs can become infected with *Hepatozoon canis* or *H. americanum*, protozoan parasites that can cause Hepatozoonosis when *Rhipicephalus sanguineus* or *Amblyomma maculatum* ticks are ingested (Sonenshine et al., 2002). Important TBDs affecting equines include borreliosis (*Borrelia burgdorferi*), Tularemia (*Francisella*

tularensis), and Equine piroplasmiasis (*Babesia caballi* and *Theileria equi*) (Sonenshine et al., 2002).

In the United States, ticks transmit a greater number of etiological agents of vector-borne diseases than any other arthropod (Sonenshine, 1993). For many years, ticks and TBD were by and large considered a veterinary problem. However, since the identification of *Borrelia burgdorferi* as the causative agent of Lyme disease in 1982 (Burgdorfer et al., 1982; Burgdorfer, 1984) there has been a dramatic rise in the identification of human TBD incidence. Between 1982 and 2004 there have been 15 new tick-borne bacterial agents discovered or recognized as human pathogens (Parola and Roul, 2005). Some of the TBDs important to human health present in the United States are human babesiosis (*Babesia microti*, and *B. divergens*), Human monocytotropic ehrlichiosis (*Ehrlichia chaffeensis*), and Lyme disease (*Borrelia burgdorferi*) (Sonenshine et al., 2002; Neer and Harrus, 2006).

Tick Control and TBD Prevention

Currently, there is no single effective vaccine against any TBD, leaving tick population control as the optimal method of reducing the impact of these diseases on the livestock industry. At present this is accomplished with the use of chemical acaricides. Although this is the most commercially successful tick control method to date, there are many drawbacks associated with acaricides, including resistance to the active ingredient, contamination of environment, detrimental effect to non-target organisms, inefficiency regarding application, and chemical persistence. Since the use of acaricides began in the

early 20th century, several chemical classes have been used against ticks only to have resistance develop. Oftentimes different active ingredients act on the same target and resistance subsequently develops. Those that have remained effective against ticks are very persistent in the environment, with residues being found in animal food products (Graf et al., 2004; George et al., 2004). Others, such as organophosphates, are non-persistent but are closely related to chemicals that negatively affect vertebrates, such as nerve gases (Ware, 2000).

Even when chemical control methods are effective and non-harmful to non-target organisms, they have the tendency to be very inefficient. For optimal use, acaricides must maintain a certain quality, quantity, and method of application. Not following the manufacturer's protocol can allow for the persistence of ticks on the host. Acaricides are often mixed and administered by each livestock facility, so it is difficult to achieve consistent application. Most acaricides need to come in contact with the tick to be effective, which is difficult to accomplish with any method other than dipping vats. Even this technique has its drawbacks, as dipping requires all animals to be assembled in one location for treatment. This is time-consuming, costly, and stressful on the animals. Furthermore, the durability of these chemicals is short, so frequent reapplication is necessary (Ghosh et al., 2007; George et al., 2004). Due to these serious drawbacks, there has been significant interest in the development of more sustainable, less toxic alternatives to the chemical control (Sonenshine et al., 2006).

Vaccination of Animals against Tick Feeding

Vaccination against tick infestations has emerged as a viable and sustainable alternative to acaricide use (Jongejan and Uilenberg, 2004; Sonenshine, 1993; Willadsen et al., 1995). Advances in tick vaccine development were expertly reviewed by Willadsen (2004, 2006, and 2008), Mulenga et al., (2000, 2001a), and Maritz-Olivier et al. (2007). Anti-tick vaccines have been shown to be a practical, cost-effective and environmental friendly alternative to chemical control. While resistance to acaricides can be developed after one point mutation in tick genes, there must be a major change in protein expression for resistance to an anti-tick vaccine to arise, thus reducing the need for the constant development of new products (Willadsen, 2004). Vaccines are also non-polluting, so animal food products will remain untainted, and residual effects will not remain in the environment (de la Fuente and Kocan, 2006). The commercialization of vaccines to *R. microplus* against two midgut antigens, Bm86 (TickGard) or Bm91 (Gavac), in Australia and Cuba, respectively, confirmed the practicality of immunizing against ticks (Kemp et al., 1989; Willadsen et al., 1989). However, a very effective tick vaccine has yet to be developed. The major limiting step preventing the development of a commercially viable tick vaccine is the identification and validation of target tick proteins that completely prevents tick feeding when disrupted (Willadsen, 2006).

Some of the most promising targets for an anti-tick vaccine are serpins. The serpin superfamily is comprised of hundreds of proteins that have been identified in many different species, including mammals, arthropods, plants, and even viruses (Gettins, 2002; Gettins et al., 1996). As a general rule, serpins function in eukaryotes to

regulate the proteolytic pathways, specifically inflammation, coagulation, and complement activity (Silverman et al., 2001; Gettins et al., 1996). Although most of the characterized serpins occur in man, at the turn of the last decade there has been a concerted effort to determine if tick-encoded serpins function to modulate the host's immune system.

Serpins and Tick Feeding Regulation

Ticks are long-term feeders. They accomplish feeding by lacerating host tissue and small blood vessels to create a feeding site and then suck up the blood from the hematoma that forms in the feeding site (Sonenshine, 1993). This method of feeding stimulates the host's tissue repair response, including inflammation and blood coagulation, to stop further blood loss. To complete feeding, ticks secrete bioactive enzymes that prevent inflammation and blood clotting, thus ensuring continued blood flow to the feeding site (Nuttall et al., 2006). Given that the host's primary lines of defense to tick feeding activity, inflammation and blood coagulation are regulated by serpins (Huntington, 2006; Gettins, 2002), it has been hypothesized that ticks may utilize serpins to evade the host's immune response (Mulenga et al., 2001a). An increasing number of serpin-encoding cDNAs have been indentified in several tick species of medical and veterinary importance (Mulenga et al., 2001b, 2003, 2007, 2009; Imamura et al., 2005, 2006, 2008; Pervot et al., 2006, 2007; Sugino, 2003). In a recent study, 17 *Amblyomma americanum* (Lone Star Tick) presumptive serpins that are expressed during the first five days of feeding were described (Mulenga et al., 2007). The objective

of this study was to biologically and biochemically characterize one of the 17 sequences, S6 to gain insight into its role in the facilitation of blood meal acquisition by *A. americanum*.

Significance of Research

Amblyomma americanum (Lone Star Tick), is the most prevalent tick throughout the southeastern and south-central United States. This species is particularly widespread in Texas and Oklahoma, but it is also distributed along the Atlantic Coast up to New York and Maine (James et al., 2001; Sonenshine et al., 2002). A voracious feeder, *A. americanum* will parasitize almost any vertebrate, including birds, reptiles, or mammals. The adult stage is closely associated with white-tailed deer but will feed on a variety of mammals and ground-dwelling birds (Kollars et al., 2000). With all stages of Lone Star Ticks readily feeding on man, *A. americanum* is the cause of most human tick bites in the southern parts of the United States (James et al., 2001). *A. americanum* has always been considered a major pest of both humans and livestock and it is major vector of pathogens, *Borrelia lonestari*, *Francisella tularensis*, and several *Ehrlichia* species (Sonenshine et al., 2002; Childs and Paddock, 2003).

CHAPTER II
TEMPORAL AND SPATIAL CORRELATION OF mRNA AND PROTEIN
EXPRESSION PATTERNS DURING THE FIRST FIVE DAYS OF *AMBLYOMMA*
AMERICANUM FEEDING CYCLE

Introduction

The tick feeding process is compartmentalized into a series of behavioral and physiological changes that starts with attainment of appetite and ends in satiation (Walade and Rice, 1982). After attainment of appetite and the tick engages the host to start feeding, the act of blood meal uptake is broadly categorized into 3 steps: the preparatory feeding phase (PFP), Slow Feeding Phase (SFP), and Rapid Feeding Phase (RFP). The PFP occurs during the first 24-36 hours (h). The first step is the insertion of its hypostome into host skin, followed by the secretion of an adhesive substance (cement) that glues itself onto host skin, followed by the creation of the feeding lesion. This is followed by the SFP during the next 7-10 days in the case of adult ticks, and is when transmission of most tick-borne pathogens starts (Skotarczak, 2009; Uilenberg, 2006), the ticks feeds in moderation to obtain nutrients required for intermolt growth (growth of new cuticle and visceral organs). The tick intermolt growth prepares the body mass to imbibe and accommodate large volumes of host blood during the RFP that occurs during the last 24 h of feeding (Sonenshine, 1993).

To decipher molecular mechanisms regulating the tick feeding process, genomics and proteomics based procedures have been used to determine differential gene

(Carvalho et al., 2010; Nene et al., 2002; Ribeiro et al. 2006; Anisuzzaman et al., 2009) and protein (Yamada et al., 2009; Anisuzzaman et al., 2009; Saito et al., 2009; Hajdusek et al., 2008; Gao et al., 2009) expression profiles during the tick feeding process. These studies have routinely involved extraction of messenger RNA and total proteins from unfed and ticks that have fed for different periods. In this way numerous genes that are differentially down or upregulated, shut off or induced in response to tick feeding have been characterized (Mulenga et al., 2007; Narasimhan et al., 2007). These data have revealed insight into candidate tick proteins that may regulate different phases of the tick feeding process.

In this study, the author adopted similar approaches to establish mRNA and protein expression profiles *A. americanum* S6 during the first 5 days of tick feeding. The author's interest in examining the expression of S6 during the first 5 days was motivated by long-term goals of this research to identify key tick proteins that regulate early stages of tick feeding.

Materials and Methods

Ticks

Ticks used in these experiments were obtained from an *A. americanum* L. colony from the laboratory of Dr. Pete Teel from the Entomology Department of Texas A&M University, College Station, Texas. This colony is maintained by feeding on chickens at the larval and nymphal stage and on cattle at the adult stage.

Tick dissections, total RNA extractions, cDNA synthesis, and protein extraction

Adult female ticks were placed in cells attached to a naive calf that had been pre-infested with adult males three days prior to encourage synchronized feeding. After 24 h, all unattached ticks were removed from cells. Attached ticks were sampled every 24 h, starting at 24 h after attachment. Ticks were collected for two purposes: total RNA extraction from whole ticks and from dissected tissues as well as for protein extraction from the same tissue samples. Tick samples were collected at the unfed, 24, 48, 72, 96, and 120 h tick feeding time points. Three adult female ticks were processed individually for RNA and protein extraction at each time point. To homogenize, each tick was placed on a glass slide, chopped with a sterile razor blade, and then placed in the TRIzol total RNA extraction reagent (Novagen, Carlsbad, CA, USA). Samples were stored at -80°C until used for RNA extractions.

For tissue dissections, 8 ticks were collected at each time point (24, 48, 72, 96, and 120 h post-attachment). Dissections were routinely done as previously described by Mulenga et al., (2001b). Ticks were placed under a dissecting scope on a hanging drop glass slide filled with Diethylpyrocarbonate(DEPC) treated water. Using a sterile razor blade and soft tissue forceps, the dorsal shield was removed. Tick organs, including the salivary glands (SG), midgut (MG), ovaries (OV), and remaining carcass tissue (CA), were dissected using forceps or 18 gauge needles. All dissected tissues were pooled in groups of eight for each time point. The whole tissue samples were placed in TRIzol reagent and stored at -80°C until used for RNA extractions.

To extract total RNA, whole tick and tissue samples were thawed at room temperature and homogenized using a Sonic Dismembrator Model 100 (Thermo Fisher Scientific). Total RNA was extracted and re-suspended in RNAase free water according to the manufacturer's protocol provided with the total RNA extraction reagent, TRIzol. Total RNA was quantified using a nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA (400 ng) was used to synthesize oligo dT primed cDNA synthesis using the Verso cDNA kit (Thermo Fisher Scientific). The resulting cDNA was then quantified as described above and stored at -20°C.

To isolate total proteins, organic phases from total RNA extractions (above) were retained. These organic phases were treated with 0.3% ethanol to precipitate genomic (g) DNA. Subsequently, total proteins were precipitated using isopropanol. The resulting pellet was washed 4 times in 0.3 M guanidine hydrochloride in 95% ethanol solution. The pellet was then dried in a Savant DNA 120 SpeedVac® Concentrator (Thermo Fisher Scientific) for 20-30 min and reconstituted in 1% sodium lauryl sulfate (SDS) nuclease-free water at 50°C.

Tick saliva collection

Tick saliva was collected from 30 ticks at 24 h, 48 h, and 96 h using the published protocol from Ribeiro et al. (2004). Ticks were placed dorsal-side up on tape, and sterile glass micropipettes were placed over the tick's hypostome. To induce salivation, 5 µL of pilocarpine (50 mg/mL in 95% ethanol) was applied to the scutum

using a pipette. The ticks were placed in an incubation chamber at 35°C for 3 h. The saliva was collected from the micropipettes by washing in PBS and stored at -80°C.

Temporal and spatial analysis of transcript expression patterns of S6 during the first 5 days

To determine temporal and spatial expression patterns 200 ng of cDNA from whole tick or dissected tick organs, respectively, was used in a PCR reaction containing GoTaq® Green PCR Master Mix (Promega, Madison, WI, USA), forward and reverse S6 primers, S6 FWD and S6 REV (Table 2.1) at 0.1 µM final concentration in a 30 µL reaction. The cycling conditions were an initial denaturation of 95°C for 2 minutes, followed by forty amplification cycles of 95°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and a final extension of 72°C for 5 minutes. For sample load control, a similar reaction was repeated using forward and reverse actin primers, actin FWD and REV (Table 2.1) at 0.1 µM final concentrations in a 30 µL reaction. The cycling conditions were an initial denaturation of 95°C for 2 minutes, 40 amplification cycles of 95°C for 45 seconds, 58°C for 30 seconds, and 72°C for 1 minute, and a final extension of 72°C for 5 minutes. Eight µL of each PCR reaction product were electrophoresed along with a 1Kb DNA ladder (Promega) at 50 V on a 2% agarose gel containing 1 µg ethidium bromide in Tris-acetate-EDTA (TAE) buffer. The densities of the resulting bands were analyzed using the ImageJ analyzer software (available through the Resource Centre for Healthcare Technologies at <http://rsbweb.nih.gov/ij/>) to determine the relative

levels of transcription. The variations between template concentrations were normalized according to the following formula:

$$Y=V+V(H-X)/X$$

where Y stands for the normalized mRNA density, V is the observed S6 PCR band density in individual samples, H is the highest tick actin PCR band density among tested samples, and X is the tick actin PCR band density.

Construction of recombinant (r) serpin 6 expression plasmid

To construct the expression plasmid, the S6 coding region (without the 5' terminal region coding for the signal peptide) was unidirectionally sub-cloned into pRSET A vector (Novagen). A sense and anti-sense primer pair, S6-FWD and S6-REV (Table 2.1), with added respective restriction enzyme sites, *Bam*HI and *Hind*III were used to amplify the S6 mature protein coding region. The pRSET A expression vector and S6 fragment were sequentially digested with *Bam*HI and *Hind*III (New England BioLabs, Ipswich, MA, USA). For digestion with the *Hind*III the following reaction was composed in a 1.7 mL microcentrifuge tube (VWR, West Chester, PA, USA) for both the S6 coding region and the pRSET A expression vector: 20 μ L of the target template (PCR product or vector), 5 μ L of the 10X *Bam*HI unique buffer, 5 μ L of the 10X BSA, 0.5 μ L of *Bam*HI, and 19.5 μ L nuclease-free water. Each of these reactions was incubated overnight at 37°C. The entire reaction was electrophoresed at 100V on a 2% agarose gel that contained 1 μ g ethidium bromide in TAE buffer and subjected to routine gel purification using StrataPrep DNA Gel Extraction Kit (Stratagene, La Jolla, CA,

USA). Five μL of the extracted product was electrophoresed on a 2% agarose gel to confirm DNA elution. The second restriction enzyme digestion was completed as above but with the use of *HindIII* and buffer 2 (New England BioLabs). The digestion was again confirmed by electrophoresis on a 2% agarose gel and purified using StrataPrep DNA Gel Extraction Kit using the previously described protocol. The digested pRSET A vector and S6 insert were ligated in a 10 μL reaction using the T4 ligase enzyme (Promega). This reaction was incubated at 4°C overnight. Seven μL of the reaction was transformed in Subcloning Efficiency™ DH5 α ™ Competent Cells (Invitrogen) using routine heat shock methods (30 seconds at 42°C) following the manufacturer's recommendations and then plated on agar plates containing ampicillin at 75 $\mu\text{g}/\text{mL}$ final concentration.

Table 2.1 DNA primers used for serpin 6 amplification, transcriptional analysis, and plasmid construction

Designation	Sequence	Orientation
Actin FWD	5' GGACAGCTACGTGGGCGACGAGG 3'	Sense
Actin REV	5' CGATTCACGCTCAGCCGTGGTGG 3'	Antisense
S6 FWD	5' CTGCTATCAGCGAGAGCACGCA 3'	Sense
S6 REV	5' TCTGCGTGAAATTTCTGTCTATTCTGGA 3'	Antisense
Serp6-FWD	5' GGATCCGACGATGCACTGCTGGCCAAAGCTC 3'	Sense
Serp6-REV	5' AAGCTTGACCTTACCATTTAGTCTTATTCTGCGTG 3'	Antisense

The plates were incubated at 37°C overnight. The transformants were chosen using blue-white selection. The white colonies were further screened for the S6 insert by a PCR reaction containing GoTaq® Green Master Mix (Promega), spiked with a portion of the bacteria colony and forward and reverse S6-specific primers (Table 2.1) at 0.1 µM final concentration in a 10 µL reaction. The cycling conditions were an initial denaturation of 95°C for 2 minutes, 40 amplification cycles of 95°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and a final extension of 72°C for 5 minutes. The colonies that contained the insert were used to inoculate 6 mL of Luria Bertani (LB) broth with ampicillin at 75 µg/mL final concentration and incubated with shaking at 37°C overnight. The plasmid was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega) and product insertion was confirmed on a 2% agarose gel as previously described. The S6-pRSETA expression plasmid was transformed in *E. coli* BL21 (DE) pLysS cells using routine heat shock methods and plated on agar plates as described above. The resulting colonies were further screened for the S6 insert by PCR as described above.

Expression and affinity purification of rS6

For rS6 expression an insert positive colony was used to inoculate 5 mL of LB broth containing ampicillin at a 75 µg final concentration, and incubated with shaking at 37°C overnight. This was used as a starter culture to inoculate a 500 mL culture. When the culture reached an OD600 of 0.6 (using Genesys 10uv Spectrophotometer, Thermo Fisher Scientific), the expression of rS6 was induced by adding isopropyl β-D-

thiogalactoside (IPTG) for a 0.2 mM final concentration. The reaction was incubated for 6 h at 37°C. Subsequently, the culture was pelleted by centrifugation (3,750xG for 15 minutes) in 50 mL conical tubes, resuspended in 5 mL phosphate buffered saline (PBS), and lysed by sonication. The insoluble fraction was pelleted and re-suspended in 5 mL of denaturing binding buffer (8 M urea, 100 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole), incubated at room temperature for 30 minutes, and centrifuged for 10 minutes at 18,000xG. The supernatant was filtered using a 0.2 µm syringe filter (Thermo Fisher Scientific) and retained for protein purification.

The rS6 was purified by affinity chromatography using the 1M HiTrap™ Chelating HP column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA) that was charged with 1 mL of 100 mM NiCl₂ and equilibrated with 5 mL of the denaturing binding buffer. The filtered supernatant was applied to the column and purified by washing with 5 mL of each of the following: denaturing binding buffer, 50 mM imidazole denaturing wash buffer (8M urea, 100 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole) and 100 mM imidazole denaturing wash buffer (8 M urea, 100 mM Tris-HCl, 500 mM NaCl, 100 mM imidazole). The protein was eluted from the column by applying the 500 mM imidazole elution buffer (8 M urea, 100 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole) and collected in 0.5 mL fractions. Recombinant protein expression was routinely verified by electrophoresis on a sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel under reducing conditions with Coomassie blue staining as detailed below.

SDS-PAGE electrophoresis and Coomassie blue staining

To visualize the results of the recombinant protein expression and purification, non-induced soluble and insoluble fractions and induced soluble and insoluble fractions and purified rS6 were run on a 12.5% acrylamide gel under denaturing conditions. The samples were heated to 95°C for 5 minutes with 4x sample buffer containing SDS and dithiothreitol (DTT). Ten μL of each sample and 7 μL of the All Blue Precision Plus Protein Standard™ (Bio-Rad, Hercules, CA) was electrophoresed at 135V and 12 mA for 2 h. The proteins were stained using Coomassie brilliant blue staining (40% Distilled Water, 10% Acetic Acid, 50% Methanol, and 2.5g of Coomassie-Blue powder) to verify expression and purification.

Production of rabbit polyclonal antibodies to rS6

Polyclonal antibody to the rS6 protein was produced in rabbits by a fee-for-service company (Pacific Immunology, Ramona, CA). The protein was quantified by a Bradford Protein Assay using Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific). Approximately 4 mg of affinity purified rS6 purified protein were electrophoresed on two one well 12.5% SDS-PAGE and stained through Coomassie brilliant blue. The target protein band was excised using a razor blade and the gel strip was sent out for polyclonal rabbit immunization. Therefore, pre-immune and immunized rabbit sera were available for use in immunoblotting analyses.

Temporal and spatial expression patterns of S6 native protein during the first five days of tick feeding

To determine S6 temporal and spatial protein expression profiles, total protein extracts of whole tick, dissected organs of unfed and fed ticks from 24 through 120 h, and tick saliva collected from ticks fed for 24, 48 and 96 h were subjected to western blotting analyses using polyclonal antibodies to rS6. Tick protein extracts and rS6 (serving as a positive control) were resolved on a 12.5% polyacrylamide gel under reducing conditions. Proteins in the gel were then transferred onto an Immobilon™ PVDF membrane (Millipore, Billerica, MA, USA) using the Xcell SureLock™ Mini-Cell XCell II™ Blot Module (Invitrogen). The membranes were washed in 20 mL of PBS-tween 20 (PBST) (0.05%) and then blocked overnight at 4°C by incubation in 5% blocking solution (1g dried skim milk added to 20 mL of PBST). The blocked membranes were washed at room temperature 6 times for 5 minutes each with 20 ml PBST. Following washing, the membranes were incubated for 1 h at room temperature (RT) in rabbit pre-immune or immune serum to rS6 (1:660, V/V) in blocking solution. After appropriate washing with PBST, the membranes were subsequently incubated for 1 h at RT with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Millipore) (1:1000, V/V) in blocking solution. After 6 washes in PBST, membranes were incubated with the metal enhanced DAB chromogenic substrate (Thermo Fischer Scientific) according to manufacturer's recommendations. After development of protein bands to desirable intensity, the membranes were washed in Milli-Q water to remove the substrate solution.

Results

S6 mRNA is ubiquitously expressed in unfed and partially fed ticks through 5 days

Results summarized in Figures 2.1 and 2.2 show that S6 is ubiquitously expressed as revealed by its amplification in all tested tick organs from unfed ticks through day 5 of feeding. Results summarized in Figures 2.1A and 2.1B revealed that when examined in whole animals, S6 mRNA displays a peak in response to tick feeding activity. Visual PCR band intensity (Figure 2.1A) and normalized band densities (Figure 2.1B) revealed that S6 transcript abundance is upregulated by 2 fold between 48 and 72 h time points and then downregulated by 3 fold between 72 and 120 h time points. In contrast when examined at organ level, S6 displays an expression profile of where expression transcript abundance in SG, MG and CA is 3.7, 3.4, and 1.7 fold respectively up- regulated between 24 and 96 h time points, before its starts to go down by the 120 h time point (Figure 2.2A and 2.2B).

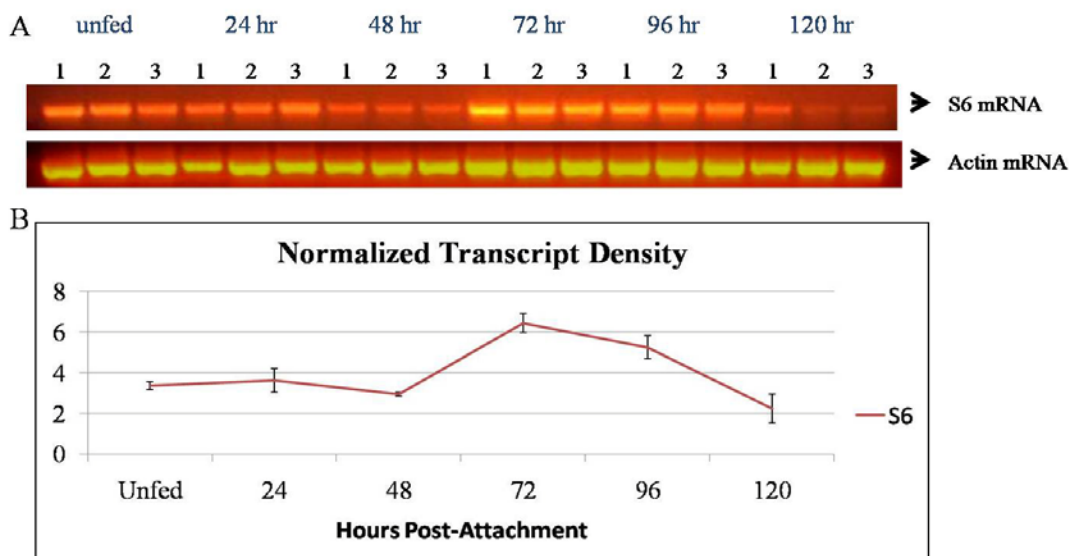


Figure 2.1 Temporal mRNA expression profile in individual whole ticks through 5 days post-attachment. (A) 3 ticks from unfed, 24, 48, 72, 96, and 120 h feeding time points were processed individually for total RNA extraction and subjected to 2-step semi-quantitative RT-PCR to amplify the S6 fragment and tick actin (load control). (B) Densities of S6 and tick actin PCR bands were determined using a web based ImageJ online program. Densities were normalized as described in the material and methods section using the following formula: $Y = \frac{V + V(H - X)}{X}$, where Y stands for the normalized mRNA density, V is the observed S6 PCR band density in individual samples, H is the highest tick actin PCR band density among tested samples, and X is the tick actin PCR band density. Means and standard error of means (SEM) of the 3 PCR band densities were determined using the imageJ analyzer program and the plotted in Microsoft Office Excel 2007. The plotted normalized densities showed a peak at 72 h.

rS6 predominantly expresses as an insoluble protein when induced at 37°C

Figure 2.3 summarizes the expression and affinity purification rS6 in *E. coli* cells. SDS-PAGE electrophoresis and Coomassie brilliant blue staining of the supernatant and pellet fractions of recombinant *E. coli* lysate revealed that the protein expressed appeared at the expected ~47kDa molecular weight and that rS6 was successfully purified. When induced with IPTG at 37°C, rS6 was found to be predominantly present in the insoluble fraction (Figure 2.3).

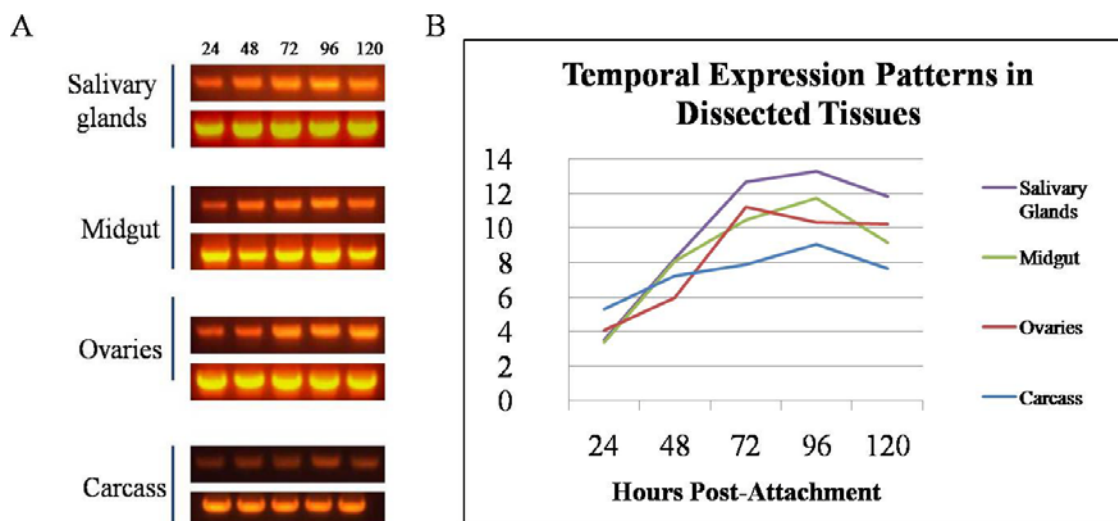


Figure 2.2 Spatial and temporal mRNA expression profile in dissected tissue through 5 days post-attachment. (A) Spatial S6 mRNA expression in dissected tissue from 8 individuals fed from 24 h – 120 h were subjected to 2-step semi-quantitative RT-PCR to amplify the S6 fragment and tick actin. (B) Densities of S6 and tick actin PCR bands were determined as described in figure 2.1 and S6 transcript abundance was normalized. S6 displays an expression profile in SG, MG, and CA is 3.7, 3.4, and 1.7 fold respectively upregulated between 24 and 96 h time points and declines by 120 h.

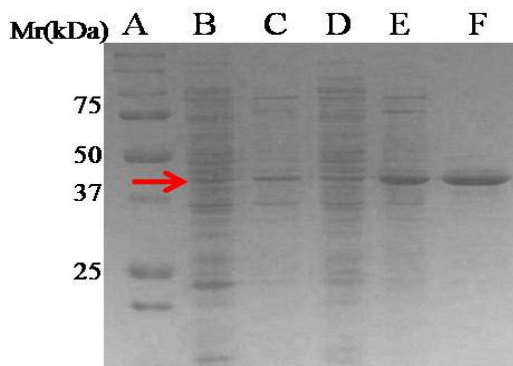


Figure 2.3 Expression and affinity purification of insoluble recombinant (r) S6. Expression of rS6 was induced for 6 h at 37°C by adding IPTG to 1mM final concentration. Samples were separated into soluble (supernant) and insoluble (pellet) fractions. These fractions were subjected to SDS-PAGE electrophoresis with Coomassie blue staining using a 12.5% acrylamide gel under denaturing conditions. rS6 expressed at the expected size of 47 kDa in increased amounts in the pelleted fraction in the induced culture. Purification through affinity chromatography was completed under denaturing conditions. Lanes A-F denote marker (A), uninduced soluble (B) and pellet (C), induced soluble (D) and pellet (E), and affinity purified rS6 (F).

Native S6 protein is ubiquitously expressed and downregulated in response to tick feeding

Western blot analyses confirmed that rS6 was immunogenic in rabbits as revealed by specific reactivity with rS6 and native S6 proteins (Figures 2.4, 2.5, and 2.6). To investigate if the observed transcription profiles in Figures 2.1 and 2.2 correlated with protein production, tick proteins extracted from the same animals that were used for RNA extraction were subjected to western blotting analyses using antibodies to rS6 as summarized in Figures 2.4 and 2.5. It is interesting to note that in whole animals, the temporal expression profile of the native S6 protein from unfed ticks through the 48 h feeding time point apparently correlated with the mRNA expression profiles (Figure 2.1). However from the 72 to the 120 h time point, protein production is apparently not correlated with mRNA expression patterns. At the mRNA level, S6 transcript abundance appears to reach its peak at the 72 h time point and then starts to drop (Figure 2.1B). In contrast native S6 protein abundance at the 72 h time point (Figure 2.4B) is weaker than the 24 h time point. When examined in dissected tick organs, the native S6 protein expression profile (Figure 2.5) appears not to correlate with mRNA expression profiles in dissected tick organs (Figure 2.2). At the mRNA level S6 is weakly expressed in all organs of 24 h fed ticks (Figure 2.2A and 2.2B). In contrast, native S6 is strongly expressed in organs of 24 h fed ticks (Figure 2.5 A-D).

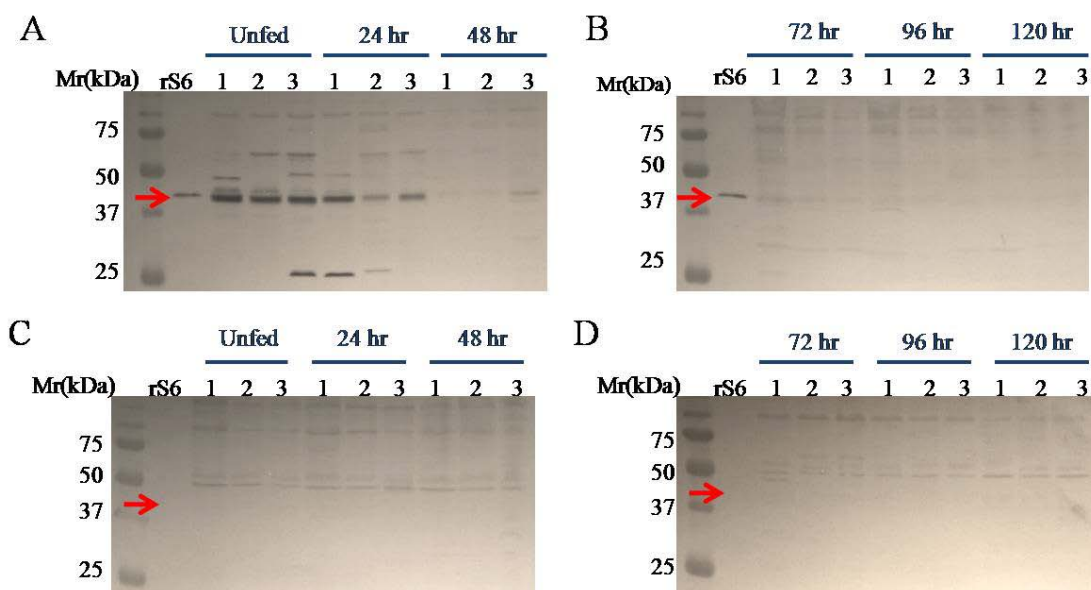


Figure 2.4 **Temporal expression analysis of the native S6 protein.** Protein was individually extracted from three ticks per indicated feeding time point and subjected to western blotting analysis using the antibody to rS6 (panels A and B) or pre-immune serum (C and D). Arrowhead denotes native S6 protein.

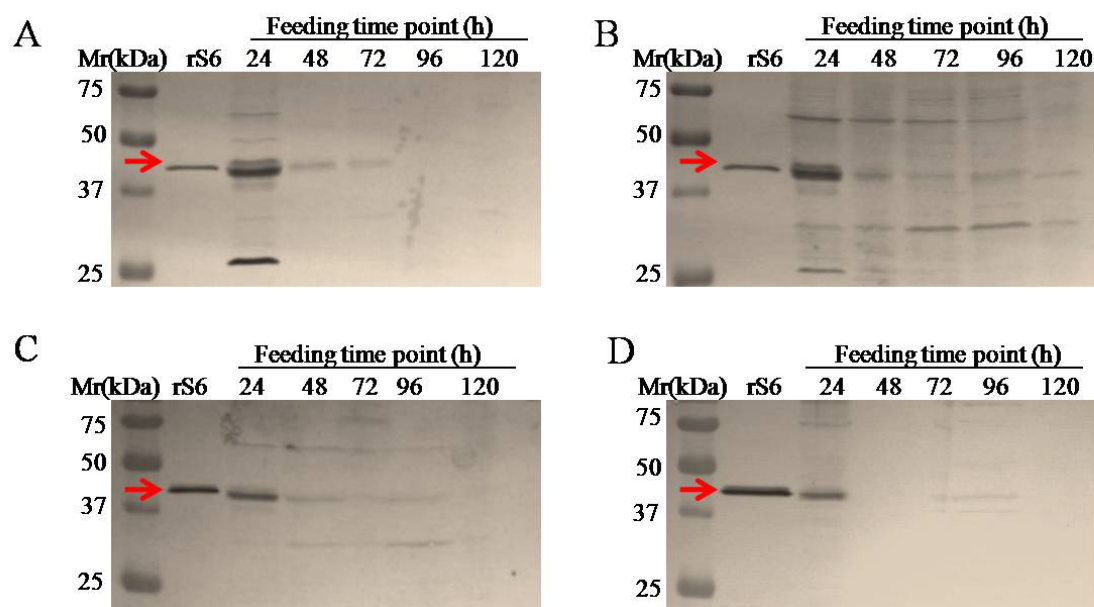


Figure 2.5 **Spatial and temporal expression analysis of the native S6 protein.** Total protein extracts of salivary gland (A), midgut (B), ovary (C) and other (D) were subjected to western blotting analysis using antibodies to rS6 (positive control). Arrowhead denotes native S6 protein.

S6 is potentially secreted into the host during tick feeding

Results summarized in Figure 2.6 suggest that S6 is potentially injected into the host during tick feeding. Antibodies to rS6 specifically reacted with the expected ~45 kDa S6 protein band on 48 h tick saliva immunoblot. The presence of S6 in saliva at 48 h correlates with both the temporal mRNA transcript and protein expression patterns in whole ticks (Figures 2.1 and 2.4) and the spatial expression of S6 mRNA and protein in the salivary glands at 48 h. The presence of S6 in tick saliva is proxy that it is secreted into the host during tick feeding

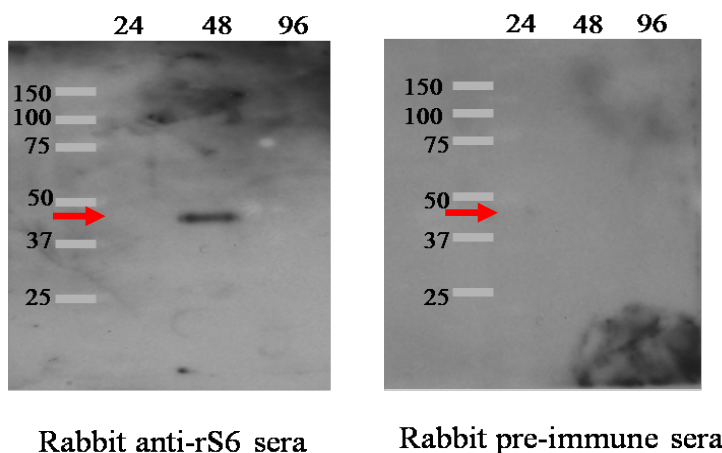


Figure 2.6 **Spatial expression analysis of S6 by western blotting analysis of tick saliva.** Saliva was collected by applying pilocarpine on the tick cuticle then collecting the secreted saliva using a capillary tube. Through western blotting analysis it was discovered that S6 is expressed in saliva collected at the 48 h time point, which indicates that this is a potentially secreted protein during the SFP of the tick feeding process. This is the first tick-encoded serpin confirmed to be present in saliva.

Discussion

The expression of S6 in salivary glands and midguts of *A. americanum* ticks and the high amino acid identity to other tick sequences (Mulenga et al., 2007) elicited the interest to gain deeper insight into the biological association of this protein to the tick

feeding process. Differential transcription patterns of where certain tick genes are down or upregulated, induced or shut down in response to tick feeding activity is a commonly observed phenomenon (Carvalho et al., 2010; Nene et al., 2002; Anisuzzamand et al., 2009). Broad interpretations of these data are that genes are induced or upregulated in response to tick feeding activity are likely to be associated with blood meal feeding regulation. On the other hand, biological functions of genes that are shut down or downregulated in response to feeding are thought to be either restricted to a particular tick feeding stage or not associated with tick feeding regulation (Almazán et al., 2003; de la Fuente et al., 2006; Hatta et al., 2010; Smith et al., 2009). Although the observed transcription pattern of S6 appears not to fit any previously described patterns, the strong expression of the S6 native protein during the first 24-48 h feeding time points of feeding strongly indicates that this protein regulates molecular events at the start of tick feeding, attachment onto host skin and creation of the feeding lesion (Sonenshine, 1993).

Although ticks can cause damage to their hosts, they are mostly known for their role as vectors of tick-borne disease agents. Given that transmission of most tick-borne disease agents occurs after 48 h post attachment, a desirable tick vaccine will be one that blocks early stage tick feeding events. From this perspective, it was exciting to note that the S6 protein was strongly expressed during the 24-48 h time point when tick attaches onto host skin and creates its feeding lesion (Sonenshine, 1993) make this protein a desirable target antigen. In immunizing animals with recombinant proteins, a major drawback may be that the conferred antibody response may not be reactive with native proteins. From this perspective, it is interesting to note that rS6 was immunogenic and

specifically reacted with native the S6 protein. The significance of this is that rS6 can be used as an immunizing antigen. The expression of S6 in multiple tick organs as revealed by spatial RT-PCR and western blotting analyses is consistent with previous studies that show that both vertebrate and invertebrate serpins tend to be ubiquitously expressed (Gettins, 2002; Silverman et al., 2004; Huntington, 2006; Law et al., 2006; Irving et al., 2002, 2006) . The ubiquitous expression pattern of S6 may underscore the significance of this protein in regulating tick physiology and thus make S6 an attractive target protein which if disrupted will compromise tick feeding success.

In hypothesizing that tick encoded serpins play key role(s) in regulating evasion of host defense reactions by ticks (Prevot et al., 2007; Mulenga et al., 2001a, 2003, 2008, 2009), the underlying assumption is that serpins are injected in the host during tick feeding. In previous studies this assumption was supported by expression of serpin mRNA in the tick salivary glands (Prevot et al., 2007, 2006; Mulenga et al., 2003, 2008, 2009). In this study, specific reactivity of antibodies to rS6 on immunoblots of experimentally harvested tick saliva strongly supports that tick injection of S6 into the host occurs during tick feeding. This data for the first time provides direct evidence that serpins are part of tick proteins that injected into the tick feeding site to regulate tick feeding.

Anti-tick protein antigens from saliva, such as S6 in this study have traditionally been classified as “exposed” in that they interact with host immune response factors during tick feeding. Conversely, target antigens based on non-tick saliva protein antigens are classified as “concealed” (Willadsen, 2004). In designing anti-tick vaccines against

animals, an important consideration is that natural tick infestations must provoke an anamnestic (production of antibodies caused by the second experience with the same antigen) antibody response in immunized animals to avoid the need for repeat immunizations. The expectation is that in animals immunized with tick saliva protein vaccine antigens, tick saliva proteins injected into the host during subsequent tick infestations will act as booster injections and the host immune memory will trigger a strong tick immunity response. Thus the apparent secretion of S6 into the host during *A. americanum* feeding makes this molecule an attractive putative target antigen warranting further study.

CHAPTER III
BIOCHEMICAL CHARACTERIZATION TO DETERMINE IF rS6 IS AN
INHIBITORY SERPIN

Introduction

Serpins make up the largest superfamily of protease inhibitors that have been identified in species of mammals, arthropods, plants, and viruses (Irving et al., 2000; Gettins, 2002; Gettins et al., 1996; Huntington, 2006). In eukaryotes where data on the biology of serpins has accumulated, this protein family is known to regulate important proteolytic pathways, blood coagulation, food digestion, fertilization, inflammation, and complement activity (Silverman et al., 2001; Gettins et al., 1996). The physiological balance between serpins and their protease substrates is critical in the maintenance of homeostasis, demonstrated by the many serious human diseases that result from imbalances, deficiencies, and mutations in serine proteases or serpins (Gettins et al., 1996; Gettins 2002; Huntington, 2006 Potempa et al., 1994; Silverman et al., 2001). This is a strong indication of the vital physiological role(s) these proteins play in a variety of organisms, including ticks.

Serpins were originally characterized as inhibitors of serine proteases, hence the acronym “serpin” (Gettins, 2002). However, other serpins with inhibitory functions against cysteine proteases and those with no inhibitory functions have been identified and characterized. Serpins with inhibitory functions against cysteine proteases such as the inhibitor of caspases regulate apoptosis, a body defense mechanisms to eliminate

infected cells (Liphaus and Kiss, 2010) and play a vital role in preventing joint degradation by inhibiting cysteine proteases from breaking down collagen in bone (Cawston and Young, 2010). Some non-inhibitory serpins function to regulate homeostasis by serving as substrates for enzymes. For example, angiotensinogen acting as a substrate for renin, and the end product being major moderator of salt and water homeostasis and an effective vasoconstrictor (Morgan et al., 1996). As another example, serpins secreted from the endometrium of pregnant ewes inhibit mitogen and peripheral blood lymphocytes to stop abortion caused by natural killer cell activity (Skopets et al., 1995). On this basis, the superfamily name of “serine protease inhibitor or serpin” has become a misnomer; not all members function to inhibit serine proteases (Silverman et al., 2001) but still have very important biological functions. Although similar in activity, cysteine inhibitors differ from their serine inhibiting counterparts in that the protease targets a cysteine residue (Barrett and Rawlings, 2001).

Serpins that function as serine protease inhibitors are dramatically different from other classes of serine protease inhibitors by their size of at least 350-450 amino acids, and by undergoing a major conformational change to form a stable, often irreversible covalent complex with their target (Egelund et al., 1998; Gettins et al., 1996). The inhibitory reaction by serpins starts with the target protease recognizing the amino acid residues in the serpin’s reactive center loop (RCL) as a binding site so that the serpine acts as a pseudo substrate. When the protease attempts to cleave the RCL at the active site, a covalent bond is formed between the serpin and protease. This causes the serpin’s conformation to go from stressed to relaxed, locking the two proteins together

indefinitely in an irreversible serine protease-serpin covalently bonded complex, effectively inhibiting the activity of both proteins (Whisstock et al.1998; Gettins et al., 1996; Gettins, 2002; Huntington 2006; Irving et al., 2006; Silverman et al., 2001; Whisstock et al., 1998). The serpin and target protease complex, which is removed from circulation within hours by membrane receptors (Gettins, 2002) has been shown to be resistant to SDS boiling. Validation of the serpin's ability to form a complex with the protease is routinely done by the serpin and target protease complex formation assay (Iwanaga et al., 2003; Dobo et al., 2006; Zhao et al., 2005). In this assay candidate recombinant or purified native serpins are co-incubated with commercially available proteases. The formed serpin and target protease complex is then visualized by SDS-PAGE with Coomassie (Dobo et al., 2006; Zhao et al., 2005) or western blotting analysis (Zhao et al., 2005). In this study I adopted similar approaches to determine whether or not *E. coli* expressed rS6 had inhibitory functions against archetypes of the serine protease protein family, chymotrypsin and trypsin.

Materials and Methods

Expression and affinity purification of soluble rS6

When induced at 37°C, rS6 expresses as inclusion bodies as described in Chapter II. Given the limitations of incorrect folding associated with proteins expressed as inclusion bodies, rS6 was expressed at 18°C to encourage expression of the recombinant protein in a soluble fraction. Protein expression and purification was conducted as described in Chapter II. A positive colony was used to inoculate 5 mL of LB broth

containing 75 µg final concentration of ampicillin and incubated with shaking at 18°C for 48 h. This colony was used as a starter culture to inoculate a 500 mL culture. When the culture reached an OD600 of 0.6 by spectrophotometric measurement, the expression of soluble rS6 was induced by adding IPTG to 0.2 mM final concentration and incubating it for 12 h at 18°C. The induced bacterial culture was pelleted by centrifugation, resuspended in 5 mL of 1X PBS, and lysed by sonication. The lysate was separated by centrifugation and 5 mL of 2X native binding buffer (100 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole) was added to the soluble fraction. This mixture was incubated at room temperature for 30 minutes and centrifuged for 10 minutes at 18,000xG. The supernatant was filtered using a 0.2 µm syringe filter and retained for protein purification.

The rS6 was purified by affinity chromatography as described in Chapter II with minor modifications. The following buffers were used for purification: native binding buffer, 50 mM imidazole denaturing wash buffer (100 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole), 100 mM imidazole native wash buffer (100 mM Tris-HCl, 500 mM NaCl, 100 mM imidazole) and 500 mM imidazole elution buffer (100 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole). The eluted protein was collected in 0.5 mL fractions and recombinant protein expression was routinely verified by SDS-PAGE electrophoresis under reducing conditions and stained with Coomassie blue as described in Chapter II. Protein concentration was quantified by a Bradford protein assay (Coomassie Bradford Protein Assay Kit). The protein was then dialyzed against trypsin and chymotrypsin

digestion buffer, 100 mM Tris HCl containing 10 mM CaCl₂ (pH 7.8) to prepare for the complex formation assay below.

rS6 and target protease complex formation assay

Commercially available chymotrypsin and trypsin, the two archetypes of the serine protease family, were used in the complex formation assay. α -chymotrypsin from bovine pancreas Type II, lyophilized powder (Sigma-Aldrich, St. Louis, MO, USA) was reconstituted in 1 mM HCl containing 2 mM CaCl₂ (pH 7.8) to form a 2 mg/mL concentration. The chymotrypsin complex formation assay was composed in separate 0.5 mL PCR tubes, each consisting of 11 μ g of rS6, 10 μ g of α -chymotrypsin, and 5 μ L of the digestion buffer recommended by the protease manufacturer (100 mM Tris-HCl containing 10 mM CaCl₂ [pH 7.8]). The reaction mixtures were incubated at the optimal temperature (37°C) for 0, 5, 10, 15, and 20 minutes. This complex formation assay was repeated at suboptimal (25°C, 20°C, 15°C, and 4°C) temperatures and visualized by SDS-PAGE with Coomassie blue staining as described in Chapter II.

The complex formation assay was repeated using trypsin. TPCK-treated trypsin (to remove chymotrypsin) from bovine pancreas (Sigma-Aldrich) was reconstituted in 100mM Tris-HCl with a final 1 mM CaCl₂ concentration (pH 8.5) to form a 2 mg/mL concentration. As before, assays, were composed in separate 0.5 mL PCR tubes, each consisting of 11 μ g rS6, 10 μ g of trypsin, and 5 μ L of the digestion buffer recommended by the protease manufacturer (100 mM Tris-HCl with a final 1 mM CaCl₂ concentration [pH 8.5]). The assays were incubated at the optimal temperature (37°C)

and suboptimal (25°C, 20°C, 15°C, and 4°C) temperatures for 0, 5, 10, 15, and 20 minutes.

Visualization of rS6 and target protease complex

After incubation, the assays were heated at 95°C for 5 minutes in denaturing 4X sample buffer containing SDS and DTT. Each assay was electrophoresed on a 12.5% denaturing SDS-PAGE gel as described in Chapter II along with 10 µg of the protease and 15 µg of rS6 as positive controls. The proteins were stained using Coomassie brilliant blue to visualize any complex formation. The stained SDS-Page gels were examined for any molecular weight shift, indicating the formation of a rS6-target protease stable complex.

Results

rS6 expresses in both the soluble and insoluble fractions when expressed at 18°C

Results summarized in Figure 3.1 show that at the suboptimal temperature, rS6 expresses both in the soluble fraction of the lysate and in an inclusion body. The supernatant and pellet fractions of recombinant *E. coli* lysate were analyzed by SDS-PAGE electrophoresis and Coomassie brilliant blue staining, revealing that the protein was detected at the expected ~47 kDa size in both soluble and pellet fractions. The soluble fraction was successfully purified and used in complex formation assays.

E. coli expressed rS6 does not form an irreversible complex when incubated with chymotrypsin or trypsin

Results summarized in Figure 3.2 show that when incubated with chymotrypsin or trypsin at 37°C, 25°C, 20°C, 15°C, and 4°C for 0, 5, 10, 15, and 20 min there was no rS6 and target protease complex formation. Except at the 0 min time point, rS6 was degraded and cleared in each of the assays (Figure 3.2).

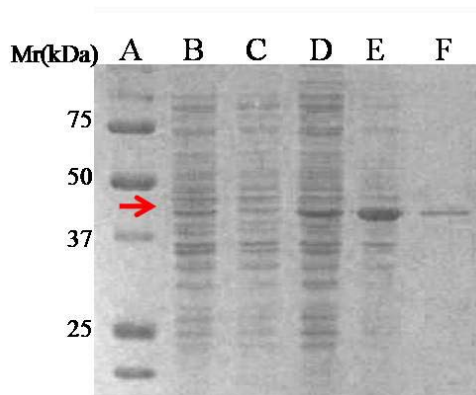


Figure 3.1 Expression and affinity purification of soluble recombinant (r) S6. Expression of rS6 was induced for 12 h at 18°C by adding IPTG to 1mM final concentration. *E. coli* cells disrupted by sonication were centrifuged and separated into soluble (supernatant) and insoluble (pellet) fractions. These fractions were subjected to SDS-PAGE electrophoresis with Coomassie blue staining using a 12.5% acrylamide gel under denaturing conditions. rS6 expressed at the expected size of 47kDa in the supernatant fraction in the induced culture. Purification through affinity chromatography was completed under denaturing conditions. Lanes A-F denotes marker (A), uninduced soluble (B) and pellet (C), induced soluble (D) and pellet (E), and affinity purified rS6 (F).

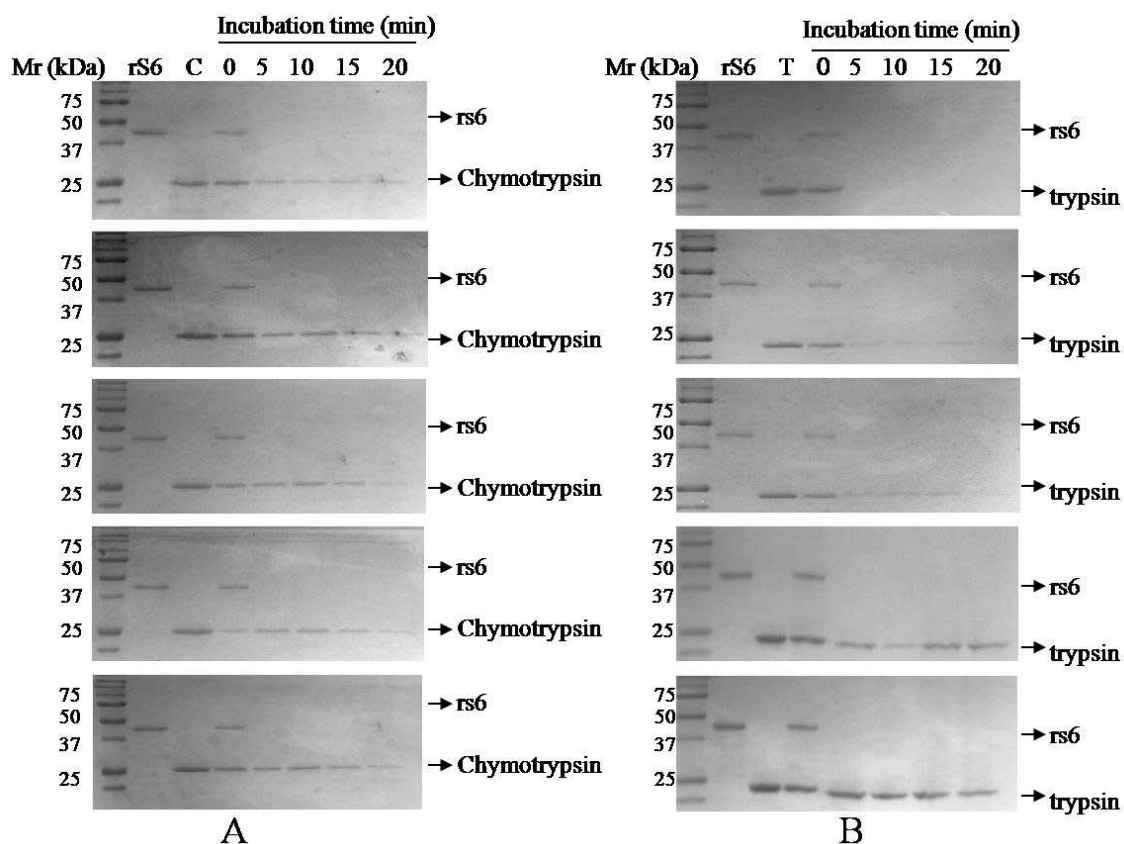


Figure 3.2 **rS6 complex formation assay.** Affinity purified soluble rS6 was co-incubated with chymotrypsin (A) or trypsin (B) at the indicated temperatures for 0, 5, 10, 15, and 20 minutes. Each sample along with rS6 and the serine protease (controls) were run on a 12.5% SDS-Page denaturing gel (Mr[kDa] = molecular marker, rS6=recombinant Serpin 6, C= chymotrypsin, T= trypsin, 0= 0 minutes, 5= 5 minutes, 10 = 10 minutes, 15= 15 minutes, and 20= 20 minutes.) There was no irreversible complex formation in any of the assays. The rS6 and portions of the protease were degraded and cleared by the protease present in each of the assays at all of the incubation times after 0 minutes.

Discussion

Previous sequence and structure-based alignment analyses that predicted that S6 was a putatively inhibitory serpin (Mulenga et al., 2007) elicited interest in the validation of complex formation functions of this protein. My data in Chapter II of this thesis strongly supports that S6 is secreted into the host during tick feeding and suggests that it may interact with vertebrate derived proteases. Although serpins with and without inhibitor functions against cysteine proteases have been identified, the majority of serpins are inhibitors of chymotrypsin and trypsin-like serine proteases (Gettins, 2000). On this basis, I chose to characterize inhibitor functions of rS6 by screening against α -chymotrypsin and trypsin. Although the failure of rS6 to form complexes with either chymotrypsin or trypsin may indicate a lack of inhibitory functions for S6, I am interpreting these findings with caution. The inability for rS6 to function as an inhibitor of chymotrypsin and trypsin might have more to do with the expression of rS6 in *E. coli* rather than a true evaluation of its biochemical function. One of most common posttranslational modifications affecting the biological activity of serpins is glycosylation, specifically N and O-type glycosylation (Gettins, 2002), the former being added to the nitrogen atom of Asn side chains in the endoplasmic reticulum (ER) and the latter being added by the Golgi apparatus to oxygen atoms of hydroxyls of Asn and Thr residues (Gettins, 2002). This process does not occur in a similar fashion in bacterial organisms, making recombinant expression of properly glycosylated proteins difficult. The cellular structure of eukaryotic and prokaryotic cells are very different, and prokaryotes lack internal membrane-bound organelles such as the ER and the Golgi

apparatus which are responsible for glycosylation in many higher organisms. Glycosylation in prokaryotes has just recently been discovered (Mescher et al., 1974, 1976; Upreti et al., 2003). The identification of glycosylation mechanism outside of the cellular membrane (Brooks, 2004; Upreti et al., 2003) revealed how different this process is in bacteria when compared to their eukaryotic counterparts.

Additionally, comparisons between the eukaryotic cell lines (yeast, mammalian, and insect cells) used for recombinant protein expression reveal that they all have different glycosylation mechanisms (Brooks, 2004) causing recombinant proteins to not fold or function as their native counterparts. Considering this, a more efficient method for assessing the biological function of S6 would be to conduct additional inhibitory assays using native tick S6 protein procured by immunoprecipitation using the antibodies to rS6 to extract the protein from whole tick tissue.

The apparent failure of rS6 to inhibit chymotrypsin or trypsin may also be explained by the possibility that S6 may have a cysteine rather than a serine protease inhibitor. It is also possible that S6 has a non-inhibitory function at the tick host interface. In future experiments I suggest including cysteine protease complexing assays.

Despite these inconclusive results as to the inhibition ability of rS6, this experiment cannot be considered a full characterization of the functionality of S6. None of tick-encoded serpins characterized thus far have been experimentally demonstrated to be secreted at the tick-host interface. As stated in Chapter II, S6 has been found to be present in tick saliva, indicating that it is the only described serpin that is potentially secreted into the host. It can therefore be surmised that the direct role of S6 in regulating

tick feeding remains to be ascertained and additional experiments need to be conducted to determine what is the biological function of S6.

CHAPTER IV
RNA INTERFERENCE TO MEASURE THE EFFECTS OF POST-
TRANSCRIPTIONAL GENE SILENCING OF S6 AND S6/S17 ON TICK FEEDING
SUCCESS

Introduction

The limiting step towards development of animal vaccines against tick feeding is the discovery and target validation of effective target antigens that when disrupted will compromise tick feeding success. In order to assess the significance of S6 expression in *A. americanum* feeding, post-transcriptional gene silencing using RNA interference (RNAi) mediated gene knockdown was conducted. Fire et al., (1998) were the first to describe *in vivo* RNAi, noting that injection of dsRNA into the nematode *Caenorhabditis elegans* caused gene silencing throughout the organism and that was also inherited by its progeny. The RNAi pathway is a naturally occurring phenomenon in which the introduction of dsRNA induces the degradation of mRNA, which prevents further translation from taking place. The mechanism of RNAi is initiated by the injection of dsRNA that correlates with the target gene. The injected dsRNA is digested into small interfering RNAs (siRNAs) by the Dicer enzyme. The resulting siRNAs migrate to their complementary mRNA molecules while guiding the RNA-induced silencing complexes (RISCs) that slices and destroys the target mRNA, thus inhibiting any further translation. The confidence in the use of RNAi as a method for studying gene function in ticks has recently been strengthened by the discovery of proteins and several conserved domains

related to the RNAi pathway identified in *R. microplus* by the screening for homologous genes using comparisons to *C. elegans* and *Drosophila melanogaster* genomes (Kurreck, 2009).

RNAi methodology has been successfully used to illuminate the biological significance of numerous genes that play an important role in tick feeding success in *Amblyomma americanum* (Mulenga and Khumthong, 2010; de la Fuente et al., 2010), *Ixodes scapularis* (Kocan et al., 2007; Aljamali et al., 2003), *I. ricinus* (Hajdusek et al., 2010), *Haemaphysalis longicornis* (Alim et al., 2009; Liao et al., 2008), *Dermacentor variabilis* (Kocan et al. 2007), and *R. microplus* (Kocan et al., 2007) to assess the effects of gene silencing on tick feeding, reproduction, and progeny viability.

In this study, the author utilized the RNAi silencing method to examine the biological significance of S6 in *A. americanum* tick feeding success and reproduction. Ability to attach onto host skin to start feeding, mortality, engorgement mass (EM, an index of amount of blood taken in by the tick), and fecundity (ability to covert blood meal to eggs) were assessed to measure the effects of S6 silencing on tick feeding success and reproduction.

Materials and Methods

Ticks

Ticks used in these experiments were obtained from an *Amblyomma americanum* L. colony from the laboratory of Dr. Pete Teel from the Entomology Department of

Texas A&M University, College Station, Texas. This colony is maintained by feeding on chickens at the larval and nymphal stage and on cattle as adults.

Generation of dsRNA

When compared to the published serpin sequences in *A. americanum* (Mulenga et al., 2007) S6 was shown to be very similar to S17. Thus for double stranded RNA (dsRNA) synthesis, I targeted two regions, the 3' region unique to S6 to achieve the silencing of the 5' region conserved between S6 and S17 (S6/S17) to silence both S6 and S17 and 3' region to silence S6 alone. Templates for dsRNA synthesis of S6/17, S6, and green fluorescent protein (GFP, negative control) were amplified in 30 μ L of reaction of the GoTaq® Green Master Mix (Promega) containing 0.1 μ M final concentrations of forward and reverse primers with added T7 promoter sequences (Table 4.1). The cycling conditions were an initial denaturation of 95°C for 2 minutes, followed by 40 amplification cycles of 95°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and a final extension of 72°C for 5 minutes. The size of each amplicon was verified by electrophoresis along with a 1Kb DNA ladder (Promega) at 100 V on a 2% agarose gel containing 1 μ g ethidium bromide in Tris-acetate-EDTA (TAE) buffer and purified using StrataPrep DNA Gel Extraction Kit as previously described in Chapter II. The purified DNA template was quantified using a nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies).

Table 4.1 Primers used from generation of dsRNA and validation of S6/S17 dual silencing and S6 silencing. T7 promoter sequence in bold.

Designation	Sequence	Orientation
T7 S6-Specific FWD	5' TAATACGACTCACTATAGGG CTGCTATCAGC GAGAGCACGCA 3'	Sense
T7 S6-Specific REV	5' TAATACGACTCACTATAGGG TCTGCGTGAAATTTCTG TCATTCTGGA 3'	Antisense
T7 Conserved FWD	5' TAATACGACTCACTATAGGG CCACTTCGCCG TGAAGCTCCTC 3'	Sense
T7 Conserved REV	5' TAATACGACTCACTATAGGG GCACCTGCGTG GAGTGCCTAG 3'	Antisense
T7 GFP FWD	5' TACGACTCACTATAGGG TCACGAACTCCAGC AGGACCATGTGATC 3'	Sense
T7 GFP REV	5' TAATACGACTCACTATAGGG ACGTAAACGGCC ACAAGTTCAGCGTGTC 3'	Antisense
S6/S17FWD	5' CCACTTCGCCGTGAAGCTCCTC 3'	Sense
S6/S17 REV	5' GCACCTGCGTGGAGTGCCTAG 3'	Antisense

dsRNA was synthesized *in vitro* using the MEGAscript RNAi Kit according to instructions by the manufacturer (Ambion, Austin, TX, USA). Five transcription reactions were assembled at room temperature for both of the targets and the GFP using 2 µg of linear DNA template, 10X T7 reaction buffer, and 2 µL each of the ATP, CTP, GTP, UTP, and T7 enzyme mix in a 30 µL reaction. The transcription reactions were incubated overnight at 37°C. For the annealing step each of the reactions was incubated at 75°C in a water bath for 5 minutes then allowed to cool to room temperature. 1/400th of the dsRNA was verified by electrophoresis along with a 1Kb DNA ladder (Promega) at 100 V on a 2% agarose gel containing 1 µg ethidium bromide in Tris-acetate-EDTA (TAE) buffer. A nuclease digestion was used to remove any remaining DNA or ssRNA in each of the 5 reactions for the S6, S6/S17, and GFP. The 50 µL digestion reactions were assembled on ice and included 20 µL dsRNA, 5 µL 10X digestion buffer, and 2 µL of both the DNase I and RNase enzymes and incubated on ice for an hour. All of the

reactions were purified by adding 50 μL 10X binding buffer, 250 μL of 100% ethanol, and 150 μL nuclease-free water to each of the reactions and applying the binding mixture to the provided filter cartridge and centrifuging at 18,000xG for 2 minutes. The filters were washed using centrifugation as described above. The 50 μL of the elution solution was applied to the filter and incubated for 2 minutes at 65°C. The eluted dsRNA was collected by centrifugation for 2 minutes at 18,000xG. The elution step was then repeated. 1/400th of the purified dsRNA was verified on a 2% agarose gel containing 1 μg ethidium bromide in TAE buffer. The eluates for each target were combined and quantified using a nanodrop ND-1000 Spectrophotometer. The reactions were concentrated to a 2 $\mu\text{g}/\mu\text{L}$ final concentration through using a Savant DNA 120 SpeedVac® Concentrator.

Tick injections with dsRNA and feeding

For each of the controls and treatments, 50 unfed female ticks were injected with 1 μL (2 $\mu\text{g}/\mu\text{L}$) of S6/17, S6 or GFP (control) dsRNA, or TE diluent buffer using 33 gauge half-inch needles attached to a 10 μL gastight syringe (Hamilton, Reno, NV, USA). The ticks were kept at 22°C overnight to observe any mortality resulting from the injection. The treatment groups and an uninjected (naive) group were placed in separate cells pre-infested with males that were adhered on the back of the calf using livestock identification cement (Nasco, Fort Atkinson, WI). Unattached females were removed from the cells 48 h later. The attached ticks were allowed to feed until detachment.

Detached females were collected every 24 h, weighed, placed in separate containers at 22°C, and allowed to oviposit.

Corroboration of RNAi-mediated silencing

To determine if tick injections with dsRNA triggered disruption of the target gene, three ticks per treatment were collected 48 h post-attachment. These ticks were individually processed for total RNA extraction using the TRIzol reagent. Extracted total RNA was treated with RQ1 DNase to eliminate genomic DNA contamination according to instructions by the manufacturer (Promega). DNase treated RNA was then subjected to two-step semi-quantitative RT-PCR as described in Chapter II, using PCR primers targeting the S6 specific domain, S6-Specific FWD and REV, (Table 2.1) or the domain conserved between S6 and 17, S6/S17 FWD and REV (Table 4.1). Tick actin forward and reverse primers (Table 2.1) were used for sample load control. Ten μ L of the PCR reactions was analyzed by electrophoresis along with a 1 Kb DNA ladder at 50 V on a 2% agarose gel containing 1 μ g ethidium bromide in TAE buffer to qualitatively corroborate silencing.

Assessment of effect of RNAi on tick feeding and fecundity

To assess the effects of S6 mRNA silencing on tick feeding success and fecundity, tick-feeding parameters were recorded, including attachment rates (the number of attached was determined by subtracting the number of unattached ticks from the total number of ticks that were placed on the animal), mortality (number of ticks

dead after 48 h subtracted from number of ticks placed on animal), engorgement mass (EM) (mass of engorged tick after spontaneously detachment), and the egg mass conversion ratio (EMCR) (the mass of the egg clutch divided by EM). To assess the effect of silencing on fecundity, ticks were incubated at 25°C for 4 weeks to lay eggs. The EMCR was done to determine the ability of the tick to convert its blood meal to eggs. To analyze the statistical significance of the differences observed between the silenced and control groups a one-way ANOVA and post-ANOVA pair-wise comparisons using Tukey's HSD test was conducted with the help of the statistics department. The web based Grubbs test (<http://www.graphpad.com>) was used to identify outlier samples which were subsequently removed from the analysis.

Results

Validation of RNAi-mediated silencing by RT-PCR

The MegaScript *in vitro* RNA synthesis kit was used to successfully synthesize and purify S6/17(fragment targeting S6 and S17), S6 and GFP double stranded RNA (dsRNA). Semi-quantitative two-step RT-PCR expression analysis was used to confirm if microinjections of the RNAi-mediated silencing of S6 mRNA as summarized in Figure 4.1. In both of the dsRNA-injected samples with the S6 and the S6/S17, S6 transcript failed to be amplified in each of the ticks tested whereas actin was amplified, thus indicating that that silencing of S6 mRNA was achieved in the treatment groups (Figure 4.1). The amplification of S6 and the S6/S17 regions in the three control groups

indicate that neither injection trauma nor the diluent buffer affected the S6 mRNA levels of the target gene.

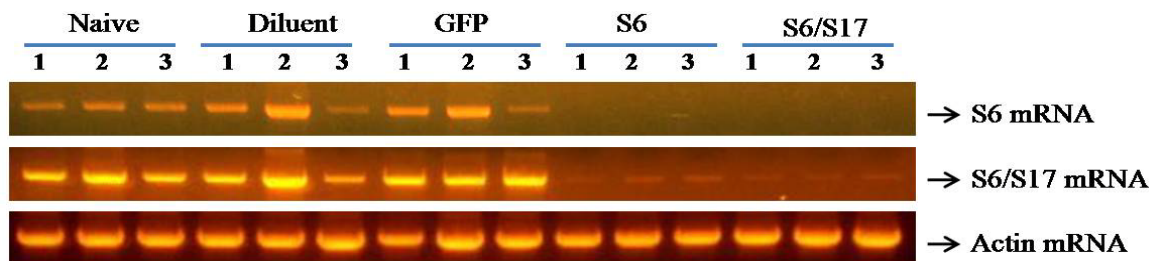


Figure 4.1 Validation of silencing by RT-PCR on individual ticks post-attachment. 3 ticks from each group were processed individually for total RNA extraction and subjected to 2-step semi-quantitative RT-PCR to amplify the S6, S6/S17, and tick actin fragment (load control). Results indicate that S6 and S6/S17 was successfully silenced in both of the treatment groups.

The effect of S6 and S17 dual silencing or S6 alone on A. americanum tick feeding efficiency

Based on mortality and attachment rates summarized in Table 4.2, the dual silencing of S6 and S17 and S6 alone was not lethal and did not affect the ability of ticks to attach onto host skin and start feeding as confirmed by Chi-square analysis (results not shown). To assess the effect of silencing on blood meal acquisition EM of spontaneously detached ticks were determined: Naive range 306.8-998.2 mg, (N=46); diluent range: 105.4 – 828.9 mg, (N=32); GFP range: 200.7-843.3 mg, (N=38); S6 range: 277.0-935.2 mg, (N=39); S6/S17 range: 97.0-871.9 mg (N=38). When subjected to Grubbs analysis, no outlier samples were identified. A one-way ANOVA revealed that there were significant differences ($F_{4, 185} = 8.88$, $P < 0.0001$) among mean EM summarized in Figure 4.2. The post hoc pair-wise comparison using the Tukey's HSD

Table 4.2 Overview of feeding and fecundity parameters of ticks subjected to RNAi for S6 and S6/S17.

Treatment Groups	Number Infested on host	Number attached 48h post-infestation	EM (mg)	Mortality (%)	Number of Ovipositing Ticks	EMCR (mg)
Naive Control	50	45	655.07	0	45	370.63
Diluent Control	50	46	466.69	0	46	318.08
GFP Control	50	50	548.20	0	49	280.37
S6	50	48	609.25	0	44	289.01
S6/S17	50	50	491.74	0	45	266.54

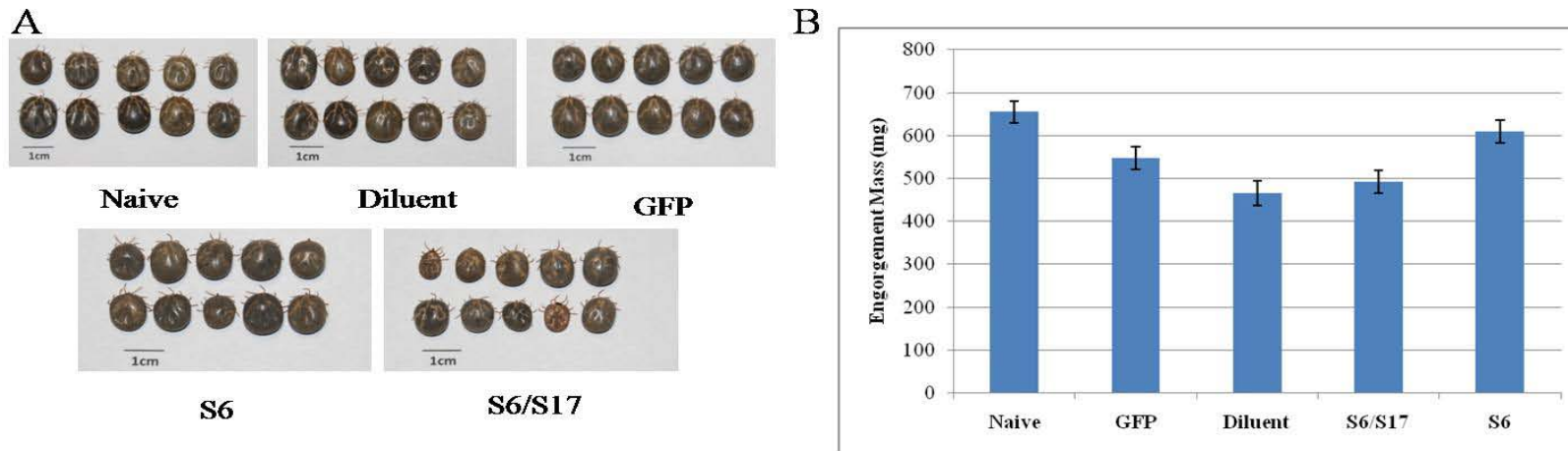


Figure 4.2 Analysis of the effect of silencing S6 and S6/S17 transcript on blood meal acquisition and fecundity. 50 unfed female ticks were injected with dsRNA S6, S6/S17, GFP, and diluent buffer. Each group of ticks were fed on calf and weighed after detachment. (A) Spontaneously detached ticks were photographed to document the physical phenotype. (B) Spontaneously detached ticks were individually weighed to determine the EM. The EM means for each group were subjected to one-way ANOVA and Tukey's HSD pairwise comparison to determine the statistical significance between treatments. Mean EM of treated and controls were not statistically different.

test revealed that that dual S6 and S17 silencing caused ticks to obtain significantly smaller blood meals (mean EM= 491.742±26.449) when compared to non-injected control ticks (mean EM = 655.079±36.301, P<0.0001). However, the mean EM of S6/S17 silenced ticks was not statistically different from GFP-dsRNA (548.197±37.405, P=0.558) or diluent (466.691±39.119, P=0.968) treatments. Similarly, silencing of S6 alone (609.25±26.11) showed did not show a significant reduction in *A. americanum* tick feeding efficiency when compared to non-injected (655.079±36.053, P=0.7092), GFP-dsRNA (548.197±37.164, P=0.472) or diluent (466.691±38.889, P=0.0029) control groups. It is important to note that mean EM of GFP-dsRNA and diluent injected control ticks were also significantly lower than the non-injected naive control (P=0.030 and <0.0001, respectively).

Dual silencing of S6 and S17 or S6 alone did not affect tick fecundity

To assess the effect of RNAi silencing on the fecundity of *A. americanum*, engorged ticks were allowed to oviposit for 28 days and EMCR was determined. Through the Grubb's test analysis it was determined that there was one outlier in each of the egg mass conversion ratio data sets. One way ANOVA revealed that there were significant differences ($F_{4,165}=4.203$, $P=0.003$) among mean EMCR average summarized in Figure 4.3. Consistent with EM data, Tukey's HSD post-hoc pair-wise analysis of mean EMCR revealed that dual silencing of S6/S17 (0.267±0.198) significantly reduced tick fecundity when compared to non-injected controls (0.371±0.028, $P=0.003$), but not when compared to GFP-dsRNA (0.280±0.029,

$P=0.990$) or diluent (0.318 ± 0.030 , $P=0.430$) injected controls. Likewise, the EMCR for S6 silenced ticks (0.290 ± 0.020) was statistically significant compared to the naive control (0.371 ± 0.030 , $P=0.032$), but not when compared to GFP-dsRNA (0.280 ± 0.029 , $P=0.998$) and diluent (0.318 ± 0.030 , $P=0.868$) injected controls.

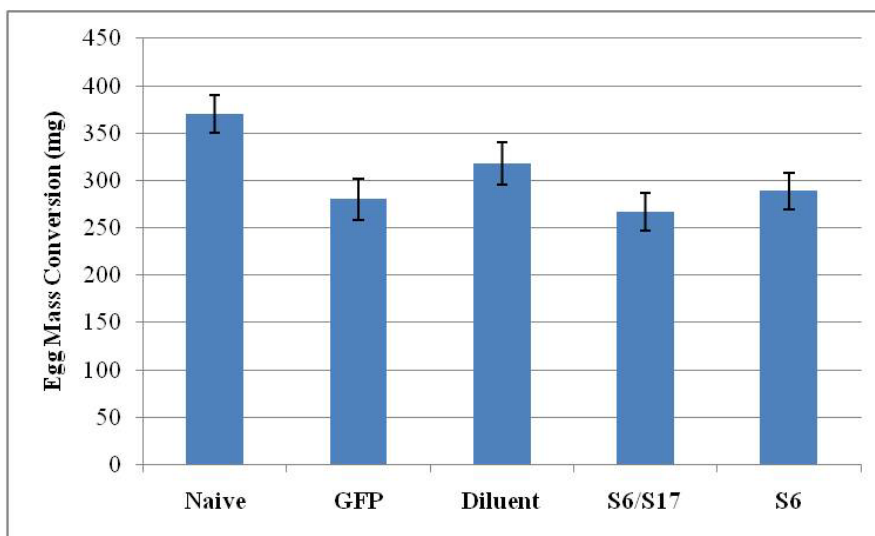


Figure 4.3 Effects of RNAi silencing of S6 and S6/S17 on fecundity measured as egg mass conversion ratio. After detached females were collected and weighed, they were placed in separate containers at 22°C to encourage oviposition. For each detached female, the mass of the egg clutch was divided by the EM to determine the egg mass conversion ratio. The egg mass conversion (EMCR) ratio was analyzed by a one-way ANOVA and the Tukey's HSD test to determine the significance in mean EMCR. Mean EMCR of treated and controls were not statistically different.

Discussion

Based on studies described in Chapter II of this thesis it was determined that biological functions of S6 may be associated with regulation of the preparatory tick feeding phase. The ubiquitous expression patterns of S6 at both mRNA and protein levels were suggestive of the importance of this molecule to tick feeding success. Thus to validate these observations, the significance of S6 biological functions in regulating *A. americanum* feeding and reproduction was determined using RNAi silencing methodology as previously described (Mulenga and Khumthong, 2010). Although complete silencing was achieved as revealed by lack of amplification of the S6 transcript in all dsRNA injected ticks, silencing of S6 alone or the dual silencing of S6 and S17 did not prevent *A. americanum* ticks from attaching onto host skin and start feeding. With exception of tick-borne viruses which may be transmitted within the first few minutes after the tick attaching onto host skin (Nutall and Labuda, 2008), data in several studies indicate that animal and human tick-borne pathogens such as *Theileria parva* (Bishop et al., 2004), *Babesia bigemina* (Bock et al., 2004), *Rickettsia rickettsii* (Burgdorfer, 1975), *Borrelia burgdorferi* (Burgdorfer, 1982), and *B. microti* (Bock et al., 2004) are transmitted after ticks have been feeding for 2-3 days. Considering this, it is a logical argument that an effective target anti-tick antigen is one that prevents ticks from attachment and the initiation of feeding and implicitly would block tick-borne disease transmission.

Given my findings that ticks were able to attach and initiate feeding despite the complete disruption of the S6 transcript, one may argue against S6 as being a suitable

target anti-tick vaccine antigen. On the basis of the inherent limitations of *in vivo* RNAi silencing as used in my study, the failure to stop ticks from initiating feeding in S6 silencing is being interpreted with caution. Since data presented in Chapter II of this thesis shows S6 protein is expressed in unfed ticks, there is a possibility that, despite the complete disruption of the S6 mRNA, its protein was still available and functional. The consequence of this is that the S6 protein that existed prior to RNAi silencing would be available to mediate tick attachment onto host skin and initiation of blood meal feeding.

In future studies, I suggest using the immunization and challenge infestation approach to evaluate the vaccine capacity of S6. My data in Chapter II shows that rS6 is immunogenic in rabbits and thus it can be used as an immunizing antigen. The immunization and challenge experiments would be to test the effect of antibodies to rS6 on tick feeding, testing if they will bind to and interfere with biological functions of native S6. By feeding ticks on immunized animals, the effect of the S6 protein present in unfed ticks will be eliminated, as from initiation of feeding the antibodies will be available to bind native the S6 protein. The idea of immunizing animals against S6 as a method of preventing tick feeding is further strengthened by the protein's apparent secretion into the host during tick feeding as demonstrated in Chapter II of this thesis. Although host antibodies can cross the tick midgut barrier (Brossard and Rais, 1984), antibody concentrations of ~20-50% (Brossard and Rais, 1984; Ben-Yakir et al., 1987) or 1000-3000 fold (Ben-Yakir, 1989) lower than titers in the host immune serum can actually interact with a non-tick saliva target protein. The implication of this is that if non-tick saliva proteins or concealed antigens are targeted in immunizations, the ability

of ticks to eliminate or destroy antibodies effectively reduces the protective level of tick resistance. This will not be so in the case of targeting S6 in that antigen-antibody interactions will occur at the tick host interface and not in the tick. A limited number of studies have shown that tick-encoded serpins have been proven to be potential vaccine candidates through host immunization with recombinant proteins derived from tick-encoded serpins from *H. longicornis* (Sergino et al., 2003; Imamura et al., 2005), *R. appendiculatus* (Imamura et al., 2006, 2008) and *I. ricinus* (Prevot et al., 2007). Although several of these serpins have been confirmed as being concealed antigens (Sergino et al., 2003; Imamura et al., 2005, 2006), when used in immunization and challenge experiments, all have shown to have a negative effect on tick feeding success and fecundity, seen in a reduction of EM and increase in mortality.

It is also important to note that there are at least 15 other *A. americanum* serpins that are expressed together with S6 and S17 during the same tick feeding period (Mulenga et al., 2007). It is possible that silencing of S6 and S6/S17 did not account for the redundancy of other serpins. The redundancy of function amongst these 15 other serpins may be another reason why dual silencing of S6 and S17 or S6 alone did not prevent ticks from starting to feed. To overcome the problem of redundancy, using peptide immunogenic regions that are conserved among all or a majority of the *A. americanum*-encoded serpins as immunizing antigens might confer an immune response blocks the functions of all serpins.

A fully fed female takes in host blood that is estimated at between 200-300 times the unfed tick weight (Sonenshine, 1993). Given the inconsistent observations in my

final EM data, it is unclear whether or not S6 plays a role in regulating blood meal uptake by ticks. Although the mean EM in dual silencing of S6 and S17 or S6 alone were significantly smaller than the non-injected control groups, they were significantly larger than or equal to control individuals respectively injected with diluent buffer or GFP-dsRNA. Based on my findings, injection of diluent buffer alone potentially affected tick feeding. To clarify the observed inconsistencies in my EM data, it will be desirable to repeat the experiment using a different diluent.

After blood meal uptake, ticks convert their blood meal to egg mass (Sonenshine, 1993). Consistent with my EM data, the effect of dual S6 and S17 silencing or S6 alone significantly affected the fecundity of *A. americanum* when compared to non-injected controls individuals, but not diluent or GFP-dsRNA injected control individuals. In summary, based on data presented here the biological significance of S6 in *A. americanum* feeding and reproduction remains unknown. Further studies are needed to conclusively determine the significance of S6 in tick feeding.

CHAPTER V

CONCLUSION

Anti-tick vaccines are a sustainable, non-contaminating, and cost efficient alternative to chemical tick control methods. This has encouraged the search for viable target antigens that play a critical role in the physiology of the tick. The goal of this study was to establish the biological relationship of S6 to the tick feeding process and characterize the function of S6.

The transcription and protein expression profiles of S6 show that it is present during the PFS and the SFS, indicating that it possibly plays a role in the establishment and maintenance of the feeding lesion and assists in blood meal acquisition. Transcriptional analysis reveals that S6 mRNA is ubiquitously expressed in unfed ticks through 5 days of the feeding, with PCR density analysis of transcription patterns show a peak at 72 h after attachment. When whole ticks were analyzed by western blotting analysis, it was found that native S6 protein is ubiquitously expressed and downregulated in response to tick feeding, with correlation between transcription and protein expression profiles only consistent from the unfed stage to 48 h. Protein expression is downregulated from 72 h to 120 h, indicating that S6 is not utilized during the latter part of the feeding cycle.

To determine the spatial distribution of S6 during the first 5 days of feeding, mRNA and protein expression patterns in the SG, MG, OV, and CA were investigated. S6 mRNA abundance in dissected tick organs showed a 3.7, 3.4, and 1.7-fold upregulation from 24 h to 96 h in the SG, MG, and CA, respectively before

downregulating at 120 h. S6 protein expression in dissected tick tissues similarly show downregulation in response to tick feeding, with S6 being identified in SG, MG, OV, and CA from 24 h until 72 h. The presence of S6 in the salivary glands during the first 3 days after attachment indicates that S6 might be one of the secreted molecules that play a critical role in tick feeding. To determine the likelihood of S6 being a secreted protein, tick saliva was analyzed for the presence of native S6.

S6 was found to be present in tick saliva after 48 h of feeding. This result indicates that S6 is most likely an exposed molecule that is injected into the host to facilitate blood meal acquisition. Exposed antigens oftentimes play a critical role in tick feeding physiology, and therefore inhibiting their function by way of immunization is likely to have a negative effect on feeding success. Additionally, when compared to their concealed counterparts, exposed antigens are viewed as a more feasible vaccine target because of their ability to elicit a strong immune response. S6 was found to be immunogenic proceeding immunizations with rS6, indicating that antibodies to the native tick-encoded S6 can effectively be produced.

RNAi-mediated post-translational gene knockdown was used to determine the role of S6 in regards to obtaining a blood meal. Dual silencing of S6/S17 and S6 silencing had no significant negative effect on tick feeding and fecundity success, however limitations of the *in vivo* RNAi methodology prevented this technique from determining how the tick utilized S6 and S17. RNAi does not clear the organism of existing protein, and as S6 is present at the unfed stage there is a chance the protein was still present and functional in the treated ticks. Although this methodology is frequently

used in acarology to establish the function of unknown genes and to assess their viability as target antigens, it is not an accurate method for evaluating the biological significance of proteins that are expressed prior to dsRNA injection. Given this limitation, the author suggests conducting an immunization and challenge infestation experiment as a more effective method of evaluating the potential of S6 and S6/S17 as a vaccine target against *A. americanum*. The presence of 15 other serpins identified in *A. americanum* during the beginning of the feeding cycle suggests a redundancy of function amongst these proteins. To account for this, peptides from immunogenic regions consistent amongst all or the majority of the serpins should be used to immunization to fully assess the effect of vaccination against all tick-encoded serpins.

Based on sequence analysis and protein expression profiles there was a strong indication that S6 might be one of the many immunomodulatory molecules secreted into the tick-host interface to suppress the immune response. To determine if S6 functions as an inhibitory protein, rS6 was co-incubated with serine protease archetypes chymotrypsin and trypsin and samples were analyzed on a SDS-PAGE for complex formation. No complex formation was observed, but the results were inconclusive. The lack of inhibition observed could be due to the incorrect folding related to recombinant protein expression. As an alternate approach, native S6 could be collected from tick tissues using immunoprecipitation for use in future inhibitory assays. Although most serpins inhibit serine proteases, some inhibit cysteine proteases or do not function as inhibitors. To better characterize the function of S6, I propose additional assays using cysteine proteases in complex formation assays.

In conclusion, this study has provided important information regarding the development of an anti-tick vaccine. Because of its occurrence in various tissues during the PFS and SFS indicates that S6 plays a critical physiological role in tick feeding. Additionally, S6 is the first serpin identified in tick saliva. The presence of S6 in saliva collected at 48 h indicates that it is likely secreted into the host during feeding. To better assess the ability of S6 as a target antigen, further work is needed to determine the characterization of S6 and the effect of immunization with S6 on tick feeding.

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