BIOINFORMATIC AND BIOCHEMICAL CHARACTERIZATION OF *AMBLYOMMA AMERICANUM* (ACARI: IXODIDAE) SERINE PROTEASE INHIBITORS (SERPINS)

A Dissertation

by

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

DOCTOR OF PHILOSOPHY

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May 2016

Major Subject: Veterinary Pathology

ABSTRACT

Ticks bites are a source of morbidity and mortality for humans and animals. Chemicals have been insufficient for tick control whereas an anti-tick vaccine may be an effective alternative. Hosts use serpins to control their immune pathways therefore ticks may inject serpins into the host for the same purpose. *Amblyomma americanum* transcriptome data were analyzed to determine extent and diversity of serpins. Candidate serpins were assayed for function against 17 proteses and for ablity to affect blood coagulation and platelet aggregation. Spliced serpin constructs consisting of putatively immunogenic regions of selected serpins were designed for expression and vaccine trials.

A. americanum expresses 122 unique serpins. The greatest diversity of serpins were in males and in feeding ticks. Forty percent were conserved in other ticks. *A. americanum* serpins 4 and 8 were selected for characterization as representatives of similar serpin clusters. These serpins bound host antibodies to tick saliva indicating they are immunogenic and used in feeding. Serpin 8 inhibited inflammatory proteases cathepsin G and proteinase 3. Serpin 4 inhibited inflammatory proteases cathepsin G and chymase, and the cysteine protease papain. Neither serpin affected blood coagulation however, serpin 4 delayed platelet aggregation. A spliced serpin construct was cloned in bacteria and yeast cells, however recombinant proteins failed to express. Results indicate serpins 4 and 8 function in the vertebrate host as counter-defense serpins and thus may

be useful as anti-tick vaccine candidates, however their relative importance in tick feeding physiology will need to be addressed using an alterantive protocol.

DEDICATION

This work is dedicated to my children, Avary, Alexa, and Kaylee, to whom I hope to provide a more enriched life as a result of my higher education. I would also like to dedicate this work to my mom Kim Spradling (Thompson), who always pushed me to succeed in my academics.

ACKNOWLEDGEMENTS

I would like to primarily thank my committee chair, Dr. Albert Mulenga for betting on me and accepting me into his laboratory. His guidance and support led me to reach one of my life's greatest accomplishments. I would also like to thank my committee members, Dr. Patricia Pietrantonio, Dr. Karen Snowden, Dr. Pete Teel, and Dr. Michele Slotman, for all of their guidance and support throughout the course of my graduate program.

Thanks also go to my colleagues and all of the friends that have given me advice and emotional support, and to the Veterinary Pathology Department, College of Veterinary Medicine and Biomedical Sciences, faculty, and staff for funding opportunities, and for enhancing my experience here at Texas A&M University and keeping me on track through my program. I also want to extend my gratitude to the National Institutes of Health (NIH), grants from which provided the funding for my PhD research.

Finally, I would like to thank my family and close friends for all of the emotional and financial support, as well as assistance in childcare while I pursue my passions.

NOMENCLATURE

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
SERPIN	Serine Protease Inhibitor
H	Hour
CDC	Centers for Disease Control

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CHAPTER I

INTRODUCTION

Tick Biology and Medical and Veterinary Importance

Ticks (Order: Acari, Superfamily: Ixodoidea) are parasitic arthropods that are obligatory hematophagous. While there are three families of ticks; Ixodidae, Argasidae, and Nutallielidae, the Ixodidae, or hard ticks, so called because of the hard appearance of their exoskeleton, collectively cause the largest health and economic burden worldwide. There are four stages to their life cycle: the inactive egg stage, and the active and blood-feeding larval, nymphal, and adult stages. Ticks parasitize a wide variety of vertebrates including reptiles, birds, and mammals. At least 39 species of ixodid ticks are commonly found on animals and at least 60 species of ixodid ticks have been cited as found feeding on humans, with 23 commonly found on humans (Dantas-Torres et. al., 2012; Estrada-Peña and Jongejan, 1999). Ticks of the family Ixodidae may be classified as one-, two-, or three- host ticks. One-host ticks spend their entire life cycle on the same host; two-host ticks spend both immature stages of their life cycle on the host, finding a new host only at the adult stage; and three-host ticks change hosts with each molt. Unlike many other hematophagous parasites, ticks are unique in the duration of time they spend on their host, which can last for days at the immature larval and nymphal stages, and for weeks at the adult stage. Ticks are telmophagous, feeding from a pool of blood created in the skin following laceration of the blood vessel. Consequent to their hematophagous

lifestyle and their prolonged feeding times, tick parasitism manifests itself into a variety of negative consequences for their hosts. Tick feeding has been documented to cause severe direct effects including paralysis and toxicosis from injected tick toxins, allergic reactions, anemia and exsanguination in heavy infestations, reduced livestock productivity, damage to skin impacting the economic value of hides, and death (Jongejan and Uilenberg, 2004; Estrada-Peña and Jongejan, 1999; Klompen et. al., 1996). Wildlife also suffer from the direct effects of tick parasitism. For example, in the northern United States, moose populations with heavy winter tick (*Dermacentor albipictus*) infestations average 33,000 ticks per moose (with up to 100,000 per moose also documented) (Severud and DelGiudice, 2015). Heavy infestations in these moose leads to a loss of fur and often death (Samuel, 2004). However, of primary concern perhaps is the transmission of disease-causing pathogens by ticks. Ticks may transmit a wide diversity of pathogenic organisms to their hosts including viruses, bacteria, protozoa, and helminthes (Sonenshine and Roe, 2014).

In livestock production, ticks are considered the most important arthropod vector of disease agents (Sonenshine, 1991; de la Fuente et. al., 2008). In livestock production, an estimated 80% of the world's cattle are affected by disease-causing tick-borne pathogens. The most serious diseases in livestock caused by tick-borne pathogens are babesiosis, anaplasmosis, theileriosis, and heartwater (Jongejan and Uilenberg, 2004; Marcelino et. al., 2012). In domestic pets, notable disease caused by tick bites are Lyme, cytauxzoonosis, canine ehrlichiosis, hepatozoonosis, rickettsiosis, and anaplasmosis (Smith et al., 2012; Rizzi et al., 2015; Baneth et al., 2015; Dantas-Torres et al., 2012).

Humans, while frequently parasitized by ticks, are considered only an incidental host, and for many years ticks and diseases stemming from their parasitism were primarily a veterinary problem. However, following the description of *Borrelia burgdorferi* as a causative agent for Lyme disease in the 1980's there was a paradigm shift regarding the impact of ticks in public health (Burgdorfer et. al., 1982; Johnson et. al., 1984). Between 1982 and 2001, the list of human diseases caused by tick-vectored pathogens ballooned with the addition of 15 new bacterial pathogens (Parola and Raoult, 2001). While in 2011, the global count of diseases in humans caused by tick-vectored pathogens was 17 (Day, 2011), this number has been on the rise. It was previously reported that there are 10 human diseases caused by tick-vectored pathogens in North America (Dantas-Torres et al., 2012). Today however, that list has increased to 15 (Tick-borne diseases of the United States, 2016; Savage et. al., 2013; Dantas-Torres et al.; 2012), and ticks rank just behind mosquitoes in their medical burden to humans (Sonenshine, 1991; de la Fuente et. al., 2008a). In the U.S., the most common diseases in humans caused by tick-vectored pathogens are Lyme disease, Rocky Mountain spotted fever (RMSF), human monocytic ehrlichiosis, human granulocytic anaplasmosis, tularemia, and babesiosis (Martin and Romero, 2012), although diseases caused by Powassan and Heartland viruses are also of great concern due to the morbidity and potential lethality of the ensuing diseases (Piantadosi et al., 2015).

Amblyomma americanum Biology and Medical and Veterinary Importance

Amblyomma americanum, long considered a nuisance tick species, has become one of the most important vectors in the United States, and is the most important tick pest in the southern United States, both in veterinary medicine and in public health (Brannan et al., 2014; Childs and Paddock 2003). *A. americanum* is a three-host tick and an aggressive biter with a reputation of indiscriminately parasitizing a wide variety of vertebrate hosts in all active stages of its life. Humans are no exception, where it is not uncommon for *A. americanum* to parasitize humns in geographic regions suffering from heavy population numbers of this species (Mixson et. al., 2006; James et. al., 2001). While currently found only in North America, recent years have seen the geographic range of *A. americanum* expand from strictly southeastern regions of the United States to the northeast and Midwestern regions (Keirans and Lacombe, 1998; Mixson et. al., 2004). Recent surveys for *A. americanum* distributions have classified this species as established in 32 states throughout the Southeast, South Central, and Midwest regions, and all along the eastern seaboard as far north as Maine (Springer et al., 2014).

In humans, *A. americanum* is responsible for the transmission of *Ehrlichia chaffeensis, Ehrlichia ewingii, Francisella tularensis, Rickettsia amblyommii,* and the causative agent of southern tick-associated rash illness (STARI) (Schulze et. al., 2011; Telford III and Goethert, 2004; Telford III et al., 2008; James et al., 2001; Goddard 2009; Tick-borne Diseases of the United States, 2016). While any disease caused by bacteria of the genus *Ehrlichia* may be called ehrlichiosis, infection with *E. chaffeensis* is also called human monocytic ehrlichiosis. Like many diseases caused by tick-borne

pathogens, ehrlichiosis symptoms are flu-like in nature including fever, fatigue, and muscle aches (Ehrlichiosis, 2013). Ehrlichiosis can be fatal, and since 2008 there have been thousands of reported cases of this disease (Nichols Heitman et al., 2016). *Francisella tularensis* is a serious bacterial pathogen, and the associated disease, tularemia, causes varying symptomology that may include ulceration at the bite site and lymph node swelling (Tularemia, 2015; Signs & Symptoms, 2015). In 2014, there were 180 cases of tularemia reported to the CDC and over 200 cases reported in 2013. Most cases of tularemia occur in Arkansas, Oklahoma, Kansas, and Missouri (Statistics, 2015). *R. amblyommii* is a spotted fever group (SFG) rickettsial species with undetermined pathogenicity in humans (Sayler et al., 2014). However, its implication in human disease comes from the fact that it has been detected in RMSF-like rashes after bites from *A. americanum* (which does not transmit the causative agent of RMSF), while RMSF has been reported in areas where *A. americanum* is the predominant tick species (not the *Dermacentor* species that are responsible for RMSF) (Sayler et al., 2014). Another incomplete epidemiological mystery is the southern tick-associated rash illness (STARI), where the disease is obvious but the pathogen has yet to be isolated. STARI is diagnosed as a Lyme-like bullseye rash appearing at the bite site of *A. americanum* ticks (rather than *Ixodes scapularis* ticks) (Masters et al., 2008; Southern tick-associated rash illness, 2015).

Additionally, *A. americanum* has been shown to carry Heartland virus; this new virus having been first detected in 2009 and subsequently found in 13 states (Heartland Virus, 2014; McMullan et. al., 2012; Savage et al., 2013). In the six years since its

discovery, Heartland virus infection has been confirmed in ten cases and two of those infected have died (Heartland Virus, 2014; McMullan et. al., 2012; Savage et al., 2013; Muehlenbachs et al., 2014; Wormser and Pritt, 2015; Williams, 2014). Accumulating evidence suggests that this virus is more common than currently appreciated, and that more cases have likely occurred but have been overlooked or alternatively diagnosed (Doucleff, 2015). In humans, diseases caused by tick-vectored pathogens may be significantly under-diagnosed due to the non-specific symptoms (flu-like symptoms) that accompany many of the diseases, together with the fact that tick attachment sometimes goes undetected even in humans.

Because animals are unable to groom themselves to the extent that humans usually do, the health burdens from tick parasitism on animals extend beyond tick pathogen transmission. In cattle, parasitism by *A. americanum* alone results in lower weight gain, damage to hides, problems with fertility, secondary infections at the bite site, and even death; resulting in estimated total economic losses of more than $165 million USD to the industry (Brannan et al., 2014; Polito et al., 2013). Domestic pets not only suffer the direct effects of *A. americanum* parasitism, but may also be subject to pathogenic organisms vectored by this tick. In domestic cats, *A. americanum* transmission of the protozoan *Cytauxzoon felis* results in a variety of clinical signs including dehydration, anorexia, lethargy, depression, and most cats die from infection (Reichard et. al., 2010; Rizzi et al., 2015). *A. americanum* also affects wildlife; its predilection towards white-tailed deer as a host results in the transmission of *Theileria cervi*, which can be fatal due to anemia and leukocytosis (Wood et al., 2013; de la

Fuente et. al., 2008; Waldrup et. al., 1992). There are few vaccines available that protect against the pathogens transmitted by ticks, therefore, prevention of disease by these pathogens is primarily achieved through tick control.

Current Tick Control Methods: Insufficiencies and the Vaccine Alternative

Ticks are currently controlled primarily through the use of acaricidal chemicals (Bissinger and Roe, 2010; George et. al., 2004; Redondo et. al., 1999, Pound et. al., 2000). The most common chemicals used against ticks include organophosphates including coumaphos, ethion, diazinon, and chlorfenvinphos; amidines including amitraz; pyrethroids including cypermethrin, deltamethrin, and cyhalothrin; macrocyclic lactones including ivermectin; and spinosad (Ghosh et al., 2007; Lopez-Arias et al., 2014; Shyma et al., 2012; Guerrero et al., 2012). Even though acaricides have proven effective in the short term, they do not offer permanent solutions due to a variety of serious limitations. Several acaricides and repellents have shown effective against *A. americanum* specifically, including deltamethrin (Schulze et al., 2001) spinosad, amitraz, permethrin, and chlorpyrifos (Miller et al., 2011), and the repellent DEET (Carroll et al., 2008).

One of these limitations is acaricide resistance which has been documented in ticks repeatedly in a pattern described as ‘paralleling’ the development of acaricides (Alonzo-Diaz et al., 2013; Shyma et al., 2012; Miller et al., 2001). Ticks may develop acaricide resistance through three mechanisms: target site insensitivity, enhanced, insensitivity enhanced metabolism, or reduced penetration (Guerrero et al., 2012). In the

target site insensitivity mechanism, a genetic mutation in susceptible ticks leads to a change in the target site which no longer binds the pesticide resulting in resistance towards the drug (Guerrero et al., 2012; Rosario-Cruz et al., 2009). For example, in *R. microplus* amino acid changes in the target site for pyrethroids, the voltage-gated sodium channel, has led to resistance in some populations of this tick species against this class of acaricides (He et al., 1999). More recently and also in *R. microplus*, evidence suggests a similar gene mutation in the RmβAOR gene, which codes for the octopamine receptor, is responsible for resistance to amitraz (Baron et al., 2015; Corley et al., 2013). Metabolic acaricide resistance may involve changes in the tick's ability to metabolize the acaricide or in the ability of the tick to sequester the drug (Guerrero et al., 2012). Studies indicate this type of resistance may be involved in *R. microplus* resistance to coumaphos through enhanced cytochrome P450 monooxygenase (cytP450) activity (Guerrero et al., 2007; Li et al., 2003). Resistance by a reduced penetration of acaricides into ticks has only been documented in laboratory ticks (Nolan and Schnitzerling, 1986; Schnitzerling et al., 1983). Extensive development of acaricide resistance and the tendency for ticks to develop simultaneous resistance to multiple classes of acaricides undermines their use in tick control. Unfortunately, the cost of developing new acaricides is steep, estimated at over $100 million (Graf et al., 2004; Willadsen, 2008; de la Fuente et al., 2007). Furthermore, acaricide use has generated concerns surround contamination of the environment and animal food products (George et al., 2004; Shapiro et. al., 1986).

As a result, it has become clear that acaricide use as a long-term control method will be insufficient and must be supplemented or replaced with a method(s) that

overcomes these limitations. There are currently few alternatives to acaricidal chemicals. While there are many documented natural predators of ticks including birds, spiders, beetles, and ants (Samish and Rehacek, 1999), their contribution has not been enough to keep tick populations below the acceptable threshold for prevention of direct and indirect effects of ticks in veterinary and public health. Some evidence suggests ticks may be controlled through the use of tick pathogens such as entomophagous fungi or nematodes (Samish et al., 2004; Kirkland et al., 2004; Kaaya and Hassan, 2000) or the manipulation of endosymbionts for tick control (Ghosh et al., 2007), however these ideas have yet to materialize into successful control alternatives. In the case of entomophagous fungi and nematodes, the primary limiting factor is the lack of an appropriate formulation (Samish et al., 2004). However, their proposed application in tick habitat may not be feasible for the control of certain tick species, such as three-host ticks, which spend the majority of their life off-host and would be difficult to target in the environment.

A validated alternative or complementary tick control strategy is the immunization of hosts against tick feeding (Rodríguez et. al. 1995; Willadsen et. al., 1995; Kumar et. al., 2009; Canales et. al., 2009; Merino et. al., 2011; Mulenga et. al., 1999; Galaï et. al., 2012; Saimo et. al., 2011; Sugino et. al., 2003; Prevot et. al., 2007). The idea of immunizing animals (cattle) against tick-vectored pathogens, or even tick infestation, by deliberately exposing those animals to tick feeding is more than 100 years old (Johnson and Bancroft, 1919; Willoughby, 1904; Dalrymple et al., 1899). However, it was not until tick antigens could be selectively singled-out for purification that an anti-

tick vaccine could reach levels of protection well beyond pre-exposure to tick feeding or immunization with tick homogenates (Mulenga et al., 1999; Kemp et al., 1986; Johnston et al., 1986). Through successive fractionation and purification a key antigen of *R. microplus* midguts was identified; Bm86 (de la Fuente et al., 2007). As a midgut protein, Bm86 was described as a 'concealed' antigen because the protein is not presented to the host during feeding as in the case of salivary proteins (Sonenshine and Roe, 2014). The use of concealed antigens was based on the idea of targeting essential proteins in ticks (Nuttal et al., 2006). Vaccine trials in cattle using this 650 amino acid antigen resulted in less female ticks feeding to repletion, a reduced engorgement weight, and a lower fecundity for ticks (de la Fuente et al., 2007).

The Bm86 antigen was protective enough to result in the first two commercialized anti-tick vaccines: GAVAC (Bm86) and TickGARD (Bm86 and Bm95) (Rodríguez et. al. 1995; Willadsen et. al., 1995). These vaccines have been used to successfully control ticks in Australia, Mexico, Cuba, Brazil, and Colombia (de la Fuente et al., 2007). TickGard is not commercialized but Gavac is still in use today (Schetters et al., 2016; Suarez et al., 2016). The application of these vaccines generated exciting results, including a reduction in the need for acaricidal use and disease incidence in cattle. The economic impact of this vaccine was an estimated savings in the millions of dollars for the cattle industry (de la Fuente et. al., 2007; Redondo et. al., 1999).

Despite the many successes, these vaccines are limited by several factors. Among the issues surrounding the vaccines are their limited use to primarily ticks in the

genus *Boophilus* (now *Rhipicephalus*) (de la Fuente et. al., 1999), thus lacking broad utility across tick species. Another cited reason for their limited success is a lack of a knockdown effect as seen with acaricide use (de la Fuente et. al., 2007), which gives the impression that vaccination does not work as well as chemical alternatives. The difficulties experienced with GAVAC/TickGARD, however, have only encouraged efforts to find a better replacement(s). In the years since its discovery and commercialization, research endeavors have continued in pursuit of additional anti-tick vaccine target antigens. As a result, a variety of promising antigens have been discovered and their experimental use in vaccine trials have further demonstrated the ability of anti-tick vaccines to reduce tick infestation, tick survival, engorgement weights, ova deposition, and target transcript abundance, among other parameters (Kumar et. al., 2009; Canales et. al., 2009; Merino et. al., 2011; Mulenga et. al., 1999; Galaï et. al., 2012; Saimo et. al., 2011; Sugino et. al., 2003).

Many of these studies have continued with the idea of using concealed antigens. In mice immunized with *H. longicornis* P27/30 recombinant protein, larvae had a much lower attachment rate (31.1%) than controls (98%) and larvae, nymphs, and adults took longer to feed than controls (You, 2004). Using recombinant embryo protein from *R. microplus*, *Boophilus* Yolk pro-Cathepsin (BYC), a vaccine efficacy of 25.24% was reported in bovine trials (Leal et al., 2006). More recently, the recombinant protein of a Bm86 homologue found in *Hyalomma anatolicum* (Haa86) was used in calf immunization trials that resulted in 36.5% of females failing to attach and feed as compared to 10.1-12.4% for controls (Azhahianambi et al., 2009). A vitellogenin-

degrading cysteine endopeptidase (VTDCE) was extracted and purified from *R. microplus* eggs and in bovine trials this vaccine efficiency using this protein was reported at 21% (Seixas et al., 2008). Although expressed in salivary glands, Hq05 from *Haemaphysalis qinghaiensis* was determined to be a concealed antigen and in vaccine trials ticks feeding on immunized sheep engorged to a lower average weight, laid 40% less eggs, and laid eggs that hatched 37% less than controls (Gao et al., 2009). In another trial, *Amblyomma hebraeum* 75% of females failed to engorge on rabbits immunized with recombinant male testicular protein voraxin (Weiss and Kaufman, 2004), while for *R. appendiculatus* recombinant protein of the voraxin homolog, voraxin-α, was used to immunize rabbits resulting in lower engorgement weights, laid fewer eggs, while 38.6% did not lay eggs at all (Yamada et al., 2009). In *R. microplus* recombinant subolesin was used to immunize cattle with a vaccine efficacy of 37-44% (Shakya et al., 2014).

Manny of the newly sought after anti-tick vaccine antigens, however, are not concealed antigens. With the idea that salivary gland proteins can be used as vaccine antigens and subsequently serve as a natural booster to vaccination each time a host is subsequently exposed to a tick (Willadsen, 2004) these proteins are being heavily explored. For example, a salivary gland protein antigen from *Haemaphysalis longicornis*, p29, was tested as a vaccine in rabbits and resulted in reduced female engorgement weights as well as 40-56% mortality in immatures (Mulenga et al., 1999). Rabbits immunized with *H. longicornis* HL34 recombinant saliva proteins resulted in increased tick mortality during and after feeding (Tsuda et al., 2001). In vaccine trials using guinea pigs, immunization with the *R. appendiculatus* putative cement protein 64P

resulted in infestation rates reduced by 48% for nymphs and 70% for adults (Trimnell et al., 2002). Zhou et al., (2006) found a low attachment rate and high mortality rate for *R. haemaphysaloides* nymphs and a high mortality rate for adults feeding rabbits immunized with the recombinant saliva protein RH50. In an example of cross-resistance potential of anti-tick vaccine candidates, recombinant *H. longicornis* glutathione S-transferase salivary gland protein was used to immunize cattle, while cattle were then challenged with *R. microplus* larvae infestation with a reported 57% efficacy of the vaccine (Parizi et al., 2011). In another vaccine trial using a cocktail of antigens, cattle were immunized with three previously validated anti-tick vaccine antigens (VTDCE, BYC, and GST-HI) and challenge-infested with *R. microplus*. Immunized cattle gained more weight and suffered less engorging females than non-immunized controls, and the effect of the vaccine cocktail was greater than vaccination with individual components alone (Parizi et al., 2012).

Several other studies, though reporting an overall vaccine efficacy, failed to find a statistical difference between parameters of control and treatment groups (Parizi et al., 2011; Seixas et al., 2008; Leal et al., 2006). While many studies report a high impact of vaccination (Trimnell et al., 2002; Weiss and Kaufman, 2004; You, 2004), results of other studies were more moderate in their impact on tick feeding. Since vaccines employing a single antigen usually do not have sufficient efficacy, one possibility for improvement is to use a cocktail of antigens (Willadsen, 2008), as at least one study has begun to do (Parizi et al., 2012). However, as yet there are no further candidates for anti-tick vaccine commercialization. Thus, the hunt for the right anti-tick vaccine

candidate(s) continues and one family of proteins has been particularly advocated for: the serine protease inhibitor (serpin) family (Mulenga et al., 2001).

Serine Protease Inhibitors (Serpins)

In general, the balance between proteases and protease inhibitors is essential to regulation of normal homeostasis across life: from microbes, to plants, to animals. In vertebrates, many critical physiological pathways such as blood coagulation, immune complement activation, and inflammation are mediated by proteases, particularly those of the serine catalytic type (Qureshi et al., 2012, Demetz and Ott, 2012, Loebbermann et al., 2012, Zhou et al., 2012, Héja et al., 2012, Forneris et al., 2012, Ichinose, 2012). In turn, most of these serine proteases are controlled by **ser**ine **p**rotease **in**hibitors (serpins). Where protease inhibitors fail to control proteases, disease may ensue (Kanoh et al., 2012). Among the diseases stemming from dysfunctional serpin activity are cirrhosis, emphysema, blood coagulation disorders, and dementia (Stein and Carrell, 1995; Davis et al., 1999; Gooptu and Lomas, 2009; Mocchegiani et al., 2011; Benson and Wilkes, 2012; Bosche et al., 2012; Gatto et al., 2013). Because serpins are used in vertebrate systems to shut off immune pathways, it was hypothesized that ticks use their own serpins to shut down the immune systems of their vertebrate hosts during feeding (Mulenga et al., 2001). Serpins were suggested as anti-tick vaccine candidates as a result of their role in vertebrate physiology

Serpins differ from other protease inhibitors because of their larger size of ~350-400 amino acid residues and mechanism of inhibition. Serpins are classified as trapping

inhibitors, forming an irreversible complex with their target protease (Rawlings et al., 2004, and Gubb et al. 2010). In serpin-protease interactions, the target protease initiates the interaction with the inhibitor. The target protease binds a bait pseudo-substrate peptide region in the serpin referred to as the reactive center loop (RCL) (Khan et al., 2011, Bird and Whisstock 2011, Huntington, 2011). The cleavage of the serpin RCL (Bird and Whisstock 2011) triggers a conformational change that leads to the protease being irreversibly trapped. Serpins are also characterized by a secondary structure fold consisting of 7-9 α-helices and 3 β-sheets, the consensus N-terminus motif NAVYKFG (Gettins, 2002), and an exposed consensus 21 amino acid RCL (Gettins, 2002). Within the RCL region (E17-[E/K/R]16-G15-[T/S]14-X13-[AGS]12-9-X8-1-X1'-4') the first eight amino acid residues are conserved across taxa, while the remaining 13 residues tend to be unique to each serpin (Gettins, 2002). Numbering of the 21 amino acid residues in the RCL is based on the numbering system by Schechter and Berger (1967), where residues on the amino-terminal side of the scissile bond, (P1-P1) are not primed while those on the carboxy-terminal side are primed. Protease cleavage of serpins occurs at the P1-P1 scissile bond.

Serpins in Ticks and as Anti-tick Vaccine Candidates

Although serpins in other organisms have been reported in the literature for decades (Tewksbury, 1983; Carrell and Owen, 1985; Pannekoek et al., 1986), their presence in ticks was first reported in the literature in 2001 (Mulenga et al.). Prior to advances in DNA technology, proteases inhibitors had to be detected by purifying them

from crude tick protein extracts (Limo et al., 1991, Vermeulen et al., 1988, Willadsen and McKenna, 1983). With the recent wave of genomics and transcriptomics, however, multiple putative serpins have now identified from multiple tick species including *I. scapularis, I. ricinus, A. maculatum, A. maculatum, R. microplus, R. appendiculatus, H. longicornis,* and *H. haemaphysaloides,* (Leboulle et al., 2002a; Nene et al., 2002; Sugino et al., 2003; Mulenga et al., 2003a,b, 2008; Imamura et al., 2005, 2006; Ribeiro et al., 2006; Prevot et al., 2007; Chalaire et al., 2011; Yu et al., 2013). With the recent sequencing of the *I. scapularis* genome as well as many tick transcriptomes (Gulia-Nuss et al., 2016; Pagel Van Zee et al., 2007; Francischetti et al., 2005; Anderson and Valenzuela, 2008; Aljamali et al., 2009; Donohue et al., 2010; Ribeiro et al., 2011; Bissinger et al., 2011; Richards et al., 2015; Tan et al., 2015) the full picture of serpins in ticks is beginning to emerge. At least 45 serpins are predicted to be expressed in *I. scapularis* on the basis of unique putative RCLs (Mulenga et al., 2009). Karim et al. (2011) report at least 32 different serpin transcripts expressed in *A. maculatum*. Tirloni et al., (2014) described 18 serpins in *R. microplus* and a further four were described shortly after (Rodriguez-Valle et al., 2015).

In *A. americanum,* an analysis of cDNAs determined that this tick species expresses at least 17 different serpin transcripts (Mulenga et al., 2007). In this dissertation, that number has expanded to 122 unique *A. americanum* serpins (Porter et al., 2015). Tick encoded serpins conform to the consensus structure of serpins (Mulenga et al., 2003b, 2008, 2009). Knowledge about tick serpins is accumulating, however, the full picture of their roles in tick physiology remains speculative. In ticks (and in other

parasitic species), protease inhibitor function is viewed from two perspectives: one being their significance in regulating the homeostasis of the parasitic organism itself, and the other being significance in regulating interactions between the parasite and its host. Accordingly, serpin function must not only be validated but also characterized as targeting either endogenous or exogenous (host) proteases.

Research Significance

Emerging functional data suggesting a role of tick serpins in facilitating tick counter-defense against the vertebrate immune system and a potential for their use as anti-tick vaccine antigens warrants further investigation into the scope and function of tick serpins. The objectives of this dissertation serve to fill several knowledge gaps in tick serpin research. This dissertation serves to determine the diversity and count of serpins in *A. americanum*. By determining the extent and diversity of serpins in *A. americanum*, this dissertation will generate a comprehensive list of serpins expressed in this species with which vaccine candidates may be selected. Due to the limited time and funding for research in combination with a steadily-increasing veterinary and public health burden of ticks and the pathogens they transmit, it is imperative to set a prioritization plan for investigating the potential of serpins as anti-tick vaccine antigens.

Within the scope of ixodid serpins, this dissertation serves to set a prioritization plan for selecting candidates for empirical validation of vaccine efficacy. Although originally identified strictly as inhibitors of serine proteases (Hunt and Dayhoff, 1980) subsequent studies have shown that certain serpins are non-inhibitory, although they

perform equally important functions such as hormone delivery, cancer pathogenesis, angiogenesis, and neuronal function (Lee et al., 2012, Tanaka et al., 2011, Hoshina et al., 2010, Carrell and Zhou, 2011). Therefore, it is necessary to validate the putative inhibitory function of in silico-annotated serpins. In comparing expression data, in silico functional predictions, and intra- and inter-specific sequence comparisons that have been empirically validated, this dissertation contributes to developing a prioritization plan. Ultimately the real test of vaccine utility lies in immunization trials. By combining bioinformatics, biological and biochemical characterization, and vaccine trials this dissertation completes the scope of empirical analysis required to qualify or disqualify serpin candidates from anti-tick vaccine inclusion.

In addition to the potential of serpins as useful anti-tick vaccine antigens, this family of proteins may be applicable in a medical context. The potential medical utility of serpins stems from the variety of human diseases caused by deficiencies in blood coagulation and platelet aggregation pathways including hemophilia A and B, and von Willebrand's disease (Dahlbäck, 2005). An even larger list of diseases associated with aberrant immune function has been documented including allergic rhinitis, asthma, myasthenia gravis, Graves' disease, multiple sclerosis, inflammatory bowel disease, and type I diabetes (Abbas et al., 2011). Because ticks secret pharmacologically active components into their host that have evolved to function efficiently in vertebrate systems, they are potentially attractive candidates for medical application. Indeed, the so called medicinal leech, *Hirudo medicinalis* yielded a potent thrombin inhibitor that was developed into a drug to treat coagulation disorders (de Kort et al., 2005; Markwardt,

2002). Biochemical characterization is the first step in determining the activity and potency of serpins against immune system-modulatory proteases. Therefore, this dissertation also serves to further validate the potential use of *A. americanum* serpins in a medical context.

CHAPTER II

BIOINFORMATIC ANALYSES OF MALE AND FEMALE *AMBLYOMMA AMERICANUM* TICK EXPRESSED SERINE PROTEASE INHIBITORS (SERPINS)[*]

Introduction

Ticks are among the most successful ectoparasites of humans and animals. In livestock production, an estimated 80% of the world's cattle are affected by tick-borne diseases (TBD), of which the most important include theileriosis, babesiosis, anaplasmosis, and Heartwater (Marcelino et al., 2012). In addition to pathogen transmission, tick feeding has been documented to cause severe direct effects including paralysis, exsanguination, reduced livestock productivity, and damage to skin impacting the economic value of hides (Klompen et al., 1996; Jongejan and Uilenberg, 2004). In public health, ticks have been implicated in the transmission of 17 diseases affecting humans, and the list continues to grow (Day, 2011; Dantas-Torres et al., 2012; Savage et al., 2013).

A. americanum has emerged among the most important tick species in public health in the United States. *A. americanum* transmits multiple TBD agents, including *Ehrlichia chaffeensis*, *E. ewingii*, *Rickettsia amblyommii*, *Francisella tularensis*, the as

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yet undescribed causative agent of southern tick associated rash illness (STARI), *Cytauxzoon felis*, *Theileria cervi*, and the emerging human Heartland virus (Waldrup et al., 1992; James et al., 2001; Childs and Paddock, 2003; Telford and Goethert, 2004; Telford et al., 2008; de la Fuente et al., 2008; Goddard, 2009; Schulze et al., 2011; Savage et al., 2013). For many years, this tick was mainly distributed in the southeastern United States, but has now been reported as established in 32 states throughout the Southeast, South Central, and Midwest regions as well as along the eastern seaboard as far north as Maine (Springer et al., 2014). Its emerging geographic expansion and role as a vector of many important human disease agents makes *A. americanum* an important consideration in strategies to improve public health.

With a lack of effective vaccines against TBD agents, the prevention of these infections in humans and animals depends on the control of ticks, which is currently acaricide based. However, acaricide use comes with many disadvantages, including the threat of food and environmental contamination and resistance development to these chemicals by ticks. A promising alternative strategy to the chemical control of ticks is to vaccinate hosts against the ticks themselves. The prerequisite to the development of an effective vaccine however, is the identification of effective molecular targets against tick feeding success and/or pathogen transmission. Among the emerging candidates for vaccine target antigens are members of the serine protease inhibitor (serpin) family.

In animals, pathways critical to life, such as blood coagulation, complement activation, and inflammation are tightly regulated by serpins (Moore et al., 1993; Gettins, 2002; Tekin et al., 2005; Huntington, 2006, 2011; Gatto et al., 2013).

Furthermore, dysfunctional serpin activity in humans has been cited to cause numerous diseases including cirrhosis, emphysema, blood coagulation disorders, and dementia (Stein and Carrell, 1995; Davis et al., 1999; Gooptu and Lomas, 2009; Mocchegiani et al., 2011; Benson and Wilkes, 2012; Bosche et al., 2012; Gatto et al., 2013). The tick feeding style of lacerating host tissue and imbibing host blood which bleeds into the feeding site, is expected to provoke tissue repair and immune response mechanisms such as platelet aggregation, inflammation, blood clotting, and complement pathways (Ribeiro, 1989; Wikel et al., 1994), all of which are serpin-regulated. Thus, to complete feeding, ticks have to overcome serpin-regulated host defense pathways. From this perspective, itis conceivable that ticks may utilize serpins to block these host defenses against tick feeding (Mulenga et al., 2001, 2002).

Serpin-encoding cDNAs have now been cloned from several tick species (Nene et al., 2002; Sugino et al., 2003; Mulenga et al., 2003a,b; Imamura et al., 2005, 2006; Ribeiro et al., 2006; Prevot et al., 2007; Chalaire et al., 2011; Yu et al., 2013). On the basis of unique putative functional domain reactive center loops (RCLs), at least 45 serpins are expressed in *I. scapularis* (Mulenga et al., 2009). Similarly, Mulenga et al. (2007) and Karim et al. (2011) have reported at least 17 and 32 different serpin transcripts expressed in *A. americanum* and *A. maculatum*, respectively. Data are now emerging which support the idea that some tick-encoded serpins are functional inhibitors associated with counter defense against anti-tick responses in the host, such as inflammation, complement activation, platelet aggregation, and blood clotting (Imamura et al., 2005; Prevot et al., 2009; Chmelar et al., 2011, 2012; Mulenga et al., 2013a, b). In

other studies, a significant reduction in feeding efficiency has been observed in ticks which fed on animals immunized with recombinant tick serpins (Imamura et al., 2005, 2006, 2008; Prevot et al., 2007; Kaewhom et al., 2009; Jittapalapong et al., 2010) suggesting a prime importance of serpins in tick feeding physiology.

An observation in our lab is that while overall amino acid conservation levels for serpins are around 35–45%, there are some serpins which show much higher conservation across all tick species investigated. We believe that highly conserved serpins could play crucial role(s) in tick physiology. The goal of this study was two-fold: first, to identify serpin transcripts expressed in unfed and fed *A. americanum* ticks, and second, to conduct a global intra- and inter-tick species bioinformatic analysis of *A. americanum* serpins (AAS) and other tick serpins. This study has described 57 and 33 AAS sequences that were respectively found only in male and female ticks, and a further 30 that were found in both. Nearly half of the serpin sequences expressed in *A. americanum* are predicted to regulate pathways important to all tick species, as they show 58–97% amino acid conservation in both metastriate and prostriate ticks. Although this study is descriptive, data presented here provide a foundation for further in depth studies on the roles of serpins in tick physiology.

Materials and Methods

Identification and sequence analysis of *A. americanum* serpin (AAS) transcripts

AAS sequences used in this study were obtained by data mining of de novo assembled *A. americanum* transcriptomes (unpublished data), serendipitously while attempting to

clone other targets, and from GenBank (Mulenga et al., 2007). *A. americanum* transcriptomes were assembled from Illumina sequence reads (BioProject accession number PRJNA226980) of 24 and 96 h fed female phage display cDNA expression libraries, unfed and fed male and unfed and 24 h fed female whole ticks, as well as 48, 96 and 120 h fed tick dissected salivary gland (SG) and midgut (MG) tissues, using two approaches. In the first approach libraries were individually assembled with source library information for each contig retained, and in the second approach reads from all sources were combined and assembled (unpublished). Mining and identification of putative AAS sequences was accomplished in two steps. In the first step, assembled contigs were subjected to batch BLASTX screening against tick sequences in GenBank. In the second step, contig sequences with matches to serpin sequences were manually inspected to confirm the presence of two consensus amino acid motifs: the reactive center loop (RCL) "p17 [E]-p16 [E/K/R]-p15 [G]-p14 [T/S]-p13 [X]-p12-9 [AGS]-p8-1 [X]-p1-4" in the C-terminus, and the 'NAVYFKG' motif in the N terminus (Carrell et al., 1987; Miura et al., 1995; Gettins, 2002). Sequences with a unique RCL sequence were identified as new and assigned an AAS number. Sequences without an RCL region were declared partial in the C-terminus region. For these sequences, two comparisons were made using Bl2seq-BLASTP (NCBI). First, these sequences were compared to each other to cluster contigs representing the same serpin. Next, we compared these clusters to AAS containing an RCL to eliminate redundancy between these groups. In addition to consensus amino acid motifs and secondary structure, a typical serpin ranges from 350 to 450 amino acids long (Gettins, 2002). Thus, sequences that had a starting

methionine and were at least 350 amino acid residues long were considered putatively full-length. Full-length sequences were subjected to SignalP Version 4 web server to detect signal peptides (Petersen et al., 2011).

Relative AAS transcript abundance

To get insight into relative abundance, Illumina reads were mapped back to assembled contigs using the map reads to reference option in CLC genomics workbench version 6.4.2. Relative abundance values were adjusted to account for contig size and total library reads using the following equation: eY = (nYNXL1X/nXNYLY) × eX, 18 where eX and eY represent normalized relative abundance levels of AAS transcript in libraries X and Y, NX and NY represent the total number of reads in libraries X and Y, nX and nY represent the number of reads related to the specific AAS transcript in libraries X and Y, and LX and LY represent the length of the contig related to the specific AAS transcript in libraries X and Y.

Phylogeny and comparative sequence analysis among AAS sequences

To determine relationships between AAS sequences, a guide phylogeny tree was constructed using the neighbor-joining method in MacVector version 12 DNA analysis software (MacVector Inc., Cary, North Carolina). Sequences were first aligned using T-coffee, then a phylogeny tree out-rooted from human antithrombin (CAA48690), was constructed using the neighbor-joining method set to the default bootstrap setting of 1000 replications and differences adjusted using the absolute # differences setting.

Subsequently, AAS sequences that clustered together on the phylogeny tree were subjected to pairwise sequence alignment analyses using MacVector. For partial sequences, amino acid identity levels were determined based on available sequences.

Comparative analyses of AAS to other tick serpin sequences

To investigate relationships among all available tick serpins, AAS and other tick serpins from publically available databases were subjected to phylogeny analysis and multiple sequence alignment analyses using alignment tools at NCBI and MacVector version 12. This analysis was done at the whole amino acid sequence and RCL levels. At the whole amino acid sequence level, amino acid sequences were subjected to batch pairwise comparisons using Bl2seq-BLASTP (NCBI) to identify AAS orthologs in other tick species. The serpin RCL is an important functional domain, which determines what protease is regulated by a candidate serpin. Thus, to investigate the relationship of AAS sequences with other tick serpins at the functional level, the neighbor-joining method in MacVector (MacVector Inc.), was used to construct guide phylogeny trees using putative RCLs. To manage the huge dataset, RCLs were first divided into four groups based on charge and polarity characteristics of the amino acid residue at the putative P1 site: polar basic, polar acidic, polar uncharged, and hydrophobic. A separate tree was constructed for each group. Next intra-clade pairwise alignments of RCLs were performed using MacVector to determine identity levels.

Results

Amblyomma americanum *male and female ticks express large numbers of serpin transcripts*

Data mining of transcriptomes from fed and unfed male and female whole ticks, as well as dissected 48, 96 and 120 h female SG and MG transcriptomes, identified 28 and 57 AAS sequences respectively found only in females and males, respectively, and an additional 30 found in both (Table 1). Mulenga et al. (2007) described 17 AAS (here after identified as AAS1–17) sequences that were expressedin120h fed ticks. Of these 17 AAS sequences, this study found 7 sequences in both males and females, and five AAS sequences in females, while the remaining five were not found at all. Taken together, these studies show the total number of AAS sequences found only in female ticks to be 33 (Table 1). Please note that AAS 77 and 78 were found only in the combined *A. americanum* transcriptome were source library information for assembled contigs was not retained, and thus source information is unknown. Overall, 87 and 63 AAS sequences were found in male and female ticks, respectively. Assignment of AAS identification numbers was done arbitrarily in three steps. In the first step, assembled *A. americanum* transcriptomes were subjected to batch BLASTX scanning against tick serpin sequence entries at NCBI. This analysis identified 388 contigs that encoded putative serpins. A typical serpin is characterized by a unique RCL region (Gettins, 2002). Thus in the second step, we conducted a manual inspection of all 388 contigs and found 61 previously unknown AAS RCL sequences that did not show identity to previously described AAS1–17 (Mulenga et al., 2007). AAS sequences

Table 1: Updated list of *Amblyomma americanum* serine protease inhibitor transcripts

Table 1a: AAS sequences found in male ticks

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
44	51298-AAFM	Partial	73	9903-AAFM	Partial	95	39404-AAFM	Partial
46	Cluster 46	Partial	74	25484-AAFM	Partial	96	40959-AAFM	Partial
48	Cluster 48	Full, NSP	75	26362-AAFM	Partial	97	41223-AAFM	Partial
49	34700-AAUM	Partial	79	1446-AAFM	Partial	98	41535-AAFM	Partial
50	Cluster 50	Partial	80	11697-AAFM	Partial	99	41587-AAFM	Partial
52	1447-AAFM	Partial	81	2964-AAFM	Partial	100	42502-AAFM	Partial
53	2068-AAFM	Full, NSP	82	16896-AAFM	Partial	101	43086-AAFM	Partial
55	Cluster 55	Partial	83	19557-AAFM	Partial	102	47122-AAFM	Partial
56	5603-AAFM	Full, NSP	84	19764-AAFM	Partial	103	51931-AAFM	Partial
57	7054-AAFM	Full, NSP	85	3838-AAFM	Partial	104	52784-AAFM	Partial
58	9916-AAFM	Full, NSP	86	4992-AAFM	Partial	105	56624-AAFM	Partial
59	12570 AAFM	Full, NSP	87	26648-AAFM	Partial	106	58458-AAFM	Partial
60	13872 AAFM	Full, NSP	88	27587-AAFM	Partial	107	60174-AAFM	Partial
61	38562 AAFM	Partial	89	31297-AAFM	Partial	111	44923-AAUM	Partial
62	48164 AAFM	Partial	90	33489-AAFM	Partial	113	9412-AAFM	Partial
64	16123-AAFM	Partial	91	34304-AAFM	Partial	114	1445-AAFM	Partial
68	Cluster 68	Partial	92	36187-AAFM	Partial	120	Multiple	Partial
70	5478 AAUM	Partial	93	37787-AAFM	Partial	121	Multiple	Partial
71	54612-AAFM	Partial	94	38659-AAFM	Partial	122	7341-AAFM	Partial

Table 1: Continued.

Table 1b: AAS sequences found in female ticks

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
1	PC	Full, SP	15	PC	Full, SP	42	27458-SG120	Partial
2	PC	Full, SP	16	PC	Full, SP	43	25306-MG48	Partial
3	PC	Full, SP	17	PC	Full, SP	45	6976-MG120	Partial
4	PC	Full, SP	19	Cluster 19	Full, SP	65	54692-AAUF	Partial
5	PC	Full, SP	20	Cluster 20	Full, SP	69	22634-AAFF	Partial
6	PC	Full, SP	22	Cluster 22	Partial	76	^	Full, NSP
7	PC	Full, NSP	26	Cluster 26	Partial	109	Cluster 109	Partial
8	PC	Full, SP	29	Cluster 29	Partial	112	59401-AAUF	Partial
9	PC	Full, SP	32	11832-MG120	Partial	115	42294-MG120	Partial
10	PC	Full, SP	36	Cluster 36	Full, NSP	116	40934-SG48	Partial
11	PC	Full, NSP	37	Cluster 37	Partial	117	38175-SG48	Partial
12	PC	Full, SP	38	14471-SG96	Partial	118	47325-SG48	Partial
13	PC	Full, SP	40	32557-SG96	Partial			
14	PC	Full, SP	41	16611-SG120	Partial			

Table 1c: AAS sequences found in both male and female ticks

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
18	Cluster 18	Full, NSP	31	Cluster 31	Full, SP	63	Cluster 63	Partial
21	Cluster 21	Full, SP	33	444-PL2	Partial	66	Cluster 66	Partial
23	Cluster 23	Full, SP	34	Cluster 34	Full, NSP	67	Cluster 67	Full, NSP
24	Cluster 24	Partial	35	Cluster 35	Partial	72	Cluster 72	Partial
25	Cluster 25	Full, NSP	39	Cluster 39	Full, SP	108	Cluster 108	Partial
27	Cluster 27	Full, SP	47	Cluster 47	Full, NSP	110	Cluster 110	Partial
28	Cluster 28	Full, NSP	51	Cluster 51	Partial	119	Cluster 119	Partial
30	Cluster 30	Full, SP	54	Cluster 54	Partial			

Table 1: Continued.

Table 1d: AAS sequences with no source information

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
77	^	Partial	78	Clust1-1-153	Partial

Cluster = AAS has more than one representative contig; Full = full length open reading frame, SP = signal peptide is present, NSP = no signal peptide; ^ = contig sequence was too short for GenBank submission (<200bp).

encoding the 61 new RCLs were assigned the identifications of AAS18–78, according to the order in which they were discovered. 233 of the 388 contigs did not have RCL regions. These sequences were subjected to intra-contig comparisons using the bl2seq-BLASTN function at NCBI. This analysis identified 44 contig sequences that encoded previously unreported AAS sequences and were designated as AAS79–122 (Table 1). This brought the total number of unique transcripts identified in this study to 105. A typical serpin molecule is 350–450 amino acids long (Gettins, 2002). On this basis, 40 of the 122 AAS sequences were determined to have complete open reading frames (ORF), as well as the consensus serpin amino-terminus motif (NAVYFKG), and the start methionine. Of the 40 AAS ORFs, 23 are predicted to have signal peptides (Table 1).

Majority of female AAS transcripts expressed in fed salivary glands and midgut tissues

Figure 1A–C summarizes the different AAS sequences found in MG and SG of 48, 96 and 120h fed female ticks. Data previously reported for AAS1–17 are indicated with an asterisk in Figure 1A, and data for MG and SG at 120 h is taken from Mulenga et al. (2007). Of the 63 AAS sequences found in female tick transcriptomes to date, 48 have been found in 48, 96, and 120 h MG and/or SG. Of these 48, 10 and 12 AAS sequences were respectively found only in MG or in SG, while the remaining 26 were found in both (Figure 1A). This translates to a total of 36 and 38 different AAS sequences found in MG and in SG, respectively. Of the 36 AAS found in MG, seven (AAS1, 4, 7, 8, 21, 31, and 109) were found at all-time points, four (AAS27, 28, 72, and 110) were found at both 48 and 96 h time points, and four (AAS19, 20, 22 and 43), one (AAS108), and 16 (AAS9–18, 23, 29, 32, 36, 45, and 115) were found at the 48, 96, or 120 h time points, respectively (Figure 1B). Likewise, of the 38 AAS found in SG

(Figure 1C), five (AAS7, 19, 21, 23, and 110) were found at all-time points, seven (AAS3, 9, 25, 27, 28, 31, and 39) were found at the 96 and 120 h time points, and five (AAS20, 36, 116, 117, and 118), five (AAS24, 26, 37, 38 and 40), and 12 (AAS1, 2, 5, 8, 10–17, 41, and 42) were found in 48, 96, or 120 h SG, respectively.

Relative AAS transcript abundance

We successfully mapped reads back to de novo assembled AAS contigs. This analysis identified 20 of 122 AAS transcripts with variable sequence reads in different libraries (Figure 2A and B). For 8 (AAS25, 28, 39, 51, 66, 108, 110, and 119) of the 20 AAS sequences, differences between sequence reads were minimal and we concluded that these apparently occurred in equivalent abundance in all libraries. Figure 2A summarizes differential abundance for ten (AAS19, 21, 23, 27, 29, 30, 31, 50, 54, 67, 72, 121) in unfed and fed, male and female whole tick libraries. Of ten AAS detected at unfed and fed time points, five are more abundant in unfed ticks (AAS19, 30, 31, 54, and 67), four are more abundant in fed ticks (AAS21, 29, 50, and 121), and one (AAS72) showed a mixed pattern with abundance in unfed females, but not in unfed males, and a transcript increase in fed male ticks (Figure 2A). It is also notable that AAS19, 21, 29, and 30 are abundant in female ticks, while AAS50, 67, and 121 are predominant in male ticks. Of the 31 AAS sequences summarized in Figure 1, we determined differential abundance in SG and MG for five transcripts, AAS19, 21, 23, 27, and 31 (Figure 2B), with differences for the remaining sequences being minimal to negligible. While AAS23

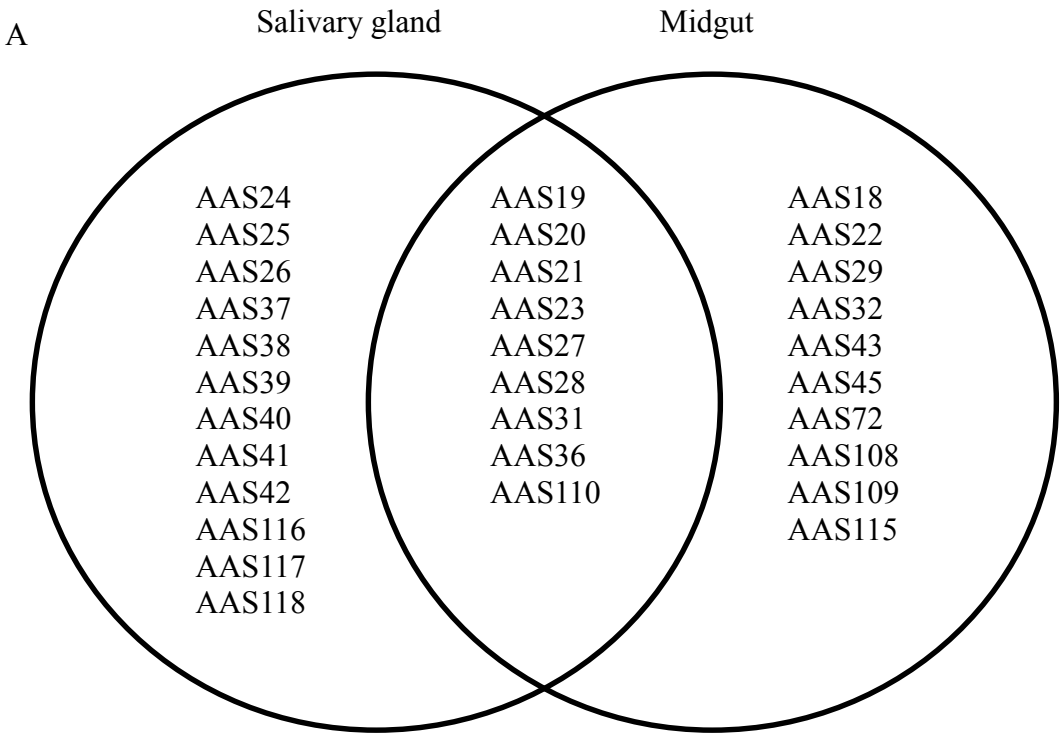

Figure 1. Adult female *A. americanum* salivary gland (SG) and midgut (MG) expressed serpin (AAS) transcripts. (A) total AAS sequences found in MG and SG and both at all tested time points, (B) apparent temporal and spatial distribution of AAS transcripts found in MG (B), and SG (C). Please note that 17 previously characterized SG and MG expressed AAS transcripts [42] are not included here.

B

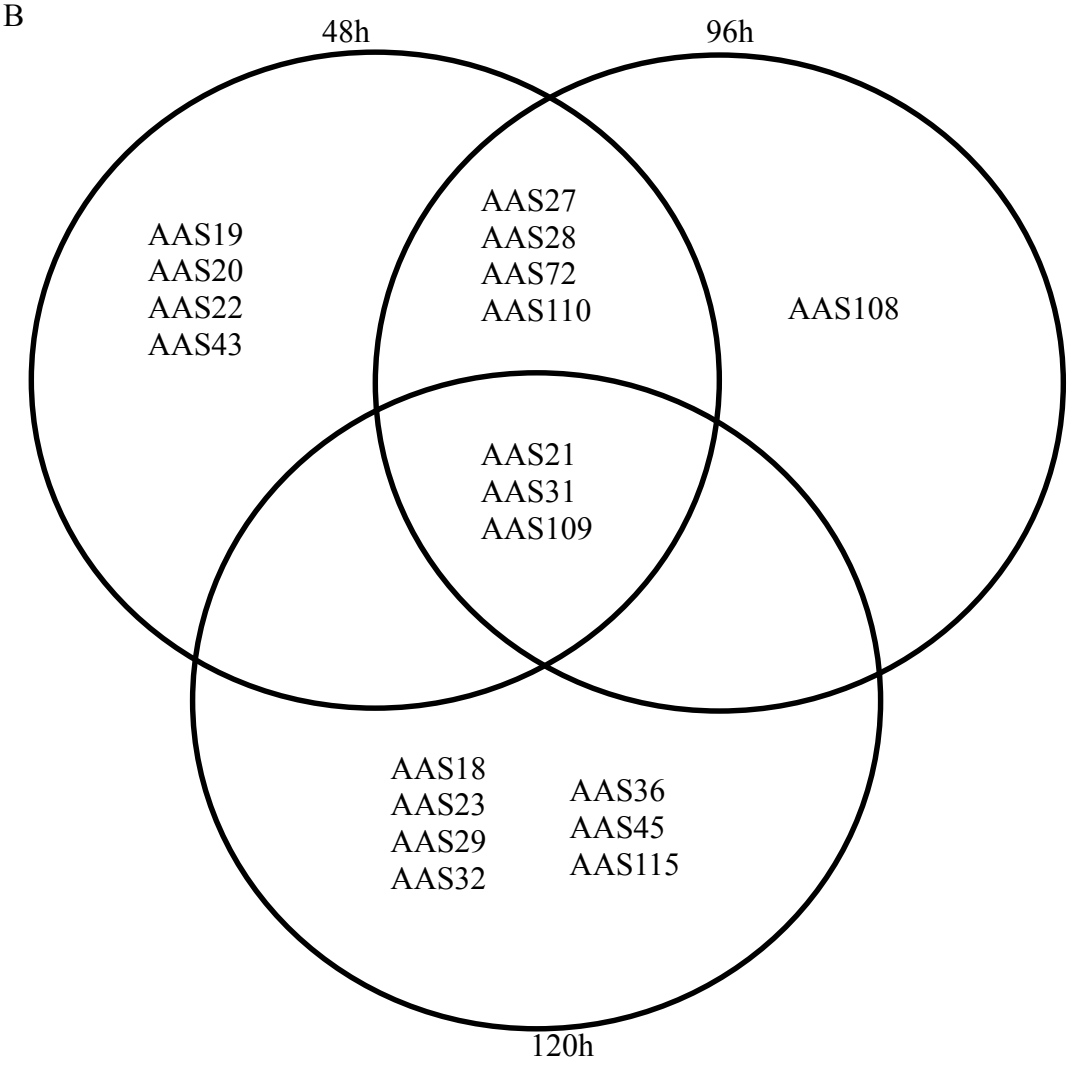

Figure 1. Continued.

C

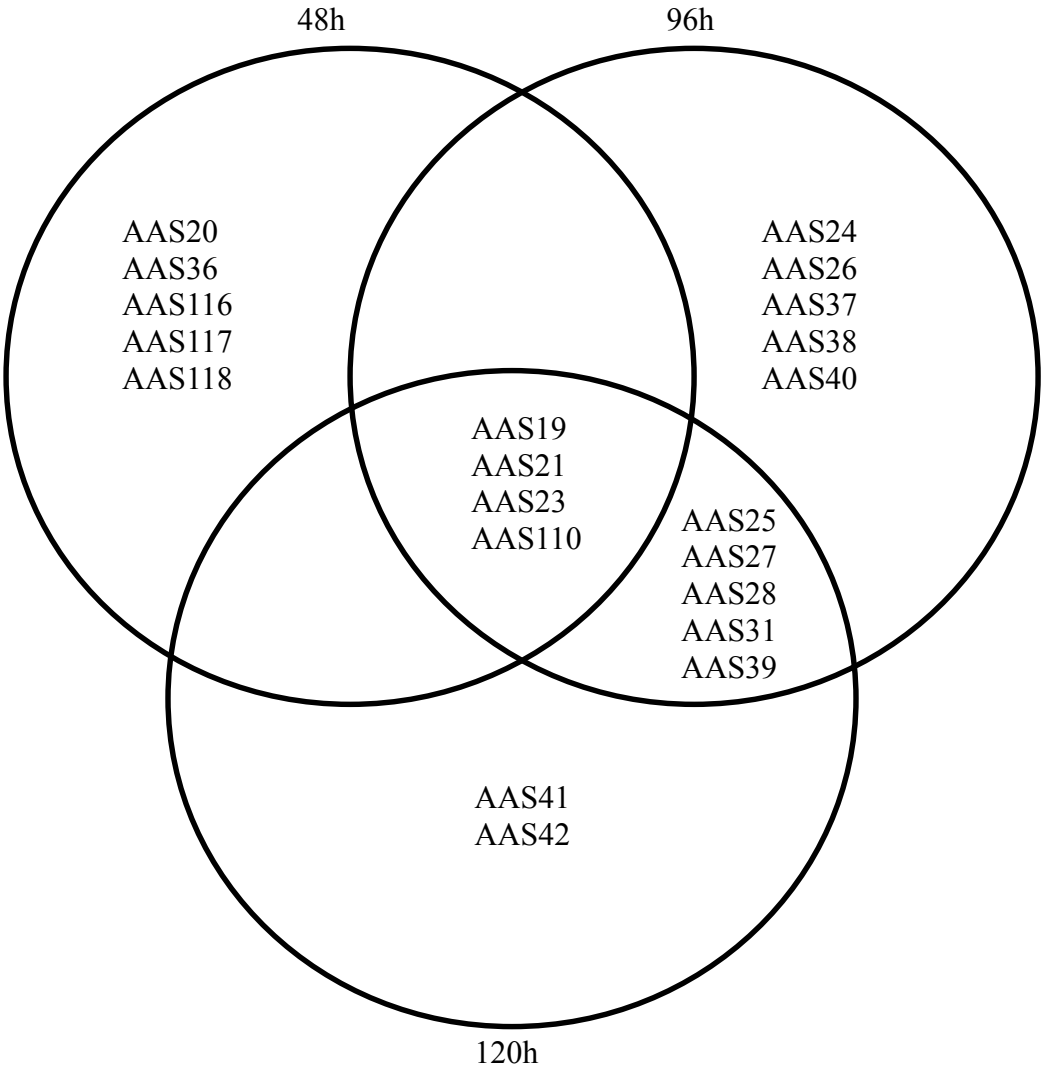

Figure 1. Continued.

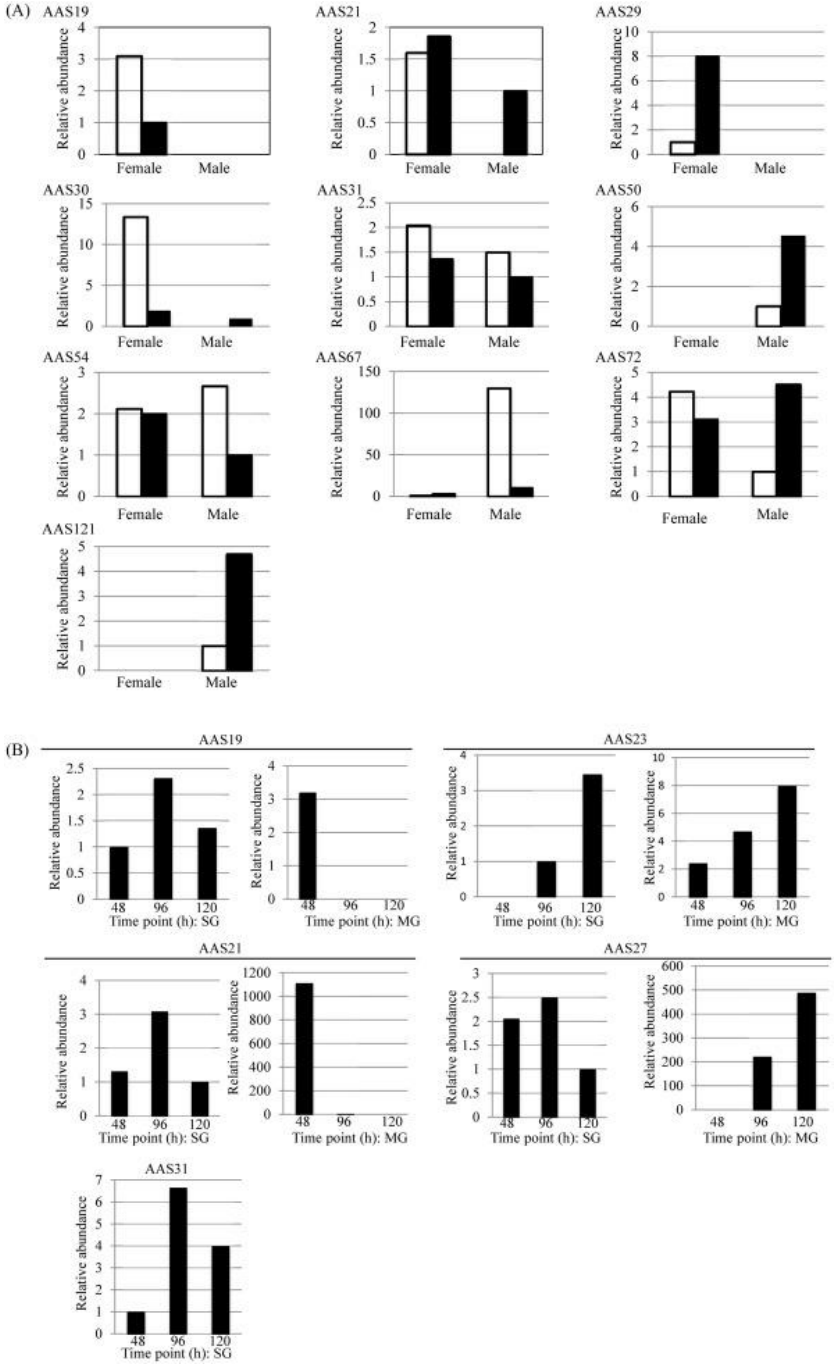

Figure 2. Relative abundance of *A. americanum* serpin (AAS) transcripts.
Sequence reads were mapped back to *de novo* assembled contigs in different libraries. Relative abundance values in: (A) unfed (empty bars) and fed (black filled bars) male and female ticks, and (B) dissected 48–120 h salivary glands (SG) and midguts (MG) were calculated using the formula described in Materials and methods.

transcript abundance increases with feeding in both SG and MG, the remaining show an apparent dichotomous pattern. Transcripts for AAS19, 21, 27, and 31 are highest at the 96 h time point, and lower at the 120 h time point in SG. In MG, AAS19 and 21 are abundant at 48 h, and absent or low at 96 and 120 h time points, while AAS27 and 31 increase with feeding (Figure 2B).

A diversity of seventeen amino acid residues is predicted at P1 sites of AAS putative RCLs

Table 2 lists 78 predicted reactive center loops (RCLs) from 27 (Table 2A) and 22 (Table 2B) AAS sequences found in female and male ticks, the 27 found in both (Table 2C), and 2 of an undetermined source (Table 2D). Please note that Table 2A includes previously characterized AAS1–17 (Mulenga et al., 2007). We would like to note that the RCL regions for AAS68 and 71 (Table 2B), and AAS77 (Table 2D) are partial (marked with an asterisk in Table 2), however based on the available sequence we were still able to conclude these RCLs to be unique. Additionally, while the predicted RCL for AAS8 and 9, 4 and 12, and 13 and 15 are identical, these sequences differ in the N-terminus region by 13–25 amino acids (Mulenga et al., 2007). Numbering of amino acid residues in the RCL is based on the standard nomenclature developed by Schechter and Berger (1967), in which amino acid residues at the N terminal end of the scissile bond (P1–P1) are not primed and those on the C-terminal end are primed: ““p17 [E]-p16 [E/K/R]-p15 [G]- p14 [T/S]-p13 [X]-p12-9 [AGS]-p8-1 [X]-p1-4” (Miura et al., 1995

Table 2: Reactive center loops (RCLs) of *A. americanum* serpins

Table 2A: Predicted RCLs found in female ticks

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
1	EEGTVAAGVTSVRVKQ**K**HSAR	19	EEGSEAAAVTGFVIQL**R**TAAF
2	EEGTVAAGVTSVRVKP**K**SFAR	20	EVGTRAVAATEAQFVS**K**SLVH
3	EEGTGAAGVPSVGGKP**K**SFAR	22	EEGSEAAGATGVIFYT**K**SAIV
4	EEGTIAAAVTGLSFVP**I**SALH	26	EEGTEAAAATATVAMF**G**SAPS
5	EEGTVAAAVTGLSVTP**L**VVPP	29	EEGTEAAAATAVTVVD**G**CMPR
6	EEGTVATAVTGISLAL**S**ALHT	32	EDGTEPEVAPTNAVFQ**P**AVRT
7	EEGTEAAAATGIAMML**M**CARF	36	EEGTEAAAATGMRIQL**K**TRVK
8	EEGSQAAAVTGVIIYT**Q**SAFV	37	EEGTEAAAATAVVMMC**R**SAAM
9	EEGSPATAVTGVIMYT**Q**SAFV	38	EEGTIATAVTGLSFAP**I**SALH
10	EEGSQAAAVTGVIIYT**Q**SAFV	40	EKGRAAAGVAARAFYT**R**AGDH
11	EEGTVPTAVPGILLVG**L**VARH	41	EEGSEAAGATGVVFVE**L**IAVR
12	EEGTIAAAVTGLSFVP**I**SALH	42	EKGTEAAAATAVMMMA**C**CMSA
13	EEGTVAAAVTGLSFPH**L**VVPP	43	EDGTEPEAAATNAVFQ**P**AVRT
14	EEGTVATAVTGISLVA**L**SALH	45	EEGTEAAAATGVVMMC**D**SLPM
15	EEGTVAAAVTGLSFPH**L**VVPP	65	EEGSETDSATLMRISG**K**AXCE
16	EEGTAAEAVTGLSITP**L**AVPP	69	EEGTIAAAVTGLSFVA**T**ASFN
17	EEGTVAAAVTGLSSIA**L**SSVG	76	EEGTIAAAVTGSLFRA**H**LGSP

Table 2b: Predicted RCLs Found in male ticks

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
44	EEGTEAAAATAIITTE**C**CIMP	59	EEGSWPVTYTEHVLST**G**DPVT
46	EEGSEAAGATGVIFVE**T**IAVR	60	ENGSSAAAVTGTTLHK**S**VHVP
48	EEGTEAAAGSASILTR**R**DAVE	61	EGGTDASSATAMTSLA**C**SATM
49	EDGTDSEAARANEVPE**P**AFSI	62	EEGTEPEAATANALIE**S**AGSI
50	EEGSETDSATLMRISG**K**AAEE	64	ARGGRAVSNEVQSTTS**A**TTAA
52	ENGTVAAAASAAIGVG**S**AGPS	68	EDGTVAASTAALAFHA**D**RPF*
53	EGGTEPGPATAGEASA**P**AGPE	70	EEGTIATAVTGLSFVA**T**ASFN
55	ENGTVAAAATAAEGGS**S**SGIM	71	EVGTKA*
56	EEGLGEACRPPNPLPA**T**MIAF	73	GAGGRPPSSNDSREAG**T**SPAK
57	ENGTGAPAAEGSIYSP**A**FRRR	74	ERSTSRMPKYTGAQGA**P**GTSS
58	EEGTEAAAATGMTLMM**C**GAMV	75	RRGPKTVAAQAVAKEA**A**RTAK

Table 2: Continued.

Table 2c: Predicted RCLs found in both male and female ticks

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
18	EEGSEAAGATAVIFFT**R**GGSS	34	ENGTKAAAATTALGSN**S**FYVP
21	EKGTEAVALSSGIIRH**S**KTPG	35	EKGTVAAASAAAAGGS**S**LYNP
23	EEGTVAAAVTSIRMRM**K**SSRR	39	EKGTVASASTVAIIVS**R**IGTP
24	EEGTVATAVTGLSFVA**T**ASFN	47	EEGTEAAAATAMPAAN**S**CEMF
25	EEGTEAAAATAVVMMC**Y**SLPM	51	EEGTEAAAATAIIATE**C**CIMP
27	EEGTEAAAASGVVGVN**R**IGID	54	EDGVEGLFLTPLIMMC**Y**AGVS
28	EEGTEAAAATGMVAMA**R**CAII	63	EDGNEIARTSALVTEV**V**SKVA
30	EEGTVATAVTGLSNTR**I**LDDS	66	EKGRAAAGVAARTFYT**R**AGDH
31	EEGSEAAAVTGVVINT**R**TIGG	67	EEGSEAAAATAVLIET**R**SDVP
33	ENGTVAAAASAALLVG**S**AGPN	72	EEPVRLDLHVSTLRLA**E**RTDL

Table 2d: Predicted RCLs, source undetermined

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
77	EEGSQATAVTGVIIYT**Q**S*	78	EEGTEAAAATSVVMMC**D**SLPM

Amino acid residues at putative P1 site are bolded; * indicates a partial RCL sequence.

Gettins, 2002). Molecular analysis predictions of the P1 site assume that there are 17 amino acid residues between the beginning of the RCL hinge region (P17), and the scissile bond (P1–P1'), (Hopkins and Stone, 1995). Based on these conventions, a diversity of 17 different amino acid residues is predicted at the P1 sites of AAS1–78 (except for AAS71 which has a partial RCL excluding the P1 site, Table 2B). The P1 residues for AAS1–78 have the following charge and polarity properties: 27 (~35%) are polar uncharged [S (10/26), C (5/26), T (6/26), Q (4/26), Y (2/26)], 25 (~32%) are hydrophobic [L (8/25), I (4/25), P (5/25), G (3/25), M (1/25), A (3/25), V (1/25)], 21 (27%) are polar basic [R (11/21), K (9/21), H (1/21)], and four residues (~5%) are polar acidic [D (3/4), E (1/4)] (Figure 3). It is noteworthy that 11 of the 22 AAS sequences found in MG and SG have basic residues at the predicted P1 site.

Some AAS sequences are highly identical

To gauge the relationships between AAS sequences, amino acid sequences were subjected to phylogeny analysis, except for AAS87 and 109, which were too short for an informative alignment (Figure 4). We would like to note that due to the large number of sequences, we split the tree into two parts, Figure 4P1 and P2. As shown in Figure 4, 68 of the 122 AAS sequences segregated into 19 clusters labeled A–S, and the remaining sequences did not cluster. Of the 19 clusters, eight have more than two sequences: E (AAS8–10, 18, 22, 41, 46, and 67), F (AAS4–6, 11–17, 24, 30, and 112), G (AAS1–3, 23), H (AAS75, 87, and 95), M (AAS32, 43, and 62), O (AAS33, 35, 52 and 79), P (AAS28, 36, 47, and 108), and R (AAS7, 25, 26, 37, 45, and 78), and the remaining 11 clusters have single pairs. When subjected to pairwise sequence alignment analysis, sequences in clusters A, B, D, E, F, G, K, M, O, P, R, and S showed variable amino acid identity levels of 15, 96, 93, 66–98, 61–98, 40–93, 98, 84 (excluding AAS62), 66–98, 56–78, 61–98, and 96%, respectively (Figure 4P1 and P2). In the remaining clusters (marked with asterisks), amino acid identity levels were below 15%. We would like to caution here that some sequences being compared are partial, and therefore the picture of these relationships might be incomplete. From pairwise alignment analyses, two general patterns emerged. In the first pattern, differences between two sequences were scattered throughout the alignment (not shown). In the second pattern, found between AAS25 and AAS45, and AAS33 and AAS52, differences were restricted to the C-terminus region within the RCL (not shown).

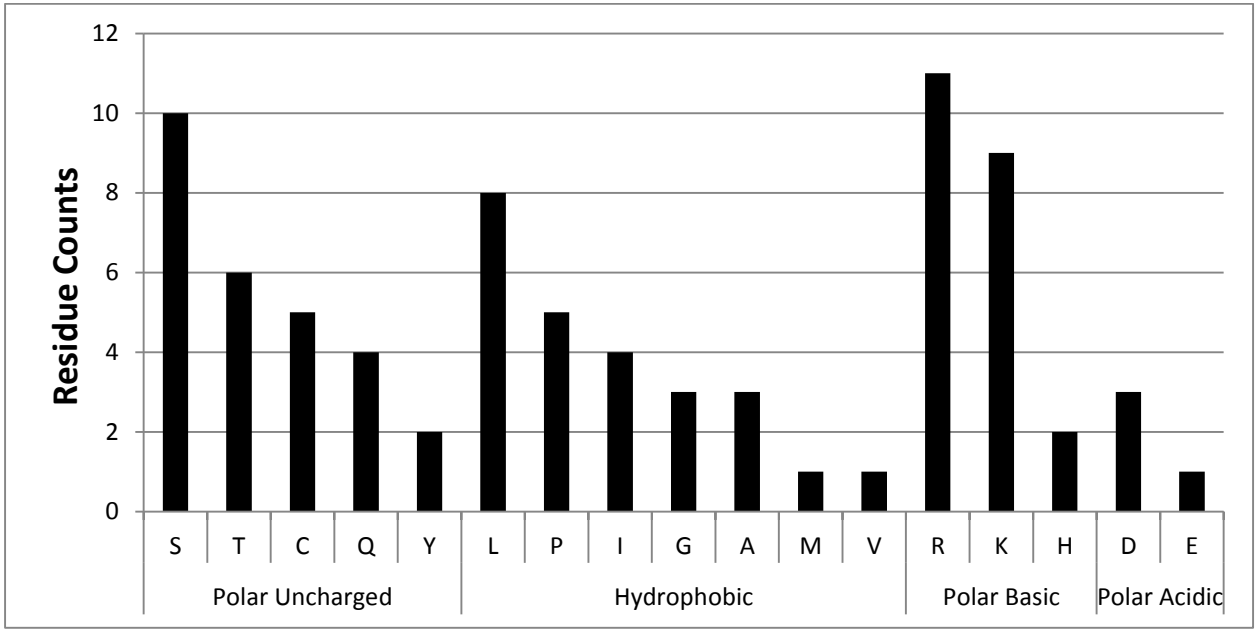

Figure 3. Diversity and counts for amino acid residues at the putative P1 position in reactive center loops of the first 78 *A. americanum* serpin sequences. Amino acid residues at putative P1 sites were determined based on molecular analysis predictions of the P1 site that assume that there are 17 amino acid residues between the beginning of the RCL hinge region (P17), and the scissile bond (P1-P1').

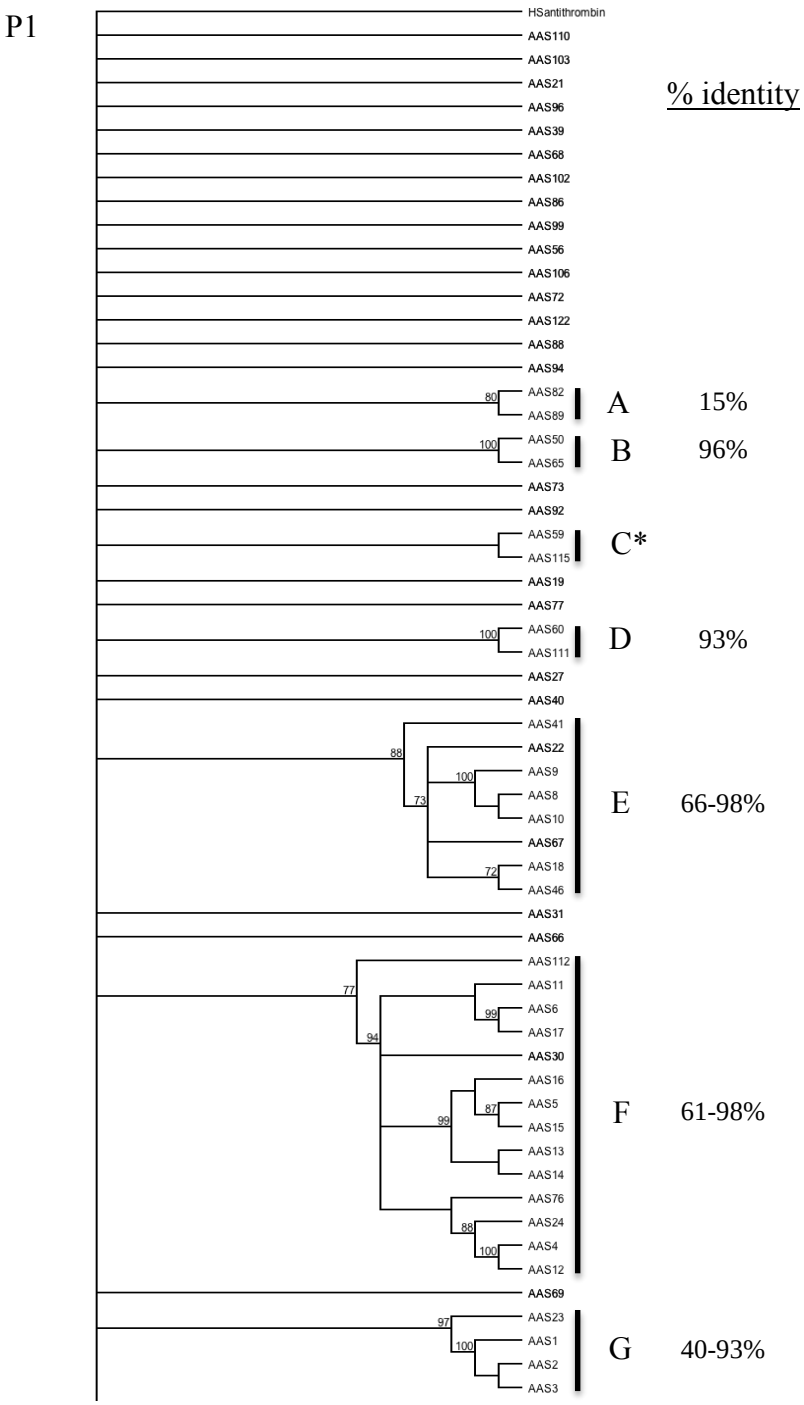

Figure 4. Relative abundance of *A. americanum* serpin (AAS) transcripts. Sequence reads were mapped back to *de novo* assembled contigs in different libraries. Relative abundance values in: (A) unfed (empty bars) and fed (black filled bars) male and female ticks, and (B) dissected 48–120 h salivary glands (SG) and midguts (MG) were calculated using the formula described in Materials and methods.

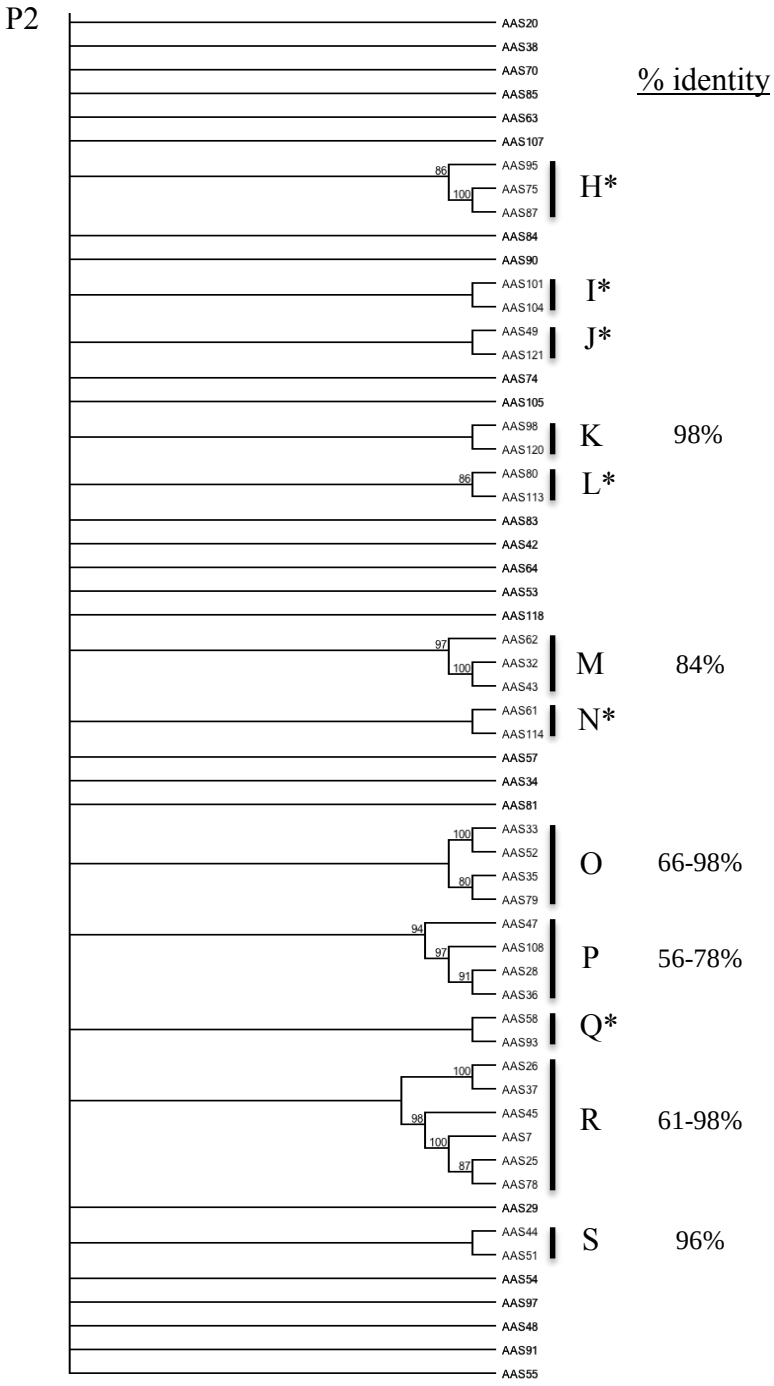

Figure 4. Continued**.**

Close to half of AAS sequences have orthologs in other tick species

To determine if any AAS sequences in this study had orthologs in other tick species, inter-species comparisons were performed. A search for other tick serpin sequences in publically available databases retrieved 165 serpin sequences across nine tick species (Table 3). Pairwise comparisons of these serpins to AAS1–122 identified 50 AAS sequences that were conserved in other tick species. To manage the high number of sequences, the data are presented in separate tables: *A. americanum* versus *A. maculatum* (Karim et al., 2011) and *A. variegatum* (Ribeiro et al., 2011) (Table 4A), and versus *R. pulchellus* (direct submission), *R. appendiculatus* (Mulenga et al., 2003b), *R. microplus* (Tirloni et al., 2014), The Gene Index Project, http://compbio.dfci.harvard.edu/tgi/], *R. haemaphysaloides* (direct submission), *H. longicornis* (Imamura et al., 2005), *I. scapularis* (GenBank direct submissions, The Gene Index Project), and *I. ricinus* (Leboulle et al., 2002b) (Table 4B). As shown in Table 4A, AAS4–6, 8–19, 22–27, 29, 30, 41, 46, and 47 show ≥75–96% amino acid identity to *A. maculatum* serpin sequences AEO35533, AEO35520, AEO34312, AEO34447, AEO34313, AEO34314, AEO32541, AEO34349, AEO34279, AEO34218, AEO34217, AEO33019, and AEO32217, while the remaining sequences showed <75% amino acid conservation. Likewise, in Table 4b, AAS19–22, 41, 42, and 65 show amino acid identities of ≥75–91% to 7 *R. pulchellus*serpins: JAA54307, JAA54309, JAA543410, JAA54167, JAA54314, JAA54313, respectively, while the rest showed amino acid identities of <75%. Additionally, AAS7 shows 75 and 77% identity to *R. microplus* EST89704 and *R. pulchellus* JAA54312, and AAS19 and 21 show 82–96% identity to *R.*

Table 3: Other tick serpins downloaded from publically available databases

Source tick species	Accession#	Source tick species	Accession#	Source tick species	Accession#
Amblyomma maculatum	AEO35533	*R. microplus*	AHC98664	*I. scapularis*	TC36982
	AEO35320		AHC98665		TC38196
	AEO34447		AHC98666		TC40138
	AEO34349		AHC98667		TC40197
	AE034314		AHC98668		TC40226
	AE034313		AHC98669		TC40411
	AE034312		AAP75707		TC40562
	AE034279		TC16456_2		TC40843
	AE034218		EST767976_2		TC40980
	AE032154		EST896705_3		TC41916
	AE032160		ADK62395		TC48141
	AE032217		ADK62396		TC50198
	AE032541	*Ixodes ricinus*	ABI94058		XP_002402925
	AE032759		JAA66227		EW874987
	AE032774		JAA66279		DN974443
	AE033019		JAA66964		EW860426
	AE034217		JAA67593		XP_002416236
A. variegatum	DAA34267		JAA67756		XP_002415307
	DAA34257		JAA69032		XP_002407493
	DAA34183		JAA71154		XP_002399564
	DAA34478		JAA71155		XP_002435393

Table 3: Continued

Source tick species	Accession#	Source tick species	Accession#	Source tick species	Accession#
Haemaphysalis longicornis	BAD11156		JAA71156		XP_002434763
			JAA72548		EW869079
Rhipicephalus haemaphysaloides	AFX65224		gi|310689892		XP_002434444
	AFX65225		gi|310689891		XP_002433376
R. appendiculatus	AAK61377		JAA71163		XP_002415208
	AAK61378		JAA66228		XP_002413438
	AAK61376		JAA73759		XP_002416263
	AAK61375		JAA72989		XP_002411933
R. pulchellus	JAA63611		JAA72940		XP_002411931
	JAA63258		JAA72595		XP_002402370
	JAA54387	*I. scapularis*	EW949864	*I. scapularis*	XP_002411045
	JAA54315		EW837780		XP_002401187
	JAA54314		EW881762		XP_002400954
	JAA54313		EW901007		XP_002403236
	JAA54312		EW814209		XP_002401986
	JAA54311		EW860426		XP_002415891
	JAA54310		EW882018		XP_002415890
	JAA54309		ISCW017295		XP_002415888
	JAA54308		ISCW011017		XP_002415887
	JAA54307		ISCW024435		XP_002415886
	JAA54306		ISCW006062		XP_002415308

Table 3: Continued

Source tick species	Accession#	Source tick species	Accession#	Source tick species	Accession#
R. pulchellus	JAA54167		ISCW023208		XP_002416681
	JAA53966		ISCW024387		XP_002416635
R. microplus	AHC98652		ISCW023617		XP_002408111
	AHC98653		ISCW024109		XP_002416150
	AHC98654		ISCW023621		XP_002415892
	AHC98655		ISCW010422		XP_002411932
	AHC98656	*I. scapularis*	EW851734	*I. scapularis*	XP_002402368
	AHC98657		EW959899		XP_002399745
	AHC98658		EW955724		XP_002405749
	AHC98659		AAM93649		ABJB011030283
	AHC98660		AAV80788		ABJB010229324
	AHC98661		ACI446630		ABJB011108013
	AHC98662		TC36450		
	AHC98663		TC54356		

microplus TC17409, TC22658, and EST767976, while AAS41, 42, and 54 show 65, 79 and 90% identity to partially characterized *R. appendiculatus* AAK61378, and AAK61376, and *R. microplus* TC16456, respectively. Four AAS (24, 38, 69, and 70) showed 70% or greater identity to the same partially characterized *R. appendiculatus* AAK61377. Of the 50 AAS sequences conserved in other tick species, only 11 sequences (AAS19–21, 25, 37, 42, 44, 45, 54, 66 and 78) appear to be conserved in prostriate ticks (4b). Except for AAS19, which showed 81 and 82% amino acid identity to *I. ricinus*ABI94058 and *I. scapularis*XP_00245308, respectively, all other AAS sequences showed 58–70% conservation with prostriate tick sequences (b).

Table 5 summarizes the 29 AAS RCL sequences which show at least 62% sequence conservation in other tick species. Preliminary manual inspection of RCLs showed high identities between sequences where the predicted P1 site is of the same charge and polarity, therefore all 212 tick serpin sequences for which an RCL could be determined were first divided into one of the four groups: polar uncharged, polar basic, polar acidic, and hydrophobic. RCL sequences (Figure 5) were then subjected to phylogeny analysis (not shown). Lastly, RCLs from all tick species were subjected to pairwise sequence alignments. Identity levels for the 29 highly conserved AAS RCLs, (AAS4, 7, 12, 14, 18–23, 25–29, 31, 37, 38, 42, 44, 45, 47, 50, 52, 54, 58, 65, 69, and 70) ranged from approximately 62–100% (Table 5). Of these 29 RCLs, seven (AAS7, 19–21, 23, 27, and 42) are conserved in both metastriate and prostriate ticks, with the remaining being conserved only in metastriate ticks. Seven AAS RCLs (AAS18, 20–22, 27, 42, and 50) showed 95–100% conservation with at least one RCL from another

Table 4a: *A. americanum* serpins (AAS) that have orthologs in other *Amblyomma* spp ticks

AAS ID	Other tick best match	% ID
1	Amac-AEO32759	**71**
2	Amac-AEO32759	**71**
3	Amac-AEO32759	**70**
4	Amac-AEO34312	**79**
	Amac-AEO34279	**89**
5	Amac-AEO34312	**75**
	Amac-AEO34279	**77**
6	Amac-AEO34312	**75**
	Amac-AEO34279	**82**
7	Amac-AEO35533	**87**
	Amac-AEO35520	**86**
	Amac-AEO32774	**72**
	Amac-AEO32541	**65**
	Amac-AEO32217	**66**
	Amac-AEO32160	**70**
	Amac-AEO32154	**71**
8	Amac-AEO34447	**82**
	Amac-AEO34313	**64**
	Amac-AEO33019	**71**
9	Amac-AEO34447	**79**
	Amac-AEO34313	**62**
	Amac-AEO33019	**69**
10	Amac-AEO34447	**82**
	Amac-AEO34313	**64**
	Amac-AEO33019	**72**
11	Amac-AEO34447	**63**
	Amac-AEO34313	**69**
12	Amac-AEO34447	**78**
	Amac-AEO34313	**89**
13	Amac-AEO34447	**74**
	Amac-AEO34313	**78**
14	Amac-AEO34447	**74**
	Amac-AEO34313	**80**
15	Amac-AEO34447	**73**
	Amac-AEO34313	**77**
16	Amac-AEO34447	**72**
	Amac-AEO34313	**75**
25	Amac-AEO35533	**81**
	Amac-AEO35520	**76**
	Amac-AEO32774	**70**
	Amac-AEO32541	**63**
	Amac-AEO32217	**64**
	Amac-AEO32160	**67**
	Amac-AEO32154	**70**
26	Amac-AEO35533	**64**
	Amac-AEO35520	**64**
	Amac-AEO32774	**62**
	Amac-AEO32541	**77**
	Amac-AEO32217	**79**
	Amac-AEO32160	**62**
	Amac-AEO32154	**61**
27	Amac-AEO34349	**81**
28	Amac-AEO34314	**62**
29	Amac-AEO35533	**62**
	Amac-AEO35520	**59**
	Amac-AEO32774	**67**
	Amac-AEO32160	**61**
	Amac-AEO32154	**65**
30	Amac-AEO34312	**76**
	Amac-AEO34279	**76**
36	Amac-AEO34314	**59**
37	Amac-AEO35533	**67**
	Amac-AEO35520	**65**
	Amac-AEO32774	**71**
	Amac-AEO32541	**70**
	Amac-AEO32217	**73**
	Amac-AEO32160	**68**
	Amac-AEO32154	**71**
41	Avar-DAA34478	**73**
46	Avar-DAA34478	**73**
	Amac-AEO34447	**69**
	Amac-AEO34313	**76**
	Amac-AEO33019	**75**

Table 4a: Continued

AAS ID	Other tick best match	% ID	AAS ID	Other tick best match	% ID
17	Amac-AEO34447	**75**	47	Amac-AEO34314	**79**
	Amac-AEO34313	**79**			
17	Amac-AEO34447	**75**	67	Amac-AEO34313	**70**
	Amac-AEO34313	**79**		Amac-AEO33019	**74**
18	Amac-AEO34447	**68**			
	Amac-AEO34313	**76**			
18	Amac-AEO34447	**68**			
	Amac-AEO34313	**76**			
19	Amac-AEO32217	**91**			
	Amac-AEO34218	**91**			
19	Amac-AEO32217	**91**			
	Amac-AEO34218	**91**			
	Avar-DAA34478	**71**			
22	Amac-AEO34447	**69**			
	Amac-AEO34313	**74**			
	Amac-AEO33019	**86**			
22	Avar-DAA34478	**71**			
	Amac-AEO34447	**69**			
	Amac-AEO34313	**74**			
23	Amac-AEO33019	**86**			
	Amac-AEO32759	**66**			
24	Amac-AEO34312	**77**			
	Amac-AEO34279	**86**			

Amac = *A. maculatum,* Avar = *A. variegatum, %* amino acid identities are bolded

Table 4b: AAS amino acid identity to serpins in metastriata and prostriata ticks

AAS ID	Other tick best match	% ID
1	Rhaem-AFX65224	**61**
	Rapp-AAK61377	**69**
	Rmic-AAP75707	**68**
	Rmic-TC16466	**73**
4	Rhaem-AFX65224	**59**
	Rapp-AAK61377	**66**
	Rmic-AAP75707	**65**
	Rmic-TC16466	**67**
	Hlong-BAD11156	**73**
6	Rapp-AAK61377	**68**
	Rmic-AAP75707	**66**
	Rmic-TC16466	**69**
	Hlong-BAD11156	**69**
7	Rhaem-AFX65224	**74**
	Rmic-TC24850	**69**
	Rmic-EST89704	**75**
	Rpulc-JAA54306	**72**
	Rpulc-JAA54314	**69**
	Rpulc-JAA54313	**69**
	Rpulc-JAA54312	**77**
	Hlong-BAD11156	**74**
8	Rpulc-JAA54310	**68**
25	Rapp-AAK61377	**66**
	Rapp-AAK61375	**62**
	Rmic-TC24850	**66**
	Rmic-EST89704	**69**
	Rpulc-JAA54306	**70**
	Rpulc-JAA54314	**63**
	Rpulc-JAA54313	**66**
	Rpulc-JAA54312	**71**
	Rpulc-JAA54311	**64**
	Ir-CAB55818.2	**61**
	Is-XP_002434444	**59**
	Ir-00245308	**70**
	Ir-EW874987	**70**
26	Rmic-TC24850	**61**
	Rmic-EST89704	**61**
	Rpulc-JAA54306	**61**
	Rpulc-JAA54314	**66**
	Rpulc-JAA54313	**63**
	Rpulc-JAA54312	**62**
	Rpulc-JAA54311	**66**
29	Rmic-TC24850	**60**
	Rmic-EST89704	**62**
	Rpulc-JAA54310	**74**
45	Rpulc-JAA54306	66
	Rpulc-JAA54314	71
	Rpulc-JAA54313	71
	Rpulc-JAA54312	68
	Ir-XP_02399745	61
	Ir-XP_002408111	60
46	Rapp-AAK61378	64
	Rpulc-JAA54312	73
47	Rpulc-JAA63611	59
	Rpulc-JAA62387	60
50	Rpulc-JAA54309	76
54	Rmic-TC16466	90
	Is-XP_002401187	58
65	Rpulc-JAA54309	80
66	Rpulc-JAA54310	70
	Is-XP_002415891	59
67	Rpulc-JAA54310	68
69	Rapp-AAK61377	70
	Rmic-AAP75707	70
	Hlong-BAD11156	68
70	Rapp-AAK61377	71
	Rmic-AAP75707	71
	Hlong-BAD11156	73

Table 4b: Continued

AAS ID	Other tick best match	% ID
9	Rpulc-JAA54310	**66**
10	Rpulc-JAA54310	**66**
11	Rmic-TC16466	**62**
	Hlong-BAD11156	**62**
12	Rmic-TC16466	**72**
	Hlong-BAD11156	**74**
13	Rmic-TC16466	**67**
14	Rmic-TC16466	**70**
15	Rmic-TC16466	**66**
16	Rmic-TC16466	**66**
17	Rmic-TC16466	**69**
18	Rpulc-JAA54310	**72**
19	Rmic-TC22658	**95**
	Rmic-EST67697	**82**
	Rpulc-JAA54307	**87**
	Ir-ABI94058	**81**
	Is-XP_00245308	**82**
20	Rpulc-JAA54310	**79**
	Is-XP_002401986	**58**
21	Rmic-TC17409	**96**
	Rpulc-JAA63258	**91**
	Ir-JAB72483	**67**
	Is-XP_002401986	**70**
30	Rapp-AAK61377	**66**
	Rmic-AAP75707	**66**
	Rmic-TC16466	**68**
	Hlong-BAD11156	**70**
36	Rpulc-JAA63611	**69**
	Rpuc-JAA62387	**68**
37	Rmic-EST89704	**65**
	Rpulc-JAA54306	**63**
	Rpulc-JAA54306	**60**
	Rpulc-JAA54314	**67**
	Rpulc-JAA54313	**64**
	Rpulc-JAA54312	**63**
	Rpulc-JAA54311	**67**
	Ir-CAB55818.2	**60**
	Is-ACI46630	**61**
38	Rapp-AAK61377	**71**
	Rmic-AAP75707	**70**
39	Ir-XP_002401986	**68**
40	Rpulc-JAA54310	**67**
41	Rapp-AAK61378	**65**
	Rpulc-JAA54310	**76**
78	Rpulc-JAA54306	65
	Rpulc-JAA54313	61
	Rpulc-JAA54312	70
	Rpulc-JAA54314	63
	Rmic-AHC98652	62
	Rmic-AHC98653	68
	Rmic-AHC98662	61
	Rapp-AAK61376	61
	Rhaem-AFX65225	68
	Is-XP_00240811	61
	Is-XP_002399745	61
	Ir-JAA66228	61
	Ir-JAA72595	61
	Ir-JAA66227	61
114	Rpulc-JAA54310	75
121	Rpulc-JAA63611	67
	Rpulc-JAA62387	67

Table 4B: Continued

AAS ID	Other tick best match	**% ID**	AAS ID	Other tick best match	**% ID**
22	Rapp-AAK61378	**61**	42	Rapp-AAK61376	**79**
				Rpulc-JAA54314	**79**
23	Rhaem-AFX65224	**71**		Rpulc-JAA54313	**77**
	Rpulc-JAA54310	**75**		Is-XP_002403236	**61**
				Is-XP_002407493	**61**
24	Rapp-AAK61377	**71**	44	Rpulc-JAA54314	**59**
	Rmic-AAP75707	**71**		Ir-XP_02399745	**58**
	Rmic-TC16466	**73**			

Rapp = *R. appendiculatus,* Rmic = *R. microplus,* Rhaem = *R. haemaphysaloides,* Rpulc = *R. pulchellus,* Ir = *I. ricinus,* Is = *I. scapularis,* and Hlong = *H. longicornis. %* amino acid identities are bolded

Table 5 Cross-tick species conserved *A. americanum* serpin reactive center loops

Serpin ID	Conserved RCL amino acid sequence	*% ID	Serpin ID	Conserved RCL amino acid sequence	*% ID
AAS4*	EEGTIAAAVTGLSFVP**I**SALH	-	AAS26	EEGTEAAAATATVAMF**G**SAPS	-
Amac-AE034279	EEGTIATAVTGLSFVA**L**SALS	81	*Amac*-AE032541	EEGTEAAAATAVIMVF**G**SASS	76
Rapp-AAK61377	EEGTIATAVTGLGFVP**L**SAHY	76	*Amac*-AE032217	EEGTEAAAATAVIMLF**G**SASS	76
Rmic-AAP75707	EEGTIATAVTGLGFVP**L**SVHY	71	AAS27	EEGTEAAAASGVVGVN**R**IGID	-
Rmic-AHC98654	EEGTIATAVTGLGFVP**L**SAHY	71	*Is*-EW860426	EEGTEAAAASGVVAVN**R**LIGV	66
Rmic-AHC98661	EEGTVAAAVTGLFVRP**T**APLP	71	*Is*-XP_002411045	EEGTEAAAASGVVMTR**R**AIEG	75
Rmic-AHC98655	EEGTIAAAVTGLFVMP**S**SSLY	81	*Amac*-AE033019	EEGTQAAAASGVVGVN**R**IGIE	90
Hlong-BAD11156	EEGTVATAVTGISFIP**L**SAHY	67	*Rpulc*-JAA54308	EEGTEAAAVSGVVSVN**R**IGIE	86
AAS7	EEGTEAAAATGIAMML**M**CARF	-	*Rmic*-AHC98657	EEGTEAAAVTGVIGVN**R**IGIE	81
Amac-AE035320	EEGTEAAAATGMAFML**L**SARF	81	AAS28	EEGTEAAAATGMVAMA**R**CAII	-
Ir-JAA66227	EEGTEAAAATAIPIML**M**CARF	86	*Rmic*-AHC98669	EEGTEAAAATGMVAMA**R**CASM	90

Table 5: Continued

Serpin ID	Conserved RCL amino acid sequence	*% ID
Ir-JAA72595	EEGTEAAAATAVPVMF**C**CAIF	66
Rhaem-AFX65225	EEGTEAAAATAITMMT**Y**CARF	81
Rapp-AAK61376	EEGTEAAAATAITMMT**Y**CARF	81
Rpulc-JAA54312	EEGTEAAAATAITMMT**Y**CARF	81
AAS12	EEGTIAAAVTGLSFVP**I**SALH	–
Amac-AE034279	EEGTIATAVTGLSFVA**L**SALS	81
Rapp-AAK61377	EEGTIATAVTGLGFVP**L**SAHY	71
Rmic-AAP75707	EEGTIATAVTGLGFVP**L**SVHY	71
Rmic-AHC98654	EEGTIATAVTGLGFVP**L**SAHY	71
Rmic-AHC98661	EEGTVAAAVTGLFVRP**T**APLP	71
Rmic-AHC98655	EEGTIAAAVTGLFVMP**S**SSLY	81
Hlong-BAD11156	EEGTVATAVTGISFIP**L**SAHY	67
AAS14	EEGTVATAVTGISLVA**L**SALH	–
Amac-AE034279	EEGTIATAVTGLSFVA**L**SALS	81
Rapp-AAK61377	EEGTIATAVTGLGFVP**L**SAHY	67
Rmic-AAP75707	EEGTIATAVTGLGFVP**L**SVHY	62
Hlong-BAD11156	EEGTVATAVTGISFIP**L**SAHY	
AAS18	EEGSEAAGATAVIFFT**R**GGSS	–
Amac-AE034313	EEGSEAAGATAVIFFT**R**GGIP	90
Avar-DAA34478	EEGSEAAGATAVIFFT**R**GGAP	90
Rmic-AHC98668	EEGTIAAAVTGLFVMP**S**SSLY	71
AAS19	EEGSEAAAVTGFVIQL**R**TAAF	–
Rpulc-JAA54307	EEGSEAAAVTGFVIQL**R**TAAF	100
Amac-AE034218	EEGSEAAAVTGFVIQL**R**TAAF	100
Amac-AE034217	EEGSEAAAVTGFVIQL**R**TAAF	100

Serpin ID	Conserved RCL amino acid sequence	*% ID
AAS29	EEGTEAAAATAVTVVD**G**CMPR	–
Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	71
AAS31	EEGSEAAAVTGVVINT**R**TIGG	–
Rmic-AHC98667	EEGSEAAAVTGVTINT**R**TTTG	81
Rmic-AHC98657	EEGTEAAAVTGVIGVN**R**IGIE	71
AAS37	EEGTEAAAATAVVMMC**R**SAAM	–
Rpul-JAA54314	EEGTEAAAATAVMMMA**C**CMSS	66
Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	66
Rmic-AHC98669	EEGTEAAAATGMVAMA**R**CASM	71
AAS38	EEGTIATAVTGLSFAP**I**SALH	–
Amac-AE034279	EEGTIATAVTGLSFVA**L**SALS	86
Rapp-AAK61377	EEGTIATAVTGLGFVP**L**SAHY	81
Rmic-AHC98654	EEGTIATAVTGLGFVP**L**SAHY	71
AAS42	EKGTEAAAATAVMMMA**C**CMSA	–
Rpulc-JAA54314	EEGTEAAAATAVMMMA**C**CMSS	90
Amac-AE032774.1	EKGTEAAAATAVMMVA**C**CLSI	86
Amac-AE032154.1	EKGTEAAAATAVMMVG**C**CLSI	81
Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	86
Rmic-AHC98652	EEGTEAAAATAVMMAA**C**CLSS	81
Rapp-AAK61375	EEGTEAAAATAVMMAA**C**CLSS	81
Is-XP_002405749	EEGTEAAAATAMVMLC**C**MSFP	76
AAS44	EEGTEAAAATAIITTE**C**CIMP	–
Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	66
AAS45	EEGTEAAAATGVVMMC**D**SLPM	–
Rmic-AHC98669	EEGTEAAAATGMVAMA**R**CASM	71

Table 5: Continued

Serpin ID	Conserved RCL amino acid sequence	*% ID
Ir-JAA72548	EEGSEAAAVTGFVIQL**R**TAAF	100
Ir-ABI94058	EEGSEAAAVTGFVIQL**R**TAAF	100
Is-XP_002415308	EEGSEAAAVTGFVIQL**R**TAAF	100
Rmic-TC22658	EEGSEAAAVTGFVIQL**R**TAAF	100
AAS20	EVGTRAVAATEAQFVS**K**SLVH	–
Rpulc-JAA54167	EVGTRAVAATQAQFVS**K**SLVH	95
Is-ISCW016489	EEGTRAVAATQAQFVS**K**SLVQ	90
Rmic-AHC98664	EVGTRAVAATQAQFVS**K**SLVH	95
AAS21	EKGTEAVALSSGIIRH**S**KTPG	–
Rpulc-JAA63258	EKGTEAVALSSGIIRH**S**KTPG	100
Rmic-AHC98658	EKGTEAVALSSGIVRH**S**KTPG	95
Is-XP_002401986	EQGTEAVALSSGIVRH**S**RPPE	95
Ir-JAB72483	EQGTEAVALSSGIVRH**S**RPPE	90
Rmic-AHC98658	EKGTEAVALSSGIVRH**S**KTPG	95
AAS22	EEGSEAAGATGVIFYT**K**SAIV	–
Amac-AE034349	EEGSEAAGATGVIFYT**K**SMVV	90
Rpulc-JAA54315	EEGSEAAAATAVIFYT**K**SAAV	86
Rmic-AHC98668	EEGTIAAAVTGLFVMP**S**SSLY	90
AAS23	EEGTVAAAVTSIRMRM**K**SSRR	–
Is-XP_002434763	EKGTVAAAVTSISMRM**G**SSLA	76
AAS25	EEGTEAAAATAVVMMC**Y**SLPM	–
Rmic-AHC98653	EEGTEAAAATAVTLMT**Y**CARI	66
Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	66

Serpin ID	Conserved RCL amino acid sequence	*% ID
AAS47	EEGTEAAAATAMPAAN**S**CEMF	–
Amac-AE034314	EEGTEAAAATAMLASN**S**CERF	86
AAS50	EEGSETDSATLMRISG**K**AAEE	–
Rpulc-JAA54309	EEGSEADSATLLRISG**K**AAEE	90
Rmic-AHC98660	EEGSEADSATLLRISG**K**AAEE	90
AAS52	ENGTVAAAASAAIGVG**S**AGPS	–
Rpulc-JAA54315	EEGTEAAAATAAVGAG**S**AGPS	76
AAS54	EDGVEGLFLTPLIMMC**Y**AGVS	–
Rmic-AHC98663	EDGVEGLFLTPLIMMC**Y**AGVS	100
AAS58	EEGTEAAAATGMTLMM**C**GAMV	–
Rmic-AHC98669	EEGTEAAAATGMVAMA**R**CASM	71
AAS65	EEGSETDSATLMRISG**K**A-CE	–
Rpulc-JAA54309	EEGSEADSATLLRISG**K**AAEE	81
Rmic-AHC98660	EEGSEADSATLLRISG**K**AAEE	88
AAS69	EEGTIAAAVTGLSFVA**T**ASFN	–
Rmic-AHC98661	EEGTVAAAVTGLFVRP**T**APLP	71
AAS70	EEGTIATAVTGLSFVA**T**ASFN	–
Rmic-AHC98654	EEGTIATAVTGLGFVP**L**SAHY	66

Amac = *A. maculatum,* Is = *I. scapularis,* Ir = *I. ricinus,* Rapp= *R. appendiculatus*, Rmic = *R. microplus,* Rpulc = *R. pulchellus,* Rhaem = *R. haemaphyssaloides,* Avar = *A. variegatum*, Hlong= *H. longicornis.* Amino acid residues at P1 sites are bolded.

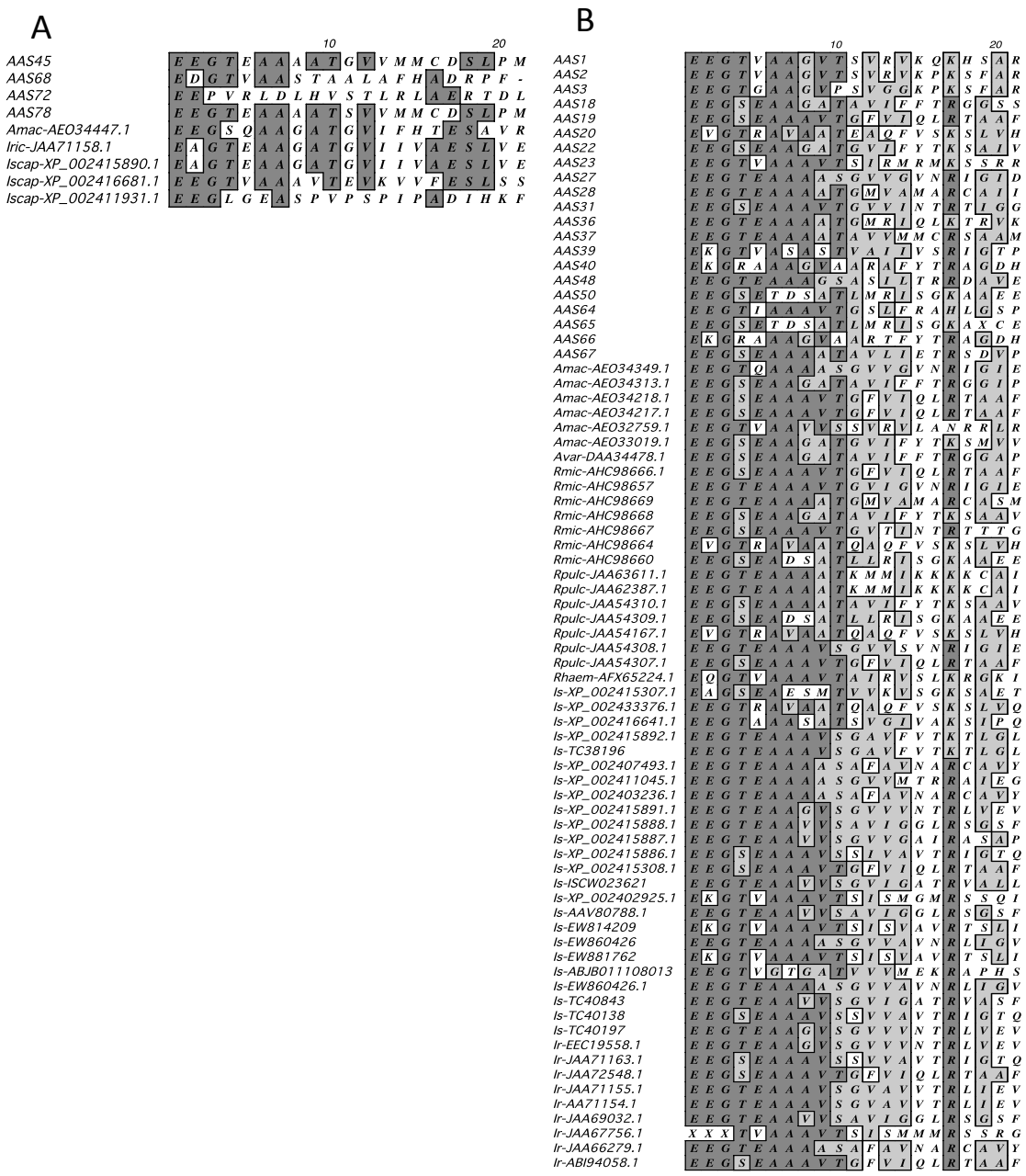

Figure 5. Multiple sequence alignment of tick serpin reactive center loops (RCL). Predicted RCLs in serpins found in this study and other tick serpins downloaded from GenBank were subjected to multiple sequence alignment using T-coffee in MacVector version 12. Asterisk (*) sign denotes predicted amino acid residues at P1 sites: (A) polar acidic, (B) polar basic, (C) hydrophobic, and (D) polar uncharged. Identical amino acid residues are shaded gray. AAS – *Amblyomma americanum* serpins, Amac – *A. maculatum*, Avar – *A. variegatum*, Rmic – *Rhipicephalus microplus*, Rpulc – *R. pulchellus*, Rhaem – *R. haemaphysalis*, Rapp –*R. appendiculatus*, Hlong – *Haemaphysalis longicornis*, Is – *Ixodes scapularis*, Ir – *I. ricinus.*

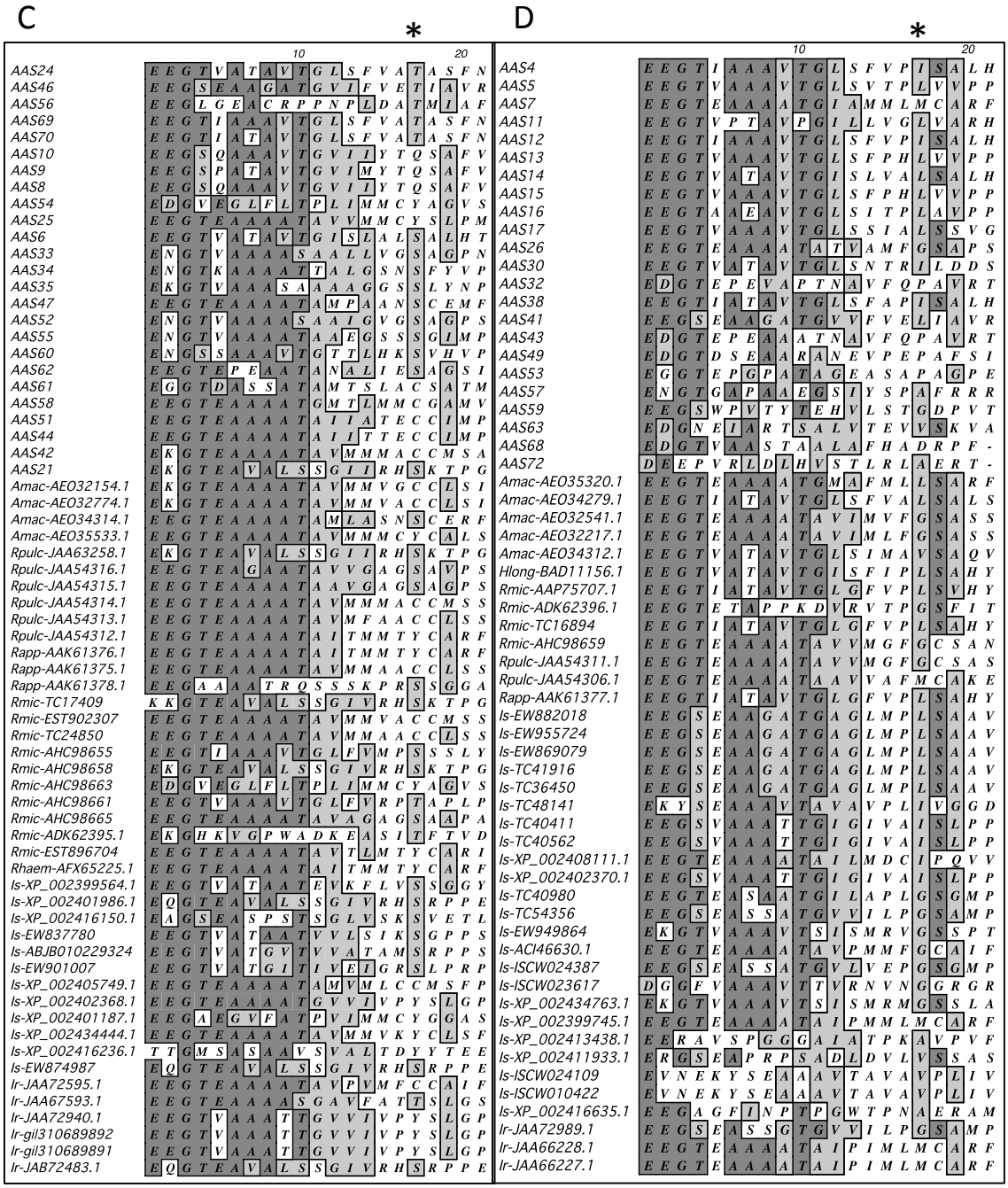

Figure 5. Continued.

species. For AAS25, RCL identity to its partially characterized ortholog in *R. appendiculatus* (AAK61375), was higher, at 81% identity, than for whole sequence identity, at 62%. Three AAS RCLs (AAS7, 19, and 20) showed >80% identity to at least one RCL from a prostriate species. The identities between remaining RCL sequences ranged between ~62 and 76% (Table 5). Itis notable that the RCL for AAS19 is 100% identical to serpin RCLs across several tick species in multiple genera, and including both metastriate and prostriate ticks. Of the 29 conserved AAS RCLs, 38% (11/29) (AAS18–20, 22, 23, 27, 28, 31, 37, 50, 65) have basic amino acid residues, 35% (10/29) (AAS21, 25, 42, 44, 47, 52, 54, 58, 69, and 70) have polar uncharged residues, and 24% (7/29) (AAS4, 7, 12, 14, 26, 29, 38, and 42) have hydrophobic residues, while only one sequence, AAS45, has a polar acidic residue at the putative P1 site. It is interesting to note that for the 10 most highly inter-species conserved RCL sequences (those which are ≥90%), the majority (7/10) have basic P1 residues, while the remaining three have polar uncharged P1 residues.

Discussion

This study provides an update of unique serpin-coding sequences expressed in unfed and fed *A. americanum* male and female ticks. While this is the first report of a very large number of serpin transcripts from male ticks, data presented here are not unusual. High numbers of serpin sequences were reported in ticks *I. scapularis* (Mulenga et al., 2009), *R. pulchellus* (direct submission), and *A. maculatum* (Karim et al., 2011), in mosquitoes *Anopheles gambiae*, *Culex quinquefasciatus*, and *Aedes aegypti*

(Rawlings et al., 2012), in *Bombyx mori* (Zou et al., 2009), *Tribolium* (Zou et al., 2007), *Drosophila* (Reichhart, 2005), in mouse and human genomes (Puente and López-Otín, 2004; Gatto et al., 2013; Heit et al., 2013), and in *Arabidopsis* and *Oryza sativa* (Fluhr et al., 2012). Such large serpin counts across a great diversity of taxa indicate the importance of this protein family in regulating homeostasis in most branches of life. While serpin counts in other tick species do not approach the number for *A. americanum* as reported here, the discrepancy is likely due to the fact that this is the first analysis of tick transcriptomes of its scope with both male and female tissues at various time points having been explored.

Although based on the design of this study we are unable to conclude expression patterns, the observation of a greater diversity of AAS transcripts in male than female ticks is interesting. We speculate that the high diversity of AAS transcripts found in male ticks could be explained by the timing of reproductive physiological changes. Male metastriate tick species such as *A. americanum* feed for only a short time prior to completion of spermatogenesis and mating (Kiszewski et al., 2001). On the other hand, many female reproductive activities including vitellogenin synthesis and deposition into oocytes, ovulation, fertilization, and oviposition do not occur until after several days of feeding (Kiszewski et al., 2001; Sonenshine and Roe, 2014). The majority of AAS which were found exclusively in males in this study, were from male ticks fed for at least three days and were potentially ready to mate, while female ticks in this study were fed up to 120 h with reproductive activities just beginning. As a result, it is possible that there are

additional serpins important for female reproductive physiology that are expressed at later feeding time points than those identified in this study.

Although empirical data will be needed, it is interesting to note that AAS50 and 121 both found in male ticks were abundant after feeding when the male tick has entered its physiologically reproductive state. It will be interesting to investigate expression of serpins in the immature stages, where there is a lack of sexual dimorphism. This will inform on which serpins are indeed involved in sex-specific physiology, and which are involved in other feeding-related physiology such as host-defense modulation. Evidence in insects, *Drosophila* and *Aedes aegypti*, indicated that serpins are among the male reproductive gland proteins transferred to females during mating (Coleman et al., 1995; Sirot et al., 2008). Although there are distinct differences between biology of ticks and insects, it is plausible that some male *A. americanum* serpins found in this study could serve as reproductive proteins. The SG and MG represent two major organs through which the tick interacts with its host and with pathogens. Thus, the identification of 31 previously unknown AAS transcripts in SG and MG is interesting. Although relative expression analysis done in this study is limited, the expression patterns of AAS21 and AAS27 are notable. Based on relative abundance determined in this study AAS21 abundance is 1000-fold higher at 48 h MG compared to SG, while AAS27 increases 500 fold at 120 h in MG more than SG. It will be interesting to investigate role(s) of AAS21 and AAS27 in tick feeding. Another important goal of this study was to identify *A. americanum* tick serpins that are expressed in both male and female metastriata ticks.

We believe that these could represent those that are important to tick feeding regulation. Despite obvious differences in their biology, both male and female ticks must interact with host defense mechanisms before mating. Indeed, there is evidence that like females, male ticks express anti-inflammatory molecules such as histamine-binding proteins (Paesen et al., 1999; Bior et al., 2002), which are potentially involved in facilitating male tick feeding. Thus, the 16 AAS transcripts found in both male and female ticks, and in 48–120 h SG and MG could represent those that are important to tick feeding success.

The high number of AAS sequences in this study could be explained by gene duplication and exon shuffling in the RCL region as suggested by high amino acid identity among some AAS sequences that clustered together on the phylogeny tree and those that showed differences restricted to the RCL region. Serpin diversity by gene duplication and subsequent divergence has been reported in a number of organisms including humans (Heit et al., 2013), mice (Borriello and Krauter, 1990; Hancock, 2005) and *B. mori* (Zou et al., 2009). In *An. gambiae* serpin genes clustering phylogenetically were found in clusters on the same chromosome, indicating that they could be duplicated genes (Suwanchaichinda and Kanost, 2009). Similarly, in *I. scapularis* 11 highly identical serpins were found on the same supercontig (Mulenga et al., 2009). The observation of differences restricted to the RCL has been observed in transcripts that are products of alternatively spliced exons. This phenomenon was reported in *Manduca sexta* (Jiang and Kanost, 1997), in *B. mori* (Zou et al., 2009), and in *Ctenocephalides felis* (Brandt et al., 2004). We also observed a very curious pattern between AAS4 and 12, and AAS13 and 15, in which RCLs were identical with differences restricted to

outside of the RCL (Mulenga et al., 2007). Whether or not these observations are consistent with events in vivo requires further investigation. However, itis interesting to note that in this study we observed a similar pattern between serpin sequences in other tick species: *A. maculatum* AEO34217 and AEO34218, and *R. pulchellus* JAA62387 and JAA63611, where the RCL is the same and differences in sequences are outside of the RCL region. We speculate that if consistent with events in vivo, AAS transcripts that share the same RCL sequence could function as redundant proteins, or could regulate the same protease under different spatio-temporal conditions.

Within the RCL region, the amino acid at the P1 site is considered most important in determining the target protease(s) that is/are regulated by a candidate serpin (Gettins, 2002; Huntington, 2006, 2011). Accordingly, the observation that 17 different amino acid residues were predicted at P1 sites of putative RCLs identified in this study indicates the potential diversity of proteases that may be regulated by these serpins. Our analysis of AAS RCL P1 sites showed a near-even distribution of residues across charge/polarity types with the exception of polar acidic similar to *M. sexta*, *B. mori* (Zou et al., 2009), plants (Roberts et al., 2004; Roberts and Hejgaard, 2008), and humans (Cassar and Hunter, 2013) where polar acidic P1 residues in serpins appear to be rare. Prediction of the P1 site amino acid residue is not in itself sufficient to determine the protease that may be regulated by a candidate serpin; empirical evidence is required. However, there is ample evidence that serpins with basic residues at the P1 site regulate trypsin and trypsin-like proteases (Gettins, 2002; Li et al., 1999; Leboulle et al., 2002a, b; Prevot et al., 2006). Thus it is interesting that the most highly conserved AAS RCLs

in this study have basic P1 residues. From the perspective of tick feeding biology, serpins with basic P1 residues could represent those that ticks use to evade trypsin-like protease-mediated host defense pathways such as blood clotting. We would like to caution the reader here that in both plants and in non-hematophagous organisms such as *Drosophila*, the majority of serpins have basic P1 amino acid residues (Reichhart, 2005; Fluhr et al., 2012). It is also interesting to note that two tick anti-coagulant serpins, HLS2 (orthologous to AAS12), in *H. longicornis* (BAD11156) (Imamura et al., 2005), and Iris in *I. ricinus* (AJ269658) (Prevot et al., 2006), contain hydrophobic residues at their P1 sites. Further experiments are therefore required in order to determine proteases/pathways that may be regulated by the serpins described in this study.

In nature, different tick species may infest the same animal host and implicitly, these different tick species will face the same host defense mechanisms. It is conceivable that different tick species could utilize conserved proteins to interact with the same host, such as the 50 cross-tick species conserved AAS sequences identified in this study. It is notable that only ~22% (11/50) were conserved in both metastriate and prostriate tick species. These could be particularly interesting candidates for further functional studies given that in general, amino acid identities between metastriate and prostriate tick proteins sequences are low. In particular, AAS25, which was detected in fed males and females, and in SG, is orthologous to Iris, found in SG and saliva of *I. ricinus*. Iris was demonstrated to inhibit lymphocyte proliferation, as well as the immune system cytokines IFN- and IL-6 (Leboulle et al., 2002a, b). Similarly, highly conserved AAS

proteins represent very interesting candidates for development of universal anti-tick vaccines as advocated (Maritz-Olivier et al., 2007).

The RCL is important to serpin function (Gettins, 2002), and thus it is interesting to note that some AAS RCLs are 90–100% conserved in other tick species. These serpins could be involved in regulating target proteases that are important to all ticks. Based on our inter-species comparative sequence analysis, it is interesting to note that AAS sequences with polar basic P1 sites, followed by polar uncharged P1 sites, were likely to be conserved in other tick species. It is noteworthy that hydrophobic P1 residues, though prevalent, tended to show little conservation in other tick species. This suggests that proteases that can interact with hydrophobic Pl residues may be involved in species-specific physiology, and might be diverging at a fast rate. Although the present study is descriptive, it contributes significantly to the picture of serpin transcript diversity expressed by male and female *A. americanum* ticks. Additionally, based on the data in this analysis, the picture of serpin transcript diversity in other tick species is likely to be far from complete.

CHAPTER III

BIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *AMBLYOMMA AMERICANUM* SERINE PROTEASE INHIBITORS 4 (AAS4) and 8 (AAS8)

Introduction

In Chapter II, bioinformatic analyses revealed that *A. americanum* expresses no less than 122 unique serpin sequences, 48 of which are expressed in salivary gland (SG) or midgut (MG) tissues (Porter et. al., 2015; Mulenga et. al., 2007). AAS4 and 8, originally named lospin 4 and 8 (GenBank ABS87356.1 and ABS87360.1), were first identified as being among 17 serpins that are expressed in female *A. americanum* during feeding: in SG and MG for AAS8 and in SG, MG, carcass (CA), and ovaries (OV) for AAS 4 (Mulenga et al., 2007). Additionally, bioinformatic analyses of transcriptome data in Chapter II shows AAS8 was found in MG 48h-, 96h-, and 120h- while AAS4 is expressed in SG 48h and in MG 48h-, 96h-, and 120h- post-tick attachment time points (Porter et al., 2015). AAS4 was also found in both unfed and fed ticks.

The SG and MG are important organs in tick feeding physiology. To feed, ticks insert their mouthparts into the host and lacerates the vascularture, stimulating the host blood coagulation and platelet formation cascades as diagramed in Figure 6. Products of the tick SG are involved in establishing the feeding site and injecting proteins involved in the counterdense of these host defences. The SG also participates in the elimination of excess water during feeding and is a passageway for tick-borne pathogens migrating from the tick to the host. The tick MG is a repository for imbibed host blood and

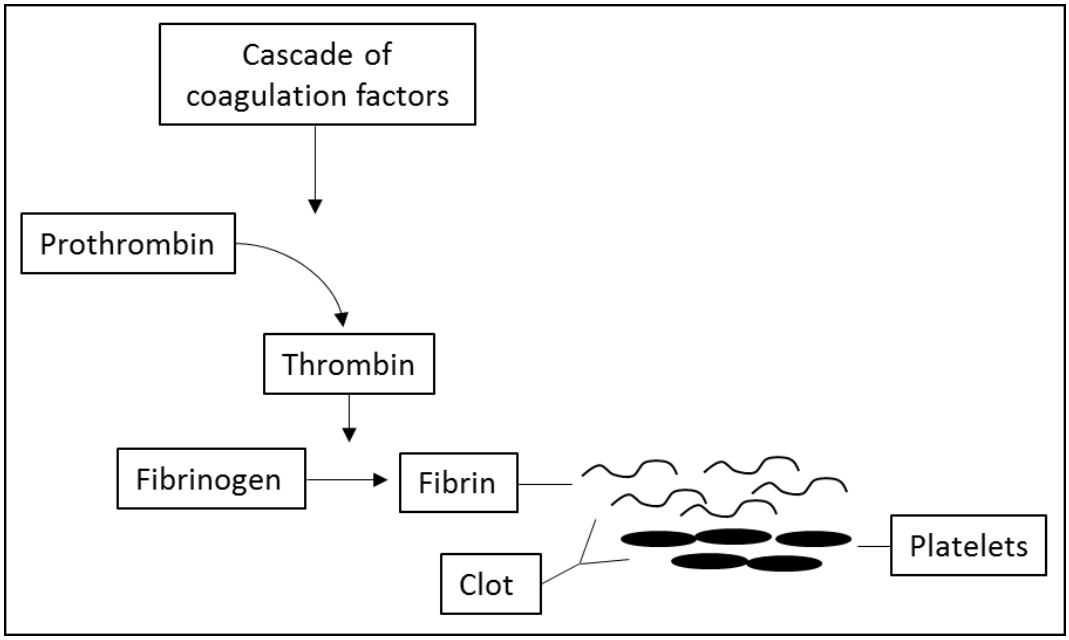

Figure 6. Diagram of vertebrate blood coagulation and platelet cascades. The diagram shows that a cascade of blood coagulation inhibitors results in the converstion of Prothrombin to Thrombin which converts fibrinogen to fibrin that covers platelets, reinforcing the clot.

pathogens (Sonenshine and Roe, 2014). Thus, expression of AAS4 and AAS8 in both SG and MG suggested the potential for these two serpins to play a role in the physiology of tick feeding. However, empirical analyses are needed to confirm this prediction.

Most interestingly, BLASTP analysis in Chapter II revealed seven sequences from five other tick species with identity to AAS4 above 50% (Table 1). AAS4 was 79 and 89% identical to *A. maculatum* AEO34312 and AEO34279, respectively. Outside of the *Amblyomma* genus, AAS4 was 65 and 67% identical to *R. microplus* serpins AAP75707.1 and TC16466. One ortholog was found in *R. appendiculatus*, serpin-3 (AAK61377.1) with 70% identity, one in *R. haemaphysaloides* (AFX65224.1) with 59% identity, one in *Haemaphysalis longicornis,* serpin-2 (BAD11156) at 73% identity

(Porter et al., 2015). In this chapter, an updated BLASTP screen revealed four additional sequences from two tick species with identity to AAS4 above 50% (Table 1): *R. microplus* serpins RmS10 (AHC98661.1), RmS4 (AHC98655.1), and RmS3 (AHC98654.1), at 64, 69, and 72% identity, respectively, and *Haemaphysalis flava* at 74%. Likewise, BLASTP analysis in Chapter II revealed 4 sequences from 2 other tick species with identity to AAS8 above 50% (Table 1): 64, 71, and 82% for *A. maculatum* AEO34313, AEO33019, AEO34447, respectively, and 68% for *R. pulchellus* JAA54310 (Porter et al., 2015). In this chapter, an updated BLASTP screen found four additional sequences from three tick species with identity to AAS8 at 50% or higher: *R. microplus* RmS17 (AHC98668) at 68%, *R. appendiculatus* serpin-4 (AAK61378) at 54%, and *I. ricinus* serpin-1 precursor (ABI94055) at 50% and serpin-2 precursor (ABI94056.2) at 50%. Serpins with high amino acid conservation across tick genera are particularly interesting as potential candidates for inclusion in anti-tick vaccines because of the potential to have broad-spectrum activity against many tick species. One of the major limitations of the only successful anti-tick vaccine, Bm86, is that it has narrow tick species specificity (Sonenshine and Roe, 2014).

Data are accumulating that support the idea that some tick-encoded serpins are functional inhibitors of exogenous proteases and are associated with counter-defense against anti-tick responses in the host, such as inflammation, complement activation, platelet aggregation, and blood clotting (Imamura et al., 2005; Prevot et al., 2009; Chmelar et al., 2011, 2012; Mulenga et al., 2013a, b). For example, Prevot et al., (2006) characterized a serpin in *I. ricinus* (Iris) ticks as an elastase inhibitor functioning in the

inhibition of coagulation, fibrinolysis, and platelet adhesion. In another study, Iris was also characterized to bind monocytes/macrophages and to prevent these cells from secreting tumor necrosis factor-α (TNF-α) (Prevot et al., 2009). A second serpin from *I. ricinus* (IRS-2) from saliva was later characterized to inhibit cathepsin G and chymase, and to prevent neutrophil migration and cathepsin G- and thrombin-induced platelet aggregation (Chmelar et al., 2011). *I. scapularis* serpin 1E1 was characterized as a thrombin and trypsin inhibitor that affects blood clotting and platelet aggregation (Ibelli et al., 2014).

In *R. microplus*, a serpin (Rmi-serpin) was characterized to inhibit trypsin (Rodriguez-Valle et al., 2011). Also in *R. microplus*, RmS-1 and -6 are chymotrypsin inhibitors, and RmS-3 is a chymotrypsin and elastase inhibitor (Rodriguez-Valle et al., 2015). *R. microplus* serpin 15 (RmS-15) was characterized as a physiological thrombin inhibitor that prolongs plasma clotting time (Xu et al., 2016). Yu et al., (2013) described *R. haemaphysaloides* serpins 1 and 2 (RHS-1 and -2) as chymotrypsin inhibitors and inhibitors of blood coagulation as assayed in the activated partial thromboplastin time (aPTT) assay. *A. americanum* serpin 6 (AAS6) was described as having cross-class inhibitory properties against trypsin, chymotrypsin, chymase, and elastase serine proteases, as well as the cysteine protease papain (Mulenga et al., 2013b). This serpin prolonged plasma clotting time and displays inhibitory properties in platelet aggregation. Another *A. americanum* serpin, AAS19, was characterized as a trypsin, plasmin, factor Xa, and factor XIa inhibitor that prolongs plasma clotting time and inhibits platelet aggregation (Kim et al., 2015a). A serpin from *H. longicornis* (HLS-2) was

characterized to prolong blood coagulation time in an aPTT assay, but interestingly was concluded as inhibiting endogenous serpins due to not binding anti-tick rabbit antibodies in a western blot (Imamura et al., 2005). These data support the hypothesis that serpins play a role in regulating host defense pathways against tick feeding. In this dissertation, biological and biochemical analyses were performed to determine if yeast and/or insect cell-expressed recombinant AAS4 and AAS8 were inhibitory serpins and if they function in tick feeding physiology.

Materials and Methods

Expression of recombinant AAS4 & AAS8 in Sf9 insect cells

The pIB/V5-His expression plasmid and the *Spodoptera frugiperda* (Sf), cell expression system (Invitrogen, Carlsbad, CA, USA) were chosen for recombinant AAS4 and 8 expression. Recombinant proteins expressed in the Sf9 system have the native signal peptide (SP) cleaved; thus it is not required to remove the nucleotide sequence encoding the SP from the open reading frame (ORF) to express the mature recombinant protein in this system. To construct the expression plasmid, the AAS4 ORF was sub-cloned into pIB-V5 His expression plasmids using the following primer set: 5'-**ggtacc**GCAATG**G**TCTCCAAGTTGG-3' and 5'-**ggatcc**AAGACTGTGCACTTCACCG-3' and the AAS8 ORF was sub-cloned into pIB-V5 His expression plasmids using the following primer set: 5'-**ggtacc**GTAATG**G**CGGGTAGCCGT-3' and 5'- **ggatc**CGAGGTGGTTCACTTGACC-3'. Restriction enzyme sites are lowercase and bolded. Restriction sites used for Sf9

expression for both AAS4 and 8 were *Kpn*I and *BamH*I. Sf9 constructs include the Kozak consensus sequence [(G/A) NNATG**G**], and reverse primers for both systems exclude the stop codon to allow for translation through the histidine tag.

AAS4 was amplified from template cDNA of *A. americanum* females using the following 2-step PCR cycling conditions: 5 min at 94°C, 30 sec at 94°C, 51°C for 30 sec, 72°C for 90 sec for eight cycles, followed by 30 sec at 94°C, 60°C for 30 sec, 72°C for 90 sec, for 30 cycles. AAS8 was amplified from template cDNA of *A. americanum* females using the following 2-step PCR cycling conditions: 5 min at 94°C, 30 sec at 94°C, 57°C for 30 sec, 72°C for 90 sec for eight cycles, followed by 30 sec at 94°C, 63°C for 30 sec, 72°C for 90 sec for 30 cycles. PCR products were separated on a 2% agarose gel with bands of the expected size excised and purified using the StrataPrep DNA gel extraction kit (Agilent Technologies, Santa Clara, CA, USA). Purified DNA was ligated into the pGEM-T vector (Promega), using the pGEM-T TA cloning kit as per the manufacturer's instructions. Following ligation, pGEM-T-AAS4 or pGEM-T-AAS8 plasmids were transformed into *Escherichia coli* DH5α competent cells (Life Technologies) using the routine heat shock method and plated on lysogeny broth (Lennox recipe: 1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, 1.5% agar, distilled water to 1L) agar plates prepared with ampicillin at 100µg/mL, with overnight incubation at 37°C. Ten colonies were selected for PCR verification of plasmid insertion using the following pGEM-T primers: T7, 5′-(TAATACGACTCACTATAGGG)-3′ and SP6: 5′-(TATTTAGGTGACACTATAG)-3′. PCR-verified insert-positive colonies were used to inoculate overnight cultures in 7mL

of Super Optimal Broth (SOB; 2% w/v bacto-tryptone, 0.5% w/v yeast extract, 8.56mM NaCl, 2.5mM KCl, distilled water to 1L pH 7.0), with ampicillin (100µg/mL). Simultaneously, empty pIB/V5-His vector plasmids were amplified by inoculating SOB media with zeocin at 25µg/mL.

Both pGEM-T-AAS4 and pGEM-T-AAS8 and empty pIB/V5-His plasmids were purified from *E. coli* cells using the Isolate II Plasmid Mini Kit (Bioline, London, UK) and digested using 1µl (10 units) each of the appropriate restriction enzymes (New England Biolabs, Ipswich, MA, USA) in a 100µL reaction volume to generate AAS4 or AAS8-encoding cDNA inserts and pIB/V5-His plasmids that were digested with the same enzymes. Prepared cDNA inserts were ligated into pIB/V5-His plasmids using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The ligation reaction was incubated at 16°C overnight and transformed into *E. coli* DH5α competent cells using the routine heat shock method. Ten colonies were selected for PCR verification of plasmid insertion using pIB/V5-His OpIE2 primers: 5'-CGCCACGATCTGGTAAACAC-3' and 5'-GACAATACAAACTAAGATTTAGTCAG-3'. PCR-verified insert-positive colonies were processed for plasmid purification using routine procedures, as previously described. Purified plasmids were sequenced using the OpIE2 and the BigDye Terminator Kit to verify proper cloning of AAS4 and AAS8 and to confirm the insert was cloned in the pIB/V5-His expression plasmids in-frame with the 6xHistidine tag.

Following verification of sequences, the pIB/V5-His-AAS4 and pIB/V5-His-AAS8 expression plasmids were purified as previously described. Prior to transfection,

2mL cultures of Sf9 cells were prepared in each of three wells of a 6-well plate, grown overnight at 28ºC. For transfection, 1µg of purified pIB/V5-His-AAS4 and -8 plasmids was diluted in 100µl SF900 II serum free media (SFM) (Invitrogen, Carlsbad, CA, USA) and incubated at room temperature (RT) for 30min. Simultaneously, 8µl of Cellfectin II reagent (Invitrogen, Carlsbad, CA, USA) was incubated with 100µl SF900 II SFM at RT for 30min. Cellfectin II reagent and pIB/V5-His-AAS4 and pIB/V5-His-AAS8 reactions were combined and after mixing gently the combined reaction was incubated at RT for 15min. After incubation, all 200µl of the combined reaction was added to Sf9 cells carefully in a drop-wise manner. Following transfection Sf9 insect cells were maintained in the 6-well plate in SF900 II SFM and incubated at 28°C. To confirm expression, one well was analyzed at each of 48h, 72h, and 96h. Collected culture samples were centrifuged at 1,500xg for 10min to separate media from cells. Media was concentrated by adding 4 volumes of ice-cold acetone, vortexing briefly, and incubating overnight at -20ºC. The following day, precipitated recombinant (r) protein was harvested by centrifugation at 10,000xg for 30min, acetone was decanted, and pelleted protein was dried completely at room temperature. After drying, protein was resuspended in 50µl of PBS. Both the concentrated media and cell lysates were subjected to western blot analysis for recombinant (r) proteins rAAS4 and rAAS8 expression, detected using horseradish peroxidase (HRP)-conjugated antibodies to the carboxy-terminus histidine tag (Life Technologies). After confirming expression, transfected cells were cultured to confluence in a T75 flask. Once confluent, positively transfected cells were selected by supplementing SF900 II SFM media with blasticidin at 50µg/mL. Selected cells were

cultured to confluence in medium containing blasticidin at 10µg/mL. At confluence, media was collected and cells sub-cultured to new T75 flasks every two days. Media was concentrated using Jumbosep columns with 10K membrane inserts (Pall Corporation, Port Washington, NY, USA). Concentrated protein was dialyzed against PBS in the same column. Dialyzed protein was diluted with 2x affinity purification binding buffer (100mM Tris-HCl/500mM NaCl/5mM Imidazole, pH 7.4) to a 1x concentration and filtered through a 0.2µM filter in preparation for purification.

Expression of recombinant AAS4 & AAS8 in X-33 yeast cells

Cloning of the AAS4 and AAS8 ORFs using the *Kpn*I and *Sac*II restriction sites for both AAS4 and AAS8into pPicZα-A expression plasmids and transformation of *Pichia pastoris* X-33 cells (Cereghino and Cregg, 2000) with pPicZα-A-AAS4 and pPicZα-A-AAS8 was previously performed in the lab by Tae Kim (unpublished), as specified by the manufacturer (Invitrogen, Carlsbad, CA, USA). After confirming successful expression and determining optimal culture time, large-scale expression was performed in this dissertation. First, a primary seed was grown by inoculating 10mL of Buffered Glycerol Complex Medium (BMGY) from successfully expressing colonies and incubated overnight at 28ºC in an incubator/shaker shaking at 250rpm. The following day a secondary seed was inoculated by combining the 10mL culture with 250mL of BMGY and grown a second night under the same conditions. On the third day, cells from the overnight culture were separated from media by centrifugation and resuspended to Buffered Methanol Complex Medium (BMMY) as previously described.

Cultures in BMMY were incubated for five days as determined optimal by pilot expression. On the fifth day, cells were separated from media by centrifugation and discarded. Media was concentrated by ammonium sulfate precipitation to an 80% saturation overnight at 4ºC (Jiang et al., 2004). Concentrated protein was pelleted by centrifugation and resuspended in PBS. The resuspension was dialyzed against PBS, diluted with 2x affinity purification binding buffer (100mM Tris-HCl/500mM NaCl/5mM Imidazole, pH 7.4) to a 1x concentration and filtered through a 0.2µM filter in preparation for purification.

Affinity purification of rAAS4 and 8

Expressed rAAS8 from both systems, and rAAS4 from X-33 were affinity-purified using the affinity chromatography His Trap columns (GE Healthcare Life Sciences) under native conditions (Hoffman and Roeder, 1991; Schmitt et al., 1993). A preliminary pilot expression was performed to determine optimal purification conditions. First, the purification column was washed with 5mL deionized, autoclaved water to flush out storage solution. The column was then charged by binding 5mL 100mM NiCl2 to the chromatography matrix inside the column followed by 25mL 1X binding buffer (20mM Tris, 1M NaCl, 10mM imidazole, pH 7.4) to optimize column conditions for protein binding. Next, rAAS4 and rAAS8 diluted in an equal volume of 1X binding buffer were applied to the column. The column was then washed with a series of buffers with increasing concentrations of imidazole: 5mM, 25mM, 50mM, 100mM, 300mM, and 500mM. All volumes passing out of the column following application (buffer and any

unbound protein) were designated as ‘eluates.’ Eluates following protein and wash buffer applications were subjected to western blot analysis with rAAS4 and 8 elutions detected using antibodies to the histidine-tag. After determining optimal elution conditions, large scale expression was performed until all rAAS4 and 8 was purified. Eluted rAAS4 and 8 were concentrated on a Microsep column (Pall Corporation, Port Washington, NY, USA) and dialyzed on the same column to 20mM Tris-HCl/150mM NaCl, pH 7.4. Dialyzed protein was quantified using the BCA kit (Thermo Fisher Scientific, Waltham, MA, USA) (Hill and Straka, 1988) in which light emissions from samples of known BSA concentrations are used to plot a regression line and readings from samples of unknown concentration are used in the regression equation to calculate the unknown concentration values. The microplate protocol for this kit was used and samples were read on the Synergy H1 plate reader (BioTek Instruments Inc., Winooski, VT, USA). A pure rAAS4 product was verified by SDS-PAGE with silver staining, which has a high sensitivity to non-specifically detect proteins (Merril et al., 1984). Using this staining method, any contaminating proteins will be detected. Western blot analysis using antibodies to the histidine tag (Pogge von Strandmann et al., 1995) was performed to confirm observed band is the expected recombinant protein.

Deglycosylation assays

Sequence analysis of AAS4 and AAS8, using the NetNGlyc 1.0 server, showed there are two N-glycosylation sites predicted for this protein (Mulenga et al., 2007). To determine if rAAS4 and rAAS8 are N-glycosylated in the recombinant Sf9 and X-33

expression systems, deglycosylation treatments were conducted using PNGase F or deglycosylation enzyme mix. **P**eptide **N**-**g**lycosidase **F** (PNGase F) was first reported from *Flavobacterium meningosepticum* as an enzyme that cleaves oligosaccharide chains that are asparagine (N)-linked (Plummer et al., 1984). The deglycosylation mix contains PNGase F and four further enzymes: *O*-Glycosidase, Neuraminidase, β1-4 Galactosidase, and β-N-acetylglucosaminidase. The activity of these four enzymes results in the removal of saccharides linked to either serine or threonine amino acids.

For the deglycosylation mix assay, ~5µg of rAAS4 and 8 in 9µl aliquots were combined with 1µl of 10X Glycoprotein Deglycosylation Buffer in a 0.2mL Eppendorf tube and incubated at 100ºC for 10mins. The reaction was then cooled on ice, followed by the addition of 2.5µl of 10X G7 reaction buffer, 2.5µl of 10%NP40, 9µl of deionized, autoclaved water, and 1µl of deglycosylation enzyme mix or water for the control reaction. Reactions were mixed gently and centrifuged in a microfuge to collect liquids to the bottom of the tube. Reactions were incubated for four hours at 37ºC in the Thermal Cycler System 9700 (Applied Biosystems, Foster City, CA). For the PNGase F assay, similar concentrations of rAAS4 and rAAS8 as above were mixed with 1µl of Glycoprotein Denaturing Buffer in a 0.2mL Eppendorf tube and incubated at 100ºC for 10mins. The reaction was then cooled on ice, followed by the addition of 2µl of 10X G7 reaction buffer, 2µl of 10% NP40, 5µl of deionized, autoclaved water, and 1µl of PNGase F enzyme (New England Biolabs, Ipswich, MA, USA) or water for the control reaction. Reactions were mixed gently and centrifuged in a microfuge to collect liquids to the bottom of the tube. Reactions were incubated for one hour at 37ºC in the Thermal

Cycler System 9700 (Applied Biosystems, Foster City, CA). Results were analyzed by SDS-PAGE and western blot analysis using antibodies to the histidine tag.

Screening against tick-exposed rabbit antibody

The criteria for selecting protein antigens for use as an anti-tick vaccine are that the protein be secreted into the vertebrate host and that it be immunogenic. To determine if AAS4 and AAS8 were secreted and immunogenic, western blotting analysis was performed using rabbit antibodies to tick saliva proteins from *A. americanum* that were fed for 48h. These antibodies were generated in New Zealand white rabbits that were repeatedly infested for up to 48h (Chalaire et al., 2011). Ticks were allowed to attach for 48h before being manually detached, and replaced with unfed ticks in a process that was repeated until an antibody response in rabbits was confirmed. Affinity-purified rAAS4 and rAAS8 were electrophoresed on a 10% sodium-dodecyl polyacrylamide gel (SDS-PAGE) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking the membrane with 0.05% skim milk diluted in 1X phosphate-buffered saline (PBS)/0.05% TWEEN detergent, the membrane was cut with a razor blade to separate it into individual lanes containing recombinant protein. Lanes were incubated in rabbit antibody to tick saliva protein or pre-immune sera at various dilutions: 1:25, 1:50, or 1:100 for rAAS8 and 1:50, 1:250, and 1:1000 for rAAS4. Membrane strips were incubated for 1h at room temperature. Subsequently membranes were washed in PBS-tween (three times at 5 min each) to remove unbound antibodies. Washed membranes were incubated in horseradish peroxidase (HRP)

conjugated goat anti-rabbit IgG (Millipore, Billerica, MA, USA) diluted in blocking buffer for 1h at RT. After one hour of secondary antibody binding, membranes were washed of any excess antibody and then exposed to 1X 3,3'-diaminobenzidine tetrahydrochloride (DAB) and Stable Peroxide Substrate Buffer (Kruger 1994) (DAB Substrate Kit) (Thermo Fisher Scientific, Waltham, MA, USA) which reacts with HRP to create a brownish stain on the membrane, or developed using the Amersham ECL Western Blotting Detection Kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Heparin binding

Comparative secondary structure modeling predicted that AAS8 had basic patches capable of binding heparin (Mulenga et al., 2007). Therefore, to determine if putative heparin biding sites on AAS8 were functional, a heparin-binding assay was performed. The assay was performed according to the heparin column manufacturer. For the assay, ~15µg of AAS8 was diluted in 5mL of binding buffer (10mM sodium phosphate buffer, pH 7.0) and filtered through a 0.45µM filter. The sample was then bound to a HiTrap Heparin HP 1mL affinity-chromatography column (GE Healthcare, Bio-Sciences, Pittsburgh, PA, USA) that was pre-washed with 10mL of binding buffer, and sample eluates were collected. The column was then washed with 10mL of binding buffer and eluates were collected. To elute any AAS8 bound to the column, 10mL of elution buffer (10mM sodium phosphate, 1.5M NaCl, pH 7.0) was passed through the column and the eluent was collected. Eluates and the eluent were concentrated on a Microsep column (Pall Corporation, Port Washington, NY, USA) and buffer exchanged

to PBS on the same column. Samples were analyzed by SDS-PAGE and western blot analysis using antibodies to the histidine tag.

Inhibitor function profiling

Some serpins have been described that serve non-inhibitory roles, such as chaperone or transport functions (Law et al., 2006) and some serpins inhibit proteases not of the serine catalytic type. Therefore, the purpose of this assay was to assess whether or not AAS4 and AAS8 function as inhibitors of serine proteases. Purified rAAS4 and AAS8 were screened against a panel of proteases as listed in Table 6. This list comprises 16 serine proteases and one cysteine protease with known functions in blood clotting, platelet aggregation, and/or other vertebrate defense pathways. For this assay, 1μM (~5μg) rAAS4 or rAAS8 in 20mM Tris/150mM NaCl, pH7.4 was pre-incubated with each protease, with final protease concentrations as listed in Table 6. The reaction buffer (20mM Tris/150mM NaCl/0.1% Bovine Serum Albumin (BSA), pH7.4) was added to the reaction up to a volume of 80μl. BSA is added to the buffer to stabilize the proteases. After vortexing briefly, reactions were incubated for 15 min at 37°C and then aliquoted into in a 96-well plate. Chromogenic substrate at concentrations indicated in Table 6, were added to the reaction to 200mM final substrate concentration. The appropriate substrate was added to all reaction wells simultaneously using a multi-channel pipette. Reactions were immediately read using the Synergy H1 plate reader (BioTek Instruments Inc., Winooski, VT, USA) at A_{405nm} every 30 seconds for 30 minutes at 37°C. Raw data was exported and analyzed in Prism 6 software (GraphPad

Table 6: List of vertebrate proteases against which AAS were screened

Protease	Final Concentration	Activity	Protease	Final Concentration	Activity
Trypsin (Bovine Pancreas)	21.01 nM	No	Thrombin (Bovine Plasma)	2.71 nM	No
α-Chymotrypsin (Bovine Pancreas)	2 nM	No	Factor VIIa		No
Plasmin (Human Plasma)	11.63 nM	No	Factor IXa (Bovine)	31.44 nM	No
Elastase (Porcine Pancreas)	6.18 nM	No	Factor Xa	4.65 nM	No
Chymase (Human Recombinant)	4.50 nM	No	Factor XIa (Human)	3.69 nM	No
Proteinase 3 (Human Neutrophil)	779.31 nM	Yes	Factor XIIa (Human, α-)	2.03 nM	No
Cathepsin G (Human)	216.67 nM	Yes	Urokinase (Human Recombinant)	9.26 nM	No
Papain	2.14 μM	No	Tissue Plasminogen Activator (Human)	4.73 nM	No
Kallikrein (Porcine Pancreas)	19.05 nM	No			

Software, La Jolla, CA, USA). Residual protease activity was calculated using the Michaelis-Menten kinetics equation: V_{max} (V_h)/V_{max} (V_0) versus inhibitor $100 - V_{max}$ (V_i)/V_{max} (V_0) × 100 where V_{max} (V_i) = protease activity in

The effect of rAAS4 on platelet aggregation time was assessed using the purified platelets method. Fresh rabbit blood was collected into 8.5 mL vacutainers containing 1.5 mL anti-coagulant acid citrate dextrose solution (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 200xg for 20 min at 18°C. The platelet-rich plasma top layer was decanted into 15mL conical tubes and centrifuged at 800xg for 20 min at 18°C. Supernatant was discarded and pellet was washed in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1% glucose, 0.25% BSA). The pellet, containing platelets, was resuspended in Tyrode buffer to an OD650 of 0.05. In 50µl reactions, rAAS4 alone, rAAS4 with cathepsin G, or cathepsin G alone were incubated at 37°C for 15 min followed by the addition of platelets pre-warmed at 37°C for 5 min. Clotting time was measured on the Synergy H1 plate reader (BioTek Instruments Inc., Winooski, VT, USA) at OD650 for 15 mins at 37°C. Several concentrations of rAAS4 were assayed: 2µM, 1µM, 0.5µM, and 0.25µM. The platelet aggregation agonist, cathepsin G, was used at a concentration of 0.6µM. Readings of optical density (O.D.) were fitted to the one-phase decay equation in PRISM (GraphPad) to determine the O.D. plateau. The percent reduction in platelet aggregation was calculated using the following formula: $Y = 100 - (\Delta Pn)/\Delta PC) * 100$, where Y = % is the reduction in platelet aggregation, ΔPn and ΔPC = a respective rAAS-treated and positive

control plateau O.D. subtracted from O.D. of the blank plateau. Data are presented as mean ± S.E.M. of duplicated platelet aggregation assays.

Effects on blood clotting

The effect of rAAS4 and rAAS8 on blood coagulation time was assessed using four assays: recalcification time assay (RCT), which measures effects on blood clotting factors in their entirety, prothrombin time (PT), which measures effects on clotting factors important to the extrinsic blood clotting activation pathway, activated partial thromboplastin time (aPTT), which measures effects on factors in the intrinsic blood clotting activation pathway, and thrombin time (TT), which measures effects on the common blood clotting activation pathway as previously described (Mulenga et. al., 2013a, 2013b).

In the RCT assay, the reaction volume was adjusted to 200µL with Tris-HCl reaction buffer (20mM Tris-HCl, 150mM NaCl, pH 7.4) and mixed with 50µL universal human reference plasma (Thermo Fisher Scientific, Waltham, MA, USA) pre-incubated for 15 minutes at 37°C with or without 1 µM rAAS4 or rAAS8 in a 96-well microplate prior to the addition of 10µL of 150mM calcium chloride (pre-warmed) to induce clotting. RCT was determined using the Synergy H1 plate reader (BioTek Instruments Inc., Winooski, VT, USA) at A_{405nm} every 20 seconds for 20 minutes at 37°C.

The PT, aPTT, and TT assays were performed according to instructions by the Pacific Hemostasis manufacturer of the clotting activation reagent with modifications (Thermo Fisher Scientific, Waltham, PA). For the PT assay, the reaction volume was

adjusted to 200µL with Tris-HCl reaction buffer, and 100µL thromboplastin (Pacific-Hemostasis-Prothrombin Time Reagent; Thermo Fisher Scientific, Waltham, MA, USA) was pre-incubated with or without 1 µM rAAS4 or rAAS8 at 37°C for 15 min before adding 50µL universal human reference plasma. For the aPTT assay, the reaction volume was adjusted to 200µL with Tris-HCl reaction buffer and 50µL universal human reference plasma was pre-incubated with 1 µM rAAS4 or rAAS8 at 37°C for 15 min before adding 50µL aPTT reagent (Thermo Fisher Scientific, Waltham, MA, USA) followed by the addition of 50µL of 25mM calcium chloride to induce clotting. For the TT assay, the reaction volume was adjusted to 100µL with Tris-HCl reaction buffer and 25µL of thrombin time reagent (Thermo Fisher Scientific, Waltham, MA, USA) was pre-incubated with or without 1 µM rAAS4 or rAAS8 at 37°C for 15 min. Following incubation, 50µL universal human reference plasma was added to the reaction. PT, aPTT, and TT assays for rAAS8 were monitored and recorded using the KC1 Delta semi-automated coagulation analyzer (Trinity Biotech, Parsippany, NJ). PT, aPTT, and TT assays for AAS4 were monitored and recorded using the Synergy H1 plate reader (BioTek Instruments Inc., Winooski, VT, USA) where an increase in OD is directly proportional to an increase in clotting.

Results

Recombinant (r) AAS4 and rAAS8 are ~50kDa glycoproteins

To test the hypothesis that AAS4 and AAS8 are functional serpins involved in host immune system counter-defense at the tick feeding site, the recombinant (r) proteins

were expressed for functional assays. Figure 7 summarizes the cloning of AAS4 and AAS8 ORFs into pIB-V5-His, the expression of recombinant (r) AAS8 in Sf9 cells, and the expression of rAAS4 and rAAS8 in X-33 cells. While both AAS4 and AAS8 expression constructs were successfully ligated into the insect cell expression vector pIB-V5 (Figure 7a and b) only rAAS8 was successfully expressed in the insect *S. frugiperda* Sf9 eukaryotic expression system (Figure 7c), while rAAS4 could not be expressed in the insect cell system. Prior to this dissertation, both rAAS4 and rAAS8 were successfully cloned and expressed in yeast *Pichia pastoris* X-33 cells. In this

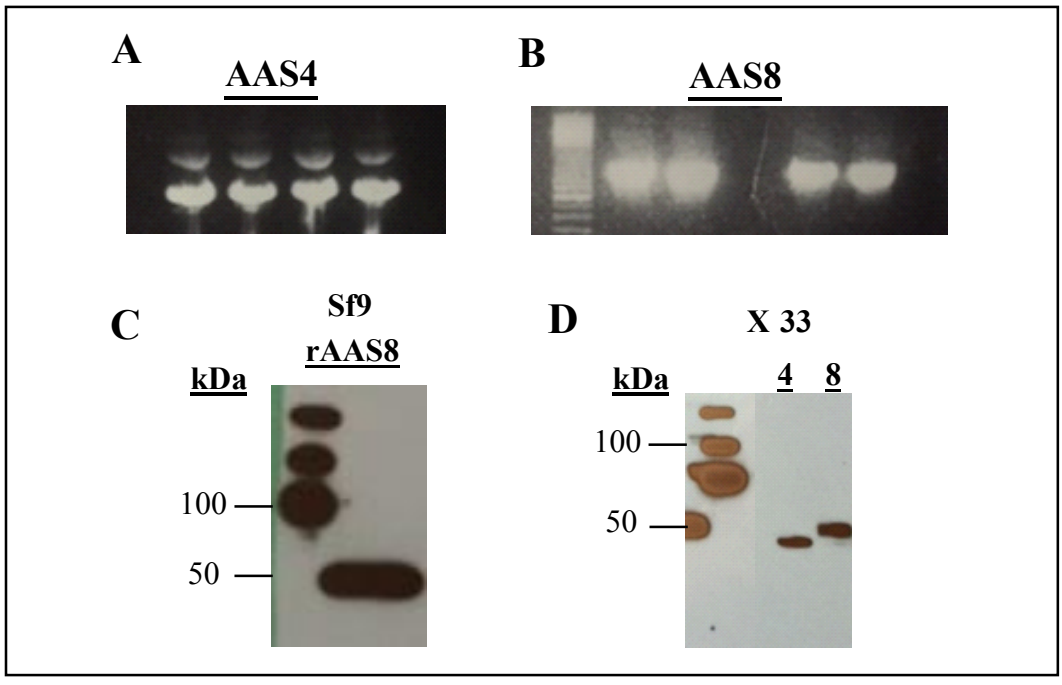

Figure 7. Cloning and expression of rAAS4 and rAAS8. Panel A and B show PCR verification of AAS4 and AAS8 construct successful ligation into pIB-V5 expression vector for insect cell expression. Panel C shows chemiluminescence detection of insect cell Sf9-expressed rAAS8 using anti-His tag antibodies. Panel D shows chemiluminescence detection of yeast cell X-33-expressed rAAS4 and rAAS8 using anti-His tag antibodies.

dissertation, experiments were performed with X-33-expressed rAAS4 (since Sf9 expression failed), and both X-33- and Sf9-expressed rAAS8 (Figure 7c and d). Both rAAS4 and rAAS8 were successfully affinity-purified as revealed by silver and Coomassie brilliant blue staining and western blot analysis using antibody to the histidine tag (Figure 8a and b; Figure 9a and b). Mature AAS4 (GenBank ABS87356.1) and AAS8 (GenBank ABS87360.1) are 381 and 375 amino acid residues long, respectively, with a calculated molecular weight (Mw) of 41.422 and 41.362 kDa,

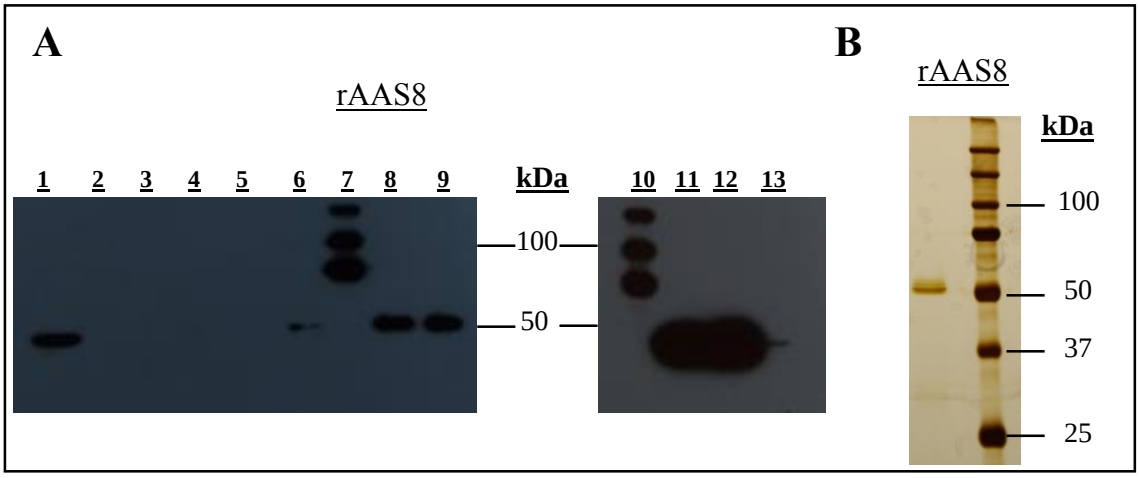

Figure 8. Affinity-purification of rAAS8. Panel A shows a silver stained gel of affinity-purified rAAS8. The figure shows that rAAS8 elutes from the affinity-purification as a single band at a molecular weight (Mw) of ~50 kDa, which is much higher that the calculated Mw for rAAS8 of 41.362 kDa. Panel B shows western blot analysis of rAAS8 affinity-purification wash and elution eluates using anti-His tag antibody. Lanes 7 and 10 are marker lanes. Lane 1 is pre-purified protein. Lane 2 is column eluate after applying rAAS8. Lanes 3-6 are 5, 25, 50, and 100 mM imidazole washes. Lanes 8, 9, and 11-13 are the five serial fractionated eluates from the 300 mM imidazole wash. Western blot detection is very sensitive and detected elution of rAAS8 not only in the 3rd, 4th, and 5th fractions of the 300 mM imidazole washes, but also in the 1st and 2nd fractions, and in the 100 mM imidazole wash.

respectively (Mulenga et al., 2007). In the pIB-V5 expression system ~3 kDa is added due to the polyhistidine tag, and in the pPicZα-A expression system ~2.5 kDa is added due to the polyhistidine tag. This increases the predicted Mw to 43.9 kDa for yeast-expressed rAAS4, and 43.862 and 44.362 kDA for yeast-expressed and insect cell-expressed rAAS8, respectively. In Figures 7 and 8, both rAAS4 and rAAS8 migrated at around 50 kDa (Lanes 1, 6, 8, 9, 11, and 12 for AAS8; Lanes 2, 5, and 8), which is higher than the calculated Mw, even with the tag. For rAAS4, a second band was also

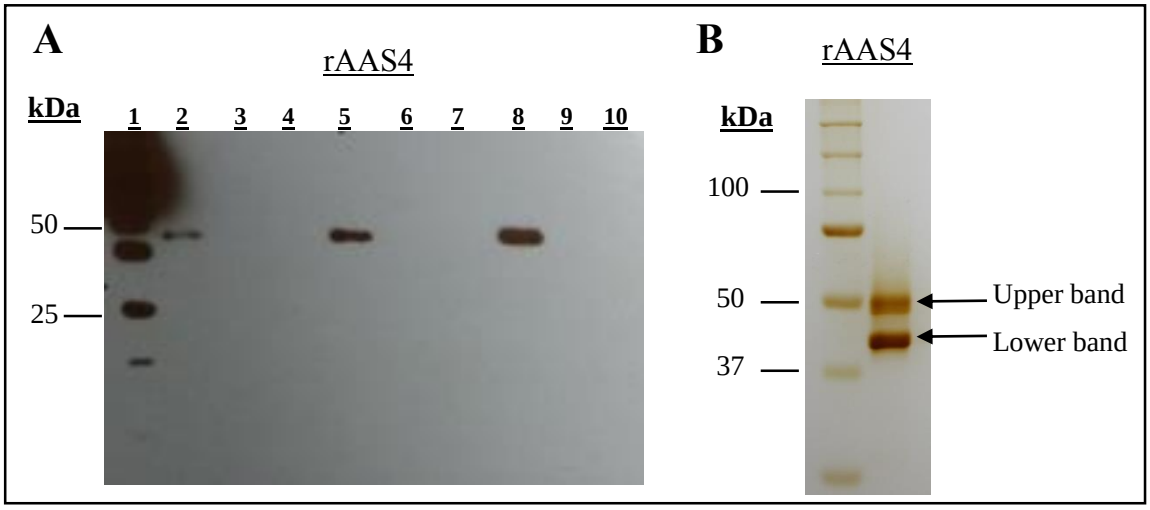

Figure 9. Affinity-purification of rAAS4. Panel A shows western blot analysis of rAAS4 affinity-purification wash and elution eluates using anti-His tag antibody. Lanes 1 is a marker lane. Lane 2 is pre-purified protein. Lane 3 is column eluate after applying rAAS4. Lanes 3-5 are 5, 50, and 100 mM imidazole washes. Lanes 6-10 are the five serial fractionated eluates from the 300 mM imidazole wash. Note that rAAS4 elutes in the 100 mM imidazole wash and in the 3rd fraction of the 300 mM wash. Panel B shows a silver stained gel of affinity-purified rAAS4. The figure shows that rAAS4 elutes from the affinity-purification column as two bands, one at the calculated molecular weight (Mw) for rAAS4 (~41.422 kDa) and another band at ~50kDa.

consistently observed below the 50 kDa band, as shown in Figure 9b. Previous NetNGlyc analysis revealed three and two putative N-linked glycosylation sites in AAS4 and AAS8, respectively (Mulenga et. a., 2007). Additionally, the affinity-purified recombinant proteins migrated at higher than expected Mws. To confirm in silico glycosylation predictions and to determine if the observed migration at ~50kDa for the recombinant proteins was due to N- or O-linked glycosylation post-translational

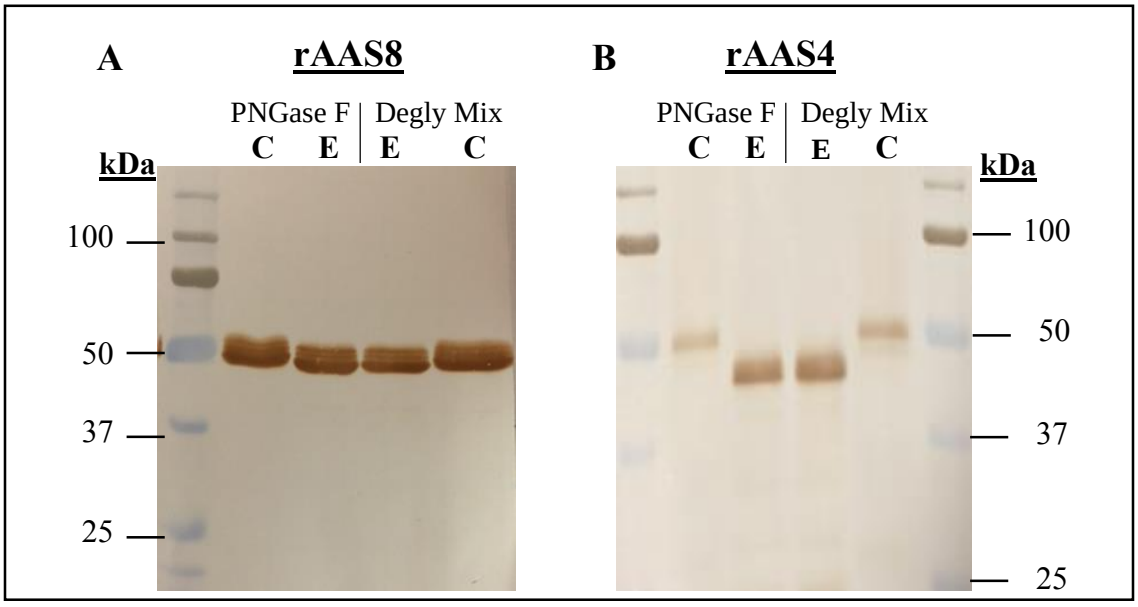

Figure 10. Deglycosylation of rAAS4 and rAAS8. Panel A shows the deglycosylation of rAAS8 and Panel B shows the deglycosylation of rAAS4. Reactions were with PNGase F enzyme (E) or control without enzyme (C) and deglycosylation mix with enzyme (E) or control without enzyme (C). The figures shows removal of N-linked sugar moeities from rAAS4 and 8 by PNGase F as seen by the downward molecular weight (Mw) shift, and no further downward shift in Mw with N- and O-linked sugar moeity removal using deglycosylation mix. These results indicate that there is no O-linked glycosylation of rAAS4 or rAAS8. The more pronounced downward shift in Mw for rAAS4 after PNGase F treatment supports the prediction of three N-linked glycosylation sites for this protein over the two predicted N-linked glycosylation sites for AAS8. The moderate downward shift in Mw for rAAS8 indicates an incomplete deglycosylation or that other post-translational modifications are present.

modification, affinity-purified rAAS4 and rAAS8 were subjected to deglycosylation assays. As predicted by NetNGlyc 1.0, Figure 10a and b shows that both rAAS4 and rAAS8 decrease in molecular weight (Mw) when treated with PNGase F, which removes N-linked sugar moieties, as compared to the control reaction with no PNGase F. The decrease in Mw for rAAS8 was a very moderate, while for rAAS4 the Mw shift was very dramatic (Figure 10a and b). Similarly, both rAAS4 and rAAS8 decrease in Mw when treated with deglycosylation mix, which removes both N- and O-linked sugar moieties. However, there is no additional downward Mw shift when treated with deglycosylation enzyme mix as compared to the PNGase F treatment showing that rAAS4 and rAAS8 do not have O-linked glycans (Figure 10a and b). These data confirm the in silico predictions of N-linked glycosylation sites for AAS4 and AAS8 (Mulenga et al., 2007). These data also confirm that for rAAS4, the migration at 50 kDa is due to N-linked glycosylatinon, while for rAAS8 N-linked glycosylation only partially explains the higher than expected migration of this recombinant protein.

Both AAS4 and AAS8 are immunogenic and injected into the host during tick feeding

To determine if AAS4 and AAS8 are immunogenic proteins and whether or not native AAS4 and AAS8 are injected into the host during tick feeding, rAAS4 and rAAS8 were subjected to western blot analysis using rabbit antibodies to tick saliva proteins as summarized in Figure 11a and b. Both rAAS4 and rAAS8 specifically bound antibodies from rabbits repeatedly parasitized by *A. americanum* ticks for up to 48h, at 1:1000 and 1:100 dilutions, respectively. However, for rAAS4, it was observed that rabbit antibodies

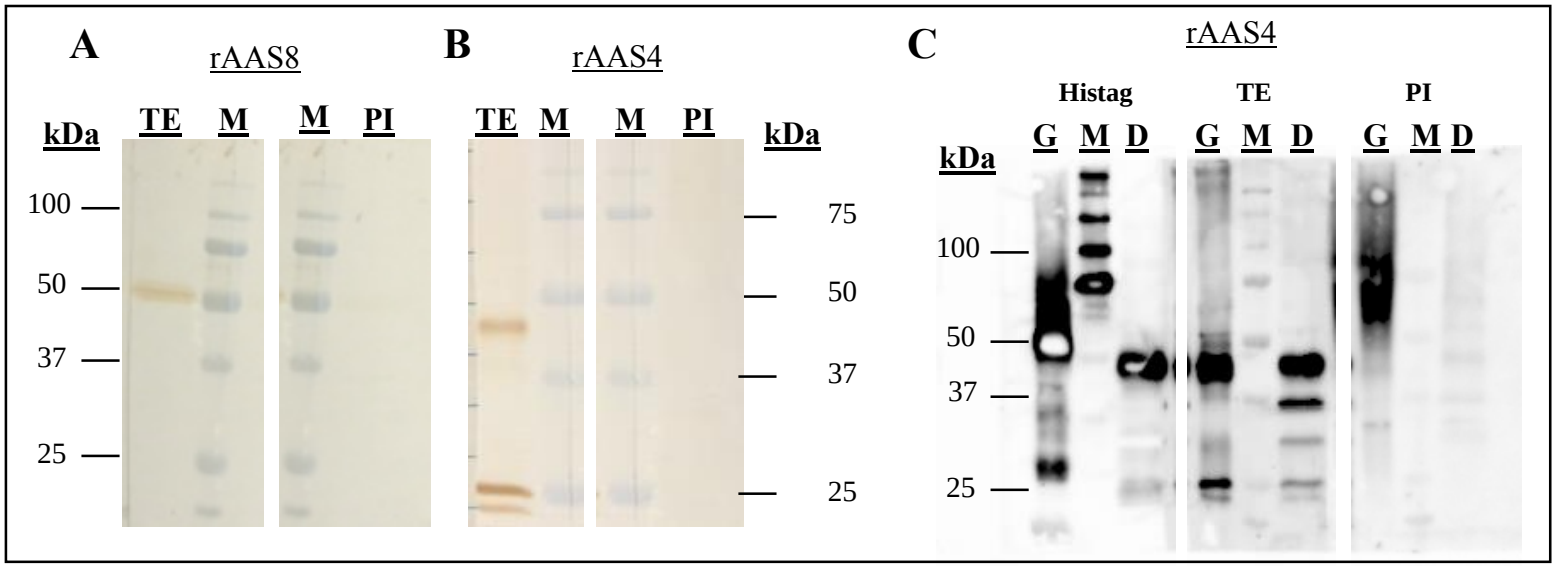

Figure 11. Binding to rAAS4 and rAAS8 of rabbit antibody to tick saliva proteins. Panel A shows binding to rAAS8 of antibody from tick-exposed rabbits (TE) at a dilution of 1:100, while antibody from pre-immune rabbits (PI) at the same dilution did not bind rAAS8. The marker is indicated (M). Panel B shows binding to rAAS4 of antibody from tick-exposed rabbits (TE) at a dilution of 1:1000, while antibody from pre-immune rabbits (PI) at the same dilution did not bind rAAS4. TE antibody bound to a band corresponding in molecular weight (Mw) to the non-glycosylated form of the recombinant protein, therefore the assay was repeated with both glycosylated (G) and de-glycosylated (D) rAAS4, Panel C. Using his-tag antibody the bands corresponding to glycosylated and de-glycosylated rAAS4 can be compared to bands where TE antibody bound to rAAS4. TE antibody bound only to a band at the same Mw as de-glycosylated rAAS4 and not to a band at the same Mw as glycosylated rAAS4, indicating ticks secrete only non-glycosylated AAS4 into the feeding site.

to tick saliva proteins bound the lower Mw protein band that is similar in size to deglycosylated rAAS4 shown in Figure 10b. To verify these data, both glycosylated and de-glycosylated rAAS4 were subjected to western blot analysis using antibodies the histidine tag and antibodies to tick saliva proteins. As shown in Figure 11c, the antibody to the histidine tag bound the diffuse protein band (50-75kDa) in glycosylated (G) lane and a single protein (under 50 kDa) in the de-glycosylated rAAS4 lane (D). Interestingly, rabbit antibodies to tick saliva proteins did not bind the high Mw diffuse protein band, rather it bound a single protein band in glycosylated (G) similar in Mw to the de-glycosylated form (D).

rAAS4 and AAS8 inhibit proteases in inflammation pathways

To test the hypothesis that AAS4 and AAS8 function in counter-defense against the host immune system, affinity-purified rAAS4 and rAAS8 were subjected to inhibitor function profiling against the proteases listed in Table 6. Proteases were chosen based on their known roles in host defense pathways including blood coagulation and platelet aggregation, inflammation, and other immune system pathways. The inhibitory activities of rAAS4 and rAAS8 at 1 μM final concentration were assessed against proteases at concentrations indicated in Table 6. For rAAS8, inhibitory activity was not detected for 15 of 17 proteases however, inhibitory activity was detected for two inflammatory proteases, proteinase 3 and cathepsin G, as shown in Figure 12. Proteinase 3 was moderately inhibited (41.13 ± 7.5%) by rAAS8, as was cathepsin G by 14 ± 9.33%. No inhibitory activity was detected for 14 of 17 proteases by rAAS4,

however, inhibitory activity was detected for three proteases: papain, chymase, and cathepsin G as shown in Figure 13. Inhibition of papain was most pronounced at 67 ± 1.08%, followed by cathepsin G by 56.30 ± 4.48%, while inhibition of chymase was the lowest at 31.54 ± 3.65%. Both chymase and cathepsin G are involved in the vertebrate inflammatory response.

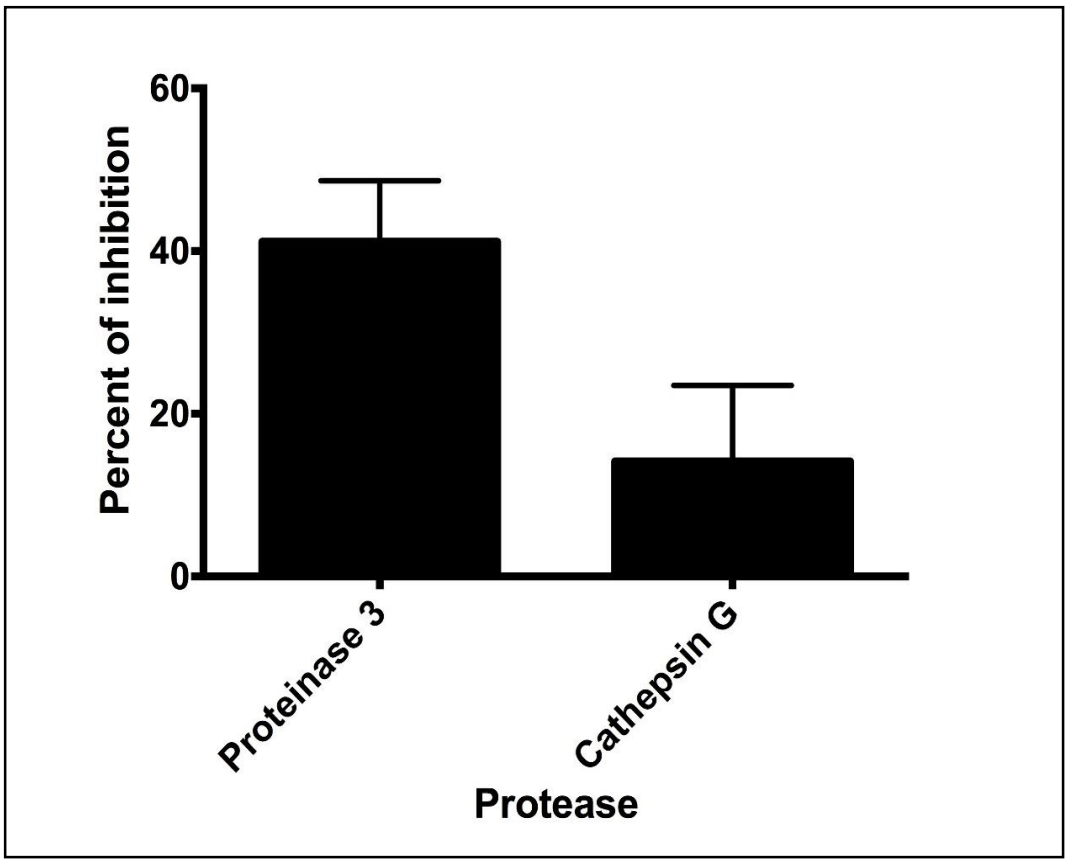

Figure 12. Inhibition of proteinase 3 and cathepsin G by rAAS8. The figure shows that rAAS8 inhibits proteinase 3 at 41.13±7.5%, and human cathepsin G at 14±9.33%. Each reaction was 100 µl in volume with 1µM final concentration of rAAS8, proteinase 3 at a final concentration of 779.31 nM, and cathepsin G at a final concentration of 216.67 nM. Assays were performed in triplicate and data are shown as mean values from triplicate

Recombinant AAS8 binds to heparin

Heparin is a polysaccharide that can bind to serpins and change their inhibitory properties (Bjork and Lindahl, 1982; Radulović et al., in preparation). Data in this dissertation indicate AAS8 is among the proteins injected by ticks into the feeding site

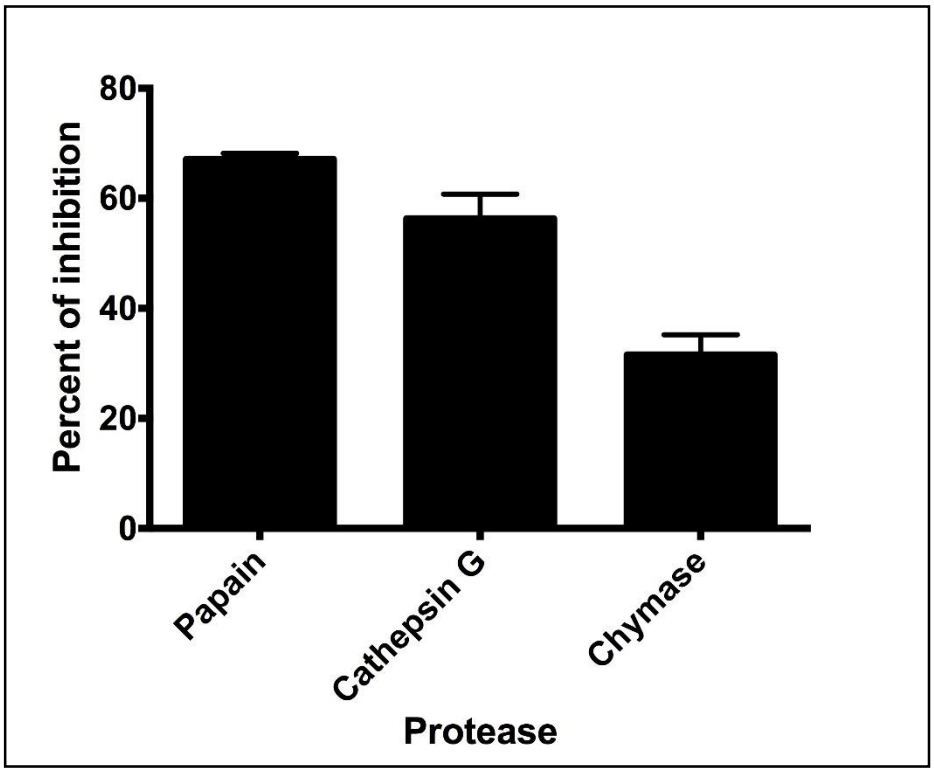

Figure 13. Inhibition of papain, cathepsin G, and chymase by rAAS4. The figure shows that rAAS4 inhibits papain at 67±1.08%, human cathepsin G at 56.30±4.48%, and human chymase at 31.54±3.65%. Each reaction was 100 µl in volume with 1 µM final concentration of rAAS4 with papain at a final concentration of 2.14 µM, cathepsin G at a final concentration of 216.67 nM, and chymase at a final concentration of 4.50 nM. Assays were performed in triplicate and data are shown as mean values from triplicate assays.

where they would be exposed to blood and blood components such as heparin. Since exposure to heparin may affect the function of AAS8 and since previous predictions in our lab indicated that AAS8 may have heparin binding sites (unpulished), the ability of rAAS8 to bind heparin was tested. As shown in Figure 14, rAAS8 was detectable in the elution buffer that passed through the heparin column, indicating that the protein bound heparin. As expected, rAAS8 was not detectable in wash buffer that passed through the column nor in the buffer that passed through the column when rAAS8 was applied. This

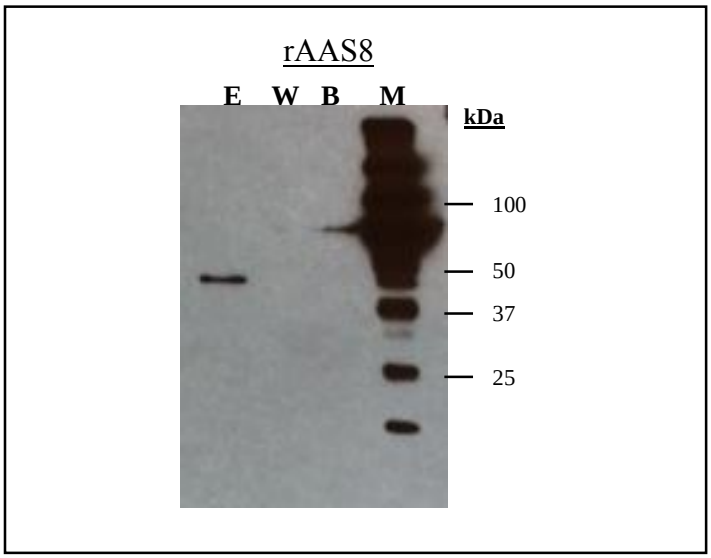

Figure 14. Binding of rAAS8 to heparin. The figure shows chemiluminescence detection of rAAS8 in protein application, wash, and elution eluates from a heparin column. Lane 1 is the eluent, lane 2 is the wash buffer after rAAS8 binding, and lane 3 is eluate from binding rAAS8 to the heparin column. Detection of rAAS8 occurred only in elution eluate showing that all rAAS8 applied to the column was bound and eluted only after application of elution buffer.

shows that all rAAS8 that was applied to the column was retained inside the column and eluted out of the column only with the application of elution buffer. Interstingly, it has been shown that serpin affinity for heparin binding is at least in some cases dependent on extent of serpin glycosylation (McCoy et al., 2003) therefore it will be interesting to determine if native AAS8 is also glycosylated to the extent of rAAS8.

AAS4 and AAS8 do not affect the blood coagulation cascade, but AAS4 moderately inhibits cathepsin G-induced platelet aggregation

To test the effect of rAAS4 and rAAS8 on anti-hemostasis, four blood coagulation assays were performed: Recalcification Time (RCT), Prothrombin Time (PT), Thrombin Time (TT), and Activated Partial Thromboplastin Time (aPTT). However, there was no effect on blood clotting time (rAAS8) or plasma clotting time (rAAS4) when rAAS4 and rAAS8 were added to the reaction as compared to controls. Similarly, there was no difference in platelet aggregation between reactions with rAAS8 and controls. Taken together, these data indicate that rAAS8 may not function in anti-hemostasis. However, a difference in platelet aggregation time was observed for reactions with added rAAS4 as compared to controls. As shown in Figure 15, cathepsin G-induced platelet aggregation was reduced by 60.22%. These data suggest that AAS4 but not AAS8 inhibit cathepsin G strongly enough to affect platelet aggregation. The observed inihibition of platelet aggregation by rAAS4 as shown in Figure 15 is consistent with inhibitor function profiling for this protein (Figure 13) where rAAS4 inhibited cathepsin G activity by 56.5%.

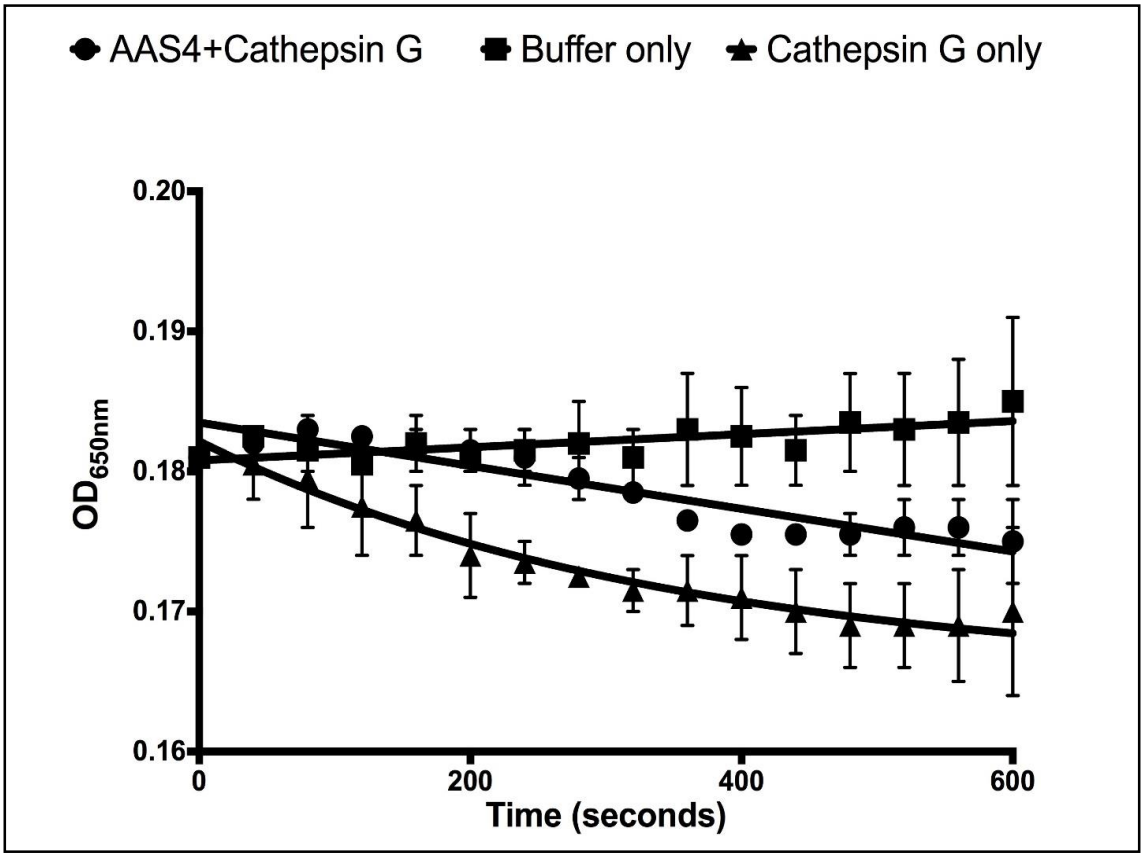

Figure 15. Effect of rAAS4 on platelet aggregation. The figure shows that rAAS4 at 1μM final concentration inhibits cathepsin G-induced platelet aggregation by 60.22%. In the assay, cathepsin G at a final concentration of 0.7 μM was used to induce clotting of purified platelets in a 50 μL reaction, with or without rAAS4. Platelet aggregation was measured on a plate reader at an OD of 650nm for 10 min at 37ºC and was performed in duplicate. Data are presented as the mean ± S.E.M. of duplicated assays.

Discussion

Post-translational modifications (PTMs) including glycosylation may be critical for proper protein functionality. As a result, eukaryotic expression systems such as the *S. frugiperda* Sf9 insect cell and *P. pastoris* X-33 yeast cell expression systems are the preferred choice for recombinant expression of eukaryotic proteins. Indeed, the

observation of rAAS4 and rAAS8 running at higher than calculated molecular weights combined with the Mw shifts in the deglycosylation assay results show that rAAS4 and rAAS8 are post-translationally modified in these expression systems. However, unexpectedly, after deglycosylation treatment rAAS8 still did not migrate within the range of the calculated Mw for this protein. This could be a result of incomplete deglycosylation since PNGase F and the deglycosylation mix cannot always remove all glycans, or could be an indication that there are other PTMs for this recombinant protein such as phosphorylation, acetylation, formylation, ubiquination, or methylation. Most of these PTMs would result in molecular weight shifts only in the Dalton range, however, ubiquitin for example is an 8.8 kDa protein and attaches to lysine residues (Parker et al., 2010), of which AAS8 has 13 in its amino acid sequence.

Also unexpectedly, antibodies to tick saliva proteins did not bind to glycosylated rAAS4 protein. Instead, antibodies bound to a band corresponding to the calculated Mw for the recombinant protein, and to a band migrating at the same Mw as the deglycosylated rAAS4 at to the lower band observed in silver-stained affinity-purified rAAS4. This lower band was confirmed by peptide mass fingerprinting to be the recombinant AAS4 (unpublished). Taken together, these data suggest that ticks secrete a non-glycosylated AAS4 during feeding. If AAS4 functions at the feeding site in a non-glycosylated form, this would be in contrast to the idea that expression in eukaryotic systems is necessary for expressing properly post-translationally modified proteins, and it may be worthwhile to repeat AAS4 assays using non-glycosylated protein. This might be accomplished by expressing the recombinant protein in a prokaryotic system such as

the *E. coli* BL21 system. Nevertheless, both AAS4 and AAS8 are immunogenic proteins that are secreted into the host during feeding as evidenced by the fact that the vertebrate host generates specific antibodies against these proteins. Furthermore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) data have also suggested the presence of both AAS4 and AAS8 in saliva of *A. americanum* ticks that fed on rabbits (Kim et al., *in preparation*). In proposing that ticks use serpins to evade host defenses, the assumption is that these serpins are injected into the host during tick feeding, therefore, these data support the hypothesis that these proteins are involved in tick feeding physiology. From the perspective of their inclusion in an anti-tick vaccine, the fact that rabbit antibodies to tick saliva proteins do not bind to AAS4 glycans is positive in that the linear peptide may be sufficient to specifically generate a protective immune response.

In silico annotations are only a first step in determining protein function. Further functional characterization is necessary, particularly for serpins, which may or may not function as inhibitors. Additionally, some serpins inhibit proteases of the cysteine catalytic type. Therefore, biological and biochemical analyses were used to determine if AAS4 and AAS8 are functional inhibitors of serine or cysteine proteases and to determine if these proteins function in counter-defense against the vertebrate immune system.

Several studies have characterized tick serpins as having anti-hemostatic function (Prevot et al., 2006; Chmelar et al., 2011; Mulenga et al., 2013b; Yu et al., 2013; Ibelli et al., 2014; Kim et al., 2015a; Xu et al., 2016). For rAAS4 and rAAS8, however, no delay

in blood clotting time was observed in PT, aPTT, TT, and RCT assays as compared to controls. While rAAS8 inhibited the serine protease cathepsin G which can initiage platelet aggregation, inhibition of cathepsin G was marginal at only 14%. Consequently, it is not surprising that rAAS8 did not affect platelet aggregation and is therefore unlikely to affect hemostasis by itself. However, in combination with other serpins that can affect platelet aggregation, such as rAAS4, hemostasis may effectively be stopped. Unfortunately from the perspective of their use in anti-tick vaccines, this would make their effect too partial for high vaccine efficacy unless administered as a mixture of serpins.

Recombinant AAS8 also bound to heparin, which is a polysaccharide of the glycosaminoglycan (GAG) family found in the secretory granules of mast cells (Rabenstein 2002), and if heparin binds to serpins, inhibitory properties of the serpin may be enhanced or even negated (Bjork and Lindahl, 1982; Radulović et al., in preparation). Heparin, through its activation of antithrombin, it is a critical player in the inhibition of the blood clotting cascade (Whisstock et al., 2000). Heparin has been shown to bind many serpins (Pratt and Church, 1993; others) and has the potential to rearrange the reactive center loop and change the activity of the serpin (Whisstock et al., 2000). Since heparin is a normal component of blood, it could impact the function of tick serpins that are injected into the feeding site. Therefore, future experiments should investigate the impact of heparin binding on the ability of AAS8 to inhibit cathepsin G and proteinase 3.

Analyses in this chapter revealed that both rAAS4 and rAAS8 have inhibitory activities against proteases associated the host's inflammation system. Interestingly, in addition to cathepsin G, AAS8 also inhibited the serine proteinase 3. Both cathepsin G and proteinase 3 are found in the granules of neutrophils, and are both in the chymotrypsin family (Korkmaz et al., 2008). Neutrophils are the first to arrive at sites of inflammation, and their proteases function in innate immunity and regulation of inflammation as well as tissue remodeling (Korkmaz et al., 2008). In humans, cathepsin G and proteinase 3 are have bactericidal properties and cathepsin G alters chemokines, cytokines, and cell receptors through proteolysis (Bank and Ansorge, 2001). For example, cathepsin G cleaves the receptor PAR4, leading to platelet activation. It is not surprising that AAS8 can inhibit both proteinase 3 and cathepsin G since they are very similar in sequence and function, having derived from a gene duplication event, and can both be inhibited by the same endogenous inhibitor α2-macroglobulin (Korkmaz et al., 2008).

In screening rAAS4, protease inhibition was detected not only for cathepsin G but also for chymase. Chymase is found in the granules of mast cells that reside in vertebrate tissues and are part of the immune arsenal against parasites (Tchougounova et al., 2005). Chymase can also activate IL-1β leading to inflammation (Mizutani et al., 1991) and is a potent chemoattractant for monocytes and neutrophils (Tani et al., 2000). Given these roles for chymase, it is easy to see why tick feeding would be facilitated by secreting inhibitors of chymase which would block vertebrate inflammation and other anti-parasitic defenses. It is not surprising that rAAS4 inhibited cathepsin G and

chymase since in humans cathepsin G and chymase are both inhibited by the serpin α-1 antichymotrypsin (Kalsheker, 1996) More interestingly, *I. ricinus* salivary protein serpin 2 (IRS-2) is also an inhibitor of both chymase and cathepsin G (Chmelar et al., 2011). This protein inhibits neutrophil inflammation in a mouse model, and like AAS4, effects cathepsin G-induced platelet aggregation. Both IRS-2 and AAS4 are found in SG, MG, and OV tissues during feeding. The functional similarity of AAS4 and IRS-2 is particularly interesting because BLASTP screening of AAS4 against non-redundant tick sequences in GenBank shows IRS-2 has a low identity to AAS4 of only 42%, with only 12/21 amino acids matching in the RCL region. These data demonstrate that amino acid identity between serpins may not be predictive of similarity in biological function. From the perspective of using *in silico* analysis for functional predictions, these data underline the importance of additional functional characterization to validate those predictions.

Interestingly, rAAS4 was a cross-class inhibitor, showing inhibition of not only serine proteases but also the cysteine proteinase papain. Papain has been shown to polarize the innate immune response to the Th2 cytokine profile (Tang et al., 2010; Pulendran and Artis, 2012), which is noteworthy because the Th2 response is induced against parasites including ticks (Mejri et al., 2001). On the other hand, several studies have investigated the Th2 polarization during tick parasitism and have interpreted this as a deliberate counter-defense mechanism by ticks (Skallova et al., 2008; Kovář et al., 2002; Ferreira and Silva, 1999). Nevertheless, the Th2 response is characterized by a cytokine profile that promotes production of the anti-parasitic antibody class, IgE (Abbas et al., 2011), therefore, it remains unclear why ticks would favor a polarization to

a Th2 response, or why ticks would simultaneously secrete promoters and inhibitors of this pathway. Future studies should investigate whether the application of AAS4, *in vitro* or *in vivo*, inhibits polarization to the Th2 profile.

Results in this chapter suggest that AAS4 and AAS8 are secreted into the host and function in counter-defense against the vertebrate immune system. As such they are candidates for further analyzing their utility in anti-tick vaccines. However, whether or not AAS4 or AAS8 are determined to be effective targets in tick control, the fact that they showed inhibition of inflammatory proteases makes them interesting proteins from a medical perspective. Of note, dual inhibitors of both cathepsin G and chymase, which is the inhibitor profile of AAS4, are considered of therapeutic value in inflammatory diseases like chronic obstructive pulmonary disorder (COPD) and asthma (Maryanoff et al., 2010). Dozens of human diseases are attributable to aberrant or hyper-active immune system function including asthma, allergies, arthritis, Crohn's diseases, and multiple sclerosis (Abbas et al., 2011). Pharmaceuticals that can be used to dampen the immune system could result in the reduction of morbidity and mortality for thousands of lives. To this end, proteins like AAS4 and AAS8 that show anti-immune function should be investigated for their potential use as immune-modulatory drugs.

CHAPTER IV

ANALYSIS OF *AMBLYOMMA AMERICANUM* SERPIN AAS4, 6, AND 8 SPLICED CONSTRUCT IN IMMUNIZATION TRIALS

Introduction

In preliminary studies, AAS4 (GenBank ABS87356.1), 8 (GenBank ABS87360.1) (*unpublished*), and 6 (GenBank ABS87358.1) (Chalaire et. al., 2011) were found in the saliva of fed female ticks, and bound to rabbit antibodies developed against tick saliva proteins. These data indicated that AAS4, 6, and 8 are injected into the host during tick feeding and are able to induce an immune response.

Several studies have already shown that immunization with recombinant serpins can confer protective anti-tick immunity. In *H. longicornis* two serpins, HLS1 and 2, have been assayed for vaccine efficacy in separate studies. Vaccination of rabbits with HLS2 resulted in 43-44.6% mortality in adult and nymphal ticks (Imamura et al., 2005); while vaccination of rabbits with HLS1 resulted in 11. 2% and 43.9% mortality in adult and nymphal ticks, respectively (Sugino et al., 2003). In a vaccine trial combining *R. appendiculatus* serpins RAS-1 and -2, ticks feeding on vaccinated cattle showed 61.4%, 28%, and 43% mortality for nymphs, females, and males, respectively (Imamura et al., 2006). When cattle were immunized with a mixture of *R. appendiculatus* serpins RAS-3, RAS-4, and a putative tick cement protein, *Rhipicephalus* Immune-dominant Molecule 36 (RIM36) (Bishop et al., 2002), mortality rates were 39.5% as compared to 12.8% in the control (Imamura 2008). In 2010, Jittapalapong et al. immunized rabbits against an

R. microplus serpin and found that adult females fed longer but weighed less on average than ticks on control rabbits that fed for shorter periods. The female ticks in this study that had fed on immunized rabbits also had 67% mortality post-engorgement without laying eggs. In another study, the efficacy of vaccination with *I. ricinus* serpin Iris showed 33.6% mortality of nymphs and reduced engorgement for nymphs and adults on immunized rabbits (Prevot et al., 2007).

In *A. americanum* challenge infestations of rabbits immunized with AAS19, 60% of ticks in a second challenge infestation failed to lay eggs, while in both first and second challenge infestations ticks feeding on immunized rabbits had smaller average engorgement weights (Kim et al., 2015b). The combined results of these studies not only demonstrates the importance of serpins in tick feeding but also shows that immunizations using recombinant serpins can confer protection against tick feeding and serve to reduce overall tick populations. To address whether AAS4, 6, and 8 are suitable candidates for inclusion in an anti-tick vaccine, the purpose of this dissertation chapter was to assess tick feeding and egg laying performance for ticks fed on rabbits previously immunized against a spliced construct of serpin putative immunogenic regions from AAS4, 6, and 8. The rationale for immunizing with a construct involving three different antigens is, (1) to attempt to synergize the anti-tick effects of the three separate antigens, and (2) to allow delivery of equimolar amounts of each of the three antigens. Furthermore the use of spliced construct proteins in subunit vaccine design is a previously validated strategy (Soria-Guerra et al., 2011).

Materials and Methods

Design and synthesis of AAS-4-6-8 spliced construct open reading frame

To design the AAS4-6-8 spliced construct (AAS4-6-8sc), the corresponding amino acid sequences were submitted to the open source Immune Epitope Database and Analysis Resource (IEDB) (http://www.iedb.org/) epitope prediction software to predict epitopes in these sequences. Amino acid sequences were first screened in SignalP version 4.1 (http://www.cbs.dtu.dk/services/SignalP/) to detect the signal peptide sequence. The corresponding amino acid sequence was removed before screening for immunogenicity. The rationale for this is that the host is exposed only to the mature protein.

Epitope prediction algorithms have been empirically validated as having high accuracy (Moutaftsi et. al., 2006). B-cell activation first requires T-cell activation, (Abbas et al., 2011) therefore, the inclusion of T-cell antigens is a strategic component to vaccine design. Thus AAS4, 6, and 8 sequences were submitted to both T- and B- cell epitope prediction algorithms. All B-cell epitopes were included in the spliced construct. For T-cell epitope predictions, every possible consecutive nine amino acid string is given a score of immunogenicity. T- and B-cell immunogenic regions were mapped onto the full AAS4, 6, or 8 amino acid sequence and regions showing low immunogenicity were removed, splicing together only the putatively immunogenic regions.

In order to initiate the response of B cells against an antigen, more than one B-cell receptor must bind the same epitope. To this end, several studies have shown that using tandemly repeating epitopes enhances the titers of neutralizing antibodies in

vaccinated animals (Zhang et al., 2002; Shao et al., 2011; Chen et al., 2014). Therefore, in this dissertation, one putative immunenic region was selected from each of AAS4, 6, and 8 for tandem duplication in the spliced construct. The chosen sequences were ones that were predicted to be immunogenic for both B- and T-cells. The coding regions corresponding to the spliced immunogenic regions were combined to form a spliced ORF construct using the MacVector DNA analysis software. The primary use of expressed proteins was for immunization, which does not require proteins in native configuration. Therefore, the quick and inexpensive prokaryotic bacterial expression method was chosen for recombinant protein expression. The commercial service GeneArt (Life Technologies) was used to optimize the spliced construct sequence for expression in *Escherichia coli*, including the restriction enzyme sites *Kpn*I and *BamH*I. The GeneArt service was used to synthesize and clone the synthetic construct into the pMA-RQ plasmid. Since the expression plasmid pRSET-A has an N-terminal histidine tag, a stop codon was also included in the AAS4-6-8sc ORF construct at the C-terminus to stop transcription at the end of the coding sequence and not have transcription continue further after the protein coding sequence.

Expression of AAS-4-6-8 spliced construct in bacterium E. coli

The recombinant AAS-4-6-8 spliced construct (rAAS4-6-8sc) in pMA-RQ from GeneArt was transformed into *E. coli* DH5α competent cells using the routine heat shock method as previously described for AAS4 and 8 in Chapter III. Following transformation, ten colonies were selected for PCR-verification of successful

transformation of pMA-RQ-AAS-4-6-8 into DH5α cells using the pMA-RQ M13 primers: 5'- TTGTAAAACGACGGCCAG-3' and 5'- CATGGTCATAGCTGTTTCC-3'. Following PCR-verification, plasmids were amplified and purified as previously described in Chapter III. After purification, plasmids were sequenced and submitted to the Gene Technologies Laboratory (GTL), Department of Biology, Texas A&M University, for Sanger sequencing to verify the correct spliced construction from GeneArt.

After sequence confirmation, purified pMA-RQ-AAS4-6-8sc plasmids were digested with *BamHI* and *KpnI* in a 100 µl reaction using 1 µl of each restriction enzyme, 10 µl of 10X Digestion Buffer (New England Biolabs), and 1 µl of BSA as required for enzyme stability. Simultaneously, empty pRSET-A plasmids were digested with the same restriction enzymes. Digestion reactions were incubated overnight at 37ºC. The following day, digestion reactions were electrophoresed on a 1% agarose gel to separate inserts from plasmids. Bands corresponding to the expected size of the insert of 1,684 base pairs (bp) or pRSET-A digested plasmids at 2,343 bp, were excised from the agarose gel using a razor blade and the insert DNA was purified from the gel slices using the Wizard Plus SV Minipreps plasmid purification kit (Promega). Digested AAS4-6-8sc inserts were ligated to digested pRSET-A expression plasmids using T4 DNA Ligase (New England Biolabs) with overnight incubation at 16ºC. The following day, pRSET-A-AAS4-6-8sc plasmids were transformed into DH5α competent cells as previously described. Insert-positive colonies were identified by PCR using the pRSET-A T7 primers 5'-AATACGACTCACTATA-3' and 5'-

CCACCGCTGAGCAATAACTAG-3'and positive colonies were used to inoculate overnight cultures grown in an incubator at 37ºC, with shaking at 225 rpm. The following day, plasmids were harvested from cells and purified as previously described. Purified plasmids were used in a Big Dye PCR reaction and submitted for sequencing at GTL to ensure proper cloning of AAS4-6-8sc in-frame with the start codon and N-terminal histidine tag. After sequence confirmation, purified pRSET-A-AAS4-6-8sc plasmids were transformed into BL21 pLysS De3 expression cells (Promega) using the same protocol for DH5α transformation as previously described in Chapter III. Insert-positive colonies were identified using pRSET-A T7 primers and positive colonies were used for pilot expressions.

For the pilot expression, seed cultures were grown overnight in 3 mL of the nutritionally rich bacterial growth media Terrific Broth (12 g/l peptone, 24 g/l yeast extract, 9.4 g/l dipotassium phosphate, 2.2 g/l potassium phosphate, Research Products International Corp.) with ampicillin at 50 µg/mL, at 37°C in an incubator/shaker. The following day, the seed was used to inoculate 20 mL of Terrific Broth and grown at 37°C with shaking until the culture reached an A_{600} of 0.4-0.6 as measured on the DU 640B spectrophotometer (Beckman Coulter). Expression of recombinant AAS4-6-8sc was induced by the addition of 1M Isopropyl β -D-1 thiogalactopyranoside (IPTG) to a final concentration of 1 µM. Following induction, the culture was grown for 16 h. Cells from collected samples were harvested by centrifugation at 10,000 g for 10min. The supernatant was discarded and the pellet was resuspended in 1 mL of Phosphate Buffered Saline (PBS). A 100 µl aliquot of BugBuster 10X (Millipore) was added to the

resuspension and then incubated for 20 min at room temperature (RT) with occasional inversion to mix. Following incubation, samples were placed on ice and subjected to five 3-second rounds of sonication using the Sonic Dismembrator Model 100 (Thermo Fisher Scientific).

After sonication, a 2 µl (2 units) aliquot of DNase I (Thermo Fisher) was added to each sample and incubated for 1h at 37ºC. Following incubation, samples were centrifuged at 10,000 g for 15 min at 4ºC. Supernatant, representing the soluble fraction, was collected and the pellet was resuspended in 100 µl of 1X Denaturing Buffer (100 mM NaH2PO4, 10 mM Tris-HCl, 8 M urea, pH 8). The resulting pellets were incubated for 30 min at RT followed by centrifugation at 10,000 g for 15 min at 4ºC. Supernatant was collected representing the insoluble fraction. All samples were analyzed by SDS-PAGE and western blot analysis using antibody to the histidine tag and chromogenic detection as previously described for AAS4 and 8 in Chapter III.

Expression of AAS4-6-8 spliced construct in yeast Pichia pastoris

The same ORF for AAS4-6-8 spliced construct (AAS4-6-8sc) was redesigned for expression in the pPicZα-A and *P. pastoris* expression system. For unidirectional cloning into pPicZα-A, the *KpnI* (GGTACC) and *NotI* (GCGGCCGC) restrictions were added to the ORF with no stop codon since the histidine tag for pPicZα-A is in the C-terminus. The expression construct was ordered from GeneArt (Life Technologies) in the pMA-RQ plasmid. Plasmids were amplified in *E. coli* and sequence verified, as previously described for pMA-RQ-AAS4-6-8sc. Purified plasmids were digested with

KpnI and *NotI* enzymes (New England Biolabs) and ligated to pPicZα-A (Invitrogen) for expression as previously described in Chapter III for AAS4 and 8. Following ligation, transformation to DH5α competent cells and sequencing to verify proper cloning was performed as previously described. Sequencing-verified pPicZα-A-AAS4-6-8sc plasmids were transformed into *Pichia pastoris* X-33 strain cells as previously described, followed by pilot expressions as previously described.

Results and Discussion

AAS 4, 6, and 8 are predicted as highly immunogenic

AAS4, 6, and 8 were predicted to be immunogenic throughout their sequences, as shown in Figure 16. Of all three serpins, AAS8 showed the highest number of immunogenic regions with a total of 20 predicted B-cell epitopes. AAS4 and 6 had 17 predicted B-cell epitopes each. Overall sequence immunogenicity for AAS4, 6, and 8 is also shown in Figure 16. The highest scoring nine amino acid T-cell epitopes were between 0.8124 and 5.3122. Scores for T-cell epitopes were also higher for AAS8 as shown in Table 7, with the top T-cell scores being 5.3122, 3.2479, and 2.2892. The highest T-cell epitope scores for AAS4 were 4.0434 and 2.3377 and for AAS6 were 4.06, 2.3377, and 2.0485. Only the highest scoring epitopes were selected for inclusion in the sliced construct design. The spliced amino acid construct is shown in Figure 17. The ORF size was 1,684 bp and 561aa long with a predicted molecular weight of 64.3 kDa including the histidine tag.

AAS4

ENDDALLAKAHNHFAVKLLKHLATQNPSSNVFFSPTSIAAAFGMAYAG
ARGSSEAELASVLGHDQVGLTEKSRVLAAYKHLLELMSSPNVTLEVAN
MVLAQNNFQIAESYIQQLHDIFDAELRSVDFANEGPRVAAEVNAWVRG
KTRGKIDGILPEGQPLDMILFIVNAVYFKGAWVTKFDPANTENKPFLN
LGTTEVSKPAMHLTRRFPYTRLGALHAAAVEIPYSGDRFSMVVLLPDS
PTGLAALREGLSLDVLQDVGSKLIFNEVVLRLPKFEMSLRYGLVPAMR
ALGLNVVFGGGANFTGISESTLVRISDAVHKAAVEVNEEGTIAAAVTG
LSFVPISALHAPLPPIQFTVDRPFLYYIRDRSNNRILFIGEVHSL

AAS6

ETDDALLAKAHNHFAVKLLKHLAAQNPSSNVFFSPTSIAAAFGMAYTGAGGSSE
AELASVLGHTEVGLTERPRVLAAYKQLLELTSSPNVTLELANLVLAQNNFEVAESY
KQQLRDVFDAELRSVDFANEGSRVAADVNAWVRGKTRGKITSILPEGQSLDVIL
FILNAVYFKGTWLTQFDPSQTKDKPFLNQGTTEVSKPAMNLRRRFPYTHLDALH
AGAVEIPYSGDRFSMVVLLPDSPTGLATLRDGLSLAVLEDIDSKLSFREVVLRLPKF
DMSLRYSLVPAMRALGLNVVFGGGANFSAISESTQIYISDAVHKASVEVNEEGT
VATAVTGISLALSALHTPPPPIQFIVDRPFLYYIRDRSNNRILFIGEVQSL

AAS8

QEEQKVANANNEFGFRLLQKIPASSEVNVFFSPYSVSTALAMAYAGARGETQQE
LYDSLAYSSAGLAPDHVPNAHAQHTQALKSPSSTLLVANTAVVQEGYNVLRVY
LQTLNQSFSAEVSTTNLADEQSLRSINEWVKHHTDGKIERLLSEPLSSDARFVLLN
AIYFKGLWNTPFHSASTFKASFFNAGTERVEVDMMHGQITAGYARDDETNSDV
VDLPYAGLDYSMTIVRPRDRTGADALRQVLTRQVFRRFLSELSETVVNVALPKFKI
EGEYKLRRPLSLLGVSKAFNKDEADFSGINGSRDLFVHDVVHKAVVEVNEEGSQ
AAAVTGVIIYTQSAFVGTPFVVNHPFLFFIRNRQTGDVLFAGQVNHL

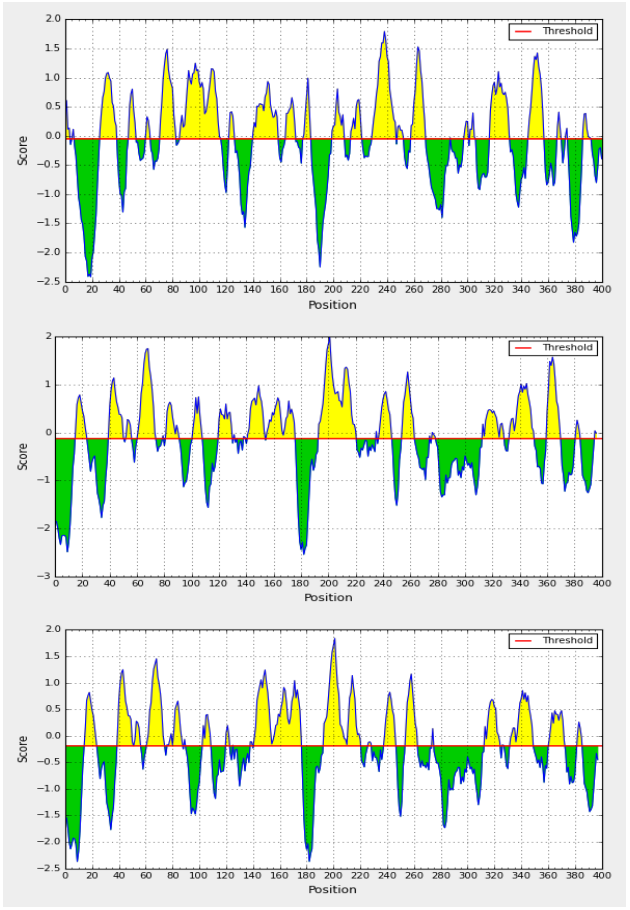

Figure 16. Predicted T- and B-cell immunogenic regions for AAS4, 6, and 8. The figure shows highlighted regions in the amino acid sequences of AAS4, 6, and 8 that were predicted to be antigenic by publically available computer algorithms. T-cell epitopes are indicated in blue, B-cell epitopes are indicated in green, and yellow areas indicate regions predicted to be antigenic to both. To the right of the sequences is a map of the antigenicity across the sequence where yellow peaks are indicative of high predicted antigenicity and green peaks indicate low predicated antigenicity.

TQNPSSNVFIAAAFGMAYAGARGSSEAEVGLTFANEG
PRVAARGKTRGKIDGILPEGQPILFIVNAVYKFDPANTE
NKTEVSKHAAAVEIPYSGDHAAAVEIPYSGDHAAAVE
IPYSGDHAAAVEIPYSGDDSPTGLANFTGIVEVNEEGT
IAAPLPPIQFTVDRPFLYYETDDALLAKAQNPSSNVFT
GAGGSSEAESPNVFANEGSRVAARGKTRGKPEGQQF
DPSQTKDKPFLNQGTTEVSKPANFSAISESTQIYANFS
AISESTQIYANFSAISESTQIYANFSAISESTQIYVEVNE
EGTVATLHTPPPPIQFIVDRPFLYYQEEQKVANANPASS
EVNVFFSPYPASSEVNVFFSPYPASSEVNVFFSPYPASS
EVNVFFSPYPASSEVNVFFSPYSVSTALAMAYAGARG
ETQQELYSSAGLAPDHVPNAHAQHTQALKSPSSNTAV
VQEGYSAEVSTTNLADEKHHTDGPLSSDARFVLLTER
VGYARDDETNSDVVDLPYGYARDDETNSDVVDLPYG
YARDDETNSDVVDLPYGYARDDETNSDVVDLPY

Figure 17. Spliced amino acid construct of AAS4, 6, and 8 predicted epitopes. Putatively immunogenic amino acid regions were spliced together into one sequence. AAS4 is indicated in blue, AAS6 in green, and AAS8 in red. The first tandemly duplicated is underlined and repated four times.

Because the spliced construct at 1,684 bp was similar in nucleotide length to the pMA-RQ plasmid at 2,343 bp, the digested insert and plasmid did not separate well in agarose gel electrophoresis as shown in Figure 18a. Therefore, an additional digestion of pMA-RQ was required with *SmlI* (New England Biolabs). After pMA-RQ digestion by *SmlI*, plasmid fragments were well separated from the band corresponding to AAS4-6-8sc as shown in the figure. Successful ligation of AAS4-6-8sc into pRSET-A and pPICZα-A and transformation into BL21 and X-33 cells is shown in Figure 18b and c.

Table 7: Putative T-cell epitopes and IEDB scores for AAS4, 6, and 8

Serpin	Epitope	Score
AAS4	IAAAFGMAY	2.3377
	ILFIVNAVY	0.8272
	HAAAVEIPY	0.8977
	FTVDRPFLY	1.7274
	TVDRPFLYY	4.0434
AAS6	ETDDALLAK	1.3692
	IAAAFGMAY	2.3377
	ILFILNAVY	0.8541
	AISESTQIY	2.0485
	ISESTQIYI	0.8124
	IVDRPFLYY	4.0600
AAS8	EVNVFFSPY	1.6841
	ASSEVNVFF	1.3000
	VSTALAMAY	3.2479
	NTAVVQEGY	2.2892
	SSDARFVLL	1.0924
	NSDVVDLPY	5.3122

Expression of immunogenic AAS-4-6-8 spliced construct was unsuccessful

The predicted molecular weight of rAAS4-6-8sc was 64.3 kDa. When the soluble and insoluble fractions of BL21-expressed protein were analyzed by western blot with chromogenic detection as shown in Figure 19, bands at this predicted molecular weight could not be detected. Pilot expressions were performed for six positive colonies, however, no bands at the expected molecular weight could be detected as shown in Figure 19a-d. Expression results were variable, with either no protein expression detected (Figure 19a), or bands appeared that were not of the expected molecular weight (Figure 19b-d). One possibility is that the recombinant protein was truncated during expression, and this could explain the results seen in Figure 19b and c. As an alternative strategy, the ORF for AAS4-6-8sc was re-synthesized for expression in *P. pastoris* X-33

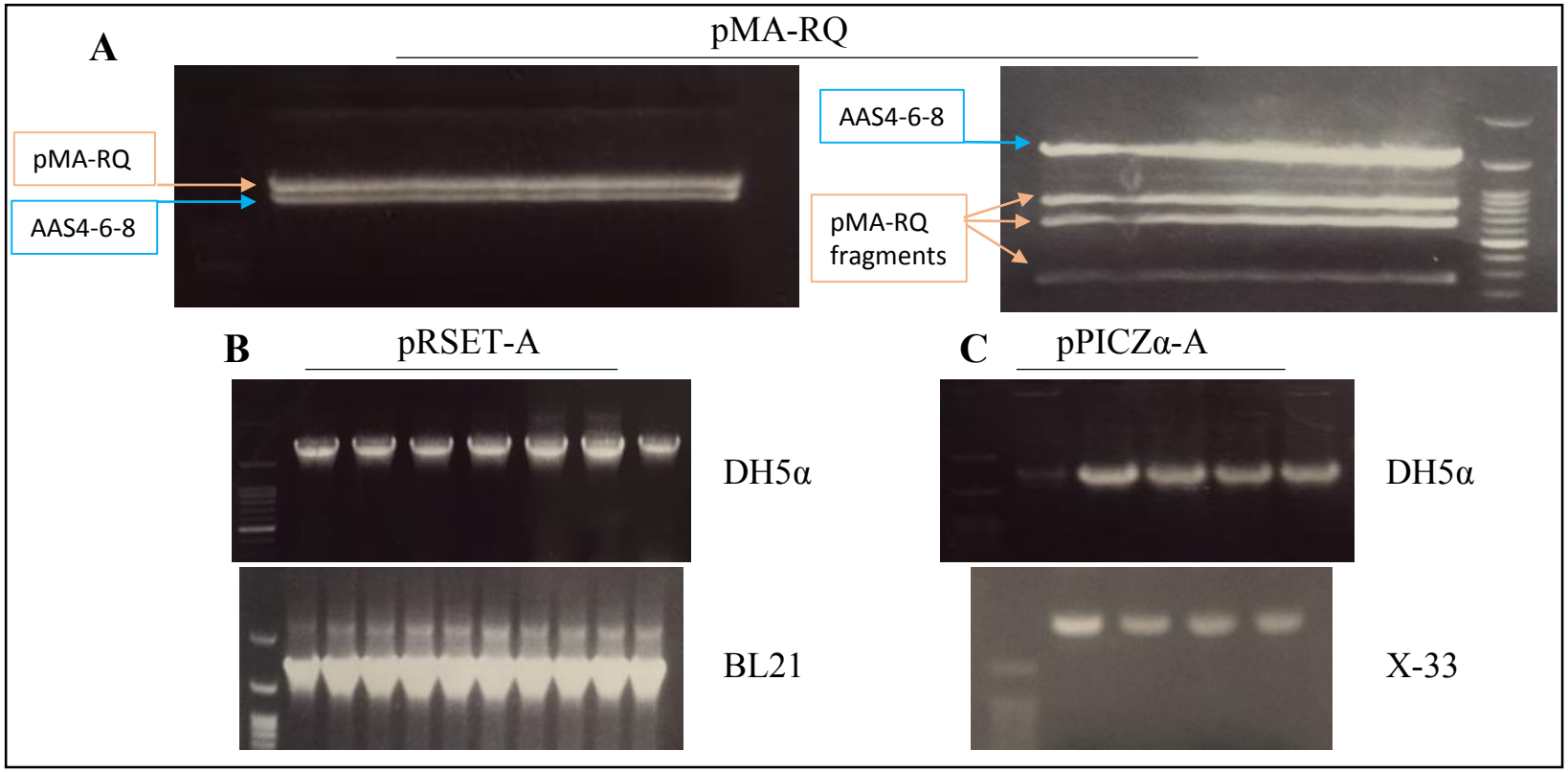

Figure 18. Cloning of AAS4-6-8sc into pRSET-A and pPICZα-A for recombinant protein expression. The left picture in panel A shows pMA-RQ plasmid digestion with KpnI and BamHI with AAS4-6-8 insert release. As indicated by the orange arrow, the plasmid band at 2.3 kb is not well separated from the insert band, indicated by the blue arrow, which is 1.6 kb. The right picture in Panel A shows pMA-RQ plasmids after further digestion with SmlI. The insert, indicated by the blue arrow, is well separated from the resulting plasmid fragments, indicated by the orange arrows. Panel B shows pRSET-A-AAS4-6-8 successfully transformed into *E. coli* DH5α and BL21 cells. Panel C shows pPICZα-A-AAS4-6-8 successfully transformed into *E. coli* DH5α and X-33 cells.

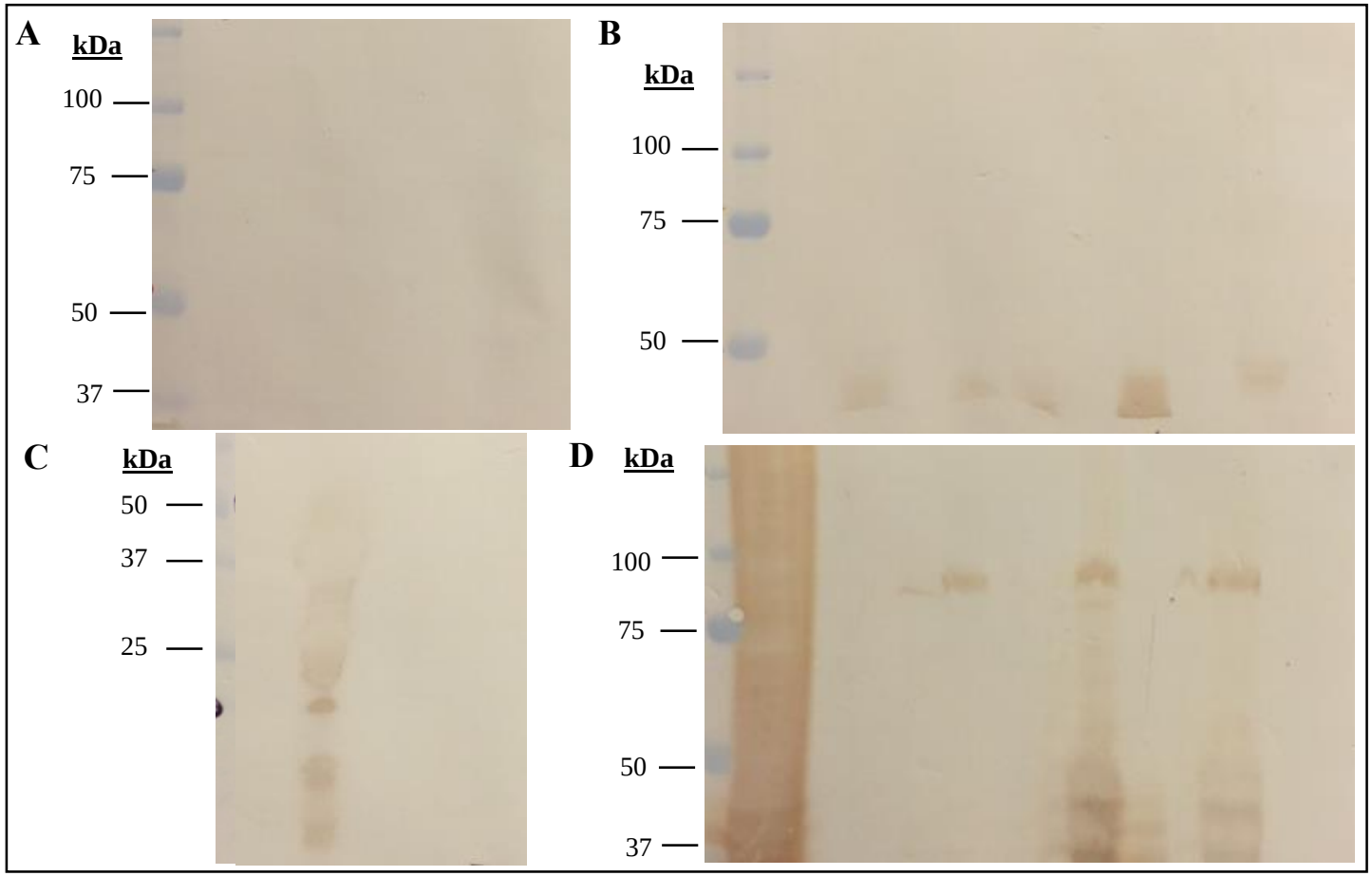

Figure 19. Western blot analysis of AAS4-6-8sc expression in *E. coli* BL21. As the figure shows, pilot expressions of positively transformed BL21 colonies resulted in either no protein expression as shown in Panel A, expression of protein smaller than the expected size of 64.3 kDa as shown in Panels B and C, or expression of protein larger than the expected size as shown in Panel D.

from GeneArt. Although successful transformation of pPicZα-A-AAS4-6-8sc into *P. pastoris* was confirmed as shown in Figure 18c, western blot analysis of pilot expressions was negative.

Protein expression in bacterial systems can fail for many reasons. It is possible that *E. coli* cells eject plasmids during culture. It is suggested that the use of carbenicillin instead of ampicillin could be a solution to this problem. Also, expressed protein can be toxic to the host cell, although the expression system used in this dissertation, BL21 pLysS DE3 is among the tighter regulation systems to control for this problem (Thermo Fisher, short URL: https://goo.gl/PGICkH). Another possibility is that the protein coded for by spliced amino acid fragments was not stable and therefore could not be expressed in any system. The fact that expression in both BL21 and X-33 systems was unsuccessful supports this idea.

Failure to express the recombinant AAS4-6-8 spliced construct prevented proceeding with the remaining immunization and tick challenge experiments in this chapter as outlined in my proposal. An alternative strategy would be to design an ORF that includes longer stretches of the native amino acid configuration, or perhaps to reduce or eliminate the tandem antigen repeats that were designed into the ORF. Another possibility would be to break the ORF into small fragments. These fragments could be amplified individually from the pre-existing ORF, and expressed one at a time. These individual fragments could then be used in immunization trials as a cocktail.

CHAPTER V

CONCLUSIONS

Serpins arose as interesting targets for anti-tick vaccine inclusion when it was hypothesized that they may play a role in tick evasion of host defense pathways. While some studies had begun to find serpins in ticks, the full picture of tick serpins remained speculative. Whereas only 17 serpins had been found in *A. americanum* by 2007 (Mulenga et al.), analysis of sequences deposited in GenBank suggested a much larger number of serpins for *I. scapularis*, leaving the serpin count in ticks as under-explored.

In this research, an extensive analysis of transcripts in fed and unfed *A. americanum* male and female ticks, as well as MG and SG tissues across feeding time points revealed serpins to be a very large family in this species. One notable observation is the presence of most serpin transcripts in several different tick tissues. This indicates that the same serpins function in multiple places in the tick and are not spatio-temporally restricted. This is in-line with accumulating evidence that tick serpins are often inhibitory against multiple proteases (Chmelar et al., 2011; Mulenga et al., 2013b; Ibelli et al., 2014; Rodriguez-Valle et al., 2015; Kim et al., 2015a). On the other side, this data indicates that between ticks and their vertebrate hosts there are many serine proteases-mediated pathways to be controlled. While serpins in ticks are being investigated by many research groups, tick proteases remain understudied. Alternatively, it could be that many serpins are simply redundant rather than having different targets. This would be an

indication that the role serpins play are critical to the point where redundancy is required to ensure proper functionality of those pathways. Unfortunately, redundancy undermines efforts to silence these targets as a means for biological characterization and as a means to control tick parasitism. At the very least, it may be necessary to take an exhaustive survey of serpins within a tick species and subsequently target highly similar serpins as a cluster.

Biochemical characterization of just two of the 122 serpins in *A. americanum* supports the hypothesis that ticks use at least some of the serpins they express to counter-defend against the vertebrate defense pathways. Specifically, data in this dissertation indicate a role for AAS4 and 8 in anti-neutrophil and/or anti-mast cell activity, suggesting that *A. americanum* ticks use these proteins as part of their arsenal of anti-inflammatory proteins. These proteins were also found to be immunogenic and therefore not only injected into the host, but also make good candidates for assessing their utility in anti-tick vaccines.

It will also be interesting to investigate whether there are patterns in serpin amino acid sequences that predict protease inhibitor profiles. As was observed for AAS4, annotations by BLAST searches alone do not provide reliable predictions with regard to the complete serpin inhibitor profile. For example, AAS4 was 73% similar to serpin-2 in *H. longicornis* which has been characterized as a thrombin inhibitor however, AAS4 inhibited chymase, papain, and cathepsin G. As more data become available on which proteases are inhibited by certain serpins, in silico analyses may become predictive. It will also be particularly useful to have the genome for *A. americanum* and other

metastriate tick species. This will allow an analysis of the serpin gene to serpin transcript analysis and could reveal if there are alternative splicing mechanisms for tick serpins as there are in other organisms (Jiang et al., 1997; Krüger et al., 2002). Furthermore, an available genome would facilitate transcriptome assemblies and an assessment of variability for tick serpins.

Data in this and other research show (Radulović et al., in preparation) certain blood components may affect the way that serpins function in the host. AAS8 bound to heparin, and while the functional significance of this binding remains uninvestigated, heparin binding to serpins in other studies shows that the binding may affect the way the serpin functions, taking away or adding, or reducing or increasing the inhibition (Radulović et al., in preparation). Furthermore, there are a variety of heparins each of which could have a different effect on serpin function. Future studies should investigate the protease inhibitor profile of AAS8 while bound to heparin to assess how functionality for this protein changes after binding.

In conclusion, this dissertation has contributed to building a more complete picture of the extent and variability of the serpin family in ixodid ticks, as well as established the nomenclature for serpins in *A. americanum*. This research has also contributed towards further elucidating serpin function in tick feeding physiology by characterizing AAS4 and 8 as anti-inflammatory tick saliva proteins. Some of the most important remaining questions to be resolved include the significance of AAS8 binding to cofactor heparin, whether or not these proteins play dual functions in inhibiting endogenous and exogenous serpins, whether similar serpins derive from the same gene,

and whether or not their inclusion in vaccines can result in protection from tick feeding and/or pathogen transmission.

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