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THESIS

MOLECULAR CLONING AND EXPRESSION OF SERPIN GENE FROM SALIVARY GLANDS OF *RHIPICEPHALUS MICROPLUS*

PAITOON KAEWHOM

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Tick salivary gland proteins have potential for application as novel tick control agents. The serine protease inhibitors secreted from salivary glands of the tick may be used in an anti-tick feeding vaccination. In the present study, the recombinant serpin (rserpin) protein of cattle tick's salivary glands was expressed and determines the immunogenicity of rserpin in rabbits. Serpin cDNA was cloned from the *Rhipicephalus microplus* salivary glands by RT-PCR and analyzed its nucleotide and deduced amino acid sequence. The results showed that the 1,200 bp open reading frame of serpin could encode a protein with 399 amino acid residues. By comparison with other serpins available in the GenBank database, the amino-acid sequence in the reactive center loop (RCL) of the cloned serpin showed a 95% and 100% identity to those of the *Rhipicephalus microplus* and *Rhipicephalus appendiculatus*, respectively.

The rserpin protein expressed in *Pichia pastoris* was used to immunize rabbits 3 times at 2 weeks interval at 100 μg rserpin per rabbit. SDS-PAGE analysis of crude supernatant has shown a distinct band of approximately 45 kDa. Western blot analysis also gave a specific band approximately the same size as that of SDS-PAGE. By ELISA, all immunized rabbits generated antibodies against rserpin at the first week of immunization and reached its peak within the seventh week. The antibody titer was analyzed by ANOVA and showed significantly differences between the control groups (group 1: PBS and group 2: WT protein) and the immunized group from the third week of immunization through the end of the experiment. This result indicated that rserpin had the strong immunogenicity and might be the candidate antigen for the anti-tick vaccine. However, it is necessary to perform a clinical trail with target animals as natural hosts.

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Student's signature Thesis Advisor's signature

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

TMB	=	Tetra-methylbenzidine
DAB	=	Diaminobenzedine
PBST	=	Phosphate buffer saline
PBS	=	Phosphate buffer saline
BSA	=	Bovine serum albumin
µl	=	microliter
ml	=	milliliter
µg	=	microgram
mg	=	milligram
bp	=	base pair
mM	=	milimolar
M	=	molar
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse Transcriptase Polymerase Chain Reaction
SDS-PAGE	=	Sodium Dodecyle Sulfate Polyacrylamide Gel Electrophoresis
ELISA	=	Enzyme linked immunosorbent assay
rpm	=	round per minute
°C	=	Degree Celsius
OD	=	Optical Density
cDNA	=	Complementary deoxyribonucleic acid
RCL	=	Reactive Center Loop
TSG	=	Tick salivary gland
ANOVA	=	Analysis of Variance
E. coli	=	*Escherichia coli*
P. pastoris	=	*Pichia pastoris*

MOLECULAR CLONING AND EXPRESSION OF SERPIN GENE FROM SALIVARY GLANDS OF *RHIPICEPHALUS MICROPLUS*

INTRODUCTION

The tropical cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* is a one-host tick that causes economically important ectoparasites of livestock in tropical and subtropical countries. This tick is important as a vector of diseases of domestic and wild animals since they can cause the great production losses resulting from direct tick feeding and its cost incurred from control of both ticks and the pathogens they transmit (WHO. 1997). Therefore, tick control is a continuing global priority. The conventional method of tick control and the application of chemical acaricides, has several disadvantages, such as high cost, environmental pollution, chemical residues in animal products and development of drug resistance. Vaccination might be the best way to overcome tick infestation problems (Mulenga *et al.,* 2000) since advantages of the vaccine over the chemical control are low cost, friendly to environment, less human health risks and lower incidence of drug resistances (Willadsen, 1997). Currently, two commercial vaccines are available for *R. microplus*. Both vaccine based on the Bm86 antigen was registered in Australia (TickGARD) and Cuba (GRVAC). The major effect of those vaccines is the successive reduction in tick numbers due to the reduction of female fertility (Willadsen *et al.,* 1995). The use of concealed tick antigens for artificial immunization of cattle has been successful enough to constitute the basis of a commercial vaccine (Willadsen *et al.,* 1995) but there are still drawbacks that should be addressed (Sahibi *et al.,* 1997). One is the fact that immunity to non-salivary gland antigens may not prevent tick feeding and; therefore, it does not prevent hide damages or the transmission of tick-borne infections. On the other hand, salivary gland components are believed to be involved in establishment and regulation of the tick feedings as well as pathogen transmission (Ribeiro, 1987)

Two sources of candidate vaccine antigens have been identified as 'exposed' antigens that are secreted into tick saliva during attachment and feeding on a host and as 'concealed' antigens that are normally hidden from the host immunity (Mulenga *et al.,* 2000). Recently, the third group of antigens has been developed by the combination of the both exposed and concealed antigens. This group has the effects on all stages and a wide variety of tick species. It also showed transmission-blocking and protective activity against a tick-borne pathogen (Nuttall *et al.,* 2006).

Secreting saliva of ticks during feeding contain pharmacologically active molecules that might modulate host immune responses. Immuno-modulating effect has not only facilitated tick feeding but also enhanced the transmission of pathogens from ticks into hosts. On the other hand, host immunization by tick saliva antigens from can induce anti-tick resistances (Kovar, 2004). The serpins (serine protease inhibitors) is one of tick salivary proteins that are important for regulation of inflammation, blood coagulation, fibrinolysis, and complement activation (Rubin, 1996). For instances, serpins of arthropod hemolymph are likely to protect itself from other infections such as fungal or bacterial protease. Serpins also have the other roles in cytokine activation (Polanowski and Wilusz, 1996).

The appearance of immunity in animal repeatedly bitten by ticks indicated the possibility of tick vaccination using tick antigen. Serpins is a possible candidate for the development of the vaccines. Serpins obtained from *Haemaphysalis longicornis* delay coagulation time and inhibit thrombin activity (Mulenga *et al*., 1999). In 2000, Mulenga and others proposed that tick serpins can be used as vaccine antigens. Andreotti *et al*., (2002) showed that the vaccination of cattle against serpins from *B. microplus* caused a significant decrease of reproduction of both tick number and egg weight.

An anti-tick vaccine is considered as an alternative method of cattle tick control in Thailand. Jittapalapong *et al.* (2004) immunized dairy cows with crude proteins from midgut and salivary glands from adult female *B. microplus* and showed that it could reduce tick burdens, engorged female weight, tick oviposition period and

egg mass weight. Recombinant proteins of potential tick salivary gland molecules should be focused on anti-tick vaccine development. In order to gain a success of tick vaccine development in Thailand, cloning, expression of serpin gene, and a determination of serpin immunogenicity from tick salivary gland may be useful for effective anti-tick vaccines that also control tick-borne diseases.

OBJECTIVES

1. To clone the serine protease inhibitor genes from cattle tick, *Rhipicephalus microplus*'s salivary glands.

2. To express the recombinant serpin proteins in *Pichia pastoris*.

3. To determine the antigenicity of the recombinant serpin protein.

LITERATURE REVIEW

1. Overview of *Rhipicephalus microplus*

1.1 Morphology and biology of ticks

Ticks are members of the same phylum (Anthropoda) as insects but they are in a different class (Arachnida). The major difference is the body of a tick is composed of two sections, while insects have three sections. Adult ticks which have four pairs of legs, while insects have only three sets of legs. Ticks are divided taxonomically into two main families including hard ticks (*Ixodidae*) and soft ticks (*Argasidae*) (Campbell, 2002). *R. microplus* is belong to the family *Ixodidae*. Hard ticks have a dorsal shield (scutum) with their forward-protrude mouthparts (capitulum). These ticks have a hexagonal basis capitulum (figure 1). The spiracular plate is rounded or oval and the palps are very short, compressed, and ridged dorsolaterally. Males have adanal and accessory shields. The anal groove is absent or indistinct in females, and faint in males. There are no festoons or ornamentation. Adults *R. microplus* have a short, straight capitulum. The legs are pale cream with a wide space between the first pair of legs and the snout. The body is oval to rectangular and the shield is oval and wider at the front, while the snout is short and straight. The nymphs of this species have an orange–brown scutum. The body is oval and wider at front and the body color is brown to blue–gray, with white at the front and sides. *R. microplus* larvae have a short, straight capitulum and a brown to cream body with 6 legs. (Aiello and Mays, 1998).

Ticks may also be classified on the basis of life cycle as one, two or three-host ticks. Most species feed on blood three times during their life cycle. *R. microplus* is a one–host tick (figure 2); all stages are spent on one animal. Eggs hatch in the environment and the larvae crawl up on grass or other plants to find a host. This tick remains on one host during the larval and nymphal stages, until they become adults, and females drop off the host after engorging to lay their batch of eggs (Vredevoe, 2005). In the summer, *R. microplus* can survive for as long as 3–4 months without feeding, but in cooler temperatures, they may live without food for up to 6 months. Newly attached ticks are usually found on the softer skin inside the thigh, flanks, and forelegs, as well as on the abdomen and brisket (Aiello and Mays, 1998).

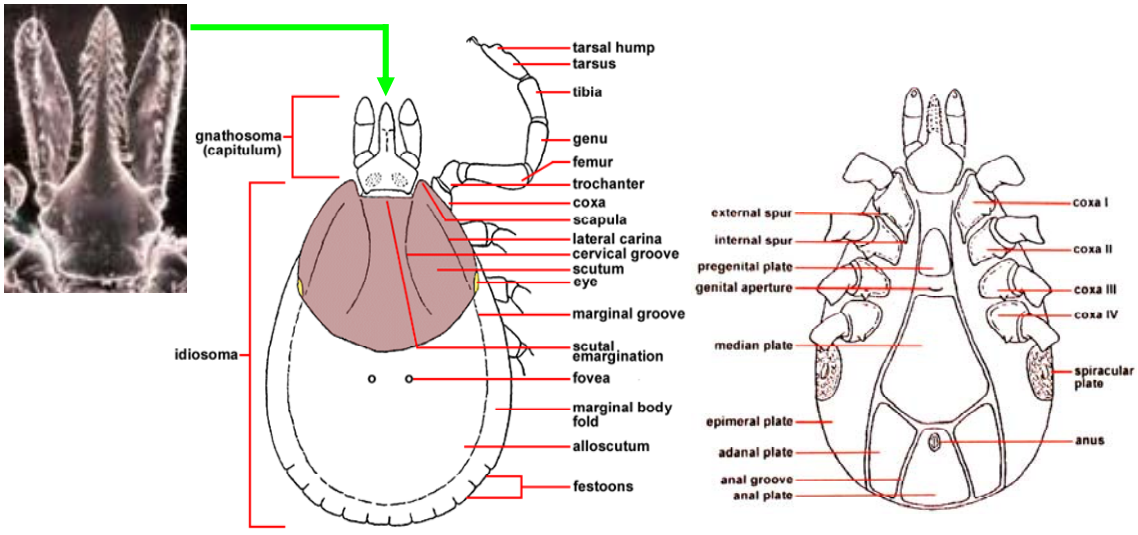

Figure 1 External structure of hard ticks is visible from top and bottom view.

Source: Aiello and Mays (1998)

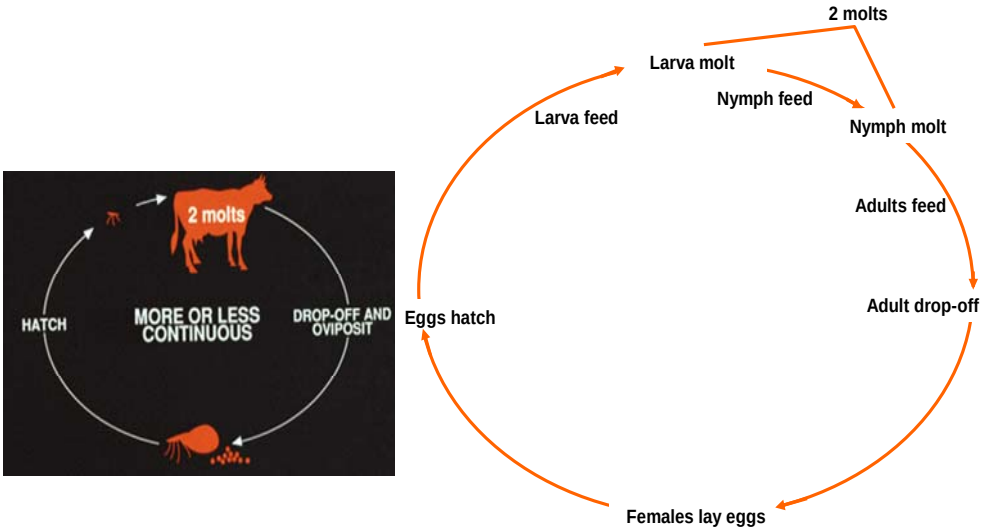

Figure 2 Life cycle of one-host tick, *R. microplus*.

Source: Vredevoe (2005)

1.2 Veterinary importance

1.2.1 Tick-borne diseases transmission

R. microplus can be found on many hosts such as cattle, buffalo, horses, donkeys, goats, sheep, deer, pigs, and dogs. It can transmit babesiosis (*Babesia bigemina* and *Babesia bovis*) and anaplasmosis (*Anaplasma marginale*) and theiloriosis in cattle (Campbell, 2002).

1.2.2 Economic losses

R. microplus is the most serious ectoparasite of livestock since they cause serious livestock losses in tropical and subtropical countries. Heavy tick burdens on animals can lower production and damage hides (Aiello and Mays,

1998). These ticks affect livestock in several ways (Campbell, 2002), including skin irritation, allergic response, loss of blood lead to reduced weight gains and weight loss, transmission of diseases, tick paralysis from tick toxin in saliva and damage to carcasses, fleece or hide from tick infestations. *R. microplus* has been caused the mojor loss in the livestock industry (Mulenga *et al.,* 2000). Despite the magnitude of the tick problem to livestock, studies that relate tick numbers to economic losses are rare in the literature (Campbell, 2002).

1.3 Tick control

1.3.1 Acaricides control

Control of ticks with acaricides may be directed against the free-living stages in the environment or against the parasitic stages on hosts. Control of ixodid ticks by acaricide treatment of vegetation has been done in specific sites in recreational areas in the world, to reduce the risk of tick attachment to animal and people. This method has not been recommended for wider use because of environmental pollution and the cost of treatment on large areas. Treatment of hosts with acaricides to kill attached larvae, nymphs, and adults of ixodid ticks and larvae of argasid ticks has been the most widely used control method. In the first half of the century, the main acaricide was arsenic trioxide. Subsequently, organochlorines, organophosphates, carbamates, amidines, pyrethroids, and avermectins have been used in different parts of the world (Aiello and Mays, 1998).

Acaricides are the most commonly application to livestock by dipping or spraying, with dipping being considered as the most effective one. In recent years, several other means of acaricide application have been developed, including slow release of systemics from implants and boluses, slow release of conventional acaricides from impregnated ear tags, pour-ons (which are applied on the back and spread rapidly over the entire body surface), and spot-ons (which are

similar but have less ability to spread) (Queensland Government's Policy. 2005) but, the disadvantages of acaricides used such as toxicity, high cost, contamination of food animal products and environment, and acaricide's resistant ticks (Aiello and Mays, 1998).

1.3.2 Rotation of pastures or pasture spelling

Rotation of pastures or pasture spelling has been used in the control of the one-host tick, *R. microplus*. The method could also be applied to other one-host tick, in which the duration of the spelling period is determined by the relatively short life span of the free living larvae. However, it has minimal application to mutihost because of the long survival periods of the unfed nymph and adults (Queensland Government's Policy, 2005).

1.3.3 Breed resistance

The first attempts to evaluate natural resistance of cattle against *R. microplus* were made in Brazil using *Bos indicus* cattle (Melendez *et al.*, 1998). Later studies showed that cattle resistance to ticks was due to the mounting of a protective immune response against ticks by bovine hosts, and this response was heritable for the cattle progeny (Robert and Kerr, 1976). In addition, it is well known that resistance to *R. microplus* varies, not only between individual bovines, but also from breed to breed, even in *Bos indicus* (Zebu cattle) as in *Bos taurus* (European cattle) herds. In general, pure or cross-bred Zebu cattle are worldwide acknowledged as more resistant to *R. microplus* and other ticks than European cattle (*Bos Taurus*) (Melendez *et al.*, 1998). The tick resistance of Zebu breeds and their crosses is being increasingly exploited as a means of control of the parasitic stages. The introduction of Zebu cattle to Australia has revolutionized the control of *R. microplus* on that continent. Use of resistant cattle as a means of tick control is also becoming important in Africa and the Americas, to develop tick-resistant cattle breeds after cross-breeding *B. taurus* x *B.*

indicus. Among these, cattle breeds developed in the tropics are the Sahiwal, Belmont Red, Bradford, Bonsmara, N'Dama and Boran (De Castro and Newson, 1993).

1.3.4 Anti-tick vaccines

The first experimental vaccination against ticks was carried out over a decade. Since then, progress has been slow, although in the early 1990s commercial release of a recombinant vaccine against *R. microplus* is significant (Willadsen and Jongejan, 1999). The nature of naturally acquired protective immunity against ticks is poorly understood, particularly in domesticated ruminant hosts. A recent advance of potentially great importance has been the production, using biotechnology, of a promising vaccine against *R. microplus*. The immunogen was known as a concealed tick antigen, not normally encountered by the host. The immune mechanism that it stimulates was different from that stimulated by exposure to ticks (i.e. tick feeding). The antigen was derived from a crude extract of partially engorged adult female ticks. It stimulated the production of an antibody that damaged tick-gut cells and killed ticks or drastically reduced their reproductive potential (Queensland Government's Policy, 2005).

The anti-tick vaccine is currently commercially available in Australia and Cuba. The Bm86 antigen based on a tick gut protein and produced by recombinant technology. Bm86 is a membrane bound protein located on the surface of gut cells in *R. microplus*. It is an 86 kDa glycoprotein with 66 half cystein residues and ~20 kDa glycosylation, and was shown to be an effective antigen in vaccination trails when tested as a native protein. It has been expressed in *Escherichia coli,* in insect cells using a baculovirus vector and in *Pichia pastoris*. It is present in ticks of different strains and is effective in all its recombinant forms, from inclusion bodies and particles, and as a soluble antigen. Bm86 can be produced on a large scale and is the sole antigen in the commercial vaccines TickGARD^{TM} Plus in Australia and GAVAC^{TM} Plus in Cuba. Vaccination of cattle with recombinant Bm86 leads to a reduction in the number of engorging female ticks and their weight and fecundity by stimulates production of specific antibodies in cattle, which damage the gut of

engorging ticks (Willadsen *et al.,* 1995). Taken together, these effects reduce the production of lavae by up to 90 % per generation in the most susceptible tick isolates. Some field isolates are less vaccine susceptible although, in Australia at least, this effect is not unacceptably large. Variation in the Bm86 gene sequence between tick isolates has been found, but its impact on vaccine efficacy is still not clear. With the recombinant vaccine, the greatest effects are on female fecundity. In practical term, this means that control of tick populations is most evident after at least one tick generation, and continual reintroduction of ticks to a vaccinated herd should be avoided (Willadsen and Jongejan, 1999). As a 'concealed' antigen, antibody titers to Bm86 are not boosted by tick infestation and sustained tick control demands booster vaccinations, not prevents tick feeding, and the transmission of tick-borne infections because its are regulated by salivary gland components, exposed antigens (Ribeiro, 1987).

2. Physiology and function of tick salivary gland

The salivary glands (figure 3) are the organs of osmoregulation in ticks and, as such, are critical to the biological success of ticks both during the extended period off the host and also during the feeding period on the host. Absorption of water vapour from unsaturated air into hygroscopic fluid produced by the salivary glands permit the tick to remain hydrated and viable during the many months between blood-meals. When feeding, the tick is able to return about 70% of the fluid and ion content of the blood-meal into the host by salivation into the feeding site (Bowman and Sauer, 2004). Saliva in feeding ticks is rich in bioactive components and exhibits a range of pharmacological properties. Factors identified in saliva or salivary glands include cement to help anchor the mouthparts to the host, various enzymes and inhibitors, histamine agonists and antagonists, prostaglandins, various anti-platelet aggregatory, anticoagulatory and anti-vasoconstrictory factors, anti-hemostatic factors, and immuno-modulating factors (Sauer *et al.,* 1995).

A secretion from the salivary glands allows ticks to absorb water from the air during the lengthy periods off their hosts and factors are counteract the host immune response and hence play a significant role in the success of tick feeding (Ramakrishnan *et al.*, 2005). The importance of the multifunctional salivary glands to tick survival and vector competency makes the glands a potential target for intervention. Continuing advances in tick salivary gland secreted protein will be cloned, and produced their protein with special emphasis on how they act on hosts (Bowman and Sauer, 2004).

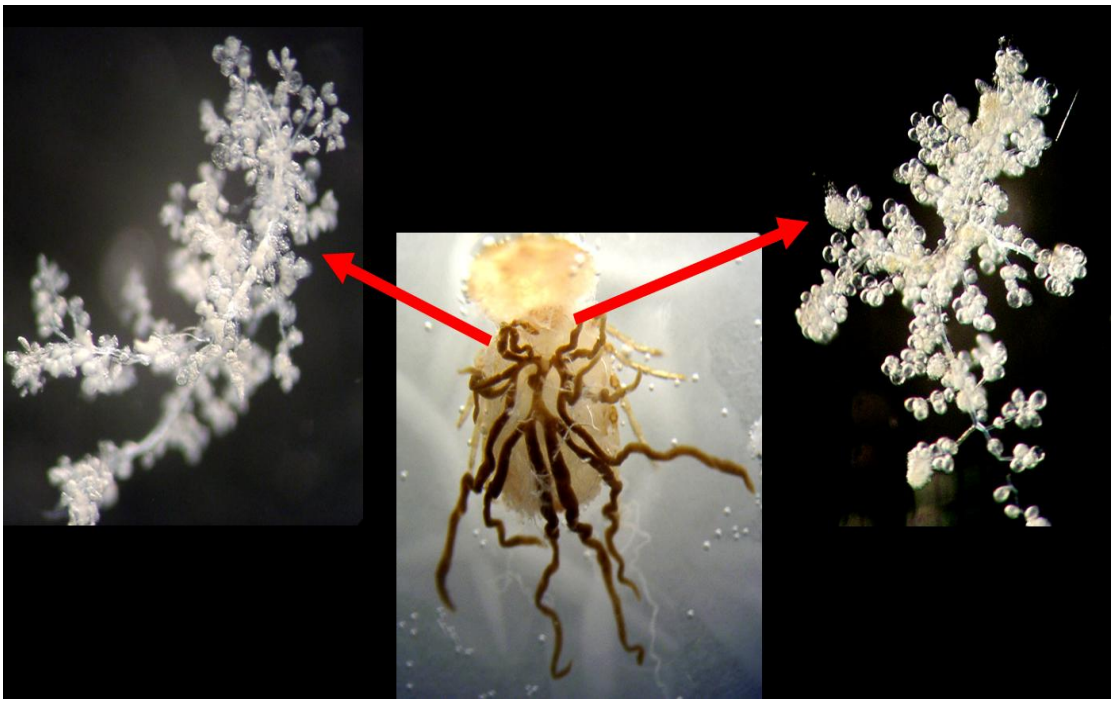

Figure 3 Photograph of the salivary glands within a female *R. microplus*.

3. Candidate antigens for an anti-tick vaccine

The possibility of vaccinating hosts against blood-feeding arthropods using antigens derived from salivary gland, gut, and other tissues of tick. Vaccine antigens are divided into two main sources of protein including exposed and concealed antigens. Exposed antigens are tick proteins injected into the host which stimulate with the host defense system during the course of tick feeding, while concealed antigens are the exact opposite (Mulenga *et al.,* 2000). Blood-sucking arthropods could be controlled by raising antibodies against molecules such as hormones in the host, and that during blood feeding, parasites will ingest these antibodies (Galun, 1975). The use of concealed tick antigens for artificial immunization of cattle has been successful enough to constitute the basis of a commercial vaccine (Willadsen *et al.,* 1995) but there are still drawbacks that should be addressed (Sahibi *et al.,* 1997). A vaccine against the cattle tick, *R. microplus*, using a recombinant antigen, has been tested under field conditions (Kay and Kemp, 1994). Control of *R. microplus* represents one of the most significant advances to date in the use of concealed antigens to immunize cattle against ticks. Three concealed antigens, Bm86 (Willadsen *et al.,* 1989) Bm91 (Riding *et al.,* 1994) and BMA7 (McKenna *et al.,* 1998) have been identified from the cattle tick *R. microplus*. A vaccine based on Bm86 became the first ever anti-arthropod vaccine when it was commercialized (Willadsen *et al.,* 1995). The other two molecules were later identified in order to enhance the vaccine capacity of the Bm86-based vaccine. The anti-tick response conferred by Bm91 or BMA7 alone was less striking when compared to that induced by recombinant Bm86. However, when either of the two antigens (Bm91 or BMA7) is used in a cocktail with Bm86, the induced anti-tick immunity is much more efficient than that observed with the commercial vaccine (single protein) (McKenna *et al.,* 1998; Willadsen *et al.,* 1996). These cocktail vaccines directed against ticks induced an immune response which directly attacked tick gut cells. The results also showed that some tick die while the others engorged but reduced in their fertility. Although the potential of salivary gland molecules as target vaccine antigens their molecular identity and the function of the majority of these molecules are remained to be clarified. Advancing in characterization of these antigens have been considered based on their ability to

induce a skin hypersensitivity reaction in tick-resistant hosts, modulate the host immune response, inhibit the host blood coagulation cascade (Table 1) and reactivity of salivary gland immunogens with anti-tick immune serum (Mulenga *et al.,* 2000). For instance, a study, by Shapiro *et al.* (1987) isolated immunogens from *Rhipicephalus appendiculatus* salivary gland extracts and immunized naïve rabbits. The result showed that it had induced immunogenic resistance in rabbits reacted with two protein band, 90 kDa and 30 kDa. In addition, study of Mulenga *et al.* (1999) *Haemaphysalis longicornis* saliva protein has been cloned, sequenced and expressed *in vitro* full length cDNA and recombinant p29 (rp29) protein was able to confer resistance against ticks infestation in naïve rabbits.

The fact of immunity from non-salivary gland antigens may not prevent feeding of ticks and, therefore does not prevent damage to the hides, or the transmission of tick-borne infections. On the other hand, salivary gland components are believed to be involved in establishment and regulation of the tick feeding site as well as pathogen transmission (Ribeiro, 1987).

Table 1 Characterization of candidate natural antigens base on their ability.

Tick – resistant/sensitized hosts	Protein size (kDa)/characterization
1. Tick proteins capable of an inducing immediate[a] /delayed[b] hypersensitivity reaction	
B. microplus / cattle	[a]30 kDa; esterase and carbohydrate splitting enzyme [a]60 kDa, esterase activity [a]24 kDa, not determined [a]18 kDa, trypsin inhibitor
H. analoticum / rabbit	[a]96 kDa, glycoprotein with nonspecific esterase and aminopeptidase activity [ab] 103 kDa glycoprotein [ab] 130 kDa, glycoprotein with acid phosphatase activity
A. americanum / guinea pigs	[a] 20 kDa, induce anti-tick immunity in naïve guinea pigs
R. appendiculatus / rabbit	[b]90 kDa cement protein
H. longicornis / rabbit	[a]84 kDa (p84) may belong to trypsin-like serine proteinase
2. Tick saliva proteins modulating host immune response.	
I. scapularis / human	49 kDa, inhibits lysis of rabbit blood by the human alternative pathway of complement
I. scapularis / mice	5 kDa, inhibits ConA-induced stimulation of murine T cells
R. sanguineus / mice	Two molecules, < 3 kDa and between 3 and 10 kDa, inhibit ConA induced stimulation of murine T cells.
3. Tick proteins inhibiting the host coagulation cascade.	
R. appendiculatus	65 kDa/factor Xa
H. trucuntum	16 kDa/factor Xa and thrombin
A. variegatum	17 kDa/factor Xa
O. moubata	6 kDa/factor Xa, 17 kDa/collagen-induced platelet aggregation, 15 kDa/ADP-stimulated platelet aggregation, 6 kDa/platelet adhesion to collagen
O. savignyi	7 kDa/factor Xa, 12 kDa/factor Xa, 14 kDa/factor Xa, 12.4 kDa/thrombin-induced platelet aggregation, 67 kDa/collagen and ADP-induced platelet aggregation

Source: Mulenga *et al.* (2000)

4. Serine protease inhibitors (serpins)

The serpins (serine protease inhibitors) are a superfamily of proteins found in plants, virus, invertebrates (exo and endo parasites of mammals) and vertebrates. The various serpins in mammals are thought to have evolved through gene duplication and divergence, giving rise to a large number of serpin genes within an organism, each encoding a protein with a unique reactive region and physiological function (Table 2). Serpins can be divided into two groups based on their inhibitory activity: one group of serpins interact in a stoichiometric manner with the active site of serine proteases (SPs), and non-inhibitory group, including thyroxine-binding protein, angiotensinogen and ovalbumin (Salzet *et al.,* 1999)

Table 2 Examples of naturally occurring serpins.

Serpin	Active site	Target enzyme	Pathology
Antithrombin	IAGR-SLNP	Thrombin, factor Xa	Thrombosis
C1-inhibitor	VALR-TLLV	C1s, kallikrein	Angioedema
α1-inhibitor	AIPM-SIPP	Neutrophil elastase	Haemorrhage, Emphysema
α2-antiplasmin	MSAR-MSLS	Plasmin	Haemorrhage

Source: Salzet *et al.* (1999)

4.1 Gene and protein of serpins

Database searching provides evidence for 1,000 serpin sequences. A simple BLAST analysis with an anonymous serpins amino acid sequence will yield hundreds of “hit” with high significance. Phylogenetic analysis reveals strong statistically support for grouping serpins into 16 clades (A-P). The remaining serpins are orphans, but these should form additional clades as more serpins sequences are identified (Table 3) (Peter, 2002).

Most serpins are 300-500 amino acids in size, variation is due mostly to different N- or C- terminal extensions and interhelical loops but virtually all contain a core structure of 380 amino acids. Due to the absolute requirement of a mobile protein folds into a well-conserved, metastable tertiary structure consisting of three β-sheets, nine α-helices and a reactive site loop (RSP). Many serpins, especially those that are secreted via the classical ER-Golgi pathway, also are variably glycosylation. A core of about 380 residues is present in all serpins, with minor modifications due to small insertions or deletions of DNA that may introduce loops or lengthen or shorten α-helices, however, there is considerable variation in size among different serpins resulting from N-type or O-type glycosylation. Thus, recombinant serpins synthesized will depend on the ability of the host synthesize, serpins molecules in the active conformation and the need for post translational modifications such as glycosylation (Gary, 2004).

Table 3 Serpin clades.

Clade name	letter
α_1-proteinase inhibitor	A
Intracellular, ov-serpin	B
Antithrombin	C
Heparin cofactor II	D
α_2-antiplasmin, PEDF (pigment epithelium derived factor)	E
C1 inhibitor	F
HSP47	G
Neuroserpin	H
Horseshoe crab	I
Insect	J
Nematode	K
Blood fluke	L
Viral SPI1-2/CrmA-like	M
Viral SPI3-like	N
Plant	O
Unclassified (orphans)	P

Source: Peter (2002)

4.2 Actions of serpins

Serpins constitute a part of an important enzymatic regulatory mechanism of inflammation. They regulate the activity of serine proteases derived from inflammatory cell and are involved in enzymatic processes such as coagulation, complement activation and fibinolysis. Serpins inhibit serine proteases by an irreversible suicide substrate mechanism when the interaction proceeds down the inhibitory arm of a branched pathway. The mechanism of proteinase inhibition, being dependent on a mechanism rather than a more normal specificity of interaction mechanism, give clear advantages in allowing inhibition of many serine proteinases, even of different folds. At the same time, the importance of the initial recognition of proteinase in determining the rate of inhibition allows for regulation of these rates by use of both the reactive center loop and exosite interaction, while modulation of the relative fluxes along different branches of the reaction pathway allows modulation of outcome. In addition, the conformational changes that accompany reaction allow for opportunities of signaling, whether the serpins is an inhibitory or noninhibitory one (Peter, 2002).

4.3 Invertebrate serpins

Both serine proteases and serpins have been identified in exo and endo-invertebrate parasites of mammals, whose immune regulatory mechanism, i.e. a conserved parasitic survival strategy to interfere with the host immune response (Figure 4) evolved much earlier than had previously been thought. This finding is just as significant as the recent discovery of invertebrate immune signaling molecules (Salzet *et al*., 1999). They hypothesize that serpins evolved specifically to limit immune activation by curtailing the enzymatic generation of stimulatory signals (Chopin *et al*., 1997; 1998). It is unlikely that serpins evolved recently, because the serine protease proinflammatory molecules are also present in invertebrates and require the down regulating processes of the serpins to maintain a dynamic balance (Salzet *et al*., 1999). Furthermore, the relatively primitive parasitic invertebrates have

co-evolved highly specific mechanism such as blocking blood coagulation and antibody production. For example, two groups of low molecular weight serpins have been found in leeches and other haematophagous animal (Krezel *et al*., 1994). The first group inhibits the activation of mammalian blood clot formation: inhibitors of thrombin (e.g. hirudin, triatomin) or factor Xa (e.g. antistasin, ixodine) as ixodine found in hard tick, *Ixodes ricinus* (Salzet *et al*., 1999). The second group including, inhibitors of elastase and cathepsin G, act on the extracellular matrix (Nekarda *et al*., 1998). Serpins from tick salivary glands may be involved in facilitation of tick feeding and digestion of blood meal as well as disease transmission. Member of serpins gene family may represent one of the most interesting candidate target antigens for tick vaccine development because of their role in regulation of several physiological functions such as the blood clotting cascade, clot resolution, the inflammatory response and complement activation (Mulenga *et al.*, 2003).

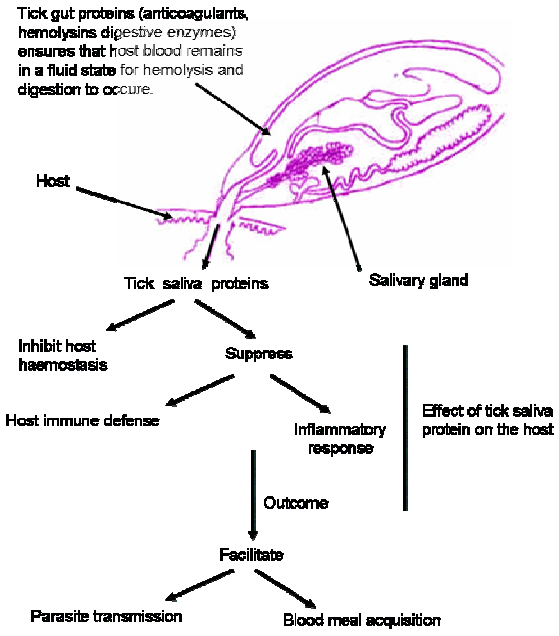

Figure 4 Schematic representation of the postulated role of tick saliva and midgut proteins in tick feeding and disease transmission.

Source: Mulenga *et al.* (2001)

The Figure 4 summarizes the postulated effect of tick saliva proteins on the host's immune response factors. In order to complete feeding successfully, ticks secrete an array of bioactive saliva proteins which modulate the host's homeostatic balance. Additionally tick gut proteins are also essential in ensuring that the imbibed blood remains in a fluid sate and available for digestion (Mulenga *et al.,* 2001).

5. Yeast expression system for production recombinant proteins

Yeasts are the potential eukaryotic expression system. They have been used for the large scale production of intracellular and extracellular protein of human, animal and plant origin since the early 1980s for (Cereghino *et al*., 1999). The only yeast expression system available was based on the common baker's yeast, *Saccharomyces cerevisiae*. This yeast has been studied intensively at the genetic and biochemical levels because of its importance in the food and beverage industry. Because of its industrial importance, there is extensive experience in large-scale fermentation of this organism. Furthermore, the yeast appears well-adapted for secreting expressed antigens into the culture supernatant, which facilitates isolation and also posttranslationally modify protein (Mark, 1998).

Many of the techniques developed for *Saccharomyces* applied to *Pichia* include transformation by complementation, gene disruption and gene replacement. The genetic nomenclature used for *Saccharomyces* has been applied to *Pichia*. For example, histidinol dehydrogenase is encoded by the *HIS4* gene in both *Saccharomyces* and *Pichia* and also cross-complementation between gene products. Several wild-type genes from *Saccharomyces* complement comparable mutant gene in *Pichia* (Invitrogen. 2004).

More recently, *Pichia pastoris* has become popular for use as an expression system. *Pichia* is not significantly different from *Saccharomyces* in its ability to posttranslationally modify protein. However, it is possible to express proteins at much greater levels in *Pichia* than in *Saccharomyces* due to the presence of the powerful alcohol oxidase promoter, *AOX1* and *AOX2* (Mark, 1998). Two genes in *P. pastoris* code for alcohol oxidase. The majority of alcohol oxidase activity in the cell is attributable to the product of the *AOX1* gene. Expression of the *AOX1* gene is tightly regulated and induced by methanol to very high levels, typically ≥ 30% of the total soluble protein in cell grown with methanol. The *AOX1* gene has been isolated and a plasmid-borne version of the *AOX1* promotor is used to drive expression of the gene of interest encoding the desired heterologous protein (Koutz *et al*., 1989; Tschopp *et al*., 1987a; Ellis *et al*., 1985). While *AOX2* is about 97% homologous to *AOX1*, growth on methanol is much slower than with *AOX1*. This slow growth on methanol allows isolation of Mut^s strains (Cregg *et al*., 1989; Koutz *et al*., 1989).

In comparison to *S. cerevisiae*, *Pichia* may have an advantage in the glycosylation of secreted proteins because it may not hyperglycosylate. Both *S. cerevisiae* and *Pichia pastoris* have a majority of N-linked glycosylation of high-mannose type; however, the length of the oligosaccharide chains added posttranslationally to proteins in *P. pastoris* (8-14 mannose residues per side chain) is much shorter than those in *S. cerevisiae* (50-150 mannose residues) (Grinna and Tschopp, 1989; Tschopp *et al*., 1987b). Very little O-linked glycosylation has been observed in *P. pastoris*, this is predicted to be less of a problem for glycoproteins generated in *P. pastoris*, because it may resemble the glycoprotein structure of higher eukaryotes (Cregg *et al*., 1993).

As a eukaryote, *Pichia pastoris* has many of the advantages of eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *S. cerevisiae*. It is faster, easier and less expensive to use than eukaryotic expression systems and generally gives higher expression levels. As a yeast, it shares the advantages of 10- to 100-fold

higher heterologous protein expression levels. These features make *Pichia* very useful as a protein expression system (Invitrogen, 2004).

There are variety of vectors and hosts commercially available for expressing proteins in *Pichia*. One vector, pPICZα is for secreted expression and contain the Zeocin^{TM} resistance gene for positive selection in *E. coli* and *Pichia*. The pPICZα vector is a circular vector at a 3.6 kb in length, contain pUC origin for allows replication and maintenance of the plasmid in *E. coli* and contain Unique restriction sites (*Sac* I, *Pme* I, *Bst*X I) that permit linearization of the vector at the *AOX1* locus for efficient integration into the *Pichia* genome. This vector is C-terminal purification and detection tag, contains six histidine residues (6xHIS) (Figure 5). This histidine patch has been shown to have high affinity for divalent cations. The recombinant protein can be easily purified on metal chelating resin (Invitrogen, 2004).

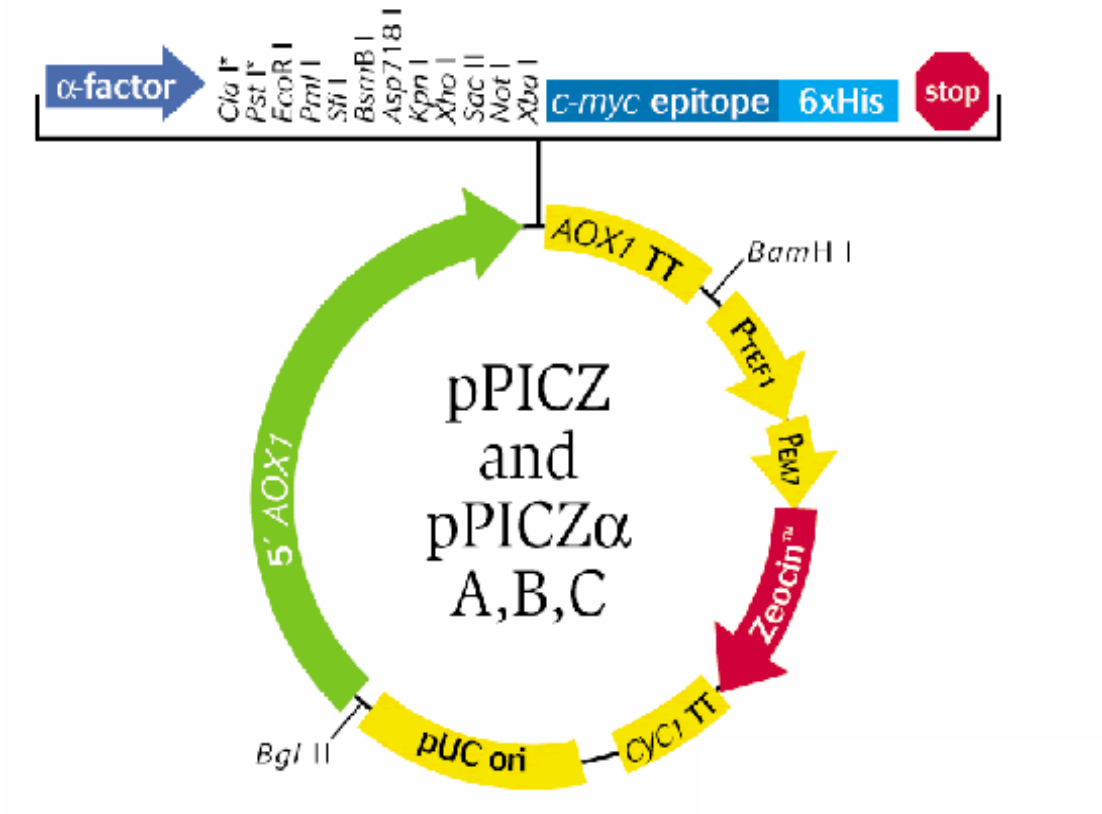

Figure 5 A pPICZα expression vector.

Source: Invitrogen (2004)

MATERIALS AND METHODS

1. Tick and salivary gland dissection

The cattle ticks (*R. microplus*) were collected from cattle in ChiangRai and RoiEt provinces (n = 400). Only female ticks were used in the experiments. Dissection of ticks was done under light microscope as described by Jittapalapong *et al.*, (2004). Briefly, partially fed ticks were submerged in phosphate buffer saline (PBS; pH 7.4) and held down with a pair of soft tissue forceps. The dorsal cuticle was excised and the salivary glands were separated by 18-guage needles. Following dissection, the tissues were transferred into RNA stabilizer reagent (Invitrogen®, USA), and kept frozen at -80 ^{o}C until use.

2. RNA extraction and RT-PCR

Total RNA were extracted from female adult *B. microplus* salivary glands by the acid phenol-chloroform method (Chomczynsky and Sacchi, 1987). RT-PCR was performed according to the two-step RT-PCR protocol (Invitrogen®). Briefly, the first strand cDNAs were obtained by reverse transcription using 50 ng of total RNA from tick salivary glands, 13 µl of distil water, 10 mM dNTPs, 2.5 µM Oligo-dT primers, 4 µl of reverse transcriptase buffer, 0.1 M DTT, 1 U Superscript III reverse transcriptase and 1 U RNase inhibitor (Finnzymes®, USA) at 50 ^{o}C for 50 min. The resulting cDNA was amplified by polymerase chain reaction (PCR) using a specific forward primer containing a *Kpn I* restriction enzyme site 5'-GGTACCATGCTCG CCAAATTTCTCTTTCTCG-3' and a specific reverse primer containing an *Xba I* restriction enzyme site 5'-TCTAGAACTAGTGTGTTAACCTCTCCGATGAAA-3'. Polymerase chain reaction was performed for 35 cycles at 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, and 72 ^{o}C for 2 min and final extension at 72 ^{o}C for 7 min in a solution 100 µl of 10 µl cDNA templates, 10 µl buffer (20 mM Tris-HCl (pH8.4), 50 mM KCl), 20 mM dNTPs, 150 mM $MgCl_2$, 0.6 pgmol of sense and anti-sense primer and 3.5 U DyNAzyme EXT DNA polymerase (Finnzymes®, USA) in a Primus 96 plus thermocycler.

3. Construction of yeast expression vectors for serpin

The serpin coding sequences were amplified by PCR and purified using QIAquick gel extraction kit (QIAGEN®, Germany). The purified products were then digested with *Kpn I* and *Xba I* restriction enzyme, and then subcloned into the pPICZαA expression vector (Invitrogen®, USA). The obtained plasmid, pPICZαA-serpin, was transformed into either *E. coli* DH5α competent cells (GIBCO-BRL, USA). The *E. coli* positive clones were selected on LB plates containing 25 μg /ml Zeocin, and identified the present of serpin gene by PCR or restriction analysis.

4. DNA sequencing and computer-assisted sequence analysis

A single colony of *E. coli* positive clone was selected and subcultured in LB media. After an overnight growth, plasmid DNA was purified from bacteria culture using QIAprep spin miniprep kit (QIAGEN®, USA) and confirmed by restriction analysis. Nucleotide sequencing was performed by the BioService Unit (BSU), National Science and Technology Development Agency (NSTDA), Thailand. The nucleotide sequence of recombinant serpin plasmids were then translated to amino acid by DNASIS program. Alignment of nucleotide and deduced amino acid sequences of our serpin and other serpins available in the GenBank database (accession number AY312432, AY035779, AY035780, AY035781, AY035782, AB162827, CAB55818 and CAC22469) were done using Clustal W program version 1.83. The obtained serpin sequence was also blast using Blastp program version 2.2.15.

5. Transformation of recombinant plasmid into *Pichia pastoris*

The KM71 strains (Mut^S, Methanol utilization slow) of *P. pastoris* were generated. These strains were used to prepare *Pichia* competent cells. The recombinant plasmids were used to transform *P. pastoris* strain KM71 competent cells by electroporation protocol (Invitrogen®, USA). The *P. pastoris* positive clones were selected on YPD plates containing 100 μg /ml Zeocin, and checked by PCR technique.

6. Expression of rserpin protein in *P. pastoris*

6.1 Protein induction

The overnight growth of recombinant *P. pastoris* were inoculated into 500 ml of BMGY (buffered complex glycerol medium) at a concentration of 1:10 and shaken at 250 rpm at 30 ^{o}C until culture reaches an OD_{600} = 2-6 (approximately 16-18 hours). The Cells were harvested by 4,000 rpm for 10 minutes at 4 ^{o}C and decanted supernatant and cell pellet resuspend in BMMY medium (buffered complex methanol medium) using 1:5 of the original culture to induce expression and return to incubator to continue growth. 100% methanol adds to final concentration of 3% methanol every 24 hours to maintain induction. For secreted expression, the supernatant was transferred to a separate tube and stored at -80 ^{o}C until used.

6.2 Sodium dodecyl sulfate polyacrylamide gel electrophorsis (SDS-PAGE)

Fifteen microliters of induced recombinant *P. pastoris* supernatant was added to 5 μl of SDS sample buffer (250 mM Tris HCl, 20% glycerol, 4% SDS, and 0.006% bromophenol blue), boiled for 5 minutes and centrifuged at 4,000 rpm for 2 minutes. The supernatants were loaded into 10 % SDS-PAGE gel and electrophoresed for 90 minutes at 110 voltages. The gel was then stained with staining solution (0.25 % Coomasie Brilliant Blue, 45% methanol, 45% distilled

water, and 10% acetic acid) for 10 minutes and subsequently destained with destaining solution (45% methanol, 45% distilled water, and 10% acetic acid).

6.3 Western blot analysis

The recombinant serpin (rserpin) were first separated in 10 % SDS-PAGE gel and electro-transferred onto nitrocellulose membrane at 400 mAmp for 300 minutes. The membrane was blocked with 5% skim milk at 4^{o}C for overnight. The membranes were then incubated with 1:1500 mouse anti-histidine antibody (Sigma) or 1:100 Bovine anti-TSGP (Tick salivary glands protein) serum for an hour at room temperature. Subsequently, the membranes were washed and incubated with 1:200 goat anti-mouse IgG peroxidase (KPL, USA) or 1:3000 goat anti-bovine IgG conjugated peroxidase (Sigma, USA) for an hour at 37^{o}C. After washing, the membranes were incubated with 3 ml of 0.6 mg/ml diaminobenzidine (DAB) (Sigma, USA) containing 0.03% H_2O_2 for 5-10 minutes at room temperature. The rserpin protein was visualized as a brown band on nitrocellulose membrane.

7. Animal immunization

7.1 Protein preparation

The rserpin antigen was partially purified for immunization. It was separated by 10% SDS-PAGE and stained with copper stain. Specific protein band compared with molecular protein marker was cut by blade and ground by glass bar in 500 µl of 0.1% SDS. The polyacrylamide gel was separated by centrifuge at 13,000 rpm for 5 minutes, and supernatant was transferred to microcentrifuge tube. The protein precipitation was done by adding 4 volumes (V/V) of cool acetone and incubated overnight at -80 ^{o}C. Protein collection was approached by centrifuge at 13,000 rpm for 15 minutes. Acetone was discarded by gently aspiration and completely dried at room temperature. Protein was resuspended with phosphate buffer saline (PBS) and measured concentration by spectrophotometer. Bovine serum

albumin (BSA) was used to standard curve for approximated recombinant protein concentration.

7.2 Rabbit immunization

Fifteen male and female New Zealand white rabbits (2 months, 2-2.5 Kg.) were singly separated. They were randomly divided into three groups with 5 each: group 1 was immunized by 500 µl of phosphate buffer saline (PBS), group 2 was immunized by 500 µl of wild type protein (only pPICZαA vector was transformed into *Pichia pastoris*), and group 3 was immunized by 500 µl contained 100 µg of rserpin and adjuvant. The vaccine contains 100 µg rserpin protein mixed with the equal volume of 10% Montanide 888 in mineral oil and well merged by ultra homogenizer. Each group was immunized three times at 2 week interval. The rabbits were bled weekly before and after immunization.

8. Enzyme linked immunosorbent assay (ELISA)

Rabbit sera were tested for antibodies against rserpin protein by ELISA technique and data were analyzed in term of mean optical intensity (OD value). Indirect ELISA was performed to determine the antibody titer. The microplates were coated by recombinant serpin diluted (1:10) with coating buffer and incubated overnight at 4 ^{o}C. The sera were diluted (1:100) with washing buffer and subsequently, 100 µl of 1:100 each diluted sera were added into each well and incubated at 37 ^{o}C for 1 hour. The excess sera were washed out and added 100 µl of 1:3000 goat anti rabbit IgG conjugated with horseradish peroxidase into each well and incubated at 37 ^{o}C for 1 hour before washed out. Finally, 100 µl of Tetra-methylbenzidine substrate was added to each well for 5 minutes. The reaction was then stopped by adding 100 µl of 0.1 N H_2SO_4. The reaction intensity was measured by the ELISA plate reader at the absorbance of 620 nm.

9. Data analysis

The effect of vaccine was compared to the control group by measurement of the level of antibody against rserpin antigen and determined by analysis of variance (ANOVA) on completely randomized design (CRD). Means of each group's antibody were compared and significant differences between groups of immunization by Duncan's new multiple range test (DMRT) when $p< 0.05$. All analyses were carried out using the SAS program.

RESULTS

1. Cloning of serpin gene of tick salivary glands (TSG)

1.1 RT-PCR

Total RNAs were extracted from female adult *Rhipicephalus microplus*'s salivary glands and amplified by reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR products were used in a PCR reaction by serpins gene-specific primers. The PCR product is 1,200 bps in size (Figure 6.)

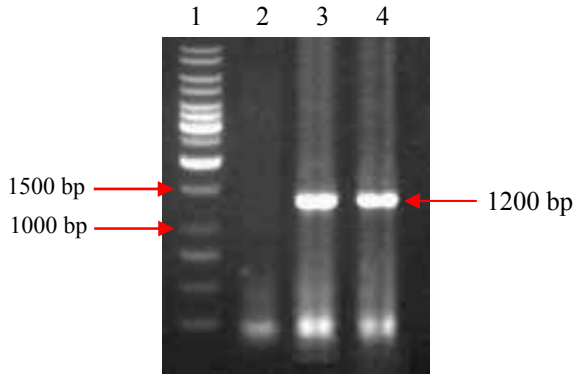

Figure 6 The PCR products of serpin gene.

The cDNA of serpin (analyzed on 1% (w/v) agarose and stained with ethidium bromide). Lane 1: 1 Kb DNA marker; lane 2: negative control; lane 3 and 4: the 1,200 bp RT-PCR product (arrow).

1.2 Cloning of serpin gene into plasmid pPICZαA

The PCR products of the serpin gene of TSG were approximately 1,200 bps in length (Figure 7), and were ligated into plasmid pPICZαA before being transformed into *E.coli* strain DH5α. The recovered white *E. coli* colonies were subsequently determined for serpin gene insertion by PCR technique. The PCR products from extracted recombinant plasmids showed the same size as those from a positive control (Figure 7) and confirmed selected colonies by *AOX1* primer (Figure 8). The restriction endonuclease assay was also performed to confirm the insertion. The digested products also had the size approximately 1,200 bps in length (Figure 9).

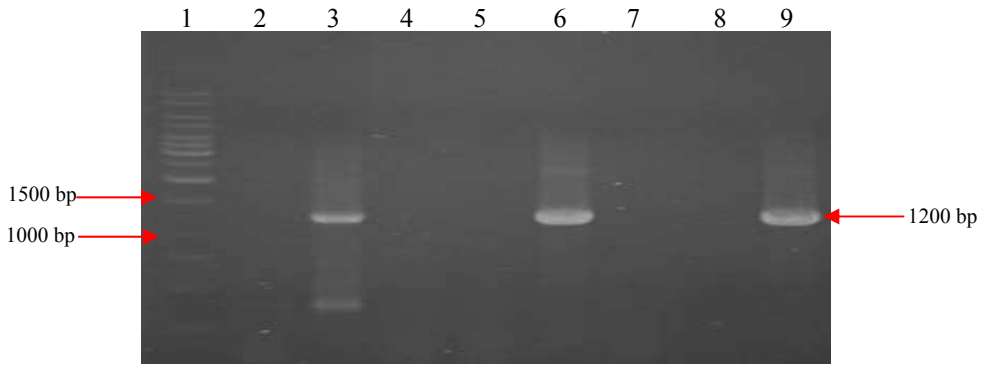

Figure 7 PCR products of the selected *E. coli* colonies.
Analysis of PCR products of serpins gene using 1% agarose gel electrophoresis. Lane 1: 1 kb DNA marker, lane 2: Negative control, lane 3: Positive control and lane 4-9: PCR product from colonies grown on selective medium.

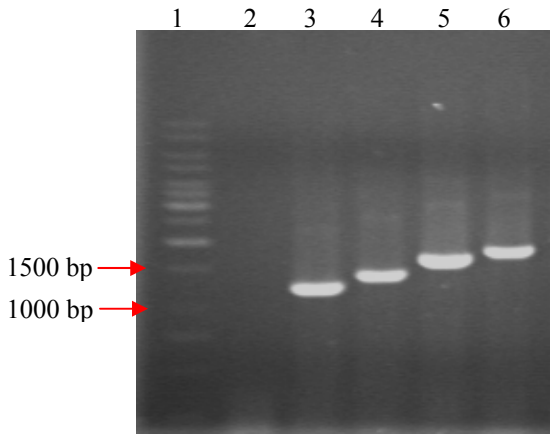

Figure 8 PCR products of serpin gene confirmed by *AOX1* primer.

Analysis of PCR products of SERPIN gene using 1% agarose gel electrophoresis. Lane 1: DNA marker, lane 2: Negative control, lane 3: PCR product of SERPIN gene using forward and reverse gene primer (1200 bps), lane 4: PCR product of SERPIN gene using forward gene and reverse 3'*AOX1* primer (1369 bps), lane 5: PCR product of SERPIN gene using forward 5'*AOX1* primer and reverse gene primer (1554 bps) and lane 6: PCR product of SERPIN gene using forward and reverse *AOX1* primer (1723 bps).

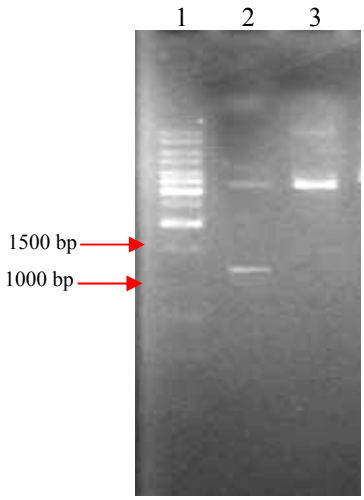

Figure 9 The restriction fragment of serpin gene.

The restriction fragment of serpin gene from restriction endonuclease assay. Lane 1: 1 kb DNA marker, lane 2: 1.2 kb band of serpin gene and 3.6 kb band of pPICZαA vector after recombinant pPICZαA was digested with restriction enzyme (*Kpn* I and *Xba* I) compared with pPICZαA (lane 3).

A serpin gene from the cattle tick (*R. microplus*) salivary glands was identified using RT-PCR. The serpin cDNA is 1,200 bp in length, which encodes a serpin protein of 399 amino acid residues (Figure 10). The deduced amino acid sequence from ChiangRai was 100% identity to that of RoiEt provinces. The molecular mass of the deduced amino acid sequence was predicted about 43.13 kDa, and predicted possible secreting signal was found at amino acid position 1-17. The N-glycosylation sites were predicted at the amino acid positions 44, 107 and 258. When compared with other known serpins available in the GenBank database, the deduced serpin amino acid sequence in the present study was 94% identity to *R. microplus* serpin (China; accession number AY312432), 93% identity to *Rhiphicephalus appendiculatus* serpin-3 (Japan: accession number AY035781), 70% identity to *Haemaphysalis longicornis* rHLS-2 (Japan; accession number AB162827) and 33% identity to *Ixodes ricinus* serpin (Belgium; accession number CAB55818). In addition, the amino acid residues in the reactive center loop (RCL) of the cloned serpin showed 95% identity to that of *B. microplus* (accession number AY312432), 35% and 80%

identity to that of *Haemaphysalis longicornis* rHLS-1 and rHLS-2 (unsubmitted and accession number AB162827) respectively, 40% identity to *Ixodes ricinus serpin* (accession number CAB55818), 30, 35, 100, and 30% identity to *Rhiphicephalus appendiculatus* serpin-1, serpin-2, serpin-3 and serpin-4, (accession number AY035779, AY035780, AY035781 and AY035782) respectively (Table 4). By using the NCBI-Blastp, serpin deduced amino acid sequence were showed 94% identity to *B. microplus* serpin (member Q72021), 93% identity to *Rhiphicephalus appendiculatus* serpin-3 (member Q8WQW9), 72% identity to *Amblyomma americanum* (member A7UI19), 67% identity to *Haemaphysalis longicornis* rHLS-2 (member Q75Q63), 37% identity to putative secreted salivary gland peptide of *Ixodes scapularis* (member Q06B72) and 36% identity to limulus intracellular coagulation inhibitor type 2 (member Q27086).

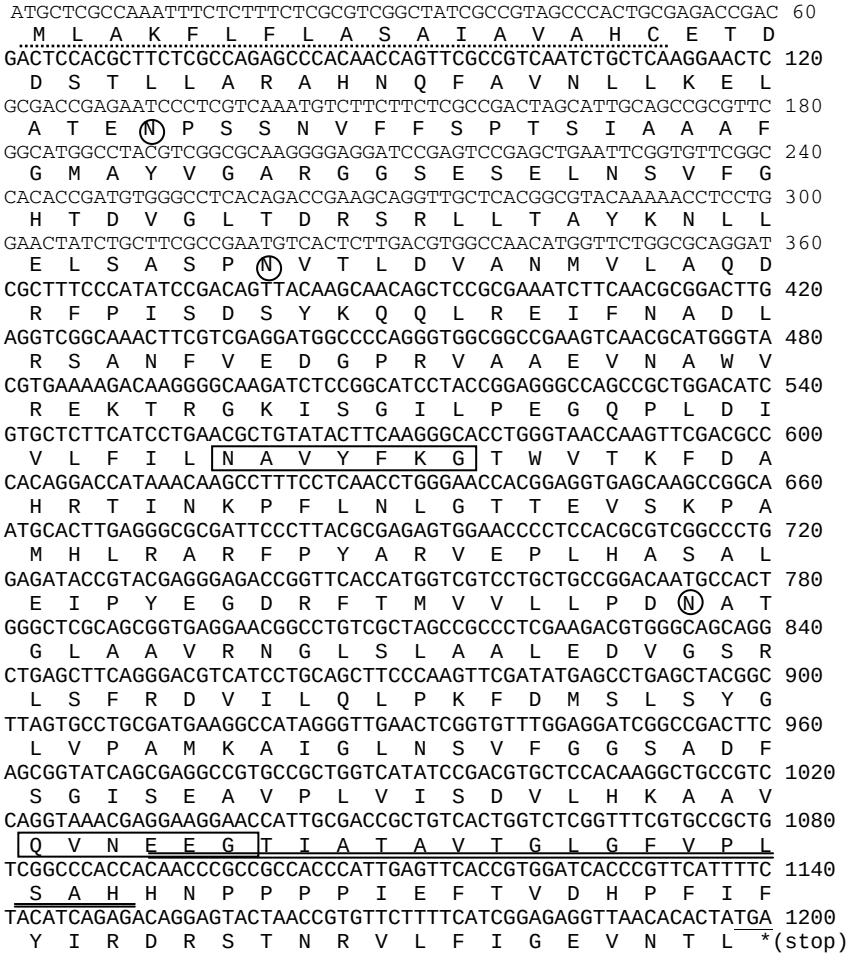

Figure 10 The deduced amino acid sequences of serpin gene.

Serpin cDNA had a 1200 bp open reading frame which encoded for 399 amino acids. The reactive center loop (RCL) indicated as a double-underlined. The predicted N-glycosylation sites were showed in circles. The consensus region of serpin was in boxes. The predicted possible cleavable signal peptide (amino acid 1-17) indicated as a dot-line. Asterisks (*) indicated as a stop codon.

Table 4 Comparison of the serpin reactive center loop (RCL) with those of other known serpins and representative serpins.

Boophilus microplus's salivary gland serpins		E E G T I A T A V T G L G F V P L S A H	%Identity
Boophilus microplus serpins		* * * * * * * * * * * * * * * * * V *	95
Rhiphicephalus appendiculatus	serpin-1	* * * * E * A * A * A V M M A A C C L S	30
	serpin-2	* * * * E * A * A * A I T M M T Y C A R	35
	serpin-3	* * * * * * * * * * * * * * * * * * * *	100
	serpin-4	* * * A A * A * R G S S S K P R S * G G	30
Haemaphysalis longicornis	rHLS-1	* * * * E * A * A S A M V A T N R C * R	35
	rHLS-2	* * * * V * * * * * * I S * I * * * * *	80
Ixodes ricinus serpin CAB55818		* * * * E * A * A * A I P A M L M C * R	40
CAC22469		* * * * E * A * A * A I P I M L M C * R	40
Intracellular coagulation inhibitor (LICI)		* * * * E * S G I S S V V A G V R * G W	30
Heparin cofactor II (HCII)		* * * * Q * * T * * T V * * M * * * T Q	65

* indicate the amino acid residues identical to those of serpins. The P1 amino acid residue indicated in box, it associate with proteases by presenting a "bait" residue, which is thought to mimic the normal substrate of the enzyme.

1.3 Preparation of transforming DNA

Digest 15 μl of recombinant plasmid DNA with the restriction enzymes (*Sac* I) and choose the enzyme that does not cut within inserted gene. Each enzyme cuts one time in the 5' *AOX1* region to linearize the pPICZαA. The restriction fragment of serpin recombinant plasmids was approximately 4,800 bps on 1% gel electrophoresis, correlated with the expected size 1,200 bps considering that the expression vector length fused with a 3,600 bps vector DNA (Figure 11). After that vector linearized was checked concentration by comparing with λ DNA digested by *Hin*d III enzyme that known concentration. The result shown, linearize recombinant plasmids concentrate was approximately 50 ng/μl (Figure 12).

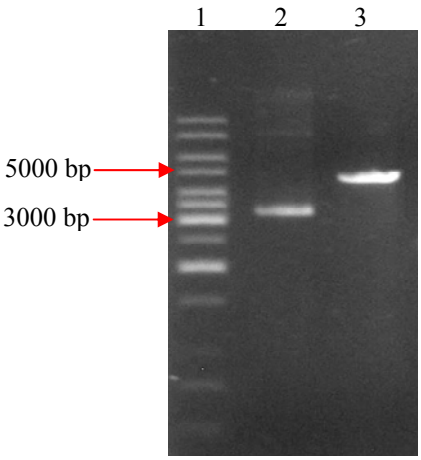

Figure 11 The linearize of serpin recombinant plasmids.
Analysis of recombinant plasmids was digested with the restriction enzymes (*Sac* I). Lane 1: 1 kb DNA marker, lane 2: uncut recombinant serpin plasmid, lane 3: linearized of recombinant serpin plasmid.

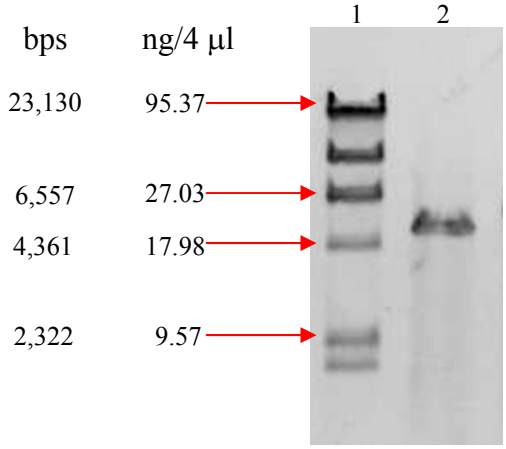

Figure 12 Estimation of linearize plasmid concentration.
Analysis of estimation of linearize plasmid concentration was checked by compare with λ DNA that known concentration. Lane 1: λ DNA, and lane 2: linearize of serpin recombinant plasmid.

1.4 Cloning of serpin gene into *Pichia pastoris*

The serpin gene from serpin recombinant plasmid was transformed into *Pichia pastoris* genome strain KM71 by electroporation. The size of PCR products of the positive clones was approximately 1723 bps of serpin gene (1,200 bps) and *AOX1* locus size (523 bps) when visualized by electrophoresis technique (Figure 13).

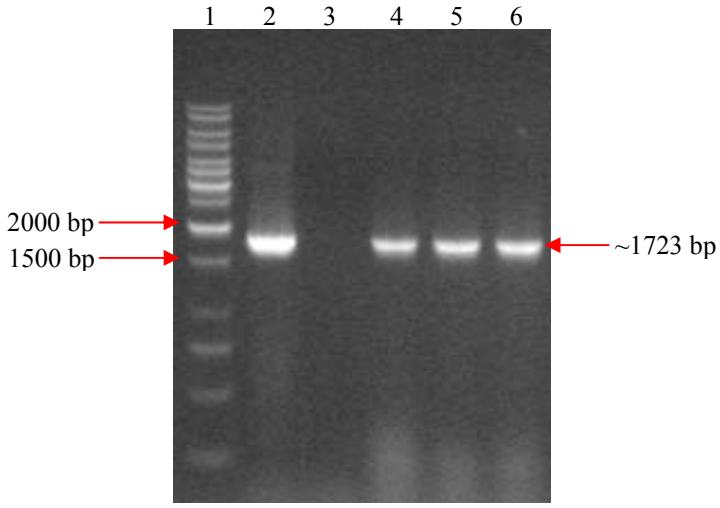

Figure 13 PCR products of the selected *Pichia pastoris* colonies.
Lane 1: 1 kb DNA marker, lane 2: positive control, lane 3: negative control and lane 4-6: PCR product (1,723) from yeast colonies grown on selective medium.

2. Expression of recombinant serpin (rserpin) protein

2.1 *In vitro* expression of rserpin

Recombinant serpin was expressed in vitro using the pPICZαA expression vector and the *P. pastoris* strain KM71 expression system. Recombinant protein was expressed as secreted into media culture. Supernatant were collected every 24 hours after induced with 3% methanol final concentration. The rserpin protein was detected at 1, 2, 3, 4, 5, 6, and 7 days post induction and peaked at 7 days (Figure 14). A molecular weight of rserpin was 45 kDa on 10% polyacrylamide gel, correlated with the expected molecular mass 42.5 kDa considering that the expression vector produced a recombinant protein fused with a 2.5 kDa vector protein (Figure 15).

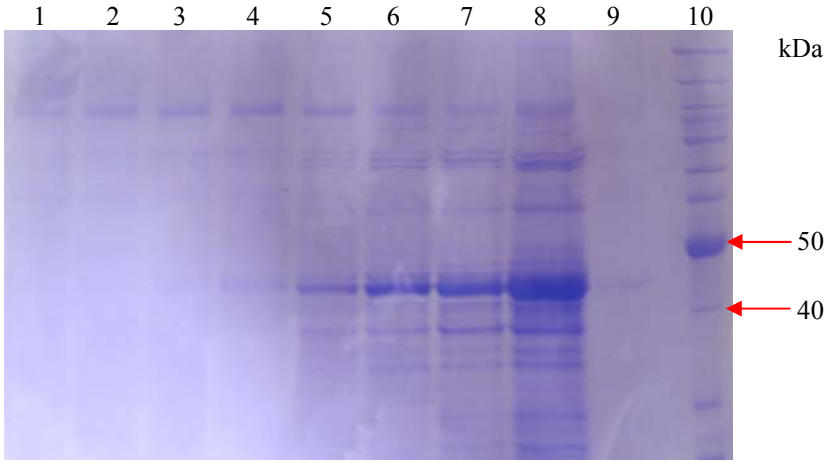

Figure 14 SDS-PAGE of rserpin from transformed *P. pastoris* induced with 3% methanol final concentrations that various time course.
10% SDS-PAGE of time course analysis for rserpin protein expression. Lane 1, 2, 3, 4, 5, 6, 7, and 8 were protein from collected supernatant at 0, 1, 2, 3, 4, 5, 6, and 7 days post induction. Lane 9: Wild type protein at 7^{th} days, lane 10: molecular protein marker.

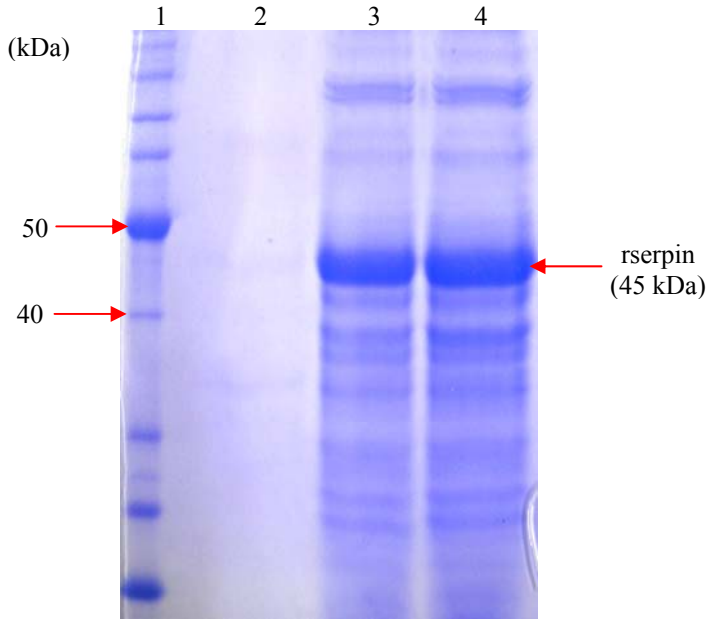

Figure 15 SDS-PAGE analysis of rserpin protein concentration (3:1) from transformed *P. pastoris* induced with 3% methanol final concentration. SDS-PAGE result demonstrated the size of rserpin protein stained by Coomassie brilliant blue. Lane 1: molecular protein marker, lane 2: Wild type protein, lane 3 and 4: 45 kDa rserpin protein (arrow).

2.2 Western blot analysis

Detection of rserpin protein was determined by Western blot technique. Supernatant were separated from media culture and routinely electrophoresed on 10% polyacrylamide gel. Following electrophoresis, the transferred proteins were electro-blotted onto a nitrocellulose membrane that was incubated in mouse-anti-histidine IgG monoclonal antibody and bovine anti-TSG protein serum polyclonal antibody. The result indicated the specific band at the same size presented in the SDS-PAGE (45 kDa) and WT-protein showed negative result (Figure 16).

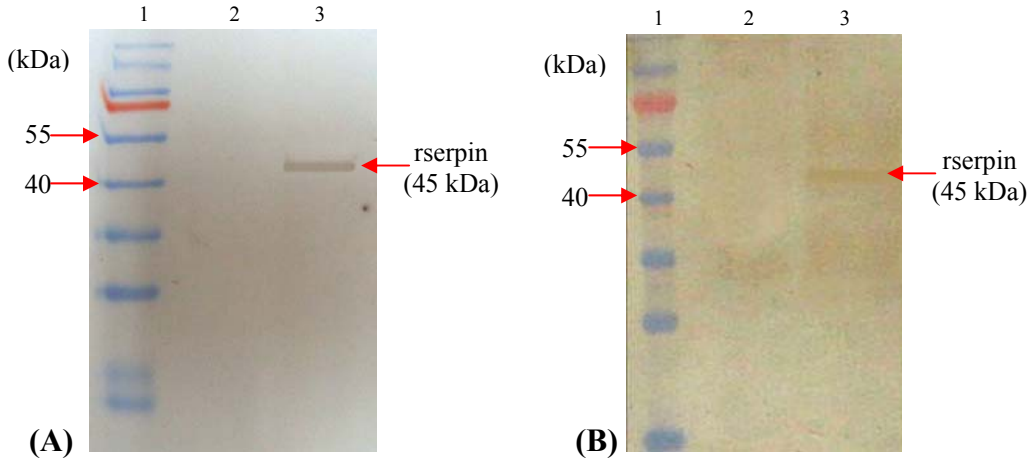

Figure 16 Western blot analysis of recombinant serpin protein.
Determination of immunogenicity of rserpin protein by Western blot with mouse anti-histidine monoclonal antibody (A) and Bovine anti-TSG protein serum polyclonal antibody (B). Lane 1: Molecular prestain protein marker, lane 2: Wild type protein, lane 3: rserpin protein (arrow).

3. Immunogenicity of rserpin

Assessment of immunogenicity of rserpin was conducted in rabbits. Rabbits were subjected to be immunized and tested for levels of antibodies against rserpin protein by ELISA and the data were analyzed before immunizations in term of mean optical density (OD). The mean of reaction intensity of antibody was 0.66 (group1), 0.62 (group 2) and 0.61 (group 3) with no significant differences among groups. In treatment groups, the levels of antibodies against rserpin were gradually increasing at the second weeks after immunization and the levels are maintained throughout experiment (Figure 17). The antibodies of treatment groups were significantly higher than of those control groups (Group 1 and Group 2) from week 3 to week 7 (Table 5).

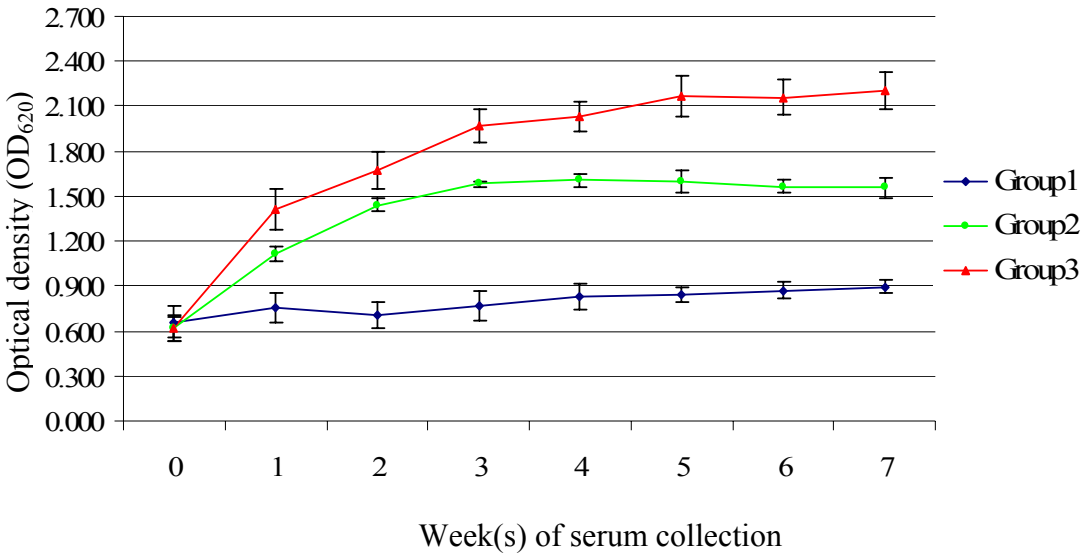

Figure 17 General Linear Model (GLM) of mean OD value of rabbit that immunized with rserpin and control group.

General Linear Model (GLM) of mean value of optical density of antibodies from sera of rabbit immunized with PBS was group 1, WT protein was group 2, and rserpin was group 3 by indirect ELISA.

Table 5 Statistic analysis of antibody responses against rserpin compared to the control group using ANOVA.

Week(s) of serum collection	Mean ± SE (n= 15)			*P* value
	Group 1 PBS	Group 2 Yeast protein	Group 3 rSerpin	
0	0.660±0.104 ^{A}	0.621±0.089 ^{A}	0.614±0.077 ^{A}	P=0.9310
1	0.751±0.097 ^{B}	1.117±0.047 ^{A}	1.412±0.131 ^{A}	P<0.0017
2	0.704±0.086 ^{B}	1.443±0.049 ^{A}	1.672±0.124 ^{A}	P<0.0001
3	0.765±0.099 ^{C}	1.580±0.022 ^{B}	1.968±0.114 ^{A}	P<0.0001
4	0.835±0.088 ^{C}	1.606±0.042 ^{B}	2.031±0.103 ^{A}	P<0.0001
5	0.841±0.048 ^{C}	1.598±0.071 ^{B}	2.166±0.132 ^{A}	P<0.0001
6	0.869±0.054 ^{C}	1.565±0.039 ^{B}	2.161±0.112 ^{A}	P<0.0001
7	0.896±0.040 ^{C}	1.558±0.069 ^{B}	2.206±0.124 ^{A}	P<0.0001

Note: Different superscript in the same row indicated significant differences between groups.

DISCUSSIONS

1. Cloning of serpin gene

Currently, serpins have been identified and genetically cloned from various tick species, such as *Rhipicephalus appendiculatus* (Mulenga *et al.,* 2003), *Haemaphysalis longicornis* (Sugino *et al.,* 2003), and *R. microplus* (Zhou *et al.,* 2003). As has been shown in previous reports, serpin was candidate capable of using as anti-tick vaccine antigens for the control of tick infestation in cattle (Sugino *et al.,* 2003; Imamura *et al.,* 2005). In this report, we describe for the first time the complete sequence of serpin from *R. microplus* collected from cattle in Thailand.

Comparison of nucleotide and the deduced amino acid sequence showed high identity to known serpins such as *R. microplus* and *R. appendiculatus* registered in GenBank, resulting 97% and 92% identity, respectively. It is interesting to mention that Blastp analysis of serpin from *R. microplus*'s salivary gland revealed amino acid similarities to serpin-2 that have the property of delay coagulation time, inhibit thrombin activity and can be used as vaccine antigens (Mulenga *et al.,* 1999) and it was similar to limulus intracellular inhibitor-2 (LICI-2). This serpins also had a function to maintain hemolymph circulation in Japanese horse shoe crab, which plays a role in regulating coagulation factor C and G (Kanost, 1999; Iwanaga *et al.,* 1998). However, amino acid sequence similarity of these serpins to LICI in the reactive center loop (RCL) was lower than that observed for heparin cofactor II that the RCL of serpins plays an essential role in the inhibition mechanism acting as a substrate for their target proteases (Felber *et al.,* 2006). Nevertheless, both LICI and HCII are the molecules involved in coagulation pathway (Imamura *et al.,* 2005). Although, the P1 (primary specificity site of the serpins) residue of serpins did not correspond to that of LICI, serpins showed the highest similarity to the *R. appendiculatus* serpin-3 (100% RCL identity). The sequence also contained two serpin consensus motifs (NAVYFKG and QVNEEG) that were also found in arthropods and mammalian serpins consensus motifs (Miura *et al.,* 1995; Han *et al.,* 2000).

2. Expression of serpin gene

The recombinant serpin has been expressed in *Escherichia coli* (Sugino *et al.,* 2003; Imamura *et al.,* 2006). Of these expression systems, *P. pastoris* has been shown to be the more efficient for protein secretion (Cregg *et al.,* 1993; Zhang *et al.,* 2005). In this study, the rserpin protein was successfully expressed in *P. pastoris* and induced antibody response in rabbits.

According to the EasySelect^{TM} *Pichia* Expression system, the induction time can influence the amount of the expression of recombinant protein. In this experiment, the optimal condition for serpin protein expression in yeast was 7 days. The molecular weight of serpin protein was calculated by summation of its amino acids (Adams, 2003) which was 42.5 kDa. The calculated molecular weight of rserpin protein expressed using recombinant *P. pastoris* was 45 kDa due to the extra molecular weight of 6 histidine in the plasmid pPICZαA (2.5 kDa). By SDS-PAGE, the rserpin protein produced by recombinant *P. pastoris* showed band at 45 kDa in size, secreted and released into media culture (Invitrogen, 2004). The result was similar to *R. appendiculatus* serpin-1 and -2 (RAS-1 and RAS-2) expressed in *E. coli* strain AD494 that molecular weight of RAS-1 and RAS-2 around 42.0 and 42.7 kDa reported by Imamura *et al*., (2006) and Sugino *et al.,* (2003). By Western blot analysis, partial purified rserpin protein was a band at ~ 45 kDa and had a specific interaction with mouse anti-histidine monoclonal antibody and bovine-anti-TSG protein polyclonal antibody. The result was similar to immunoreactivity of anti-rRAS-1 and RAS-2 sera against whole tick extracts (Imamura *et al*., 2006). Imamura *et al*., (2005) had cloned serpin gene from *Haemaphysalis longicornis*, expressed of *H. longicornis* serpin-2 (HLS2) in *E. coli* strain AD494, and immunized in rabbits. Vaccination of rabbits with HLS2 conferred protective immunity against ticks, resulting in 44.6 and 43.0% mortality in nymphal and adult ticks, respectively. These results also showed that rserpin could be an important candidate antigen as a component of an anti-tick vaccine since our result have confirmed the possibility of rserpin's immunogenicity (Imamura *et al*., 2005; Sugino *et al*., 2003).

3. Animal immunization

Rabbits were selected as an animal model to test the ability of serpin to induce antibody response. The rabbits were divided into groups and each group contained five rabbits since Halow and Lane (1988) suggested at least three rabbits per group was used to reduce error from variation of individual.

In previously experiment, rabbit immune response could be induced by immunization of RAS-1 and RAS-2 protein at 500 μg/dose (Imamura *et al*., 2006). In this study, rabbits were immunized by rserpin at 100 μg. However, various doses ranged from 50 to 1000 μg in rabbits were recommended by Halow and Lane (1988). The same dose at 100 μg was also successfully induced host responses against recombinant Bm86 production in cattle (Redondo *et al.,* 1999). Montanide adjuvant was selected to promote a strong antibody response because a subunit vaccine was less immunogenic than traditional vaccine (Aucouturier *et al.,* 2001; Hagan *et al.,* 2001; Tizard, 2000). Other adjuvant such as Complete Freunds' adjuvant was highly toxic to animal tissue (Halow and Lane, 1988). The emulsifying agent, montanide was easily mixed to protein antigen and stable. The function of montanide adjuvant can both enhance induced humoral and cell-mediated immunity (O'Hagan, 2009). The performance of these adjuvants is said to be similar to Incomplete Freunds Adjuvant [IFA] for antibody production; however the inflammatory response is usually less. The surfactants of the Montanide group undergo strict quality control to guard against contamination by any substances that could cause excessive inflammation, as has been found in Freund's adjuvant (Hanly *et al.,* 1994). Montanide adjuvant began to use in preparation of FMD vaccine since 1982. Excellent results have been recorded in the regular vaccine control tests and in the serological response of field cattle (Abaracon *et al.,* 1982).

The property of rserpin protein as an immunogen was investigated by indirect ELISA. The antibody against rserpin has been detected at 1 week after immunization and dramatically increased through the end of the experiment. The means optical density serpin group before immunize was significant lower than 1 to 7 week after

immunization, all immunized rabbits generated antibodies against rserpin at the first week of immunization and reached its peak within the seventh week. Comparison of means optical density serpin group with WT-protein group was higher than those from which was significantly different since the third week of the trial when statistical analysis using ANOVA. Because of, ELISA plates were coated by crude protein that antibodies of WT-protein rabbits were only reactivity with WT-protein but except specific signal against rserpin. Therefore, the optical level of WT-protein group has lower than serpin immunized rabbits, The result was similar to the effects of the vaccination with recombinant *H. longicarnis* serpin-1 (rHLS-1), higher OD values (0.5) than control unimmunized rabbits (0.01) after the first and second immunization (Sugino *et al*., 2003). There has been shown in several previous reports that vaccination of cattle with a combination of rRAS-1 and rRAS-2 conferred significantly reduction in the number of engorged nymphal *R. appendiculatus* and the number of molting adult ticks (Imamura *et al*., 2006). The increased mortality rate of adult ticks was correlated to the reduced amount of egg production which could lead to drastic reduction of tick population even under field condition (Imamura *et al*., 2006). Additionally, rserpin proteins have been prove as the promising antigen to induced host immunity against tick infestation (Sugino *et al*., 2003; Imamura *et al*., 2005; Andreotti *et al*., 2002).

CONCLUSION

The serpin gene of tick salivary glands collected from cattle in ChiangRai and RoiEt provinces were isolated by RT-PCR. The serpin cDNA was cloned into pPICZαA expression vector. The serpin cDNA is 1,200 bp in length, which encodes a serpin protein of 399 amino acid residues that deduced amino acid was 100% identity to each other. These deduced amino acid sequences were highly identical to the previous reported sequences serpins derived from *Rhipicephalus microplus*, *Rhiphicephalus appendiculatus* (serpin-3) and *Haemaphysalis longicornis* (rHLS-2). A recombinant serpin protein was successfully expressed in *P. pastori*s expression system. The optimum induction peroid for the expression of serpin protein was 7 days. The rserpin protein produced from recombinant *P. pastoris* showed a band at 45 kDa in size and this protein was secreted into media culture. Recombinant protein interacted specifically with bovine anti-TSG protein polyclonal antibodies and anti-histidine IgG.

The recombinant protein serpin at doses 100 μg was capable of eliciting antibody response when immunized and can be detected by ELISA and Western blot. Base on this result, our studies had established a potential use of recombinant serpin as a candidate antigen for cocktail anti-tick vaccine. However, the experiment was conducted in rabbits, so it requires further clinical trial in natural host animal to prove its efficacy and against tick infestations.

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APPENDICES

Appendix A

Chemical reagents and substances

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Chemical reagents and substances

1. *Pichia* media recipe

- 10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids)

Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 ml of water and filter sterilize. Heat the solution to dissolve YNB completely in water. Store at 4°C.

- 500X B (0.02% Biotin)

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at 4°C.

- 100X H (0.4% Histidine)

Dissolve 400 mg of L-histidine in 100 ml of water. Heat the solution, if necessary, to no greater than 50°C in order to dissolve. Filters sterilize and store at 4°C.

- 10X D (20% Dextrose)

Dissolve 200 g of D-glucose in 1000 ml of water. Autoclave for 15 minutes or filter sterilizes.

- 10X M (30% Methanol)

Mix 5 ml of methanol with 95 ml of water. Filters sterilize and store at 4°C.

- 10X GY (10% Glycerol)

Mix 100 ml of glycerol with 900 ml of water. Sterilize either by filtering or autoclaving and store at room temperature.

- 1 M potassium phosphate buffer, pH 6.0

Combine 132 ml of 1 M K_2HPO4, 868 ml of 1 M KH2PO4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at room temperature.

- YPD or YEPD Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)

1. Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water. Note: Add 20 g of agar if making YPD slants or plates.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 ml of 10X D. The liquid medium is stored at room temperature. YPD slants or plates are stored at 4°C.

- YPD (+ Zeocin) Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)
2% agar
100 µg/ml Zeocin.

1. Dissolve the following in 900 ml of water: 10 g yeast extract 20 g of peptone.
2. Include 20 g of agar if making YPD slants or plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Cool solution to ~60°C and add 100 ml of 10X D. Add 1.0 ml of 100 mg/ml Zeocin. YPD slants or plates are stored at +4°C.

- BMGY and BMMY Buffered Glycerol-complex Medium Buffered Methanol-complex Medium (1 liter)

1% yeast extract

2% peptone

100 mM potassium phosphate, pH 6.0

1.34% YNB

4 x 10-5% biotin

1% glycerol or 3% methanol

1. Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water.
2. Autoclave 20 minutes on liquid cycle.
3. Cool to room temperature, then add the following and mix well:

   100 ml 1 M potassium phosphate buffer, pH 6.0

   100 ml 10X YNB

   2 ml 500X B

   100 ml 10X GY
4. For BMMY, add 100 ml 10X M instead of glycerol.
5. Store media at 4°C.

2. <u>SDS-PAGE reagents</u>

- 2x SDS-PAGE sample buffer

2.5 ml of 4xTris HCl / SDS, pH 6.8 (250 mM Tris HCl)

2.0 ml of glycerol (20% glycerol)

0.4 g of SDS or 4 ml of 1 0%SDS (4% SDS)

0.2 ml of beta- mercaptoethanol (2% of 2-ME)

0.006% bromophonol blue

- 4x Tris HCl / SDS pH 6.8, buffer for stacking gel (250 ml)

0.5M Tris HCl ; 15 g Tris HCl

0.4% SDS; 1 g SDS

200 ml distilled water

Adjust pH using concentrated HCl , then add distilled water to 250 ml total volume

- 4x Tris HCl / SDS pH 8.8 buffer for separating gel (500 ml)

1.5M Tris HCl; 91 g Tris Base

0.4% SDS; 2 g SDS

400 ml distilled water

Adjust pH using concentrated HCI then add distilled water to 500 ml total volume

- 30% Acrylamide / Bis-acrylamide (bis-acrylamide acrylamide =1:36) to prepare 513.5 ml of solution.

150 g acrylamide

4.1 g Bis-acrylamide

Add distilled water to 513.5 ml sterilize by filter and store at 4°C.

- 10% Ammonium persulfate

100 mg ammonium persulfate

1 ml distilled water

- TEMED (N,N,N',N'-tetramethylethylenediamine) store protected from light at 4°C

- Glycine buffer

192 mM glycine

25 mM Tris base

0.1% SDS

- Coomasie Brilliant Blue stain (2 litres)

2 g Coomasie brilliant blue powder

1 litre methanol

200 ml acetic acid

800 ml distilled water

Stir for minimum 2 hours and filter through Whatman filter disc.

- Destainning solution (1 litre)

450 ml methanol

100 ml acetic acid

450 ml distilled water

3. Immunoblotting reagents

- PBS buffer, pH 7.4 (1 litre)

8.0 g NaCI

0.2 g $K_2H_2PO_4$

0.2 g KCl

1.15 g Na_2HPO_4

Distilled water adjust to 1000 ml

- PBS-Tween buffer

Add to final concentration of 0.5% Tween 20 (Sigma®)

- Blocking agent

5% skim milk in PBS- 0.5 %Tween buffer

- Serum diluting agent

2% skim milk in PBS- 0.5 %Tween buffer

- DAB (Sigma®) substrate

6 mg of DAB

10 µl of H_2O_2

990 µl of sterilize water

- Transfer buffer (1 litre)

25mM Tris; 3 g Tris base

190mM glycine; 14.4 g glycine

20% methanol; 200 ml conc. methanol (water adjust to 1,000 ml)

Appendix B

The standard methods

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The standard methods

1. Preparation of *E. coli* competent cells for transformation

1. Culture *E. coli* strain DH5α cells on LB agar plate at 37 °C overnight.
2. Pick up a large colony and culture in 1 ml of LB broth at 37°C overnight with vigorous shaking (~ 250 rpm).
3. From 500 µl of overnight culture, subculture to 100 ml of SOB medium containing 25 µg/ml kanamycin, incubate at 37°C until OD_{600} is 0.4 - 0.8 (approximately 3 - 4 hrs).
4. Store the culture on ice for 10 minutes.
5. Centrifuge at 4 °C, for 10 minutes at 3,000 rpm, discard the supernatant.
6. Gently resuspend the pellet in 33 ml of ice-cold TB and store on ice for additional 10 minutes
7. Centrifuge at 4°C, for 10 minutes at 3,000 rpm, discard the supernatant.
8. Gently resuspend the pellet with 2 ml of ice-cold TB, then add 7% DMSO (150 ul)
9. Aliquot the cell to ependorf tube each 200 ul and store at -70°C until use for transformation.

2. Phenol-Chloroform extraction of RNA and ethanol precipitation

1. RNA was extracted from 100 µl of tick salivary glands that mixed with 500 µl of denature solution and 50 µl of 2M NaAc, was shaken for 5-10 minutes
2. Add RNA phenol 150 µl and chloroform 150 µl was shaken for 5 minutes.
3. Centrifuge the sample at 13,000 rpm for 5 minutes to separate the phases.
4. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted a second time with same procedure.

5. Repeat 2-4 again.
6. Remove about 90% of the upper, aqueous layer to a clean tube, add isopropanol 550 µl and 0.5 µl of glycogen (20ng/ml), invert gently up side down and keep in – 80°C for 40 minutes
7. Centrifuge at 13,000 rpm for 10 - 15 minutes. Carefully decant the supernatant.
8. To wash the RNA pellet with 75% ethanol. Centrifuge at 13.000 rpm for 5 minutes. Decant the supernatant, and dry the pellet by air.

3. <u>QIA quick gel extraction kit protocol</u>

1. Exise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl)
3. Icubate at 50°C for 10 mins. To help dissolve gel, mix by vortexing the tube every 2-3 minutes during incubation.
4. After the gel slice has dissolve completely, check that color of the mixture is yellow
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. Apply the sample to the QIAquick column, and centrifuge for 1 minute.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. Add 0.75 ml of PE buffer to QIAquick colume and centrifuge for 1 minute.
10. Discard flow-through and place QIAquick column an additional 1 minutes at 13,000 rpm.
11. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
12. To elute DNA, add 50 ul of EB buffer (10 mM Tris-HCl, pH 8.5) to the center of QIAquick column membrane, let the column stand for 1 minute, and centrifuge for 1 minute.

4. Preparation of *P. pastoris* competent for electroporation

1. Grow 5 ml of your *Pichia pastoris* strain in YPD in a 50 ml conical at 30°C overnight.
2. Inoculate 500 ml of fresh medium in a 2 liter flask with 0.1-0.5 ml of the overnight culture. Grow overnight again to an OD600 = 1.3-1.5.
3. Centrifuge the cells at 1500 x g for 5 minutes at 4°C. Resuspend the pellet with 500 ml of ice-cold (0°C), sterile water.
4. Centrifuge the cells as in Step 3, and then resuspend the pellet with 250 ml of ice-cold (0°C), sterile water.
5. Centrifuge the cells as in Step 3, and then resuspend the pellet in 20 ml of ice-cold (0°C) 1 M sorbitol.
6. Centrifuge the cells as in Step 3, then resuspend the pellet in 1 ml of ice-cold (0°C) 1 M sorbitol for a final volume of approximately 1.5 ml. Keep the cells on ice and use that day. Do not store cells.

5. Transformation by electroporation

1. Mix 80 µl of the cells from Step 6 (above) with 5-10 µg of linearized DNA (in 5-10 µl sterile water) and transfer them to an ice-cold (0°C) 0.2 cm electroporation cuvette. Note: For circular DNA, use 50-100 µg.
2. Incubate the cuvette with the cells on ice for 5 minutes.
3. Pulse the cells according to the manufacturer.s instructions for yeast (*Saccharomyces cerevisiae*).
4. Immediately add 1 ml of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15 ml tube.
5. Let the tube incubate at 30°C without shaking for 1 to 2 hours.
6. Spread 10, 25, 50, 100, and 200 µl each on separate, labeled YPDS platescontaining 100 µg/ml Zeocin.. Plating at low cell densities favors efficient Zeocin selection.
7. Incubate plates from 3-10 days at 30°C until colonies form.

8. Pick 10-20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing 100 µg/ml Zeocin.

6. Expression of recombinant *Pichia* strains

1. Using a single colony, inoculate 100 ml of MGYH, BMGH, or BMGY in a 1 liter baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until the culture reaches an OD600 = 2-6 (approximately 16-18 hours).
2. Harvest the cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature. To induce expression, decant the supernatant, and resuspend cell pellet in MMH, BMMH, or BMMY medium using 1/5 to 1/10 of the original culture volume (approximately 10-20 ml).
3. Place in a 100 ml baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue to grow.
4. Add 100% methanol to a final concentration of 3% every 24 hours to maintain induction.
5. At each of the times indicated below transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2-3 minutes at room temperature. Time points (hours): 0, 24 (1 day), 48 (2 days), 72 (3 days), 96 (4 days), 120 (5 days), 144 (6 days) and 168 (7 days).
6. For secreted expression, transfer the supernatant to a separate tube. Store the supernatant and the cell pellets at -80°C until ready to assay.

7. Total DNA isolation from *Pichia*

1. Grow at 30°C the recombinant strain and the parent strain to an OD_{600} of 5-10 in 10 ml of minimal media such as MD or MDH. Note: *his4* strains require histidine for growth.
2. Collect the cells by centrifugation at 1500 x g for 5-10 minutes at room temperature.
3. Wash the cells with 10 ml sterile water by centrifugation as in Step 2.
4. Resuspend the cells in 2 ml of SCED buffer, pH 7.5. Make this solution fresh.
5. Add 0.1-0.3 mg of Zymolyase (mix well before adding to the cells). Incubate at 37°C for 50 minutes to achieve < 80% spheroplasting.
6. Add 2 ml of 1% SDS, mix gently and set on ice (0 to +4°C) for 5 minutes.
7. Add 1.5 ml of 5 M potassium acetate, pH 8.9, and mix gently.
8. Centrifuge at 10,000 x g for 5-10 minutes at +4°C and save the supernatant.
9. Transfer the supernatant from Step 5 above and add 2 volumes of ethanol to the supernatant. Incubate at room temperature for 15 minutes.
10. Centrifuge at 10,000 x g for 20 minutes at +4°C.
11. Resuspend the pellet **gently** in 0.7 ml of TE buffer, pH 7.4 and transfer to a microcentrifuge tube.
12. Gently extract with an equal volume of phenol:chloroform (1:1 v/v) followed by an equal volume of chloroform:isoamyl alcohol (24:1). Split the aqueous layer into two microcentrifuge tubes.
13. Add 1/2 volume of 7.5 M ammonium acetate, pH 7.5, and 2 volumes of ethanol to each tube. Place on dry ice for 10 minutes or at -20°C for 60 minutes.
14. Centrifuge at 10,000 x g for 20 minutes at +4°C and wash the pellets once with 1 ml of 70% ethanol. Briefly air dry the pellets and resuspend each one in 50 µl of TE buffer, pH 7.5. Determine the concentration of the DNA sample. The two samples can be stored separately or combined and stored at -20°C until ready for use.

8. Scale-up of expression

1. Using a single colony, inoculate 10 ml of MGYH, BMGH, or BMGY in a 100 ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until the culture reaches an OD600 = 2-6 (approximately 16-18 hours).
2. Use this 10 ml culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3 or 4 liter baffled flask and grow at 28-30°C with vigorous shaking (250-300 rpm) until the culture reaches log phase growth (OD600 = 2-6).
3. Harvest the cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend cell pellet in 1/5 to 1/10 of the original culture volume of MMH, BMMH, or BMMY medium (approximately 100-200 ml).
4. Place the culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator. Continue to grow at 28-30°C with shaking.
5. Add 100% methanol to 3% every 24 hours until the optimal time of induction is reached.
6. Harvest cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature. For intracellular expression, decant the supernatant and store the cell pellets at -80°C until ready to process. For secreted expression, save the supernatant, chill to +4°C, and concentrate it down if desired. Proceed directly to purification or store the supernatant at -80°C until ready to process further.

9. <u>SDS-polyacrylamide get electrophoresis (SDS-PAGE)</u>

Prepare the gel by the recipe as follow for 2 gels

- Seperating gel (10%)

H_2O	3.3 ml
4x Tris HCI/SDS pH 8.8	2.0 ml
30% Acry/0.8%bis-Acrylamide	2.66 ml
10% APS	26.6 µl
TEMED	6.0 µl

- Stacking gel

H_2O	2.135 ml
4x TrisHCI/SDS pH 6.8	0.875 ml
30% Acryl/0.8%bis-Acrylamide	0.455 ml
10% APS	17.5 µl
TEMED	6 µl

4.1 If not already in electrophoresis sample buffer, add an equal volume of 2X sample buffer to all samples and boil for 5 minutes.

4.2 Run electrophoresis (110 volt, 90 minutes, constant ampere)

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