Cloning and Characterization of cDNA Encoding a Serine Protease Inhibitor from Salivary Glands of Thai Cattle Tick (*Boophilus microplus*)

Paitoon Kaewhom¹, Theerapol Sirinarumitr², Sirirak Chantakru³ and Sathaporn Jittapalapong⁴*

**ABSTRACT**

Tick salivary gland (TSG) proteins have been an evidence for application as novel tick control agents. The serine protease inhibitors (serpins) secreted from TSG may be used in an anti-tick feeding. In this study, we cloned serpin cDNA from TSG of *Boophilus microplus* by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and analyzed its nucleotide and deduced amino acid sequences. Our results showed that the 1,200 bp open reading frame of serpins could encode a protein with 399 amino acid residues. By comparison with other serpin available in the GenBank database, the amino-acid sequence, in the reactive center loop (RCL) of the cloned serpin, demonstrated a 95% and 100% identity to those of the *B. microplus* and *Rhipicephalus appendiculatus*, respectively. This is the first report on a cloning of cDNA encoding a serpin derived from *B. microplus* in Thailand. Based on this result, Thai serpin genes were homologous to the other serpins gene and confirmed a potential use of recombinant Thai serpin as the candidate antigen for vaccine to immunize cattle against *B. microplus* infestations.

**Key words:** serpins, salivary gland, *Boophilus microplus*

**INTRODUCTION**

*Boophilus microplus*, the tropical cattle tick, is a one-host tick that causes economically important ectoparasite as vector of important disease pathogens of livestock in tropical and subtropical countries including Thailand. The conventional chemical control has several disadvantages such as high cost, environmental pollution, chemical residues in animal products and development of tick resistance. Therefore, anti-tick vaccine is the best alternatives to control tick due to their low cost, environmental safety, lack of human health risks and delayed onset of resistance. The major effect of current vaccines is the successive reduction in tick numbers due to the reduction of female’s fertility. Two sources of candidate vaccine antigens have been identified as exposed antigens that are secreted in tick saliva during attachment and feeding on a host (Willadsen, 1997). The other is concealed antigens normally hidden from the host (Mulenga *et al.*, 1997).

¹ Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus Nakornpathom 73140, Thailand.
² Department of Pathology, Faculty of Veterinary Medicines, Kasetsart University, Bangkok 10900, Thailand.
³ Department of Anatomy, Faculty of Veterinary Medicines, Kasetsart University, Bangkok 10900, Thailand.
⁴ Department of Parasitology, Faculty of Veterinary Medicines, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: fvetspj@yahoo.com
Recently, the third group of antigens has been developed using the combined properties of both exposed and concealed antigens resulting in adverse affects to adults and immature stages of a wide variety of tick species. It also showed transmission-blocking and protective activity against a tick-borne pathogen (Nuttall et al., 2006).

Normally, ticks are secreting saliva containing pharmacologically active molecules and modulating host immune response. Tick saliva affected immuno-modulation at the attachment site facilitates both tick feeding and enhances the success of transmission of pathogens from tick into hosts. On the other hand, host immunization with tick saliva antigen can induce anti-tick resistance (Kovar, 2004). The serpins (serine protease inhibitors) is one of tick saliva proteins that have important regulators of serine protease. Serpins have a role in inflammation, blood coagulation, fibrinolysis, and complement activation (Rubin, 1996). For examples, serpins of arthropod hemolymph are functioning in protecting arthropods from infections by fungal or bacterial protease and in regulating endogenous protease involved in prophenol oxidase activation, or cytokine activation (Polanowski and Wilusz, 1996).

The immunity developed in animal repeatedly infested by ticks indicated the possibility of tick vaccination using TSG antigens. Serpins is also recognized as a promising candidate antigen since there were evidences of delaying coagulation time and inhibit thrombin activity and caused a significant decrease of reproduction of both tick number and egg weight (Mulenga et al., 1999; Andreotti et al. 2002). To achieve a success of anti-tick vaccine development in Thailand, this study was aimed to clone and characterize the cDNA encoding a serpin from Thai cattle tick (B. microplus) salivary glands.

**MATERIALS AND METHODS**

**Tick and salivary gland dissection**

*B. microplus* were collected from cattle in Chiang Rai and Roi Et provinces (n = 400). Only partial fed female ticks were used in the experiment. 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 salivary glands were separated by pin-point forceps. Following dissection, the tissues were transferred into RNA stabilizer reagent (Invitrogen®, USA), and kept frozen at -80 °C until use.

**RNA extraction and RT-PCR**

Total RNAs were extracted from *B. microplus*’s salivary glands using the acid phenol-chloroform method (Chomczynsky and Sacchi, 1987). RT-PCR was performed according to the two-step RT-PCR protocol. Briefly, the first strand cDNAs were obtained by reverse transcription using 50 ng of total RNA from tick salivary glands, 13 µl of distilled 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 °C for 50 min. The TSG’s cDNA was amplified by polymerase chain reaction (PCR) using a specific forward primer containing a *Kpn I* restriction enzyme site 5'-GG TACCATGCTCGCCAAATTTCTCTTTCTCG-3' and a specific reverse primer containing an *Xba I* restriction enzyme site 5’-TCTAGAAGCTTGT GTTAACCTCTCCGATGAAA-3’. Polymerase chain reaction was performed for 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min and final extension at 72 °C for 7 min in a 100 µl containing 10 µl cDNA templates, 10 µl buffer (20 mM Tris-HCl (pH8.4), 50 mM KCl), 20 mM dNTPs, 150 mM MgCl2, 0.6 pgmol of
sense and anti-sense primer and 3.5 U DyNAzyme EXT DNA polymerase (Finnzymes®, USA) in a thermocycler (Primus 96 plus).

Construction of yeast expression vectors for serpins

After amplification, the serpin coding sequences were purified using QIAquick gel extraction kit (QIAGEN®). The purified products were digested with Kpn I and Xba I restriction enzymes, and then subcloned into the pPICZaA expression vector (Invitrogen®). The obtained plasmid, pPICZaA-serpin was transformed into either *E. coli* DH5α competent cells (GIBCO-BRL) or competent KM71 *Pichia* cells (Invitrogen®). The *E. coli* positive clones were selected on LB plates containing 25 µg/ml Zeocin, and identified the presence of serpin gene by PCR or restriction analysis. The *Pichia* positive clones were selected on YPD plates containing 100 µg/ml Zeocin, and confirmed by PCR.

DNA sequencing and computer-assisted sequence analysis

A single colony of *E. coli* positive clone was selected and subcultured in LB medium. After an overnight growth, plasmid DNA was purified from bacteria culture using QIAprep spin miniprep kit (QIAGEN®) 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 the obtained 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.

RESULTS

A serpin cDNA from Thai *Boophilus microplus’* salivary glands was 1,200 bp in length (Figure 1), which encoded a serpin protein of 399 amino acid residues (Figure 2) that the deduced amino acid sequence from Chiang Rai was 100% identity to that from Roi Et. The molecular weight of the protein was predicted to be about 43.1 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 *B. microplus* serpin (accession number AY312432), 93% identity to *Rhiphicephalus appendiculatus* serpin-3 (accession number AY035781), 70% identity to *Haemaphysalis longicornis* rHLS-2 (accession number AB162827) and 33% identity to *Ixodes ricinus* serpin (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% to that of *Haemaphysalis longicornis* rHLS-1 and rHLS-2

![Figure 1](image_url) The cDNA of Thai 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 1200 bp RT-PCR product.
respectively, 40% to *Ixodes ricinus* serpin (accession number CAB55818), 30, 35, 100, and 30% identity to *R. appendiculatus* serpin-1, serpin-2, serpin-3 and serpin-4, (accession number AY035779, AY035780, AY035781 and AY035782), respectively (Table 1). By using the NCBI-Blastp, Thai serpin deduced amino acid sequence showed 94% identity to *B. microplus* (accession number Q72021), 72% to *Amblyomma americanum* (member A7U119), 67% to *H. longicornis* rHLS-2 (member Q75Q63), 37% to putative secreted salivary gland peptide of *I. scapularis* (member Q06B72) and 36% to limulus intracellular coagulation inhibitor type 2 (member Q27086).

**DISCUSSION**

Previously, serpins have been genetically cloned from several tick species, including *R. appendiculatus* (Mulenga et al., 2003), *H. longicornis* (Sugino et al., 2003), and *B. microplus* (unsubmitted and accession number AB162827), respectively. 40% to *Ixodes ricinus* serpin (accession number CAB55818), 30, 35, 100, and 30% identity to *R. appendiculatus* serpin-1, serpin-2, serpin-3 and serpin-4, (accession number AY035779, AY035780, AY035781 and AY035782), respectively (Table 1). By using the NCBI-Blastp, Thai serpin deduced amino acid sequence showed 94% identity to *B. microplus* serpin in Gen Bank (member Q72021), 93% to *R. appendiculatus* serpin-3 (member Q8WQW9), 72% to *Amblyomma americanum* (member A7U119), 67% to *H. longicornis* rHLS-2 (member Q75Q63), 37% to putative secreted salivary gland peptide of *I. scapularis* (member Q06B72) and 36% to limulus intracellular coagulation inhibitor type 2 (member Q27086).

**DISCUSSION**

Previously, serpins have been genetically cloned from several tick species, including *R. appendiculatus* (Mulenga et al., 2003), *H. longicornis* (Sugino et al., 2003), and *B. microplus* (unsubmitted and accession number AB162827), respectively. 40% to *Ixodes ricinus* serpin (accession number CAB55818), 30, 35, 100, and 30% identity to *R. appendiculatus* serpin-1, serpin-2, serpin-3 and serpin-4, (accession number AY035779, AY035780, AY035781 and AY035782), respectively (Table 1). By using the NCBI-Blastp, Thai serpin deduced amino acid sequence showed 94% identity to *B. microplus* serpin in Gen Bank (member Q72021), 93% to *R. appendiculatus* serpin-3 (member Q8WQW9), 72% to *Amblyomma americanum* (member A7U119), 67% to *H. longicornis* rHLS-2 (member Q75Q63), 37% to putative secreted salivary gland peptide of *I. scapularis* (member Q06B72) and 36% to limulus intracellular coagulation inhibitor type 2 (member Q27086).
Several evidences suggested the possible use of the recombinant serpins proteins as anti-tick vaccine antigens for the control of tick infestation in cattle (Sugino et al., 2003; Imamura et al., 2005). In this paper, we have described for the first time of the complete sequences of serpin from *Thai B. microplus*. Comparison of nucleotide and the deduced amino acid sequence showed high identity to known serpins of the other *B. microplus* and *R. appendiculatus* registered in GenBank, resulting in 97% and 92% identity, respectively. It is interesting to find that Blastp analysis of serpin of *B. microplus*’ salivary gland revealed amino acid similar to serpin-2, which delay coagulation time, inhibit thrombin activity and it is capable of using as vaccine antigens (Mulenga et al., 1999). It was also similar to limulus intracellular inhibitor-2 (LICI-2) that had been reported as a serpins to maintain hemolymph circulation in Japanese horse shoe crab, which plays a role in regulating coagulation factor C and G (Iwanaga et al., 1998; Kanost, 1999). However, amino acid sequence’s identity of these serpins to LICI in the reactive center loop (RCL) was lower than heparin cofactor II that plays an essential role in the inhibition mechanism as a substrate for their target proteases (Felber et al., 2006). LICI and HCII are normally 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% identity). The sequence also contained two serpin consensus motifs (NA VYFKG and QVNEEG) that were similar to arthropods and mammalians serpins consensus motifs (Miura et al., 1995; Han et al., 2000).

In summary, two cDNA clones of serpins from TSG collected from cattle in Chiangrai and Roi-et provinces were isolated by RT-PCR. The serpin cDNA was 1,200 bp in length, which encoded a serpin protein of 399 amino acid residues deduced amino acid was 100% identity to the other. These deduced amino acid sequences were highly identity to the previously reported sequences serpins that derived from *B. microplus*, *R. appendiculatus* (serpin-3) and *H. longicornis* (rHLS-2). Based on this result, Thai serpin genes were homologous to the other serpins gene. This study indicated a potential possible of using recombinant serpin as a vaccine against *B. microplus*. The full-length cDNA sequence obtained from this study could be further used for

| Boophilus microplus' 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 |
| Rhipicephalus appendiculatus serpin-1     | * | * | * | * | * | E | A | A | A | A | V | M | M | A | A | C | C | L | S | 30 |
| serpin-2                                  | * | * | * | * | * | E | A | 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 | 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 | A | A | A | A | I | P | A | M | L | M | C | R | 40 |
| CAC22469                                  | * | * | * | * | E | A | A | A | 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 | * | T | V | * | M | * | * | * | T | Q | 65 |

* indicated the amino acid residues identical to those of serpins. The P1 amino acid residue indicated in box and associated with proteases by presenting a “bait” residue, which is thought to mimic the normal substrate of the enzyme.

(Zhou et al., 2003). Several evidences suggested the possible use of the recombinant serpins proteins as anti-tick vaccine antigens for the control of tick infestation in cattle (Sugino et al., 2003; Imamura et al., 2005). In this paper, we have described for the first time of the complete sequences of serpin from *Thai B. microplus*.
recombinant protein expression and rabbit immunization to determine the efficacy of these proteins as a candidate antigen for anti-tick vaccine.

LITERATURE CITED


strategy. **Immunology** 20: 541-544.


