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Humoral Antibody Responses of Rabbits Immunized with Anti-Tick Vaccine Using SERPIN Recombinant Protein

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ABSTRACT

The objective of this study was to determine the immunogenicity of recombinant serine protease inhibitor (rSERPIN) in rabbits. The rSERPIN protein, expressed in *Pichia pastoris,* was used to immunize rabbits three times at two- week intervals with a dose of 100 μg rSERPIN. Dot blot and SDS-PAGE analysis of crude supernatant showed 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 in the first week after immunization, which reached its peak at the seventh week. The antibody titers were analyzed by ANOVA and showed high significance between the immunized group and the control groups (group 1, PBS; and group 2, wild type protein) from the third week after immunization through to the end of the experiment. This result indicated that rSERPIN had a strong immunogenicity and might be the candidate antigen for an anti-tick vaccine. However, it is necessary to perform a clinical trail with targeted animals as natural hosts.

Key words: serpin, salivary gland, *Rhipicephalus microplus*, *Pichia pastoris*

INTRODUCTION

Cattle tick (*Rhipicephalus microplus*) infestation is the major factor causing deterioration in livestock development in tropical and subtropical countries, due to its capability as a vector for many pathogens. Currently, the control method of ticks in Thailand is based primarily on the use of acaricides, which have limited efficacy in reducing tick infestations. The use of acaricides is often accompanied by serious drawbacks, including acaricide-resistant ticks and environmental contamination. The development of vaccines against tick infestations offers a cost-effective and environmentally favorable control method. Commercial vaccines for the control of cattle ticks are based on the Bm86 protein that is produced by recombinant technology (Willadsen *et al*., 1995). However, new antigens are needed to increase the efficacy of tick vaccines. Recent technologies have been developed involving gene discovery for tick vaccines from the tick midgut or salivary gland that are multifunctional and morphologically complex, as the salivary glands are essential to the biological survival of the tick and intricately involved in the transmission of pathogens (Sauer *et al*., 1995).

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Both serine proteases and serpins have been identified in exo and endo-invertebrate parasites of mammals. This important immune-regulatory mechanism, which is also a conserved parasitic survival strategy to interfere with the host immune response, evolved much earlier than had previously been thought. This finding is as significant as the recent discovery of invertebrate immune signaling molecules (Salzet *et al*., 1999). It was hypothesized that serpins evolved specifically to limit immune activation by curtailing the enzymatic generation of stimulatory signals (Chopin *et al*., 1997; 1998). Furthermore, the relatively primitive parasitic invertebrates have co-evolved highly specific mechanisms, such as blocking host physiological processes, blood coagulation and antibody production. For example, two groups of low molecular weight serpins have been found in leeches and other haematophagous animals (Krezel *et al*., 1994). The first group inhibits the activation of mammalian blood clot formation, i.e., inhibitors of thrombin (e.g. hirudin, triatomin) or factor Xa as ixodine, found in the hard tick, *Ixodes ricinus* (Salzet *et al*., 1999). The second group including inhibitors of elastase and cathepsin G, acts on the extracellular matrix (Nekarda *et al*., 1998). Finally, serpins from tick salivary glands are involved in the facilitation of tick feeding and digestion of the blood meal, as well as disease transmission. Members of the serpins gene family may represent one of the most interesting candidate-target antigens for tick vaccine development because of their role in the regulation of several physiological functions, such as blood clotting cascade, clot resolution, the inflammatory response and complement activation (Mulenga *et al*., 2003). Thus, serpins are possible candidates for the development of vaccines. The serpins obtained from *Haemaphysalis longicornis* delayed coagulation time and inhibited thrombin activity (Mulenga *et al*., 1999). In 2000, Mulenga and others proposed that tick serpins could be used as vaccine antigens. Andreotti *et al*. (2002) showed that the serpins vaccination of cattle against *B. microplus* caused a significant impairment of tick reproductive functions, reflected by both tick number and egg weight. The current study was aimed to immunize rabbits by using rSERPIN and determine the ability to produce an immune response by serine protease inhibitor from the salivary glands of the Thai cattle tick, *Rhipicephalus microplus,* expressed in *Pichia pastoris*.

MATERIALS AND METHODS

Salivary gland dissection and RNA extraction

The cattle ticks (*Rhipicephalus microplus*) used in this study were collected from cattle in Chiang Rai and Roi Et provinces. Dissection of partially engorged female ticks followed the procedure previously described by Jittapalapong *et al*. (2004). Following dissection, the salivary glands were transferred into RNA stabilizer reagent (Invitrogen®), and kept frozen at -80°C until use. Total RNA was extracted from female adult *R. microplus* salivary glands by the acid phenol-chloroform method (Chomczynsky and Sacchi, 1987).

RT-PCR

The first stand cDNA was synthesized from total RNA with reverse transcriptase and amplified with serpin-specific primers (forward 5′-GGTACCATGCTCGCCAAATTTCTCT TTCTC G-3′ and reverse 5′-TCTAGAACTAGTGTG TTAACCTCTCCGATGAAA-3′), which included 5′ *Kpn* I and 3′ *Xba* I restriction sites for directional cloning. The following reagents were used in 100-µL reactions: 69.5 µL distilled water, 10 µL buffer (200 mM Tris-HCl(pH 8.4), 500 mM KCl), 2 µL of each 10 mM deoxynucleotide triphosphate, 3 µL of 50 mM $MgCl_2$, 1 µL each of forward and reverse primer, 3.5 µL of DyNAzyme EXT DNA polymerase (1 U/µL) (Finnzymes, USA) and 10 µL of template cDNA. DNA was amplified in a

Primus 96^{plus} thermocycler by 35 cycles of 1 min at 94°C, 1 min 55°C and 2 min at 72°C. Amplified products were analyzed by agarose gel electrophoresis.

Construction of yeast expression vectors for serpin

The amplified serpin gene was purified using a QIA quick gel extraction kit (QIAGEN®), digested with *Kpn* I and *Xba* I and ligated into pPICZα plasmids (Invitrogen®) for secreted expression with a C-terminal purification and detection tag. This vector contained the zeocin resistance gene for positive selection in *Escherichia coli* and *Pichia pastoris*. Ligated plasmids were used to transform *E. coli* strain DH5α competent cells and positive clones were selected by screening colonies on LB agar plates containing zeocin (25 μg /mL). These recombinant plasmids were used to transform *P. pastoris* strain KM71 competent cells by electroporation and those cells were selected by PCR screening of yeast colonies on YPD agar plates containing zeocin 100 μg /mL.

Recombinant serpin protein production

The overnight growth of recombinant *Pichia pastoris* was inoculated into 500 ml of BMGY (buffered complex glycerol medium) at a concentration of 1:10 and shaken at 250 rpm at 30°C until the culture reached an OD_{600} = 2-6 (approximately 16-18 h). The cells were harvested by centrifugation at 4,000 rpm for 10 min at 4°C. The supernatant was decanted and the cell pellets were resuspended in BMMY medium (buffered complex methanol medium) using 1/5 of the original culture to induce expression and then returned to the incubator to continue growth. Some 100% methanol was added to the final concentration of 3% methanol every 24 h to maintain induction. The supernatant was subjected to SDS-PAGE and Western blot analysis. The supernatant was transferred to a separate tube and stored at -80°C until used.

Preparation of rSERPIN protein

The recombinant serpin (rSERPIN) antigen was partially purified for immunization. It was separated by 10% SDS-PAGE and stained with copper stain. Specific protein bands were compared with a molecular protein marker by cutting with a blade and grinding with a glass bar in 500 μl of 0.1% SDS. The polyacrylamide gel was separated by centrifugation at 10,000 G for 5 min and the supernatant was transferred to a new micro tube. Protein precipitation was carried out by adding four volumes (V/V) of cool acetone into the incubated solution and leaving overnight at -80°C. Protein was collected by centrifugation at 10,000 G for 15 min; the acetone was discarded and the product completely dried at room temperature. The protein was resuspended with phosphate buffer saline (PBS) and the concentration was measured by spectrophotometer. Bovine serum albumin (BSA) was used to prepare a standard curve for approximated protein concentration.

Rabbit immunization

Nine male and six female New Zealand white rabbits (2 months old, approximately 2-2.5 kg weight) were randomly divided into three groups (3 male and 2 female rabbits per group): group 1 was immunized with 500 μl of phosphate buffer saline (PBS); group 2 was immunized with 500 μl containing 100 μg of wild type protein (only pPICZαA vector was transformed into *Pichia pastoris*); and group 3 was immunized with 500 μl containing 100 μg of rSERPIN and adjuvant. The vaccine recipe involved using the rSERPIN protein mixed with an equal volume of 10% Montanide 888 in mineral oil, which was merged together by ultra sonicator to homogenize the gradient. Each group was immunized three times at two-week intervals. The rabbits were bled weekly before and after immunization (3 μl/ rabbit).

Enzyme linked immunosorbent assay (ELISA)

Assessment of immunogenicity of rSERPIN in rabbit sera were tested for antibodies against rSERPIN protein by ELISA and data were analyzed by a comparison of the mean optical density (OD) values. The expressed antibodies were performed to determine the antibody titers. The microplates were coated with rSERPIN diluted (1:10) with coating buffer to get the final intensity appropriate for detection using the ELISA reader. The plate was incubated overnight at 4°C. The sera were diluted (1:100) with washing buffer and subsequently, 100 µl of 1:100 each diluted sera was added into each well and incubated at 37°C for 1 h. The excess sera were washed out and 100 µl of 1:3000 goat anti rabbit IgG conjugated with horseradish peroxidase was added into each well and incubated at 37°C for 1 h, before being washed out. Finally, 100 µl of tetra-methylbenzidine substrate was added to each well for 5 min. The reaction was then stopped with 100 µl of 0.1 N H_2SO_4. The reaction intensity was measured by the ELISA plate reader at the wavelength of 620 nm.

Statistical analysis

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

RESULTS

In vitro expression of rSERPIN

Recombinant serpin was expressed *in vitro* using the pPICZaA expression vector and the *P. pastoris* strain KM71 expression system. Recombinant protein was expressed as secreted into media culture. A molecular weight of rSERPIN was 45 kDa on 10% polyacrylamide gel, which 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 1).

Detection of rSERPIN expression by Western blotting

Detection of rSERPIN protein was determined by the Western blot technique. Each supernatant was 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 produced a negative result (Figure 2).

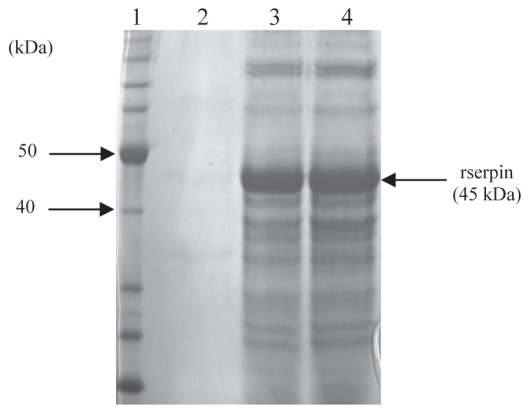

Figure 1 SDS-PAGE result demonstrating the size of the rSERPIN protein stained by Coomassie brilliant blue. Lane 1, molecular marker; lane 2, Wild type protein; lane 3 and 4, 45 kDa rSERPIN protein (arrow).

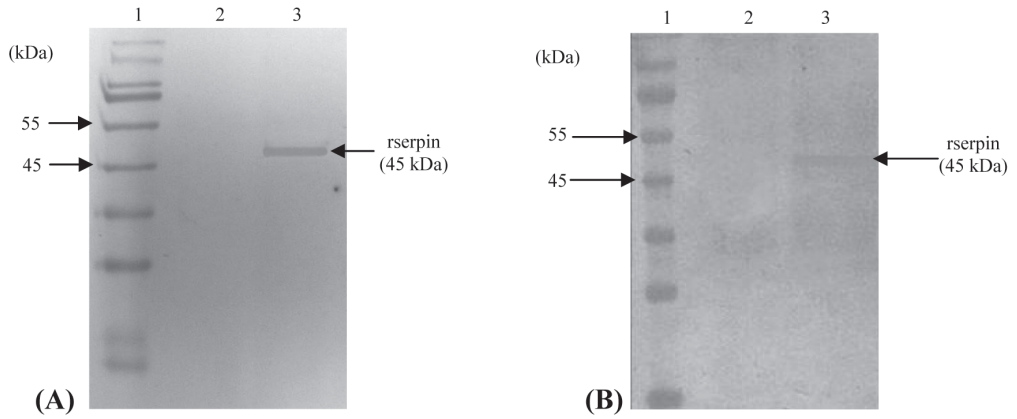

Figure 2 Determination of immunogenicity of rSERPIN protein by Western blot with: (A) mouse anti-histidine monoclonal antibody; and (B) bovine anti-TSG protein serum polyclonal antibody. Lane 1, Molecular prestain protein marker; lane 2, Wild type protein; lane 3, rSERPIN protein (arrow).

Humoral antibody response induced by rSERPIN

Assessment of immunogenicity of rSERPIN was conducted in rabbits. Rabbits were immunized and tested for levels of antibodies against rSERPIN protein by ELISA and the data were analyzed before immunization in terms of the 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 gradually increased after the second week following immunization and the levels were maintained throughout the experiment (Figure 3). 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 1).

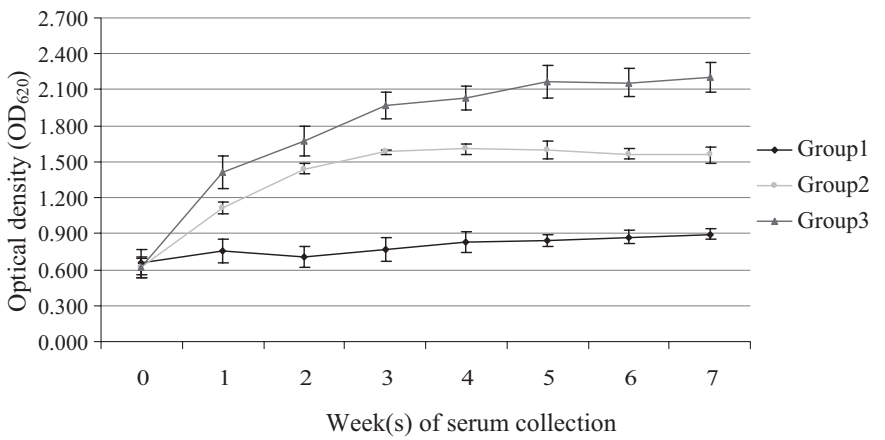

Figure 3 General linear model (GLM) of the mean value of optical density of antibodies from sera of rabbit immunized with PBS (group 1), WT-protein (group 2) and rSERPIN (group 3), using indirect ELISA.

Table 1 Statistic analysis of antibody responses against rSERPIN compared to the control group using ANOVA.

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

Note: Different superscripts in the same row indicate significant differences between groups.

DISCUSSION

Previously, the cDNA encoding serpin gene has been cloned from the salivary glands of the cattle tick, *Rhipicephalus microplu*s (Kaewhom *et al*., 2007). Analysis of the serpin gene showed a potential use of recombinant serpin as a vaccine to immunize cattle against ticks (Sugino *et al*., 2003). In this study, the recombinant serpin protein was successfully expressed in *Pichia pastoris* and induced an antibody response in rabbits.

The optimal condition for serpin protein expression in yeast was 3% final concentration of methanol induction for 7 d. The rSERPIN protein in *Pichia pastoris* was around 45 kDa in size and secreted in supernatant. Western blot analysis of partial purified rSERPIN protein showed a band at ~ 45 kDa and had a specific interaction with goat-anti-bovine polyclonal antibody against proteins from the salivary glands of the tick. These results also showed that rSERPIN could be an important candidate antigen as a component of an anti-tick vaccine, since previous results have confirmed the possibility of rSERPIN's immunogenicity (Sugino *et al*., 2003; Imamura *et al*., 2005).

The property of rSERPIN protein as an immunogen was investigated by indirect ELISA. The antibody against rSERPIN was detected one week after immunization and dramatically increased through to the end of the experiment. The means optical density of serpin group before immunization was significantly lower than 1 to 7 weeks after immunization; all immunized rabbits generated antibodies against rSERPIN at the first week of immunization and the antibodies reached a peak within the seventh week. Comparison of the mean optical density between the serpin group and the WT-protein group indicated that it was higher and significantly different at the third week of the trial, based on 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 the WT-protein group was lower than in the serpin-immunized rabbits, The result was similar to the effects reported following vaccination with recombinant *H. longicornis* serpin-1 (rHLS-1), which produced higher OD values (0.5) than in the control unimmunized rabbits (0.01) after the first and second immunization (Sugino *et al*., 2003). Several previous reports have shown that vaccination of cattle with a combination of rRAS-1 and rRAS-2 conferred a significant 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 a drastic reduction in the tick population, even under field conditions (Imamura *et al*., 2006). Additionally, rSERPIN proteins have been proven as a promising antigen to induced host immunity against tick infestations (Andreotti *et al*., 2002; Sugino *et al*., 2003; Imamura *et al*., 2005).

CONCLUSION

The recombinant protein, serpin, at doses of 100 mg was capable of eliciting an antibody response in immunization trials and could be detected by ELISA and Western blot. Based on these results, this study has established a potential use of recombinant serpin as a candidate antigen for a cocktail anti-tick vaccine. However, the experiment was conducted in rabbits, so it requires further clinical trials in natural host animals to prove its efficacy against tick infestations.

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