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The putative role of *Rhipicephalus microplus* salivary serpins in the tick-host relationship

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Abstract

Inflammation and haemostasis are part of the host's first line of defense to tick feeding. These systems are in part serine protease mediated and are tightly controlled by their endogenous inhibitors, in the serpin superfamily (serine protease inhibitors). From this perspective ticks are thought to use serpins to evade host defenses during feeding. The cattle tick *Rhipicephalus microplus* encodes at least 24 serpins, of which RmS-3, RmS-6, and RmS-17 were previously identified in saliva of this tick. In this study, we screened inhibitor functions of these three saliva serpins against a panel of 16 proteases across the mammalian defense pathway. Our data confirm that *Pichia pastoris*expressed rRmS-3, rRmS-6, and rRmS-17 are likely inhibitors of pro-inflammatory and pro-coagulant proteases. We show that rRmS-3 inhibited chymotrypsin and cathepsin G with stoichiometry of inhibition (SI) indices of 1.8 and 2.0, and pancreatic elastase with SI higher than 10. Likewise, rRmS-6 inhibited trypsin with SI of 2.6, chymotrypsin, factor Xa, factor XIa, and plasmin with SI higher than 10, while rRmS-17 inhibited trypsin, cathepsin G, chymotrypsin, plasmin, and factor XIa with SI of 1.6, 2.6, 2.7, 3.4, and 9.0, respectively. Additionally, we observed the formation of irreversible complexes between rRmS-3 and chymotrypsin, rRmS-6/rRmS-17 and trypsin, and rRmS-3/rRmS-17 and cathepsin G, which is consistent with typical mechanism of inhibitory serpins. In blood clotting assays, rRmS-17 delayed plasma clotting by 60 s in recalcification time assay, while rRmS-3 and rRmS-6 did not have any effect. Consistent with inhibitor function profiling data, 2.0 μM rRmS-3 and rRmS-17 inhibited cathepsin G-activated platelet aggregation in a dose-responsive manner by up to 96% and 95% respectively. Of significant interest, polyclonal antibodies blocked inhibitory functions of

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the three serpins. Also notable, antibodies to *Amblyomma americanum, Ixodes scapularis*, and *R. sanguineus* tick saliva proteins cross-reacted with the three *R. microplus* saliva serpins, suggesting the potential of these proteins as candidates for universal anti-tick vaccines.

Keywords

immune response; tick saliva; platelet aggregation inhibitor; cathepsin G

1. Introduction

The cattle tick *R. microplus* is one the most harmful hematophagous ectoparasites of bovines, with significant impact on the cattle industry worldwide due to its spoliation action and its role as a vector of tick-borne pathogens such as *Babesia* spp and *Anaplasma marginale*, the agents of babesiosis and anaplasmosis, respectively (Grisi et al., 2014; Jongejan and Uilenberg, 2004). Current tick control strategies rely mostly on the use of chemical acaricides, even though selection of resistant tick populations to most used acaricides has been confirmed (Guerrero et al., 2002; Guerrero et al., 2012; Pohl et al., 2011). This is recognized as a worldwide drawback to successful tick control, not to mention environment and food chain contamination hazards. Immunization of cattle against *R. microplus* and other ticks has been recognized as an alternative against tick control strategy (de la Fuente et al., 2007; Willadsen et al., 1989). Thus, in the effort to find effective targets for tick vaccine development, our research group has endeavored to understand how ticks acquire blood meal.

Tick blood feeding occurs as two steps, namely the disruption of host tissue and the suction of blood that flows into the feeding lesion, triggering a host response that includes pain, itching, blood coagulation, inflammation, complement activation, tissue repair response, and adaptive immune response (Francischetti et al., 2009; Heinze et al., 2014). Serine proteases such as pro-coagulant (thrombin, factor Xa, factor XIa, and other blood coagulation factors), pro-inflammatory (neutrophil elastase, proteinase-3, chymase, tryptase, kallikrein, cathepsin G, trypsin-like, and chymotrypsin-like), and complement proteases (factors B, factor C, factor D, and component 2) have a role in these host defense responses to tick feeding (Cattaruzza et al., 2014; Davie et al., 1979; Korkmaz et al., 2008; Matsunaga et al., 1994). Ticks successfully acquire blood meals by inoculation of saliva proteins in order to counteract host defenses to tick feeding (Francischetti et al., 2009; Ribeiro, 1987; Ribeiro and Francischetti, 2003). Proteomic analysis of tick saliva revealed that it contains a great variety of proteins with antihemostatic, anti-inflammatory, and immunomodulatory roles, among which proteinase inhibitors that belong to different families such as serpin, Kunitz-type, Kazal-type, cystatin, alpha-2-macroglobulin, thyropin, and trypsin inhibitor-like (TIL) inhibitors (Carvalho-Costa et al., 2015; Diaz-Martin et al., 2013; Lewis et al., 2015; Mudenda et al., 2014; Oliveira et al., 2013; Radulovic et al., 2014; Tirloni et al., 2014a).

Members of the serpin (serine proteinase inhibitors) superfamily are irreversible inhibitors of serine protease mediators of host defense pathways to tick feeding (Gettins, 2002). In mammals serpins are known to regulate blood coagulation cascade, fibrinolysis, wound

healing, angiogenesis, as well as inflammatory and immune responses (Rau et al., 2007; Silverman et al., 2001). This knowledge has led to the assumption that ticks inject serpins during feeding to disrupt the host homeostatic balance, as a way to prevent, slow down, and/or evade host defenses (Mulenga et al., 2001). Several tick serpin-encoding cDNAs have been cloned and characterized, including serpins from *Amblyomma americanum* (Kim et al., 2015; Mulenga et al., 2007; Mulenga et al., 2013; Porter et al., 2015), *A. maculatum* (Karim et al., 2011), *Ixodes scapularis* (Ibelli et al., 2014; Mulenga et al., 2009; Ribeiro et al., 2006), *I. ricinus* (Chmelar et al., 2011; Leboulle et al., 2002b; Prevot et al., 2006), *R. microplus* (Jittapalapong et al., 2010; Rodriguez et al., 2015; Rodriguez-Valle et al., 2012; Tirloni et al., 2014b), *R. appendiculatus* (Mulenga et al., 2003), *R. haemaphysaloides* (Yu et al., 2013), and *Haemaphysalis longicornis* (Imamura et al., 2005; Imamura et al., 2006; Sugino et al., 2003). Additionally, proteomic studies have identified serpins in saliva of blood-fed ticks, such as *R. microplus* (Tirloni et al., 2014a), *A. americanum* (Radulovic et al., 2014), *Dermacentor andersoni* (Mudenda et al., 2014), and *H. longicornis* (Tirloni et al., 2015), suggesting that the secretion of serpins is a common biologic strategy adopted by different tick species in order to counteract host's defenses during tick feeding.

Recent evidence shows that some of the tick-encoded serpins are functional inhibitors that are likely associated with tick evasion of host defense. In *A. americanum* two salivary serpins were characterized: serpin 6 (Chalaire et al., 2011; Mulenga et al., 2007), an inhibitor of papain and trypsin-like proteinases with anti-blood clotting and anti-complement activation functions (Mulenga et al., 2013), and *A. americanum* serpin 19 (AAS19), a conserved serpin among ixodid ticks that acts as a broad spectrum inhibitor of trypsin-like proteases with anti-haemostatic functions (Kim et al., 2015). In *I. scapularis*, a blood meal induced serpin inhibited thrombin and reduced platelet aggregation (Ibelli et al., 2014). In *I. ricinus* the serpin IRIS is an inhibitor of pro-inflammatory protease elastase and exhibits immunomodulatory properties (Prevot et al., 2006; Prevot et al., 2009). Likewise, *I. ricinus* serpin IRS-2 inhibited pro-inflammatory proteases cathepsin G and chymase, in addition to Th17 differentiation inhibition (Chmelar et al., 2011; Palenikova et al., 2015). In *R. haemaphysaloides* two serpins with inhibitory activity against chymotrypsin were described (Yu et al., 2013).

In a previous study, three (RmS-3, RmS-6 and RmS-17) of the 24 *R. microplus* serpin (Tirloni et al., 2014a) were found in saliva of this tick. The objective of the present study was to characterize these the three *R. microplus* saliva serpins to gain insight into their role(s) in the tick-host relationship. The data obtained confirms and builds on previous studies that characterized inhibitor function profiles of RmS-3 and RmS-6 (Rodriguez-Valle et al., 2012).

2. Materials and Methods

2.1 Ethics statement

Animals used in these experiments were housed at Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. This study was conducted according to the ethic and methodological aspects preconized by the International and National Directives and Norms by the Animal Experimentation Ethics Committee of the

UFRGS. The protocols were approved by the Comissão de Ética no Uso de Animais - CEUA – UFRGS.

2.2 Ticks and animals

R. microplus ticks (Porto Alegre strain), free of pathogens such as *Babesia spp* and *Anaplasma spp* were reared on Hereford calves (*Bos taurus taurus*), which were brought from a naturally tick-free area (Santa Vitória do Palmar, RS, Brazil; 33°32'2'' S, 53°20'59'' W) and maintained in individual sheds. Two naïve calves were infested with approximately 20,000 larvae that were 10 days old (from 1 g of *R. microplus* eggs). The larvae molt to nymphs and develop to females and males that mate. Female ticks feed to repletion and detaches from the host. The complete life cycle on the host takes roughly 21 days (Reck, Jr. et al., 2009). At 20th day, partially engorged adult females were manually detached from host.

2.3 Tick dissections, RNA and protein extractions, and cDNA synthesis

Partially engorged adult female ticks were manually and carefully removed from calves during tick feeding process. Ticks were collected, weighed, and divided in eight groups according to weight (as a measure of blood uptake index): group 1 (9.9 ± 1.6 mg); group 2 (16 ± 1.2 mg); group 3 (24.2 ± 1.9 mg); group 4 (34.8 ± 2.8 mg); group 5 (53.2 ± 2.3 mg); group 6 (84.2 ± 7.9 mg); group 7 (188.8 ± 15.4 mg); and group 8 (270.2 ± 14.9 mg). Ticks were rinsed with 70% ethanol, followed by dorsal surface dissecting with a scalpel blade to separate salivary glands (SG). Dissected SG from each group were washed with phosphate-buffered solution (PBS) in an RNase-free environment and placed in a tube containing TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Total RNA and protein were extracted according to the manufacturer's recommendations. The total RNA samples were resuspended in diethylpyrocarbonate (DEPC)-treated water and treated with DNase I (Invitrogen, Carlsbad, CA, USA). Total RNA concentration and purity were determined using a spectrophotometer. Protein samples were resuspended in 1% SDS solution and protein concentration was determined using the bicinchoninic acid method (BCA Protein Assay, Pierce, Rockford, USA) as previously described (Brown et al., 1989). Both total RNA and proteins were stored at −70 °C upon use.

2.4 Saliva collection

Tick saliva was collected as previously described (Clarke and Hewetson, 1971; Tirloni et al., 2014a). Partially engorged female ticks (varying from 20 to 200 mg) were manually and carefully removed from calves maintained in individual pens. Prior to saliva collection, any host contaminating tissue in tick mouthparts was removed using a scalpel blade and surgical forceps. Ticks were rinsed with sterile distilled water and induced to salivate by dorsal injection of 2–5 μL 2% pilocarpine (in PBS). Ticks were maintained at room temperature in a humid chamber for approximately 3 h, during which time saliva accumulated in the mouthparts was periodically collected using a pipette tip. Saliva protein concentration was determined by BCA and stored at −70 °C upon use.

2.5 Recombinant protein expression and purification of rRmS-3, rRmS-6 and rRmS-17

Serpin-encoding mature protein open reading frames of RmS-3, RmS-6, and RmS-17 (Tirloni et al., 2014b) were cloned into pPICZαC using a set of primers as described in Table S1. The pPICZαC/RmS expression plasmids were linearized with *Sac*I and electroporated into *Pichia pastoris* X-33 strain according to the manufacturer's recommendations (Life Technologies, Carlsbad, CA, USA). Transformed colonies were selected on yeast extract peptone dextrose medium (YPD) agar plates containing zeocin (100 μg/mL -Life Technologies, Carlsbad, CA, USA) and incubated at 28 °C. After five days, positive grown colonies were inoculated in buffered glycerol-complex medium (BMGY) and grown overnight at 28 °C with shaking (240 rpm). Subsequently, cells were used to inoculate buffered methanol-complex medium (BMMY) to OD_{600} of 1.0 and grown at 240 rpm at 28 °C during five days. Protein expression was induced daily during that period, by adding methanol to a 0.5 % final concentration. Recombinant proteins in spent culture media were precipitated by ammonium sulfate saturation (525 g/L of media) with stirring at 4 °C overnight. The precipitate was pelleted at 12,000 rpm for 1 h at 4 °C and resuspended in and dialyzed against buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.4). Expression of rRmS-3, rRmS-6, and rRmS-17 was confirmed resolving samples on a 12.5 % SDS-PAGE. Western blotting analysis was performed using an 1:5,000 dilution of antibody to the C-terminus hexa histidine tag (Life Technologies, Carlsbad, CA, USA), and positive signal was detected using a metal enhanced DAB chromogenic substrate kit (Thermo Scientific, Waltham, MA, USA). Recombinant proteins were affinity-purified under native conditions using Hi-Trap Chelating HP Columns (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). To evaluate purity, affinity-purified proteins were resolved on a 12.5% SDS-PAGE and stained with Coomassie brilliant blue. Affinity-purified proteins were dialyzed against 20 mM Tris-HCl, NaCl 150 mM buffer pH 7.4, protein concentration was determined by BCA and stored at −80 °C until use.

2.6 Antibody production and characterization

Rabbit anti-sera and murine monoclonal antibodies (MAbs) were raised for each serpin. To raise polyclonal antibodies against the three saliva serpins, rabbits were inoculated subcutaneously with 150 μg of either rRmS-3, rRmS-6 or rRmS-17 emulsified in oil adjuvant (Montanide 888 - Seppic and Marcol 52 – Exxon Mobil Corporation). Following this first administration, two 150-μg boosters of each recombinant serpin in the same adjuvant were applied at 15-day intervals. Antibodies from sera were purified by affinity chromatography on a protein G Sepharose resin according to the manufacturer's instructions (GE Healthcare, Pittsburgh, PA, USA).

To raise monoclonal antibodies against the three saliva serpins, six-week-old BALB/c mice were injected three times intraperitoneally with 100 μg of either rRmS-6 or rRmS-17 (Freund´s adjuvant, Sigma-Aldrich, St. Louis, MO, USA) at 15-day intervals for monoclonal antibody production (Harlow and Lane, 1988). Indirect ELISA using recombinant protein was used to obtain the sera antibodies titer. Splenic lymphocytes were fused to murine Sp2/O-Ag14 myeloma cells in the presence of PEG 3350 (Sigma-Aldrich, St. Louis, MO, USA) and cultivated in DMEM (Sigma-Aldrich, St. Louis, MO, USA) containing 20% fetal calf serum (Cultilab, Campinas, SP, Brazil) and HAT (Sigma-Aldrich, St. Louis, MO,

USA). Hybridomas growing in HAT medium were screened for specific antibodies by indirect ELISA, and those producing positive results were cloned twice using the limiting dilution technique. MAbs were purified by affinity chromatography on a protein G Sepharose resin according to the manufacturer's instructions (GE Healthcare, Pittsburgh, PA, USA) and dialyzed against PBS. Concentrations of purified MAbs and polyclonal antibodies were determined at OD_{280nm}, and the preparations were stored at −20 °C until use.

Anti-sera and MAbs specificity to native RmS targets in tick saliva was tested using western blotting and a sandwich ELISA assay. Purified rabbit IgG against rRmS-6 or rRmS-17 was used to coat 96-well polyestirene plates (310 ng/well) for 1 h at 37 °C. PBS-Tween (PBS-T) was used as a blocking solution for 1 h at 37 °C. Saliva from partially engorged *R. microplus* was added to the plate (800 ng of total saliva protein per well diluted in PBS-T) and incubated for 1 h at 37 °C, when MAbs were added to each well (100 ng/well diluted in PBS-T). After three washes in PBS-T, wells were incubated with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Sigma, St. Louis, MO, USA) for 1 h at 37 °C. After three washes in PBS-T, the reaction was visualized with 100 μL of enzyme substrate/chromogen solution (H_2O_2/ortophenylenediamine) in 100 mM citrate-phosphate buffer, pH 5.0, and the reaction was allowed to take place in the dark for 10 min. Optical density was read at 492 nm using the VersaMax tunable plate reader (Molecular Devices, Sunnyvale, CA, USA).

Purified rabbit IgG and purified MAbs were assayed in an indirect ELISA as above, with modifications to identify cross-reaction with different recombinant serpins. Briefly, 200 ng/well of either rRmS-3, rRmS-6 or rRmS-17 were used to coat 96-well polystyrene plates. After removal of unbound proteins and blocking unspecific binding sites, 1 μg/well of antibody was added to each well and incubated for 1 h at 37 °C. After three washes in PBS-T, wells were incubated with a 1:2,000 dilution of either horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA). The reaction was visualized as described above. Results were expressed as reaction percentage (the optical density from antibody raised to the antigen used as immunogen was taken as 100 %) from triplicates and were expressed as mean ± SD.

2.7 Expression analysis of salivary serpins by RT-PCR and western blotting

Expression profiles of the RmS-3, RmS-6 and RmS-17 genes in salivary glands from eight different adult female developmental stages were determined by RT-PCR and western blotting analysis. OligodT primed cDNA was synthesized from 5 μg of total RNA using the SuperScript III kit (Life Technologies, Carlsbad, CA, USA). Specific forward and reverse primers (Table S1) were used to determine transcription profile in pools of cDNA of SG from those eight groups of ticks (under item 2.3). For a constitutive gene control, forward and reverse primers targeting the 40S ribosomal protein S3a were used (Table S1). RT-PCR was carried out with 400 ng of cDNA using Taq DNA polymerase (Ludwig Biotecnologia, Porto Alegre, RS, Brazil). The reactions were performed according to the following steps: 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min and 30 s at 72 °C, with a final elongation at 72 °C for 5 min. PCR products were electrophoresed on a 1.0

% agarose gel and visualized by staining with GelRed™ (Uniscience, São Paulo, SP, Brazil). To analyze RmS-3, RmS-6, and RmS-17 protein expression profile in tick SG, total protein extracts were resolved on a 12 % SDS-PAGE (100 μg total protein per lane) and western blotting performed using rabbit pre-immune (1:500) or immune anti-sera raised for each recombinant serpin (1:500) as primary antibody. Subsequently, membranes were incubated with phosphatase-conjugated anti-rabbit IgG (Sigma–Aldrich) as secondary antibody for 1 h in a 1:5000 blocking buffer dilution, and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro blue tetrazolium) were used for the colorimetric detection.

2.8 Deglycosylation assay

Amino acid sequence analyses predicted the existence of N-linked glycosylation sites in RmS-3, RmS-6, and RmS-17 (Tirloni et al., 2014b). To determine if yeast-expressed serpins and native salivary serpins were glycosylated, affinity-purified protein and total saliva proteins were treated with a protein deglycosylation enzyme mix according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Deglycosylation was confirmed by 12.5 % SDS-PAGE stained with Coomassie brilliant blue and western blotting using antibody to the C-terminus hexa histidine tag (as described in item 2.5) or rabbit anti-sera against serpins (1:200 dilution) and 1:2,500 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA).

2.9 Inter-species cross-reactivity assay

To analyze if recombinant *R. microplus* serpins react with serum from animals infested with *R. microplus* and if they cross-react with serum from animals infested with other ticks species, rRmS-3, rRmS-6, and rRmS-17 (2 μg both glycosylated as well as deglycosylated proteins) were subjected to western blotting analysis using: (i) cattle antibodies (1:50 dilution) generated to replete-fed (allowed to feed to completion) *R. microplus* (Reck, Jr. et al., 2009), and (ii) rabbit antibodies generated to replete-fed adult *I. scapularis* (1:25 dilution), *A. americanum* (1:50 dilution), and *R. sanguineus* (1:50 dilution) tick saliva proteins. After three washes in PBS, membranes were incubated with a 1:5,000 dilution of either horseradish peroxidase (HRP)-conjugated anti-bovine or a 1:2,000 dilution of anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA). After three washes with PBS, antibody binding was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate.

Antibodies to tick-saliva proteins from replete-fed *I. scapularis* and *A. americanum* were produced as previously published (Chalaire et al., 2011; Ibelli et al., 2014). To obtain antibodies to tick saliva proteins from replete-fed *R. sanguineus*, New Zealand White rabbits were repeatedly infested (three times) with 40 adult ticks (20 males and 20 females). Two weeks after the last infestation, immune serum was collected and stored at −70 °C.

2.10 Protease inhibition assay

To get insight into inhibitory activity of rRmS-3, rRmS-6, and rRmS-17, their protease inhibitory activities were evaluated against a panel of 16 mammalian serine proteases, most

of which are related to host defense pathways against tick feeding (the amount of protease used is give in parenthesis): bovine thrombin (43 U), pancreatic porcine elastase (21.6 nM), pancreatic bovine trypsin (24.6 nM), pancreatic bovine α-chymotrypsin (96 nM), pancreatic porcine kallikrein (33 U), human chymase (10 U), human tryptase (10 U), human plasmin (10 nM) (Sigma-Aldrich, St. Louis, MO, USA), human neutrophil cathepsin G (166 nM) (Enzo Life Sciences Inc., Farmingdale, NY, USA), human factor XIa (3.68 nM), bovine factor IXa (306 nM), human factor XIIa (7.6 nM), human t-PA (32 nM), human u-PA (47.2 nM) (Molecular Innovations, Inc., Novi, MI, USA), bovine factor Xa (5.8 nM) (New England Biolabs, Ipswich, MA, USA), and human proteinase-3 (68 U) (EMD Millipore, Billerica, MA, USA). Substrates were used at 0.20 mM final concentration and purchased from Sigma-Aldrich: N α-benzoyl-DL-Arg-pNA for tryptase; N-succinyl-Ala-Ala-Pro-Phe-pNA for chymase, cathepsin G, and chymotrypsin; N-benzoyl-Phe-Val-Arg-pNA for thrombin and trypsin; and N-succinyl-Ala-Ala-Ala-p-nitroanilide for pancreatic elastase. The following substrates were purchased from Chromogenix, a daughter-company of Diapharma Inc. (Philadelphia, PA, USA): Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA for factor Xa; H-D-Val-Leu-Lys-pNA for plasmin; and H-D-Pro-Phe-Arg-pNA for kallikrein, factor XIa, and factor XIIa. The substrate CH_3SO_2-D-CHG-Gly-Arg-pNA was purchased from Enzyme Research and used for factor IXa, u-PA and t-PA. The substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-pNA was purchased from Enzo Life Sciences and used for proteinase-3. Reagents were mixed at room temperature in triplicate. Recombinant proteins (1 μM) were pre-incubated with indicated amounts of the protease for 15 min at 37 °C in 20 mM Tris-HCl, 150 mM NaCl, BSA 0.1%, pH 7.4. The corresponding substrate for each protease was added to a 100-μL final reaction volume. Substrate hydrolysis was measured at OD_{405nm} every 11 s for 30 min at 30 °C using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Acquired OD_{405nm} data were subjected to one phase decay analysis in Prism 6 software (GraphPad Software, La Jolla, CA, USA) to determine plateau values as proxy for initial velocity of substrate hydrolysis. The percent protease activity inhibition level was determined using the formula: $100 - (V_i/V_0) \times 100$ where, V_i = activity in presence of, and V_0 = activity in absence of recombinant serpins. Data are presented as mean percent of inhibition from triplicate readings and at least duplicate assays. The unit (U) definition used here is the amount of proteinase necessary for the increase of 0.001 OD_{405nm}/min.

2.11 Stoichiometry of inhibition assay

Stoichiometry of inhibition (SI) indices were determined for each serpin displaying inhibitory activity. The kinetic of substrate hydrolysis in the absence and in the presence of recombinant serpins was evaluated. Preparations were pre-incubated for 1 h with constant concentration of: (i) chymotrypsin (10 nM), cathepsin G (200 nM), and pancreatic elastase (50 nM) for rRmS-3 (molar ratio ranging from 0 to 10); (ii) trypsin (10 nM), chymotrypsin (10 nM), factor Xa (5 nM), factor XIa (5 nM), and plasmin (50 nM) for rRmS-6 (molar ratio ranging from 0 to 10 for trypsin, chymotrypsin and plasmin, and from 0 to 20 for factor Xa and factor XIa); and (iii) trypsin (10 nM), chymotrypsin (10 nM), cathepsin G (200 nM), factor XIa (5 nM), and plasmin (50 nM) for rRmS-17 (molar ratio ranging from 0 to 10 for trypsin, chymotrypsin, cathepsin G and plasmin, and from 0 to 20 for factor XIa). Protease activity was measured using colorimetric substrates specific for each proteinase as described above. Data were plotted as the protease residual activity (V_i/V_0) versus serpin:protease

molar ratio. SI or the molar ratio serpin to protease (when protease activity is completely inhibited) was determined by fitting data onto the linear regression line and the SI index was estimated from the X-axis intercept (Kantyka and Potempa, 2011).

2.12 Serpin-protease complex formation

Formation of covalent complexes serpin-protease (Huntington et al., 2000) was evaluated as follows. Affinity-purified (i) rRmS-3 was incubated with chymotrypsin (0.5 μg) and cathepsin G (0.5 μg); (ii) rRmS-6 was incubated with trypsin (1.16 μg); and (iii) rRmS-17 was incubated with trypsin (1.16 μg) and cathepsin G (0.5 μg), all in varying molar ratios (from 0 to 10). These serpin-protease incubations were prepared in buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4) and incubated for 1 h at 37 °C. SDS-PAGE denaturing sample buffer was added to the reaction mix, and incubated for 5 min at 99 °C. Samples were subjected to 12.5 % SDS-PAGE and stained with Coomassie brilliant blue.

2.13 Recalcification time

The recalcification time (RT) assay was performed with 50 μL of universal coagulation reference human plasma Thermo Scientific, Waltham, MA, USA) pre-incubated in presence (10 μM) or absence of recombinant serpins in 90 μL of reaction buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 15 min at 37 °C. Adding 10 μL of pre-warmed 150 mM calcium chloride ($CaCl_2$) triggered plasma clotting. Plasma clotting was monitored every 20 s for 30 min at OD_{605nm} using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland) (Ciprandi et al., 2006).

2.14 Anti-platelet aggregation activity

Anti-platelet aggregation activity of rRmS-3 and rRmS-17 upon cathepsin G-induced platelet aggregation was determined using platelet-rich plasma (PRP) prepared from citrated (acid citrate dextrose) whole bovine blood as previously described (Berger et al., 2010; Kim et al., 2015). To prepare PRP, fresh citrated whole bovine blood was centrifuged at 200 g for 20 min at 18 °C. Subsequently, the PRP (top layer) was transferred into a new tube and centrifuged at 800 g for 20 min at 18 °C. The pellet containing platelets was washed and diluted with Tyrode solution pH 7.4 (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 % glucose, 0.25 % BSA) until $OD_{650} = 0.15$. To determine anti-platelet aggregation activity, different amounts of rRmS-3 or rRmS-17 (varying from 3 μM to 0 μM) were pre-incubated for 15 min at 37 °C with cathepsin G (0.7 μM) in a 50-μL reaction. Adding 100 μL of pre-warmed PRP triggered platelet aggregation. Platelet aggregation was monitored every 20 s over 30 min at OD_{650nm} using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). In this assay, higher OD_{650nm} was observed in our blank (platelet only), and increased platelet aggregation was correlated with reduction in OD_{650nm}. Percentage of platelet aggregation inhibition was quantified by calculating the area under the curve and expressed as percent of the negative (only PRP and buffer) and positive control (absence of serpin). Data is presented as mean ± SEM of triplicate platelet aggregation assays.

2.15 Anti-rRmS antibodies effect upon serpin inhibitory activity

Purified rabbit IgG from anti-sera or MAbs raised against serpins were used to check if antibody-binding to serpins could block their protease inhibitory activity. rRmS-3 (55 nM), rRmS-6 (55 nM), and rRmS-17 (88 nM) were pre-incubated with purified rabbit IgG from anti-sera (varying from 0.8 to 3 μM) or purified mouse IgG from MAbs (varying from 0.3 to 1 μM) for 30 min at 37 °C before the addition of chymotrypsin (4.9 nM) for rRmS-3, or trypsin (1.6 nM) for rRmS-6 and rRmS-17, following a new 15-min incubation at 37 °C. Substrate (N-(p-Tosyl)-Gly-Pro-Lys-pNA for trypsin, and N-succinyl-Ala-Ala-Pro-Phe-pNA for chymotrypsin) was added to a 100-μL final reaction volume (final concentration 0.2 mM) and substrate hydrolysis was measured at OD_{405nm} every 11 s for 15 min at 30 °C using the VersaMax tunable plate reader (Molecular Devices, Sunnyvale, CA, USA). Data are shown as mean of two assays done in duplicates.

3. Results

3.1 Salivary serpins are glycoproteins

Data about expression and affinity purification of rRmS-3, rRmS-6, and rRmS-17 in *P. pastoris* are summarized in Fig.1A and 1B. Daily samples of yeast-expressed recombinant proteins were subjected to western blotting analysis using a specific antibody directed to the C-terminus histidine tag (Fig. 1A). Recombinant proteins were purified by affinity chromatography under native conditions and migrated according to molecular weight: ≈40 kDa for rRmS-3 and rRmS-6, and ≈50 kDa for rRmS-17 (Fig. 1B). When treated with deglycosylation enzymes, downward molecular weight shifts were observed (Fig. 1C), demonstrating that rRmS-3, rRmS-6, and rRmS-17 are glycosylated and consistent with sequence-based predictions (Tirloni et al., 2014b).

3.2 Serum from animals repeatedly infested with different tick species recognize *R. microplus* saliva serpins

Immune serum from bovine repeatedly infested with *R. microplus* recognized all three recombinant salivary serpins (Fig. 2). Both glycosylated and deglycosylated forms of rRmS-3 bound *R. microplus* antibodies, while only glycosylated rRmS-6 and rRmS-17 were recognized by bovine anti-serum to *R. microplus* saliva proteins (Fig. 2). In addition to *R. microplus*-infested bovine serum, serum from *R. sanguineus* and *A. americanum* repeatedly infested rabbits bound both glycosylated and deglycosylated forms of the three serpins. Immune serum to *I. scapularis* weakly bound to rRmS-6 (Fig. 2).

3.3 Feeding and salivary serpins expression profile

The temporal transcription analyses show that mRNA of serpins in tick salivary gland was present throughout all SG blood feeding stages investigated (Fig. 3A and Fig. 3B). In order to compare mRNA expression profiles with protein production, total tick salivary proteins were subjected to western blotting analysis (Fig. 3C). Polyclonal sera recognized all three serpins in the eight different groups of tick SG (Fig. 3C). Serpins appear as a band between 40 and 55 kDa (arrows in Fig. 3C). This molecular size is in accordance as previously published for identification of RmS-3, RmS-6, and RmS-17 in *R. microplus* saliva by GeLC-

MS/MS (Tirloni et al., 2014a). RmS-3, RmS-6 and RmS-17 were expressed at some level throughout all groups, (Fig. 3C).

3.4 *R. microplus* salivary serpins inhibit trypsin- and chymotrypsin-like proteases

The inhibitory profile of rRmS-3, rRmS-6, and rRmS-17 serpins against a panel of 16 mammalian proteases associated with host defense pathways showed a trypsinand chymotrypsin-like inhibitory pattern (Table 1). Incubation of serpins with the protease in a molar excess showed that rRmS-3 (1 μM) inhibited the activity of chymotrypsin (96 nM) by 96 %, the activity of cathepsin G (166 nM) by 78 %, the activity of pancreatic elastase (21.6 nM) by 92 %, and the activity of chymase (10 U) by 23 %. Comparatively, rRmS-6 (1 μM) inhibited the activity of trypsin (24.6 nM) by 73 %, the activity of plasmin (10 nM) by 24 %, the activity of factor Xa (5.8 nM) 32 %, the activity of factor XIa (3.68 nM) by 62 %, and the activity of chymotrypsin (96 nM) by 24 %. Also, rRmS-17 (1 μM) inhibited the activity of trypsin (24.6 nM), plasmin (10 nM), cathepsin G (166 nM), chymotrypsin (96 nM), and factor XIa (3.68 nM) by 87 %, 58 %, 78 %, 89 %, and 98 %, in that order (Table 1).

The inhibitory efficiency was evaluated using the SI (stoichiometry of inhibition) index where the kinetics of inhibition by tick serpins upon their sensible protease was tested at different molar ratios. Accordingly, the SI index for rRmS-3 was 2.0 for cathepsin G (Fig. 4A), 1.8 for chymotrypsin (Fig. 4B), and higher than 10 for pancreatic elastase (Fig. 4C). For rRmS-6, SI index was 2.6 for trypsin (Fig. 5A), near to 10 for plasmin (Fig. 5E) and higher than 10 for chymotrypsin (Fig. 5B), factor Xa (Fig. 5C) and factor XIa (Fig. 5D). For rRmS-17, SI index was 1.6 for trypsin (Fig. 6A), 2.7 for chymotrypsin (Fig. 6B), 2.6 for cathepsin G (Fig. 6C), 3.4 for plasmin (Fig. 6D), and higher than 10 for factor XIa (Fig. 6E).

The mechanisms of action of rRmS-3, rRmS-6, and rRmS-17 confirmed that they behave like typical inhibitory serpins (Fig. 7). rRmS-3 is able to form covalent complex with chymotrypsin and cathepsin G (Fig. 7A), rRmS-6 is able to form a stable complex with trypsin (Fig. 7C), and rRmS-17 is able to form stable complexes with trypsin and cathepsin G (Fig. 7B). After incubation of a protease with a serpin, the resulting complex has molecular mass increased to a value corresponding to the sum of the masses of the target protease and the cleaved serpin (complexes indicated by arrows in Fig. 7). These irreversible complexes between serpins and the target proteases were observed at similar molar ratios shown in SI assays (Figs. 4 – 6).

3.5 rRmS-3 and rRmS-17 inhibit cathepsin G-induced platelet aggregation

Cathepsin G released from activated neutrophils induces platelet aggregation via PAR4 activation (Sambrano et al., 2000; Selak et al., 1988). Since rRmS-3 and rRmS-17 were able to inhibit cathepsin G (Table 1, Fig. 4, and Fig. 6), we tested the ability of these serpins to inhibit cathepsin G-induced platelet aggregation. rRmS-3 inhibited cathepsin G-activated platelet aggregation in a dose-responsive manner by 10% at 0.5 μM to 96% at 2.5 μM (Fig. 8A and Fig. 8C). Similar result was observed for rRmS-17, which inhibited cathepsin G-activated platelet aggregation by 18% at 0.5 μM to 94% at 2.5 μM (Fig. 8B and Fig. 8C). *R. microplus* recombinant serpins did not have any effect on thrombin and ADP-induced platelet aggregation (data not shown). When subjected to blood clotting assays, rRmS-17

delayed recalcification time (RT) by 60 s (Fig. 9). However, none of these three serpins delayed plasma clotting time, aPTT, PT, and TT (data not shown).

3.6 Antibodies to RmS-3, -6 and -17 recognize native and recombinant serpins

Five monoclonal antibodies (MAb) were generated from rRmS-6-immunized mice, and one MAb was obtained from an rRmS-17-immunized mouse. MAbs and purified rabbit IgG were tested in an indirect ELISA to detect cross-reactivity among the recombinant serpins (Table 2). MAbs BrBm41 and BrBm42 reacted only with rRmS-6, while MAbs BrBm39 and BrBm43 reacted with all three recombinant proteins. Although clone BrBm40 recognizes all three serpins, it reacts poorly with rRmS-17. Though MAb BrBm44 recognizes all three recombinant serpins, reactions were weak (Table 2). The three polyclonal sera reacted with all three recombinant proteins, showing cross-reactivity ranging from 30% to 55% with two other serpins (Table 2). Two of the MAbs (BrBm39 and BrBm40) were able to recognize native serpins in the saliva, as shown by a sandwich ELISA (Table 2), although no reaction was observed when MAbs were tested with denatured saliva proteins in a western blotting assay (data not shown). Additionally, all three polyclonal sera were able to recognize serpins in the saliva, whether they were glycosylated or deglycosylated (Fig. 10). The variation in molecular sizes of glycosylated and deglycosylated saliva proteins confirms that native RmS-3, RmS-6, and RmS-17 are secreted as glycosylated proteins (Fig. 10).

3.7 Antibodies abolish serpin inhibitory activity function

Purified IgG from polyclonal serum or MAbs were used for the serpin blocking activity assay. Each serpin was pre-incubated with IgG for 30 min followed by a second incubation with the test protease, when kinetics was monitored for 15 min. Blockage was observed exclusively when purified IgG from polyclonal serum was tested (Fig. 11 and Table 3), since IgG from MAbs was not able to block serpin inhibitory function (Fig. 11). This result shows that polyclonal antibodies were able to block the inhibitory activity of rRmS-3 by approximately 66%, of rRmS-6 by 50% and of rRmS-17 by 85% (Table 3). A non-related serpin anti-serum was used as control (anti-VTDCE) (Oldiges et al., 2012) and did not affect serpin inhibitory activity(Fig. S1).

4. Discussion

In this work we demonstrate the inhibitory properties of three salivary serpins from the cattle tick *R. microplus*. RmS-3, RmS-6, and RmS-17 are inhibitors of serine proteinases of the serpin superfamily (Tirloni et al., 2014a). In addition to the confirming that these serpins are clearly present in cattle tick saliva, as found using proteomic approaches in a previous study (Tirloni et al., 2014a), here it is shown that antibodies raised against these serpins produced in *P. pastoris* are able to recognize native serpins that are present in tick saliva. Additionally, immune serum from cattle repeatedly infested with *R. microplus* recognized all three recombinant salivary serpins, proving that these proteins are injected into animals during tick feeding. Consistent with earlier literature findings, according to which some serpins are indeed glycoproteins (Gettins, 2002), the present study shows that RmS-3, RmS-6 and, RmS-17 are secreted as glycoproteins (Fig. 10).

The protease inhibitory profile of *R. microplus* salivary serpins shows that rRmS-3 has anti-chymotrypsin activity, rRmS-6 has anti-trypsin activity, and rRmS-17 has both anti-trypsin and anti-chymotrypsin activity. Taking all data together, it becomes clear that rRmS-3 and rRmS-6 have a more strict inhibitory profile, while RmS-17 has a broad-spectrum of inhibition. rRmS-3 action upon chymotrypsin and pancreatic elastase is in accordance with previous work (Rodriguez et al., 2015). However, in that study, the authors showed that rRmS-6 has only anti-chymotrypsin activity (Rodriguez et al., 2015).

Aiming to compare the inhibitory efficiency of these serpins, stoichiometry of inhibition (SI) indices were determined. This index reflects the number of serpin molecules required to form a stable inhibitory complex with a given protease. Accordingly, rRmS-3 is an efficient inhibitor of chymotrypsin and cathepsin G. Although it also inhibits pancreatic elastase at a high excess molar ratio, SI data suggests rRmS-3 is not an efficient pancreatic elastase inhibitor. As pancreatic elastase (used in this study) and neutrophil elastase have different biochemical properties (Bode et al., 1989; Sinha et al., 1987), the inhibition of neutrophil elastase cannot be ruled out, and needs further characterization. Differences in elastase inhibition by some parasitic inhibitors was shown to be dependent on the source of elastase (Jin et al., 2011). rRmS-6 efficiently inhibits trypsin, while rRmS-17 has a broad spectrum of efficient inhibition, as demonstrated using trypsin, chymotrypsin, cathepsin G. However, it is less efficient upon plasmin and factor XIa. The high SI indices observed for some serpin-protease pairs point to the possibility that some serpins need a co-factor to enhance inhibition, as demonstrated for some mammalian serpins (Gettins, 2002; McCoy et al., 2003).

Tertiary serpin structure contains typically three β-sheets, eight or nine α-helices, and a reactive center loop (RCL). RCL is a solvent exposed flexible stretch of 21 amino acid residues positioned between β-sheets sA and sC and that acts as a suicide-substrate of the specific target protease (Gettins, 2002). RCL contains the peptide bond (between residues P_1 and P_1,) that is cleaved by the target protease, while the P_1 residue in RCL is critical to define the specificity of a serpin for a particular protease (Hopkins et al., 1993; Hopkins and Stone, 1995). The predicted P_1-P_1, residues of *R. microplus* salivary serpins are Leu-Ser for RmS-3, Arg-Ile for RmS-6, and Lys-Ser for RmS-17 (Tirloni et al., 2014b), which were predicted to inhibit chymotrypsin-like (RmS-3) and trypsin-like proteinases (RmS-6 and RmS-17) (Keil, 1992). This prediction is in accordance with the inhibitory profile described here. However, the finding that rRmS-17 was able to inhibit α-chymotrypsin is intriguing, since a serpin possessing a Lys at P_1 has not been reported to inhibit chymotrypsin-like proteases, which have preference for large hydrophobic amino acids such as Phe, Tyr, or, to a lesser extent, Leu, Met, and His at the P_1 position (Keil, 1992). It is possible that RmS-17 has two close reactive sites in RCL, allowing the interaction with both trypsin-like and chymotrypsin-like proteases, as yet demonstrated for another serpin (Liu et al., 2014). The P_4-P_3, region of RmS-17 is comprised of Phe-Tyr-Thr-Lys-Ser-Ala-Val (Tirloni et al., 2014b), thus the Phe or Tyr at P_4 and P_3 position could be the real reactive site in RmS-17 for chymotrypsin inhibition.

rRmS-3 and rRmS-17 were able to inhibit cathepsin G. This protease has both trypsin- and chymotrypsin-like specificity, being able to accommodate both large, hydrophobic P_1

residues (Phe, Leu, Met) as well as positive ones (Lys or Arg) (Hof et al., 1996). Modeling studies suggest that Ser40 in cathepsin G forms hydrogen bonds with P_1' Ser, providing a structural basis for the inhibition by natural cathepsin G inhibitors such as α_1-antichymotrypsin, α_1-antitrypsin, and squamous cell carcinoma antigen 2 (SCCA-2) (Korkmaz et al., 2008). This is in accordance with both RmS-3 and RmS-17 RCL, where the P_1-P_1' residues for RmS-3 and RmS-17 are Leu-Ser and Lys-Ser, respectively. Interestingly, the RCL of RmS-3 resembles the chymotrypsin and cathepsin G cleavage site, as present in α_1-antichymotrypsin and SCCA-2 serpins (Schick et al., 1997). Since serpins are natural inhibitors of proteases with a role in host defenses against tick feeding (Rau et al., 2007), we hypothesize that this finding is the result of a parasite convergent evolution process that, in ticks, selected a serpin with biologically equivalent recognition sites present in the host's serpin, allowing tick saliva to target both cathepsin G and chymotrypsin-like proteases, mimicking proteins from the host itself.

After RCL P_1-P_1' peptide bond cleavage, serpin covalently traps and distorts the target protease, resulting in a covalent complex serpin-protease, and therefore serpins are classified as suicide inhibitors (Huntington et al., 2000). Data presented here showed that *R. microplus* salivary serpins interact with proteases via this classical suicide inhibition, as demonstrated by the formation of heat- and SDS-stable complex between serpin and protease (Fig. 8). Altogether, these results demonstrate that RmS-3, RmS-6 and RmS-17 are classical serine protease inhibitors injected in the host during tick feeding, and that they target different host proteases. However, what could be the role of these protease inhibitory profiles regarding host defense modulation?

It was demonstrated that rRmS-6 and rRmS-17 are effective inhibitors of trypsin. Although they are produced predominantly by the pancreas as a means to degrade dietary proteins, trypsin-like proteases are also expressed in the nervous system and in epithelial tissues, where they are the most powerful protease-activated receptor 2 (PAR2) activator being important factors in neurogenic inflammation and pain in the skin (Cattaruzza et al., 2014; Steinhoff et al., 2000). Thus, injection of trypsin-like inhibitors such as RmS-6 and RmS-17 in the feeding site could interfere in pro-inflammatory and algesic responses during tick feeding.

Neutrophils play an essential part as triggers of an immune response against tick feeding (Heinze et al., 2012a; Heinze et al., 2012b). athepsin G, neutrophil elastase and proteinase 3 are the most abundant molecules present in neutrophil azurophil granules (Korkmaz et al., 2008; Pham, 2006; Pham, 2008). Accordingly, with results showed here, rRmS-3 and rRmS-17 effectively inhibited neutrophil cathepsin G (Table 1 and Fig. 5 and Fig. 7). So, these serpins can be involved in modulation of neutrophil function as was observed in *I. scapularis* saliva, pointing to its importance in the tick feeding process as well as in pathogen transmission (Guo et al., 2009; Ribeiro et al., 1990). Also, rRmS-3 and rRmS-17 were able to inhibit platelet aggregation induced by cathepsin G. The ability of neutrophils to enhance aggregation of human platelets *in vitro* is linked to cathepsin G activity (Faraday et al., 2013). Altogether, inhibition of cathepsin G could be potentially associated with blockage of several relevant pathways that may result in diminished neutrophil-mediated inflammation and thrombosis during tick feeding.

Another subset of cells with an important function in tick feeding are eosinophils. It was demonstrated that eosinophils have significant chymotrypsin-like activity, and that an inhibitor of the chymotrypsin-like protease markedly inhibited eosinophil peroxidase (EPO) release from eosinophils (Matsunaga et al., 1994). Since rRmS-3 and rRmS-17 have anti-chymotrypsin activity, these serpins could inhibit chymotrypsin-like proteases from cattle eosinophils. Interesting, the importance of eosinophils for tick feeding was highlighted in studies showing hosts upon repeated tick infestation present eosinophil accumulation in the attachment site, which has been linked to tick resistance (Brown et al., 1982; Carvalho et al., 2010; Schleger et al., 1981; Ushio et al., 1993).

Classically described as a protease related to fibrinolytic system, plasmin has been reported to participate also in pro-inflammatory processes (Burysek et al., 2002; Li et al., 2007; Li et al., 2010; Li et al., 2012; Syrovets et al., 1997). Plasmin inhibition by rRmS-6 and rRmS-17 could impair these plasmin immune functions. To investigate whether tick serpins could alter the classical coagulation parameters, standard coagulation tests in the presence of tick serpins were conducted. Given that rRmS-17 interferes with recalcification time, this feature seems to have a relationship with the inhibition of factor XIa. Serpins did not significantly alter plasma clotting time, TT, or aPTT (data not shown).

As serpins are secreted into host during tick feeding, and in order to investigate the specificity of host immune response against serpins during tick infestation, sera of repeated tick-infested hosts were used to probe glycosylated as well as deglycosylated serpins. Data showed that sera from tick-infested animals recognized either glycosylated or deglycosylated serpins. Interestingly, *R. microplus* serpins were also recognized by serum from hosts infested with *A. americanum*, *R. sanguineus*, and *I. scapularis* (Fig. 2), confirming that serpins are naturally secreted during tick feeding, and that hosts are able to produce a humoral immune response against these proteins after sequential tick infestations. These data are in accordance with a previous study in which RmS-3 was recognized by serum from tick-resistant cattle (Rodriguez-Valle et al., 2012). Furthermore, data showing cross-reactivity among sera from hosts infested with different tick species suggest secretion of similar serpins into saliva of other tick species, which highlight a potential use of salivary serpins as antigens in a universal anti-tick vaccine (Parizi et al., 2012). Serpins have been identified in saliva of other tick species, including *D. andersoni*, *H. longicornis*, and *A. americanum* (Chalaire et al., 2011; Kim et al., 2015; Mudenda et al., 2014; Tirloni et al., 2015), lending strength to the hypothesis that different tick species secrete these proteins in their tick saliva, and pointing to a role in the evasion from host defenses during tick feeding (Chmelar et al., 2011; Kim et al., 2015; Leboulle et al., 2002a; Mulenga et al., 2013; Prevot et al., 2006). Weak reactivity of sera from *I. scapularis*-infested animals against *R. microplus* serpins was observed (Fig. 2). Such an observation comes as no surprise, since *I. scapularis* is a prostriate tick while *R. microplus*, *R. sanguineus*, and *A. americanum* are metastriate ticks. The presence of specific either prostriate as well as metastriate specific-protein families in salivary glands has been clearly demonstrated (Francischetti et al., 2009), suggesting differences in salivary gland contents between these groups of ticks. According to western blotting results, the anti-*R. microplus* sera reactivity was lower with deglycosylated as compared with glycosylated serpins. These results suggest that the

carbohydrate moieties of the serpin proteins participate in but are not essential for eliciting an immune response in the host, which could also be variable. Admittedly, the real importance of oligosaccharides concerning the immunogenicity of a particular glycoprotein cannot be determined without immunization experiments (Gavrilov et al., 2011). Many recombinant non-glycosylated proteins have been used as vaccine antigen, showing that glycosylation is not essential for the induction of a protective response (Imamura et al., 2005; Sugino et al., 2003).

MAbs and polyclonal sera raised against tick serpins crossreacted, though at different levels, with recombinant and native salivary serpins, suggesting that similar epitopes are present in different serpins. When poly- and monoclonal antibodies were used to assay the effect of antibodies against serpin activity, polyclonal antibodies abolished serpin inhibitory activity (Fig. 11). This is of major importance, when serpins are considered antigens in an anti-tick vaccine development. Host functional antibodies raised during immunization could block serpin inhibitory activity during tick feeding and interfere with modulatory functions of serpins, impairing blood meal acquisition and tick development. The different inhibition capacities of the MAbs and polyclonal antibodies can be explained based on the possibility that polyclonal antibodies recognize more than one epitope, while monoclonal antibodies can be recognized as a structure with unstable or non-functional epitopes (Kummer et al., 2004; Parra-Lopez et al., 2014; Saunders et al., 1998).

Analyzed together, our results and previous findings show that serpins are inoculated during tick infestation and play the role of immunogenic inductors of humoral responses. Indeed, serpins are involved in host-parasite interactions and help the parasite to evade the host immune system. In previous studies, proteins from *I. ricinus* (Prevot *et al.*, 2007), *H. longicornis* (Sugino *et al.*, 2003), and *R. appendiculatus* (Imamura *et al*., 2006; Imamura *et al*., 2008) were shown to induce the production of antibodies and to be associated with protection against tick infestation. Inducing an efficacious immune response against tick serpins could help hosts to limit and control parasite infections and, therefore, serpins may be included as suitable antigen candidates in an anti-tick vaccine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The tick *Rhipicephalus microplus* secretes three types of salivary serpins.
- *R. microplus* serpins inhibit pro-inflammatory and pro-coagulant proteases.
- RmS-3 and RmS-17 inhibit cathepsin G-induced platelet aggregation.
- Anti-serpin antibodies abolish this inhibitory activity.
- Sera against other tick species recognize *R. microplus* serpins.

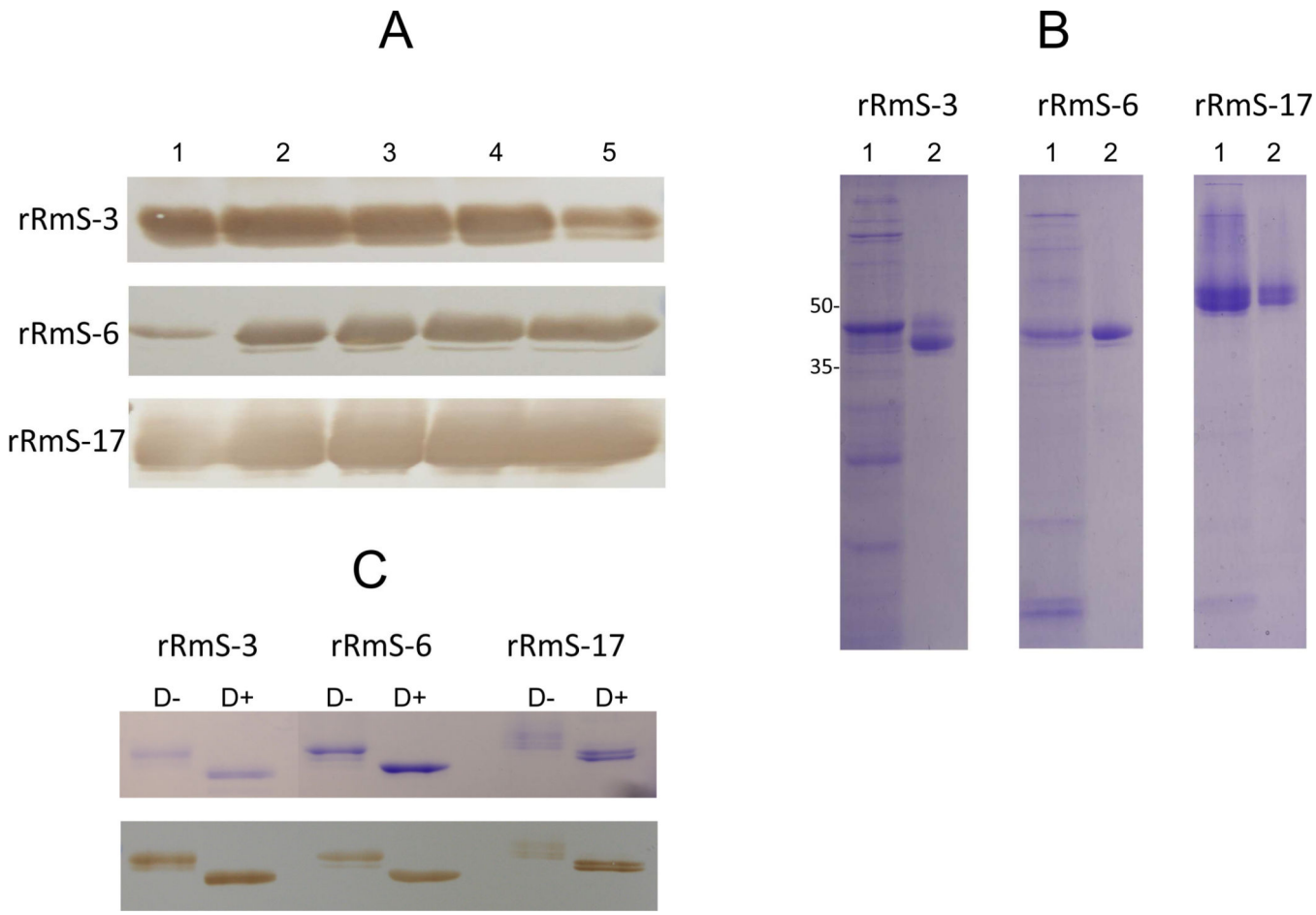

Figure 1. Recombinant expression of *R. microplus* salivary serpins in *Pichia pastoris*

(A) Daily expression levels of rRmS-3, rRmS-6, and rRmS-17 throughout five days (1–5). Recombinant proteins in spent culture media were precipitated by ammonium sulfate and verification of protein expression was performed using an antibody to the C-terminus hexa histidine tag. (B) Total expression (1) and affinity purified-(2) rRmS-3, rRmS-6, and rRmS-17 were resolved on a 12.5 % SDS-PAGE following Coomassie brilliant blue staining. (C) Recombinant serpins treated with deglycosylation enzyme mix (D+) or without treatment (D−) were resolved on a 12.5 % SDS-PAGE following Coomassie brilliant blue staining and western blotting using anti-C-terminus hexa histidine tag antibody.

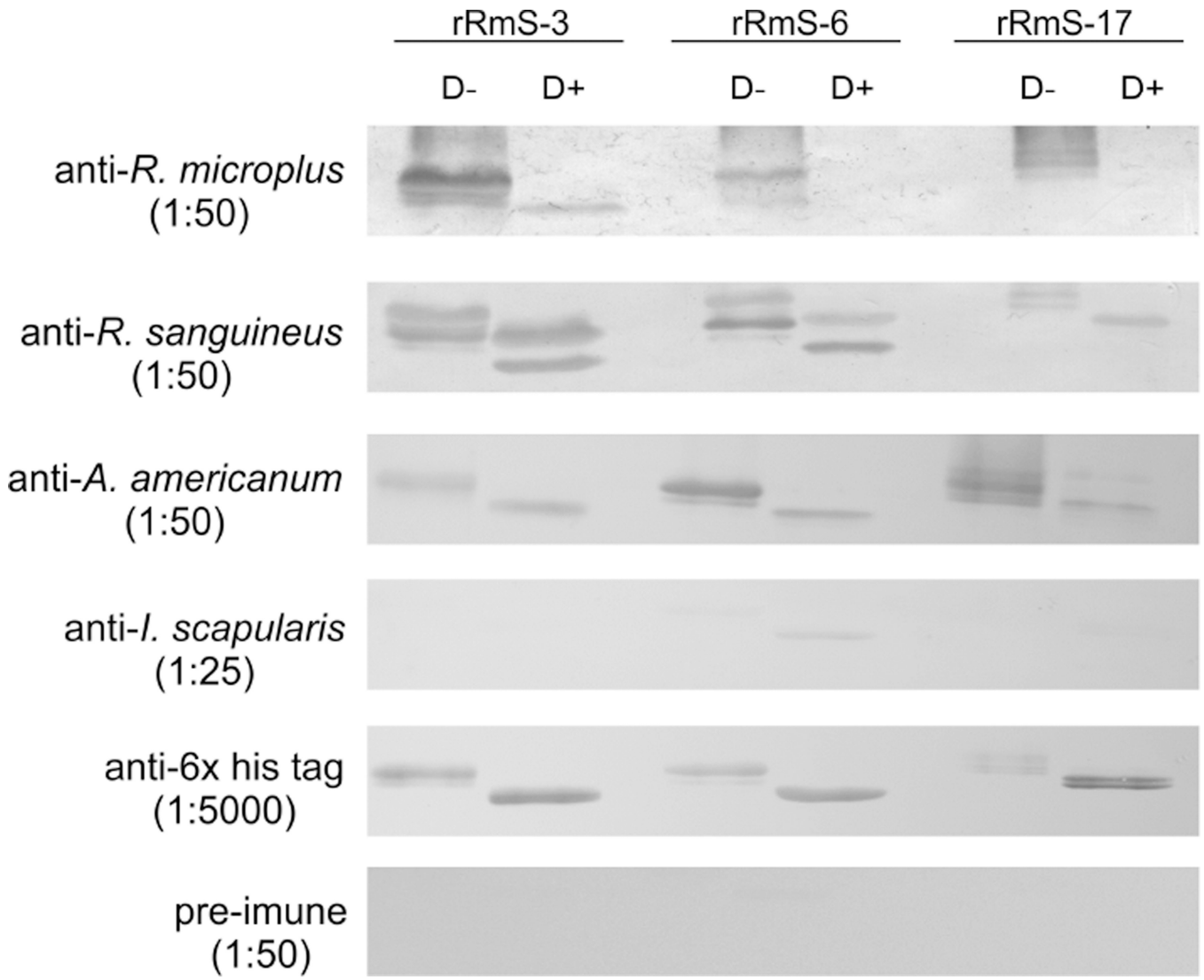

Figure 2. Inter-species cross-reactivity assay

Purified rRmS-3, rRmS-6 and rRmS-17 treated with deglycosylation enzyme mix (D+) or without treatment (D−) were resolved on a 12.5 % SDS-PAGE following western blotting analysis with: cattle serum generated by *R. microplus* tick infestation (dilution 1:50), rabbit serum generated by adult *R. sanguineus* infestations (dilution 1:50), *A. americanum* infestation (dilution 1:50), and adult *I. scapularis* infestation (dilution 1:25). Anti-C-terminus hexa histidine tag antibodies (dilution 1:5000) and rabbit pre-immune serum (dilution 1:50) were used as control.

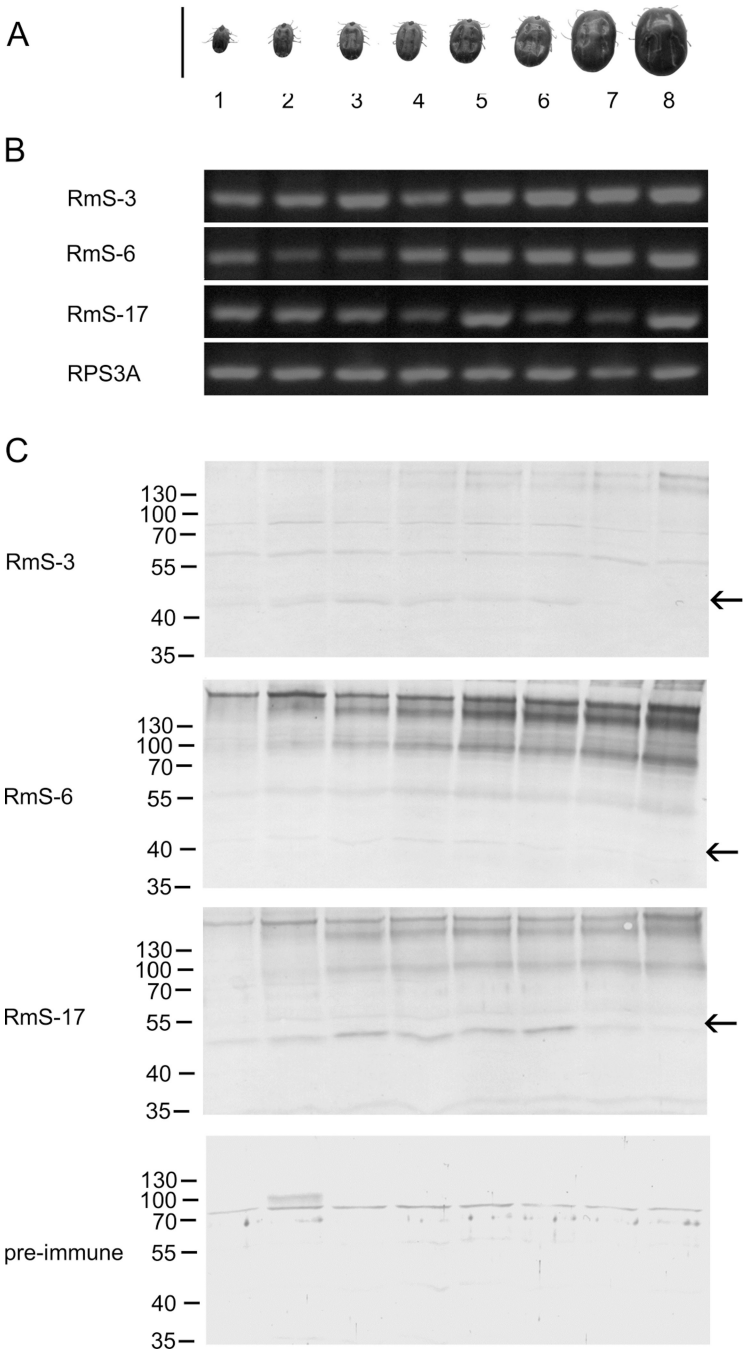

Figure 3. Transcription and expression profile of salivary serpins

(A) Comparison of the feeding stage of ticks used for serpin expression profile.. (B) Total RNA was extracted from ticks and subjected to RT-PCR to amplify RmS-3, RmS-6, and RmS-17 fragments. Tick ribosomal protein S3a was used as reference. (C) Total protein extracts of SG from eight groups of ticks were subjected to western blotting analyses using antirRmS-3, anti-rRmS-6, rRmS-17 sera as well as pre-immune serum. Arrows indicate the position of the native protein.

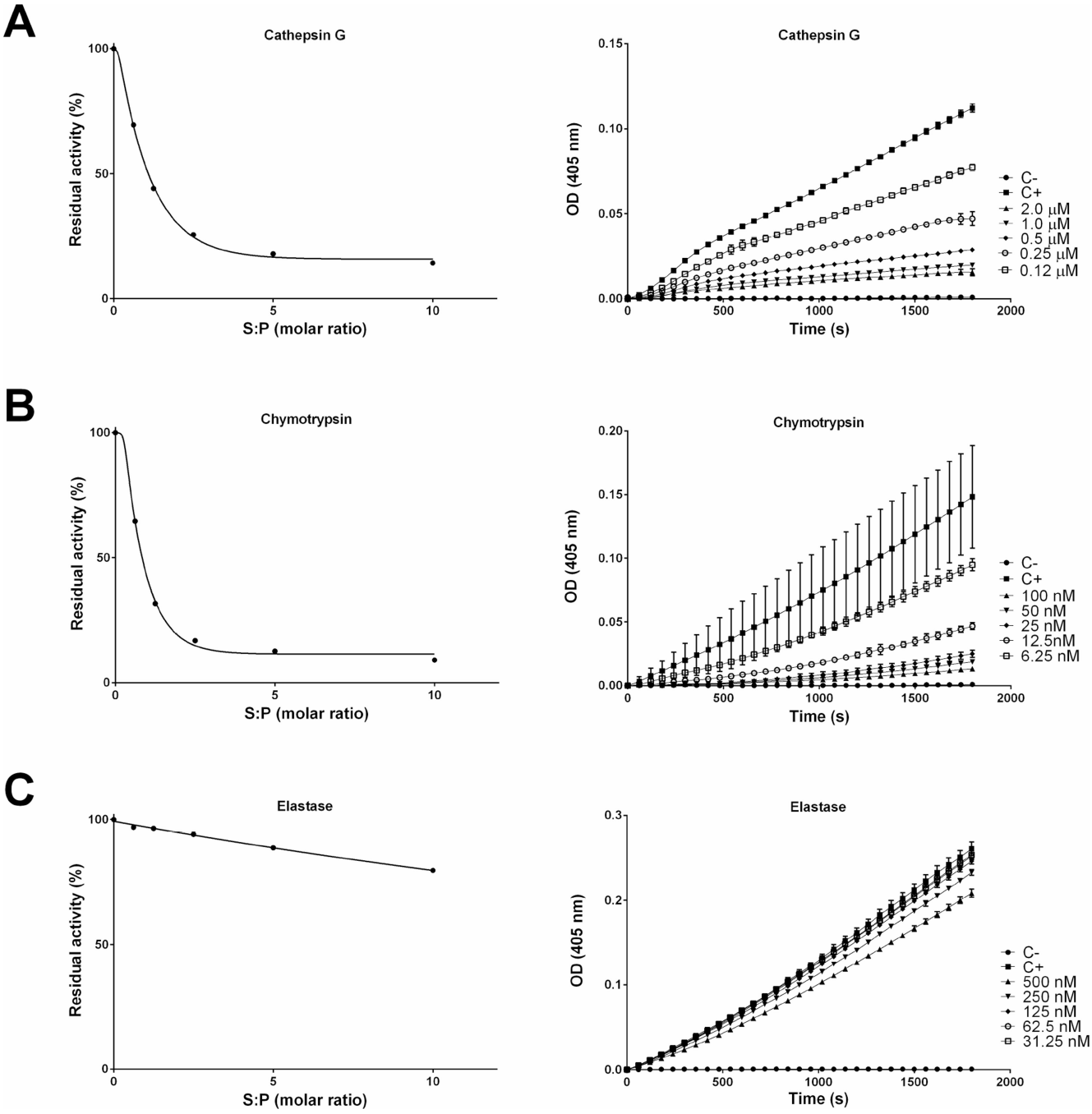

Figure 4. rRmS-3 stoichiometry inhibition (SI) assay

Residual protease activity in the presence and absence of rRmS-3 was evaluated pre-incubating serpin for 1 h at 37 °C with (A) cathepsin G (200 nM), (B) chymotrypsin (10 nM), and (C) pancreatic elastase (50 nM), resulting in molar ratios (serpin:protease) ranging from 0 to 10. Protease activity was measured using specific colorimetric substrate for each protease as described in Materials and Methods. The data were plotted as residual protease activity (V_i/V_0) versus molar ratio (serpin:protease).

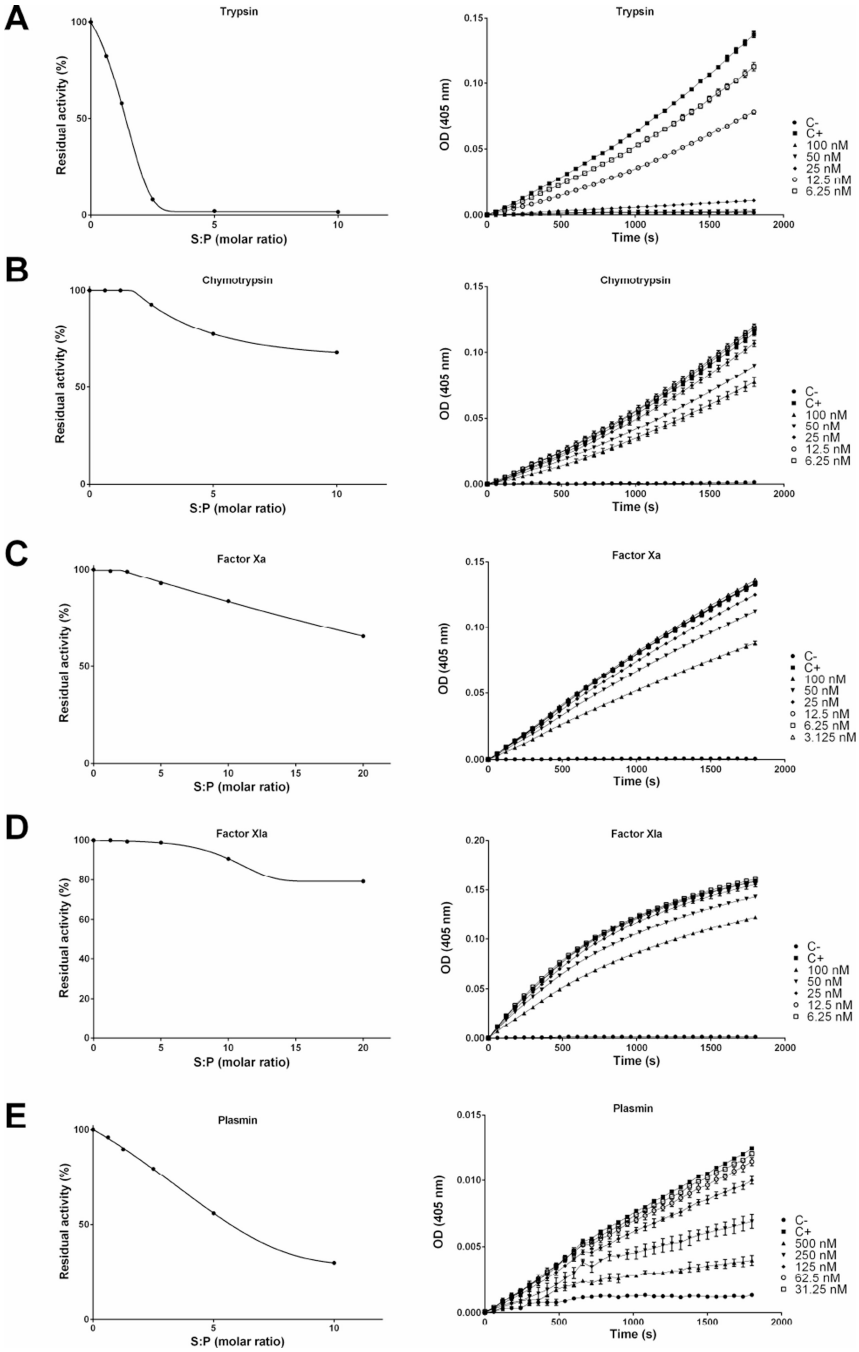

Figure 5. rRmS-6 stoichiometry inhibition (SI) assay

Residual protease activity in the presence and absence of rRmS-6 was evaluated pre-incubating serpin for 1 h at 37 °C with (A) trypsin (10 nM), (B) chymotrypsin (10 nM), (C) factor Xa (5 nM), (D) factor XIa (5 nM), and (E) plasmin (50 nM), resulting in a molar ratio (serpin:protease) ranging from 0 to 10 for trypsin, chymotrypsin, and plasmin, and from 0 to 20 for factor Xa and factor XIa. Protease activity was measured using specific colorimetric substrate for each protease as described in Materials and Methods. The data were plotted as residual protease activity (V_i/V_0) versus molar ratio (serpin:protease).

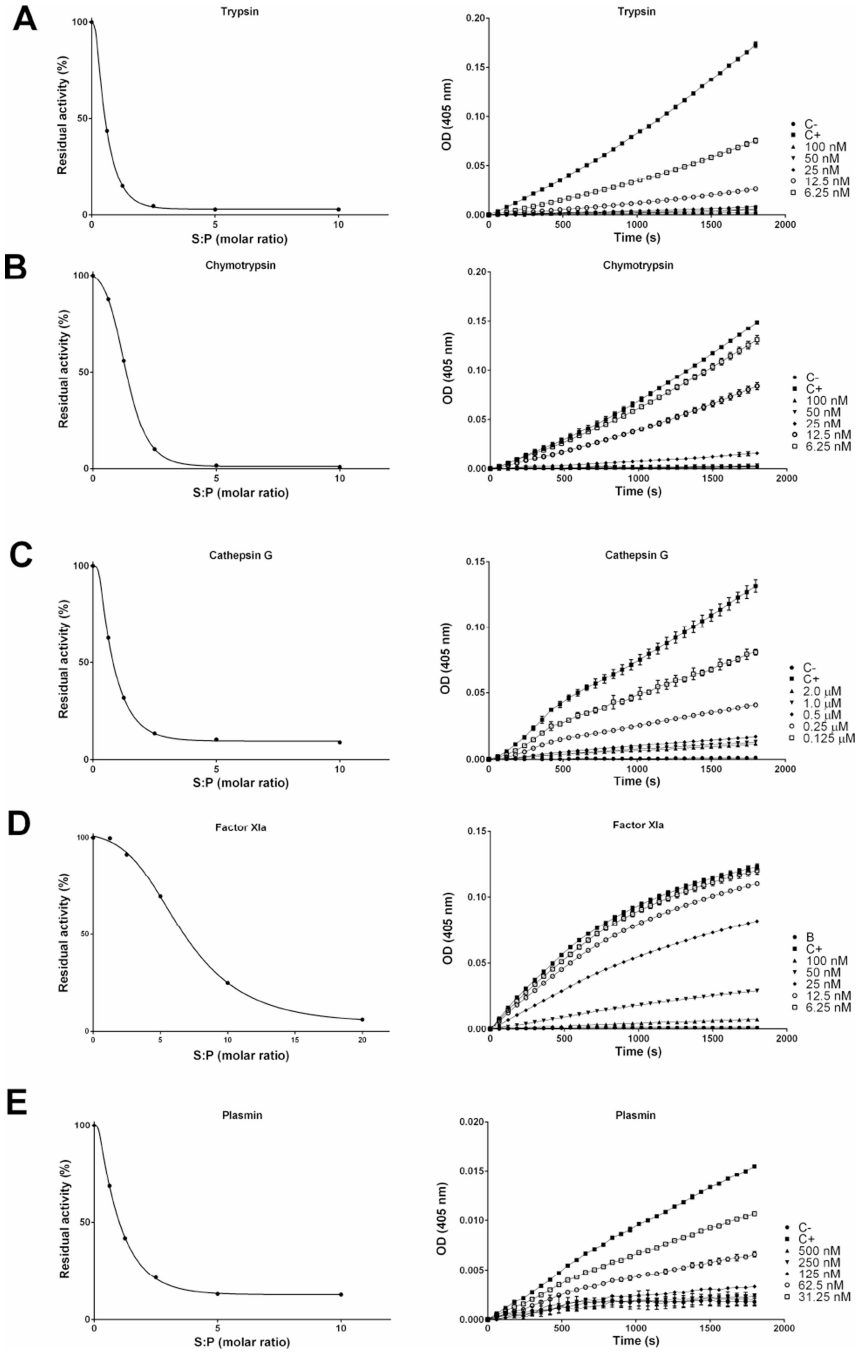

Figure 6. rRmS-17 stoichiometry inhibition (SI) assay

Residual protease activity in the presence and absence of rRmS-17 was evaluated pre-incubating serpin for 1 h at 37 °C with (A) trypsin (10 nM), (B) chymotrypsin (10 nM), (C) cathepsin G (200 nM), (D) factor XIa (5 nM), and (E) plasmin (50 nM), resulting in a molar ratio (serpin:protease) ranging 0 to 10 (for factor XIa molar ratio ranges between 0 to 20). Protease activity was measured using specific colorimetric substrate for each protease as described in Materials and Methods. The data were plotted as residual protease activity (V_i/V_0) versus molar ratio (serpin:protease).

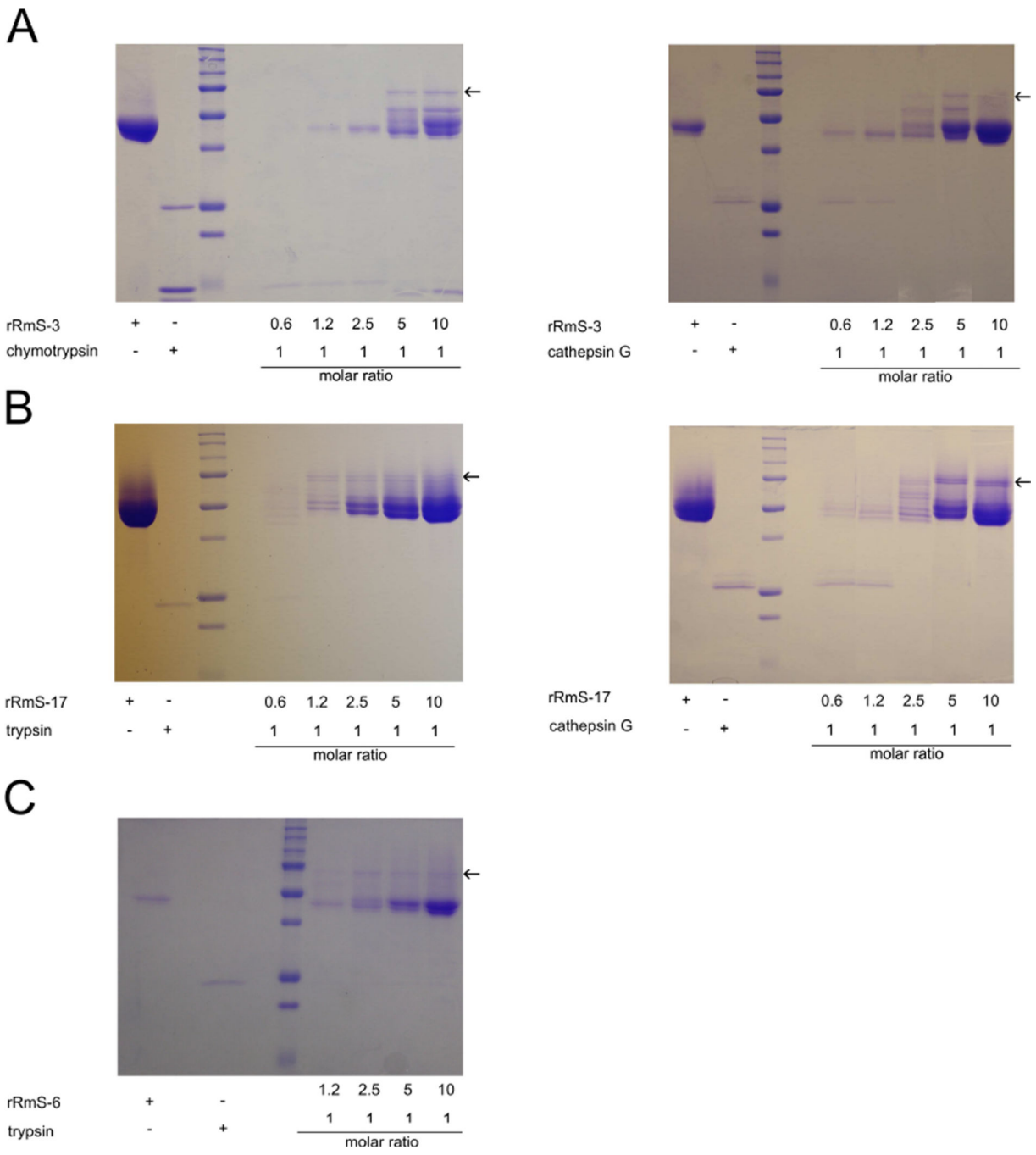

Figure 7. Heat and SDS-stable complex formation assay

Increasing amounts of recombinant serpins were pre-incubated for 1 h at 37 °C with a constant concentration of (A) chymotrypsin and cathepsin G for rRmS-3, (B) trypsin and cathepsin G for rRmS-17, and (C) trypsin for rRmS-6, resulting in molar ratios varying from 0.6:1 to 10:1 (serpin:protease). Samples were resolved on 12.5% SDS–PAGE and Coomassie blue-stained to identify SDS-stable complexes (indicated by arrows).

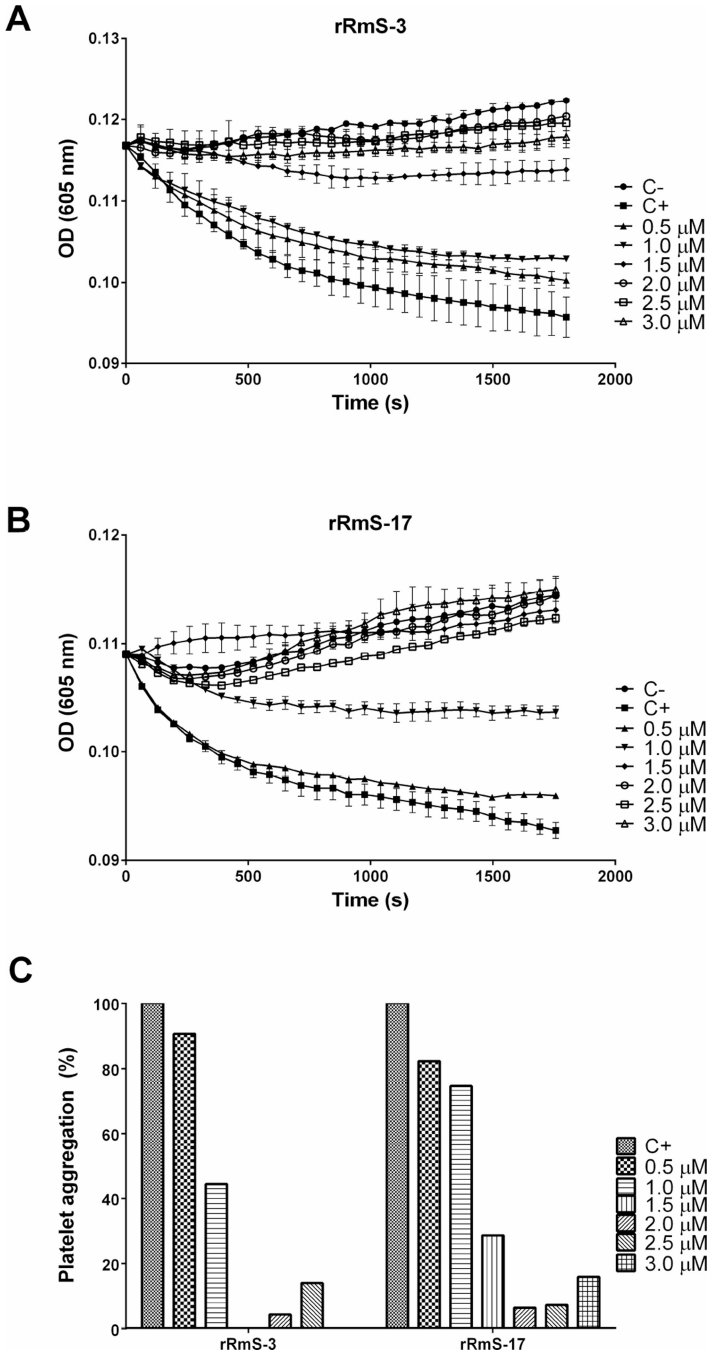

Figure 8. rRmS-3 and rRmS-17 effect upon cathepsin G-induced platelet aggregation

Platelet aggregation function induced by cathepsin G assay was done using bovine platelet rich plasma (PRP) as described in Materials and Methods. (A) Tyrode solution with varying amounts of rRmS-3 or rRmS-17 (3 µM, 2.5 µM, 2 µM, 1.5 µM, 1 µM, and 0.5 µM) were pre-incubated with cathepsin G (0.7 µM) in a 50-µL reaction for 15 min at 37 °C. Platelet aggregation was initiated with addition of 100 µL pre-warmed PRP and monitored at 20-s intervals over 30 min at OD_{650nm}. (B) Percent of reduction of cathepsin G-induced platelet aggregation inhibited by rRmS-3 and rRmS-17.

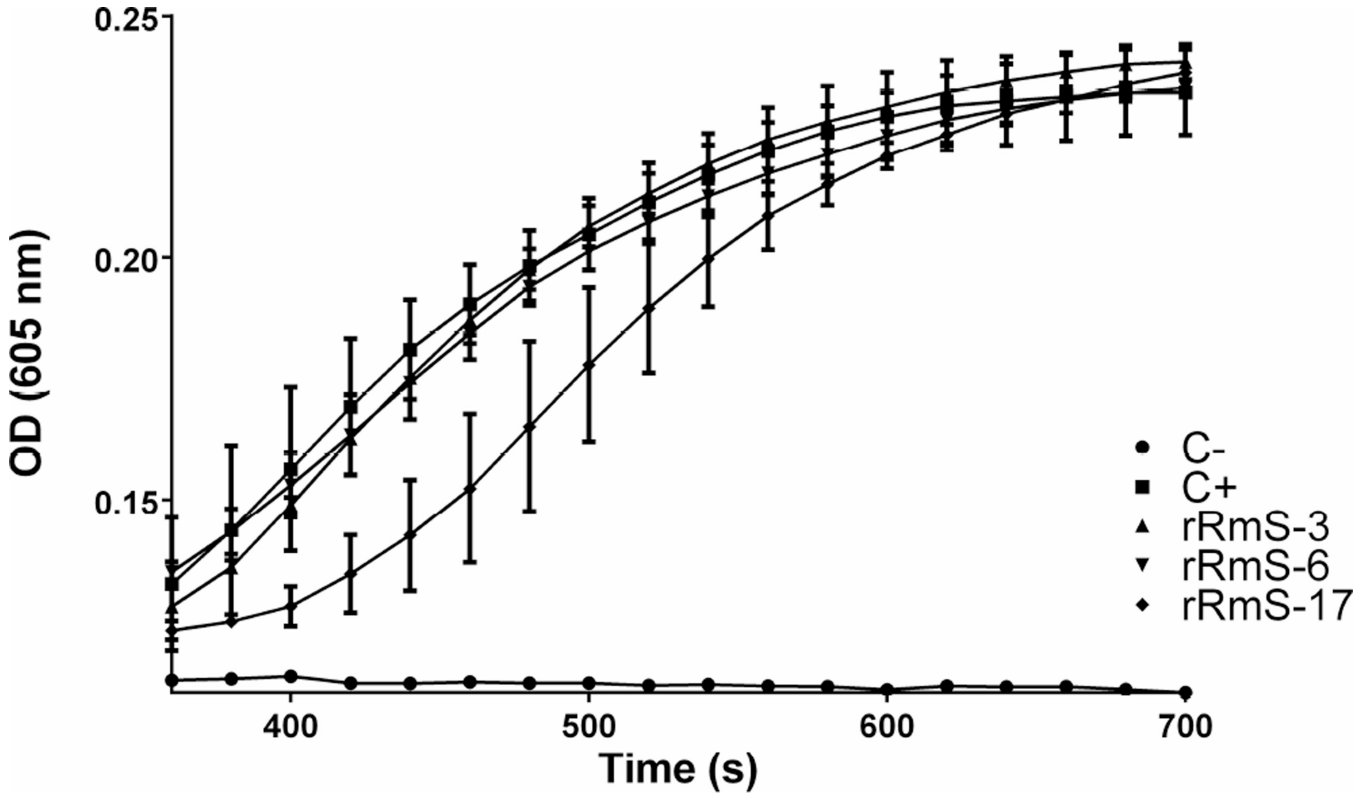

Figure 9. Effect of *R. microplus* serpins upon plasma recalcification time

Human reference plasma (50 μL) was incubated with rRmS-3 (10 μM), rRmS-6 (10 μM), and rRmS-17 (10 μM) in 90 μL of Tris–HCl reaction buffer for 15 min at 37 °C followed by the addition of 150 mM $CaCl_2$ (10 μL). Clotting was measured every 20 s for 30 min.

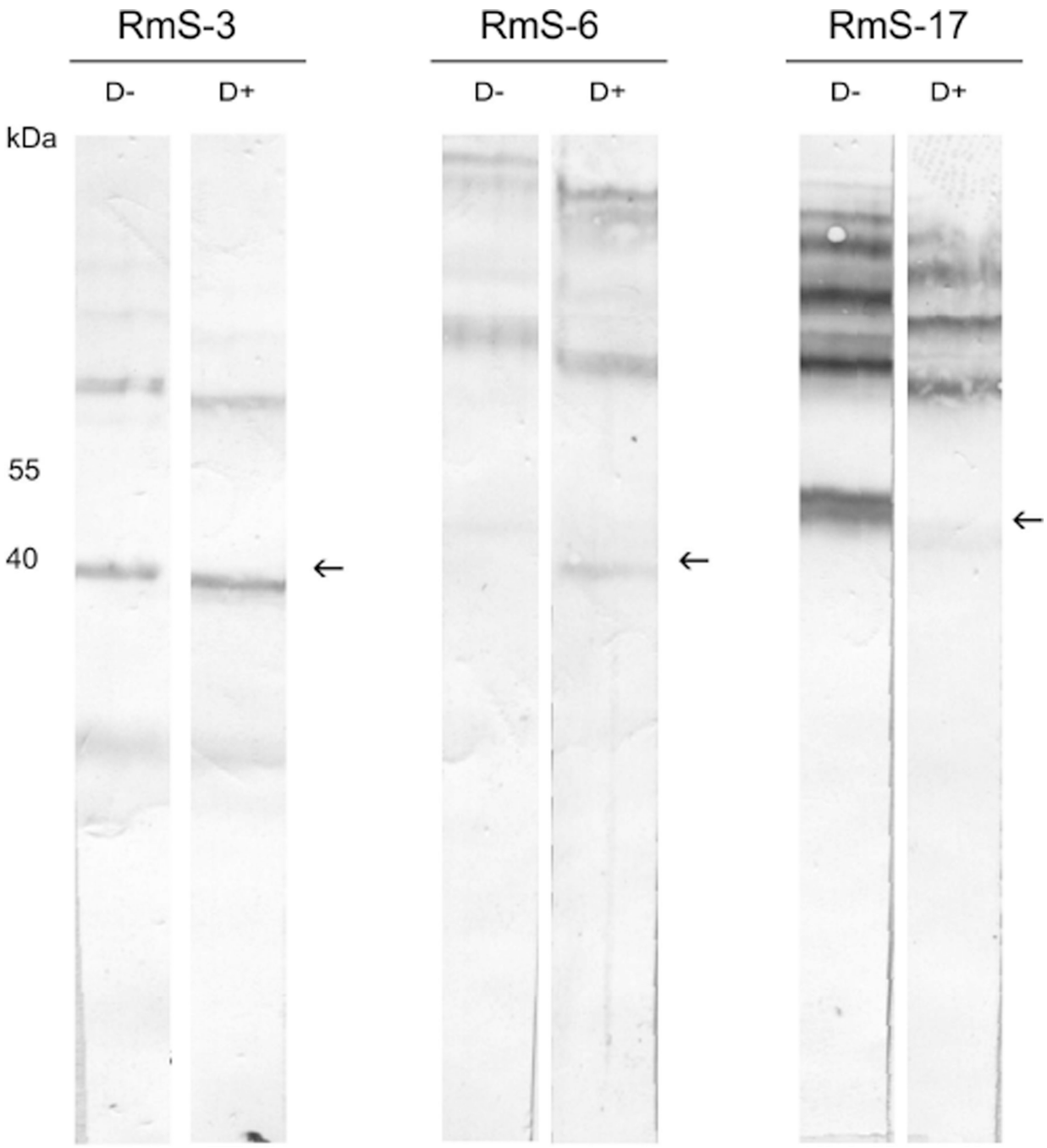

Figure 10. Reactivity of anti-rRmS antibodies with RmS-3, RmS-6 and RmS-17 from tick saliva

Pilocarpine-induced tick saliva were harvested as described in Material and Methods. Saliva total protein treated with deglycosylation enzyme mix (D+) or non-treated (D−) were resolved on a 12 % SDS-PAGE following western blotting using polyclonal sera (A) anti-rRmS-3, (B) anti-rRmS-6, and (C) anti-rRmS-17.

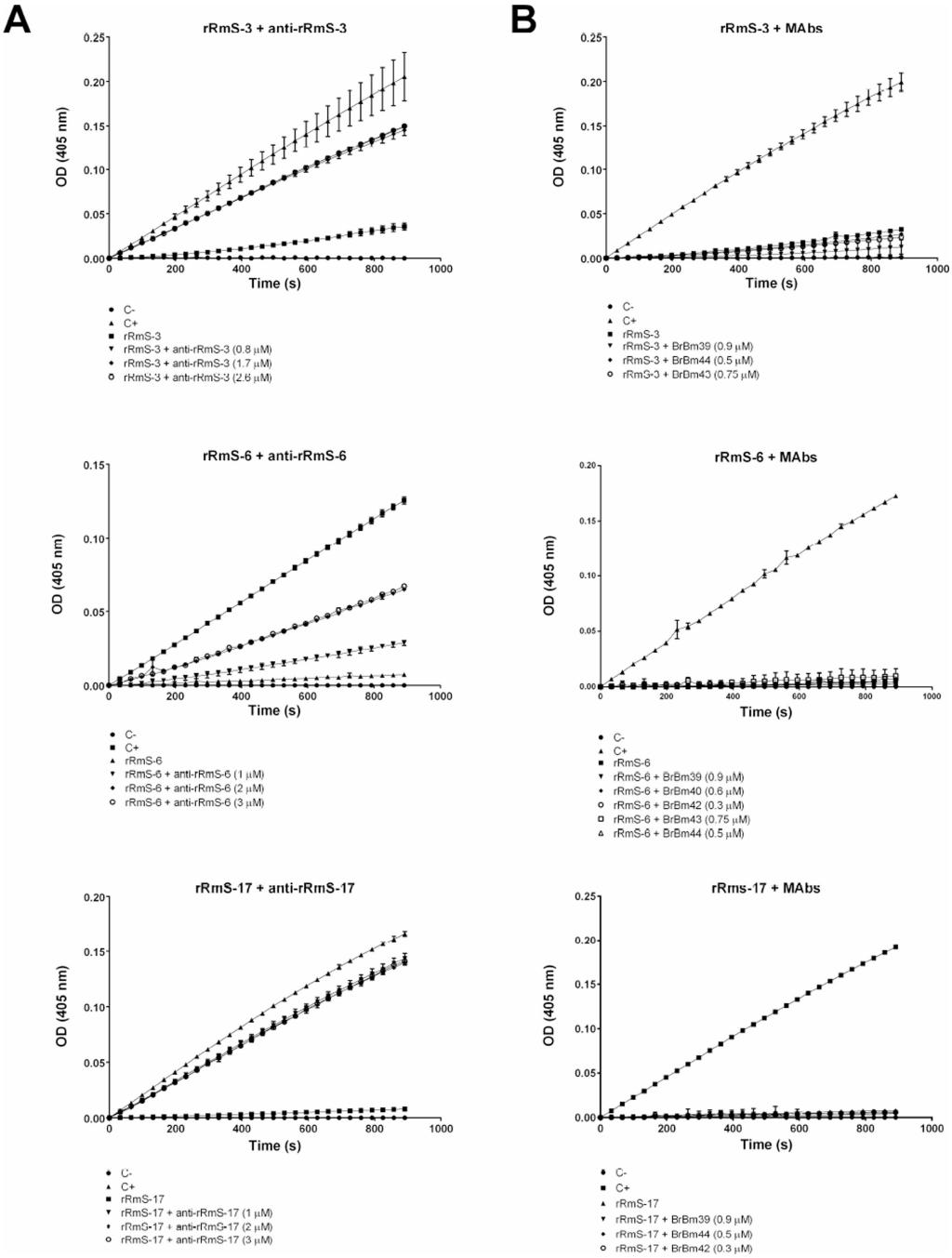

Figure 11. Effect of antibodies upon serpin inhibitory activity

Purified IgG from (A) rabbit polyclonal serum or (B) from mice MAbs (anti-rRmS-3, anti-rRmS-6, and rRmS-17) were incubated with rRmS-3 (55 nM), rRmS-6 (55 nM) or rRmS-17 (88 nM) at 37 °C for 30 min before the addition of chymotrypsin (4.9 nM) for rRmS-3, or trypsin (1.6 nM) for rRmS-6 and rRmS-17, following a new 15-min incubation. Protease kinetics was monitored for 15 min every 11 s.

Table 1

R. microplus salivary serpins inhibitory profile.

Protease	**Serpin**		
	RmS-3 (1 μM)	**RmS-6 (1 μM)**	**RmS-17 (1 μM)**
Chymotrypsin (96 nM)	96.94 ± 1.07	24.29 ± 7.39	89.76 ± 2.97
Factor IXa (300 nM)	n.i	n.i	n.i
Cathepsin G (166 nM)	78.74 ± 0.20	n.i	78.84 ± 0.29
Factor XIa (3.68 nM)	n.i	62.86 ± 11.10	98.02 ± 1.24
Trypsin (24.6 nM)	n.i	73.52 ± 11.91	87.03 ± 4.80
Factor Xa (5.8 nM)	n.i	32.76 ± 10.68	n.i
Elastase (21.6 nM)	92.62 ± 2.40	n.i	n.i
Plasmin (10 nM)	n.i	24.13 ± 4.98	58.39 ± 13.52
Chymase (10 U)	23.36 ± 4.64	n.i	n.i
Factor XIIa (7.6 nM)	n.i	n.i	n.i
Thrombin (43 U)	n.i	n.i	n.i
u-PA (47.2 nM)	n.i	n.i	n.i
Tryptase (10 U)	n.i	n.i	n.i
t-PA (32 nM)	n.i	n.i	n.i
Proteinase-3 (68 U)	n.i	n.i	n.i
Kallikrein (33 U)	n.i	n.i	n.i

n.i: not inhibited or inhibition rate lower than 20%.

Table 2

Antibodies reactivity with native salivary and recombinant serpins.

Antibody	Immunogen	Cross-reactivity (%)*			
		Recombinant			Native
Monoclonal		**rRmS-3**	**rRmS-6**	**rRmS-17**	**Saliva**
BrBm39	rRmS-6	79 (±8)	100	69 (±20)	91 (±5.5)
BrBm40	rRmS-6	51	100	30	106 (±19)
BrBm41	rRmS-6	NR**	100	NR**	NR**
BrBm42	rRmS-6	NR**	100	NR**	NR**
BrBm43	rRmS-6	76 (±22)	100	49 (±11)	NR**
BrBm44	rRmS-17	Weak**	Weak**	Weak**	NR**
Polyclonal					
Anti-RmS-3	rRmS-3	100	47 (±10)	40 (±9)	+***
Anti-RmS-6	rRmS-6	35 (±10)	100	31 (±6)	+***
Anti-RmS-17	rRmS-17	46 (±10)	56 (±14)	100	+***

* Compared to primary reactivity. Results are shown as mean (±SD)

** **NR** = No reaction; **Weak** = low reaction with all three serpins tested

*** + : please refer to Figure 10 for western blotting reactions

Table 3

Antibodies effect on serpin inhibitory activity.

Antibody	Serpin activity inhibition (%)*
rRmS-3	
rRmS-3	0
rRmS-3 + anti-rRmS-3 (0.8 μM)	66.7
rRmS-3 + anti-rRmS-3 (1.7 μM)	64.2
rRmS-3 + anti-rRmS-3 (2.6 μM)	66.8
rRmS-6	
rRmS-6	0
rRmS-6 + anti-rRmS-6 (1 μM)	18.9
rRmS-6 + anti-rRmS-6 (2 μM)	48.8
rRmS-6 + anti-rRmS-6 (3 μM)	50.4
rRmS-17	
rRmS-17	0
rRmS-17 + anti-rRmS-17 (1 μM)	85.6
rRmS-17 + anti-rRmS-17 (2 μM)	84.1
rRmS-17 + anti-rRmS-17 (3 μM)	84.3