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Inhibition of bovine platelet aggregation by salivary gland proteins/peptides of *Rhipicephalus (Boophilus) microplus* ticks

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Abstract

The cattle tick *Rhipicephalus (Boophilus) microplus* is a haematophagous ectoparasite that has a huge economic and health impacts. They act as a potential vector for the transmission of pathogenic micro-organism such as *Babesia* spp. and *Anaplasma* spp. leading to considerable economic losses for cattle farming throughout the world. They deposit their saliva having bioactive molecules at the site of their attachment to a host in order to inhibit haemostasis so as to suck host blood successfully. Therefore, the study was planned to isolate anti-platelet aggregating peptides from salivary gland extract (SGE) of *Rhipicephalus (Boophilus) microplus* ticks. Female ticks were dissected out and homogenized in HEPES buffer under the ice to prepare salivary gland extract. Proteins were fractionated by gel filtration chromatography using Sephacryl S-200 column. Total protein concentration in fractions was estimated. Isolated bovine platelets were treated with Gly-Pro-Arg-Pro amide (negative control) and with salivary gland fractions and then stimulated with thrombin (positive control), for identification of proteins/peptides having anti-platelet aggregating activities. A total of 9 fractions having the protein concentration of 0.88 to 61.3µg/ml showed platelet aggregation inhibition ranged from 27.1-34.7 %. The results suggest that the fractions of *Rhipicephalus (Boophilus) microplus* salivary glands possess thrombin induced anti-platelet aggregating activity and which could be further exploited for raising anti-tick vaccine and also for the therapeutic purpose.

Keywords: *Rhipicephalus (Boophilus) microplus* salivary gland, gel filtration chromatography, thrombin, anti-platelet aggregating proteins/peptides

Introduction

Rhipicephalus (Boophilus) microplus is the haematophagous ectoparasite affecting livestock worldwide leading to considerable economic losses for cattle farming mainly due to the blood loss, decreased milk and damage to hides in tropical and subtropical areas of the world [13, 20, 8]. Infestation by this parasite transmits pathogenic micro-organisms, such as *Babesia* spp. and *Anaplasma* spp. [22] thus responsible for causing tick-borne diseases like Babesiosis and Bovine erythrocytic anaplasmosis [23]. Hence, control of tick infestation requires immediate attention and intense research for improved livestock production.

Ticks need blood meals to develop from one stage to the next and for reproduction. Therefore, they use their mouthparts to penetrate the skin of its host and generate a feeding cavity from which they feed on blood [6]. While feeding, ticks secrete bioactive molecules that modulate the host immune system and hemostasis, which aids in the hematophagy and in the transmission of pathogens [19, 24, 6]. These include inhibitors of platelet adhesion to collagen [14], direct collagen inhibitors [9], catechol and apyrases oxidases which are able to counteract the physiologic pro-haemostatic mechanisms that are triggered to avoid blood loss. Analyses of these anti-haemostatic molecules may be useful tools in cell biology and could be exploited for the anti-tick vaccine [21, 3] or as therapeutics for prevention and treatment of blood vascular disease. Therefore, the study was planned to isolate and fractionate the anti-platelet aggregating salivary gland proteins/peptides of *Rhipicephalus (Boophilus) microplus* and to see their effects on bovine platelet aggregation.

Materials and Methods

Ethical approval

The research was conducted after due approval from Institutional Animal Ethics Committee.

Sample collection

Rhipicephalus (Boophilus) microplus female ticks were collected and identified as per the key is given by Miranpuri and Gill (1983).

Dissection of ticks and collection of salivary glands

After washing with normal saline ticks were immobilized individually on a petri-dish kept on ice by glue and then incised along the dorsal-lateral margin using fine scalpel blade under a stereoscopic dissection microscope (Magnus MSZ-TR). Salivary glands were removed by fine tip forceps, transferred into HEPES saline buffer, pH 7.0 and stored in liquid nitrogen till analyzed.

Extract preparation

Salivary glands (one hundred pairs) were pooled and homogenized using tissue homogenizer (T10 basic ULTRA-TURRAX®, India) under ice. The homogenate was centrifuged at 12,000 X g for 7 min at 4°C, supernatant removed, filtered through Millex-GV Syringe Filter Unit, 25 mm PVDF. 22 µm Sterile with Vent. The resulting filtrate was diluted to 2 ml with 50 mM Tris-Cl, pH 8.3 and then used for fractionation, isolation and identification of anti-platelet aggregating factors from *Rhipicephalus (Boophilus) microplus* salivary glands.

Isolation of anti-platelet aggregating factors from the salivary glands

The filtrate was applied to a Sephacryl S-200 gel filtration column (1 cm × 60 cm) equilibrated with 50mM Tris-HCL, pH 7.5 with 100mM KCL and eluted with 40 mM Tris-Cl, pH 7.5, fractions each of 1.5 ml were collected. The column was calibrated with molecular-weight markers from Sigma (alcohol dehydrogenase, 150 kDa; albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome C, 12.4 kDa; and the void volume determined with Blue Dextran, 2000 kD). The approximate molecular weights of proteins were determined using a standard curve of V_e/V_0 against log molecular weight.

Estimation of protein concentration in fractions

The protein concentration in each fraction was estimated Bradford (1976), using bovine serum albumin as standard.

Platelets preparation

Platelets were prepared from buffalo calves blood maintained at Department of Veterinary Physiology & Biochemistry, LUVAS, HISAR by using 0.1M trisodium citrate as an anticoagulant in 9:1. Platelet-rich plasma (PRP) was obtained by centrifugation at 1000rpm. Then PRP was centrifuged at 4000rpm to get the platelet pellet. The platelet pellet was washed twice with Tyrode buffer 'A' (with EGTA) and then the final pellet was resuspended in the Tyrode buffer 'B' (without EGTA), in a volume adjusted to give an OD of 0.15 at 650 nm.

Platelet aggregation assay

To estimate the effects of proteins/peptides present in isolated fractions on platelet aggregation, the method followed by Francischetti *et al.* (2000) with little modification was used. Platelets were incubated with Gly-Pro-Arg-Pro (1mM) amide as an antagonist and isolated protein fractions for 10 min at 37 °C in 96 well flat bottom plate. Then the aggregation was initiated by adding thrombin (0.5nM) as an agonist. Changes in platelet aggregation were monitored at 650nm at every 5

minute interval for 20 minutes.

Results

Total protein concentration of one hundred and twenty gel filtration chromatographic fractions of *Rhipicephalus (Boophilus) microplus* salivary gland ranged from 0.88 to 61.3 µg/ml (Figure 1). Then the fractions having proteins were further analyzed for anti-platelet inhibitory activities, stimulated with thrombin. Effects of various *Rhipicephalus (Boophilus) microplus* salivary protein fractions on bovine platelet aggregation inhibition are shown in Figure 2. Fraction nos. 22, 23, 25, 27, 31, 36, 38, 39 and 51 having total protein concentration 48.1, 61.3, 33.6, 39.7, 41.3, 10.1, 6.7, 0.88 and 16.9 µg/ml respectively, showed platelet aggregation inhibition activities nearly similar to that of antagonist-induced aggregation inhibition and was much higher as compared to agonist-induced platelet aggregation. Percent platelet aggregation inhibition by *Rhipicephalus (Boophilus) microplus* salivary gland fractions ranged from 27.1-34.7% as compared to antagonist peptide where 29.5% inhibition was observed (Figure 3). Some of the fractions proteins/peptides inhibition was comparatively less while some showed more inhibition compared to the known antagonist. The effects of individual protein fractions having the anti-platelet aggregating activities are shown in Figure 4. Proteins/peptides present in different fractions behaved differently in terms of platelet aggregation inhibition when compared to that of thrombin-stimulated (agonist) platelet aggregation and antagonist-induced platelet inhibition. Fraction nos. 36 and 51 had slightly higher inhibitory activities than the known antagonist. The % inhibition in relation to total protein concentration in fraction nos. 36 and 51 i.e. 10.1 and 16.9 µg/ml was 34.7 and 34.2% respectively.

Discussion

The current study demonstrated that *Rhipicephalus (Boophilus) microplus* salivary gland selected fractions inhibit bovine platelet aggregation which was stimulated by thrombin and peptide (Gly-Pro-Arg-Pro) known to have platelet aggregation inhibitory activities. Thrombin is considered a 'strong and multiple' activator stimulating blood platelets at different receptor sites [1]. These receptors are well known proteins namely platelet activating receptors-1 (PAR-1) and platelet activating receptors-4 (PAR-4) [18, 11]. PAR-1 and PAR-4 receptors which are mainly found in activated platelets for platelet plug formation at the site of vascular injury and thereby preventing the conversion of fibrinogen to fibrin. The mechanism of inhibitory peptide which was used in present study is through inhibition of fibrin polymerisation and because of this property thrombin inhibitors are predominantly used in anti-thrombotic therapy (anticlotting) and prophylaxis of acute myocardial infarction, deep venous thrombosis and pulmonary embolism and also for the prevention of arterial reocclusion following endarterectomies and endovascular stent deployment [7]. So there were possibilities of using inhibitory peptides present in the ticks salivary gland fractions in prophylaxis and treatment of above mentioned disease. Some of the fractions showed comparable inhibitory effect than the known inhibitory peptide while others showed the slightly more effects to that of inhibitory peptide. Similarly other thrombin inhibitors purified from soft and hard ticks, for example, savignin from the soft tick *Ornithodoros savignyi* [17] and an inhibitor from the hard tick *Boophilus microplus* [10] were shown to inhibit thrombin-

induced platelet aggregation in platelet-rich plasma, indicating that both aggregation and clotting are prevented by the inhibitors. Also, the purified inhibitor AV 16/3 from salivary gland extract of partially engorged *A. variegatum* females possessed an antithrombin effect on human blood platelets with hirudin-like activity [15]. IxscS-1E1, a blood meal-induced serine protease inhibitor from *I. scapularis* (Ixsc) tick saliva was found to inhibit 23.4% thrombin induced aggregation [12]. Thrombin inhibitors were also isolated from snake venom BmooAi, *Bothrops moojeni*, which inhibited platelet aggregation [4]. In *Rhipicephalus*

(*Boophilus*) *microplus* maximum inhibitory activity was found to be in the range of 27.1-34.7 % in different fractions. Therefore, the inhibitory effects of proteins/peptides present in different fractions can be either due to inhibition of fibrin polymerization or due to inhibition of action of inhibitory proteins/peptides on thrombin exosites. *Rhipicephalus* (*Boophilus*) *microplus* tick salivary gland contain significant amount of platelet aggregation inhibitory proteins/peptides which can be further exploited for raising anti-tick vaccines as well as for therapeutic purposes.

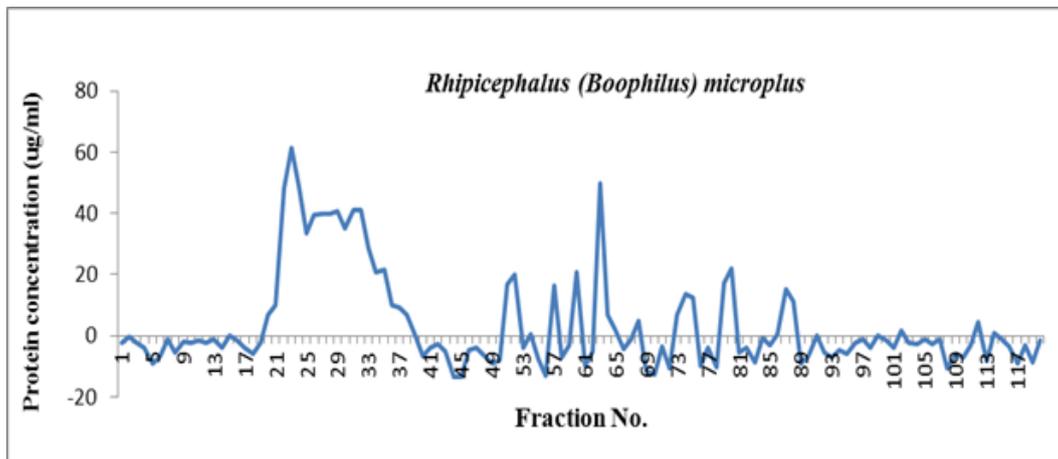


Fig 1: Concentrations of total protein in *Rhipicephalus* (*Boophilus*) *microplus* salivary gland fractions collected by gel exclusion chromatography.

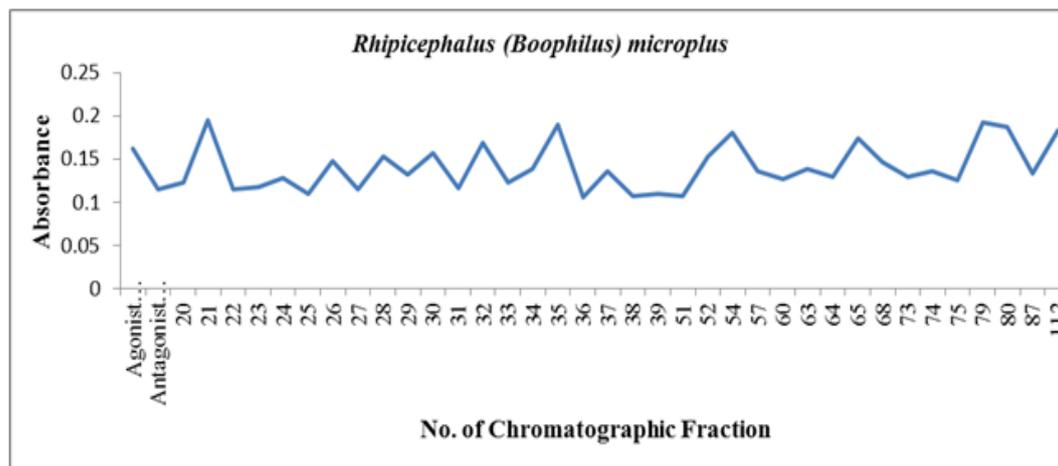


Fig 2: Effects of *Rhipicephalus* (*Boophilus*) *microplus* salivary gland protein fractions on Bovine platelet aggregation.

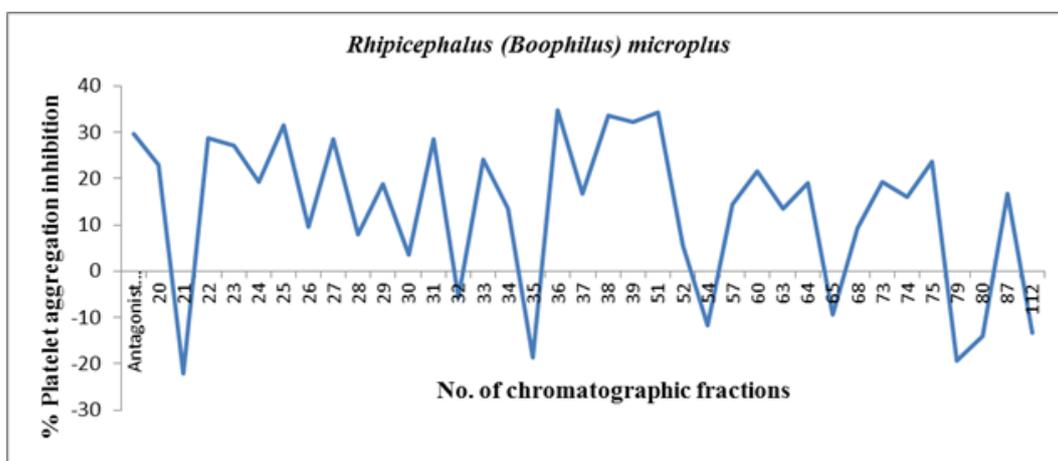


Fig 3: Effects of *Rhipicephalus* (*Boophilus*) *microplus* salivary gland protein fractions on Bovine platelet aggregation (%).

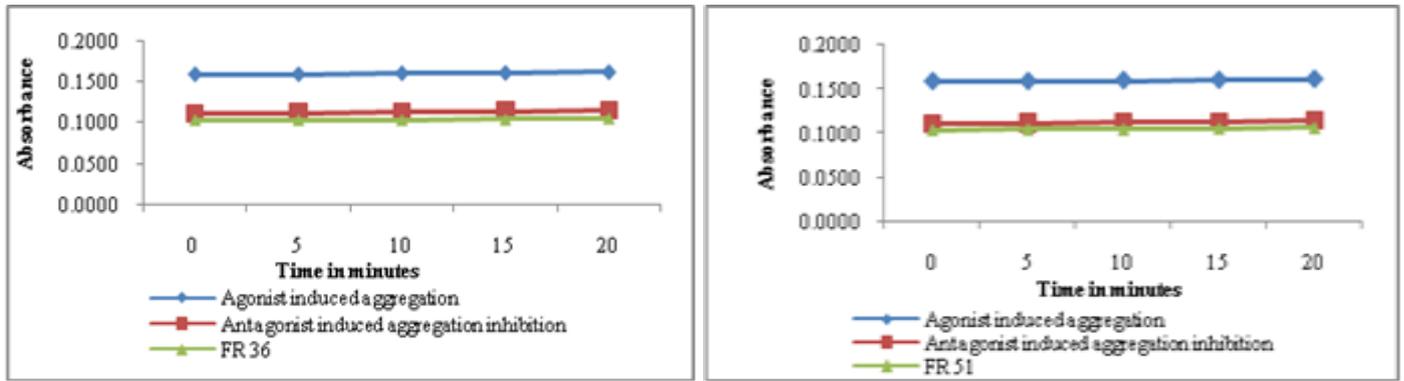


Fig 4: Effects of *Rhipicephalus (Boophilus) microplus* salivary gland individual protein fractions on Bovine platelet aggregation inhibition after stimulation with thrombin (0.5nm)

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