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Electrophoretic Characterization of Anti-platelet Aggregating Proteins/Peptides from Salivary Gland of *Rhipicephalus (Boophilus) microplus*

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A B S T R A C T

Ticks are economically devastating arthropod ectoparasites of livestock that exclusively feed on host blood. Ticks saliva is a crucial weapon against host-defense systems. Several small fragmented attempts are made by researchers to characterize these biomolecules. Being extremely small in size and quantity, numerous such molecules escape the deep insight of biochemists employing routine gel electrophoresis followed by Coomasie brilliant blue (CBB) staining. In a systematic manner, the present study aimed to isolate and electrophoretically characterize the salivary gland anti-platelet aggregating proteins/peptides from *Rhipicephalus (Boophilus) microplus* ticks. Salivary glands dissected out from female ticks were homogenized and fractionated into 120 fractions using gel filtration chromatography. All fractions were screened for anti-thrombotic activity. Nine fractions which inhibit thrombin induced platelet aggregation were electrophoretically characterized with SDS-PAGE followed by silver staining of gel. Amongst all 9 fractions which showed antiplatelet aggregating activity, protein band of approximate 64.1kDa was found predominant indicating towards peptide/protein with thrombin induced platelet aggregation inhibitory activity. Experiment was repeated many folds to evaluate the presence of the 64.1kDa bands in active fractions. Subsequently, the potential biomolecule identified need further purification and characterization to explore its therapeutic and immunization potential.

Introduction

Ticks are economically devastating obligate hematophagous ectoparasites that infest mammals, birds, reptiles and amphibians. These are classified into two major families, one is Ixodidae also called as hard ticks, other is Argasidae also called as soft ticks and third minor family is Nuttalliellidae which contains only a single species, *Nuttalliella namaqua.* In India, due to its wide diversity in climatic zones which is favourable for the survival and propagation of such arthropod vectors, the large livestock population can become the potential hotspot for different tick-borne diseases. The ixodid ticks *Rhipicephalus*

(boophilus) microplus species is endemic in India infesting dairy animals. These ticks transmit pathogens like *Babesia, Anaplasma* etc. to cause diseases bovine babesiosis and anaplasmosis. The control of tick infestation mainly depends on the use of chemical acaricides which are easily available and indiscriminately used all over India (Sharma *et al.,* 2012). The global economic losses by tick infestation through direct production losses and the associated cost of treatment has been estimated at US$14,000–18,000 million loss annually; while in India tick-borne diseases in livestock alone accounts for US$498.7 million loss per annum (Minjauw and McLeod, 2003).

During evolutionary development, blood-feeding arthropods have evolved anti-haemostatic mechanisms to counteract their host immune system. However, the cocktail of anti-haemostatic compounds in tick saliva differs between species (Kazimírová and Štibrániová, 2013). Thrombin plays acentral role in hemostasis, it cleaves specific peptide bonds in fibrinogen to form fibrin, induces platelet aggregation. Thrombin mainly acts through two receptors present on platelet membrane, namely, platelet activating receptors-1 and platelet activating receptors-4 (Ofosu, 2003; Huntigton, 2005). Research into the mechanisms by which ticks inhibit host haemostasis has led to the discovery and characterization of a variety of compounds with diverse biological activities and potential use in development of novel pharmaceuticals (Kazimírová, 2007; Francischetti *et al.*, 2009; Koh and Kini, 2009; Chmelar *et al.*, 2012). In addition to discovery of new drug candidates, studies on tick anti-haemostatics contribute to our understanding of the host parasite interactions (Kazimírová and Štibrániová, 2013). So keeping in view above facts the present study was aimed to characterize the proteins/peptides present in salivary gland of *Rhipicephalus (Boophilus) microplus*.

Materials and Methods

Collection of ticks

Adult female ticks of *R. (B.) microplus* were collected from villages around Hisar district of Haryana.

Tick dissection and collection of tick salivary glands

Adult female of *R. microplus* ticks were washed with normal saline and immobilized individually on a petri dish using glue with their dorsal surface upward. Using fine scalpel blade and fine tip forceps, ticks were incised along the dorsal-lateral margin, and the dorsal integuments were removed under a stereoscopic dissection microscope. Then the salivary glands were removed by the method of Wu *et al.,* (2010). A total number of one hundred pairs of salivary glands from *R. microplus* female ticks were collected in HEPES Saline buffer, pH 7.0 and stored in liquid nitrogen at -196 degree Celsius till further analysis.

Extract preparation

Salivary glands (one hundred pairs) were homogenized under ice using tissue homogenizer (T10 basic ULTRA-TURRAX) in HEPES saline buffer, pH 7.0. The homogenate was centrifuged at 12000 X g for 7 min at 4°C. The supernatant was further filtered through Millex-GV sterile syringe filter having, 25 mm PVDF and .22 µm vent. Then the supernatant was diluted to 2 ml with 50 mM Tris-Cl, pH 8.3, which was then fractionated by gel filtration chromatography.

Fractionation and isolation of proteins/peptides

The fresh salivary gland extract (500µl) from *R. microplus* ticks were applied to Sephacryl

S-200 gel filtration column (1cm×60cm) equilibrated with 50 mM Tris-HCL, pH 7.5 with 100mM KCL. Elution was performed with 40 mM Tris-HCl, pH 7.5 and fractions were collected each of 1.5ml. The molecular mass determination of unknown peptides in the fractions was done by comparing the V_e/V_o for the protein in question to the V_e/V_o of protein standards of known molecular mass (Cytochrome c from horse heart 12.4 kDa sigma C7150, Carbonic anhydrase from bovine erythrocytes 29 kDa sigma C7025, Albumin from bovine serum 66 kDa sigma A8531, alcohol dehydrogenase 150 kDa sigma A8656, β-Amylase from sweet potato 200 kDa sigma A8781) [V_e was elution volume and V_o was void volume]. The V_o of the column was the volume of effluent required for the elution of Blue dextran (molecular mass of ~ 2,000 kDa, sigma-D4772).

Estimation of total protein in fractions

The protein concentrations in fractions were estimated by the method of Bradford (1976)at 595 nm using ELISA reader using BSA as standard.

Isolation of bovine platelets

Blood sample was collected from jugular vein of buffalo calves maintained by the Department of Veterinary Physiology and Biochemistry at LUVAS animal farm in vials containing 0.1M trisodium citrate as anticoagulant in the ratio of 9:1. Samples collected were centrifuged at 1000 rpm to get the platelet rich plasma (PRP). The PRP was removed in another centrifuge tube and was centrifuged at 4000 rpm to get the platelet pellet. Supernatant was discarded and pellet was washed two times with tyrode buffer 'A'. Finally the pellets having the platelets was reconstituted with tyrode buffer 'B' so that the final concentration of platelets was 2 x 10^8/ml and OD of 0.15 at 650 nm.

Platelet aggregation assay of fractions having proteins/peptides

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 in each well were incubated with antagonist Gly-Pro-Arg-Pro amide (1mM) and salivary glandfractions having proteins/peptides for 10 min at 37 °C.Then aggregation was initiated by thrombin (0.5nM). The absorbance was noted at 650 nm at every 5min interval for 20 mintues.

Electrophorectic separation and characterization of anti-platelet aggregating proteins/peptides

Proteins/peptides in fresh crude salivary extracts as well as in fractions having anti-platelet aggregating activities were electrophoretically separated by using 12% discontinuous SDS-PAGE (Hames, 1998) on vertical gel apparatus (Bio-Rad Mini-PROTEAN). Gels were stained by silver staining (Morrissey, 1981) and the approximate molecular weight of various proteins/peptide bands were calculated with reference to the standard marker proteins using gel documentation system.

Results and Discussion

A total of 120 fractions of salivary gland extract of *Rhipicephalus (boophilus) microplus* ticks were collected. These 120 fractions were further tested for platelet aggregation inhibitory activity by using thrombin as agonist and Gly-Pro-Arg-Pro amide as antagonist and found that only 9 fractions showed platelet aggregation inhibitory activities. Platelets have a central role in hemostasis. Thrombin is a key enzyme that catalyses conversion of fibrinogen into fibrin clot, activates blood coagulation factors

and blood platelet reactions. So in order to counteract the host hemostatic mechanism blood-sucking invertebrates have evolved various mechanisms that interfere with blood coagulation of their hosts (Markwardt, 1994; Arocha-Piñango *et al.,* 1999). Hirudin, a single chain peptide of 65 residues, was isolated from the leech *Hirudo medicinalis* and is currently the most potent natural direct inhibitor of thrombin (Salzet, 2001). A number of distinct platelet aggregation inhibitors have been described in various blood-sucking insects such as *Rhodnius prolixus, Ixodes scapularis, Ornithodoros moubata* etc.

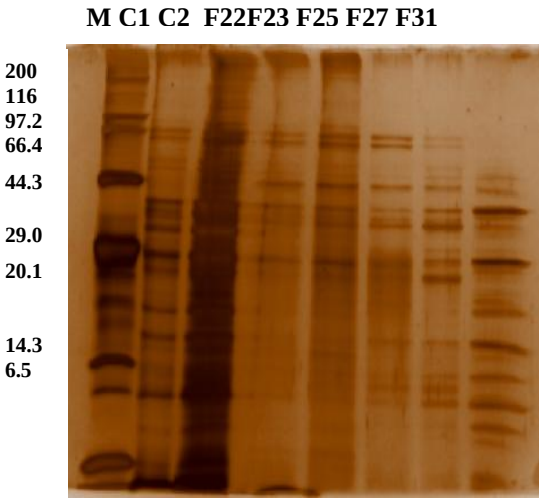

Figure.1 Electrophoretic protein profiling of fractions of *Rhipicephalus (Boophilus) microplus*in12%SDS PAGE stained with silver staining.Track-M,bands of molecular weight marker;track-C1,diluted crude SGE of *Rhipicephalus (Boophilus) microplus*;tracl-C2, crude SGE of *Rhipicephalus (Boophilus) microplus* track-F22,fraction22;track-F23, fraction23;trackF25,fraction25;track-F27,fraction27; track-F31,fraction31.

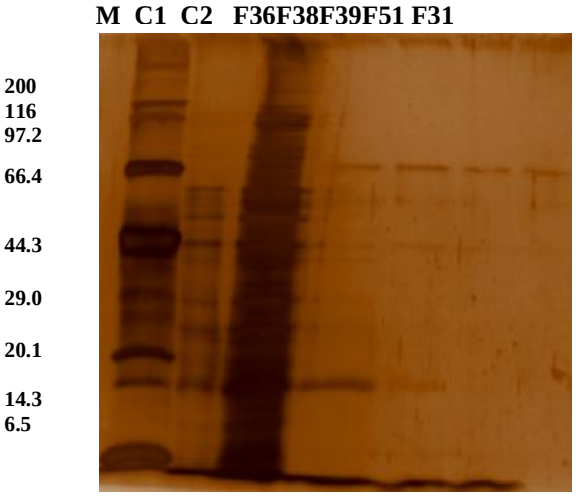

Figure.2 Electrophoretic protein profiling of fractions of *Rhipicephalus (Boophilus) microplus* in12%SDS PAGE.Track-M,bands of molecular weight marker;track-C1,diluted crude SGE of *Rhipicephalus (Boophilus) microplus*;tracl-C2, crude SGE of *Rhipicephalus (Boophilus) microplus* track-F36,fractio36;track-F38, fraction38;trackF39,fraction39;track F51,fraction51. The gel was stained with Silver staining.

Table.1 Approximate molecular weight (kDa) of the protein bands of *Rhipicephalus (Boophilus) microplus* salivary gland protein fractions.

Mol. Wt. (KDa)											
S.No.	**Marker**	**Crude**	**Fraction 22**	**23**	**25**	**27**	**31**	**36**	**38**	**39**	**51**
1	200	-	-	-	-	-	-	-	-	-	-
2	-	174.5, 132.9	-	-	-	-	-	-	-	-	-
3	116	-	-	-	-	-	-	-	-	-	-
4	-	106.1	-	-	-	-	-	98.3	-	-	-
5	97.2	-	-	-	-	-	-	-	-	-	-
6	-	92.1, 81.1, 74.3, 70.5,	94.6, 88.5, 74.3, 69.5	92.7, 87.3, 73.8, 68.6	91.5, 86.1	91.5, 85.6, 72.4, 67.2		92.4	-	-	-
7	66.4	65.4	64.5	63.9	63.3	63.3	66.8	66.4	66.0	65.2	64.1
8	-	58.0, 53.3, 45.5	57.2, 52.6, 49.3	56.4, 51.8	56.1, 51.4	55.9, 51.1, 44.9	62.2, 55.6, 51.1, 44.7	57.5, 53.7, 48.8	-	-	-
9	44.3	-	-	-	-	-	-	-	-	-	-
10	-	41.3, 37.7, 35.9, 32.1, 30.4	43.1, 40.9, 31.9, 30.3	42.7, 40.5, 36.9, 31.5, 29.9	42.4, 40.3, 31.3	42.8, 40.1, 31.1	42.7, 41.3, 37.5, 36.5, 30.8	40.7, 36.9	40.4	-	-
11	29	-	-	-	-	-	-	-	-	-	-
12	-	21.4	-	25.3	21.0	23.7, 20.3	23.4	28.0, 23.6	-	-	-
13	20.1	-	-	-	-	-	-	-	-	-	-
14	-	19.6, 16.7	19.5	19.9, 14.6		18.8	18.5, 16.5, 15.3	18.0	17.9	-	-
15	14.3	-	-	-	-	-	-	-	-	-	-
16	-	9.9	-	-	-	-	-	-	-	-	-
17	6.5	-	-	-	-	-	-	-	-	-	-

These molecules include direct collagen inhibitors, inhibitors of platelet adhesion to collagen (Karczewski *et al.*, 1995), apyrases and catechol oxidases. In addition to thrombin inhibitor, NO-releasing molecules, fibrinogen-receptor antagonists and specific

inhibitors of collagen-induced platelet aggregation (Noeske-Jungblut *et al.,* 1994; Keller *et al.,* 1993) have also been reported. Analyses of anti-platelet aggregating proteins/peptides may be useful tools in cell biology and may also have potential for therapeutic applications.

Further, electrophoretic characterization of proteins/peptides present in these 9 fractions showing platelet aggregating inhibitory activities were done. The protein bands in all the 9 fractions ranged from 9.9 kDa to 174.5 kDa. Out of these 9 fractions, some of the fractions i.e. fraction no. 22, 23, 25, 27, 31, 36, 38 and 39 (Figure 1 and 2) were found to have multiple protein bands of different molecular weight (Table 1) while only single protein band of approximate molecular weight 64.1 kDa was found in fraction no. 51 (Figure 2).

This single protein might be responsible for inhibition of thrombin induced platelet aggregation in *Rhipicephalus (Boophilus) microplus.* Similarly Wang *et al.,* (1996) also reported a novel inhibitor of human platelet aggregation, named variabilin, from salivary glands of the hard tick *Dermacentor variabilis* with a mass of 4985.5 daltons. Similarly in *Bothrops jararaca*, a glycoprotein with molecular weight of 61 kDa was reported by De Morais *et al.,* (2009). The *Boophilus microplus* anticoagulant Protein' (BmAP), with 60,000 Da, blocked thrombin active site (Horn *et al.,* 2000), while microphilin, with 1,770 Da, interacted with thrombin exosite I (Ciprandi *et al.,* 2006). BmGTI isolated from gut of *Boophilus microplus,* molecular weight is estimated to be in the range of 26 kDa, this protein was different from BmAP and microphilin, two *Boophilus microplus* salivary anticoagulants (Ricci *et al.,* 2007). Novel antithrombin molecules was identified from the ixodidae tick, *Haemaphysalis longicornis* madanin 1 and 2, were 7-kDa proteins (Iwanaga *et al.,* 2003). A 30-kDa salivary allergen from *Aedes* (aegyptin) was also reported, which were shown to specifically block collagen-induced human platelet aggregation (Calvo *et al.,* 2007). So the protein/peptide found in fraction no. 51 in the present study could be exploited further to purify it. The mechanism of action how these peptides are inhibiting platelet aggregation still need to be studied. Theses proteins/peptides can be used either in therapeutics or as a source of antigen for the development of vaccine.

Out of total of 120 fractions collected, only 9 salivary fractions of *Rhipicephalus (boophilus) microplus* ticks showed thrombin induced platelet aggregation inhibition. On electrophorectic characterization by SDS-PAGE of these 9 fractions, some of the fractions were found to have multiple protein bands of different molecular weight while only single protein band of approximate molecular weight 64.1 kDa was found in fraction no. 51. So these promising anti-platelet aggregating proteins present in fraction no. 51 could be further purified and exploited for its mechanism of action of inhibition of platelet aggregation which can be further usedfor therapeutic purposes as well as for raising anti-tick vaccine.

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