PARCIAL PURIFICATION OF SERINEPROTEASE INHIBITOR FROM *BOOPHILUS MICROPLUS* LARVAE

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SUMMARY: The hard tick, *Boophilus microplus*, has been a problem for the cattle production in Brazil because of its ability of spoliation and disease transmission and also because of the high costs of the current control technology. Tick proteins can be used as tools to develop models of tick control. Toward that objective, a Serine protease inhibitor from *B. microplus* larvae was preliminarily characterized. Unfed larvae were the source of a 2% protein solution in 5 mM tris-HCl, 20 mM NaCl, pH 7.4. The inhibitor chromatographically purified by affinity on trypsin-Sepharose, and ion-exchange on a Resource Q column, resolved in one major active peak, approx. 18 Kda proteins. Trypsin inhibition was Ki 0.4 nM , elastase inhibition was Ki 3.5 nM and plasma Kallikrein with Ki 35 nM. The inhibitor did not change prothrombine time (PT) and thrombine time (TT), but prolonged activated partial thromboplastin time (APTT) 3 times more. As the studied protein appears in larvae that had no contact with host blood, once injected into cattle they may act as antigens. The antibodies from that process would hinder the preying ability inactivating the inhibitor and its role in the fixation process.

KEY WORDS : *Boophilus microplus*, larvae, control, blood clotting, APTT, tick.

INTRODUCTION

The hard tick, *Boophilus microplus*, has been a problem for the cattle beef production in Brazil because of its ability to spoilage and transmit diseases and also because of the high costs of current control technology. Ticks are blood sucker arthropods, widely distributed, and important vectors of arbovirusis, rickettsiosis, spirochetosis and protozoans to men and domestic animals (KAUFMAN,1989).

Tick control is still a challenge for the cattle beef producer because of the high costs, the environmental risks and the available techniques; it means a billion dollar loss per year (HORN & ARTECHE, 1985). As there isn't any policy for tick control in Brazil, farmers are compelled to use individual control practices, responding for a great part of the beef and dairy cattle production costs.

Inappropriate management is one of the ways to provoke a rise in the population of the chemical acaricide resistant tick. This resistant selection leads farmers to use doses higher then those recommended by the industry, thus, affecting the environment with toxic waste; mainly water sources, meat, and milk and dairy products (BULLMAN *et alii*, 1996; LIMA, 1997; EUA, 1997).

The cost of putting a new chemical acaricide on the market is estimated at 20 to 50 million dollars reducing the possibility of having new products in the near future and increasing the efforts to extend the lifetime of the available chemical acaricides. On the other hand, the organizations engaged in environmental protection create the possibility of introducing management strategies with technical innovations of low environmental impact (VILLELA, 1992).

To face this new demand, the industry tries to adjust its methods to provide safer products: no chemical substances, no cholesterol and so on, like "green meat" (ROCHA, 1997), known as that which is produced from animals raised and fed in the field with a minimum of chemical products and reduced production costs. It means that it is necessary to search for alternatives to tick chemical control with less contamination risks to food and environment.

The *B. microplus* larvae present at least three forms of

trypsin inhibitors, previously reported by WILLADSEN & RIDING (1979 e 1980). They described them as doubled-head inhibitors able to inhibit two enzyme molecules at the same time, for example, trypsin and chymotrypsin (WILLADSEN & RIDING, 1979).

Research on the *B.microplus* inhibitor has pointed out bovine trypsin and chymotrypsin (WILLADSEN & RIDING, 1979) as acting in various phases of life. The results show that the concentration of the inhibitors decreases from the time the larvae are eggs in relation to the their development. The inhibitors also influence blood clotting, changing the prothrombine time and the activated partial thromboplastin in bovines (WILLADSEN & RIDING, 1980).

Those inhibitors, found in many tick specie eggs, *B. microplus* included, are considered egg toxins and have immunological relationships (VERMEULEN *et alii*, 1988).

Previous studies suggest the relation between those inhibitors and an important physiologic activity at the moment of larvae eclosion, in its interaction with the hosts, at the unfed larvae fixing moment, and with internal processes of the parasite itself (WILLADSEN & RIDING, 1980).

During the infestation of animals, sensitive or not, previously exposed to ixodides, a quick neutrophil infiltration occurs, gradually increasing the presence of eosinophil and basophil. The prostaglandin vasodilation action, present in saliva, associated with the histamine coming from the mast cell degranulation and other granulocytes, induces tissue lise and blood afflux.

In susceptible animals, according to RIBEIRO (1989), the ixodides might be benefited by the host reaction, especially during the final ingurgitation phase, with the quick improvement of saliva secretion, increasing capillary permeability and blood afflux. In resistant animals, the number of granulocytes increases significantly at the ixodides fixing place. The great afflux of basophils, followed by the histamine release of other immune response mediators and antigenic complex, induces parasite rejection.

Antigens coming from adult ixodide saliva extracts have demonstrated promising effects in animal immunization against those parasites just as antigens coming from its intestine cells present similar action (JOHNSON *et alii*, 1986; OPDEBEECK *et alii*, 1988).

Larvae that do not feed and are situated close to the bovines skin die quickly and lose, on the average, 6 mg from its weight in a 12 hour period because the environment close to the bovine skin disserts the larvae. Furthermore, there is additional stress in the larvae because they need short and frequent fixations when attacking resistant animals (KEMP *et alii*, 1976). That is why this life phase can be considered strongly vulnerable to the strategic use of control methods.

Success in the feeding process by way of ixodides embodies a set of events, one of the most important factors being the host susceptibility. In general, the mouth size and the saliva cement guarantee the fixation process.

Some of the ixodides genres have short mouth parts, penetrating only the skin's surface: Boophilus, Rhipicephalus, Anocenter and Haemanphisalis. In those species, according to MOORHOUSE & TATCHELL, (1966), the saliva gland plays a preponderant role in the host fixation.

Histochemical and histological studies have demonstrated little influence of the citolitic enzymes present in saliva during the fixation process (BINNINGTON & KEMP, 1980; BOURDEAU, 1982; KAUFMAN, 1989). However, some esterases and phosphatases have been identified (FOGGIE, 1959; ARTHUR, 1965; BALASHOV, 1972). The fixation lesion caused by B. microplus is induced by the host defense cell afflux TATCHELL & MOORHOUSE (1970)

TATCHELL & MOORHOUSE (1970) demonstrated that the lesion at the *Rhipicephalus sanguineus* fixation place in dogs was provoked by leukocyte degranulating.

The object of this study is to confirm the presence of, to isolate, to purify and to characterize the serine proteinase inhibitor found in unfed *B. microplus* larvae; and also to better know the action mechanism of those inhibitors, mainly related to the blood clotting process and to the host x ectoparasite interaction. The study of these inhibitors might provide knowledge to subsidize new ixodide control models.

MATERIALS AND METHODS

Larvae extract preparation

0,5 g *B. microplus* larvae were ground in a morthar with 25 mL 5mM tris HCl buffer (pH 7.4) containing 20mM NaCl. It corresponds to a 2% protein concentration. The extract was centrifuged at 7000 rpm for 15 min at 4%. The resultant supernatant was used in affinity chromatography.

Tripsin-Sepharose affinity chromatography

12 mL of supernatant from the larvae extract were applied on a tripsin-Sepaharose column, prepared as instructed by the manufacturer, and pre-equilibrated with a tris HCl 50 mM, pH 8.0 buffer. After that, the column was washed with the same buffer to 280 nm, below 0.03. This step was followed by a new wash using the above described buffer, adding a 0.15 M NaCl. After the washing steps, the trypsin inhibitors were eluted with a 0.5 M KCl solution (pII 2.0). The fractions (1 mL) containing the inhibitor activities were pooled in a single fraction. It was kept frozen at 20° C to be used in further assays.

Inhibitor isolation by ion exchange chromatography on "High Trap Q"

The active material from trypsin-Sepharose was applied on a "High Trap Q" column (FPLC system), and the bound inhibitor was eluted by a linear NaCl gradiente (0 – 0,8M). The identified inhibitor was stored at –20° C.

Polyacrilamide gel eletrophoresis containing SDS (15%) (SDS-PAGE)

The method was described by LAEMMLI (1970). In the

present study, a 15% separation Polyacrilamide gel was used. The eletrophoresis time was around 4 hours at environment temperature in a 35 mA electric current. Bromophenol blue indicated the running end. The polyacrilamide gel was dyed by a comassie blue solution G-250.

Trypsin inhibition using Ac-Phe-Arg-pNA as substrate

Trypsin inhibitory activity was measured by the remaining enzyme activity on the Ac-Phe-Arg-pNA_substrate (0.5mM) in a tris-HCl 0.1 buffer, pH 8.0, as described by ERLANGER *et alii* (1961). During the inhibition assays, trypsin was pre-incubated with different inhibitor concentrations, following this, the substrate was added and the hydrolyze was followed by the absorbencies in 405 nm. The trypsin used in this assay was entitled by NPGB, CHASE & SHAW (1970).

The other serinoproteinases were analyzed using the specific chromogenic substrates and optimal conditions for each enzyme.

RESULTS AND DISCUSSION

In this study the isolated inhibitor was partially purified by affinity chromatography on trypsin-Sepharose associated to an ion exchange chromatography on "High Trap Q" FPLC system. The chromatography presented two peaks which were separated and identified (Fig. 1).

The peak identified as A was isolated from the other peaks in a "High Trap Q" column at a 0.56 M NaCl concentration. The B peak showed in Figure 1 did not present any activity in the further assays. The A and B peaks presented a proteic band of approximately 18 and 10kDA respectively in a polyacrilamide gel eletrophoresis containing SDS (15%) (Fig. 2).

This larval inhibitor type is rapidly lost at the initial stages of the parasite life cycle, suggesting that the inhibitor is secreted into the host. (WILLADSEN & RIDING, 1980). These and other evidences have suggested that these inhibitors are important in the fixation process and in feeding success (WILLADSEN & McKENNA, 1983).

This inhibitor was used, in the present study, in inhibition assays for many proteinases presenting the dissociation constants (Ki) shown on Table 1.

The bovine trypsin (10.4) nM) was pre-incubated in different inhibitor concentrations weighing 18 kDa, molecular weight. After that, the enzyme residual activity was obtained by adding the Ac-Phe-Arg-pNA (0,25 mM) substrate with absorbent readings at 405 nm showing a 0.4 nM Ki (Fig. 3).

The human plasmatic kallikrein (0.62 nM) was pre-incubated in different inhibitor concentrations. After that, the enzyme residual activity was obtained by adding the S2302 (H-D-Pro-Phe-Arg-pNA) (0,25 mM)substrate with absorbent readings at 405 nm. The inhibition curve referring to the 18 kDA, molecular weight inhibitor, presented a 35 nM Ki (Fig. 4).

The human neutrophil elastase (13nM) was pre-incubated in different inhibitor concentrations. After that, the enzyme residual activity was obtained by adding the Meo-Suc-Ala-Ala-Phe-Val-pNA (125µM) substrate with absorbent readings at 405 nm. The 18Kda inhibitor presented a 3,5 nM Ki (Fig. 5).

In relation to chymotrypsin, inhibition activity was observed for a 18Kda inhibitor but the Ki value was not determined, and the papaine a cisteine protease was not inhibited. The data is shown on Table 1.

The *B.microplus* trypsin inhibitors are present in the eggs and larvae but the isolation of different forms of *B.microplus* suggests that they are, at least, stage-specific (WILLADSEN & McKENNA, 1983).

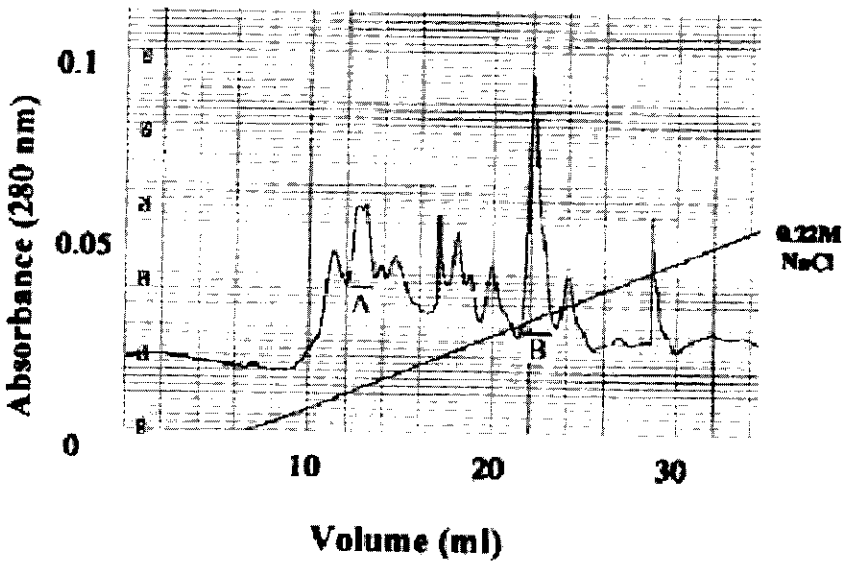

Fig. 1 – Ion exchange chromatography on High Trap Q of BmTIs purified in trypsin-Sephrose.

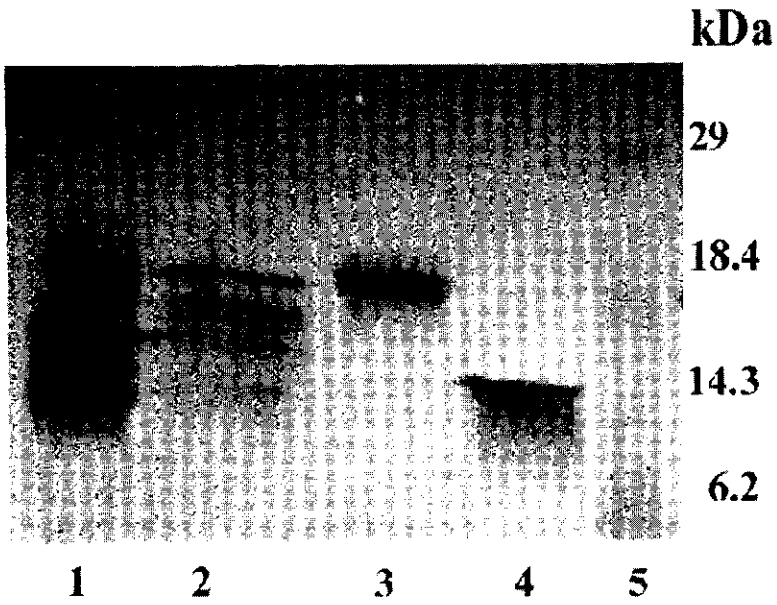

Fig. 2 – SDS-PAGE 15% Lanes: 1- Inhibitor purified by trypsin-Sepharose; 2- Inhibitors that flow through of High Trap Q; 3- BmTI-A from High Trap Q elution; 4- BmTI-B; 5- Low Mr markers.

Table 1– Dissociation constants – Ki(nM) of inhibitor for different serine proteinases.

Enzyme	Substrate [mM]	K_i (nM)
Bovine tryipsin	*Ac.Phe-Arg-Pna*	0,4
Bovine quimotripsin	Suc-Phe-*Pna*	n.d
Human factor Xa	N-Bz-Ile-Glu-Gly-Arg-*Pna* (S 2222)	n.i
Human Plasma Kallikrein	H-D-Pro-Phe-Arg-*pNA* (S 2302)	35
Human neutrophil elastase	MeO.Suc-Ala-Ala-Phe-Val-*pNA*	3,5

n.d. - not determined
n.i. – not inhibited

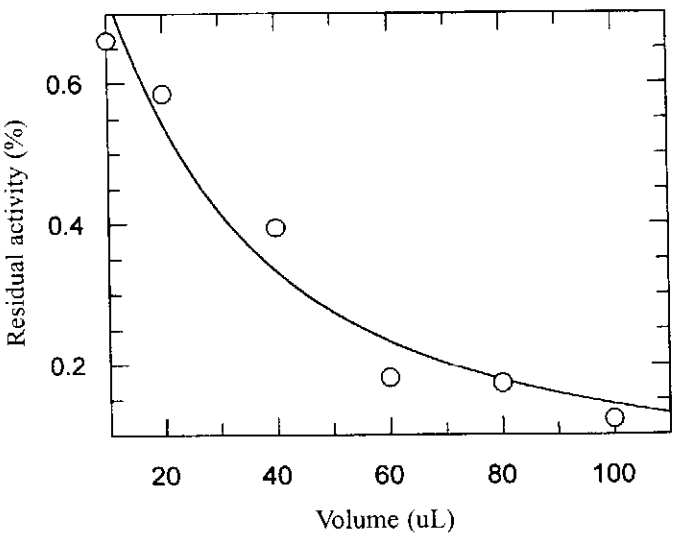

Fig. 3 – Trypsin inhibition curve using 18kDa inhibitot.

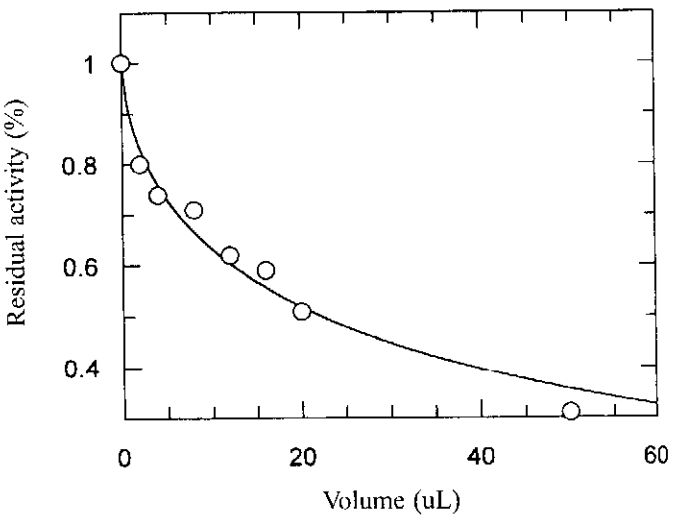

Fig. 4 – Human plasma kallikrein curve using the 18kDa inhibitor.

The 18Kda inhibitor did not interfere in the following blood clotting time: prothrombin time (PT), or thrombin time(TT), but the ativated-partial-thromboplastin-time(APTT) was prolonged 3 times more (Table2). These results confirm that the inhibitor might be important in the blood clotting inhibition by plasmatic kallikrein as it does not inhibit factor Xa.

The inflammatory process, originated by the lesion, release, at the bite local, the defense factors such as immunoglobulin and the complement by vase-active substances, and the phagocyte cells by chemotactic factors which, in normal conditions, are confined to the vases.

The Hageman Factor (factor XII) is activated as a result of the vascular endothelium lesion activating the blood clotting cascade and forming the fibrin clot. At the same time, it activates the so-called plasminogen activator which, in turn, generates the plasmin responsible for the fibrin destruction. During this process, the plasmin releases peptide fragments which have chemotactic effects on the neutrophils.

The Hageman Factor, the neutrophil plasmin and the macrophages activate the pre kallikrein. The activated kallikrein activates the kininogen to kinin which has an important role in the inflammatory process and its response is related to the vase-dilation, the vascular permeability increase, and to the pain.

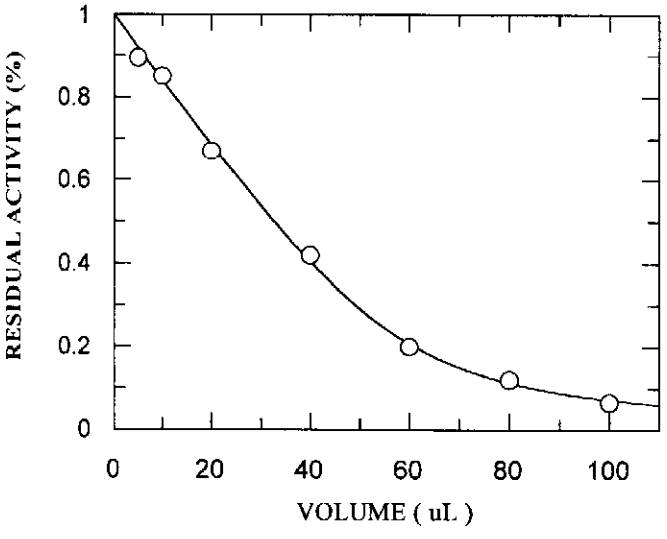

FIG. 5. Human neutrophil inhibitor curve using the 18kDa inhibitor.

TABLE 2. Coagulation tests using the 18kDa inhibitor.

	Prolonged time
TT (Thrombin time)	n.p.
PT (Prothrombin time)	n.p.
APTT (Activated-partial-thromboplastin time)	3*

n.p – not prolong
* When compared with blood clotting time in the normal plasma

Kallikrein is also released from the indirect action of the platelets and by neutrophiles from which kallikrein is considered a chemotactic factor and one of the main tupe I hipersensibility mediators.

The fibrinogen fragments and the elastase and colagenase released from the neutrophils, when in the death process, are monocytes chemotactic factors.

The neutrophils, which are attracted by the complement cascade products, stick to the immunity complex and phagocyte them rapidly. During this process, the neutrophils release their enzymes, by degranulation, and consequently, these enzymes are freed to the tissues nearby. The freed enzymes are the collagenases, that break the collagen fibers; the neutral proteases, that destroy the fundamental substances and the basal membrane; and the elastases that destroy the elastic tissue (TIZARD, 1994).

The capillary permeability increase, as a result of the prostaglandin released by the ixodides, was demonstrated by DICKINSON *et alii* (1976) and HIGGS *et alii* (1976) for B. microplus, and the maximum vase-dilation activity occurs at the final engorging phase. RIBEIRO *et alii* (1985) demonstrated the prostaglandin E_2 (PGE_2) in the Ixodes dammini saliva.

These results suggest that this inhibitor has an important role in blocking the blood clotting by the kallikrein inhibition of the immediate immune response by the neutrophil elastase inhibition during the B.microplus larvae fixation process in bovines. This blocking is profitable for the tick in the fixation process to the host and for feeding at the initial stages of the parasite phase cycle.

The role of this inhibitor still needs to be better explained, but its potential as an antigen and its use as a control tool of the natural infestation by ticks indicate a way to be studied.

SUMÁRIO

O carrapato *Boophilus microplus* causa grandes prejuízos econômicos à exploração pecuária brasileira em função da espoliação dos bovinos e do alto custo operacional das técnicas atuais de controle. Proteínas extraídas a partir de carrapatos podem ser usadas como ferramentas no controle dos mesmos. Com esse objetivo, um inibidor de serinoproteases foi isolado e parcialmente caracterizado. A partir de larvas não alimentadas foi preparada uma solução a 2% de proteína em 5 nM tris-HCL, 20 mM NaCl, pH 7.4. O inibidor, cromatograficamente purificado por afinidade tripsina-sepharose e troca iônica em uma coluna Resource Q, resultou em um pico de atividade, correspondendo a uma proteína de 20 Kda de peso molecular quando submetida a eletroforese em gel de poliacrilamida. A inibição de tripsina resultou em um Ki de 0,4 nM; elastase com um Ki de 3,5 nM; e calicreína plasmática com Ki de 34,6 nM. O inibidor não alterou o tempo de protrombina (PT) e tempo de trombina (TT), mas prolongou em 2,7 vezes o tempo de tromboplastina parcialmente ativada (APTT). O inibidor estudado aparece em larvas de

carrapato que não tiveram contato com o hospedeiro e, uma vez injetado em bovinos, ele pode atuar como antígeno. Os anticorpos gerados nesse processo podem impedir a habilidade de fixação do carrapato por meio de inativação desse inibidor e seu papel no processo de fixação.

PALAVRAS-CHAVE: *Boophilus microplus*, larva, controle, coagulação sangüínea, APTT, carrapato.

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(Received 5 December 1998, Accepted 13 March 1999)