

Assessment and partial purification of serine protease inhibitors from *Rhipicephalus (Boophilus) annulatus* larvae

Avaliação e purificação parcial dos inibidores da serina protease de larvas do *Rhipicephalus (Boophilus) annulatus*

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Abstract

Ticks are rich sources of serine protease inhibitors, particularly those that prevent blood clotting and inflammatory responses during blood feeding. The tick *Rhipicephalus (Boophilus) annulatus* is an important ectoparasite of cattle. The aims of this study were to characterize and purify the serine protease inhibitors present in *R. (B.) annulatus* larval extract. The inhibitors were characterized by means of one and two-dimensional reverse zymography, and purified using affinity chromatography on a trypsin-Sepharose column. The analysis on one and two-dimensional reverse zymography of the larval extract showed trypsin inhibitory activity at between 13 and 40 kDa. Through non-reducing SDS-PAGE and reverse zymography for proteins purified by trypsin-Sepharose affinity chromatography, some protein bands with molecular weights between 13 and 34 kDa were detected. Western blotting showed that five protein bands at 48, 70, 110, 130 and 250 kDa reacted positively with immune serum, whereas there was no positive reaction in the range of 13-40 kDa. Serine protease inhibitors from *R. (B.) annulatus* have anti-trypsin activity similar to inhibitors belonging to several other hard tick species, thus suggesting that these proteins may be useful as targets in anti-tick vaccines.

Keywords: *Rhipicephalus (B.) annulatus*, serine protease inhibitors, zymography.

Resumo

Carrapatos são uma rica fonte de inibidores da serina protease, particularmente aqueles que previnem coagulação e respostas inflamatórias durante a alimentação com sangue. O carrapato *Rhipicephalus (B.) annulatus* é um ectoparasita importante de bovinos. O objetivo deste estudo foi caracterizar e purificar os inibidores da serina protease presentes no extrato de larva do *R. (B.) annulatus*. Os inibidores foram caracterizados através de zimografia reversa uni e bidimensional e purificados com cromatografia de afinidade em uma coluna de sepharose-tripsina. A análise do extrato de larva pela zimografia reversa uni e bidimensional mostrou atividade inibitória de tripsina entre 13 e 40 kDa. Através de SDS-PAGE e zimografia reversa para proteínas purificadas pela cromatografia por sepharose-tripsina, algumas bandas de proteínas com pesos moleculares entre 13 e 34 kDa foram detectadas. Western blotting mostrou que cinco bandas de proteínas a 48, 70, 110, 130 e 250 kDa reagiram positivamente com o soro imune, enquanto não houve reação positiva nas bandas 13-40 kDa. Inibidores da serina protease do *R. (B.) annulatus* têm atividade antitripsina semelhante àquelas dos inibidores de outras espécies de carrapatos duros, sugerindo, assim, que essas proteínas podem ser úteis como alvo de vacinas contra carrapatos.

Palavras-chave: *Rhipicephalus (B.) annulatus*, inibidores da serina protease, zimografia.

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Introduction

Ticks are the most frequent ectoparasites that infest mammals, birds, reptiles and amphibians (SAUER et al., 1995). In Iran, *Rhipicephalus annulatus* (formerly *Boophilus annulatus*) is one of the most common ticks in cattle. This tick has also been recorded in some regions of Europe and the Americas, such as; Portugal, Spain, Italy, Greece, Turkey and Mexico; furthermore, it is common in northern Africa.

Ticks cause serious economic losses among cattle because of their blood-sucking activities and consequent transmission of some tick-borne diseases such as babesiosis and anaplasmosis (ESTRADA-PEÑA et al., 2004). Therefore, controlling them has become a priority in tropical and subtropical regions. Tick control is mainly based on acaricides. Because of the disadvantages of acaricides, which including pollution of the environment and food chain and also acaricide resistance (PREVOT et al., 2007), alternative control methods such as vaccination are promising. Development of vaccines relies on obtaining more knowledge about tick proteins. Proteases and their inhibitors may be candidates for designing vaccines.

Ticks' success in blood sucking is due to the anti-hemostatic and anti-inflammatory substances present in tick saliva (AROCHA-PIÑANGO et al., 1999). These insects are rich source of different serine protease inhibitors, which are distributed in tick tissues over the course of development of ticks' life cycle (VERMEULEN et al., 1988). Proteinase inhibitors have long been recognized in eggs and larvae of *R. (B.) microplus*, and have been seen to inhibit trypsin, chymotrypsin, elastase and plasma kallikrein (TANAKA et al., 1999). Interestingly, some inhibitors have been shown to prolong the activated partial thromboplastin time (APTT) and prothrombin time (PT) for bovine blood (IBRAHIM et al., 2001). These inhibitors, which are present in different ticks' tissues, can also protect these arthropods from infection by different pathogens (KANOST, 1999).

In this study, serine protease inhibitors in unfed larvae of *R. (B.) annulatus* were characterized by means of one and two-dimensional reverse zymography and purified using trypsin-Sepharose affinity chromatography.

Materials and Methods

Ticks

The *R. (B.) annulatus* ticks were handled as described by Brown et al. (1984). The tick larvae were obtained by incubating engorged females at 85% relative humidity and 28 °C. Two healthy seronegative four-month-old Holstein calves were provided for tick infestation. Each calf was infested with about 10,000 *R. (B.) annulatus* larvae. The larvae from the engorged ticks were collected ten days after eclosion and stored at -20 °C.

Protein extraction

Tick larvae (0.1 g) were ground in a mortar on ice with 0.15 M PBS (pH 7.2). The homogenate was centrifuged at 10,000 g for

10 minutes at 4 °C and the supernatant was filtered and collected. The protein assay was performed as described by Bradford (1976).

Casein agar plate assay

This assay was done as described by Yuan and Cole (1987), with some modifications: 0.5 g of agar in 46 ml of 0.5 M Tris buffer (pH 6.5) was boiled and then cooled to approximately 50 °C, and 4 ml of casein (0.5% in 1.0 M NaOH) was added to it. This medium was divided between plates (15 ml) and allowed to harden. Some wells were then constructed in agar and were filled with 50 µl of a mixture of eggs and unfed tick larval extract and trypsin (200 µg/ml). The plates were incubated overnight at room temperature. Bright zones appeared around the wells after adding 3 ml of acetic acid, thereby indicating the proteolytic activity.

Serine protease inhibitor purification using affinity chromatography

The trypsin-Sepharose column was prepared as specified by Hudson and Hay (1989), with some modifications. Sepharose (7 ml) was mixed with cyanogen bromide (250 mg in 2 ml of distilled water) and the pH was adjusted to 11 using NaOH (0.2 M) for 10 min. The Sepharose was then washed with 0.1 M borate saline buffer (pH 8.3). Trypsin (50 mg in 10 ml of borate saline buffer) was added to the activated Sepharose and was kept at 4 °C overnight, then washed with PBS (0.15 M, pH 7.2). The trypsin-Sepharose was mixed with glycine (1 M, pH 8.0) for one hour and was washed with 40 ml of PBS. Then, the trypsin-Sepharose was poured into the column and larval protein (30 mg) was applied to the column, with incubation at room temperature for 45 minutes, followed by washing with PBS. The inhibitors that had bonded were eluted with 0.1 M glycine buffer (pH 2.5) containing 4 M urea and were immediately neutralized with 2 M Tris-HCl buffer (pH 8). The fractions were pooled and concentrated using centrifugal filter tubes (Eppendorf) with a molecular cutoff of 4 kDa.

SDS-PAGE and western blotting

Electrophoresis on 12% SDS polyacrylamide gel in the absence of 2-mercaptoethanol (2-ME) was carried out as described by Laemmli (1970), using 10 µg protein. The gels were stained with Coomassie blue and silver. Western blotting was performed as prescribed by Wang and Nuttall (1994). Gel containing resolved proteins was transferred electrophoretically to nitrocellulose membrane. The membrane was blocked for 45 min, by means of PBS containing 2.5% Tween 20, and all the washing steps were carried out with PBS containing 0.05% Tween 20 (3 × 5 min). The membrane was incubated in serum derived from calves that had been experimentally infested with *R. (B.) annulatus* larvae (1:50 in PBS containing 0.05% Tween 20) for 1 hour at room temperature. It was then washed and incubated with anti-bovine IgG coupled with horseradish peroxidase (1/1000 in PBS) for 30 min at room temperature. After washing, the color development step was performed with diaminobenzidine containing H₂O₂.

One-dimensional reverse zymography

Serine protease inhibitors were separated using polyacrylamide gel electrophoresis (gel contained 0.1% gelatin) and the Laemmli buffer system. 20 μ l of samples containing 1000 μ g of protein per ml were mixed with 20 μ l of sample buffer without reducing reagents and loaded (10 μ l). After electrophoresis, the gel slab was incubated in 0.1 M Tris-HCl buffer (pH 8.0) containing trypsin (10 μ g/ml) for 2 h at room temperature. The gels were then stained with Coomassie blue R-250 (PRUETT et al., 2008).

Two-dimensional reverse zymography

Isoelectric focusing (IEF) was performed as described by Yatsuda et al. (2003), using 7-cm immobilized pH gradient (IPG) strips (non-linear pH range 3-10). Samples containing 300 μ g of proteins were diluted in rehydration buffer (total volume 120 μ l) and used to rehydrate the strips for 18 h at room temperature. IEF was performed in a Protean IEF cell (Bio Rad) under conditions recommended by the manufacturer. The IPG strips were then equilibrated for 20 min at room temperature in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris; pH 8.8). The equilibrated IPG strips were transferred onto the gel, and SDS-PAGE for reverse zymography was carried out.

Results

Casein agar plate assay

In order to confirm the presence of serine protease inhibitors, the casein agar plate assay method was applied. As is shown in Figure 1, the absence of bright zones around wells 1 and 2 in plate A after adding acetic acid indicated that there was inhibition of proteolytic activity due to presence of trypsin inhibitor in tick egg

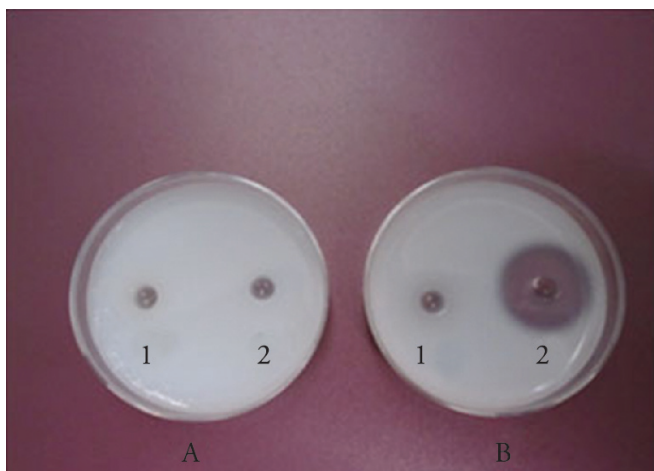


Figure 1. Proteolytic inhibition activity on agar plates containing casein as substrate: well containing mixture of unfed tick larvae extract and trypsin (A-1); well containing mixture of tick egg extract and trypsin (A-2); well containing PBS as negative control (B-1); well containing trypsin as positive control (B-2).

and larval extracts. The presence of a bright zone around well 2 in plate B was due to trypsin proteolytic activity.

SDS-PAGE

To profile the tick proteins, larval extract was resolved by means of non-reducing SDS-PAGE, and eight sharp bands with molecular weights between 40 and > 250 kDa were detected (Figure 2).

Through the non-reducing SDS-PAGE method for *R. (B.) annulatus* larval extract using silver staining, several bands with molecular weights between 12 and > 250 kDa were observed (Figure 3).

Western blotting

The serum immunoreactivity of the calves that had been experimentally infested with tick larvae was detected by means of western blotting. Five protein bands at 48, 70, 110, 130 and 250 kDa produced positive reactions with immune serum (Figure 4).

Reverse zymography

To detect serine protease inhibitors, reverse zymography of larval extracts was applied. Analysis on one-dimensional reverse zymography of larval extracts showed that there was trypsin inhibitory activity between 13 and 40 kDa. The blue zones with a bright background that were seen using Coomassie blue staining were indicative of trypsin inhibitory activity (Figure 5).

Analysis on two-dimensional reverse zymography of larval extracts showed that was trypsin inhibitory activity mainly between

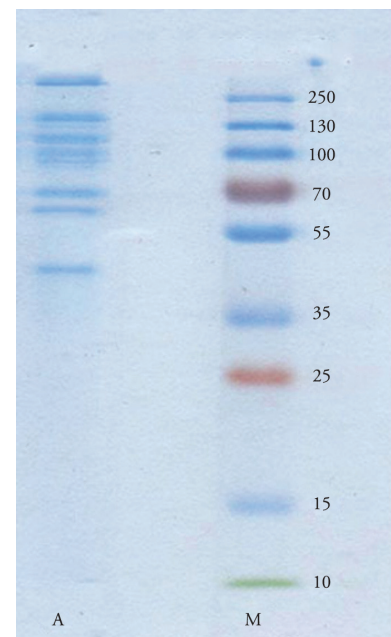


Figure 2. Non-reducing SDS-PAGE (12%) profile of unfed *R. (B.) annulatus* larval extract (Coomassie blue staining): tick larval extract (lane A); molecular weight standards (lane M).

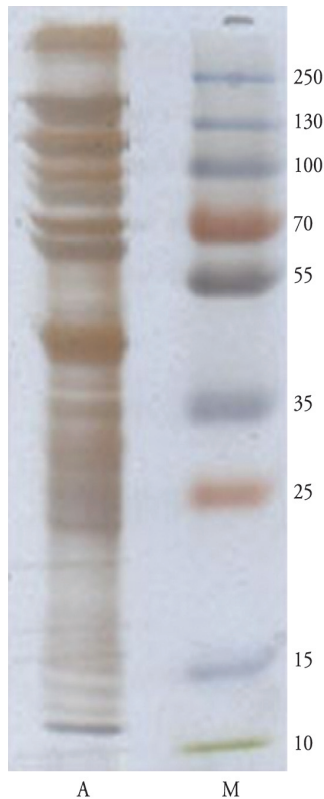


Figure 3. Non-reducing SDS-PAGE (12% gel) on *R. (B.) annulatus* larval extract, using silver staining: tick larval extract (lane A); molecular weight standards (lane M).

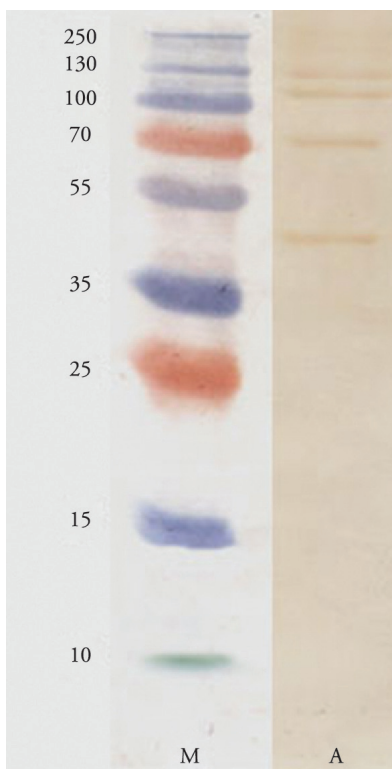


Figure 4. Unfed *R. (B.) annulatus* larval extract analyzed using western blotting: molecular weight standards (lane M); tick larval extract (lane A).

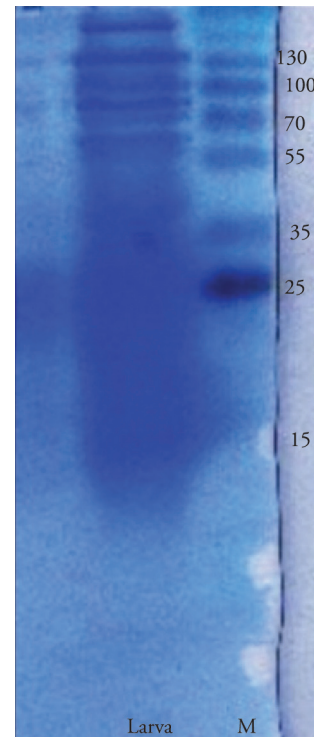


Figure 5. One-dimensional reverse zymography analysis on serine protease inhibitors of *R. (B.) annulatus* larval extracts: molecular weight standards (M). The gel was stained using Coomassie blue R 250.

13 and 40 kDa, at different isoelectric pH values (3-10). The blue zones with a bright background that were seen using Coomassie blue staining were indicative of trypsin inhibitory activity (Figure 6).

Using non-reducing SDS-PAGE for proteins that were purified by means of trypsin-Sepharose affinity chromatography, some protein bands with molecular weights between 13 and 34 kDa were detected through Coomassie blue and silver staining (Figure 7).

Reverse zymography for proteins that were purified by means of trypsin affinity chromatography showed some blue zones in a bright field, with molecular weights between 13 and 34 kDa, through Coomassie blue staining (Figure 8).

Discussion

R. (B.) annulatus is an important tick in meso-Mediterranean climates. In Iran, this tick is distributed in the northern provinces and has an important role in transmission of some pathogens. Novel control methods such as vaccination have been developed recently. Any new biochemical and physiological information about ticks may help in developing new vaccine. Tick larvae have been shown to be important sources of trypsin and chymotrypsin inhibitors (WILLADSEN; RIDING, 1979; WILLADSEN; MCKENNA, 1983; TANAKA et al., 1999; ANDREOTTI et al., 2001). There are reports suggesting that serine protease inhibitors may be candidates for use in vaccines and that such vaccines would be effective (MULENG et al., 2001; IMAMURA et al., 2005). The inhibitory activities of these proteins relating to blood

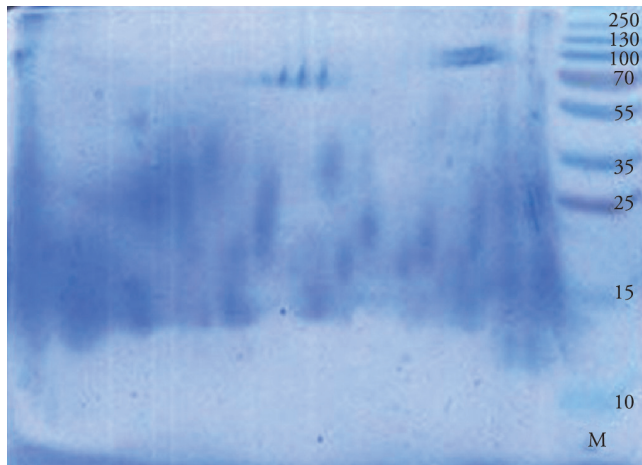


Figure 6. Two-dimensional reverse zymography analysis on serine protease inhibitors of *R. (B.) annulatus* larval extracts: molecular weight standards (M). The gel was stained using Coomassie blue R 250.

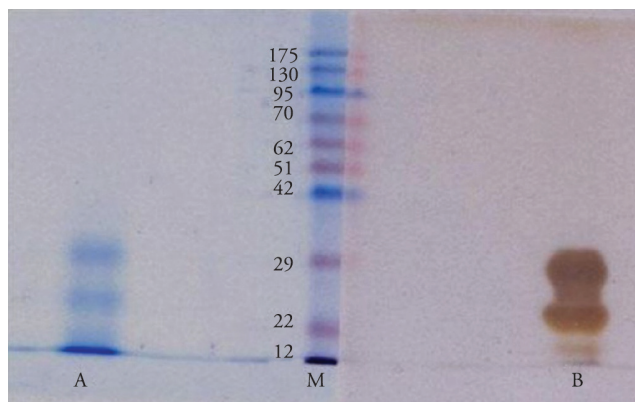


Figure 7. SDS polyacrylamide gel electrophoresis (12%) on *R. (B.) annulatus* larvae serine protease inhibitors purified by means of trypsin-Sepharose: stained using Coomassie blue (A); stained using silver (B); molecular weight standards (M).

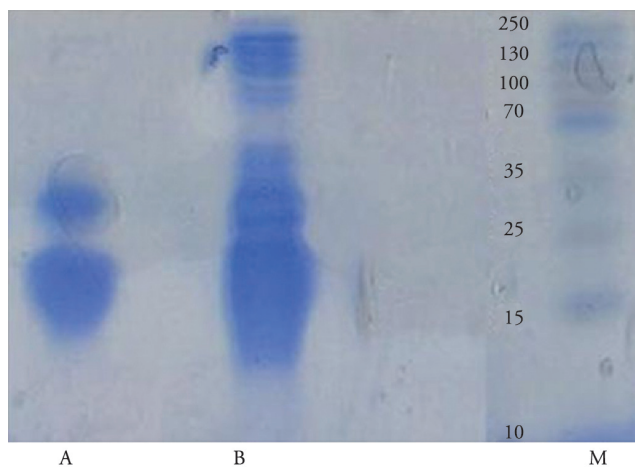


Figure 8. One-dimensional reverse zymography analysis on serine protease inhibitors of *R. (B.) annulatus* larval extracts purified by means of trypsin-Sepharose affinity chromatography (A) and whole larval extract (B); molecular weight standards (M). The gel was stained using Coomassie blue R250.

clotting and inflammatory responses may facilitate ticks' blood sucking process.

In the present study, non-reducing SDS-PAGE was firstly performed on *R. (B.) annulatus* larval extract and eight sharp bands with molecular weights between 40 and > 250 kDa were obtained using Coomassie staining. Several bands with molecular weights between 12 and > 250 kDa were observed using silver staining. In the second step, we characterized and purified serine protease inhibitors from *R. (B.) annulatus* larval extracts. Through one and two-dimensional reverse zymography, larval extracts showed trypsin inhibitory activity mainly between 13 and 40 kDa, at different isoelectric pH values (3-10). The purified serine protease inhibitors presented molecular masses between 13 and 34 kDa.

Serine protease inhibitors are classified into at least 18 families based on the primary and three-dimensional structures and inhibition mechanisms (LASKOWSKI; QASIM, 2000). Six of these families, comprising Kazal, BPTI-Kunitz, α -macroglobulin, serpin, pacifastin and Bombyx, are present in invertebrate hemolymph and also in saliva (KANOST, 1999; PHAM et al., 1996; SIMONET et al., 2002). Sasaki et al. (2004) purified serine protease inhibitors with molecular weights between 6 and 18 kDa from *R. (B.) microplus* larvae by means of trypsin-Sepharose affinity chromatography. All fractions presented trypsin inhibitory activity, and some of them also inhibited human plasma kallikrein and human neutrophil elastase. Based on amino acid sequence data, these authors classified them as members of the Kunitz-type serine proteinase inhibitor family. In addition, other results have shown that serine proteinase inhibitors from *R. sanguineus* with molecular weights between 8 and 18 kDa have inhibitor activity similar to that of *R. (B.) microplus* inhibitors (SANT'ANNA AZZOLINI et al., 2003). The variations in the molecular weights of serine protease inhibitors in different tick species may be due to the presence of different genes encoding these proteins, which may contain variable numbers of Kunitz domains (CORRAL-RODRÍGUEZ et al., 2009). Alternatively, they may be due to proteolytic processing of large Kunitz-type inhibitors during larval development (SASAKI et al., 2004).

An efficient immune response against serine proteinase inhibitors and neutralization of their enzymatic activity may influence blood feeding and tick survival (PREVOT et al., 2007). Our western blot results suggested that the calves that had been experimentally infected with ticks did not produce antibodies against serine proteinase inhibitors (in the range of 13-40 kDa). This result was in agreement with Andreotti et al. (2001) and Pruett et al. (2008). Lack of antibody induction against serine protease inhibitors in cattle during tick infestation may be due to the low amounts of these inhibitors introduced for immune response stimulation in the host. It may also be due to bovine unresponsiveness to them as a result of natural exposure via saliva, or due to antibody production at very low concentrations that are undetectable with the western blot method (PRUETT et al., 2008).

Animals have been immunized using purified serine proteinase inhibitors from different tick tissues and larvae in several studies. Experimental challenges performed on animals previously immunized with tick larvae have shown that the animals acquired significant protective immunity against ticks, with reduction of the

biological parameters that would favor ticks (ANDREOTTI et al., 2001; PREVOT et al., 2007).

Development of new vaccines relies on characterization of tick proteins. Serine protease inhibitors have fundamental physiological roles in ticks and play an important role in tick-host interactions. Therefore, these proteins can be considered to be potential candidates for use in vaccines. It seems that further studies are needed in order to characterize and identify tick larval proteins.

Acknowledgements

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