Available online at www.sciencedirect.com
ScienceDirect
Experimental Parasitology 114 (2006) 40–46
Experimental Parasitology
www.elsevier.com/locate/yexpr

Boophilus microplus: Its saliva contains microphilin, a small thrombin inhibitor

Alessandra Ciprandi [a,b], Simone Kobe de Oliveira [a], Aoi Masuda [a,c], Fabiana Horn [d], Carlos Termignoni [a,b,*]

[a] *Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil*
[b] *Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil*
[c] *Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil*
[d] *Departamento de Biofísica, Universidade Federal do Rio Grande do Sul, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil*

Received 10 October 2005; received in revised form 8 February 2006; accepted 9 February 2006
Available online 4 April 2006

Abstract

Saliva of the cattle tick *Boophilus microplus* contains two thrombin inhibitors, BmAP and microphilin. This work presents the purification and characterization of microphilin. It was purified from the saliva by gel filtration, ultrafiltration through a 3 kDa cut-off membrane and affinity chromatography in a thrombin–Sepharose column. Analysis by mass spectrometry showed a molecular mass of 1770 Da. Microphilin is the smallest salivary thrombin inhibitor peptide known to date. It inhibits fibrinocoagulation and thrombin-induced platelet aggregation with an IC_{50} of 5.5 μM, is temperature resistant and its inhibitory activity was abolished by protease K treatment. Microphilin did not inhibit the amidolytic activity of the enzyme upon a small chromogenic substrate, but inhibited the hydrolysis of a substrate that binds both catalytic site and exosite I. Therefore, we propose that microphilin blocks thrombin at exosite I.

Index Descriptors and Abbreviations: Thrombin inhibitor; Tick saliva; Tick anticoagulant; *Boophilus*; Antithrombin

1. Introduction

Ticks, as any other hematophagous animal, must overcome the efficient and sophisticated host hemostatic system in order to have their blood meal. Indeed, their saliva contains an amazingly complex and diverse assortment of salivary components to avoid host hemostasis (Mans and Neitz, 2004; Ribeiro and Francischetti, 2003). Redundancy is extremely common in arthropod antihemostatic mechanisms. Among antihemostatic molecules present in saliva, one single molecule may target more than one coagulation factor (or agonists of platelet aggregation) (Andersen et al., 2004; Ehebauer et al., 2002; Mans et al., 1998, 2002b; Ribeiro et al., 1991); conversely, a coagulation factor may be the target of different salivary molecules (Ibrahim et al., 2001; Iwanaga et al., 2003). Some ticks are able to inhibit the coagulation extrinsic pathway (Ehebauer et al., 2002; Francischetti et al., 2002), but usually they inhibit thrombin (Hoffmann et al., 1991; Horn et al., 2000; Ibrahim et al., 2001; Iwanaga et al., 2003; Kazimirova et al., 2002; Lai et al., 2004; Mans et al., 2002a; Nienaber et al., 1999; Zhu et al., 1997), or its activator, factor Xa (Joubert et al., 1995; Limo et al., 1991; Waxman et al., 1990).

Thrombin is the major serine proteinase in blood coagulation. It hydrolyzes fibrinogen to form the fibrin-clot, induces platelet aggregation and activates other coagulation factors to reinforce the coagulation cascade. Thrombin has high substrate specificity, determined by the selectivity of its deep active site and by a highly positively charged region in its surface, called exosite I, which participates in the interaction between the enzyme and substrates (Rydel et al., 1991). Some molecules inhibit thrombin by binding to

* Corresponding author. Fax: +55 51 33167309.
E-mail address: ctermignoni@cbiot.ufrgs.br (C. Termignoni).

0014-4894/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2006.02.010

exosite I, thus preventing its interaction with its natural substrates. The catalytic site may remain free to bind and hydrolyze small peptides; in some cases, the binding of an inhibitor to exosite I actually increases thrombin activity with respect to these small synthetic peptides (Francischetti et al., 1997).

The cattle tick *Boophilus microplus* is prevalent in several of the major world beef production areas, causing significant problems for the industry. Each tick can ingest up to 1 mL of blood, so that tick infestations cause bovine weight loss and a consequent decrease in beef and milk production. In addition, *B. microplus* is the vector of babesiosis and anaplasmosis. We have previously described that *B. microplus* saliva has a 60 kDa thrombin inhibitor able to inhibit thrombin activity with respect to large and small substrates, i.e., it inhibits the catalytic site (Horn et al., 2000). Here we show that saliva of this tick also contains a molecule able to inhibit thrombin by binding to its exosite I. Indeed, this is the smallest salivary thrombin inhibitor described to date.

2. Materials and methods

2.1. Materials

All materials used were of analytical grade. Milli-Q (Millipore, Bedford, EUA) water was used in all experiments. Thrombin–Sepharose affinity resin was prepared by coupling bovine thrombin (previously treated with *H*-D-Phe-Pro-Arg-chloromethylketone, from Bachem, Bubendorf, Switzerland) to CNBr-activated Sepharose 4B, as described by the manufacturer (Amersham Biosciences, Buckinghamshire, UK). α-Cyano-4-hydroxycinnamic acid, fibrinogen and BSA (bovine serum albumin) were purchased from Sigma–Aldrich (St. Louis, USA). Chromogenic substrate S-2238 (*H*-D-Phe-Pip-Arg-pNA; pip = L-pipecolyl) was purchased from Chromogenix (Milano, Italy). The fluorogenic substrate Abz-LDPRSFRL-RNKNDKYEPFWEDEENKQ-EDDnp was synthesized by Dr. Luiz Juliano (Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil).

2.2. Saliva collection

Adult fully engorged female ticks that had spontaneously detached from the bovine were collected, rinsed, fixed to glass plates with adhesive tape and induced to salivate with injection of 5 μL pilocarpine (2% w/v in PBS). Ticks were maintained in a humid chamber and saliva was collected for a period of 2 h (approximately 1 μL per tick) with a small vacuum apparatus. Saliva was stored at −20 °C until use.

2.3. Purification of microphilin

2.3.1. Size-exclusion chromatography

Saliva (100 μL) was injected onto a TSK G2000SW gel filtration column (0.75 × 30 cm, LKB UltroPack, Bromma, Sweden) previously equilibrated with 20 mM CH_3COONH_4, pH 6.0, using a high-pressure liquid chromatography pump (LKB) at room temperature with a flow rate of 0.5 mL/min. Column fractions (1.0 mL) were tested for anticoagulant activity using the recalcification time assay.

2.3.2. Ultrafiltration through a 3 kDa cut-off membrane

The fractions corresponding to the microphilin anticoagulant activity were pooled and ultrafiltered in Centricon-3 (Amicon, Millipore, Bedford, USA). The ultrafiltrate containing microphilin was concentrated in Centrivap (Labconco, Kansas, USA).

2.3.3. Thrombin–Sepharose affinity chromatography

The fraction <3000 Da was applied onto a thrombin–Sepharose column pre-equilibrated in 20 mM Tris–HCl pH 7.5 with a flow rate of 0.5 mL/min at room temperature. After 2.5 h, the column was washed with equilibration buffer (fractions 1–7), followed by elution of the bound inhibitor with 20 mM Tris–HCl, pH 7.5, 1 M NaCl. Fractions were dialyzed, concentrated, and tested for inhibition of fibrinogen clotting.

2.4. Protein determination

Protein concentration was estimated by reading the absorbance at 280 nm in a quartz 96-well microplate using a SpectraMax microplate reader (Molecular Devices Corporation, Sunnyvale, USA) and corrected to a 1.0 cm light pathway.

2.5. Laser desorption mass spectrometry

The molecular mass of the peptide eluted from thrombin–Sepharose was determined by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) using MicroMass TofSpec SE (Manchester, UK) and matrix made of α-cyano-4-hydroxycinnamic acid.

2.6. Treatment with protease K

A partially purified microphilin (after the ultrafiltration step; 6 μg) was incubated with or without protease K (86 nM) in 10 mM Hepes, pH 7.4, 150 mM NaCl at room temperature for 2 h. The anticoagulant activity was then tested using the recalcification time assay.

2.7. Characterization of the inhibitor

Inhibition activity assays were performed at 37 °C using either a SpectraMax (Molecular Devices, Sunnyvale, USA) microplate reader, as described (Horn et al., 2000), or a FMax microplate reader (Molecular Devices, Sunnyvale, USA), both equipped with a software module for kinetic analysis (SOFTMax PRO, Molecular Devices, Sunnyvale, USA).

2.7.1. Recalcification time

Citrated bovine plasma (20 μL) was incubated with 55 μL of 10 mM Hepes, pH 7.4, 150 mM NaCl, with or without the sample to be tested, at 37 °C for 10 min. The coagulation cascade was triggered by addition of 25 μL of pre-warmed 40 mM $CaCl_2$, 150 mM NaCl, after which the microplate was immediately agitated. Fibrin-clot formation was followed by reading the absorbance at 650 nm at 11 s intervals.

2.7.2. Fibrinogen-clotting assay

Thrombin (20 nM) was incubated with microphilin (1.06, 2.11, 4.22, or 8.45 μM) in 50 mM Tris–HCl, pH 7.5, BSA 0.1% for 10 min at 37 °C prior to the addition of fibrinogen (200 μg). Fibrin-clot formation was followed by reading the absorbance at 650 nm at 11 s intervals.

2.7.3. Amidolytic assays using chromogenic substrate

Thrombin (4.8 nM) was incubated with microphilin (25.5 or 51 μM) in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ for 10 min at 37 °C. S-2238 (*H*-D-Phe-Pip-Arg-pNA) (200 μM) was added and the substrate hydrolysis measured at 405 nm in a SpectraMax microplate reader at 11 s intervals.

2.7.4. Amidolytic assay using fluorogenic substrate

Thrombin (4.8 nM) was incubated with microphilin (10.2, 20.4, 30.6, or 40.8 μM) in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, for 10 min at 37 °C. Abz-LDPRSFRLRNKNDKYEPFWEDEENKQ-EDDnp (14 μM) was added and the substrate hydrolysis was measured at 320/430 nm in a FMax microplate reader at 30 s intervals.

2.7.5. Platelet aggregation assays

Assays were performed as described (Horn et al., 2000). Briefly, rabbit platelet-rich plasma was centrifuged at 650*g* for 10 min and the pellet washed twice in Tyrode–protein buffer (5 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1% glucose, and 0.25% BSA). The final pellet was resuspended in the same buffer, in a volume adjusted to give an OD of 0.15 at 650 nm. Assays were done in 96-well microplates using a SpectraMax microplate reader to monitor aggregation of the washed platelets. Fifty microliters of platelet suspension was induced to aggregate with thrombin (0.5 nM) that had been previously incubated or not (controls) for 10 min at 37 °C with microphilin (1.69, 3.38, 6.76, or 10.14 μM). The decrease in absorbance at 650 nm was recorded for 20 min at 11 s intervals.

3. Results

3.1. Anticoagulant purification

The two anticoagulant activities of *B. microplus* saliva were separated by size-exclusion chromatography (Fig. 1A). The first peak with anticoagulant activity corresponds to BmAP, a 60 kDa-thrombin inhibitor already characterized (Horn et al., 2000). The second peak with anticoagulant activity was eluted from the column with an apparent molecular mass of 40 kDa (a column calibration was done with proteins of known molecular sizes). When this preparation was ultrafiltered through a 3 kDa-molecular weight cut-off membrane, the activity was recovered in the ultrafiltrate; this material was named microphilin. The ultrafiltrate was then applied onto a thrombin–Sepharose column, and the activity eluted with 1 M NaCl (Fig. 1B). This protocol yielded, from 2 mL of saliva, 60 μg of microphilin, as estimated by absorbance at 280 nm. The purity of the preparation and the low molecular mass of the anticoagulant were confirmed by mass

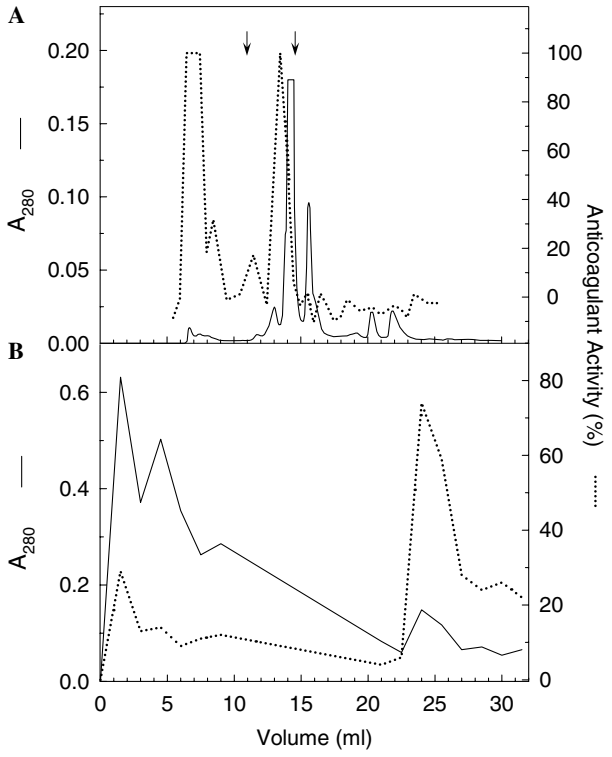

Fig. 1. Purification of microphilin. (A) One hundred microliters of tick saliva was applied onto a G2000SW gel filtration column (0.75 × 30 cm) equilibrated and processed with 20 mM CH_3COONH_4, pH 6.0. Protein elution was monitored by absorbance at 280 nm (solid line), and anticoagulant activity was measured using recalcification time assay (dotted line). Lack of fibrin-clot formation during the time of the assay (20 min) was taken as 100% relative anticoagulant activity. The elution volumes of BSA (M_r, 66,000) and ovoalbumin (M_r, 45,000) are indicated by arrows (left and right, respectively). Ribonuclease A (M_r, 13,700) eluted at 55 mL. (B) The fractions of gel filtration corresponding to the second peak of anticoagulant activity were pooled and ultrafiltered in Centricon-3. The ultrafiltrate was concentrated under vacuum and applied onto a thrombin–Sepharose column equilibrated in 20 mM Tris–HCl, pH 7.5. The column was washed with equilibration buffer (fractions 1–7, 2 mL/fraction) and eluted with 20 mM Tris–HCl, pH 7.5, 1 M NaCl (fractions 8–14). Protein elution was monitored by absorbance at 280 nm (solid line), and inhibition of thrombin activity was measured using the fibrinogen-clotting assay (dotted line). Fibrinogen-clot formation in the absence of inhibitor was taken as 0% thrombin inhibition and a control without thrombin as 100% inhibition.

spectrometry on MALDI-TOF, which showed two peaks, of 1755 and 1770 Da (Fig. 2), which are possibly isoforms of microphilin.

3.2. Thermal stability

Compared to the activity of the inhibitor (tested in the fibrinogen-clotting assay) immediately after thawing (taken as 100%), microphilin retained 100% of its activity after incubation at room temperature for 30 min, 80% of its inhibitory activity after 30 min at 37 °C and 80% of its activity after 5 min at 100 °C.

3.3. Digestion by protease K

The small size of microphilin and its thermal stability raised the possibility that the inhibitor was not a peptide. To test this, we treated a partially purified microphilin preparation (after the ultrafiltration step) with protease K. This enzyme, in the concentration and incubating conditions used, partially abolished microphilin anticoagulant activity (Fig. 3), indicating that the inhibitor is a peptide.

3.4. Antithrombin activity characterization

Like BmAP, microphilin inhibits thrombin. The purified microphilin inhibited cleavage of fibrinogen (200 μg) by thrombin in a dose-dependent manner (Fig. 4), with an IC_{50} of 5.5 μM (Fig. 4 inset).

Microphilin also inhibited thrombin–induced platelet aggregation with an IC_{50} of 5.8 μM (Fig. 5 and inset).

We then tested the effect of the inhibitor on thrombin amidolytic activity using a synthetic substrate, S-2238 (*H*-D-Phe-Pip-Arg-pNA). Fig. 6 shows that microphilin did not inhibit thrombin activity with respect to this small synthetic substrate at an inhibitor/enzyme molar excess in which fibrinogen-clotting and platelet aggregation were strongly inhibited. This suggests that microphilin does not interact with thrombin catalytic site. We therefore, tested a synthetic substrate that was sufficiently long to reach both the catalytic site and the anion-binding exosite I of thrombin (Abz-LDPRSFRLRNKNDKYEPFWEDEENKQ-EDDnp). Thrombin activity with respect to this long substrate was inhibited by microphilin (Fig. 7), indicating that microphilin interacts with thrombin exosite I. This

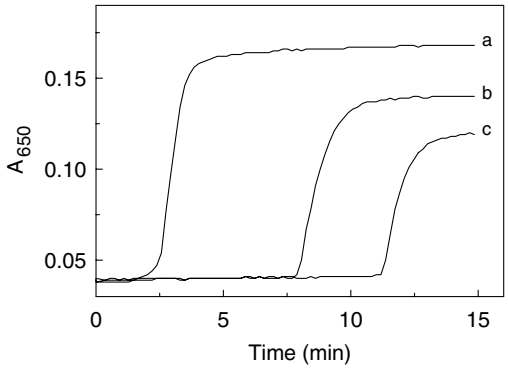

Fig. 3. Anticoagulant activity of microphilin after treatment with protease K. Partially purified microphilin (6 μg), previously incubated with (curve b) or without (curve c) protease K (86 nM) in 10 mM Hepes, pH 7.4, 150 mM NaCl at room temperature for 2 h, was tested using the recalcification time assay. Curve a: coagulation of citrated bovine plasma (triggered by addition of 25 μL of pre-warmed 40 mM $CaCl_2$, 150 mM NaCl) without inhibitor.

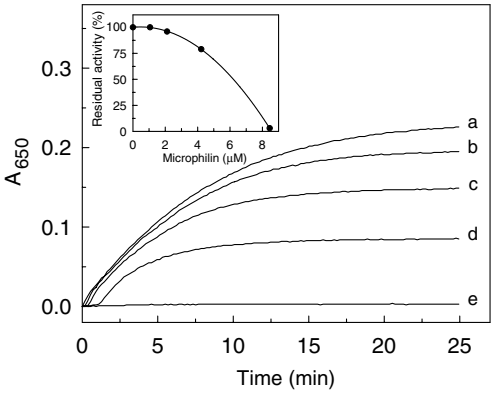

Fig. 4. Inhibition of fibrinogen-clot formation by microphilin. Thrombin (20 nM) was incubated with (a) 0 μM; (b) 1.06 μM; (c) 2.11 μM; (d) 4.22 μM; or (e) 8.45 μM of microphilin for 10 min at 37 °C prior to the addition of fibrinogen (200 μg). Fibrin-clot formation was followed by reading the absorbance at 650 nm. Inset: dose–response curve of fibrinogen-clotting inhibition by microphilin. Residual activity of thrombin was calculated as the initial velocity variation (in %), taking clotting in the absence of inhibitor as 100% thrombin activity.

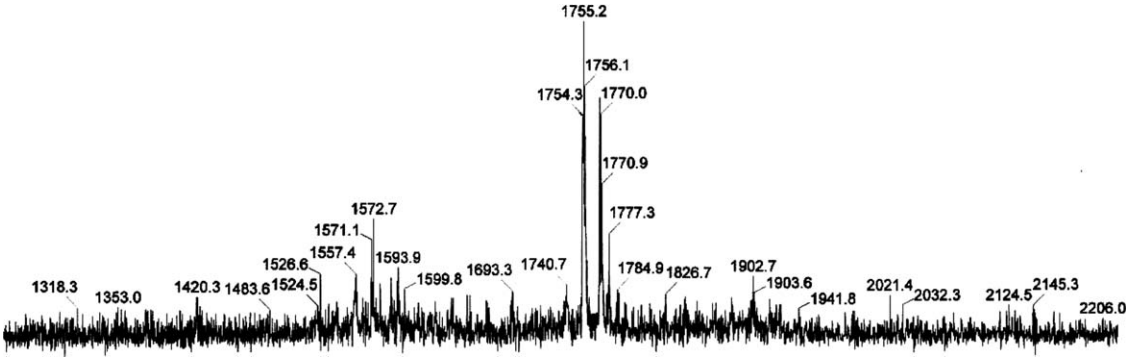

Fig. 2. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry of microphilin. The analysis was done in a Micromass TofSpec SE (Manchester, UK) using positive polarity, 20.000 V.

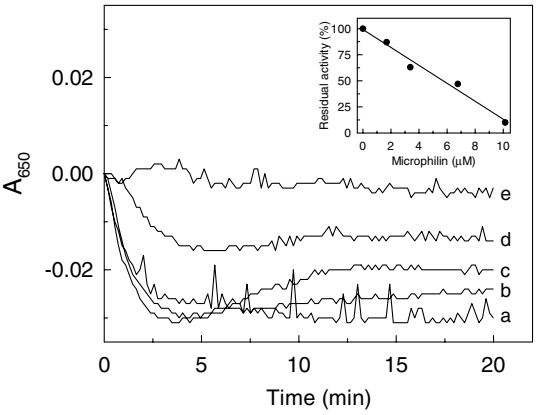

Fig. 5. Effect of microphilin on thrombin-induced platelet aggregation. Washed platelets (50 μL) were induced to aggregate with thrombin (0.5 nM) previously incubated for 10 min with (a) 0 μM; (b) 1.69 μM; (c) 3.38 μM; (d) 6.76 μM; or (e) 10.14 μM of microphilin in Tyrode buffer. Platelet aggregation was monitored by the decrease in absorbance at 650 nm. Inset: dose–response curve of thrombin-induced platelet aggregation inhibition by microphilin. Residual activity was calculated as the absorbance variation (in %) after a 800 s incubation, taking aggregation induced by thrombin in the absence of inhibitor as 100%.

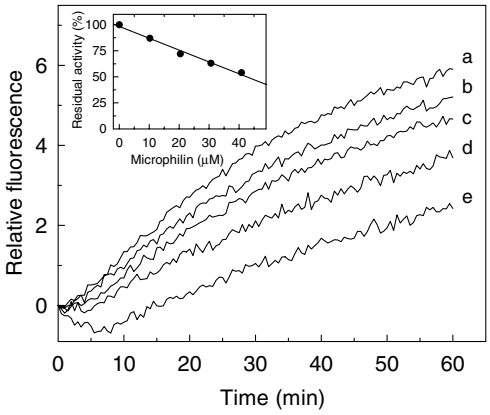

Fig. 7. Inhibition of thrombin activity with respect to a long fluorogenic substrate. Thrombin (4.8 nM) was incubated with (a) 0 μM; (b) 10.2 μM; (c) 20.4 μM; (d) 30.6 μM; or (e) 40.8 μM of microphilin, followed by addition of the fluorogenic substrate Abz-LDPRSFRLRNKNDKYEPF-WEDEENKQ-EDDnp (14 μM). Fluorescence measurements were performed in a FMax microplate reader; excitation and emission wavelengths were 320 and 430 nm, respectively. Inset: dose–response curve of thrombin inhibition by microphilin. Residual activity of thrombin was calculated as the initial velocity variation (in %), taking initial velocity in the absence of inhibitor as 100% thrombin activity.

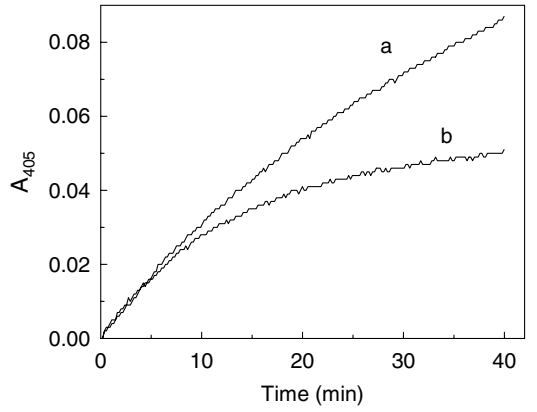

Fig. 6. Effect of microphilin on thrombin activity with respect to S2238. Thrombin (4.8 nM) was incubated with microphilin (a: 51 μM, b: without inhibitor) for 10 min at 37 °C, followed by addition of S2238 (300 μM). Substrate hydrolysis was measured at 405 nm.

inhibition was dose-dependent and the IC_{50} using this substrate was 42.3 μM (Fig. 7 inset).

4. Discussion

In this work, we report the purification and characterization of a small antithrombin inhibitor—microphilin—present in the saliva of the cattle tick *B. microplus*. Microphilin, when whole saliva was fractionated in size-exclusion chromatography, eluted from the column with an apparent molecular mass of ~40 kDa. However, the activity was able to pass through a 3000 MWCO membrane, indicating that it has a molecular mass lower than 3 kDa. Thus, microphilin migrates in the gel filtration column associated with a higher molecular weight protein. The small size of microphilin was confirmed by mass spectrometry. The activity purified by thrombin-affinity chromatography showed two components, corresponding to molecular masses of 1755 and 1770. Sensitivity to protease K showed that the inhibitor is a peptide. According to its molecular mass, microphilin is expected to contain between 10 (if all Trp) and 29 (if all Gly) amino acid residues. Microphilin is the smallest salivary thrombin inhibitor found so far. Comparing all species of hematophagous animals, thrombin inhibitors smaller than microphilin were observed only in the camel tick *Hyalomma dromedarii* (Ibrahim et al., 1998). Nevertheless, these small thrombin inhibitors were obtained from tick embryos, and the presence of such molecules in saliva remains to be determined. Other small anticoagulant peptides include the antithrombin inhibitors from saliva of the tsetse fly *Glossina morsitans* (3.5 kDa; Cappello et al., 1996) and from salivary glands of the mosquito *Anopheles albimanus* (anophelin, 6.5 kDa; Valenzuela et al., 1999).

Microphilin inhibited thrombin activity with respect to its natural substrates, namely fibrinogen and thrombin receptors on platelets, but was unable to inhibit the hydrolysis by thrombin of a small synthetic substrate (S-2238). In fact, microphilin slightly favored S-2238 hydrolysis by thrombin (Fig. 6). Indeed, it is well known that the binding of an inhibitor to exosite I can allosterically increase the activity of some enzymes of the coagulation cascade upon small synthetic peptides (Francischetti et al., 1997; Monteiro, 2005). These results clearly indicate that microphilin interacts with thrombin via a site distinct from the active site. This hypothesis was confirmed when we tested a longer synthetic substrate, designed to interact with both active site and exosite I (R. Zingali, personal communication), whose hydrolysis by thrombin was inhibited by microphilin. As microphilin does not inhibit the hydrolysis of small

substrates, which bind only to the active site, the possibility that microphilin inhibitory action comes from its interaction with the heparin-binding site can be eliminated, because in this case the activity upon small substrates would also be inhibited. We therefore concluded that the thrombin site blocked by microphilin is exosite I, which is crucial for interaction of both fibrinogen and platelet thrombin receptors with thrombin, while the active site remains fully accessible to tripeptide chromogenic substrates. Hirulog, a peptide derived from the hirudin C-terminal region, which is highly effective in inhibiting the hydrolytic activity of thrombin upon its natural substrates, also inhibits thrombin by association with exosite I (Maraganore et al., 1990; Naski et al., 1990). Anticoagulants with a similar mode of action are calcaratin, from the tick *Boophilus calcaratus* (Motoyashiki et al., 2003), triabin, from the bug *Triatoma pallidipennis* (Noeske-Jungblut et al., 1995), and madanins, from the tick *Haemaphysalis longicornis* (Iwanaga et al., 2003). Microphilin could also bind to thrombin's heparin-binding site (exosite II), which, similar to exosite I, is also positively charged.

Since exosite I, with which microphilin interacts, is highly positively charged, we propose that microphilin would be rich in acidic residues. Elution of microphilin from the thrombin–Sepharose column at high salt concentrations (which disrupts eletrostatic interactions) is consistent with this proposal. A highly charged small peptide would also explain our observations that the inhibitor tended to interact strongly with DEAE chromatographic resins. Unfortunately, due to the limited quantities of saliva, we do not have sufficient quantities of microphilin to obtain a clear result from amino acid sequencing. Despite that, determination of microphilin amino acid sequence is mandatory and new attempts are currently under way.

Similarly to BmAP (Horn et al., 2000), microphilin has an IC_{50} within the micromolar range and has to be in molar excess to inhibit thrombin. Therefore, like BmAP, microphilin is not a tight-binding inhibitor, in contrast to the inhibition kinetics exhibited by some anticoagulants from other hematophagous animals (Friedrich et al., 1993; Noeske-Jungblut et al., 1995). As *B. microplus* crude saliva is very effective in inhibiting coagulation of plasma (5 μL of saliva abolished the coagulation of 20 μL of bovine plasma for more than 60 min) (Horn et al., 2000), an interesting possibility is that both thrombin inhibitors act cooperatively and this should be investigated.

Both BmAP and microphilin target thrombin, and the high anticoagulant activity of *B. microplus* saliva can result from the action of these two inhibitors. Besides microphilin and BmAP, *B. microplus* saliva may contain inhibitors of other coagulation factors, such as inhibitors of the tenase or the prothrombinase complexes. Saliva from hematophagous animals often contain more than one antihemostatic substance, targeted to different coagulation factors and/or to agonists of platelet aggregation. In conclusion, *B. microplus* saliva has, against the same target, two apparently very distinct inhibitors, the 60 kDa BmAP, that blocks thrombin active site, and the 1.7 kDa microphilin, that blocks thrombin exosite I.

Acknowledgments

We would like to thank Maria Aparecida Juliano for the mass spectrometry analysis and Luiz Juliano for supplying us with fluorogenic substrate. We acknowledge the financial support from FAPERGS, CAPES, CNPq, and PRONEX/FAPERJ. A.C. was recipient of a CNPq Master Degree scholarship.

References

Andersen, J.F., Gudderra, N.P., Francischetti, I.M., Valenzuela, J.G., Ribeiro, J.M., 2004. Recognition of anionic phospholipid membranes by an antihemostatic protein from a blood-feeding insect. Biochemistry 43, 6987–6994.

Cappello, M., Bergum, P.W., Vlasuk, G.P., Furmidge, B.A., Pritchard, D.I., Aksoy, S., 1996. Isolation and characterization of the tsetse thrombin inhibitor: a potent antithrombotic peptide from the saliva of *Glossina morsitans morsitans* [published erratum appears in American Journal of Tropical Medicine and Hygiene 1996 Jul;55(1):118]. American Journal of Tropical Medicine and Hygiene 54, 475–480.

Ehebauer, M.T., Mans, B.J., Gaspar, A.R., Neitz, A.W., 2002. Identification of extrinsic blood coagulation pathway inhibitors from the tick *Ornithodoros savignyi* (Acari: Argasidae). Experimental Parasitology 101, 138–148.

Francischetti, I.M., Monteiro, R.Q., Guimaraes, J.A., Francischetti, B., 1997. Identification of glycyrrhizin as a thrombin inhibitor [published erratum appears in Biochemical and Biophysical Research Communications 1997 Aug 8;237(1):203]. Biochemical and Biophysical Research Communications 235, 259–263.

Francischetti, I.M., Valenzuela, J.G., Andersen, J.F., Mather, T.N., Ribeiro, J.M., 2002. Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, *Ixodes scapularis*: identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. Blood 99, 3602–3612.

Friedrich, T., Kroger, B., Bialojan, S., Lemaire, H.G., Hoffken, H.W., Reuschenbach, P., Otte, M., Dodt, J., 1993. A Kazal-type inhibitor with thrombin specificity from *Rhodnius prolixus*. Journal of Biological Chemistry 268, 16216–16222.

Hoffmann, A., Walsmann, P., Riesener, G., Paintz, M., Markwardt, F., 1991. Isolation and characterization of a thrombin inhibitor from the tick *Ixodes ricinus*. Pharmazie 46, 209–212.

Horn, F., dos Santos, P., Termignoni, C., 2000. *Boophilus microplus* anticoagulant protein: an antithrombin inhibitor isolated from the cattle tick saliva. Archives of Biochemistry and Biophysics 384, 68–73.

Ibrahim, M.A., Ghazy, A-H.M., Maharem, T.M., Khalil, M.I., 1998. Purification and characterization of peptide anticoagulants from the embryos of the camel tick; *Hyalomma dromedarii* (Acari: Ixodidae). Journal of the Egyptian German Society of Zoology 27, 287–305.

Ibrahim, M.A., Ghazy, A.H., Maharem, T., Khalil, M., 2001. Isolation and properties of two forms of thrombin inhibitor from the nymphs of the camel tick *Hyalomma dromedarii* (Acari: Ixodidae). Experimental and Applied Acarology 25, 675–698.

Iwanaga, S., Okada, M., Isawa, H., Morita, A., Yuda, M., Chinzei, Y., 2003. Identification and characterization of novel salivary thrombin inhibitors from the ixodidae tick, *Haemaphysalis longicornis*. European Journal of Biochemistry 270, 1926–1934.

Joubert, A.M., Crause, J.C., Gaspar, A.R., Clarke, F.C., Spickett, A.M., Neitz, A.W., 1995. Isolation and characterization of an anticoagulant present in the salivary glands of the bont-legged tick, *Hyalomma truncatum*. Experimental and Applied Acarology 19, 79–92.

Kazimirova, M., Jancinova, V., Petrikova, M., Takac, P., Labuda, M., Nosal, R., 2002. An inhibitor of thrombin-stimulated blood platelet aggregation from the salivary glands of the hard tick *Amblyomma variegatum* (Acari: Ixodidae). Experimental and Applied Acarology 28, 97–105.

Lai, R., Takeuchi, H., Jonczy, J., Rees, H.H., Turner, P.C., 2004. A thrombin inhibitor from the ixodid tick, *Amblyomma hebraeum*. Gene 342, 243–249.

Limo, M.K., Voigt, W.P., Tumbo-Oeri, A.G., Njogu, R.M., Ole-MoiYoi, O.K., 1991. Purification and characterization of an anticoagulant from the salivary glands of the ixodid tick *Rhipicephalus appendiculatus*. Experimental Parasitology 72, 418–429.

Mans, B.J., Gaspar, A.R., Louw, A.I., Neitz, A.W., 1998. Apyrase activity and platelet aggregation inhibitors in the tick *Ornithodoros savignyi* (Acari: Argasidae). Experimental and Applied Acarology 22, 353–366.

Mans, B.J., Louw, A.I., Neitz, A.W., 2002a. Amino acid sequence and structure modeling of savignin, a thrombin inhibitor from the tick, *Ornithodoros savignyi*. Insect Biochemistry and Molecular Biology 32, 821–828.

Mans, B.J., Louw, A.I., Neitz, A.W., 2002b. Savignygrin, a platelet aggregation inhibitor from the soft tick *Ornithodoros savignyi*, presents the RGD integrin recognition motif on the Kunitz-BPTI fold. Journal of Biological Chemistry 277, 21371–21378.

Mans, B.J., Neitz, A.W., 2004. Adaptation of ticks to a blood-feeding environment: evolution from a functional perspective. Insect Biochemistry and Molecular Biology 34, 1–17.

Maraganore, J.M., Bourdon, P., Jablonski, J., Ramachandran, K.L., Fenton, J.W., 1990. Design and characterization of hirulogs: a novel class of bivalent peptide inhibitors of thrombin. Biochemistry 29, 7095–7101.

Monteiro, R.Q., 2005. Targeting exosites on blood coagulation proteases. Anais da Acadamia Brasileira Ciencias 77, 275–280.

Motoyashiki, T., Tu, A.T., Azimov, D.A., Ibragim, K., 2003. Isolation of anticoagulant from the venom of tick, *Boophilus calcaratus*, from Uzbekistan. Thrombosis Research 110, 235–241.

Naski, M.C., Fenton, J.W., Maraganore, J.M., Olson, S.T., Shafer, J.A., 1990. The COOH-terminal domain of hirudin. An exosite-directed competitive inhibitor of the action of alpha-thrombin on fibrinogen. Journal of Biological Chemistry 265, 13484–13489.

Nienaber, J., Gaspar, A.R., Neitz, A.W., 1999. Savignin, a potent thrombin inhibitor isolated from the salivary glands of the tick *Ornithodoros savignyi* (Acari: Argasidae). Experimental Parasitology 93, 82–91.

Noeske-Jungblut, C., Haendler, B., Donner, P., Alagon, A., Possani, L., Schleuning, W.D., 1995. Triabin, a highly potent exosite inhibitor of thrombin. Journal of Biological Chemistry 270, 28629–28634.

Ribeiro, J.M., Endris, T.M., Endris, R., 1991. Saliva of the soft tick, *Ornithodoros moubata*, contains anti-platelet and apyrase activities. Comparative Biochemistry and Physiology A 100, 109–112.

Ribeiro, J.M., Francischetti, I.M., 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. Annual Review of Entomology 48, 73–88.

Rydel, T.J., Tulinsky, A., Bode, W., Huber, R., 1991. Refined structure of the hirudin–thrombin complex. Journal of Molecular Biology 221, 583–601.

Valenzuela, J.G., Francischetti, I.M., Ribeiro, J.M., 1999. Purification, cloning, and synthesis of a novel salivary anti-thrombin from the mosquito *Anopheles albimanus*. Biochemistry 38, 11209–11215.

Waxman, L., Smith, D.E., Arcuri, K.E., Vlasuk, G.P., 1990. Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. Science 248, 593–596.

Zhu, K., Bowman, A.S., Brigham, D.L., Essenberg, R.C., Dillwith, J.W., Sauer, J.R., 1997. Isolation and characterization of americanin, a specific inhibitor of thrombin, from the salivary glands of the lone star tick *Amblyomma americanum* (L.). Experimental Parasitology 87, 30–38.