



## Expression and functional characterization of boophilin, a thrombin inhibitor from *Rhipicephalus (Boophilus) microplus* midgut

Tatiane Sanches Soares<sup>a</sup>, Renata Midori Okuta Watanabe<sup>a</sup>, Anita Mitico Tanaka-Azevedo<sup>b</sup>, Ricardo José Soares Torquato<sup>a</sup>, Stephen Lu<sup>a</sup>, Ana Carvalho Figueiredo<sup>c</sup>, Pedro José Barbosa Pereira<sup>c</sup>, Aparecida S. Tanaka<sup>a,\*</sup>

<sup>a</sup> Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua 3 de Maio 100, 04044-020 São Paulo, SP, Brazil

<sup>b</sup> Laboratório de Fisiopatologia, Instituto Butantan, São Paulo, SP, Brazil

<sup>c</sup> IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, 4150-180 Porto, Portugal

### ARTICLE INFO

#### Article history:

Received 17 November 2011

Received in revised form 17 January 2012

Accepted 22 January 2012

#### Keywords:

Kunitz-type inhibitor

Tick

Anticoagulant

Elastase inhibitor

RNAi silencing

### ABSTRACT

*Rhipicephalus (Boophilus) microplus* is an ectoparasite responsible for an important decrease in meat, milk and leather production, caused both by cattle blood loss and by the transmission of anaplasmosis and babesiosis. *R. microplus* is a rich source of serine protease inhibitors, including the trypsin inhibitors BmTI-A and BmTI-6, the subtilisin inhibitor BmSI, and the recently described thrombin inhibitor, boophilin. Boophilin is a double Kunitz-type thrombin inhibitor, with the unusual ability to form a ternary complex with a second (non-thrombin) serine proteinase molecule. The large-scale expression and purification of boophilin and of its isolated N-terminal (D1) domain in *Pichia pastoris*, its expression profile, and the effect of RNAi-mediated gene silencing in tick egg production are reported. Full-length boophilin and D1 were expressed at 21 and 37.5 mg/L of culture, respectively. Purified boophilin inhibited trypsin ( $K_i$  0.65 nM), neutrophil elastase ( $K_i$  21 nM) and bovine thrombin ( $K_i$  57 pM), while D1 inhibited trypsin and neutrophil elastase ( $K_i$  of 2.0 and 129 nM, respectively), but not thrombin. Boophilin gene silencing using RNAi resulted in 20% reduction in egg weight production, suggesting that the expression of boophilin in this life stage would be important but not vital, probably due to functional overlap with other serine proteinase inhibitors in the midgut of *R. microplus*. Considering our data, boophilin could be combining with other antigen in a vaccine production for tick control.

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### 1. Introduction

The cattle tick *Rhipicephalus (Boophilus) microplus* is widely distributed in tropical and subtropical regions, being responsible for the transmission of the causative agents of babesiosis and anaplasmosis, with a significant

economic impact in cattle production by reducing weight gain and milk production (Sonenshine, 1991).

Blood sucking animals produce a considerable number of active molecules in their salivary glands (e.g. anticoagulants, vasodilators and platelet aggregation inhibitors) that interfere with homeostasis in their vertebrate hosts. In particular, these haematophagous parasites vitally depend on blocking the blood coagulation cascade in order to facilitate the acquisition and digestion of their blood meal (Ribeiro, 1995). Thrombin (or coagulation factor IIa) plays a vital role in blood clotting by promoting platelet aggregation and by converting fibrinogen to fibrin at the end of the pathway (Davie et al., 1991). Thrombin is a serine protease, which

**Abbreviations:** D1, boophilin domain 1; D2, boophilin domain 2; Boophilin, full-length boophilin (domains 1–2).

\* Corresponding author. Tel.: +55 1155764445; fax: +55 1155723006.

E-mail address: [tanaka.bioq@epm.br](mailto:tanaka.bioq@epm.br) (A.S. Tanaka).

contains two functionally important structural features, besides the active site: the surface areas enriched in basic residues known as exosite I (Bode et al., 1992) and exosite II (Arni et al., 1994; Sheehan et al., 1993). As thrombin has key roles in the intrinsic and extrinsic pathways of blood coagulation, thrombin inhibitors are the most often identified anticoagulant molecules in blood sucking organisms (Francischetti et al., 2008).

Among blood sucking animals, ticks are rich sources of serine protease inhibitors, many of them belonging to the BPTI-Kunitz family (Azzolini et al., 2003; Mans et al., 2008; Sasaki et al., 2004), such as BmTIs (*Boophilus microplus* trypsin inhibitor) from larvae and eggs, which target trypsin, chymotrypsin, neutrophil elastase, plasma kallikrein and plasmin (Andreotti et al., 2001, 2002; Sasaki et al., 2004; Sasaki and Tanaka, 2008; Tanaka et al., 1999); the tick anticoagulant peptide (TAP), a factor Xa inhibitor from *Ornithodoros moubata* (Waxman et al., 1990); and the thrombin inhibitors ornithodorin (from *O. moubata* (van de et al., 1996)), savignin (from *Ornithodoros savignyi* (Mans et al., 2002b)), monobin (from *Argas monolakensis* (Mans and Ribeiro, 2008)) and boophilin (from *R. microplus* (Macedo-Ribeiro et al., 2008)). Boophilin is a doubled-headed Kunitz inhibitor displaying a P1 Lys residue at the canonical reactive loop of its N-terminal Kunitz domain. However, boophilin inhibits thrombin in a non-canonical manner, inserting its N-terminal segment into thrombin's active site, while its C-terminal Kunitz domain binds to the exosite I of the protease (Macedo-Ribeiro et al., 2008).

Given the important role of Kunitz-type inhibitors in the *R. microplus* life cycle and the high specificity of boophilin for thrombin, we expressed and purified full-length mature boophilin and its N-terminal Kunitz domain in large scale using a *Pichia pastoris* system. We also profiled boophilin gene expression and evaluated the effect of RNAi gene silencing in tick egg production.

## 2. Materials and methods

### 2.1. Animals

*R. (Boophilus) microplus* (*Babesia* spp.-free) ticks were supplied by Dr. Itabajara da Silva Vaz Junior (Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, RS, Brazil).

### 2.2. Materials

The pPICZαB vector and *P. pastoris* strain KM71H were purchased from Invitrogen (Carlsbad, CA, USA) and used following the supplier's instructions. DNA sequencing was performed using the BigDye Terminator V3.1 Cycle Sequencing Kit on an ABI 377 or ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA). The substrates S2484 (Pyro-Glu-Pro-Val-pNa) and S2238 (HD-Phe-Pip-Arg-pNa), were purchased from Chromogenix (Molndal, Sweden) and tosyl-Gly-Pro-Arg-pNA from Sigma (Darmstadt, Germany). Bovine trypsin (EC 3.4.21.4) and bovine thrombin (EC 3.4.21.5) were obtained from Sigma (St. Louis,

MO, USA) and human neutrophil elastase (EC 3.4.21.37) from Calbiochem (San Diego, CA, USA).

### 2.3. Quantitative Real-Time PCR

RNA from ovary, fat body, salivary gland, gut, and hemo-cytes of engorged *R. microplus* adult females was extracted using Tryzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The cDNAs were synthesized using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA). Quantitative PCR was performed using two specific primers designed based on the boophilin sequence with GenBank accession number AJ304446: *Boophilinfw* (5'-CAG AGA AAT GGA TTC TGC CGA CTG CCG GCA-3') and *Boophilinrev* (5'-ACA CTC CTC TAT GGT CTC GAA-3'). *R. microplus* elongation factor 1-alpha (ELF1a) specific primers – ELF1afw (5'-CGT CTA CAA GAT TGG TGG CAT T-3') and ELF1arv (5'-CTC AGT GGT CAG GTT GGC AG-3') – were used for DNA amplification control. Boophilin and ELF1a (endogenous control) were quantified using SYBR® Green PCR Master Mix in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the following conditions: 40 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. For each tissue, 50 ng of cDNA were used as template. All qPCR runs were conducted in triplicate, in three independent experiments. The amount of each mRNA was calculated according to the 2- $\Delta\Delta C_t$  method (Livak and Schmittgen, 2001). ANOVA ( $p < 0.05$ ) and the Tukey test were used in the statistical analysis.

### 2.4. Cloning of boophilin and D1 into pPICZαB

The DNA fragments encoding boophilin or D1 were amplified by PCR using a midgut cDNA preparation and the primer set *Boophilfw1std* (5'-GTA TCT CTC GAG AAA AGA CAG AGA AAT GGA TTC TGC CGA CTG CCG G-3') and *Boophilrv2ndd* (5'-CGA ATT AAT TCG CGG CCG CCT ACA TGT TCT TGC AGA CGA GTT CAC AC-3') for boophilin and *Boophilfw1std* and *Boophilrv1std* (5'-CGA ATT AAT TCG CGG CCG CCT AAG CTC CGC ACG CCT TTT GAC AAT C-3') for D1. PCR reactions were conducted in a final volume of 50 μL in 100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% (v/v) Nonidet P40, 1.5 mM MgCl<sub>2</sub>, 100 μM dNTPs, 10 pM of each primer, 5 U Taq DNA polymerase with the following parameters: 94 °C for 2 min, prior to 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min followed by 5 min at 72 °C. Boophilin and D1 DNA fragment amplification products were separated by agarose gel electrophoresis and purified using the QIAEX II gel extraction system (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified DNA fragments were digested with *Xho*I and *Not*I restriction enzymes, and ligated into the pPICZαB vector, previously digested with the same enzymes, generating the constructions *Boophilin*-pPICZαB and *D1*-pPICZαB, which were verified by automated DNA sequencing.

### 2.5. Preparation and transformation of *P. pastoris* yeast

*P. pastoris* KM71H strain was transformed with 10 μg of *Sac*I-linearized *Boophilin*-pPICZαB or *D1*-pPICZαB by

electroporation in a Gene Pulser (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The electro-porated cells were immediately suspended in 1.0 mL of ice-cold 1.0 M sorbitol and spread on MD agar plates (1.34% yeast nitrogen base (YNB), 2% dextrose,  $4 \times 10^{-5}$ % biotin) without histidine. The target gene was detected in the recombinant *P. pastoris* by PCR using 3'AOX and 5'AOX primers (Invitrogen, Carlsbad, CA, USA). Clones that were homologous recombinants with the AOX I sequence were selected.

## 2.6. Expression of recombinant Boophilin and D1

To identify positive yeast clones expressing each of the inhibitors, six isolated *P. pastoris* KM71H strains carrying the *boophilin* or *D1* gene fragment, identified by PCR, were individually inoculated in 2.5 mL BMGY medium (1.0% (w/v) yeast extract, 2.0% (w/v) peptone in 100 mM potassium phosphate buffer pH 6.0, 1.34% (w/v) YNB,  $4 \times 10^{-5}$ % (w/v) biotin and 1% (v/v) glycerol) in a 50 mL sterile tube, and grown at 30 °C for 28 h at 250 rpm. The yeast cells were harvested by centrifugation at  $3000 \times g$  for 5 min at 4 °C and resuspended in BMMY (BMGY with glycerol replaced by 0.5% (v/v) methanol) medium to an absorbance of 1.0 at 600 nm. Expression took place at 30 °C with shaking at 250 rpm for 4 days, with addition of 0.5% (v/v) methanol every 24 h. After removing the cells by centrifugation ( $4000 \times g$  for 20 min at 4 °C), the inhibitory activity of the supernatants against bovine thrombin or bovine trypsin was assessed in activity assays using a chromogenic substrate (S2238 or tosyl-Gly-Pro-Arg-pNA). Individual clones with high expression levels for boophilin or D1 were selected (data not shown).

A single *P. pastoris* colony (Mut+) expressing high levels of boophilin or D1 was selected and used to inoculate 120 mL BMGY medium in a 1 L sterile flask, and incubated at 30 °C and 250 rpm for 24 h. Expression was performed as described above and the culture supernatant was stored at 4 °C prior to purification.

## 2.7. Purification of recombinant Boophilin and D1

Recombinant boophilin or D1-containing yeast culture supernatant was loaded onto an affinity trypsin-Sepharose column previously equilibrated with 50 mM Tris-HCl buffer pH 8.0 (buffer A). Weakly bound proteins were washed out with buffer A supplemented with 0.15 M NaCl. The bound material was eluted with 0.5 M KCl pH 2.0 and the collected fractions were immediately neutralized with 1 M Tris-HCl buffer pH 8.0. Absorbance at 280 nm was also monitored. The inhibitory activity of the fractions was analyzed in protease activity assays (see below). The fractions containing inhibitory activity and displaying one main protein band in SDS-PAGE were pooled and concentrated using a 5000 MWCO membrane (Millipore, Billerica, MA, USA).

## 2.8. Determination of dissociation constant for different serine proteases

The concentration of active trypsin was determined by active site titration with *p*-nitrophenyl-*p'*-guanidino-benzoate as previously described (Chase and Shaw,

1969). The equilibrium dissociation constants of complexes formed by boophilin or D1 with bovine trypsin or neutrophil elastase were determined using the method described by Bieth (1980). Briefly, the serine proteases were incubated at 37 °C with different concentrations of inhibitors in 0.1 M Tris-HCl buffer pH 8.0 containing 0.15 M NaCl and 0.1% Triton X-100. The residual enzyme activity was measured after the addition of the chromogenic substrate tosyl-Gly-Pro-Arg-pNA or elastase substrate I (MeOSuc-Ala-Ala-Pro-Val-pNA) for trypsin and neutrophil elastase, respectively. Apparent  $K_i$  values were calculated by fitting the steady-state velocities to the equation  $(V_i/V_0 = 1 - \{E_t + I_t + K_i - [(E_t + I_t + K_i)^2 - 4E_tI_t]^{1/2}\}/2E_t)$  for tight-binding inhibitors and using a non-linear regression analysis (Morrison, 1969).

## 2.9. Thrombin inhibition assays

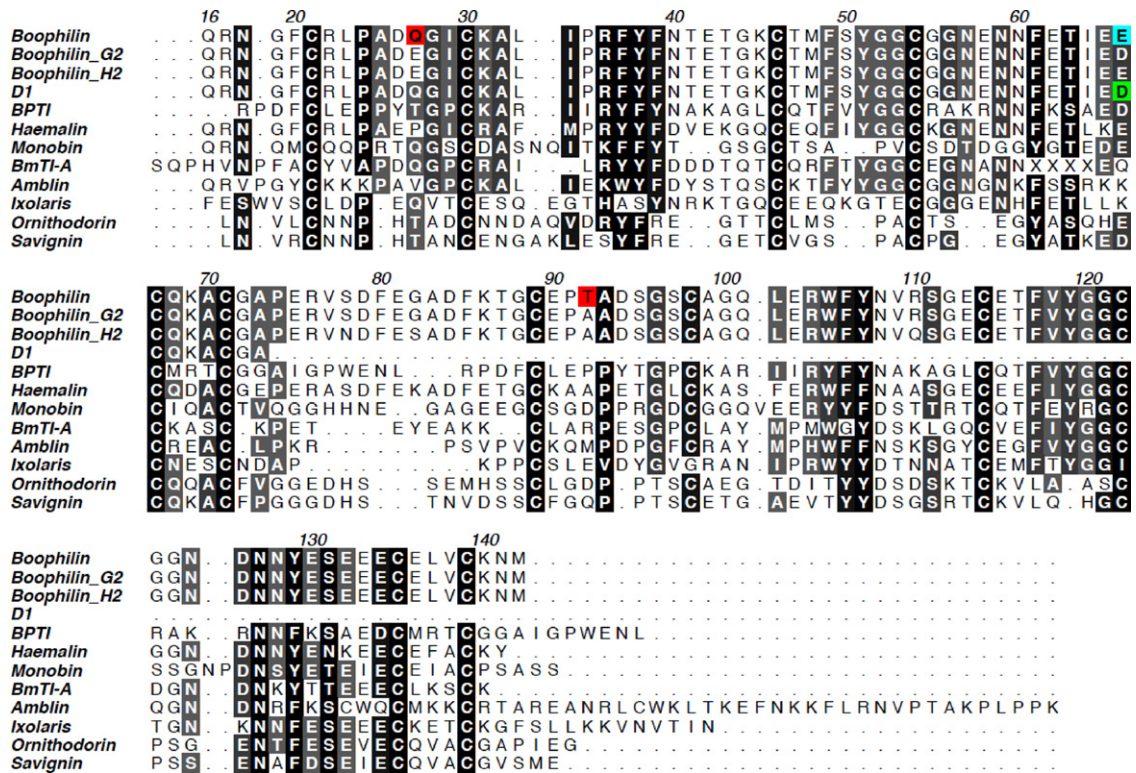
Boophilin (1.2 and 2.4  $\mu$ M) was pre-incubated with  $\alpha$ -thrombin (0.025 U) or  $\gamma$ -thrombin (1  $\mu$ g) for 10 min at 37 °C in 100 mM Tris-HCl buffer pH 8.0 containing 150 mM NaCl and 0.1% Triton X-100. The residual thrombin activity against the fluorogenic substrate Benzoyl-Phe-Val-Arg-AMC (200  $\mu$ M) was measured, after incubation in the same conditions for 20 min. The fluorescence was monitored at  $\lambda_{em} = 460$  nm and  $\lambda_{ex} = 380$  nm in a Synergy HT microplate reader (BioTek, Winooski, VT, USA) for 20 min. As a control, the same assay was performed in the absence of boophilin.

## 2.10. RNA interference

A boophilin fragment conjugated with the T7 promoter region was amplified by PCR using primers BoophRNAifw (5'-GGA TCC TAA TAC GAC TCA CTA TAG GCA GAG AAA TGG ATT CTG CCG AC-3') and BoophRNAirv (5'-GGA TCC TAA TAC GAC TCA CTA TAG TCA TGT TCT TGC AGA CGA GTT CAC-3'). PCR products were purified with the QIAEX II kit (QIAGEN, Hilden, Germany) and used as template for double-stranded RNA (dsRNA) synthesis using the T7 Ribomax<sup>TM</sup> Express RNAi system (Promega, Madison, WI, USA). The dsRNA was digested with DNase and RNase, precipitated with isopropanol, resuspended in sterile PBS, and quantified by measuring its absorbance at 260 nm. Engorged *R. microplus* females (35 individuals) were injected with 2  $\mu$ L of dsRNA-boophilin (3.5  $\mu$ g), using an insulin syringe. An identical control group was injected with 2  $\mu$ L of PBS buffer, and a third group was not injected. After dsRNA injection, all groups were kept at 22–25 °C and 95% humidity for 24 h, after which ten ticks of each group were dissected and their guts placed in Trizol reagent (Invitrogen, Carlsbad, CA, USA) for subsequent RNA extraction. Eggs of 25 ticks were collected 24 and 48 h after injection and weighed.

## 2.11. Transcription analysis of boophilin gene expression in tick gut by PCR

cDNA from *R. microplus* engorged adult female gut was prepared from all silencing gene expression experimental groups using the ImProm-II<sup>TM</sup> Reverse Transcription System (Promega, Madison, WI, USA). The sequence encoding



**Fig. 1.** Amino acid sequence alignment of boophilin with several Kunitz-type inhibitors. Full length boophilin and D1 (this work) are compared to boophilin variants G2 (UniProt entry Q8WPI3) and H2 (UniProt entry Q8WPI2), the thrombin inhibitors haemalin (UniProt entry B6ZIW0) from *Haemaphysalis longicornis* (Liao et al., 2009), monobin (UniProt entry Q09JW6) from *Argas monolakensis* (Mans et al., 2008), amblin (UniProt entry Q5VJG2) from *Amblyomma hebraeum* (Lai et al., 2004), ornithodorin (UniProt entry P56409) from *Ornithodoros moubata* (van de et al., 1996), and savignin (UniProt entry Q8WPG5) from *Ornithodoros savignyi* (Mans et al., 2002a). For comparison purposes, two molecules of BPTI (UniProt entry P00974), the bovine basic trypsin inhibitor, are also included. Invariant residues are displayed with a black background, while conserved amino acids are shaded gray. The residues of boophilin distinct from both G2 and H2 variants are highlighted in red and those where it is identical only to H2 are marked in blue. The single position where the D1 sequence differs from that of boophilin is highlighted in green. The numbering above the alignment corresponds to that of mature boophilin (Macedo-Ribeiro et al., 2008). Figure prepared with ALINE (Bond and Schuttelkopf, 2009). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

boophilin was then amplified by PCR using cDNAs as template and the specific primers *Boophilinfw* (5'-CAG AGA AAT GGA TTC TGC CGA CTG CCG GCA-3') and *Boophilinrev* (5'-ACA CTC CTC TAT GGT CTC GAA-3').

The PCR reaction (25  $\mu$ L) contained 1  $\mu$ L of cDNA sample, 25 pmol of each primer, 100  $\mu$ M dNTPs, 1.5 mM MgCl<sub>2</sub>, and 2.5 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and was performed with the following parameters: 94 °C for 5 min, 25 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min, followed by 72 °C for 5 min. For DNA amplification control a similar reaction was performed using 25 pmol of *R. microplus* elongation factor 1-alpha (ELF1a) specific primers: ELF1afw (5'-CGT CTA CAA GAT TGG TGG CAT T-3') and ELF1arv (5'-CTC AGT GGT CAG GTT GGC AG-3').

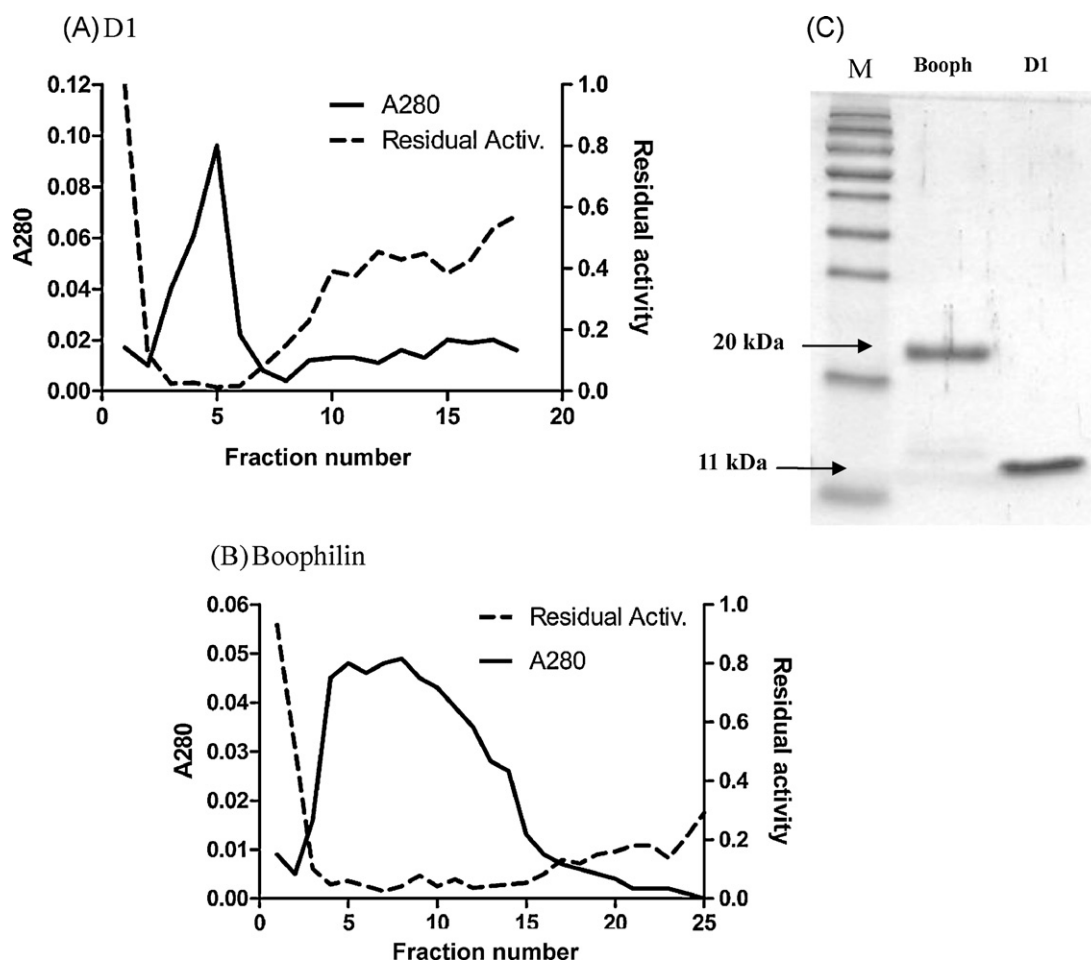
### 3. Results

#### 3.1. Cloning, expression in *P. pastoris* and characterization of recombinant boophilin

A specific tandem Kunitz domain thrombin inhibitor from *R. microplus*, named boophilin, was previously

described (Macedo-Ribeiro et al., 2008). In an attempt to produce large amounts of recombinant boophilin, the DNA fragment coding for the full-length inhibitor or for its N-terminal domain (D1) were amplified by PCR using specific oligonucleotides based on the sequence of boophilin variant G2 (EMBL accession code AJ304446.1) and cloned into the *P. pastoris* pPICZ $\alpha$ B expression vector. Positive clones for boophilin and D1 were confirmed by automated DNA sequencing and used to transform *P. pastoris* yeast. The sequence of cloned boophilin differed from that of boophilin variants G2 (EMBL accession code AJ304446.1) and H2 (EMBL accession code AJ304447.1), being closest to the former (Fig. 1). The cloned mature boophilin was distinct from both previously described variants at positions 27 (Gln instead of Glu) and 92 (Thr instead of Ala), and identical to variant H2 at position 66, while D1 was identical to variant G2 at this position. All these amino acid exchanges occur on the solvent-exposed face of the inhibitor on its complex with thrombin (Macedo-Ribeiro et al., 2008) and are therefore unlikely to affect its anticoagulant activity.

Full-length boophilin and D1 were expressed in *P. pastoris* at high levels (21 and 37.5 mg/L, respectively) and purified by affinity chromatography on trypsin-Sepharose



**Fig. 2.** Purification of boophilin and D1 expressed in *P. pastoris* yeast. (A) Affinity chromatography of D1 on a trypsin-Sepharose column. Yeast supernatant containing recombinant D1 was applied onto a trypsin-Sepharose column previously equilibrated with 0.05 M Tris–HCl buffer pH 8.0. Bound material was eluted with 0.5 M KCl pH 2.0. (B) Affinity chromatography of boophilin on a trypsin-Sepharose column. Yeast supernatant containing recombinant boophilin was purified as described for D1. (C) SDS-PAGE (15%) analysis of purified recombinant proteins. M, protein molecular mass standard (Bio-Rad); Booph, full-length boophilin (2  $\mu$ g); D1, N-terminal domain of boophilin (2  $\mu$ g). Boophilin and D1 display apparent molecular masses of approximately 20 and 11 kDa, respectively.

(Fig. 2A and B). On SDS-PAGE, purified boophilin displayed an apparent molecular mass of 20 kDa and purified D1 of 11 kDa (Fig. 2C). The inhibitory activity of boophilin against thrombin, trypsin and neutrophil elastase was assessed, and the corresponding inhibition constants ( $K_i$ ) determined (Table 1). Purified boophilin showed high selectivity to thrombin with a  $K_i$  of 57 pM, a value significantly lower than the 1.80 nM reported for boophilin produced in *Escherichia coli* (Macedo-Ribeiro et al., 2008). The second

**Table 1**  
Inhibition constants ( $K_i$ ) for different serine proteases (nM).

	Bovine trypsin	Bovine thrombin	Human neutrophil elastase
D1	2.00	n.i.	128.90
Boophilin	0.65	0.057	21.22
Boophilin <sup>a</sup>	n.d.	1.80	n.d.

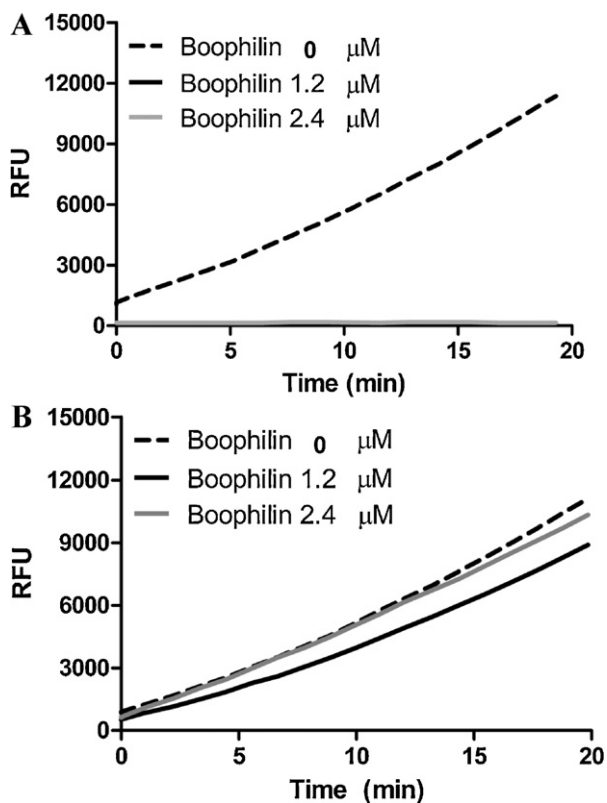
n.i., no inhibition; n.d., not determined.

<sup>a</sup> Macedo-Ribeiro et al. (2008).

Kunitz domain of boophilin displays an alanine residue at the reactive loop P1 position (Schechter and Berger, 1967), suggesting it could inhibit elastase. Both boophilin and D1 inhibited human neutrophil elastase *in vitro* with  $K_i$  values of 21 nM and 129 nM, respectively. Boophilin inhibits thrombin by binding simultaneously to the active site and the exosite 1 of the protease (Macedo-Ribeiro et al., 2008). The contribution of the interaction with the exosite 1 to the inhibitory activity of boophilin was probed by comparing its activity towards  $\alpha$ -thrombin and the exosite 1-less form,  $\gamma$ -thrombin (Fig. 3). Recombinant boophilin revealed no activity towards  $\gamma$ -thrombin, in amounts that completely abolished the amyolytic activity of  $\alpha$ -thrombin, therefore underscoring the importance of the interaction with the exosite 1.

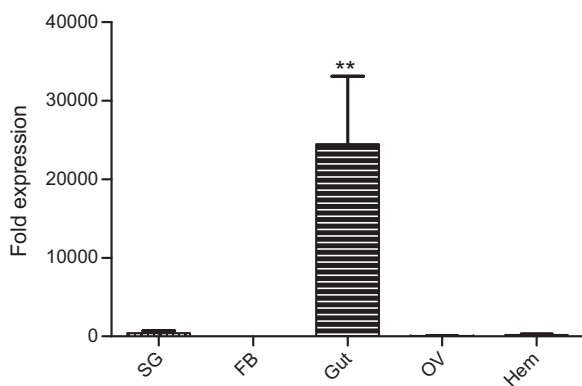
### 3.2. Boophilin gene expression analysis by qPCR

Different tissues of engorged *R. microplus* females were dissected and used for total RNA purification and cDNA



**Fig. 3.** Inhibition of alpha and gamma-thrombin by purified recombinant boophilin. (A) The residual activity of alpha (A) or gamma-thrombin (B) against the fluorogenic substrate Tosyl-Gly-Pro-Arg-AMC was determined in the presence of different amounts of recombinant boophilin.

synthesis (Fig. 4). Boophilin gene expression was mostly detected in the midgut (25,000 fold above other tissues) with minor expression levels in hemocytes, although a contamination with midgut cells during dissection cannot be discarded.



**Fig. 4.** Boophilin gene expression profile in different tissues of engorged adult *R. microplus* females by quantitative PCR. Gut (Gut), ovary (OV), hemocyte (Hem), salivary gland (SG) and fat body (FB) cDNA of fed ticks were used in qPCR experiments. All data were normalized to the expression level of ELF1a (elongation factor 1-alpha), used as an internal control. The expression level of boophilin in gut showed a significant increase when compared to other tissues (\*\* $p < 0.05$  in Tukey's Multiple Comparison Test). Error bars correspond to the standard error of the mean of three independent experiments.

### 3.3. Boophilin gene silencing using RNA interference

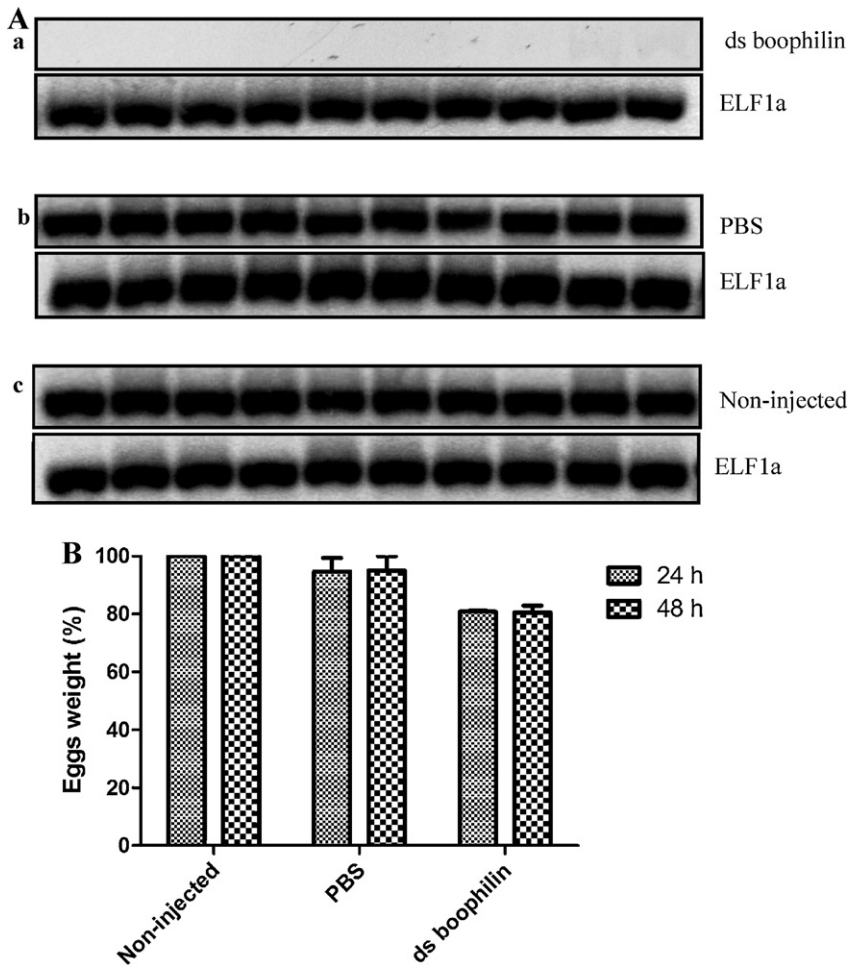
In an attempt to unveil boophilin's physiological role, a RNAi-mediated gene silencing experiment was performed. Three groups of ticks, each composed of 25 animals, were injected with either boophilin dsRNA, PBS buffer or left untreated. In comparison to the control animals, an efficient silencing of boophilin expression was achieved after boophilin dsRNA treatment (Fig. 5A). Boophilin down-regulation resulted in a decrease (~20% after 24 and 48 h) in egg production (Fig. 5B).

## 4. Discussion

Considering the important role of Kunitz-type inhibitors in the life cycle of *R. microplus* and the high specificity of the tandem Kunitz inhibitor boophilin for thrombin, full-length boophilin and its N-terminal Kunitz domain (D1) were expressed, purified and characterized. Furthermore, boophilin gene expression analysis and evaluation of the effect of RNAi silencing in egg production were also performed.

Active boophilin and D1 were efficiently expressed in *P. pastoris* and purified in a single step by affinity chromatography. Purified recombinant boophilin strongly inhibited thrombin, with a dissociation constant in the pM range. Moreover, it also displayed considerable activity against trypsin ( $K_i$  0.65 nM) and neutrophil elastase ( $K_i$  21 nM). As for purified recombinant D1, it displayed an inhibitory activity against trypsin similar to that of the full-length inhibitor ( $K_i$  2 nM), and also inhibited neutrophil elastase, although with a significantly decreased efficiency ( $K_i$  0.129  $\mu$ M), suggesting a significant contribution from the C-terminal Kunitz domain to this interaction, compatible with the presence of an alanine residue in the reactive loop P1 position. The three-dimensional structure of the thrombin-boophilin complex revealed a bidentate interaction of boophilin with the active site and the exosite I of  $\alpha$ -thrombin. The N-terminal region of the inhibitor binds to and blocks the active site of thrombin while the negatively charged C-terminal Kunitz domain of boophilin docks into the basic exosite I (Macedo-Ribeiro et al., 2008). As expected from the thrombin-boophilin complex architecture, isolated D1 does not display inhibitory activity against thrombin, confirming the fundamental contribution of the C-terminal domain-mediated interaction for thrombin inhibition. Further highlighting the importance of the exosite I for thrombin inhibition, boophilin inhibited strongly  $\alpha$ -thrombin *in vitro* but was unable to inhibit the exosite I-disrupted form of the enzyme,  $\gamma$ -thrombin.

In contrast to other previously described natural thrombin inhibitors from blood-sucking animals, boophilin may also target additional serine proteases such as trypsin and plasmin (Macedo-Ribeiro et al., 2008). The observed activity of boophilin against neutrophil elastase corroborated this hypothesis, suggesting a role other than counteracting blood coagulation in the midgut of *R. microplus*. Blood is a complex mixture of numerous soluble proteins, including plasmin precursor plasminogen, and of different cells, among which the elastase-producing neutrophils. In ticks, blood digestion lasts for several days, during and after the



**Fig. 5.** (A) Boophilin expression in *R. microplus* gut after gene silencing. (a) Ticks injected with boophilin dsRNA, each line represents the gut of one tick, showing that the silencing was efficient. Results of two independent experiments (only one was shown); (b) ticks injected with PBS buffer and (c) non-injected ticks. (B) Reduction in oviposition after RNAi-silencing of boophilin. Tick injected with PBS showed 5% of oviposition reduction while ticks injected with ds-boophilin showed 20% of oviposition reduction compared to non-injected ticks. The results are the mean of two independent experiments  $\pm$  standard deviation.

engorgement process, and it is therefore conceivable that boophilin might be used to control any plasmin or elastase activity arising in the midgut during this period, even when complexed with thrombin, avoiding unwanted tissue damage.

Boophilin amino acid sequence is 37% identical to that of hemalin (Liao et al., 2009), a thrombin inhibitor described in the tick *Haemaphysalis longicornis*. However, while hemalin was expressed in all major tissues (including salivary glands, midgut, hemocytes and fat body) of adult female ticks, boophilin was exclusively expressed in the midgut, suggesting an important role in this organ. Considering its limited expression and its multiple inhibitory activities, silencing of boophilin expression was expected to impact serine protease activity levels in the midgut and consequently the digestion process, with a visible effect in egg production. Injection of dsRNA-boophilin in engorged *R. microplus* females almost abolished boophilin transcription in the midgut and resulted in 20% reduction in egg production, a result similar to that observed for hemalin

gene silencing (Liao et al., 2009). A possible explanation for the apparently small impact of boophilin gene silencing in egg production might be the vast array of Kunitz type inhibitors present in *R. microplus*, which were not observed in the related *Rhipicephalus sanguineus* (Azzolini et al., 2003). Besides boophilin, *R. microplus* produces other Kunitz type inhibitors with activity against trypsin, plasmin, plasma kallikrein and neutrophil elastase (Sasaki et al., 2004; Sasaki and Tanaka, 2008; Tanaka et al., 1999). Interestingly, some of these inhibitor genes are highly expressed in *R. microplus* midgut, and could partially make up for boophilin decrease in the dsRNA-boophilin injected ticks, leading to a lower impact in egg production. Similar results were obtained when other Kunitz type inhibitors were silenced or anti-BmTIs were injected in engorged females (A.S. Tanaka, unpublished data). The present findings, as well as the presence of another thrombin inhibitor in the midgut of *R. microplus* (Ricci et al., 2007), highlight the high redundancy of *R. microplus* Kunitz-based serine protease inhibitor arsenal, which certainly contributes to its evident

efficiency as a bovine ectoparasite. Considering our data, and the redundancy of *R. microplus* Kunitz inhibitors we believe that boophilin may be useful as an antigen together with other tick protein in a vaccine production for tick control.

## Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; grants 05/03514-9 and 09/05405-3), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant 490574/2006-8), and INCT-Entomologia Molecular. A.S.T. was the recipient of a CNPq fellowship. This work was funded in part by Fundação para a Ciência e a Tecnologia, Portugal, through grants PTDC/BIA-PRO/70627/2006 and REEQ/564/B10/2005 (EU-FEDER and POCI 2010) and the post-doctoral fellowship SFR/BPD/46722/2008 to A.C.F. We thank Cassia A. Lima, Renato Sakai and Rafael Marchesano for helping in cloning and expression experiments.

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