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

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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

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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).