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Brasiliensin: A novel intestinal thrombin inhibitor from *Triatoma brasiliensis* (Hemiptera: Reduviidae) with an important role in blood intake

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Abstract

Every hematophagous invertebrate studied to date produces at least one inhibitor of coagulation. Among these, thrombin inhibitors have most frequently been isolated. In order to study the thrombin inhibitor from *Triatoma brasiliensis* and its biological significance for the bug, we sequenced the corresponding gene and evaluated its biological function. The *T. brasiliensis* intestinal thrombin inhibitor, termed brasiliensin, was sequenced and primers were designed to synthesize double strand RNA (dsRNA). Gene knockdown (RNAi) was induced by two injections of 15 μg of dsRNA into fourth instar nymphs. Forty-eight hours after the second injection, bugs from each group were allowed to feed on hamsters. PCR results showed that injections of dsRNA reduced brasiliensin expression in the anterior midgut by approximately 71% in knockdown nymphs when compared with controls. The reduction in gene expression was confirmed by the thrombin inhibitory activity assay and the citrated plasma coagulation time assay which showed activity reductions of ~18- and ~3.5-fold, respectively. Knockdown nymphs ingested approximately 39% less blood than controls. In order to confirm the importance of brasiliensin in blood ingestion, fourth instar nymphs were allowed to ingest feeding solution alone or feeding solution containing 15 U of thrombin prior to blood feeding. Fifty-five percent less blood was ingested by nymphs which were fed thrombin prior to blood feeding. The results suggest that anticoagulant activity in the midgut is an important determinant of the amount of blood taken from the host. The role of anticoagulants during blood ingestion is discussed in the light of this novel insight.

Keywords: Triatomine; *Triatoma brasiliensis*; RNA interference; Brasiliensin; Feeding efficiency; Thrombin inhibitor; Intestinal anti-coagulant

1. Introduction

Every hematophagous invertebrate studied to date produces at least one inhibitor of coagulation in the salivary glands and/or intestine. Most of these target one or more of the serine proteases which make up the tissue factor pathway, e.g. factor VIIa, factor Xa and/or thrombin (Ledizet et al., 2005). Among them, thrombin inhibitors have frequently been isolated from arthropods. For example, the protein Amblin from the ixodid tick *Amblyomma hebraeum* (Lai et al., 2004), ornithodorin from the soft tick *Ornithodorus moubata* (van de Locht et al., 1996) and the major salivary thrombin inhibitor from *Glossina morsitans* that is also expressed in the midgut (Cappello et al., 1998).

In triatomines, thrombin inhibition was demonstrated in the saliva (Noeske-Jungblut et al., 1995) and also seen for the intestinal rhodniin from *Rhodnius prolixus* (Friedrich et al., 1993), dipetalogastin from *Dipetalogaster maximus* (Mende et al., 1999) and infestin from *Triatoma infestans*

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(Campos et al., 2002). Infestin was found in the anterior midgut and is encoded by a unique gene incorporating seven Kazal type domains.

Triatoma brasiliensis are vessel feeding, hematophagous arthropods and one of the main Brazilian vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease. They take very large blood meals (up to 370 μl for the fifth instar nymph) which can take more than 35 min to ingest (Guarneri et al., 2003). In order to control the hemostatic system of their hosts and guarantee successful feeding and management of the ingested blood, triatomines produce several anti-hemostatic substances in their saliva and intestine. During the feeding process, saliva is released the whole time and some of it is ingested with the blood (Soares et al., 2006). The undigested blood is then stored in the wide anterior part of the midgut, in which water and ions are transported to the haemolymph and malpighian tubules, and excreted via the rectum. The concentrated blood is passed in small amounts into the digestive and absorptive part of the posterior midgut (Kollien and Schaub, 2000). It has been verified in ixodid ticks that the ingestion of blood decreased when the salivary anticoagulant was depressed (Narasimhan et al., 2004). In contrast, when salivary glands were removed from *R. prolixus*, although they pierced host skin more often than normal bugs, if they are exposed to the host for sufficient time then the salivarectomy did not affect the amount of blood ingested (Ribeiro and Garcia, 1981). Here we have investigated one aspect of this apparent contradiction. As part of a gene discovery program for *T. brasiliensis*, we have found an orthologue of the anti-thrombin gene infestin from *T. infestans*, which was termed brasiliensin. In this paper, we describe this gene and use RNA interference (RNAi) to knock down brasiliensin (Araujo et al., 2006) to determine its physiological role during blood ingestion by *T. brasiliensis*.

2. Materials and methods

2.1. Triatomine bugs

Triatoma brasiliensis were reared under controlled temperature (26 ± 2.0 °C) and humidity (65 ± 5.0%), 12/12 light/dark and fed weekly on chickens or rats. The fourth instar specimens used in the experiments had similar physiological status (7 ± 1 days after molt).

2.2. Brasiliensin gene cloning and sequencing

Total RNA was extracted from the anterior midgut of four *T. brasiliensis* using Trizol solution (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized from 1.25 μg of total RNA with Improm II (Promega) and d(T)12 following the manufacturer's instructions. First strand cDNA was used as a template in a PCR performed with primers designed from the infestin gene of *T. infestans* (Lovato et al., 2006). PCR product was analysed by electrophoresis in 1% agarose gel and the desired amplicom was cloned into the pGEM-T Easy vector. The complete gene sequence was determined on an ABI Prism 377 DNA sequencer with DYEnamicTM ET Terminator Cycle Sequencing Kit (GE Healthcare Life Sciences).

2.3. Double strand RNA synthesis

Brasiliensin cDNA was amplified by PCR using specific primers (forward 5′-gagttctacaccgggtttgc-3′ and reverse 5′-ccatctgaaccacacactgg-3′, annealing temperature (T_a) = 60 °C) conjugated with 23 bases of the T7 RNA polymerase promoter. PCR was carried out for 35 cycles (94 °C for 30 s, 60 °C for 30 s, 45 °C for 45 s) with 1 μl of the cDNA in addition to 200 nM of each primer, 200 μM deoxyribonucleotide triphosphate (dNTP) and 1 U *Taq* Phoneutria (Phoneutria, Brazil) in a final volume of 20 μl. The 575 bp PCR products, 529 bp of the brasiliensin and 46 bp of the T7 promoter sequences, were used as a template for double-stranded RNA (dsRNA) synthesis using the T7 Ribomax Express RNAi System (Promega). After synthesis, the dsRNA was isopropanol-precipitated, resuspended in ultra pure water and quantified by 260 nm wavelength spectrophotometry. The quality of the dsRNA products was verified by agarose gel electrophoresis. The dsRNA was kept at −80 °C until use.

2.4. Delivery of dsRNA

Fourth instar nymphs were injected once or twice laterally into the thoracic haemocoel with a 48-h interval between injections. Each bug from the knockdown group was injected with 15 μg brasiliensin dsRNA diluted in 2 μl of 0.9% NaCl saline solution (brasiliensin dsRNA group) while each bug from the control groups received 2 μl of saline alone (saline control group) or 2 μl of saline containing 15 μg dsRNA from the β-lactamase gene (BLA dsRNA group). Forty-eight hours after the second injection, nymphs were fed on hamsters (Araujo et al., 2006).

2.5. Verification of knockdown by PCR

RNA was extracted from anterior midguts of individual nymphs from each group 48 h after dsRNA injection and semi-quantitatively assessed by cDNA synthesis and PCR for the level of gene knockdown. PCR was performed using primers for brasiliensin (as in section 2.3.) and the 18s rRNA (RP18s: forward 5′-cctgcggcttaatttgactc-3′ and reverse 5′-gtacaaagggcagggacgta-3′, T_a = 60 °C) as a loading control. PCR was carried out as above, but for 23 cycles. The products were analysed by 2% agarose gel electrophoresis and the intensity of bands was measured by densitometry using the Alpha DigiDoc 1201™ software (Alpha Innotech).