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Research paper

A novel trypsin Kazal-type inhibitor from *Aedes aegypti* with thrombin coagulant inhibitory activity

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ABSTRACT

Kazal-type inhibitors play several important roles in invertebrates, such as anticoagulant, vasodilator and antimicrobial activities. Putative Kazal-type inhibitors were described in several insect transcriptomes. In this paper we characterized for the first time a Kazal unique domain trypsin inhibitor from the *Aedes aegypti* mosquito. Previously, analyses of sialotranscriptome of *A. aegypti* showed the potential presence of a Kazal-type serine protease inhibitor, in female salivary glands, carcass and also in whole male, which we named AaTI (*A. aegypti* trypsin inhibitor). AaTI sequence showed amino acid sequence similarity with insect thrombin inhibitors, serine protease inhibitor from *Litopenaeus vannamei* hemocytes and tryptase inhibitor from leech *Hirudo medicinalis* (LDTI). In this work we expressed, purified and characterized the recombinant AaTI (rAaTI). Molecular weight of purified rAaTI was 7 kDa rAaTI presented dissociation constant (K_i) of 0.15 and 3.8 nM toward trypsin and plasmin, respectively, and it weakly inhibited thrombin amidolytic activity. The rAaTI was also able to prolong prothrombin time, activated partial thromboplastin time and thrombin time. AaTI transcription was confirmed in *A. aegypti* female salivary gland and gut 3 h and 24 h after blood feeding, suggesting that this molecule can act as anticoagulant during the feeding and digestive processes. Its transcription in larvae and pupae suggested that AaTI may also play other functions during the mosquito's development.

1. Introduction

Proteinases are found in all living organisms and play crucial roles in various biological and physiological processes, like digestion [1], metamorphosis [2], innate immunity [3], as well as in the pathogenesis of certain pathogens [4] and blood clotting [5]. Protease inhibitors are widely found in most living systems, from bacteria to humans. Until now more than 16,000 sequences of protease inhibitors have been characterized and divided into 67 families [6]. Among the serine protease inhibitors, the best known families are Kazal, Kunitz, Bowman-Birk, α-2-macroglobulin, serpin and pacifastin [7–9]. Several Kazal-type family members have previously been described in vertebrates and invertebrates. All the Kazal-type inhibitors contain one or more Kazal domains, each characterized by a well-conserved amino acid sequence containing three disulfide bridges [7] and highly similar three-dimensional structures [10]. To date, over 100 Kazal-type inhibitors have been found and investigated in vertebrates, invertebrates and bacteria.

In invertebrates, a large number of serine protease inhibitors have been described, such as tryptase inhibitor, LDTI (Leech Derived Tryptase Inhibitor) [11], subtilisin inhibitor, infestin 1R [12], and elastase inhibitor, CmPI-II [13]. Besides serine protease inhibitor activities of Kazal-type domains, other functions were described such as the vasoactive of vasotab peptide [14], and antimicrobial activity [15]. Among these functions, some Kazal-type serine protease inhibitors identified in blood sucking animals are powerful thrombin and other blood coagulation factor inhibitors, acting to prevent clotting during host blood sucking and digestion. The first description of Kazal-type inhibitor with anticoagulant activity was the thrombin inhibitor rhodniin from the insect *Rhodnius prolixus* [16], followed by dipetalogastin from the insect *Dipetalogaster maximus* [17]. In this scenario, our group has offered

Abbreviations used: rAaTI, recombinant *A. aegypti* trypsin inhibitor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; tPA, tissue plasminogen activator; HuPK, human plasma kallikrein; fXa, activated factor X, fXIIa, activated factor XII; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time.

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a contribution, by describing the thrombin inhibitor infestin 1–2 [18] and a specific factor XIIa inhibitor, infestin 4 [19] from the kissing bug *Triatoma infestans* midgut.

Aedes aegypti is the principal vector of dengue and yellow fever viruses worldwide, mainly because of its adaptability to urban life and its highly susceptibility to dengue virus; besides, it has an almost imperceptible bite, and is able to bite several people for one blood meal [20].

Recently, *A. aegypti* genome was sequenced, facilitating further gene investigations. Ribeiro and coworkers analyzed a set of 3776 salivary glands cDNA sequences (a total of 4232 compared with previous set of 456 clones) and identified 573 new transcripts, 136 of putative secretory proteins, most of which without known function. Among those sequences, Kazal-type putative protease inhibitors were found, including the sequence gi|94468720, which was expressed in salivary glands and in carcass of female and also in whole male [21].

In an attempt to contribute to the understanding of unknown predicted protein secreted found in transcriptome approaches, the aim of the present work was the expression, purification and characterization of a putative serine protease Kazal-type domain found in the *A. aegypti* sialotranscriptome, which was named *A. aegypti* Trypsin Inhibitor (AaTI). Among the mosquito putative Kazal-type inhibitors, AaTI is the first inhibitor to be characterized.

2. Materials and methods

2.1. Animals

A. aegypti (Higgs strain) were reared in a local facility at the Institute of Biomedical Sciences, University of São Paulo, Brazil. Temperature was maintained at 26 °C, humidity at 80% and a 12/12-h photoperiod. Larvae were fed on powdered fish food (TETRAMIN). Adult mosquitoes were given continuous access to a 10% sucrose solution and 5-day-old females were fed on anesthetized mice, when required. All the experiments were carried out in accordance with the guidelines of the Institutional Ethics Review Committee (Colégio Brasileiro de Experimentação Animal – COBEA) and Animal Care of the Institute of Biomedical Sciences (Comissão de ética em experimentação animal – CEEA) of University of São Paulo, protocol # 133.

2.2. Cloning and expression of a Kazal-type inhibitor from A. aegypti salivary gland (AaTI)

Based on the AaTI sequence (GenBank accession number DQ440176), two gene-specific primers were designed to amplify AaTI gene fragment by PCR: AaTI forward 5′ – AGCTCTCGAGAAAA GAGAGAGAGGAGTTTGTGC – 3′ and AaTI reverse 5′ – TTTTCCTTTTG CGGCCGCTCAATACTCCTGTGGGATGAAG – 3′. AaTI DNA fragments were amplified using a PCR product kindly provided by Dr. José M. Ribeiro (NIH – Rockville, MD, USA). The PCR-amplified product was cloned into pPIC9 vector (Invitrogen, Carlsbad, CA) and the sequences were confirmed by DNA sequencing using "DYEnamic ET* Terminator Cycle Sequencing" kit (GE Healthcare, Chalfont St. Giles, UK). Alignment of amino acid sequence was performed using the ClustalW program [22]. AaTI recombinant protein was produced by *Pichia pastoris* yeast GS115 strain following the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Transformed yeast cells were induced with 0.5% methanol every 24 h. After 120 h, yeast cell cultures were harvested and the supernatant (containing secreted rAaTI) was stored at −20 °C.

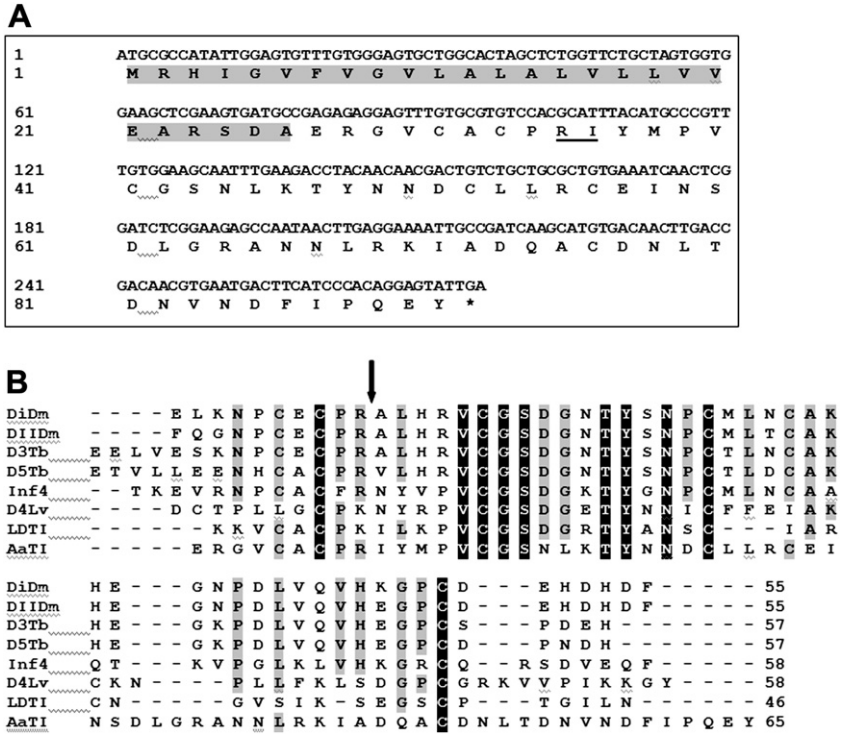

Fig. 1. (A) AaTI nucleotide sequence and its translated amino acids sequence (GenBank accession number DQ440176). The putative signal peptide is represented in gray, the putative reactive site is underlined (determined by comparison with other Kazal-type inhibitors), and Arg residue in P1 position. (B) Comparison of AaTI amino acid sequence with other Kazal-type inhibitors. The ClustalW program was used at EMBL-EBI. The arrow indicates the reactive site predicted by comparison with other Kazal-type inhibitors. Identical amino acid residues in all domains are black boxed, and amino acid residues which appear in at least five sequences are in a gray box. DIDm and DIIDm: a thrombin inhibitor (Dipetalogastin) from *Dipetalogaster maximus* (Domain I and II, respectively) [17]; D3Tb and D5Tb: Brasiliensin domains 3 and 5, respectively, from *Triatoma brasiliensis* [31], INF4: Infestin domain 4, from *Triatoma infestans* [19]; D4Lv, a Kazal-type inhibitor from *Litopenaeus vannamei* hemocytes [32] and LDTI – Leech Derived Tryptase Inhibitor, from *Hirudo medicinalis* [11].

2.3. Recombinant AaTI purification

Supernatant containing recombinant proteins was applied in an affinity chromatography column of trypsin-Sepharose. Fractions were eluted with 0.5 M KCl solution pH 2.0. The fractions containing inhibitory activity toward trypsin were pooled, concentrated and analyzed by SDS-PAGE using a 15% polyacrylamide gel [23]. The active material from trypsin-Sepharose column was purified by gel filtration chromatography, on a Superdex 75 column. To determine the N-terminal amino acid sequence, the rAaTI purified by affinity chromatography was loaded on a reverse phase chromatography (C_8 Sephasil Peptide column) using the ÄKTA Purifier System. Proteins were eluted with an acetonitrile linear gradient (0–90%) in 0.1% trifluoroacetic acid. Purified rAaTI was submitted to automated Edman degradation for N-terminal sequencing. Protein concentration was determined according to the Bradford method [24].

2.4. Characterization of serine protease inhibition by recombinant AaTI

Serine protease inhibition tests were carried out in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 0.1% Triton X-100. Tissue plasminogen activator (tPA), human plasma kallikrein (HuPK), urokinase, plasmin, thrombim, factor Xa (fXa), chymotrypsin, neutrophil elastase and factor XIIa (fXIIa) were separately pre-incubated with different concentrations of rAaTI in the buffer for 10 min at 37 °C. Then, the enzyme residual activity was tested by adding chromogenic substrate (100 μM): S2288 (HD-Ile-Pro-Arg-pNa – Chromogenix), S2302 (HD-Pro-Phe-Arg-pNa – Chromogenix), S4444 (Pyro-Glu-Gly-Arg-pNa – Chromogenix), S2251 (HD-Val-Leu-Lys-pNa – Chromogenix), Tosyl-Gly-Pro-Arg-AMC (Sigma), S2222 (Bz-Ile-Glu(g-OR)-Gly-Arg-pNa – Chromogenix), N-Suc-Ala-Ala-Pro-Phe-pNa (Fluka), S2484 (Pyro-Glu-Pro-Val-pNa – Chromogenix) and S2302 (HD-Pro-Phe-Arg-pNa – Chromogenix) for the respective enzymes. The reaction mixture was incubated at 37 °C for 10 min and the absorbance at 405 nm was determined using a microplate reader Synergy HT (BioTek).

Apparent dissociation constant (K_i) value to plasmin was calculated by fitting the steady-state velocities to the equation $(V_i/V_o = 1 - \{E_t + I_t + K_i - [(E_t + I_t + K_i)^2 - 4E_tI_t]^{1/2}\}/2E_t)$ for tight-binding inhibitors using a non-linear regression analysis [25].

To determine K_i for trypsin, the experiment was composed by six curves using different concentrations of Tosyl-Gly-Pro-Arg-pNa (50, 80, 100, 150, 200 and 500 μM). For each curve, rAaTI was used in different concentrations (0, 5.2, 13.0, 20.8, 26.0, 52.0, 130.0 and 180.0 nM) and 5.2 nM trypsin solution. The reactions were carried out in a total volume of 100 μL in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 0.1% Triton X-100, and were started

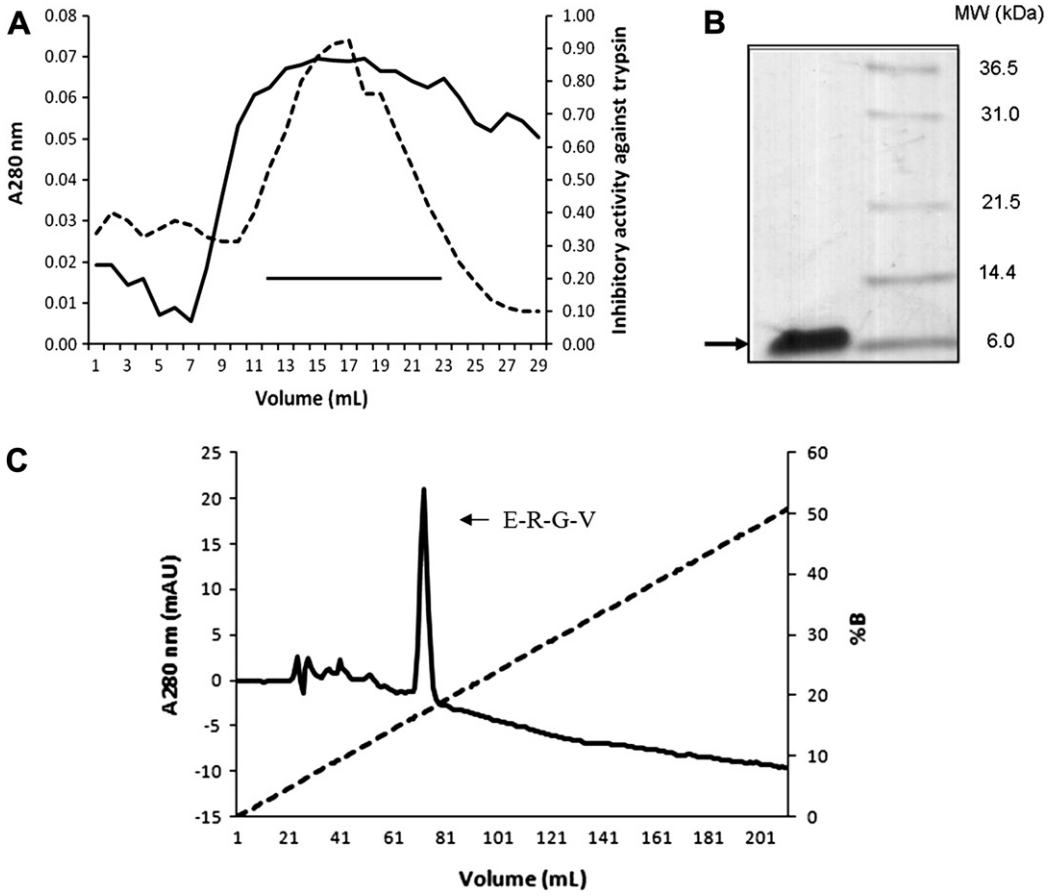

Fig. 2. Purification of rAaTI. (A) Affinity chromatography on trypsin-Sepharose column. Spotted line (- - -) shows absorbance at 280 nm and black line (—) shows inhibitory activity toward trypsin. Black bar (under the graph) corresponds to fractions which were pooled, concentrated and analyzed by SDS-PAGE. (B) SDS-PAGE 15%. Sample: rAaTI after affinity chromatography. The arrow shows rAaTI with approximately 7.0 kDa. (C) Reverse phase chromatography on Sephasil peptide C_8 column. Protein elution was performed with B buffer (0.1% acid Trifluoroacetic acid in 90% acetonitrile). The arrow shows the fraction submitted to automated Edman degradation for N-terminal sequencing, and this sequence is shown in the graph.

using trypsin. Absorbance at 405 nm was measured for 10 min. The amount of *p*-nitroaniline was calculated using a molar extinction coefficient of 9960. The enzyme activity unit was calculated as mmol of *p*-nitroaniline/min. K_i was calculated using the Lineweaver–Burk plot (1/V × 1/[S]) plotting [15,26].

2.5. Preparation of female gut extracts 3 h and 24 h after blood feeding

Female mosquitoes were dissected 3 h and 24 h after blood feeding in sodium phosphate buffered saline, pH 7.2 (PBS), according to Moll et al. [27]. The extracts were prepared with 150 and 90 guts collected from mosquitoes 3 h and 24 h after blood feeding, respectively, in the same buffer (225 μL) in 50 mM sodium acetate buffer pH 5.5 (375 μL). Gut extracts were stored at −20 °C.

2.6. A. aegypti trypsin inhibition

A. aegypti trypsin inhibitory assay was performed using 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 0.1% Triton X-100. In this work, it was considered that 1 U of trypsin-like activity was the amount of enzyme that released 1000 relative fluorescent unit (RFU)/min from a fluorogenic substrate. In the experiments, 0.45 U of enzyme from gut collected 3 h after blood feeding and 0.5 U from gut 24 h collected after blood feeding were separately pre-incubated with different concentrations of rAaTI (0–65 nM) for 10 min at 37 °C, followed by the addition of fluorogenic substrate Tosyl-Gly-Pro-Arg-MCA (100 μM). The reaction was monitored using the excitation wavelength of 380 nm and emission measured at 460 nm for 20 min at 37 °C [28].

2.7. Coagulation assays

Activated partial thromboplastin time (APTT) assay was performed using the activated partial thromboplastin time reagent (Diacelin-Diamed). Briefly, 50 μL of normal plasma was incubated with 50 μL of rAaTI (1.3 μM) and 50 μL of APTT reagent for 3 min at 37 °C. Next, 50 μL pre-heated $CaCl_2$ 25 mM was added and clotting time was measured using the coagulometer Quick Timer (Drake). The prothrombin time assay (PT) was performed using the PT reagent (Neoplastin C – Stago): 50 μL of normal plasma was pre-incubated with 50 μL of rAaTI (1.3 μM) for 1 min at 37 °C. Then, 100 μL of PT reagent was added, and clotting time was measured. Thrombin time (TT) was measured by pre-incubating 70 μL of bovine fibrinogen (2.0 mg/mL) and 70 μL of rAaTI (1.3 μM) for 1 min at 37 °C. The reaction was activated adding 70 μL of bovine thrombin (5 U/mL), and the clotting time was measured using the coagulometer Quick Timer (Drake). The statistical analysis was provided using the *t*-Student test, with $p < 0.05$.

2.8. Transcription analysis of AaTI in different tissues of A. aegypti

AaTI transcript was analyzed in female salivary gland, ovary, fat body, gut, and gut 3 h and 24 h after feeding, and 1st instar, 2nd instar, 3rd instar, 4th instar larvae and pupae. Total RNA from each tissue was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) and used to cDNA preparation with ImProm-II™ Reverse Transcription System (Promega, Madison, WI). AaTI gene was amplified by PCR using specific primers and ribosomal gene fragment was amplified using the following primers (forward primer: 5′ – GCTATGACAAGCTTGCCCCCA – 3′ and reverse primer: 5′ – TCATCAGCACCTCCAGCTC – 3′). PCR product was analyzed by 1% agarose gel.

Table 1
Apparent dissociation constants (K_i) of rAaTI and rAaTIΔ for different serine proteases.

	K_i (nM, Mean ± SEM)				
	Trypsin	Plasmin	HuPK	FXIIa	FXa
rAaTI	0.15 ± 0.01	3.8 ± 0.6	n.i.	n.i.	n.i.

HuPK – Human plasma kallikrein; FXa – factor Xa; FXIIa – factor XIIa. n.i. – not inhibited.

3. Results

3.1. Cloning and expression of AaTI

A DNA fragment of 230 bp (AaTI) was amplified by PCR using gene-specific primers, and cloned into pPIC9 vector. The fragment sequence was determined by DNA sequencing (Fig. 1A), and analyzed by BLASTX [29]. Translated amino acid sequence showed one Kazal-type domain and a predicted signal peptide according to the Signal P V2.0 program [30]. The alignment of AaTI with other Kazal-type inhibitors showed similarity with blood coagulation cascade inhibitors [12,17,31], Kazal-type inhibitor from *Litopenaeus vannamei* hemocytes [32] and LDTI (Leech Derived Tryptase Inhibitor) [11]. The putative reactive site of AaTI presented an Arg residue in P1 position (Fig. 1B).

3.2. Purification of recombinant AaTI

rAaTI, expressed in *P. pastoris* system was purified from culture supernatant using an affinity chromatography on trypsin-Sepharose

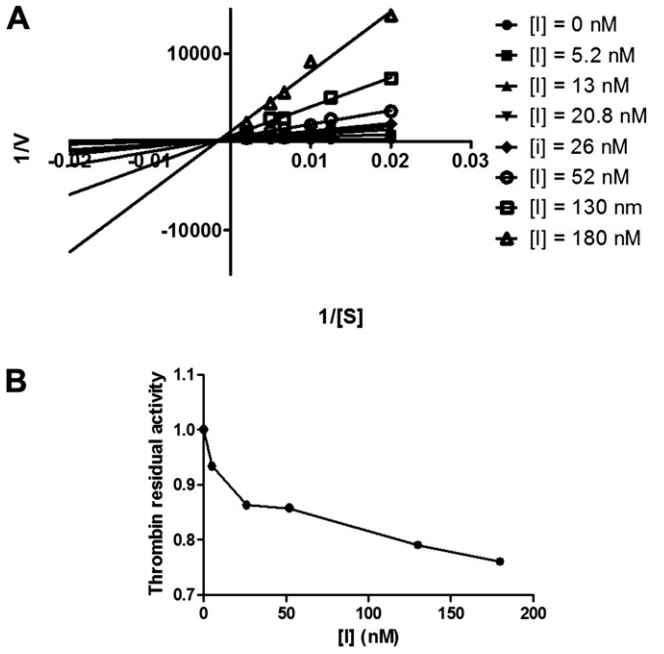

Fig. 3. (A) Lineweaver–Burk plot (1/V × 1/[S]) of trypsin inhibition assay. Reactions occurred in different substrate concentrations (50–500 μM). For each substrate concentration, increasing amount of rAaTI (0–180 nM) was used. Reactions were started by adding trypsin 5.2 nM to substrate-inhibitor mixture and K_i was calculated using the Lineweaver–Burk plot (1/V × 1/[S]) plotting. (B) Inhibition of thrombin. Thrombin was pre-incubated with different concentrations of rAaTI and then fluorogenic substrate Tosyl-Gly-Pro-Arg-AMC was added to reaction and fluorescence was monitored. The fluorescence in the presence of recombinant inhibitor was compared with reaction without inhibitor.

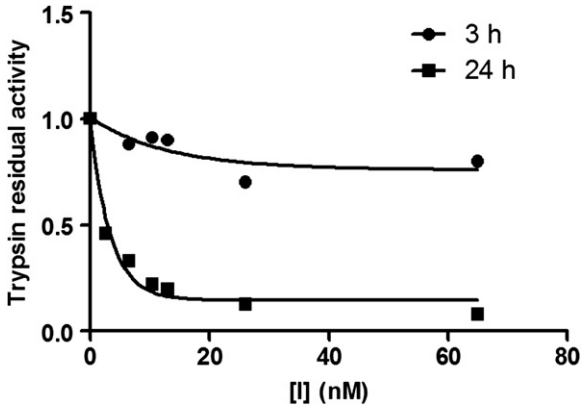

Fig. 4. Inhibition of trypsin-like enzymes from gut 3 and 24 h after blood feeding. Different concentrations of rAaTI (0–65 nM) were separately pre-incubated with 0.45 U of enzyme from 3 h after blood feeding and 0.5 U from gut 24 h after blood feeding. Then, fluorogenic substrate Tosyl-Gly-Pro-Arg-AMC was added to the mixture, and fluorescence was monitored for 20 min. Reactions with different concentrations of rAaTI were compared with reaction without inhibitor.

column (Fig. 2A). The fractions containing inhibitory activity against trypsin were pooled and submitted to SDS-PAGE (Fig. 2B). The eluted fractions from affinity chromatography were applied in a gel filtration chromatography on a Superdex 75 column (data not shown). To determine N-terminal sequence, fractions eluted from affinity chromatography were applied to reverse phase chromatography on a Sephasil Peptide C_8 column (Fig. 2C), and the major protein peak was submitted to automated Edman degradation for N-terminal sequencing. As expected, the N-terminal sequence was the same as predicted: E-R-G-V (Fig. 2C).

3.3. Characterization of serine protease inhibition by recombinant AaTI

Purified rAaTI inhibited only amidolytic activity of trypsin, plasmin and weakly inhibited thrombin activity, and it did not affect human plasma kallikrein (HuPK), factor Xiia (fXIIa), factor Xa (fXa), (Table 1), tissue plasminogen activator (tPA), urokinase, chymotrypsin and neutrophil elastase (data not shown). Dissociation constant (K_i) of rAaTI for trypsin (Fig. 3A) and plasmin was 0.15 nM and 3.8 nM, respectively. Recombinant inhibitor was able to weakly inhibit thrombin amidolytic activity (Fig. 3B). rAaTI also inhibited amidolytic activity of enzyme of gut 24 h after feeding, but it almost did not affect enzyme of gut 3 h after feeding (Fig. 4).

3.4. Coagulation assays

The presence of rAaTI anticoagulant activity was measured by APTT, PT and TT assays. rAaTI, was able to prolong PT (Fig. 5A), APTT (Fig. 5B) and TT (Fig. 5C).

3.5. cDNA analysis

rAaTI transcription was analyzed in female salivary gland, ovary fat body, gut, gut 3 h and 24 h after feeding, and early 1st instar, late 1st instar, 2nd instar, 3rd instar, 4th instar larvae and pupae. The transcript (260 bp) was observed in female salivary gland, gut, 3 h and 24 h after feeding gut and all larvae instars and pupae (Fig. 6).

4. Discussion

Kazal-type inhibitors can be found in many living systems and play several roles in the control of physiological systems, like

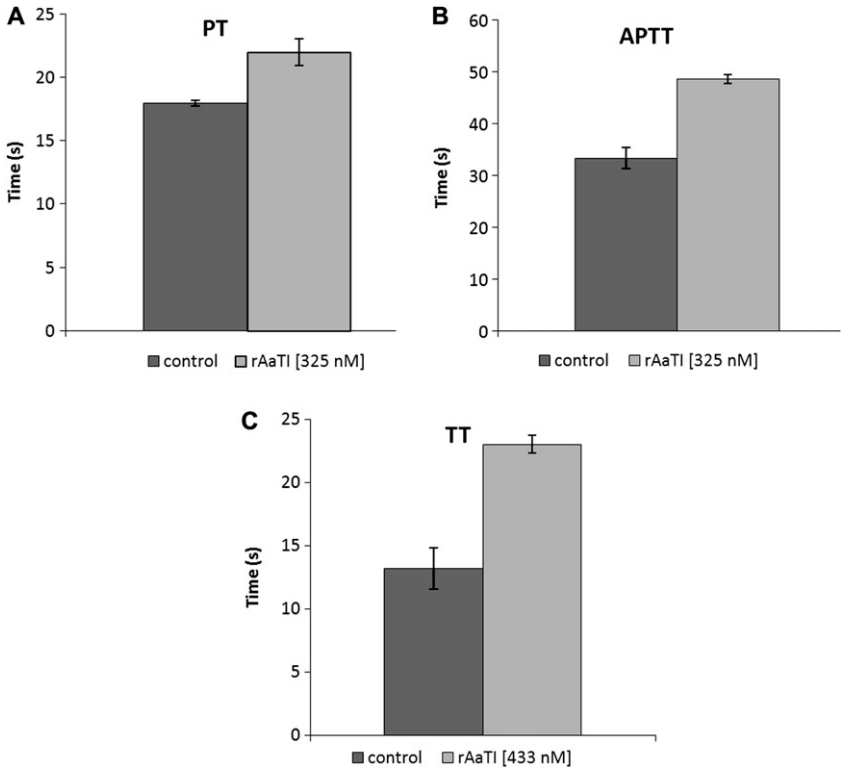

Fig. 5. Coagulation assays. (A) Prothrombin Time (PT). rAaTI (325 nM) was used in prothrombin time. (B) Activated Partial Thromboplastin Time (APTT). The assay was performed using 325 nM rAaTI. (C) Thrombin Time (TT) using rAaTI (433 nM). Results are the mean of triplicate points ± standard deviation. It was used *t*-Student test with $p < 0.05$.

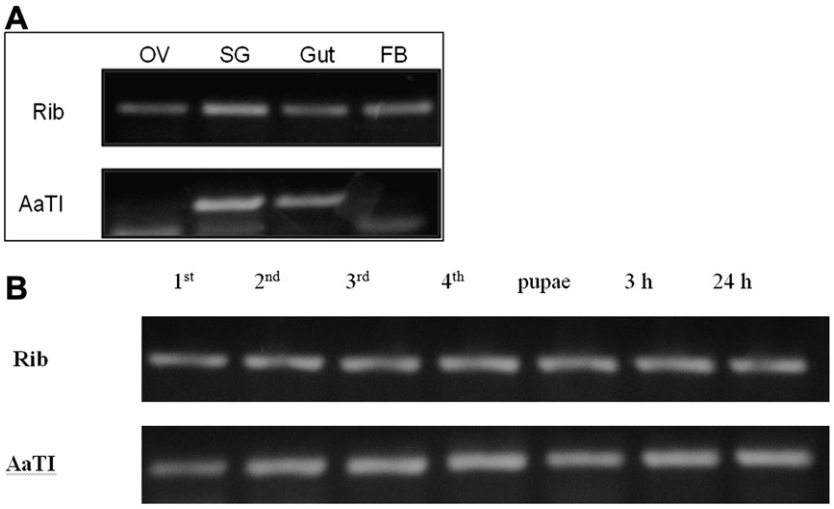

Fig. 6. Transcript analyses. (A) AaTI transcription experiment using cDNAs of non-feeding female ovary (OV), salivary gland (SG), gut and fat body (FB). (B) AaTI transcription experiment using cDNAs of female gut 3 h and 24 h after blood feeding. Rib – ribosomal control. PCR products were analyzed in 1% agarose gel.

digestion, innate immunity, and complement systems [33]. The invertebrate Kazal-type inhibitors can be single or multiple domains. rAaTI is a single domain Kazal-type inhibitor with 53 amino acids containing 6 cysteine residues. In this work we described rAaTI cloning, expression, purification and characterization. Multiple alignment of AaTI amino acid sequence with other Kazal-type inhibitors revealed high similarity to non-classical Kazal-type inhibitors such as dipetalogastin [17], infestins [12,18], LDTI [11], brasiliensin [31], and a Kazal-type inhibitor from *L. vannamei* [32]. AaTI presented Arg residue at P1 position confirming its inhibitory activity toward trypsin-like enzymes.

Purified recombinant AaTI N-terminal sequence confirmed it was corrected processed by *P. pastoris* enzymes and it molecular weight was confirmed by SDS-PAGE.

Recombinant AaTI was able to strongly inhibit amidolytic activity of bovine trypsin as a competitive inhibitor and human plasmin, but only weakly inhibited bovine and human thrombin. Considering that thrombin is a key enzyme of blood coagulation cascade and that *A. aegypti* is a hematophagous insect, an anticoagulant activity of AaTI would be an important role for this Kazal-type inhibitor. In order to confirm its possible anticoagulant activity, rAaTI was used in coagulation assays. rAaTI was shown to prolong clotting time in APTT, PT and TT, suggesting that one possible function of AaTI in female adult mosquitoes is to control the blood clotting during the feeding and storing processes. As rAaTI only weakly inhibited amidolytic activity of thrombin, and since it was able to prolong the three clotting times (PT, aPTT and TT), we suggest that rAaTI interaction with thrombin might be facilitated by plasma components. So, it was tested whether rAaTI could increase antithrombin III activity because of its charged C-terminal region. Yet, the results of this experiment were inconclusive (data not shown). We also tested rAaTI in platelet aggregation in washed platelet, but we did not see any effect (Data not shown).

Our results also suggested that AaTI may have other functions in the *A. aegypti* biology, mainly because it was transcripted in tissues of all development stages of the mosquito. Moreover, we have to consider that its expression was found in salivary gland of female and also in the carcass of female and male [21]. AaTI expression profile suggested a possible role as an inhibitor of endogenous proteases; this was reinforced by the fact that rAaTI inhibits trypsin-like activity from gut of *A. aegypti* females 24 h after feeding and weakly affected enzyme activity from gut 3 h after feeding, which confirmed the difference in the trypsin-like enzymes contents during blood digestion [34]. Hematophagous mosquitoes utilize the amino acids from blood proteins as the constituents of their eggs. Proteins are the predominant components of blood, and 24 h after feeding, 80% of the ingested proteins have been digested [35]. Midgut of *A. aegypti* females synthesizes two trypsin forms after a blood meal. Early trypsin is produced in small amounts and appears in the midgut within 1 h of the blood meal. Late trypsin is produced in large amounts and begins to appear 8–10 h after blood feeding, playing a major role in blood-meal protein digestion [34]. AaTI inhibition profile for trypsin-like enzymes measured 3 h and 24 h after blood feeding suggested that it is more selective for late trypsin than early trypsin. A possible explanation is that in the first 3 h after feeding, AaTI acts as a thrombin inhibitor to control the blood coagulation, and 24 h after feeding the coagulation factors may be already inactivated, so the inhibitor starts to neutralize the digestive enzymes.

Another possible role of AaTI would be to control other endogenous enzymes. Our present results also showed a strong inhibitory activity of rAaTI in purified 4th instar larvae trypsin with IC_{50} in pM range (Sanches et al., unpublished), corroborating our inhibition data for adult digestive trypsin-like enzymes and confirming its function as an inhibitor of endogenous trypsins of *A. aegypti* in different development stages.

In conclusion, AaTI is a trypsin Kazal-type inhibitor with thrombin coagulant inhibitory activity, and it also inhibits trypsin-like enzymes from different development stages of *A. aegypti*. AaTI may play at least two different roles in the mosquito biology: it can (i) act as an anticoagulant in the adult female salivary gland and gut, and (ii) regulate trypsin-like enzymes such as late trypsin and a trypsin purified from 4th instar larvae gut. Its wide expression distribution strongly suggests that it may have yet another role.

Acknowledgments

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for performing platelet aggregation test. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (05/03514-9) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (470070/2004-8; 575829/2008-7). R.M.O Watanabe was supported with a fellowship by FAPESP (07/56614-6).

References

[1] H. Briegel, Excretion of proteolytic enzymes by *Aedes aegypti* after a blood meal. J. Insect Physiol. 21 (1975) 1681–1684.
[2] M. Eguchi, Protein protease inhibitors in insects and comparison with mammalian inhibitors. Comp. Biochem. Physiol. B. 105 (1993) 449–456.
[3] M.R. Kanost, Serine proteinase inhibitors in arthropod immunity. Dev. Comp. Immunol. 23 (1999) 291–301.
[4] J. Travis, J. Potempa, H. Maeda, Are bacterial proteinases pathogenic factors? Trends Microbiol. 3 (1995) 405–407.
[5] E.W. Davie, K. Fujikawa, K. Kurachi, W. Kisiel, The role of serine proteases in the blood coagulation cascade. Adv. Enzymol. Relat. Areas Mol. Biol. 48 (1979) 277–318.
[6] N.D. Rawlings, F.R. Morton, C.Y. Kok, J. Kong, A.J. Barrett, MEROPS: the peptidase database. Nucleic Acids Res. 36 (2008) D320–D325.
[7] M. Laskowski Jr., I. Kato, Protein inhibitors of proteinases. Annu. Rev. Biochem. 49 (1980) 593–626.
[8] Z. Liang, L. Sottrup-Jensen, A. Aspan, M. Hall, K. Soderhall, Pacifastin, a novel 155-kDa heterodimeric proteinase inhibitor containing a unique transferrin chain. Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 6682–6687.
[9] R.A. Boigegrain, H. Mattras, M. Brehelin, P. Paroutaud, M.A. Coletti-Previero, Insect immunity: two proteinase inhibitors from hemolymph of *Locusta migratoria*. Biochem. Biophys. Res. Commun. 189 (1992) 790–793.
[10] A. van de Locht, D. Lamba, M. Bauer, R. Huber, T. Friedrich, B. Kroger, W. Hoffken, W. Bode, Two heads are better than one: crystal structure of the insect derived double domain Kazal inhibitor rhodniin in complex with thrombin. EMBO J. 14 (1995) 5149–5157.
[11] C.P. Sommerhoff, C. Sollner, R. Mentele, G.P. Piechottka, E.A. Auerswald, H. Fritz, A Kazal-type inhibitor of human mast cell tryptase: isolation from the medical leech *Hirudo medicinalis*, characterization, and sequence analysis. Biol. Chem. Hoppe-Seyler 375 (1994) 685–694.
[12] D.V. Lovato, I.T. Nicolau de Campos, R. Amino, A.S. Tanaka, The full-length cDNA of anticoagulant protein infestin revealed a novel releasable Kazal domain, a neutrophil elastase inhibitor lacking anticoagulant activity. Biochimie 88 (2006) 673–681.
[13] Y. Gonzalez, T. Pons, J. Gil, V. Besada, M. Alonso-del-Rivero, A.S. Tanaka, M.S. Araujo, M.A. Chavez, Characterization and comparative 3D modeling of CmPI-II, a novel 'non-classical' Kazal-type inhibitor from the marine snail *Cenchritis muricatus* (Mollusca). Biol. Chem. 388 (2007) 1183–1194.
[14] P. Takac, M.A. Nunn, J. Meszaros, O. Pechanova, N. Vrbjar, P. Vlasakova, M. Kozanek, M. Kazimirova, G. Hart, P.A. Nuttall, M. Labuda, Vasotab, a vasoactive peptide from horse fly *Hybomitra bimaculata* (Diptera, Tabanidae) salivary glands. J. Exp. Biol. 209 (2006) 343–352.
[15] S. Donpudsa, A. Tassanakajon, V. Rimphanitchayakit, Domain inhibitory and bacteriostatic activities of the five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon*. Dev. Comp. Immunol. 33 (2009) 481–488.
[16] T. Friedrich, B. Kroger, S. Bialojan, H.G. Lemaire, H.W. Hoffken, P. Reuschenbach, M. Otte, J. Dodt, A Kazal-type inhibitor with thrombin specificity from *Rhodnius prolixus*. J. Biol. Chem. 268 (1993) 16216–16222.
[17] K. Mende, O. Petoukhova, V. Koulitchkova, G.A. Schaub, U. Lange, R. Kaufmann, G. Nowak, Dipetaliogastin, a potent thrombin inhibitor from the blood-sucking insect. *Dipetalogaster maximus* cDNA cloning, expression and characterization. Eur. J. Biochem. 266 (1999) 583–590.
[18] I.T. Campos, R. Amino, C.A. Sampaio, E.A. Auerswald, T. Friedrich, H.G. Lemaire, S. Schenkman, A.S. Tanaka, Infestin, a thrombin inhibitor presents in *Triatoma infestans* midgut, a Chagas' disease vector: gene cloning, expression and characterization of the inhibitor. Insect. Biochem. Mol. Biol. 32 (2002) 991–997.
[19] I.T. Campos, A.M. Tanaka-Azevedo, A.S. Tanaka, Identification and characterization of a novel factor XIIa inhibitor in the hematophagous insect, *Triatoma infestans* (Hemiptera: Reduviidae). FEBS Lett. 577 (2004) 512–516.
[20] D.J. Gubler, The global emergence/resurgence of arboviral diseases as public health problems. Arch. Med. Res. 33 (2002) 330–342.
[21] J.M. Ribeiro, B. Arca, F. Lombardo, E. Calvo, V.M. Phan, P.K. Chandra, S.K. Wikel, An annotated catalogue of salivary gland transcripts in the adult female mosquito, *Aedes aegypti*. BMC Genomics 8 (2007) 6.
[22] R. Chenna, H. Sugawara, T. Koike, R. Lopez, T.J. Gibson, D.G. Higgins, J.D. Thompson, Multiple sequence alignment with the clustal series of programs. Nucleic Acids Res. 31 (2003) 3497–3500.
[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680–685.
[24] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248–254.
[25] J.F. Morrison, Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. Biochim. Biophys. Acta 185 (1969) 269–286.
[26] M. Dixon, The determination of enzyme inhibitor constants. Biochem. J. 55 (1953) 170–171.
[27] R.M. Moll, W.S. Romoser, M.C. Modrzakowski, A.C. Moncayo, K. Lerdthusnee, Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. J. Med. Entomol. 38 (2001) 29–32.
[28] G. Lipps, R. Fullkrug, E. Beck, Cathepsin B of Schistosoma mansoni. Purification and activation of the recombinant proenzyme secreted by *Saccharomyces cerevisiae*. J. Biol. Chem. 271 (1996) 1717–1725.
[29] W. Gish, D.J. States, Identification of protein coding regions by database similarity search. Nat. Genet. 3 (1993) 266–272.
[30] H. Nielsen, J. Engelbrecht, S. Brunak, G. von Heijne, A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Int. J. Neural. Syst. 8 (1997) 581–599.
[31] R.N. Araujo, I.T. Campos, A.S. Tanaka, A. Santos, N.F. Gontijo, M.J. Lehane, M.H. Pereira, Brasiliensin: a novel intestinal thrombin inhibitor from *Triatoma brasiliensis* (Hemiptera: Reduviidae) with an important role in blood intake. Int. J. Parasitol. 37 (2007) 1351–1358.
[32] F. Jimenez-Vega, F. Vargas-Albores, A four-Kazal domain protein in *Litopenaeus vannamei* hemocytes. Dev. Comp. Immunol. 29 (2005) 385–391.
[33] V. Rimphanitchayakit, A. Tassanakajon, Structure and function of invertebrate Kazal-type serine proteinase inhibitors. Dev. Comp. Immunol. (2009).
[34] F.G. Noriega, M.A. Wells, A molecular view of trypsin synthesis in the midgut of *Aedes aegypti*. J. Insect Physiol. 45 (1999) 613–620.
[35] H. Briegel, A.O. Lea, Relationship between protein and proteolytic activity in the midgut of mosquitoes. J. Insect. Physiol. 21 (1975) 1597–1604.