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Research paper

Characterization of thrombin inhibitory mechanism of rAaTI, a Kazal-type inhibitor from *Aedes aegypti* with anticoagulant activity

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ABSTRACT

Saliva of blood-sucking arthropods contains a complex mixture of anti-haemostatic, anti-inflammatory and immune-modulator compounds. Among anti-haemostatic factors, there are anticoagulants, vasodilators and platelet aggregation inhibitors. Previous analyses of the sialotranscriptome of *Aedes aegypti* showed the potential presence of a Kazal-type serine protease inhibitor in the female salivary glands, carcass and also in the whole male, which inhibitor we named AaTI (*A. aegypti* thrombin inhibitor). Recently, we expressed and characterized rAaTI as a trypsin inhibitor, and its anticoagulant activity [1]. In this work we characterized the thrombin inhibition mechanism of rAaTI. Recombinant AaTI was able to prolong prothrombin time, activated partial thromboplastin time and thrombin time. In contrast, AaTIΔ (rAaTI truncated form) and C-terminal AaTI acidic tail prolong only thrombin time. In the competition assay, rAaTI, AaTIΔ or C-terminal AaTI acidic tail–thrombin interactions seem to be affected by heparin but not by hirudin, suggesting that rAaTI binds to thrombin exosite 2. Finally, the thrombin inhibition assay of rAaTI showed an uncompetitive inhibition mechanism. In conclusion, rAaTI can probably inhibit thrombin by interacting with thrombin exosite 2, and the interaction is not mediated by the AaTI C-terminal region, since the truncated AaTIΔ form also prolongs thrombin time.

1. Introduction

Animals belonging to at least fourteen arthropod families which contain over 400 different genera and more than 15,000 species including *Aedes aegypti*, evolved independently to feed on vertebrate blood. To achieve fast blood feeding, adult female mosquitoes inject sophisticated cocktails of salivary pharmacologic reagents that affect blood clotting, platelet aggregation and vascular dilation [2–5].

A. aegypti is the principal vector of dengue and yellow fever viruses worldwide, mainly because of its adaptability to urban life and its high susceptibility to dengue virus. Besides, it has a quite imperceptible bite, and can bite several people in order to acquire just one blood meal [6].

Recently, the *A. aegypti* genome was sequenced, facilitating further gene discovery. Ribeiro and collaborators analyzed a set of 3776 Salivary Gland cDNA sequences (total of 4232 sequences when considering a previous set of 456 clones) and identified 573 new transcripts, from which 136 originate putative secretory proteins most of them without known function. Among those sequences, Kazal-type putative protease inhibitors were found including the sequence gi|94468720, which was expressed in the salivary glands and carcass of female mosquitoes, and also in the whole male [7].

In invertebrates, a huge number of serine protease inhibitors have been described, each one showing a different inhibitory activity, such as tryptase inhibitor, LDTI (Leech derived tryptase inhibitor) [8], subtilisin inhibitor, infestin 1R [9], and elastase inhibitor, CmPI-II [10]. Besides, the Kazal-type domain showed another biological function that was described as the vasoactive peptide vasotab [11]. Some Kazal-type serine protease inhibitors identified in blood-sucking animals are powerful inhibitors of thrombin and other blood coagulation factor, acting to prevent clotting during host blood sucking and digestion. The first description of a Kazal-type inhibitor with anticoagulant activity

Abbreviations: APTT, activated partial thromboplastin time; PT, prothrombin time; rAaTI, recombinant *A. aegypti* thrombin inhibitor; rAaTIΔ, truncated recombinant *A. aegypti* thrombin inhibitor; TT, thrombin time.

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was the thrombin inhibitor rhodniin from the insect *Rhodnius prolixus* [12], followed by dipetalogastin from the insect *Dipetalogaster maximus* [13]. Moreover, our group contributed in describing the thrombin inhibitor infestin 1–2 [14] and a specific factor XIIa inhibitor, infestin 4 [15] from the kissing bug *Triatoma infestans* midgut, both belonging to the Kazal family.

Thrombin is a serine protease, being a key enzyme of the blood coagulation cascade and also an important platelet aggregation activator. This enzyme is peculiar because it has two important regulatory regions besides the active site, exosites 1 and 2, which are binding sites for fibrinogen and heparin, respectively [16–18]. Thrombin inhibition can occur through interaction with the enzyme active site or with its exosites. Rhodniin, ornithodorin and boophilin inhibition mechanism is similar to hirudin, inhibiting thrombin by interacting with exosite 1 and active site [19–21] bothrojaracin binds to exosite 1 and exosite 2 [22], triabin binds only to the thrombin exosite 1 [23] and haemadin inhibits thrombin by interacting with the exosite 2 and the active site [24].

Previously, we expressed, purified and characterized a putative Kazal-type serine protease inhibitor that is present in different tissues of *A. aegypti*, which was named *A. aegypti* Trypsin Inhibitor (AaTI). In addition, we showed an anticoagulant activity of this inhibitor [1]. In the present work, our aim was the characterization of the AaTI inhibition mechanism for thrombin.

2. Materials and methods

2.1. Cloning and expression of a truncated form of Kazal-type inhibitor from A. aegypti (AaTIΔ)

Cloning and expression of rAaTIΔ (rAaTI form without the C-terminal amino acid sequence, truncated after N-52) was performed as previously described [1]. Briefly, based on AaTI sequence (GenBank accession number DQ440176), two gene-specific primers were designed to amplify AaTIΔ gene fragment by PCR: AaTIΔ forward 5′ – AGCTCTCGAGAAAAGAGAGAGAGGAGTTTGTGC – 3′ and AaTIΔ reverse 5′ – TTTTCCTTTTGCGGCCGCTCAGTTGTCACAT GCTTG – 3′. AaTIΔ DNA fragment was amplified using a plasmid construction containing AaTI DNA fragment. The PCR-amplified product was cloned into pPIC9 vector (Invitrogen, Carlsbad, CA, USA) and the sequence was confirmed by DNA sequencing using "DYEnamic ET* Terminator Cycle Sequencing" kit (GE Healthcare, Chalfont St. Giles, UK). Transformed *Picha pastoris* GS 115 strain cells were induced with 0.5% methanol every 24 h in BMMY medium. After 120 h, yeast cell cultures were harvested and the supernatant (containing secreted rAaTIΔ) was stored at −20 °C.

2.2. Synthesis of C-terminal peptide of AaTI

The C-terminal acidic peptide of AaTI (Ac – DNLTDNVNDFIPQEY – NH_2) was synthesized using a solid-phase strategy [25], and it was purified by preparative reverse phase high performance liquid chromatography on a Vydac C_{18} column. The purified peptide was characterized by mass spectrometry (LC/ESI-MS) and amino acid analysis.

2.3. Recombinant AaTIΔ purification

Recombinant protein was purified from cell culture supernatant by affinity chromatography on a trypsin–Sepharose column. Fractions were eluted with a 0.5 M KCl/0.01 M HCl solution pH 2.0. The fractions containing inhibitory activity towards trypsin were pooled, lyophilized and analyzed by SDS-PAGE on 15% polyacrilamide gels [26]. The active material from the trypsin–Sepharose column was applied onto a Superdex 75 column, a gel filtration chromatography, and the isocratic elution was made in 50 mM Tris/HCl buffer with 0.15 M NaCl pH 8.0. To determine the N-terminal amino acid sequence, the rAaTI purified by affinity chromatography was loaded onto a reverse phase chromatography column (C_8 Sephasil Peptide) connected to 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 by the Bradford method [27].

2.4. rAaTIΔ inhibitory characterization

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. Trypsin, plasmin, or thrombin was separately pre-incubated with different concentrations of rAaTIΔ for 10 min at 37 °C. Then, the enzyme residual activity was tested by addition of chromogenic substrate (100 μM). Tosyl-Gly-Pro-Arg-pNa (Sigma), S2251 (HD-Val-Leu-Lys-pNa – Chromogenix), S2238 (HD-Phe-Pip-Arg-pNa – Chromogenix), respectively, were used as chromogenic substrates. The reaction mixture was incubated at 37 °C for 10 min and the absorbance at 405 nm was determined using a Synergy HT microplate reader (BioTek).

2.5. Thrombin inhibition assay using rAaTI

To determine inhibitory activity against thrombin, six different concentrations of S2238 – HD-Phe-Pip-Arg-pNa (50, 80, 100, 150, 200 and 500 μM) in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 0.1% Triton X-100 were used. For each substrate concentration, rAaTI was used in increasing concentrations (0, 5.2, 13.0, 20.8, 26.0, 52.0, 130.0 and 180.0 nM) and 0.5 U of thrombin in a total volume of 100 μL. The reaction was started by the addition of thrombin. The absorbance at 405 nm was measured during 10 min at 37 °C. The amount of *p*-nitroaniline was calculated using a molar extinction coefficient of 9960. The enzyme activity was calculated as mmol of *p*-nitroaniline/min. K_i was calculated using the Cornish–Bowden plot ([S]/V × [I]) [28,29].

2.6. Thrombin time

Thrombin time (TT) was measured by pre-incubating 70 μL of bovine fibrinogen (3.4 mg/mL) and 70 μL of rAaTI (1.3 μM), which was previously expressed, AaTI synthetic peptide (722 nM) or rAaTIΔ (880 nM) for 1 min at 37 °C. The reaction was activated by addition of 70 μL of bovine thrombin (5 U/mL), and the clotting time was measured using the coagulometer.

2.7. Competition assay for thrombin exosite I

Thrombin (106 pM) was pre-incubated with rAaTI (1.04 μM) in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 0.1% Triton X-100 for 10 min at 37 °C. After incubation, different concentrations of hirudin were added to the thrombin and rAaTI mix, and the residual activity was measured using the fluorogenic substrate Benzoyl-Phe-Val-Arg-AMC (80 μM). The fluorescence was measured at $\lambda_{em} = 460$ nm and $\lambda_{ex} = 380$ nm (emission and excitation wavelengths, respectively) in a Synergy HT microplate reader (BioTek). As a control, the same assay was performed in the absence of rAaTI.

To verify if hirudin interferes in thrombin inhibition by rAaTI, the enzyme (106 pM) was pre-incubated with hirudin (106 pM) for 10 min at 37 °C. Then, different concentrations of rAaTI were added and incubated for 10 min at 37 °C. The reaction was monitored after addition of fluorogenic substrate Benzoyl-Phe-Val-Arg-AMC (80 μM). As a control, the same assay was performed without hirudin.

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                                                P1P1´
rhod_D1        -EGGEPCACPHALHRVCGSDGETYSNPCTLNCAKFNGK---PELVKVHDGPCE---PDEDE------ 54
inf_D1         LEEND-CACPRVLHRVCGSDGNTYSNPCTLDCAKHEGK---PDLVQVHEGPCD---PNDHDFE---- 56
LDTI-5T        ---KKVCACPRVLSLVCGSDGRTYANSCIARCN---------GVSIKSEGSCP---TGILN------ 46
AaTI           --ERGVCACPRIYMPVCGSNLKTYNNDCLLRCEINSDLGRANNLRKIADQACDNLTDNVNDFIPQEY 65
AaTI*          --ERGVCACPRIYMPVCGSNLKTYNNDCLLRCEINSDLGRANNLRKIADQACDN------------- 52
C-term           ------------------------------------------------DNLTDNVNDFIPQEY 14
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Fig. 1. Alignment of amino acid sequences of AaTI, AaTIΔ and C-terminal peptide with other thrombin inhibitor of Kazal-type family. Rhod_D1, rhodniin first domain [12]; inf_D1, infestin first domain [14]; LDTI-5T, leech derived tryptase inhibitor mutant 5T – thrombin inhibitor [35]. All identical amino acid residues are shaded in light grey. AaTI amino acid residue that is identical to one or more of other sequence is shaded in dark grey.

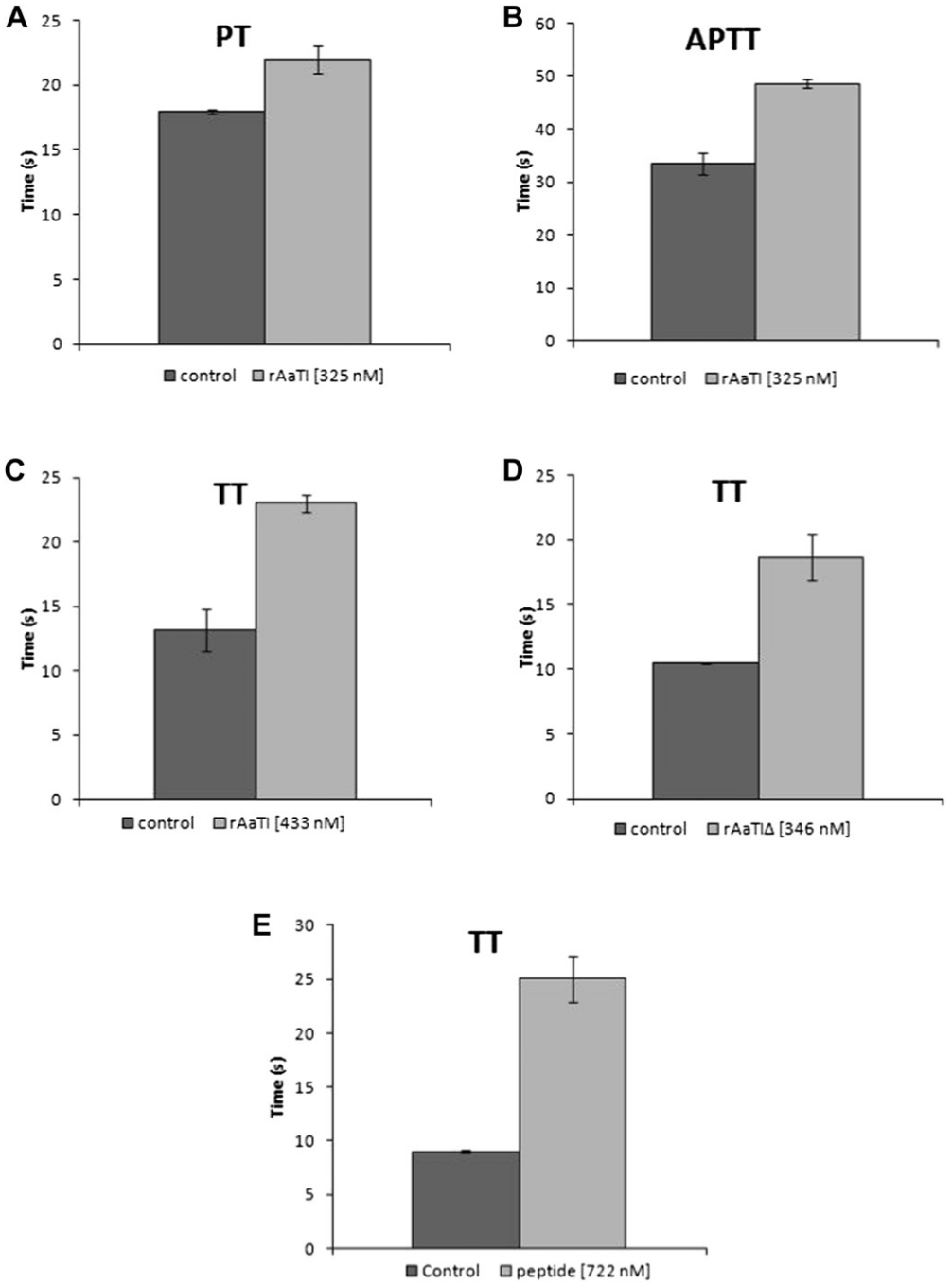

Fig. 2. Coagulation assays. (A) Prothrombin Time (PT). rAaTI (325 nM) was used in prothrombin time. (B) Activated Partial Thromboplastin Time (APTT). The assay was performed using 325 nM rAaTI. (C) Thrombin Time (TT) using rAaTI (433 nM). (D) Thrombin Time using rAaTIΔ (346 nM). (E) Thrombin Time using peptide (722 nM). Results are the mean of triplicate points ± standard deviation.

2.8. Competition assay for thrombin exosite II

To verify whether heparin and antithrombin III binding to thrombin interfered in thrombin inhibition by rAaTI, thrombin (106 pM) was pre-incubated with heparin (1 ng/mL) and antithrombin III (167 pM) for 10 min at 37 °C. Afterwards, different concentrations of rAaTI were added to the mix and incubated for 10 min at 37 °C. The enzyme residual activity was measured using the fluorogenic substrate Benzoyl-Phe-Val-Arg-AMC (80 μM). A thrombin inhibition curve was also constructed using thrombin in low concentration (2.12 pM) and rAaTI concentrations between 52 nM and 1300 nM. The residual activity towards the fluorogenic substrate was monitored using the fluorogenic substrate and the reaction was monitored using the excitation wavelength of 380 nm and emission measured at 460 nm for 20 min at 37 °C.

2.9. Inhibition assay for thrombins

Alpha-thrombin (0.025 U) or gamma-thrombin (1 μg) was pre-incubated with rAaTI (0.38–1.56 μM) in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 0.1% Triton X-100 for 15 min at 37 °C. After incubation at the same conditions (20 min), the residual activity was measured using the fluorogenic substrate Benzoyl-Phe-Val-Arg-AMC (200 μM). The fluorescence was measured at λ_{em} = 460 nm and λ_{ex} = 380 nm (emission and excitation wavelengths, respectively) in a Synergy HT microplate reader (BioTek). As a control, the same assay was performed in the absence of rAaTI.

3. Results and discussion

In this work we described the cloning, expression, purification and characterization of the AaTIΔ (AaTI without C-terminal acidic peptide). rAaTI was previously expressed, purified and characterized, and it displayed anticoagulant activity [1]. Multiple alignments of AaTI, AaTIΔ, C-terminal acidic peptide with other Kazal-type thrombin inhibitors revealed high similarity in the molecule core and also in the C-terminal end (Fig. 1). Thrombin, a key enzyme of blood coagulation cascade, possesses three functional domains: the active site, the exosite 1 and the exosite 2. The thrombin inhibitors have different mechanisms of inhibition. While hirudin [30], rhodniin [31], ornithodorin [21], savignin [32], dipetalogastin [13], boophilin [20] interact with both active site and exosite 1; triabin [33] binds only to exosite 1; haemadin [24] binds to exosite 2 and active site, and bothrojaracin [22] binds to both exosites but it does not bind to the active site.

In order to confirm the AaTI inhibitory mechanism for thrombin, we cloned the truncated form of *A. aegypti* Kazal-type serine protease inhibitor gene fragment (AaTIΔ) which encodes a protein of 5.8 kDa and theoretical pI 7.8. rAaTIΔ cloning and expression was performed as previously described for the full length protein [1]. The fragment sequence was verified by DNA sequencing, and rAaTIΔ was expressed in *P. pastoris* with an yield of 1.1 mg/L of culture medium.

rAaTIΔ, expressed in the *P. pastoris* system, was purified from the culture supernatant using trypsin–Sepharose affinity chromatography. The fractions containing inhibitory activity against trypsin were pooled, and submitted to SDS-PAGE. The eluted fractions of affinity chromatography were applied in a gel filtration

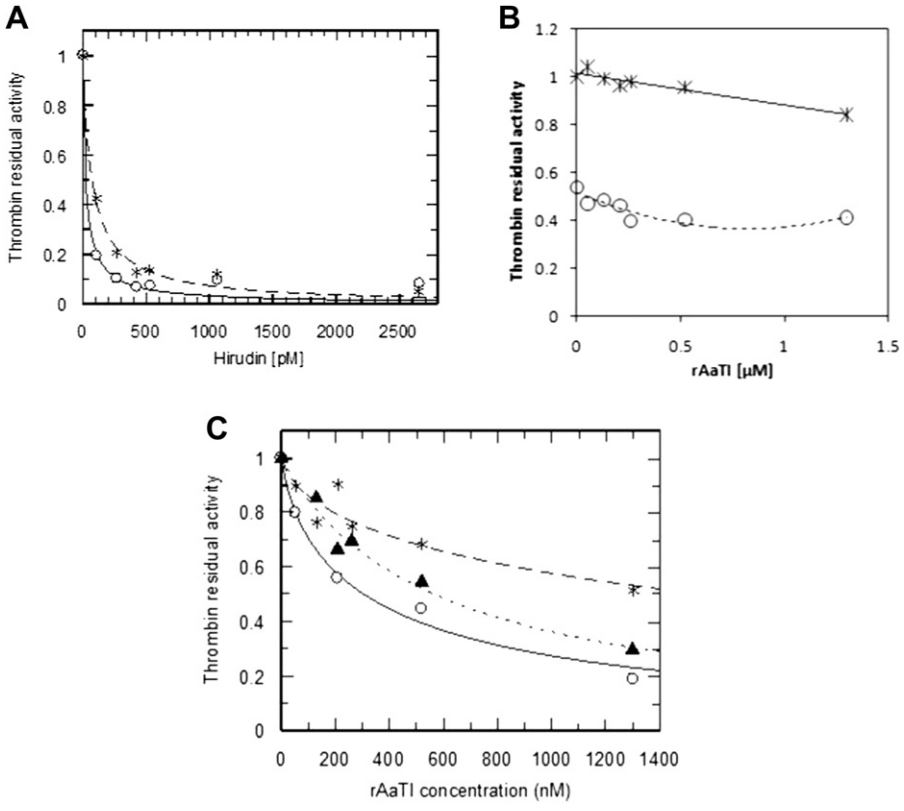

Fig. 3. Exosites competition assays. Exosite 1 competition assay. (A) Hirudin inhibitory activity against thrombin in the presence of rAaTI (1.04 μM) (-○-) and without rAaTI (-∗-). (B) rAaTI inhibitory activity against thrombin without hirudin (-∗-) and in the presence of hirudin (106 pM) (-○-). In rAaTI inhibitory activity against thrombin it was used an hirudin concentration able to inhibit 50% of thrombin to verify if its binding in exosite I could interfere in rAaTI activity. Exosite 2 competition assay. (C) rAaTI inhibitory activity in the presence and in the absence of antithrombin III and heparin. First, it was verified if rAaTI inhibitory activity against thrombin 106 pM (-∗-), then thrombin (106 pM) was inhibited by antithrombin (167 pM) in the presence of heparin (1 ng/L) (-○-). In order to simulate free thrombin (not inhibited by antithrombin), thrombin (2.12 pM) was used in the inhibitory assay (-▲-).

chromatography on a Superdex 75 column. Purified rAaTIΔ only inhibited trypsin and plasmin activities. Previous results showed that rAaTI weakly inhibited thrombin amidolytic activity while rAaTIΔ did not, suggesting that C-terminal charged peptide in rAaTIΔ may have an important role in thrombin inhibition, which negatively affects binding strength, if the AaTI has a bidentate interaction with the enzyme.

rAaTI showed similar activities to anticoagulants, containing a C-terminal charged peptide, the same as for other thrombin inhibitors, like hirudin [30], dipetalogastin [13] and rhodniin [31], suggesting that C-terminal region might be important to the rAaTI activity. rAaTI was tested in coagulation assays, and prolonged APTT, PT and TT (Fig. 2A–C) [1], which suggested that it acted as a thrombin inhibitor. C-terminal peptide and rAaTIΔ also affected the thrombin time showing that both parts of AaTI may have an important role in thrombin inhibition (Fig. 2D–E). Thrombin Time observed behavior can result from steric hindering of substrate binding. rAaTIΔ could also prolong thrombin time similarly to rAaTI, suggesting that it may bind to another site than the thrombin active site, which would allow the synthetic substrate hydrolysis but impairing fibrinogen cleavage. rAaTI and its truncated form were also tested in a thrombin-induced platelet aggregation assay, but none of them affected this process. This fact could be explained by rAaTI not binding directly to the exosite 1. This result was confirmed by exosite 1 competition assay, which showed that neither hirudin could affect the rAaTI inhibition nor rAaTI affected the hirudin inhibition of thrombin (Fig. 3A and B). In the exosite 2 competition assay, when thrombin (106 pM) was previously inhibited by antithrombin III in the presence of heparin, resulting in ~4% residual activity of thrombin (~2.0 pM) (data not shown), the inhibitory activity of rAaTI seemed to be higher when compared to the rAaTI inhibitory activity against thrombin 106 pM, showing that it weakly inhibits thrombin. In order to simulate the free thrombin (not inhibited by antithrombin III), thrombin 2.12 pM was used (Fig. 3C), and we verified that the rAaTI inhibitory activity was similar in both cases. This result suggested that rAaTI may bind to the same region where antithrombin III or heparin binds on the thrombin surface. Classical inhibition experiments showed an uncompetitive inhibition mechanism for rAaTI and thrombin (Fig. 4). This result suggests that rAaTI interacts with the enzyme–substrate complex, decreasing both K_m and V_{app}. K_i was calculated using the Cornish–Bowden plot ([S]/v × [I]), and its value was 320 nM.

In an attempt to verify the importance of thrombin exosite 1 in the rAaTI inhibitory activity, it was performed an inhibition assay using

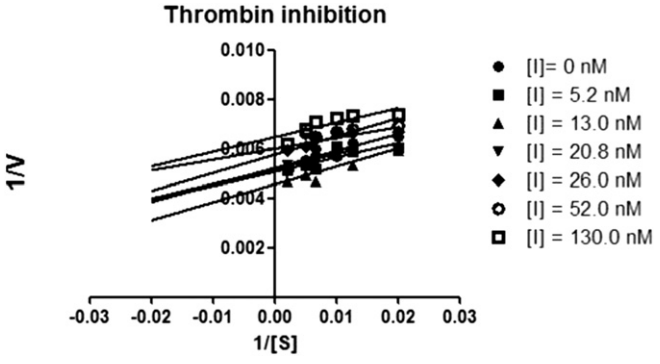

Fig. 4. rAaTI inhibition curve for thrombin. The experiment was composed by six curves using different concentrations of S2238 (50, 80, 100, 150, 200 and 500 μM). For each curve, rAaTI was used in increasing concentrations (0, 5.2, 13.0, 20.8, 26.0, 52.0, 130.0 and 180.0 nM) and thrombin 2.5 U/mL. Reactions were started by addition of thrombin to substrate-inhibitor mixture and K_i was calculated using the Cornish–Bowden plot ([S]/V × [I]).

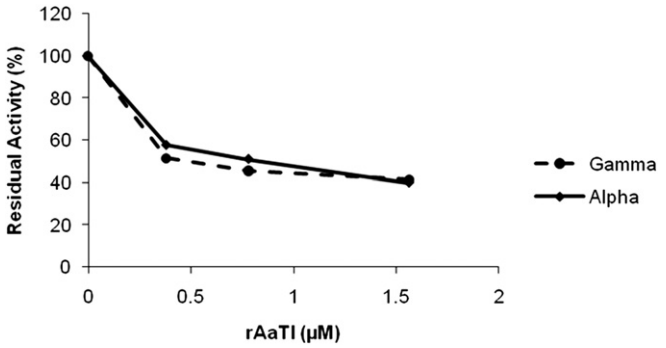

Fig. 5. rAaTI inhibition curve for alpha- and gamma-thrombin. Alpha-thrombin (0.025 U) or gamma-thrombin (1 μg) was pre-incubated with rAaTI different concentrations (0.38–1.56 μM) in 0.1 M tris buffer pH 8.0 for 15 min at 37 °C. The enzymatic activity was measured by addition of fluorogenic substrate, Benzoyl-Phe-Val-Arg-AMC (0.2 mM) and incubation for 20 min at 37 °C.

gamma-thrombin, an enzyme which has only the active site and exosite 2 [34]. Our results showed that the absence of exosite 1 did not interfere in the gamma-thrombin by rAaTI suggesting that the inhibitor does not require this site to inhibit alpha-thrombin (Fig. 5).

In addition, the AaTI transcript is found in female salivary gland and gut, suggesting also a possible role as a gut anticoagulant molecule.

On the basis of our results, we can conclude that AaTI is a serine protease inhibitor with thrombin anticoagulant activity, and it weakly interacts with the enzyme at a region other than the active site, probably to the exosite 2 of thrombin but not to the exosite 1 and may be stabilized by the C-terminal tail by electrostatic interactions.

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