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RESEARCH ARTICLE

Dimiconin, a novel coagulation inhibitor from the kissing bug, *Triatoma dimidiata*, a vector of Chagas disease

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SUMMARY

Sequence analysis of a *Triatoma dimidiata* salivary gland cDNA library resulted in the identification of two transcripts (Td60 and Td101) homologous to triabin, an inhibitor of thrombin in *Triatoma pallidipennis* saliva. In the present study, a recombinant protein of Td60, designated dimiconin, was expressed in *Escherichia coli* and its activity was characterized. The resulting protein inhibited the intrinsic but not extrinsic blood coagulation pathway, suggesting that dimiconin is not a thrombin inhibitor. Measurement of the enzymatic activity of coagulation factors using chromogenic substrates revealed that dimiconin efficiently inhibited factor XIIa (FXIIa) activity in a dose-dependent manner. In addition, pre-incubation of dimiconin with FXII effectively inhibited FXIIa activity whereas dimiconin did not affect already activated FXIIa, indicating that dimiconin inhibits the activation of FXII but not the enzymatic activity of FXIIa. These results show that dimiconin is an inhibitor of the contact phase initiated by FXII activation in the blood coagulation cascade, which differs from the bioactivity of triabin.

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INTRODUCTION

Hematophagous insects have evolved a wide set of pharmacologically active molecules to counteract host hemostatic processes (Ribeiro, 1995; Ribeiro and Francischetti, 2003; Fontaine et al., 2011). When probing in the host skin for blood feeding, they inject saliva, a cocktail of bioactive agents containing anticoagulants, vasodilators, and inhibitors of platelet aggregation induced by collagen, adenosine diphosphate (ADP), arachidonic acid or thrombin (Ribeiro, 1995; Ribeiro and Francischetti, 2003; Champagne, 2005; Andersen et al., 2005; Valenzuela, 2005). Other salivary molecules almost certainly involved in the feeding process include antihistamine, sialidase, serine protease, sodium channel blockers, immunosuppressants and pore-forming molecules (Ribeiro, 1995; Ribeiro and Francischetti, 2003; Champagne, 2005; Andersen et al., 2005; Valenzuela, 2005). As hematophagous arthropods have evolved their feeding strategy independently, different types of molecules have developed to overcome host hemostatic defenses in different species (Ribeiro, 1995; Champagne, 2005). Therefore, insect salivary components have been extensively studied in various species to understand their unique physiological activities and have attracted attention as novel candidates for natural pharmacological agents (Ribeiro, 1995; Champagne, 2005; Fontaine et al., 2011).

Triatomine bugs are large blood-sucking insects, and species belonging to the tribes Triatomini and Rhodniini are involved in the transmission of *Trypanosoma cruzi*, a causative agent of Chagas disease in Latin America (Beard, 2005). They feed directly from the blood vessel for 20–30 min efficiently regardless of the host hemostatic response, suggesting the presence of strong and unique bioactive substances in their saliva (Martínez-Ibarra et al., 2001). To discover unique pharmacologically active agents, salivary components of triatomine bugs have been explored in *Rhodnius prolixus* (Ribeiro et al., 2004), *Triatoma brasiliensis* (Santos et al., 2007), *Triatoma infestans* (Assumpção et al., 2008), *Triatoma dimidiata* (Kato et al., 2010) and *Dipetalogaster maxima* (Assumpção et al., 2011) by transcriptome analyses of the salivary gland. Characteristically, their salivary components were found to be rich in lipocalins, a large group of extracellular proteins that bind and transport small hydrophobic molecules. Lipocalins are remarkably diverse at the sequence level; however, they share sufficient similarity in the form of short characteristic conserved sequence motifs, and their structure is highly conserved (Flower, 1996). Triabin is a lipocalin protein identified from the saliva of *Triatoma pallidipennis* and functions as an inhibitor of thrombin, resulting in the inhibition of thrombin-induced platelet aggregation as well as blood coagulation (Noeske-Jungblut et al., 1995). Various lipocalins showing homology with triabin have been identified from salivary transcripts of triatomine bugs (Ribeiro et al., 2004; Santos et al., 2007; Assumpção et al., 2008; Kato et al., 2010; Assumpção

et al., 2011), and some of them have been functionally characterized: pallidipin 2 as a collagen-induced platelet aggregation inhibitor in *T. pallidipennis* (Noeske-Jungblut et al., 1994), *R. prolixus* aggregation inhibitor 1 (RPAI-1) as a platelet aggregation inhibitor in *R. prolixus* (Francischetti et al., 2000), and triafestin-1 and -2 as plasma kallikrein–kinin system inhibitors in *T. infestans* (Isawa et al., 2007).

Recently, a transcriptome-based analysis of the salivary gland of *T. dimidiata* resulted in the identification of transcripts homologous to *T. pallidipennis* triabin, an inhibitor of thrombin activity (Kato et al., 2010). In the present study, a recombinant *T. dimidiata* triabin-like protein (designated 'dimiconin') was prepared and its activity was characterized.

MATERIALS AND METHODS

Sequence analysis

The sequences were aligned with CLUSTAL W software (Thompson et al., 1994) and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura et al., 2007). Phylogenetic trees were constructed by the neighbor-joining method with the distance algorithms in the MEGA package. Bootstrap values were determined with 1000 replicates of the data sets.

Production and purification of recombinant dimiconin

A DNA fragment encoding a mature dimiconin was amplified and inserted into the *Xho*I site of an N-terminal thioredoxin (Trx)–hexahistidine (His)-tagged fusion plasmid vector, pET-32b(+) (Novagen, Drams, Germany). The *Xho*I adaptor-ligated primers used for PCR amplification of the dimiconin encoding fragments were Xho-Td60-S (5′-ttctcgagGAACACTGTGCACAAGAAAA-3′) and Xho-Td60-R (5′-ttctcgagTCACTTTAATTGAAATGATA-3′). *Escherichia coli* BL21 (DE3) cells were transformed with the recombinant plasmid and grown in Luria-Bertani (LB) medium containing ampicillin (50 μg ml^{-1}). Production of the Trx-His-tagged recombinant dimiconin was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mmol l^{-1}. The BL21 cells suspended in binding buffer (20 mmol l^{-1} sodium phosphate, 500 mmol l^{-1} NaCl, 20 mmol l^{-1} imidazol, pH 7.4) were sonicated, and recombinant protein was purified from the soluble fraction using His GraviTrap (GE Healthcare, Buckinghamshire, UK) and finally dialyzed against phosphate-buffered saline (PBS). Trx-His-tagged protein only was expressed and purified to be used as a control.

SDS-PAGE and immunoblot analysis

The samples were treated with sodium dodecyl sulfate (SDS) sample buffer [125 mmol l^{-1} Tris-HCl (pH 6.8), 4.5% SDS, 20% glycerol, 0.01% Bromophenol Blue and 10% 2-mercaptoethanol] and analyzed in a 10% polyacrylamide gel. To estimate the molecular mass of the samples, Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used. After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

For the immunoblot analysis, the proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% skimmed milk in PBS for 1 h at room temperature, the membrane was incubated overnight at 4°C with mouse anti-His antibody (GE Healthcare) or serum from a mouse repeatedly subjected to blood-sucking by *T. dimidiata*. After three washes with PBS containing 0.1% Tween 20 (PBS-T), the membrane was further incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse immunoglobulin (Zymed Laboratories, San Francisco, CA, USA) for 1 h at room temperature. After another three washes with PBS-T, the blots were developed by adding a substrate (Alkaline Phosphatase Conjugate Substrate Kit; Bio-Rad Laboratories) and visualized.

Effect of dimiconin on plasma coagulation

The effect of dimiconin on plasma coagulation was tested by examining prothrombin time (PT) and activated partial thromboplastin time (APTT). Forty-five microliters of citrated normal human plasma was mixed with 5 μl of Trx-His-tagged dimiconin or Trx-His-tag protein with a final concentration of 45, 22.5, 11.3 or 5.6 μmol l^{-1} and incubated for 3 min at 37°C. Plasma coagulation was activated for 3 min at 37°C with 100 μl of PT reagent (thromboplastin from rabbit brain; Sysmex, Hyogo, Japan) for the PT assay, or for 1 min at 37°C with 50 μl of APTT reagent (synthetic phospholipid; Sysmex) followed by 50 μl of 0.02 mol l^{-1} $CaCl_2$ for 2 min at 37°C for the APTT assay. The clot formation was measured using a CA-50 coagulometer (Sysmex).

Effect of dimiconin on the intrinsic pathway of blood coagulation

The effect of dimiconin on the intrinsic coagulation pathway was assessed based on the generation of activated coagulation factors (FIXa, FXa and FXIIa). Fifty microliters of citrated human plasma, diluted 1:9 in 20 mmol l^{-1} Hepes buffer (pH 7.35), was pre-incubated for 5 min at 37°C with 15 μl of serially diluted dimiconin. The mixture was activated by adding 5 μl of APTT reagent and incubating for 10 min at 37°C, followed by the addition of 5 μl of 0.02 mol l^{-1} $CaCl_2$. After 1 min at 37°C, 25 μl of chromogenic substrate was added to give a final concentration of 0.5 mmol l^{-1} (Decrem et al., 2009), and the amidolytic activity of the enzyme generated was determined at a wavelength of 405 nm using a microplate reader (iMark; Bio-Rad Laboratories). The chromogenic substrates used were as follows: SPECTROZYME FIXa (American Diagnostica, Greenwich, CT, USA) for FIXa, SPECTROZYME FXa (American Diagnostica) for FXa, SPECTROZYME FXIIa (American Diagnostica) for FXIIa and SPECTROZYME P.Kal (American Diagnostica) for kallikrein assay. Soybean trypsin inhibitor, an inhibitor of plasma kallikrein (SBTI; Wako Pure Chemical Industries, Osaka, Japan) was added for the FXIIa assay to give a final concentration of 20 nmol l^{-1} (Isawa et al., 2007). In a separate study, the effect of *T. dimidiata* salivary gland homogenate (SGH) on FXII was addressed.

Effect of dimiconin on FXII

The effect of dimiconin on FXII activation was assessed as follows: human FXII (final concentration, 0.2 μmol l^{-1}) (Haematologic Technologies, Inc., Essex Junction, VT, USA) was pre-incubated with serially diluted dimiconin for 5 min at 37°C in the presence of $ZnCl_2$ (0.5 mmol l^{-1}) and activated by the addition of 5 μl of APTT reagent (Sysmex) for 5 min at 37°C. The effect of dimiconin on the enzymatic activity of FXIIa was assessed as follows: human FXII (0.2 μmol l^{-1}) (Haematologic Technologies, Inc.) was activated with 5 μl of APTT reagent (Sysmex) for 5 min at 37°C in the presence of $ZnCl_2$ (0.5 mmol l^{-1}) and then incubated with serially diluted dimiconin for 5 min at 37°C. The activity of FXIIa was measured using the chromogenic substrate SPECTROZYME FXIIa (American Diagnostica).

RESULTS

Sequence analysis

By sequencing a *T. dimidiata* salivary gland cDNA library, we identified two transcripts (Td60 and Td101) coding for proteins

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CTCACAACATGAAGACGATCATTGTAGTGACAATTTTTGGAATCCTGACATGTGCATATC      60
        M  K  T  I  I  V  V  T  I  F  G  I  L  T  C  A  Y         17

CAACAGACGGTGAACACTGTGCACAAGAAAAAGCTATGGAGGACTTTGATCCTTCACGGT     120
P  T  D  G  E  H  C  A  Q  E  K  A  M  E  D  F  D  P  S  R      37

TTTTCAATGGAAAGTGGTATGTGGTTCATTATGGGAAGACAGGTCCAACTGTCTGTCAGA     180
F  F  N  G  K  W  Y  V  V  H  Y  G  K  T  G  P  T  V  C  Q      57

AATTTAGTACTAATGGAACCCAAGGTGCCCCTACCCAAATTGTTGAAACTGGCTACGACA     240
K  F  S  T  N  G  T  Q  G  A  P  T  Q  I  V  E  T  G  Y  D      77

AATTTGAAGATTACTTGAAATTCCAATGTGATGAAACTGGAAAAAAAAAATGACTATCATT     300
K  F  E  D  Y  L  K  F  Q  C  D  E  T  G  K  K  N  D  Y  H      97

ATTCTTTCAAATGCAAAAGTTATGAGTGTGGTAGTGATAATATTGAATTCGAAGTAGATT     360
Y  S  F  K  C  K  S  Y  E  C  G  S  D  N  I  E  F  E  V  D     117

TTACAGTTCTTAGTGCAAGCTATGATGACTTTGCCCTAGTTTGTAGAACTATCACATTTA     420
F  T  V  L  S  A  S  Y  D  D  F  A  L  V  C  R  T  I  T  F     137

CATCGGGTACTAAGGATAAGGATGATGAGGTATTGGTCTTAGAACGCGAGAAAACTCTTG     480
T  S  G  T  K  D  K  D  D  E  V  L  V  L  E  R  E  K  T  L     157

ATGTTATTGACAATTGTAGAAGAACATATTATCTAACTGAATTTGAAAAAATGTCCTTAT     540
D  V  I  D  N  C  R  R  T  Y  Y  L  T  E  F  E  K  M  S  L     177

CTTCCCAATTTTTATCGAAAAAGGAAAATATAACGATGTTATCATTTCAATTAAAGTGAC     600
S  S  Q  F  L  S  K  K  E  N  I  T  M  L  S  F  Q  L  K  *     196

TAGATAAATCTGGTTTAA                                             618
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Fig. 1. Nucleotide and deduced amino acid sequences of Td60, encoding a triabin-like protein from *T. dimidiata* salivary glands. The deduced amino acid sequence is shown by the single-letter code under the nucleotide sequence. The putative signal peptide is underlined.

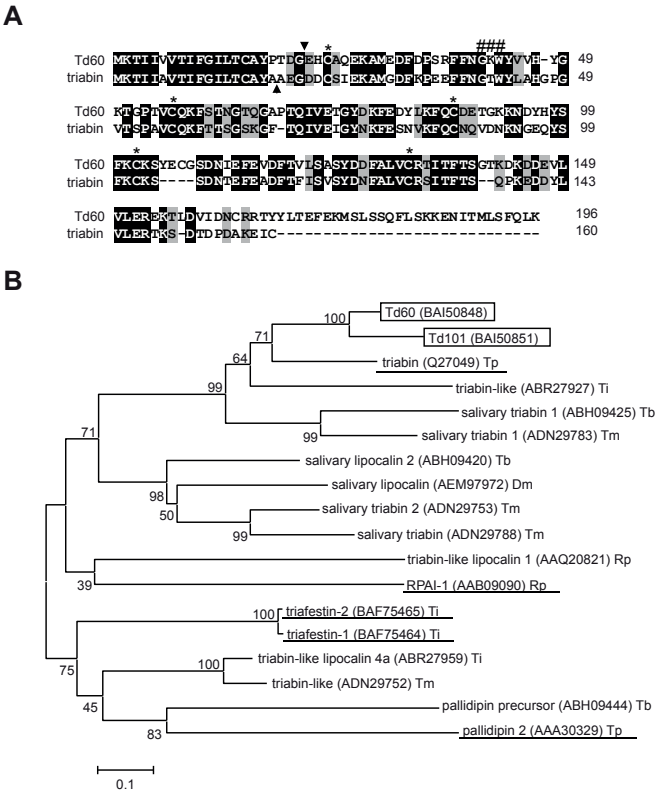

Fig. 2. (A) Sequence alignment of the salivary triabin-like protein Td60 from *T. dimidiata*, together with triabin from *T. pallidipennis*. Black- and grey-shaded amino acids represent identical and conserved residues, respectively. Dashes indicate gaps introduced for maximal alignment. Asterisks at the top of the amino acids denote conserved cysteine residues, and the GXW motif is indicated by ###. The putative signal peptide cleavage sites are shown by arrowheads. (B) Phylogenetic tree of triabin-like protein sequences from different triatomine bugs. Triabin-like protein sequences of *T. dimidiata* (Td60 and Td101) were aligned with those of representative triatomine triabin-like proteins obtained from a non-redundant protein database, and a phylogenetic tree was constructed. The functionally characterized proteins are underlined. The sequences from the database are represented by '(GenBank accession number) abbreviation of the species name'. Ti, *T. infestans*; Tb, *T. brasiliensis*; Tp, *T. pallidipennis*; Tm, *T. matogrossensis*; Rp, *R. prolixus*; Dm, *D. maxima*. The scale bar represents 0.1% divergence. Bootstrap values are shown above or below branches.

homologous to triabin, a selective thrombin inhibitor from the saliva of *T. pallidipennis* (Noeske-Jungblut et al., 1995; Fuentes-Prior et al., 1997). The first transcript (Td60) coded for a protein of 196 amino acid residues containing a 21-amino-acid signal peptide with a predicted molecular mass of 20.3 kDa and a calculated isoelectric point of 4.96 in the mature form (GenBank accession no. BAI50848) (Fig. 1). The second transcript (Td101) coded for a protein of 193 amino acid residues with a predicted molecular mass of 20.0 kDa and a calculated isoelectric point of 5.51 in the mature form (GenBank accession no. BAI50851). The amino acid sequences of Td60 and Td101 shared 62% and 58% identity with triabin, respectively. The amino acid sequence of Td60 was aligned with that of triabin. The GXW motif, which is often observed in lipocalins, was conserved in Td60, and five cysteine residues in the putative mature proteins were located at the corresponding positions (Fig. 2A). A phylogenetic analysis was performed with salivary proteins homologous to triabin from various triatomine bugs. Of these, triabin, pallidipin 2, RPAI-1 and triafestin-1 and -2 were functionally characterized; others were proteins with unknown function, although most of them were named as triabin-like on the basis of their sequence similarities. Td60 and Td101 showed closer relationships with triabin than any other triabin-like salivary proteins from various triatomine bugs including functionally characterized proteins such as pallidipin 2, RPAI-1 and triafestins (Fig. 2B). A triabin-like protein from *T. dimidiata*, Td60, was designated 'dimiconin' and its biological function was analyzed.

Production and purification of recombinant dimiconin

To characterize the biological function of dimiconin, the recombinant protein was expressed in *E. coli* as a Trx-His-tagged fusion protein and purified from the soluble fraction of the *E. coli* lysate. Trx-His-tagged dimiconin had a molecular mass of approximately 37 kDa according to polyacrylamide gel electrophoresis and reacted to anti-His antibody in the immunoblot analysis (Fig. 3A,B). The antigenicity of Trx-His-tagged dimiconin was assessed by immunoblotting with serum from a mouse repeatedly exposed to *T. dimidiata*. The immune serum reacted to the Trx-His-tagged dimiconin but not the Trx-His-tagged protein, indicating that the recombinant protein maintained the antigenicity of a *T. dimidiata* salivary protein (Fig. 3C).

Dimiconin inhibits the activation of the contact phase of the intrinsic blood coagulation pathway

Triabin inhibits thrombin activity, resulting in inhibition of thrombin-induced platelet aggregation as well as blood coagulation (Noeske-Jungblut et al., 1995). To investigate the biological function of dimiconin, its effect on plasma coagulation was examined by measuring PT and APTT. Dimiconin prolonged APTT in a dose-dependent manner (Fig. 4) but did not affect PT. This result indicated that dimiconin is an inhibitor of intrinsic blood coagulation, which is different from the bioactivity of triabin. The most apparent

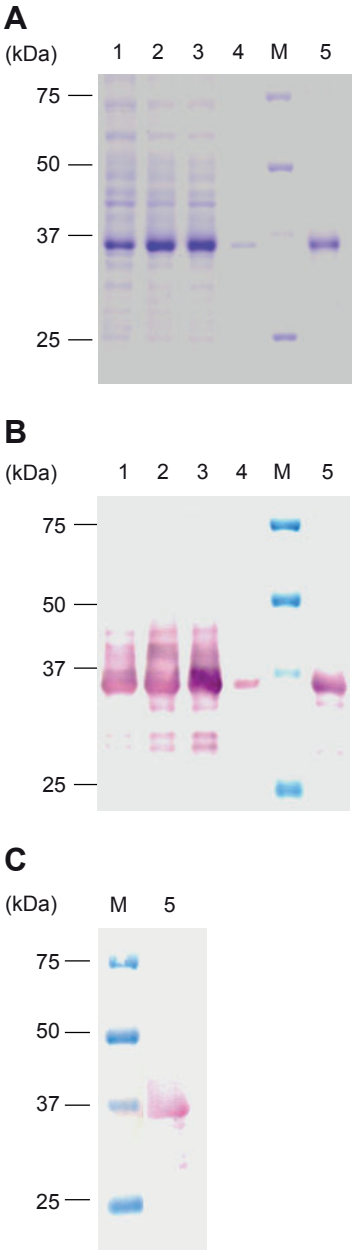

Fig. 3. SDS-PAGE and immunoblot analysis of recombinant dimiconin expressed in *Escherichia coli*. *E. coli* was transformed with Trx-His-dimiconin-expressing plasmid (lanes 1–5), and expression of the recombinant protein was induced by isopropyl β-D-thiogalactoside (IPTG). The whole cell lysates with (lane 2) or without (lane 1) IPTG induction, soluble (lane 3) and insoluble (lane 4) fractions after sonication, and purified protein (lane 5) were subjected to SDS-PAGE analysis (A). Immunoblot analyses were performed with anti-His antibody (B) or serum from a mouse repeatedly exposed to *T. dimidiata* (C). Lane M, protein molecular mass marker.

structural difference between dimiconin and triabin is observed in the C-terminal, where triabin lacks about 30 amino acids (Fig. 2A). A mutant dimiconin lacking 30 C-terminal amino acids (dimiconin ΔC30) was prepared and its activity was investigated. Similar to dimiconin, dimiconin ΔC30 prolonged APTT but not PT (data not shown), indicating that the C-terminal 30 amino acids are not responsible for the difference in activity between dimiconin and triabin.

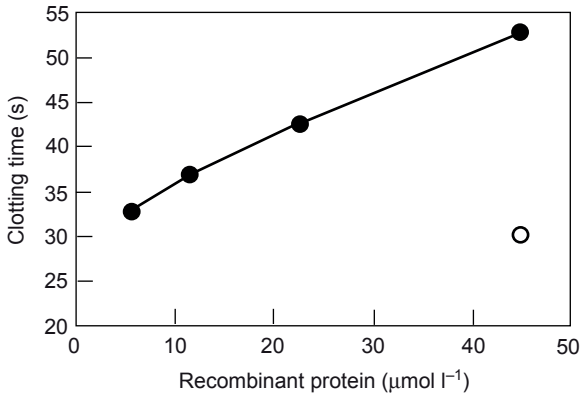

Fig. 4. Inhibition of intrinsic coagulation by recombinant dimiconin. The inhibitory effect of dimiconin on the intrinsic coagulation pathway was examined using the activated partial thromboplastin time (APTT) assay. Citrated human plasma was incubated with Trx-His-dimiconin (●) or Trx-His-tagged protein (○) and activated with APTT reagent. Clot formation was measured using a coagulometer.

The intrinsic blood coagulation pathway is initiated when FXII binds to a negatively charged surface such as that of endothelial cells, and this pathway is associated with factors FVIII, FIV, FXI, FXII, prekallikrein, and high-molecular-weight (HMW) kininogen (Mans and Neitz, 2004; Isawa et al., 2007). To characterize the mechanism involved in the anti-coagulation activity of dimiconin, an amidolytic assay was performed using chromogenic substrates specific to FIXa, FXa, FXIIa and kallikrein. The enzymatic activities of FIXa, FXa and FXIIa were markedly inhibited by dimiconin at higher concentrations, but not by a Trx-His-tagged control (Fig. 5). Dimiconin had a limited impact on kallikrein activity. At lower concentrations, an inhibitory effect was observed on FXIIa activity but the effect on FIXa and FXa activities was weak, suggesting that the primary target of dimiconin is FXII (Fig. 5). The IC_{50} value of dimiconin was estimated to be 35.7 μmol l^{-1} for FIXa, 12.9 μmol l^{-1} for FXa and 8.6 μmol l^{-1} for FXIIa. An inhibitory effect on FXIIa activity was demonstrated in the salivary gland homogenate of *T. dimidiata* (supplementary material Fig. S1). To determine whether dimiconin inhibits the activation of FXII or enzymatic activity of FXIIa, FXII was treated with dimiconin before or after activation and then the activity of FXIIa was measured. As shown in Fig. 6, pre-treatment of FXII with dimiconin inhibited FXIIa activity in a dose-dependent manner (Fig. 6A) whereas dimiconin did not inhibit the activated FXII (Fig. 6B), suggesting that dimiconin inhibits the activation of FXII but not the activity of FXIIa. Platelet aggregation induced by ADP or collagen was not affected by dimiconin (data not shown).

DISCUSSION

Recently, a large number of transcripts from the salivary glands of *T. dimidiata* were sequenced, and transcripts (Td60 and Td101) coding for a lipocalin protein homologous to triabin, a thrombin inhibitor identified from *T. pallidipennis* saliva, were identified (Kato et al., 2010). In the present study, a recombinant protein was produced from the transcript Td60, and its biological activity was characterized. The protein, dimiconin, efficiently inhibited intrinsic blood coagulation by targeting the activation of FXII, indicating that dimiconin plays an important role in the blood-feeding process in *T. dimidiata*.

Lipocalins are a family of extracellular proteins that bind and transport small hydrophobic molecules (Flower, 1996). Although

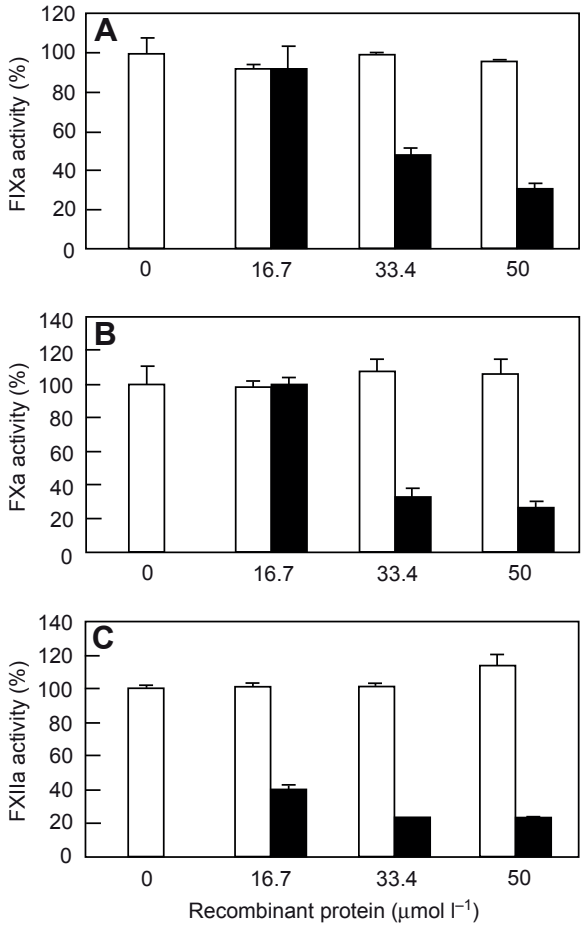

Fig. 5. Inhibitory effects of dimiconin on the enzymatic activity of FIXa, FXa and FXIIa. Citrated human plasma was incubated with various concentrations of dimiconin (■) or Trx-His-tagged protein (□), and activated with APTT reagent. The generated FIXa (A), FXa (B) and FXIIa (C) activities were measured using chromogenic substrates. The results are expressed as the mean for triplicate assays ± s.d.

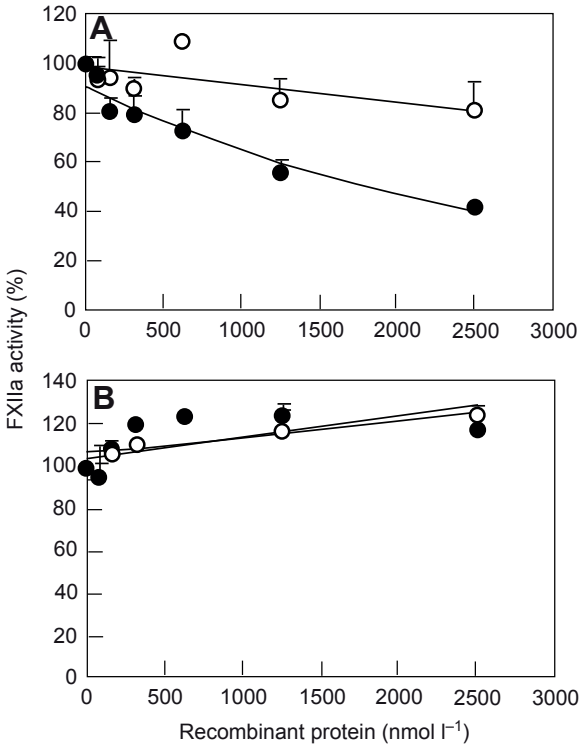

Fig. 6. Inhibitory effect of dimiconin on FXII. FXII was treated with dimiconin (●) or Trx-His-tagged protein (○) before (A) or after (B) activation and then enzymatic activity of FXIIa was measured. The results are expressed as the mean for triplicate assays ± s.d.

they display unusually low levels of overall sequence conservation, with pairwise sequence identity often falling below 20%, their three-dimensional structures are highly conserved (Flower, 1996). To date, several salivary lipocalins showing homology with triabin have been functionally characterized from triatomine bugs; pallidipin 2 from *T. pallidipennis* and RPAI-1 from *R. prolixus* as platelet aggregation inhibitors (Noeske-Jungblut et al., 1994; Francischetti et al., 2000), and triafestins from *T. infestans* as plasma kallikrein–kinin system inhibitors (Isawa et al., 2007). Interestingly, the bioactivities of these proteins were different from that of triabin. Since dimiconin showed much higher homology with triabin than any other 'triabin-like' proteins, we initially expected it to have a similar function to triabin as an inhibitor of thrombin in *T. dimidiata* saliva. However, dimiconin prolonged APTT, an indicator of intrinsic coagulation, but not PT, an indicator of the extrinsic pathway. This result was unexpected because the inhibition of thrombin activity should affect both coagulation assays. Overall, dimiconin shows a high degree of similarity to triabin at the amino acid level, but marked diversity between the two was noted in the C-terminal, where triabin lacks approximately 30 amino acids. Although a mutant dimiconin lacking this region (dimiconin ΔC30) was functionally investigated, its activity was similar to that of the original dimiconin, indicating that other amino acids are responsible for their different functions.

The intrinsic pathway involves FXII, FXI, FIX, prekallikrein and HMW kininogen. The pathway is initiated by the binding of FXII to negatively charged surfaces, resulting in the conversion of FXII into the serine protease FXIIa. FXIIa converts prekallikrein into kallikrein, and FXI into FXIa, followed by activation of FIX and then FX to generate FXa (Shan et al., 2003; Decrem et al., 2009). A common pathway leads to the generation of thrombin from prothrombin and ultimately produces insoluble fibrin from fibrinogen (Decrem et al., 2009; Fontaine et al., 2011). When the enzymatic activity of coagulation factors was measured using chromogenic substrates, FXIIa activity was found to be markedly inhibited by dimiconin in a dose-dependent manner. Although dimiconin inhibited FIXa and FXa activities efficiently at higher concentrations, the inhibition was diminished at a lower dose. These results strongly suggest that dimiconin is an inhibitor of FXII, and the downstream pathway of the intrinsic coagulation cascade, such as activation of FIX and FX, was inhibited under conditions where FXIIa activity was completely abrogated by higher doses of dimiconin. Further study showed that dimiconin inhibits the activation of FXII, but not the activity of FXIIa. Thus, dimiconin is considered to target mainly the contact phase initiated by FXII activation in the blood coagulation cascade. Triafestins from *T. infestans* saliva were characterized as a contact phase inhibitor that prevents activation of the kallikrein–kinin system by interfering with the association of FXII and HMW kininogen with biological activating surfaces (Isawa et al., 2007). Dimiconin had little effect on the enzymatic activity of kallikrein even at higher concentrations,

suggesting that it acts on the contact phase *via* a different mechanism to triafestins.

In the present study, a recombinant triabin-like salivary protein from *T. dimidiata*, named dimiconin, was produced by *E. coli* and characterized as to its inhibitory effect on the contact phase of the blood coagulation cascade. Therefore, this protein is considered to play an important role in the blood-feeding process in *T. dimidiata*. The amino acid sequences of Td101, the other triabin-like protein found in *T. dimidiata* saliva, shared 82% identity with dimiconin, suggesting that Td101 may have similar activity to dimiconin. It was reported that a deficiency of FXII is not associated with an increased spontaneous or injury-related bleeding tendency in humans and mice, and therefore FXII activation was suggested to have little effect on physiological hemostasis (Stavrou and Schmaier, 2010). However, FXII was shown to contribute to thrombus formation and pathological clotting in different disease models using FXII-deficient mice (Renné et al., 2005; Kleinschnitz et al., 2006), indicating that FXII plays an essential role in thrombus formation and may be a novel target for antithrombotic therapy. Therefore, dimiconin has potential as a pharmacological substance as well as a reagent for a wide variety of research purposes.

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REFERENCES

Andersen, J. F., Gudderra, N. P., Francischetti, I. M. B. and Ribeiro, J. M. C. (2005). The role of salivary lipocalins in blood feeding by *Rhodnius prolixus*. *Arch. Insect Biochem. Physiol.* **58**, 97-105.

Assumpção, T. C., Francischetti, I. M. B., Andersen, J. F., Schwarz, A., Santana, J. M. and Ribeiro, J. M. C. (2008). An insight into the sialome of the blood-sucking bug *Triatoma infestans*, a vector of Chagas' disease. *Insect Biochem. Mol. Biol.* **38**, 213-232.

Assumpção, T. C., Charneau, S., Santiago, P. B., Francischetti, I. M. B., Meng, Z., Araújo, C. N., Pham, V. M., Queiroz, R. M., de Castro, C. N., Ricart, C. A. et al. (2011). Insight into the salivary transcriptome and proteome of *Dipetalogaster maxima*. *J. Proteome Res.* **10**, 669-679.

Beard, C. B. (2005). Kissing bugs and bedbugs, the Heteroptera. In *Biology of Disease Vectors* (ed. W. C. Marquardt, W. C. Black, J. E. Freier, H. H. Hagedorn, J. Hemingway, S. Higgs, A. A. James, B. Kondratieff and C. G. Moore), pp. 57-65. San Diego, CA: Elsevier.

Champagne, D. E. (2005). Antihemostatic molecules from saliva of blood-feeding arthropods. *Pathophysiol. Haemost. Thromb.* **34**, 221-227.

Decrem, Y., Rath, G., Blasioli, V., Cauchie, P., Robert, S., Beaufays, J., Frère, J. M., Feron, O., Dogné, J. M., Dessy, C. et al. (2009). Ir-CPI, a coagulation contact phase inhibitor from the tick *Ixodes ricinus*, inhibits thrombus formation without impairing hemostasis. *J. Exp. Med.* **206**, 2381-2395.

Flower, D. R. (1996). The lipocalin protein family: structure and function. *Biochem. J.* **318**, 1-14.

Fontaine, A., Diouf, I., Bakkali, N., Missé, D., Pagès, F., Fusai, T., Rogier, C. and Almeras, L. (2011). Implication of haematophagous arthropod salivary proteins in host-vector interactions. *Parasit. Vectors* **4**, 187.

Francischetti, I. M. B., Ribeiro, J. M. C., Champagne, D. and Andersen, J. (2000). Purification, cloning, expression, and mechanism of action of a novel platelet aggregation inhibitor from the salivary gland of the blood-sucking bug, *Rhodnius prolixus*. *J. Biol. Chem.* **275**, 12639-12650.

Fuentes-Prior, P., Noeske-Jungblut, C., Donner, P., Schleuning, W. D., Huber, R. and Bode, W. (1997). Structure of the thrombin complex with triabin, a lipocalin-like exosite-binding inhibitor derived from a triatomine bug. *Proc. Natl. Acad. Sci. USA* **94**, 11845-11850.

Isawa, H., Orito, Y., Jingushi, N., Iwanaga, S., Morita, A., Chinzei, Y. and Yuda, M. (2007). Identification and characterization of plasma kallikrein-kinin system inhibitors from salivary glands of the blood-sucking insect *Triatoma infestans*. *FEBS J.* **274**, 4271-4286.

Kato, H., Jochim, R. C., Gomez, E. A., Sakoda, R., Iwata, H., Valenzuela, J. G. and Hashiguchi, Y. (2010). A repertoire of the dominant transcripts from the salivary glands of the blood-sucking bug, *Triatoma dimidiata*, a vector of Chagas disease. *Infect. Genet. Evol.* **10**, 184-191.

Kleinschnitz, C., Stoll, G., Bendszus, M., Schuh, K., Pauer, H. U., Burfeind, P., Renné, C., Gailani, D., Nieswandt, B. and Renné, T. (2006). Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J. Exp. Med.* **203**, 513-518.

Mans, B. J. and Neitz, A. W. (2004). Adaptation of ticks to a blood-feeding environment: evolution from a functional perspective. *Insect Biochem. Mol. Biol.* **34**, 1-17.

Martínez-Ibarra, J. A., Miguel-Alvarez, A., Arredondo-Jiménez, J. I. and Rodríguez-López, M. H. (2001). Update on the biology of *Triatoma dimidiata* Latreille (Hemiptera: Reduviidae) under laboratory conditions. *J. Am. Mosq. Control Assoc.* **17**, 209-210.

Noeske-Jungblut, C., Krätzschmar, J., Haendler, B., Alagon, A., Possani, L., Verhallen, P., Donner, P. and Schleuning, W. D. (1994). An inhibitor of collagen-induced platelet aggregation from the saliva of *Triatoma pallidipennis*. *J. Biol. Chem.* **269**, 5050-5053.

Noeske-Jungblut, C., Haendler, B., Donner, P., Alagon, A., Possani, L. and Schleuning, W. D. (1995). Triabin, a highly potent exosite inhibitor of thrombin. *J. Biol. Chem.* **270**, 28629-28634.

Renné, T., Pozgajová, M., Grüner, S., Schuh, K., Pauer, H. U., Burfeind, P., Gailani, D. and Nieswandt, B. (2005). Defective thrombus formation in mice lacking coagulation factor XII. *J. Exp. Med.* **202**, 271-281.

Ribeiro, J. M. C. (1995). Blood-feeding arthropods: live syringes or invertebrate pharmacologists? *Infect. Agents Dis.* **4**, 143-152.

Ribeiro, J. M. C. and Francischetti, I. M. B. (2003). Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu. Rev. Entomol.* **48**, 73-88.

Ribeiro, J. M. C., Andersen, J., Silva-Neto, M. A., Pham, V. M., Garfield, M. K. and Valenzuela, J. G. (2004). Exploring the sialome of the blood-sucking bug *Rhodnius prolixus*. *Insect Biochem. Mol. Biol.* **34**, 61-79.

Santos, A., Ribeiro, J. M. C., Lehane, M. J., Gontijo, N. F., Veloso, A. B., Sant'Anna, M. R., Nascimento Araujo, R., Grisard, E. C. and Pereira, M. H. (2007). The sialotranscriptome of the blood-sucking bug *Triatoma brasiliensis* (Hemiptera, Triatominae). *Insect Biochem. Mol. Biol.* **37**, 702-712.

Shan, J., Baguinon, M., Zheng, L. and Krishnamoorthi, R. (2003). Expression, refolding, and activation of the catalytic domain of human blood coagulation factor XII. *Protein Expr. Purif.* **27**, 143-149.

Stavrou, E. and Schmaier, A. H. (2010). Factor XII: what does it contribute to our understanding of the physiology and pathophysiology of hemostasis & thrombosis. *Thromb. Res.* **125**, 210-215.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596-1599.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Valenzuela, J. G. (2005). Blood-feeding arthropod salivary glands and saliva. In *Biology of Disease Vectors* (ed. W. C. Marquardt, W. C. Black, J. E. Freier, H. H. Hagedorn, J. Hemingway, S. Higgs, A. A. James, B. Kondratieff and C. G. Moore), pp. 377-386. San Diego, CA: Elsevier.