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Identification and characterization of a novel factor XIIa inhibitor in the hematophagous insect, *Triatoma infestans* (Hemiptera: Reduviidae)

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Abstract Recently, we have cloned several Kazal-type serine protease inhibitors from the midgut of the *Triatoma infestans* bug. A single gene composed of multi Kazal-type domains, in tandem, encodes these inhibitors. In this work, we describe the purification and characterization of recombinant infestins 3-4 and 4, which are potent factor XIIa inhibitors (K_i = 67 pM and 128 pM, respectively). We also identified the first native factor XIIa inhibitor from a hematophagous insect. The factor XIIa inhibitory activity of infestin 4 demonstrates extremely efficient anticoagulant activity, prolonging activated partial thromboplastin time by approximately 3 times. Our results suggest that infestins perform a very important role in the *T. infestans* midgut during meal acquisition and digestion by controlling blood coagulation by means of inhibiting thrombin and factor XIIa.

Keywords: Factor XIIa; Serine protease inhibitor; Blood coagulation; Insect; Hematophagous; *Triatoma infestans*

1. Introduction

The hemostatic system of vertebrate animals has two main mechanisms to control blood loss. Initially, activated platelets adhere and aggregate at the injury site, forming a platelet plug that reduces or blocks blood loss. In parallel, the blood coagulation cascade is activated by limited proteolysis of several factors that circulate in an inactive form in the blood. Factor XII and plasma kallikrein activation are involved in the intrinsic pathway and factor VII activation and tissue factor association in the extrinsic pathway. Both pathways converge to form a common pathway resulting in factor Xa generation, which activates prothrombin to thrombin. Finally, thrombin converts fibrinogen to fibrin net that is stabilized by factor XIIIa action [1].

Hematophagous animals have a vital need to interfere with their hosts' hemostatic system in order to obtain a blood meal. The first reports of substances from hematophagous animals able to block or delay vertebrate blood coagulation date from the XIX century [2]. Since then, many other substances from these animals have been described, among them are molecules with potent anti-clotting, anti-platelet aggregation, vasodilators and fibrinolytic activity [3–7].

Triatoma infestans (Hemiptera: Reduviidae), a vector for the protozoan parasite *Trypanosoma cruzi*, which is responsible for Chagas' disease, is an anthropophilic and exclusively hematophagous insect. This insect obtains a blood meal by injecting its maxilla into the vertebrate's skin [8]. Three potent thrombin inhibitors of the Kazal-type family have been previously described in kissing bugs (Triatominae) [9–11]. They are probably all localized in the midgut and are responsible for inhibiting clot formation in the digestive tract of these insects [10,11]. These thrombin inhibitors are composed of two non-classical Kazal-type domains in tandem but, surprisingly, their genes contain up to six Kazal-type domains that are transcribed in the same mRNA molecule. The inhibitors are then released by an unknown post transcriptional, proteolytic mechanism.

In a previous study [11], we described a recombinant infestin, encoded by a cDNA fragment containing four non-classical Kazal-type domains, obtained from the *T. infestans* midgut. Infestin 1-2 was able to inhibit blood coagulation by inhibiting thrombin activity and the four domain protein, named infestin 1-4, besides thrombin, also inhibits factor XIIa and factor Xa [11]. The factor Xa and XIIa inhibitory activities were probably localized in the infestin domains 3 and 4 that are not present in the native thrombin inhibitor (infestin 1-2).

Thrombin and factor Xa have been found to be the main targets of hematophagous animals. Factor XIIa, a serine protease of the contact phase of blood coagulation, is involved in the inflammation process [12] and is also an important activator of plasmin which is responsible for thrombus dissolution [1]. Few inhibitors that interfere in the intrinsic or extrinsic blood coagulation pathway have been described [7]. The number of factor XIIa inhibitors already described is small when compared to thrombin and factor Xa inhibitors. Among them, ecotin is from *Escherichia coli* [13], few are non-plasmatic inhibitors, and most have been isolated from plants [14–17]. Until now, no factor XIIa inhibitor from hematophagous animals was described.

In the present work, we describe the cloning and expression of the two first factor XIIa inhibitors (infestin 3-4 and infestin 4) belonging to the Kazal-type serine protease inhibitor family, and infestin 3-4 is the first factor XIIa inhibitor isolated from hematophagous insect.

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Abbreviations: APTT, activated partial thromboplastin time; BMMY, buffered methanol-complex medium; BMM, buffered minimal methanol; TFA, trifluoroacetic acid

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2. Materials and methods

2.1. Protein sequence analysis

Sequence similarity searches were performed using the Blast program [18]. Alignment of protein sequences was performed with the ClustalW program, version 1.7 [19].

2.2. Cloning, expression and purification of recombinant infestin 3-4

The infestin 3-4 gene was amplified from the vector pIC 2.1.2 [11] by PCR using the primers INF3DFWD (5′-ATCTCTAGAT AAA-AGAATAC TGAAATCATG CATATGTACA AAA-3′) and IN-F4DREV (5′-CCCAAGCTTT CAAAACTGTT CAACATTGCT GCGCTGACAA CGTCC-3′). The PCR product was digested with *Xba*I and *Hin*dIII restriction enzymes, purified and ligated into the pVT102α plasmid. Positive constructs were obtained after DNA sequencing experiments. The plasmid (pIC 5.1.1) was used to transform the *Sacchramocyes cerevisiae* S-78 strain [20] and standard yeast cultivation was carried out as previously described [11]. After yeast cultivation, cells were separated by centrifugation ($6000 \times g$, 20 min, 4 °C) and the supernatant was applied to a 5 ml HiTrap SP column (Amersham Biosciences). The bound proteins were eluted with 50 mM sodium acetate buffer, pH 5.0, containing 0–0.7 M NaCl. Fractions containing protease inhibitory activity were pooled, concentrated and applied to a Superose 12 column (Amersham Biosciences) pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 0.3 M NaCl. Fractions containing protease inhibitory activity were pooled and stored at −20 °C.

2.3. Cloning, expression and purification of recombinant infestin 4

The infestin 4 gene was amplified by PCR using pIC 5.1.1 as template and INF4DREV and INF4DFWD (5′-ATCTCTAGAT AAA-AGAGAGG TCAGAAACCC TTGCGCC-3′) as primers. The PCR product was digested with *Xho*I and *Not*I restriction enzymes and ligated into the plasmid pPIC9 (Invitrogen). The resulting plasmid (pIC 11.1.1) was linearized with *Sal*I restriction enzyme to transform competent *Pichia pastoris* GS115 yeast strain prepared according to the manufacturer's instructions. Yeast was incubated for 5 days in buffered methanol-complex medium (BMMY) medium. After fermentation, yeast cells were harvested by centrifugation ($4000 \times g$, 20 min, 4 °C). The recombinant infestin was purified by affinity chromatography on a trypsin-Sepharose column [11].

2.4. Kinetics assay and determination of equilibrium dissociation constants (K_i)

The equilibrium dissociation constants for complexes of purified infestins with bovine thrombin (E.C. 3.4.21.5), bovine trypsin (E.C. 3.4.21.4), human factor Xa (E.C. 3.4.21.6), human factor XIIa (E.C. 3.4.21.38) and human plasmin (E.C. 3.4.21.7) were determined according to Bieth [21] using the chromogenic substrates (Chromogenix): S2238 (HD–Phe–Pip–Arg–pNa) for thrombin, S2222 (Bz-Ile-Gly-Arg-pNa) for trypsin and factor Xa, S2302 (HD–Pro–Phe–Arg–pNa) for factor XIIa and S2251 (HD–Val–Leu–Lys–pNa) for plasmin. Apparent K_i values were calculated by fitting the steady-state velocities to the equation describing the tight-binding inhibitor model using non-linear regression analysis [22].

2.5. Triatoma infestans anterior midgut homogenate and native infestin purification

Bugs were provided by Dr. Ionizete G. Silva from Instituto de Patologia Tropical e Saúde Pública – UFG, Goiania, Brazil. Fed bugs were maintained at 25 °C until their anterior midguts had completed digestion of the blood meal. Insects were dissected and their stomachs stored at −20 °C until homogenization. 140 stomachs were homogenized in 10 mM sodium acetate buffer, pH 5.5, containing 50 mM NaCl (6 ml). The homogenate was centrifuged at $16\,000 \times g$ for 10 min and the supernatant was filtered through a 0.22 μm filter. Supernatant was applied to a 1 ml Resource S column (Amersham Biosciences) previously equilibrated with 50 mM sodium acetate buffer, pH 5.5, and the bound proteins were eluted with a 0–0.7 M NaCl linear gradient. Fractions containing factor XIIa inhibitory activity were pooled. This material was used in NH_2-terminal sequence and kinetic analysis.

2.6. NH_2-terminal sequence

Purified native infestin was applied to a C_8 Sephasil Peptide column (Amersham Biosciences) and the proteins were eluted with a 0–90% acetonitrile linear gradient in 0.1% trifluoroacetic acid (TFA). Protein peaks were assayed for factor XIIa inhibition and the active protein peak was blotted on a PVDF membrane and sequenced by Edman degradation using a PPSQ automatic sequencer (Shimatzu).

2.7. APTT-assay

50 μl r-Infestin 4 (1 μg) was pre-incubated with 50 μl of factor V, VII, X or XII deficient plasma (Diagnostica Stago, Asnieres-Sur-Seine, France), 50 μl of a normal human plasma pool and 50 μl of activated cephaloplastin reagent (Dade International Inc., Miami, USA) for 3 min at 37 °C. Pre-warmed 25 mM calcium chloride solution (50 μl) was then added and the clotting time recorded. This experiment was performed in triplicate.

3. Results

3.1. Cloning, expression and purification of r-infestin 3-4

Using a plasmid containing the DNA fragment that encodes infestin 1-4, the infestin 3-4 fragment was amplified and subcloned into a *S. cerevisiae* expression vector (pVT102α). S-78 yeast cells expressed approximately 1.5 mg/l of active infestin 3-4. Due to the large volume, supernatant was lyophilized and dialyzed to reduce salt, medium pigment and low molecular mass contaminants. Concentrated supernatant showed inhibitory activities to trypsin, plasmin, factor Xa and XIIa (Fig. 1). Purified material was used in enzymatic assays for dissociation constant determination.

3.2. Cloning, expression and purification of r-infestin 4

Using infestin 3-4 as template, the infestin 4 DNA fragment was amplified and subcloned into the pPIC 9 yeast expression vector (*Pichia pastoris*). Infestin 4 presented low expression using buffered minimal methanol (BMM) but using BMMY the expression level was 4 mg/l of active infestin 4. As shown in Fig. 1, r-infestin 4 also inhibited trypsin, plasmin, factor Xa and factor XIIa, similar to the r-infestin 3-4 activity. r-Infestin 4 was purified by affinity chromatography on trypsin-Sepharose and appears as a 6 kDa single band in SDS–PAGE (data not shown).

3.3. Kinectic analysis of r-infestin 3-4 and 4

Dissociation constants (K_i) for both inhibitors were determined (Table 1) using Morrison non-linear regression to the following serine proteases: trypsin, factor XIIa, plasmin, factor Xa and thrombin. Infestin 3-4 and 4 strongly inhibited factor XIIa ($K_i = 67$ and 128 pM, respectively) and also trypsin, plasmin and factor Xa (K_i in nanomolar range). The kinetic results suggested that the third domain of infestin did not contribute significantly to factor XIIa inhibition.

3.4. APTT-assay

r-Infestin 4 prolonged the coagulation time of factor XIIa in an activated partial thromboplastin time (APTT) assay by almost three times (Fig. 2), but it did not affect the APTT of factors V, VII and X, confirming that infestin 4 inhibits factor XIIa activity.

3.5. Anterior midgut homogenate and native infestin purification

Anterior midgut homogenate was used to isolate the native form of factor XIIa inhibitor. The factor XIIa inhibitor was partially purified from the midgut in an anion-exchange column (Fig. 3). Active fractions against factor XIIa were pooled, the apparent dissociation constant (K_i) to factor XIIa was determined and then the active material was applied to a

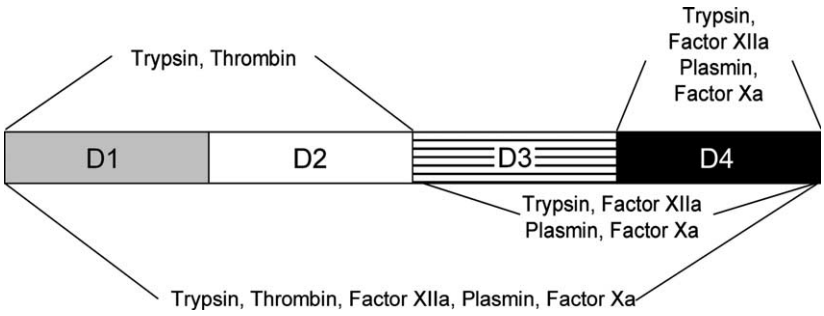

Fig. 1. Diagram of the infestin cDNA fragment. The gray, white, dashed and black boxes represent the domains 1, 2, 3 and 4, respectively. Lines indicate the target proteases of the infestin domain combination.

Table 1
Dissociation constants (K_i) of recombinant infestins for different serine proteases

	K_i (nM ± S.E.)				
	1–4[a]	1–2[a]	3–4	4	NFI
Trypsin	5.2 ± 3.2	3.1 ± 0.7	3.3 ± 2.6	11.0 ± 8.6	nd
Factor XIIa	0.078 ± 0.009	ni	0.067 ± 0.039	0.128 ± 0.016	0.088 ± 0.019
Plasmin	1.1 ± 0,2	ni	0.4 ± 0.1	2.1 ± 0.8	nd
Factor Xa	69.2 ± 4.8	ni	18.2 ± 3.8	53.0 ± 22.2	nd
Thrombin	0.800 ± 0.054	0.025 ± 0.007	ni	ni	ni

NFI, native factor XIIa inhibitor; ni, not inhibited and nd, not determined.
[a] data from Campos et al. [11] .

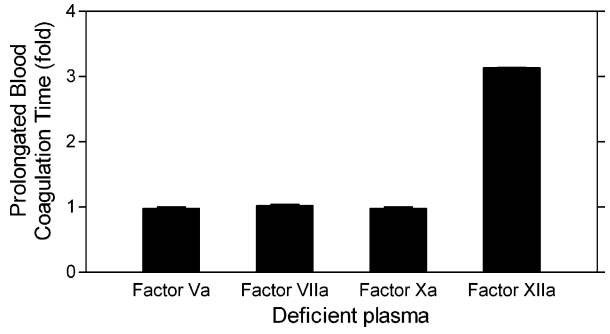

Fig. 2. Effect of r-infestin 4 on blood coagulation factors V, VIII, X and XII. Clotting time obtained by incubation of infestin 4 with factors V, VII, X and XII deficient plasmas. Normal human clotting time-APTT is 24 seconds for factor V, VII and X and 88 seconds for factor XII deficient plasmas. Bars indicate standard error.

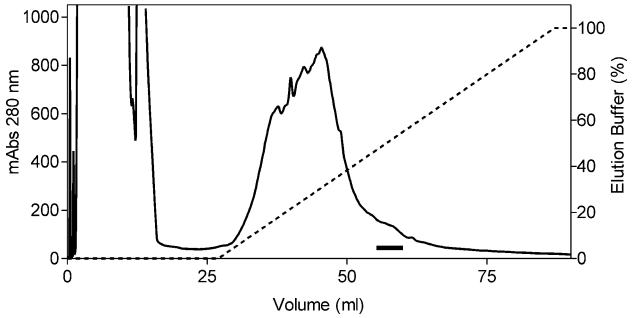

Fig. 3. Ion-exchange chromatography of native infestin with factor XIIa inhibitor activity. Midgut extract was applied to a Resource S column previously equilibrated with 50 mM sodium acetate buffer, pH 5.0. Proteins were eluted with a linear gradient of 0–0.7 M NaCl (dashed line). The horizontal line represents the fractions containing factor XIIa inhibitory activity.

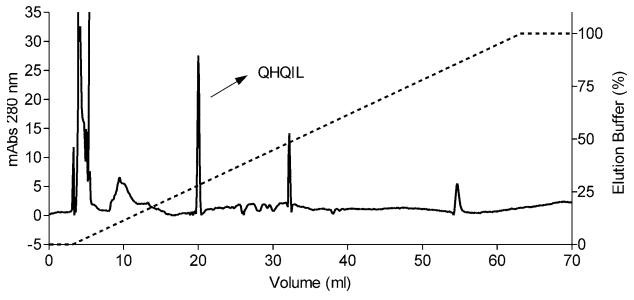

Fig. 4. Reverse-phase chromatography of native factor XIIa inhibitor. Active factor XIIa inhibitor eluted from the Resource S column was applied to a Sephasil peptide C_8 column previously equilibrated with 0.1% TFA in water. Proteins were eluted with a 0–90% acetonitrile linear gradient (dashed line). The horizontal line represents the fractions containing factor XIIa inhibitory activity. The arrow indicates the five first N-terminal amino acid residues determined by automatic amino acid sequencing.

reverse-phase column. The factor XIIa inhibitor was active after reverse-phase chromatography and it was possible to determine the protein peak corresponding to the native factor XIIa inhibitor (Fig. 4). Due to the low inhibitor concentration, only the N-terminal sequence of the native protein was determined. The amino acid sequence Gln–His–Gln–Ile–Leu corresponds to the loop region between infestin domains 2 and 3, indicating that the native form of the inhibitor is probably composed of two Kazal-type domains, infestin 3-4, and not only one domain, infestin 4 (Fig. 5).

4. Discussion

It is interesting to remember that hematophagous insects have not one, but several ways to interfere with the hemo-

static system of their hosts [23,24]. During feeding, an adult bug is able to ingest 1–2 times his own weight in blood in approximately 10 min [25]. This blood is then stored and processed in the midgut [26]. A potent thrombin inhibitor (Infestin 1-2) from the midgut of *T. infestans* has been previously described [11] and it was believed that infestin 1-2 was responsible for inhibiting the final blood cascade pathway due to its high affinity to thrombin (Ki of 43 pM). This inhibitor might participate in the digestion and emptying processes of the gut that can take up to several weeks. Infestin 1-2 is composed of two Kazal type domains, similar to other thrombin inhibitors from kissing bugs [9,10]. Infestin 1-2 is encoded by the first two domains of a DNA fragment that codes for a four Kazal-type domain protein. All four domains were expressed as infestin 1-4 and inhibited not only trypsin and thrombin, like infestin 1-2, but also plasmin, factor Xa and factor XIIa.

At that time, our hypothesis was that domains 3 and 4 were responsible for the new inhibitory activities. So the unknown infestins (3-4, 3 and 4) were cloned, expressed and characterized. The expressed r-infestin 3-4 presented the expected inhibitory activities of r-infestin 1-4 but not of r-infestin 1-2. Firstly, r-infestin 3-4 was purified on a trypsin-Sepharose column, however, the eluted inhibitor was processed in the column (data not shown) and even though it still presented some inhibitory activity, this method was discarded. Secondly, r-infestin 3-4 was purified using classical purification methods. Since r-infestin 3-4 presented several inhibitory activities, we decided to express the domains 3 and 4 separately to see which domain was responsible for each activity. Surprisingly, the expressed infestin 3 domain did not present inhibitory activity (data not shown) to the tested serine proteases. In contrast, r-Infestin 4 presented an affinity to factor XIIa comparable to r-infestin 3-4. Purified r-infestin 4 was able to prolong coagulation time in APTT by approximately 3 times, inhibiting factor XIIa. The existence of native factor XIIa inhibitor (infestin 4 or 3-4) in the midgut was our next question.

The native factor XIIa inhibitory activity was detected as expected and its amino terminal sequence determined. The amino acid sequence contained residues localized in the loop between domains 2 and 3. These data suggested that the native factor XIIa inhibitor is a double Kazal-type domain protein and its dissociation constant (K_i) for factor XIIa was similar to that of r-infestin 3-4. The low quantity of native infestin 3-4 obtained did not allow us to perform other kinetic assays.

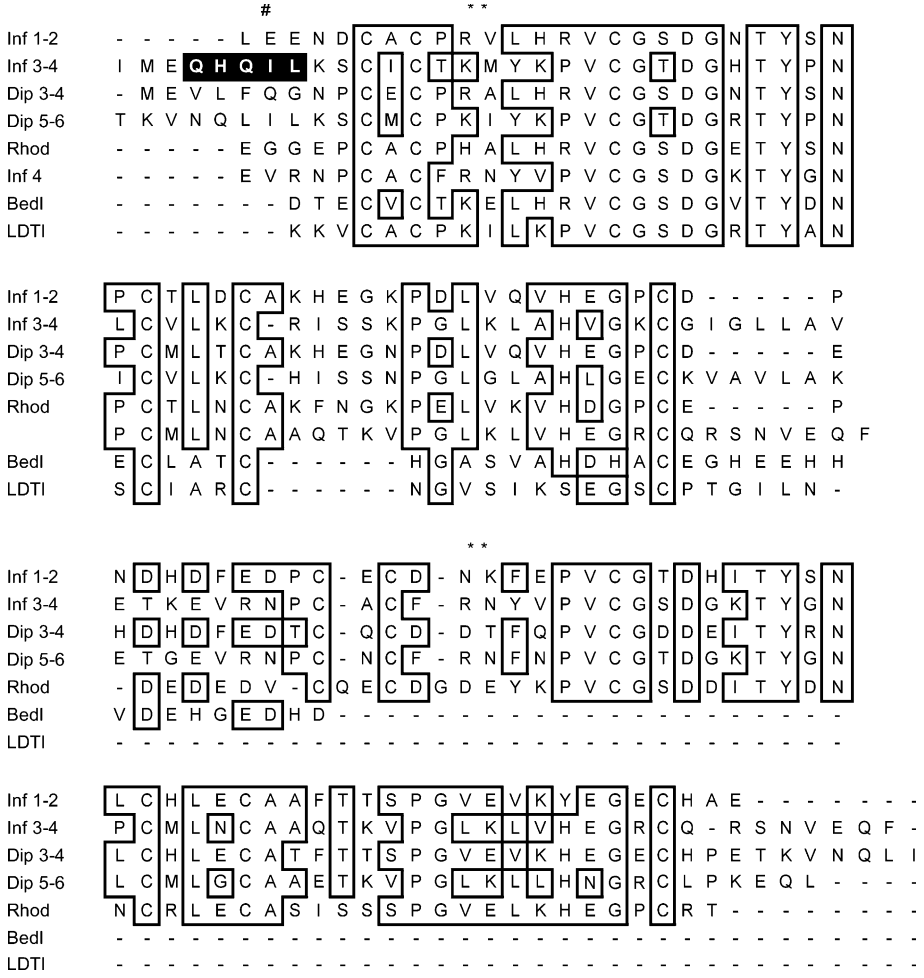

Fig. 5. Alignment of the amino acid sequence of infestins 1-2 and 3-4 with other Kazal type serine protease inhibitors. Inf 1-2, Infestin 1-2; Inf 3-4, Infestin 3-4; Inf 4, Infestin 4 (NCB Accession # AAK57342); Rhod, Rhodniin (Swiss-Prot # Q06684); Dip 3-4, Dipetalogastin; Dip 5-6, Dipetalogastin 5-6 (NCB Accession # CAA10384); Bedl, Bdellin native (Swiss-Prot # P09865); LDTI, leech derivated triptase inhibitor (Swiss-Prot # P80424); and **, Reactive site. Reactive sites were determined by similarity. # indicates the N-terminal of r-infestin 3-4. The black box indicates native factor XIIa inhibitor N-terminal sequence determined.

Our data allowed us to conclude that infestins 3-4 and 1-2 act in cooperation, blocking the beginning and the end of the blood coagulation cascade by inhibiting factor XIIa and thrombin, respectively. The presence of infestin 1-2 and infestin 3-4 demonstrated how the insect has evolved to overcome the host's hemostatic barrier. A possible explanation for infestin 3-4 strong inhibition of factor XIIa may be that the most active coagulation pathway is the intrinsic one, since *T. infestans* is preferentially a vessel-feeder [8] and therefore the extrinsic pathway may be poorly triggered. Factor XIIa is important in the intrinsic pathway initialization [1] and is strongly inhibited by infestin 3-4. If undesirable blood coagulation activity occurs, the thrombin and factor Xa generated may be inhibited by infestin 1-2 and infestin 3-4, respectively. It is interesting to note that these inhibitory activities are complementary and present in the same mRNA molecule. These inhibitors are then separated, probably by a post transcription processing.

Hematophagous animals are a rich source of anti-hemostatic substances [3,24], but few inhibitors of the intrinsic pathway have been described [27]. Among those described are a few non-plasmatic factor XIIa inhibitors, most isolated from plants [14–17], only ecotin was from *E. coli* [13] and none of them were Kazal-type inhibitors. Infestin 3-4 is the first potent factor XIIa inhibitor to be isolated from a hematophagous animal. The Kazal-type domain versatility in inhibiting serine proteases defines them as an interesting tool for specificity studies and new inhibitor developments. At this time, our group is working on the infestin processing mechanisms.

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