Process Biochemistry 46 (2011) 2317–2321

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

The first serine protease inhibitor from *Lasiodora* sp. (Araneae: Theraphosidae) hemocytes

Tatiana Soares[a], Felipe Roberto Borba Ferreira[a], Francis Soares Gomes[a], Luana Cassandra Breitenbach Barroso Coelho[a], Ricardo José Soares Torquato[b], Thiago Henrique Napoleão[a], Maria do Socorro de Mendonça Cavalcanti[c], Aparecida Sadae Tanaka[b], Patrícia Maria Guedes Paiva[a,*]

[a] *Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil*
[b] *Departamento de Bioquímica, Universidade Federal de São Paulo, São Paulo, São Paulo, Brazil*
[c] *Departamento de Ciências Fisiológicas, Universidade de Pernambuco, Recife, Pernambuco, Brazil*

ARTICLE INFO

Article history:
Received 18 April 2011
Received in revised form 9 August 2011
Accepted 17 September 2011
Available online 21 September 2011

Keywords:
Serine protease inhibitor
Antibacterial activity
Hemocytes
Lasiodora sp.
Spider

ABSTRACT

This work reports, for the first time, the purification, characterization and antibacterial activity of an elastase inhibitor from *Lasiodora* sp. hemocytes (EILaH). The hemocyte extract inhibited chymotrypsin (22%), trypsin (44%), tissue plasminogen activator (52%), urokinase (58%) and human neutrophil elastase (99%). EILaH was purified by Trypsin-Sepharose column and RP-HPLC. SDS-PAGE of EILaH revealed a molecular mass of 8 kDa and MALDI-TOF mass spectrometry revealed a single molecular mass of 8274 Da. The amino terminal sequence determined was LPC(PF)PYQQELTC. The dissociation constant (K_i) for human neutrophil elastase was 0.32 nM. Hemocyte extract exerted antibacterial effect on *Bacillus subtilis* and *Enterococcus faecalis*, while EILaH was only active against *E. faecalis*. Currently, *Lasiodora* sp. is undergoing a systematic review and this study contributes to molecular characterization of the genus. In addition, the results suggest that serine protease inhibitors expressed in *Lasiodora* sp. hemocytes may be involved in the defense against bacterial infection.

1. Introduction

Arachnids comprise the largest and, from a human standpoint, the most important and numerous class of chelicerates, among which the most common and best known are spiders, scorpions, mites and ticks. The Brazilian spider *Lasiodora* (Mygalomorphae, Theraphosidae), whose trivial names are "caranguejeira" or tarantula, is distributed in northeastern Brazil, in the rainforest [1]. Spiders are the most diverse and successful terrestrial invertebrates after insects, which are their primary prey [2]. This success is due to innate immunity, carried out mostly by hemocytes, and includes cellular as well as humoral responses [3].

In arthropods, phagocytosis, complement, antimicrobial peptides, coagulation and melanization are instances of cellular defenses, while humoral defense usually involves components released from hemocytes [4–8]. It has been suggested that a serine protease inhibitor released from blood cells protects the arthropod from microbial infection by inhibiting fungal or bacterial proteinases as well as by regulating endogenous proteinases involved in host-defense mechanisms [9–11]. *Limulus* hemocytes are able to detect the lipopolysaccharide present in Gram-negative bacteria cell wall, starting the exocytose of hemocyte large granules, which contain protease inhibitors and pseudo-serine protease with antimicrobial activities [9].

Protease inhibitors are essential for all organisms, and play a major role in controlling protein damage of self and non-self proteases [12]. They inhibit serine, cysteine and aspartic acid proteases, as well as metalloproteinases. Additionally, the best characterized families of protease inhibitors are Kazal, Kunitz and Pacifastin [13–15]. Serine protease inhibitors play important roles as modulators of several biological processes such as apoptosis, digestion, prophenol oxidase activation, blood coagulation, cellular remodeling, metamorphosis, complement system and defense against invading organisms [16–21]. Elastase inhibitors have been isolated from numerous invertebrates, including the locust *Schistocerca gregaria* [22], the shrimp *Penaeus monodon* [13,23,24], the kissing bug *Triatoma infestans* [12], the tick *Boophilus microplus* [6,25,26] and *Rhipicephalus* (*B.*) *microplus* [27].

In addition to their physiological role, protease inhibitors have important medical applications. It has been suggested that inhibitors of the neutrophil elastase could be useful in the treatment of pulmonary diseases [28]. In addition, recombinant versions

* Corresponding author. Tel.: +55 8121268540; fax: +55 8121268576.
E-mail address: ppaivaufpe@yahoo.com.br (P.M.G. Paiva).

1359-5113
doi:10.1016/j.procbio.2011.09.012

of naturally occurring inhibitors and synthetic small inhibitors are potential therapeutic agents in the treatment of respiratory diseases [28]. Since the description of antibacterial effect of serine proteinase inhibitors, a growing interest in the use of these inhibitors as potential antibiotics has emerged [29]. The secretory leukocyte protease inhibitor (SLPI), which decreases elastase activity, has multiple medical applications, including anti-inflammatory, anti-bacterial and anti-viral properties [30].

The studies on the spider immune system have focused only on antimicrobial peptides. Hemocytes defensins and ctenidins active against bacteria and fungi are released into the hemolymph upon infection [3,31]. However, to the best of our knowledge, no protease inhibitor has been purified from *Lasiodora* sp. hemocytes. In this scenario, the aims of the present study were to isolate and characterize an elastase inhibitor from *Lasiodora* sp. hemocytes (EILaH) and to evaluate its antibacterial activity.

2. Materials and methods

2.1. Spiders and sample collection

The animals used in the experiments were adult females in the intermolt stage, since the more static behaviour in captivity and larger size characteristic of this development stage facilitate manipulation and puncture, when compared to adult males. Hemolymph was collected from 16 spiders (approximately 1 ml per animal) by cardiac puncture with an apyrogenic syringe in 3% NaCl supplemented with 2 mM propanolol, to avoid coagulation. The hemocytes were obtained from plasma by centrifugation at 800 *g* for 10 min at 28 °C.

2.2. Hemocyte extract

Hemocytes were dried by lyophilisation and macerated in 100 μl of 0.15 M NaCl. Then, 900 μl of 0.15 M NaCl was added. The macerated hemocyte mass was centrifuged at 800 *g* for 10 min at 28 °C and the supernatant was collected and stored at −20 °C. This procedure was repeated several times and all the supernatants were pooled (26 ml) to obtain the hemocyte extract used for inhibitor purification.

2.3. Protein determination

Protein concentration in hemocyte extract, as well as in the samples obtained after each step of inhibitor purification, was determined using the Coomassie Blue dye binding method [32]. Bovine serum albumin was used as standard protein.

2.4. Protease inhibition assay

The protease inhibition assay was carried out by pre-incubating the hemocyte extract (100 μl; 1.8 mg/ml of protein) with the enzymes chymotrypsin, trypsin and thrombin obtained from Sigma (St. Louis, MO) as well as human neutrophil elastase (HNE), plasmin, factor Xa, tissue plasminogen activator (tPA), subtilisin A and urokinase obtained from Calbiochem (San Diego, CA). Human plasma kallikrein was prepared as previously described by Sampaio et al. [33]. After 10 min at 37 °C, specific substrates (Chromogenix, Sweden) were added to a final concentration of 0.2 mM: Suc $(Ala)_2$-Pro-Phe-pNa (4 mM chymotrypsin), Suc-Ala-Ala-Pro-Val-pNA (4 mM HNE), HD-Val-Leu-Lys-pNA (4 mM plasmin), tosyl-Gly-Pro-Arg-pNA (4 mM trypsin), Glu-Gly-Arg-pNa (10 mM urokinase), Bz-Ile-(OR)-Gly-Arg-pNA (4 mM factor Xa), HD-Pro-Phe-Arg-pNA (3.64 mM plasma kallikrein-HuPK), Boc-Gly-Gly-Leu-pNa (4 mM subtilisin), HD-Phe-Pip-Arg-pNA (4 mM thrombin) and HD-Ile-Pro-Arg-pNA (4 mM tPA). The enzyme activities in the presence of hemocyte extract (residual activities) were determined by measuring absorbance at 405 nm after incubation for 15 min at 37 °C, using a spectrophotometer (ThermoLabsystems, Finland; model iEMS). Control reactions were performed under the same conditions, without the inhibitor. One unit of protease activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per minute. Inhibition percentages were calculated as follows: % inhibition = 100 − [100 × (residual activity/activity in control)].

2.5. Isolation of elastase inhibitor by affinity chromatography on Trypsin-Sepharose and reversed-phase chromatography

The hemocyte extract exerting inhibitory activity against proteases was loaded (26 ml; 46.86 mg of protein) onto a Trypsin-Sepharose column (1 ml) equilibrated with 50 mM Tris-HCl pH 8.0 at a flow rate of 0.16 ml/min, of which 1 ml fractions were collected. Proteins were eluted with 0.5 M KCl–HCl pH 2.0 at the same flow and the pH was neutralized by adding 50 μl of 1 M Tris-HCl pH 8.0. Protein was measured by absorbance at 280 nm. The pool of eluted fractions (0.18 mg/ml of protein) was evaluated by protease inhibition assay described in Section 2.4 using the same enzymes and substrates, and a sample volume of 100 μl.

The material eluted from Trypsin-Sepharose chromatography was applied (3.06 mg of protein) in a Sephasil Peptide C_{18} column connected to an ÄKTA System (GE Healthcare) pre-equilibrated with 0.1% trifluoroacetic acid (TFA) at the flow rate of 0.5 ml/min, and 1 ml fractions were collected. Peptides were eluted with a 0–90% acetonitrile linear gradient in 0.1% TFA. The single peptide peak eluted from the column was evaluated for inhibitory activity on trypsin and elastase, using the same enzyme and substrate concentrations described in Section 2.4, and a sample volume of 5 μl (7 μg/ml of protein). Purified elastase inhibitor was named EILaH (Elastase Inhibitor from *Lasiodora* sp. Hemocytes).

2.6. SDS-PAGE analysis

Samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [34]. The concentration of acrylamide was 12% in the separating gel and 5% in the stacking gel. Gels were stained with Silver (BioAgency, Brazil). The molecular weight standards (Sigma, USA) were: β-galactosidase (116 kDa), phosphorylase b (97 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa).

2.7. Determination of dissociation constant (K_i)

The dissociation constant of EILaH with HNE (Calbiochem®) was determined according to Bieth [35]. Briefly, the 4 mM serine protease was incubated at 37 °C with increasing concentrations of EILaH in 0.1 M Tris-HCl pH 8.0 containing 0.15 M NaCl and 0.1% Triton X-100, and enzyme activity was measured after addition of chromogenic substrate Suc-Ala-Ala-Pro-Val-pNA (final concentration of 0.2 mM). Enzymatic activity was measured by absorbance at 405 nm using a Synergy HT microplate reader (BioTek). The residual activity corresponded to HNE activity in presence of EILaH. Inhibition percentages were calculated as follows: % inhibition = 100 − [100 × (residual HNE activity/HNE activity in control)]. The K_i value was calculated by fitting the steady-state velocities to the equation $(Vi/Vo = 1 - \{Et + It + K_i - [(Et + It + K_i)2 - 4EtIt]1/2\}/2Et)$ for the tight-binding inhibitor using a non-linear regression analysis [36].

2.8. Molecular identification

EILaH molecular mass determination was carried out on a TOF Spec E mass spectrometer (Micromass, Manchester, UK) operating in linear mode using the matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) method, using α-cyano-4-hydroxycinnamic acid as the matrix.

The N-terminal amino acid sequence of EILaH was determined by Edman degradation [37] using a PPSQ-23 Model Protein Sequencer (Shimadzu, Japan). Acquired data were searched against all available sequences in the NCBI non-redundant database using blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.9. Antibacterial activity

Gram-positive (*Bacillus subtilis* ATCC-6633, *Staphylococcus aureus* ATCC-6538 and *Enterococcus faecalis* ATCC-6057) and Gram-negative (*Escherichia coli* ATCC-25922 and *Klebsiella pneumoniae* ATCC-29665) bacterial strains were provided by the *Departamento de Antibióticos*, *Universidade Federal de Pernambuco*, Brazil. Stationary cultures were maintained in nutrient agar (NA) and stored at 4 °C. Bacteria were cultured in nutrient broth (NB) and incubated under constant shaking at 37 °C overnight. The cultures were adjusted turbidimetrically at a wavelength of 490 nm to 1.5×10^8 colony forming units (CFU)/ml (0.5 in McFarland scale).

The samples (hemocyte extract and EILaH) were concentrated by lyophilisation and ressuspended in 0.15 M NaCl. Aliquots (100 μl) of hemocyte extract (6.8 mg/ml of protein) or EILaH (0.455 mg/ml of protein) were diluted 1:2 in NB (100 μl) and submitted to a series of ten double dilutions, to a final ratio of 1:2048. Next, a 180 μl aliquot of each dilution was dispensed into a microtiter plate well. After, all the wells were inoculated with 20 μl of the bacterial culture and incubated at 37 °C for 24 h. The assay was performed in triplicate. Control assay contained NB medium and microorganism. After incubation, optical density was measured at 490 nm (OD_{490}) using a microplate reader. Minimal inhibitory concentration (MIC) was determined and corresponded to the lowest protein concentration at which the optical density decreased by more than 50% in comparison to the control OD_{490} [38].

Minimal bactericide concentration (MBC) was determined starting from the results of MIC assay. Inoculations (10 μl) from the wells in which the sample inhibited bacterial growth were transferred to NA plates. The number of CFU grown in plates was determined after incubation at 37 °C for 24 h. The MBC corresponded to the minimum concentration of protein in which no bacterial growth was observed.

3. Results

Lasiodora sp. hemocyte extract (1.8 mg/ml of protein) inhibited chymotrypsin (22%), trypsin (44%), TPA (52%), urokinase (58%) and HNE (99%); inhibition of factor Xa, thrombin, plasmin, HuPK and subtilisin was not detected. Hemocyte extract inhibited *E. faecalis*

Table 1
Summary of EILaH purification.

Sample	Volume (ml)	Total protein (mg)	Total activity (mU)[a]	Specific activity (mU/mg)[b]	Yield (%)[c]	Purification (fold)[d]
Hemocyte extract	26	46.86	894.4	19.08	100	1
Trypsin-Sepharose chromatography	17	3.06	40.8	13.33	4.56	0.69
EILaH	1.25	0.0087	0.6	68.96	0.07	3.61

[a] Inhibitory activity on human neutrophil elastase (HNE). One unit of elastase inhibitor unit corresponds to the number of units of HNE activity inhibited by the sample.
[b] Specific activity was calculated by the ratio of total activity (mU) and total protein content (mg).
[c] Yield corresponded to the amount of total activity in crude extract recovered after each purification stage.
[d] Purification was measured as the ratio between the specific activity in the stage and specific activity of crude extract.

and *B. subtilis* growth, and MIC values determined were 3400 μg/ml of protein. The extract did not inhibit the growth of *S. aureus*, *E. coli* and *K. pneumoniae*. Similarly, it had no bactericidal effect on *E. faecalis* and *B. subtilis*.

Elastase inhibitor (EILaH) was successfully isolated from hemocyte extract by two chromatographic steps (Table 1). The extract was applied on Trypsin-Sepharose column and the adsorbed fractions eluted with 0.5 M KCl–HCl pH 2.0 showed inhibitory activity only on trypsin and HNE (Fig. 1A). The fractions with inhibitory activity obtained from the affinity column were pooled and evaluated by SDS-PAGE, producing multiple polypeptide bands (Fig. 1C, lane 2). The pool was further purified on Sephasil C_{18} column (Fig. 1B). The eluted peptide (44 μg) showed inhibitory activity only on HNE, corresponding to EILaH. Purified EILaH was shown to be homogeneous by SDS-PAGE with a molecular mass of 8 kDa (Fig. 1C, lane 3).

EILaH was a strong neutrophil elastase inhibitor (Fig. 2A). When the equation for tight-binding inhibitors was used, the inhibition data for EILaH fitted well to the mechanism of these inhibitors, and a dissociation constant (K_i) of 0.32 ± 0.021 nM was calculated. EILaH molecular mass was confirmed by MALDI-TOF mass spectrometry, which revealed a single molecular mass of 8274 Da (Fig. 2B). The amino terminal sequence determined was LPC(PF)PYQQELTC (a single-letter code where letters in parenthesis indicate that either proline or phenylalanine may take this position). Alignment with known sequences in the NCBI non-redundant database did not indicate any similarity with other protease inhibitors. EILaH was active against *E. faecalis* (MIC of 227.5 μg/ml) and did not inhibit the growth of *B. subtilis*, *S. aureus*, *E. coli* and *K. pneumoniae*.

4. Discussion

Proteases comprise a broad range of hydrolytic enzymes which catalyze the cleavage of targeted protein substrates. Hydrolysis of peptide bonds occurs by means of nucleophilic attack, which however is governed by different catalytic mechanisms.

Protease inhibitors are important molecules for invertebrate animals to control extracellular serine protease cascades that mediate rapid defense responses upon wounding or pathogen infection. Several inhibitors belonging to the families Kunitz and Serpin have been isolated, especially from hemolymph of arthropods. In this study, it is demonstrated that hemocytes from the tarantula spider *Lasiodora* sp. contain a powerful neutrophil elastase inhibitor, here named EILaH.

The *Lasiodora* sp. hemocyte extract was a preparation rich in inhibitors of different serine proteases, and showed antibacterial activity. Yet, several have been the preparations rich in protease inhibitors that likewise showed antibacterial activity [6,39]. It has been reported that antibacterial activity of protease inhibitors involves binding to and disruption of the bacteria membrane, and that the serine proteinase inhibitor released from blood cells inhibits bacterial, as well as endogeneous proteinases that regulate immune responses [10,40].

Hemocyte extract was chromatographed on Trypsin-Sepharose matrix and the material eluted from the column inhibited only

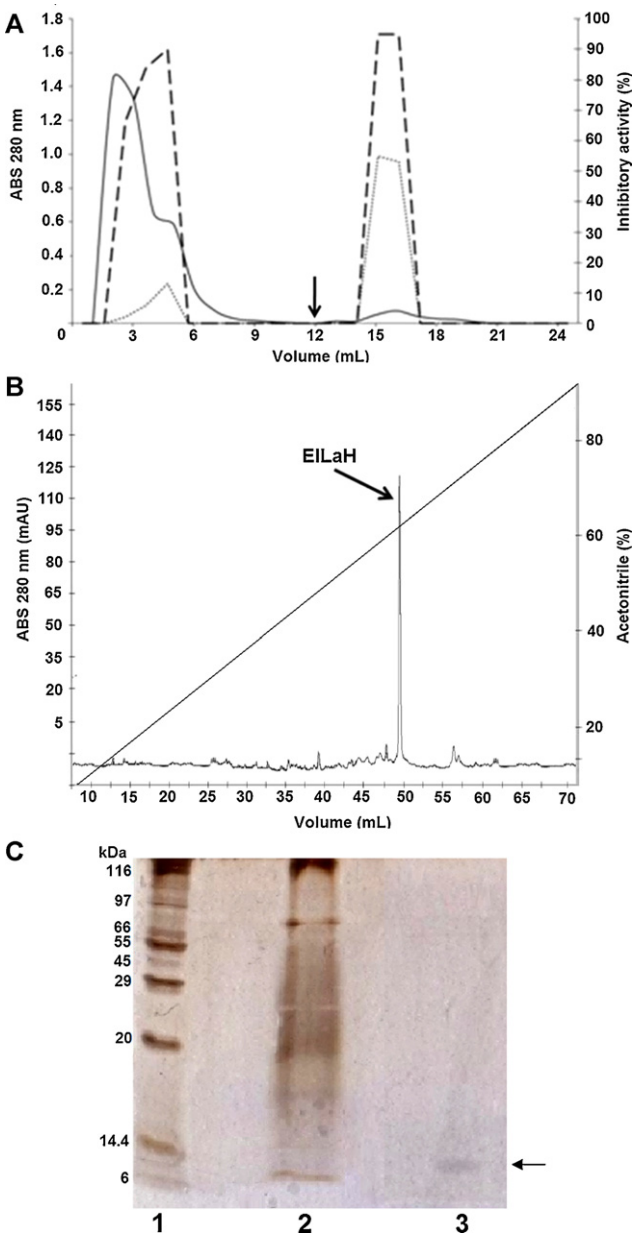

Fig. 1. Purification of EILaH from hemocytes of *Lasiodora* sp. (A) Affinity chromatography on Trypsin-Sepharose of hemocyte extract. Non-adsorbed and eluted fractions were evaluated for protein by absorbance at 280 nm (–) and for trypsin (····) and neutrophil elastase (- - - -) inhibitory activities. The arrow indicates the start of the elution step. (B) Reversed phase chromatography on Sephasil Peptide C18 column of EILaH purified from the eluate of chromatography on Trypsin-Sepharose. Protein elution was done by linear acetonitrile gradient (0–90%) and was monitored by absorbance at 280 nm. Only the fraction represented by an arrow exhibited inhibitory activity, only against human neutrophil elastase, corresponding to EILaH. (C) SDS-PAGE of molecular mass markers (1), the sample (40 μg) eluted from Trypsin-Sepharose column (2) and EILaH (80 μg) (3).

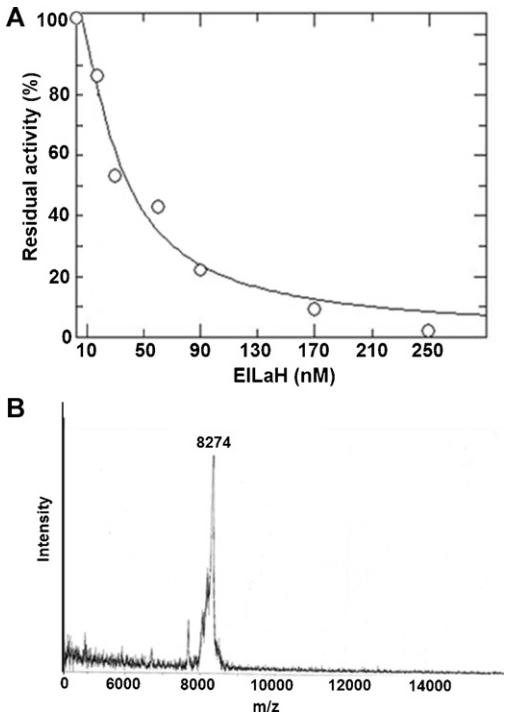

Fig. 2. Characterization of EILaH. (A) Inhibition curve used to calculate the dissociation constant (K_i = 0.32 nM) of EILaH for human neutrophil elastase. The percentage of residual HNE activity was determined by the ratio between residual activity in presence of EILaH and the HNE activity in absence of the inhibitor. (B) MALDI-TOF-MS of EILaH.

trypsin and HNE, indicating that this chromatography step was important to exclude inhibitors of other proteases present in the hemocyte extract. SDS-PAGE revealed the presence of multiple polypeptide bands, probably different trypsin inhibitors. The EILaH peptide, although present in the eluted sample from Trypsin-Sepharose colunm, was not detected by SDS-PAGE when 40 μg of protein were applied on gel, probably due to its lower concentration, when compared to other proteins in the sample. On another hand, low protein resolution and separation were achieved by electrophoresis when 80 μg of protein were used (data not shown).

The final purification of EILaH was obtained by a reversed phase chromatography and the homogeneity of preparation was demonstrated by detection of a single peptide on SDS-PAGE and a single molecular mass by MALDI-TOF, revealing the absence of contaminant molecules. These results indicate that this chromatographic step removed trypsin inhibitors other than EILaH that were present in the material from affinity column. The removal of a great number of other inhibitors may be the reason for the low activity yield after this purification stage, as shown in Table 1. SDS-PAGE separation efficiency of proteins with a molecular mass of less than 10 kDa is usually low but this method was able to detect EILaH peptide (8 kDa) as well as the serine protease inhibitor isolated from *B. microplus* (8 kDa) [41].

EILaH was active against HNE after RP-HPLC, but not against trypsin. Probably, the elution conditions promoted structural changes in EILaH, impairing its ability to bind trypsin, but did not affect the ability to inhibit HNE. Another possibility is that EILaH affinity for HNE is much higher than for trypsin, pointing to the need for high amounts of purified inhibitor required to inhibit trypsin. The EILaH structure required to inhibit HNE was resistant to denaturation by acetonitrile after RP-HPLC, similarly to the HNE/chymotrypsin inhibitor isolated from the ovary gland of *Schistocerca gregaria* [22].

Arthropods have a wide range of trapping-(which form irreversible complexes with target proteases) and tight-binding (which bind strong but reversible to proteases) inhibitors [42]. Since most serine proteases purified from invertebrates are tight-binding inhibitors, we chose this inhibition mechanism to evaluate the data obtained with EILaH. The effect of EILaH on HNE fitted well on this mechanism, and the equation used allowed the calculation of a K_i value of 0.32 nM, clearly revealing the high affinity of purified EILaH for elastase. The elastase inhibitors isolated from *B. microplus* and *S. gregaria* also showed K_i(s) in the nM range [22,41]. EILaH molecular mass (8274 Da) is similar to those of the inhibitors isolated from *B. microplus* (8000 Da) and *S. gregaria* (9229 Da).

Amino acid sequencing showed only one N-terminal sequence (LPC(PF)PYQQELTC), confirming that EILaH was not contaminated with another polypeptide chain. Partial amino acid sequences have been reported for proteins isolated from small animals. The N-terminal sequence AVFAIQDQPC was determined for the polypeptide from *Phoneutria nigriventer* spider venom responsible for the increased vascular permeability in rabbit skin, and the N-terminal sequence DHEVTS was reported for trypsin inhibitor purified from the skin secretions of frog *Kaloula pulchra hainana* [43,44].

EILaH amino acid sequence did not show any similarity with known members of the family of protease inhibitors. Similarly, neither the N-terminal sequence of the elastase inhibitor isolated from *Bufo andrewsi* skin secretions nor the whole sequence of *S. gregaria* inhibitor showed any similarity with other known serine protease inhibitors [22,45].

Antibacterial assays revealed that *B. subtilis* was sensible to hemocyte extract, but not to EILaH, and that both *Lasiodora* preparations inibited the growth of *E. faecalis*. The MIC value determined for EILaH was 15 times lower than that of hemocyte extract, indicating that purification of EILaH promoted increase in antibacterial activity. These data suggest that *Lasiodora* hemocytes contain other antibacterial agents besides EILaH active against *B. subtilis*, and that the weak bacteriostatic effect of hemocyte extract on *E. faecalis* may be associated to the low concentration of EILaH in this crude preparation. Coexistent anti-proteolytic and antimicrobial functions have also been demonstrated for elastase inhibitor isolated from human lung [46]. Nevertheless, the high MIC value, in a clinical perspective, indicates that EILaH is not suitable for practical application as a good antibacterial agent on *E. faecalis*.

Lasiodora has an open circulatory system and the presence of EILaH probably is important for the protection of animals against infection by *E. faecalis*, a pathogen of arthropods [47]. Similarly, it has been suggested that the antibacterial activity of serine protease inhibitor of epithelial and gland cells from *Hydra magnipapillata* is involved in Hydra's innate host defense [48].

Most hemocytes of other arthropods closely related to spiders contain granulocytes with internal granules that release their contents when the presence of microbial invaders is detected. The *Lasiodora* hemocytes may contain elastase as a protease involved in anti-inflammatory processes, and EILaH may have a role in regulating the elastase molecules.

The current literature includes studies about the toxins in *Lasiodora* sp. venom [49–51], but no investigation on serine protease inhibitors in hemocytes has been reported. EILaH is the first elastase inhibitor with bacteriostatic activity isolated from hemolymph cells of *Lasiodora* sp. The deleterious effect of EILaH against arthropod pathogen (*E. faecalis*) is an indicative of its participation in the innate immunity of *Lasiodora*. However, further studies are necessary to elucidate the physiological role of EILaH in this arthropod species as well as other potential medical applications.

Acknowledgements

The authors express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research grants and fellowships (LCBBC, AST, PMGP). We are also grateful to the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE), the Fundação de Amparo à Ciência e Tecnologia do Estado de São Paulo (FAPESP) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. We are grateful to Dr. Maria A. Juliano and Dr. Izaura Y. Hirata of Departamento de Biofísica, UNIFESP, for performing the mass spectrometry analysis and N-terminal amino acid sequencing, respectively. We thank Maria Barbosa Reis da Silva for technical assistance and Felix Nonnenmacher for English editing.

References

[1] Bertani R. Revision, cladistic analysis, and zoogeography of *Vitalius*, *Nhandu*, and *Proshapalopus*; with notes on other theraphosine genera (Araneae, Theraphosidae). Arq Zool 2001;36:265–356.
[2] Rash LD, Hodgson WC. Pharmacology and biochemistry of spiders venoms. Toxicon 2002;40:225–54.
[3] Baumann T, Kuhn-Nentwig L, Largiadèr CR, Nentwig W. Expression of defensins in non-infected araneomorph spiders. Cell Mol Life Sci 2010;67:2643–51.
[4] Cerenius L, Söderhäll K. The prophenoloxidase-activating system in invertebrates. Immunol Rev 2004;198:116–26.
[5] Iwanaga S, Lee BL. Recent advances in the innate immunity of invertebrate animals. J Biochem Mol Biol 2005;38:128–50.
[6] Fogaça AC, Almeida IC, Eberlin MN, Tanaka AS, Bulet P, Daffre S. Ixodidin, a novel antimicrobial peptide from the hemocytes of the cattle tick *Boophilus microplus* with inhibitory activity against serine proteinases. Peptides 2006;27:667–74.
[7] Jiravanichpaisal P, Lee BL, Söderhäll K. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 2006;211:213–36.
[8] Donpudsa S, Tassanakajon A, Rimphanitchayakit V. Domain inhibitory and bacteriostatic activities of the five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon*. Dev Comp Immunol 2009;33:481–8.
[9] Muta T, Iwanaga S. The role of hemolymph coagulation in innate immunity. Curr Opin Immunol 1996;8:41–7.
[10] Johansson MW, Keyser P, Soderhall K. Purification and cDNA cloning of a four-domain Kazal proteinase inhibitor from crayfish blood cells. Eur J Biochem 1994;223:389–94.
[11] Kanost MR. Serine proteinase inhibitors in arthropod immunity. Dev Comp Immunol 1999;23:291–301.
[12] de Marco R, Lovato DV, Torquato RJS, Clara RO, Buarque DS, Tanaka AS. The first pacifastin elastase inhibitor characterized from a blood sucking animal. Peptides 2010;31:1280–6.
[13] Fritz H, Wunderer G, Dittmann B. The isolation of porcine and human kallikrein using affinity chromatography: specific binding to water-insoluble Kunitz soybean-inhibitor-celluloses and dissociation with competitive inhibitors (benzamidine). Hoppe Seylers Z Physiol Chem 1972;353:893–900.
[14] Kato I, Schrode J, Kohr WJ, Laskowski Jr M. Chicken ovomucoid: determination of its amino acid sequence, determination of the trypsin reactive site and preparation of all three of its domains. Biochemistry 1987;26:193–201.
[15] Simonet G, Claeys I, Franssens V, De Loof A, Broeck JV. Genomics, evolution and biological functions of the pacifastin peptide family: a conserved serine protease inhibitor family in arthropods. Peptides 2003;24:1633–44.
[16] Laskowski Jr M, Kato I. Protein inhibitors of proteinases. Annu Rev Biochem 1980;49:593–626.
[17] Bode W, Huber R. Natural protein proteinase inhibitors and their interaction with proteinases. Eur J Biochem 1992;204:433–51.
[18] Laskowski M, Qasim MA. What can the structures of enzyme–inhibitor complexes tell us about the structures of enzyme substrate complexes? Biochim Biophys Acta 2000;1477:324–37.
[19] Morris MT, Coppin A, Tomavo S, Carruthers VB. Functional analysis of *Toxoplasma gondii* protease inhibitor. J Biol Chem 2002;277:45259–66.
[20] Kress H, Jarrin A, Thüroff E, Saunders R, Weise C, Busch MS, Knapp EW, Wedde M, Vilcinskas A. A Kunitz type protease inhibitor related protein is synthesized in *Drosophila prepupal* salivary glands and released into the moulting fluid during pupation. Insect Biochem Mol Biol 2004;34:855–69.
[21] Zheng QL, Chen J, Nie ZM, Lv ZB, Wang D, Zhang YZ. Expression, purification and characterization of a three-domain Kazal-type inhibitor from silkworm pupae (*Bombyx mori*). Comp Biochem Physiol B Biochem Mol Biol 2007;146:234–40.
[22] Brillard-Boudert M, Hamdaoui A, Hajjar E, Boudier C, Reuter N, Ehret-Sabatier L, Bieth JG, Gauthier F. A novel locust (*Schistocerca gregaria*) serine protease inhibitor with a high affinity for neutrophil elastase. Biochem J 2006;400:467–76.
[23] Somprasong N, Rimphanitchayakit V, Tassanakajon A. A five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon* and its inhibitory activities. Dev Comp Immunol 2006;30:998–1008.
[24] Visetnan S, Donpudsa S, Supungul P, Tassanakajon A, Rimphanitchayakit V. Kazal-type serine proteinase inhibitors from the black tiger shrimp *Penaeus monodon* and the inhibitory activities of SPIPm4 and 5. Fish Shellfish Immunol 2009;27:266–74.
[25] Sasaki SD, Cotrin SS, Carmona AK, Tanaka AS. An unexpected inhibitory activity of Kunitz-type serine proteinase inhibitor derived from *Boophilus microplus* trypsin inhibitor on cathepsin L. Biochem Biophys Res Commun 2006;341:266–72.
[26] Sasaki SD, de Lima CA, Lovato DV, Juliano MA, Torquato RJS, Tanaka AS. BmSI-7, a novel subtilisin inhibitor from *Boophilus microplus*, with activity toward Pr1 proteases from the fungus *Metarhizium anisopliae*. Exp Parasitol 2008;118:214–20.
[27] Tanaka AS, Andreotti R, Gomes A, Torquato RJ, Sampaio MU, Sampaio CA. A double headed serine proteinase inhibitor – human plasma kallikrein and elastase inhibitor – from *Boophilus microplus* larvae. Immunopharmacology 1999;45:171–7.
[28] Chugtai BAB, O'Riordan TG. Potential role of inhibitors of neutrophil elastase in treating diseases of the airway. J Aerosol Med 2004;17:289–98.
[29] Fitch PM, Roghanian A, Howie SEM, Sallenave J-M. Human neutrophil elastase inhibitors in innate and adaptative immunity. Biochem Soc Trans 2006;34:279–82.
[30] Scott CJ, Taggart CC. Biologic protease inhibitors as novel therapeutic agents. Biochimie 2010;11:1681–8.
[31] Baumann T, Kämpfer U, Schürch S, Schaller J, Largiadèr C, Nentwig W, Kuhn-Nentwig L. Ctenidins: antimicrobial glycine-rich peptides from the hemocytes of the spider *Cupiennius salei*. Cell Mol Life Sci 2010;67:2787–98.
[32] Bradford M. A rapid and sensive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[33] Sampaio CAM, Wong SC, Shaw E. Human plasma kallikrein. Purification and preliminary characterization. Arch Biochem Biophys 1974;165:133–9.
[34] Laemmli UK. Cleavege of structural protein during assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[35] Bieth JG. Pathophysiological interpretation of kinetic constants of protease inhibitors. Bull Eur Physiopathol Respir 1980;16:183–97.
[36] Morrison JF. Kinetics of the reversible inhibition of enzyme catalysed reactions by tight-biding inhibitors. Biochim Biophys Acta 1969;185:269–86.
[37] Edman P, Begg G. A protein sequenator. Eur J Biochem 1967;1:80–91.
[38] Amsterdam D. Susceptibility testing of antimicrobials in liquid media. In: Loman V, editor. Antibiotics in Laboratory Medicine. Baltimore: Williams and Wilkins; 1996. p. 52–111.
[39] Azzolini SS, Santos JM, Souza AF, Torquato RJ, Hirata IY, Andreotti R, Tanaka AS. Purification, characterization, and cloning of a serine proteinase inhibitor from the ectoparasite *Haematobia irritans irritans* (Diptera: Muscidae). Exp Parasitol 2004;106:103–9.
[40] Agarwala KL, Kawabata S, Hirata M, Miyagi M, Tsunasawa S, Iwanaga S. A cysteine protease inhibitor stored in the large granules of horseshoe crab hemocytes: purification, characterization, cDNA cloning and tissue localization. Biochemistry 1996;119:85–94.
[41] Sasaki SD, Azzolini SS, Hirata IY, Andreotti R, Tanaka AS. *Boophilus microplus* tick larvae, a rich source of Kunitz type serine proteinase inhibitors. Biochimie 2004;86:643–9.
[42] Gubb D, Sanz-Parra A, Barcena L, Troxler L, Fullaondo A. Protease inhibitors and proteolytic cascades in insects. Biochimie 2010;92:1749–59.
[43] Bento AC, Rego E, Pedroso-Mariani SR, Mancuso LC, Giglio JR, Novello JC, Marangoni S, Caracelli I, Oliveira B, Antunes E, de Nucci GE. Isolation of a polypeptide from *Phoneutria nigriventer* spider venom responsible for the increased vascular permeability in rabbit skin. Toxicon 1995;33:171–8.
[44] Zhang Y, Wang M, Wei S. Isolation and characterization of a trypsin inhibitor from the skin secretions of *Kaloula pulchra hainana*. Toxicon 2010;56:502–7.
[45] Zhao Y, Jin Y, Wei S-S, Lee W-H, Zhang Y. Purification and characterization of an irreversible serine protease inhibitor from skin secretions of *Bufo andrewsi*. Toxicon 2005;46:635–40.
[46] Simpson AJ, Maxwell AI, Govan JRW, Haslett C, Sallenavea J-M. Elafin (elastase-specific inhibitor) has anti-microbial activity against Gram-positive and Gram-negative respiratory pathogens. FEBS Lett 1999;452:309–13.
[47] Youngjin P, Kim K, Kim Y. A pathogenic bacterium, *Enterococcus faecalis*, to the beet armyworm, *Spodoptera exigua*. J Asia-Pac Entomol 2002;5:221–5.
[48] Augustin R, Siebert S, Bosch TCG. Identification of a kazal-type serine protease inhibitor with potent anti-staphylococcal activity as part of *Hydra*'s innate immune system. Dev Comp Immunol 2009;33:830–7.
[49] Kalapothakis E, Kushmerick C, Gusmão DR, Favaron GOC, Ferreira AJ, Gomez MV, de Almeida AP. Effects of the venom of a Mygalomorph spider (*Lasiodora* sp.) on the isolated rat heart. Toxicon 2003;41:23–8.
[50] Kushmerick C, de Carvalho FM, de Maria M, Massensini AR, Romano-Silva MA, Gomez MV, Kalapothakis E, Prado MAM. Effects of a *Lasiodora* spider venom on Ca21 and Na1 channels. Toxicon 2001;39:991–1002.
[51] Vieira ALG, Moura MB, Babá EH, Chávez-Olórtegui C, Kalapothakis E, Castro IM. Molecular cloning of toxins expressed by the venom gland of *Lasiodora* sp. Toxicon 2004;44:949–52.