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

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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

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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*