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Research paper

Cloning, expression and characterization of a phospholipase D from *Loxosceles gaucho* venom gland

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ABSTRACT

Loxosceles venom comprises a mixture of diverse toxins that induces intense local inflammatory reaction, dermonecrotic injury, platelet aggregation, hemolytic anemia and acute renal failure. Among several toxins in the venom, phospholipases D (PLDs), also called dermonecrotic toxins, are the most important and best studied, since they account for the main effects observed in loxoscelism. Despite their importance, biological analysis of PLDs is hampered by the minute amounts normally purified from the venom, and therefore many efforts have been made to clone those toxins. However, to date, no PLD from *Loxosceles gaucho* has been obtained in a heterologous system. Thus, in this work we show the cloning of a PLD from *L. gaucho* venom gland, named LgRec1, which was successfully expressed in a bacterial system. LgRec1 evoked local reaction (edema, erythema, ecchymosis, and paleness), dermonecrosis and hemolysis. It was also able to hydrolyze sphingomyelin and promote platelet aggregation. ELISA and Western blot analysis showed that LgRec1 was recognized by an anti-*L. gaucho* venom serum, a commercial arachnidic antivenom as well as a monoclonal antibody raised against the dermonecrotic fraction of *L. gaucho* venom. In addition, LgRec1 demonstrated to be highly immunogenic and antibodies raised against this recombinant toxin inhibited local reaction (~65%) and dermonecrosis (~100%) elicited by *L. gaucho* whole venom. Since PLDs are considered the major components accounting for the local and systemic envenomation effects caused by spiders from genus *Loxosceles*, the information provided here may help to understand the mechanisms behind clinical symptomatology.

1. Introduction

Loxosceles spider venom induces a necrotic-hemolytic syndrome in patients [1]. At least three different *Loxosceles* species of medical importance are known in Brazil (*Loxosceles intermedia*, *Loxosceles gaucho*, and *Loxosceles laeta*), and according to the Brazilian Ministry of Health around 7900 cases of loxoscelism were reported in 2010. In fact, owing to the frequency and morbidity of *Loxosceles* bites, it is considered the most important form of arachnidism in Brazil [2]. The clinical picture caused by *Loxosceles* spiders is mainly characterized by an intense inflammatory reaction and dermonecrosis at the bite site. In severe cases, massive intravascular hemolysis may result in acute renal failure, which is the primary cause of death in loxoscelism [1,3–5]. In Brazil, therapeutics include antivenom administration, associated or not with corticosteroids [6].

Studies have shown that *Loxosceles* venom is rich in proteases, hyaluronidases, hydrolases, lipases, peptidases, collagenases, phospholipases D, alkaline phosphatases and other components [3,7,8]. Phospholipases D (PLDs) – also called dermonecrotic toxins, sphingomyelinases D or SMases – are considered the major components accounting for the local and systemic effects of *Loxosceles* venom, and are thereby the most important and best studied toxins therein. Many reports have shown that native PLDs may cause massive inflammatory response, dermonecrosis, hemolysis and platelet aggregation [9–11]. Due to the importance of PLDs in *Loxosceles* envenomation, great efforts have been made to understand the mechanism of action of these toxins. However, the difficulty in obtaining large amounts of venom and isolating its toxins represent a limiting factor. Therefore, many PLDs have been cloned

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from the venom glands of *L. intermedia* [12–16] and *L. laeta* [17–20] and expressed in bacterial systems to obtain large amounts of PLDs.

Proteomic analysis of *L. gaucho* venom led to the identification of at least eleven PLDs isoforms, named loxnecrogin [21,22], but so far, none of them has been cloned and expressed to evaluate their biological activities. *L. gaucho* venom possess many important activities, such as the ability to activate keratinocytes in primary cultures to produce TNF-α [23], to induce hematological and hemostatic disturbance in rabbits [24], to cause intense inflammatory reaction [25], to hydrolyze sphingomyelin [8], and to stimulate the generation of pro-inflammatory cytokines and lipid mediators [26]. In addition, *L. gaucho* crude venom, as well as its dermonecrotic fraction promote human and rabbit platelet aggregation [11] and induce kidney damage and rhabdomyolysis [27]. Many of these activities are associated with PLDs, and despite the high homology shared by these toxins they may exhibit distinct biological activities [16]. These small differences are probably due to mutations of amino acids affecting substrate affinity or protein stability leading to variation in their activities such as dermonecrosis, platelet aggregation, hemolysis, etc. Therefore, information about sequences and activities of new PLDs will be important to establish structure/function relationship that may help to better understand the mechanisms behind clinical symptomatology caused by these molecules. So, in order to bring more information about these toxins, in this work we show the cloning, expression and biological characterization of a new recombinant PLD isolated from *L. gaucho* venom gland.

2. Material and methods

2.1. Ethics

The procedures involving animals were conducted according to national laws and policies controlled by Butantan Institute Animal Investigation Ethical Committee (protocol n° 697/10) and IBAMA (Brazilian Institute for the Environment and Renewable Natural Resources) provided animal collection permission n° 15383-2, while CGEN (Board of Genetic Heritage Management) provided the license for genetic patrimony access (02001.005110/2008). All microorganisms manipulation was approved by CTNBio (National Technical Commission on Biosecurity) (n° 2949/2011). All procedures involving human blood were approved by São Paulo Health Institute – Ethical Committee for Human Protocol: 019/2008/SES/IS/CEPIS.

2.2. Animals and venoms

Adult New Zealand white rabbits (3–4 kg) were provided by the Butantan Institute Animal House. Specimens of adult *L. gaucho* spiders were collected in São Paulo state (Brazil) and kept in quarantine for 1 week without food before venom collection. The venoms were obtained as previously described [28]. Protein concentrations were determined in duplicate by the bicinchoninic acid (BCA) assay [29]. Standard curves were constructed using bovine serum albumin (Sigma Chemicals, St Louis, MO) diluted in duplicate.

2.3. Molecular cloning of LgRec1

A hundred and fifty *L. gaucho* spiders were milked to stimulate the production of mRNAs in the venom glands. After 5 days, the spider venom glands were collected and total RNA was extracted from venom glands using the RNeasy Lipid Tissue Mini Kit (Qiagen) following the manufacturer instructions. Five micrograms of total RNA was transcribed into cDNA using the 3′RACE system (Invitrogen™) following supplier instructions. PCR amplification was conducted using Platinum® *Pfx* DNA Polymerase (Invitrogen™). The reverse primer was from the 3′RACE system kit, while the forward random primer (CRGATGWYGVDGAACG) was generated aligning the cDNA sequences of phospholipases D from *L. laeta* (SMase I-AY093599), *L. intermedia* (LiRecDT1-DQ218155 and LiRecDT2-DQ266399) and *Loxosceles reclusa* (SMase D-AY862486). The PCR product was cloned into pGEM®-T Easy Vector Systems (Promega) after A-tailing procedure as described by the manufacturer's instruction. This product was then used to transform calcium competent *Escherichia coli* DH5α cells which were subsequently plated on an LB agarose plates containing 100 μg/mL of ampicillin. White colonies were sequenced using an ABI 373A automated sequencer and Prism labeling kit (Applied Biosystems, Warrington, UK). One sequence showing high similarity with other spider phospholipases D was named LgRec1 and deposited in the GenBank under accession number JX866729.

2.4. Expression and purification of mature recombinant LgRec1

cDNA encoding for putative mature dermonecrotic protein LgRec1 were amplified by PCR with forward and reverse primers containing *Bam*HI and *Hind*III restriction site, respectively. This amplicon was subcloned in frame with a 6xHis tag sequence present in a T7 based promoter plasmid pAE that express the protein into bacteria cytoplasm [30], resulting in the pAE-LgRec1 vector. Chemically competent *E. coli* BL21 Star™(DE3)pLysS (Invitrogen™) cells were transformed with pAE-LgRec1 construction and plated on LB agarose plates containing 100 μg/mL ampicillin and 34 mg/mL chloramphenicol and grown overnight at 37 °C. Next day, a single colony was then inoculated into LB broth (containing 100 μg/mL ampicillin and 34 mg/mL chloramphenicol) and grown overnight at 37 °C. The overnight culture was then used to inoculate 250 mL of fresh LB (ampicillin/chloramphenicol) medium at 1:100 dilution. At an optical density (600 nm) of 0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce LgRec1 expression. The culture was allowed to express for 4 h before the cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C. Cells were resuspended in binding buffer (50 mM sodium phosphate pH 7.0, 300 mM NaCl and 10 mM of imidazole) and intermittently sonicated on ice for 60 s with intervals of 2 min for cooling, with total sonication time of 6 min. Cells were then harvested by centrifugation (7000 × *g*, 10 min, 4 °C), and the recombinant protein was purified from the supernatant by immobilized metal affinity chromatography (IMAC) using Talon resins (Clontech) charged with cobalt following the manufacturer recommendations. After purification the recombinant protein was dialyzed against phosphate buffered saline (PBS) and analyzed in a 12% SDS-PAGE under reducing conditions.

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sample of *L. gaucho* venom (5 μg) and LgRec1 (2.5 μg) were analyzed by SDS-PAGE (12.5%-T running gels) under reducing (2.5% DTT) and non-reducing conditions. Prior to electrophoresis, the samples were mixed with sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, and 0.02% bromophenol blue) and boiled for 5 min. After electrophoresis, proteins were stained with Coomassie Blue R-250 or by silver nitrate [31]. Pre-stained molecular mass markers (Kaleidoscope pre-stained standards (BioRad, Hercules, CA)) were used to estimate the protein molecular mass.

2.6. Molecular mass determination by MALDI-TOF MS

The molecular mass of LgRec1 was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry