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Toxins from *Lonomia obliqua* — Recombinant Production and Molecular Approach

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Additional information is available at the end of the chapter

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1. Introduction

Few species of butterflies and moths (order Lepidoptera) are involved in human envenoming [1]. Caterpillars are the larval forms of moths and butterflies. Toxins are usually found in the caterpillar's hairs and spines with defense purposes. The majority of medically important encounters with lepidopterans occur with exposure to the caterpillar's urticating hairs or spines, but hemolymph can also have toxic properties [1, 2]. A variety of clinical effects have been described, which depend on the family and species involved, ranging from local to systemic reactions [3, 4].

In most occasions, the adverse effects caused by caterpillars are self-limited and can be treated with topical antipruritics [4]. However, for the envenoming by the South American *Lonomia obliqua* caterpillars (Figure 1), named lonomism, the antilonomic serum produced at the Butantan Institute in Brazil is the only effective treatment to reestablish the coagulation parameters in poisoned patients and to avoid the complications seen in severe cases such as intracerebral hemorrhage and acute renal failure [5-10].

In 1989, an outbreak of accidents with this species became a serious public health threat in southern Brazil, with high fatality rates [5, 11-15]. Since then, many studies have been carried out to understand the pathophysiological mechanisms of envenoming [14] and to identify the toxins responsible for adverse reactions.

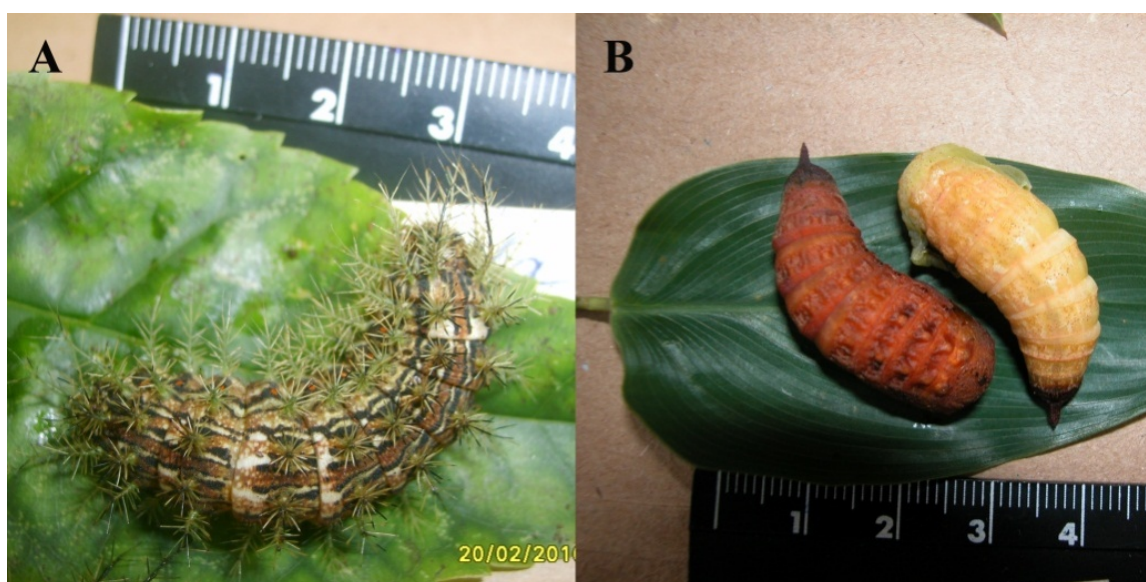


Figure 1. A) *Lonomia obliqua* caterpillar (5th instar) and B) pupa.

L. obliqua is the caterpillar that has the most studied venom, which main components have been isolated and characterized [14, 16, 17]. Table 1 lists the biological activities and toxins isolated and characterized from the bristle extract or hemolymph of *L. obliqua*. *In vivo* studies reported an antithrombotic effect caused by the bristle extract, while most *in vitro* studies reported procoagulant activities [14, 16-23]. It is well known, for a wide range of animal venoms, that procoagulant toxins can cause *in vivo* activation of the coagulation system. The hemostatic disturbances observed in the envenoming by *L. obliqua* caterpillars, result in a consumption coagulopathy (resembling a disseminated intravascular coagulation) and secondary fibrinolysis, which can lead to the hemorrhagic syndrome [6].

The principal components in the caterpillar's venom have been initially identified by isolating toxins through classical purification methods and following the main activities observed in the whole bristle extract (Figure 2). However, this approach provides knowledge restricted only to the most abundant toxins, and usually reveals that activities which are directly associated to the main symptoms and effects observed in the envenoming outcomes. Experimental assays were specifically developed to test the hemostatic and enzymatic activities of *Lonomia* toxins and their actions on the coagulation cascade. This knowledge has been valuable for description and management of the envenoming syndrome, but with the classical approach, low abundant components and unexpected effects are usually overlooked. Possible interaction of venom components, cross-reactions and secondary effects, useful to provide a systemic view of the pathways involved in the toxin's effects are often unnoticed.

In the last years, methods applied in genomic, transcriptomic and proteomic analyses have been applied with the aims of cataloging and classifying the toxins based on their structure and activity (Figure 3). Thus, it was possible to analyze the envenoming processes at the molecular level. For example, significant advance was achieved through two independent transcriptome studies, which generated a list of putative toxic proteins from *L. obliqua* bristles

Activity (toxin)	Source	MW (kDa)	Characteristics and observed effects	Reference
Prothrombin activation (Lopap)	Bristle extract	21	Serine protease, activity increased by Ca ²⁺ ; consumption coagulopathy <i>in vivo</i> ; cell survival in endothelial cell culture. Recombinant form produced in bacteria and yeast.	[18, 19, 21, 24-26]
FXa-like	Bristle extract	21	Hydrolytic activity on S-2222 chromogenic substrate, Ca ²⁺ -independent; N-terminal sequence similar to Lopap.	[27]
Factor X activation (Losac)	Bristle extract	45	Serine protease, Ca ²⁺ -independent; Cell survival in HUVEC. Recombinant form produced in bacteria.	[20-22, 28]
Phospholipase A ₂ -like	Bristle extract	15	Indirect hemolytic activity in human and rat red blood cells <i>in vitro</i> , Ca ²⁺ -independent; intravascular hemolysis <i>in vivo</i> .	[29-31]
Fibrinogenolytic (Lonofibrase)	Hemolymph	35	αβ fibrinogenase activity; enable to affect fibrin cross-linked.	[32-34]
Hyaluronidase (Lonoglyases)	Bristle extract	49 53	β-endohexosaminidase activity; degradation of extracellular matrix.	[35]
Antiapoptotic	Hemolymph	51	Activity on <i>Spodoptera frugiperda</i> (Sf-9) cell culture.	[36]
Antiviral	Hemolymph	20	Antiviral activity against measles, influenza and polio viruses. Recombinant form produced in baculovirus/insect.	[37, 38]
Nociceptive and Edematogenic	Bristle extract	NI	Nociception facilitated by prostaglandin production; edematogenic response facilitated by prostanoids and histamine.	[39]
Kallikrein-kinin system activation	Bristle extract	NI	Kinin release from low molecular weight kininogen; edema formation and fall in arterial pressure.	[40]
Platelet adhesion and aggregation	Bristle extract	NI	Direct platelet aggregation and ATP secretion; activity inhibited by <i>p</i> -bromophenacyl bromide, a specific PLA ₂ inhibitor.	[41, 42]
NI: None isolated, studies carried out using the whole venom				

Table 1. Toxins and activities described in *L. obliqua* venom.

and hemolymph [20, 43]. In addition, significant advance was achieved as a result of microarray study [44]. Moreover, by coupling proteomics and immunochemical approaches, some immunogenic components were identifying in the bristle extract, especially those related to hemostasis [9]. These components were detected by the antilonomic hyperimmune serum produced at the Butantan Institute, and abundant proteins were identified.

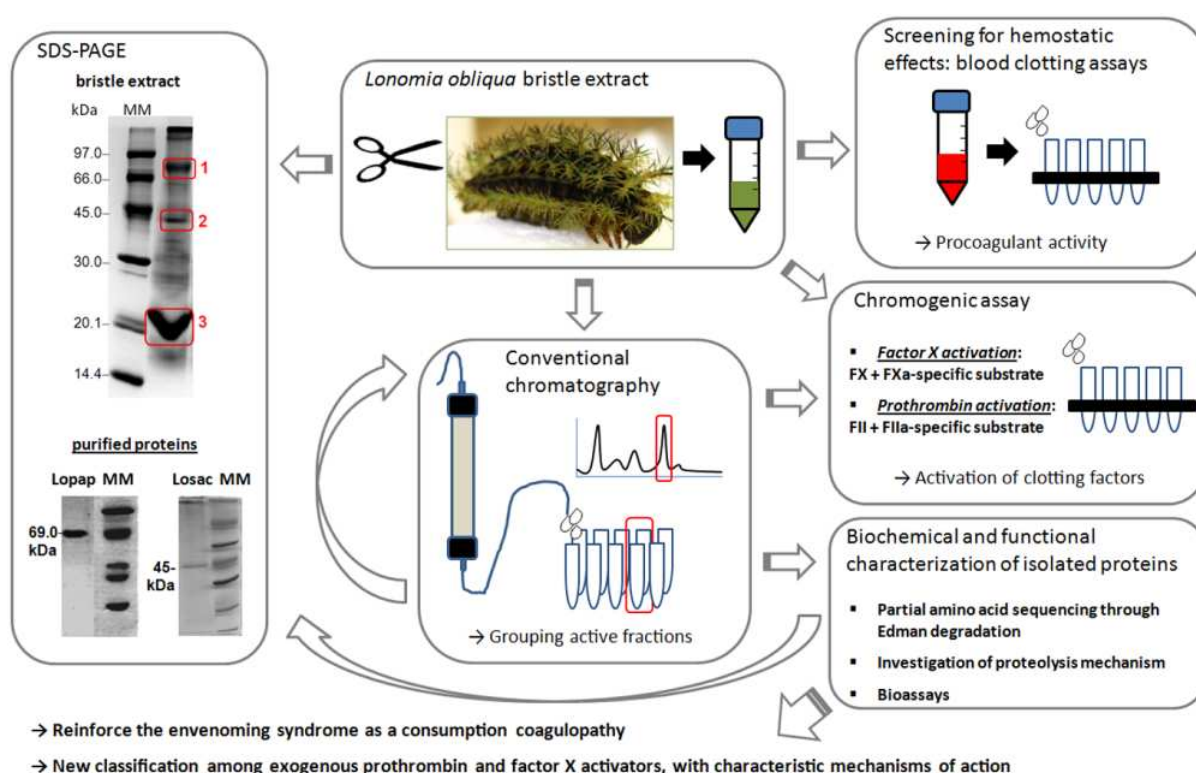


Figure 2. Schematic representation of the classical strategy employed in initial studies of the *Lonomia obliqua* venom. The bristle extract was analyzed through denaturing electrophoresis (SDS-PAGE) which showed the venom is a complex mixture of proteins. Screening assays were carried out to investigate possible effects on blood coagulation and fibrinolysis. The venom showed procoagulant activity by decreasing blood clotting time. Two procoagulant components (Lopap, a prothrombin activator and Losac, a factor X activator) were identified and isolated from the bristle extract for further characterization. The specific activity of Lopap and Losac were observed on purified coagulation factor zymogens (FII or FX) using chromogenic substrates to detect generation of active forms of clotting factors (FIIa and FXa) by these toxins. This assay was used in the purification process to identify the active fractions containing each toxin. SDS-PAGE profile shows Lopap (1- multimer, 3- monomer) and Losac (2) are abundant components in the venom. MM: molecular markers.

Production of recombinant forms of *Lonomia* toxins and discovery of new molecules are opening perspectives in the scientific area for basic and applied researches. These molecules can point out novel mechanisms of action, undiscovered molecular interactions and new classes of enzymes and inhibitors. Interesting, some venom toxins have shown multifunctional properties [19, 22, 28]. The best examples are Lopap (a prothrombin activator with high similarity with lipocalins) and Losac (a factor X activator highly similar to hemolins). Besides activation of blood coagulation, Lopap and Losac can modulate cellular functions and promote cell survival [22, 45]. Both molecules were cloned and produced in its recombinant form in yeast and/or bacteria [19, 25, 28].

Additional studies will be conducted to determine the involvement of the venom components in the envenoming syndrome and their biological significance for physiological processes of the animal, such as insect metamorphosis, which is a combination of growth/activation/differentiation/programmed cell death signals. Thus, this chapter reviews the currently

available information about *L. obliqua* venom, and focus on strategies to unveil molecular aspects of toxins and the perspectives for therapeutic and biotechnological applications.

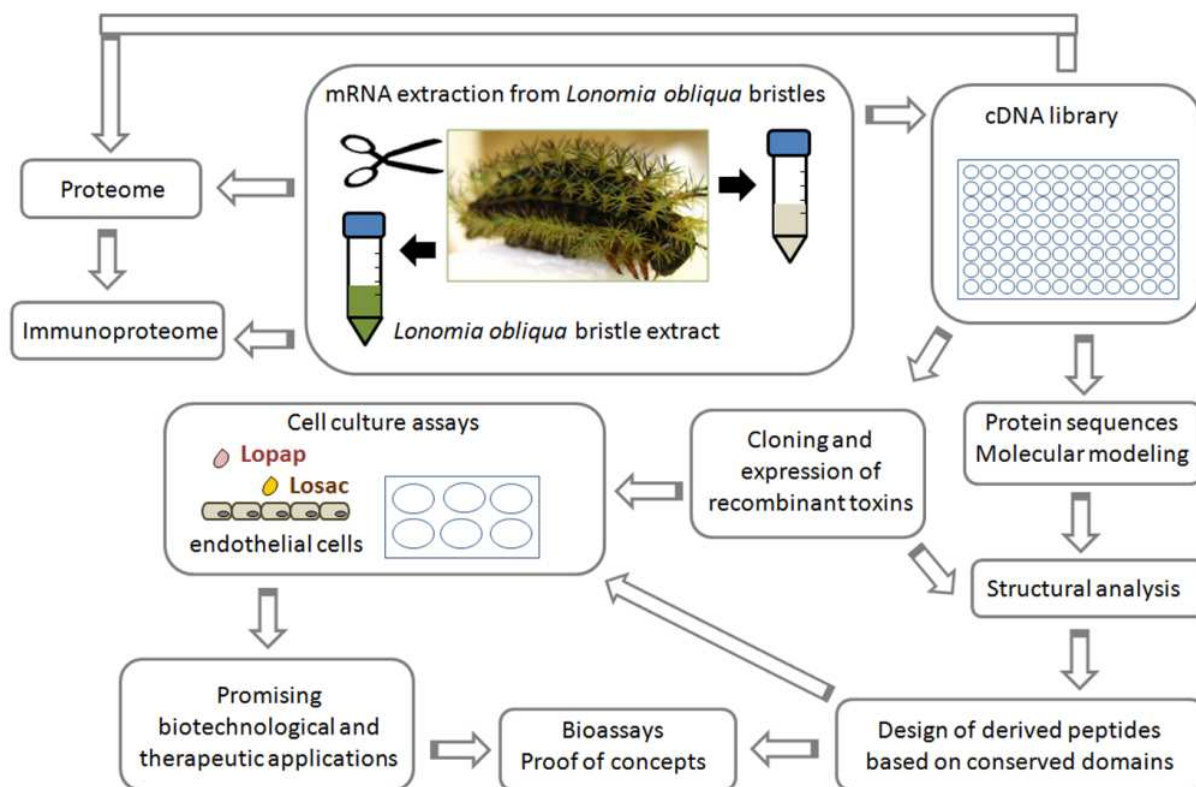


Figure 3. Schematic representation of the strategies to explore the *Lonomia obliqua* venom and toxins based on cellular and molecular approaches. Results obtained indicate promising applications for these proteins and derived peptides.

2. Molecular approach

For many years, direct purification of toxins from venoms was the best procedure to characterize them with regard to their primary structure. Then, the development of molecular approaches to characterize toxin genes represented an expansion in the understanding of the structure and function of toxin, critical for the development of new treatments directed against the venom toxins (antivenoms). Cloning of cDNAs coding for biochemically isolated toxins has improved their characterization. *Transcriptomic* allows the identification of cellular transcripts in a given cell population, while proteomic studies protein's properties and functions (expression level, structure, post-translational modification, etc.) of proteins expressed by the genome of an organism at a certain point of time. The availability of technologies for high throughput analysis has led to integrate toxin expression at mRNA and

protein level. This flow of genetic information from DNA to proteins is the base of the central dogma of molecular biology [46].

2.1. Transcriptomics of *Lonomia obliqua* bristle extract

Expressed Sequence Tags strategy is an approach to characterize the transcriptome of a cell, gland or organism and is based in all the transcript (the most abundant are the mRNAs) produced at a specific time and fully sequenced to create a representative catalogue of expressed genes [47]. Hundreds to a thousand of sequences are grouped into *contigs* or clusters based on DNA sequence information and bioinformatics analysis (Figure 4). Nowadays, the EST-based strategy is commonly employed for identifying expressed genes in species of interest [48, 49]. This approach has been successfully used to compile a lists of genes expressed in venom’s glands of a wide range of animals [50-53].

EST-strategy was used to identify the major transcripts present in *L. obliqua* bristle extract [19, 20, 43]. About 702 clusters (representing 1,278 independent clones) were assembled and characterized as lipocalins, hemolins, serine proteases, serine protease inhibitors, serpins, tumor suppressors, ribosomal, structural and cell cycle proteins as shown in Table 2 [20]. Most of the transcripts represent proteins involved in the animal physiology. Those sequences were deposited in data bank (NCBI GenBank accession numbers: CX815710–CX817210, CX820335–CX820336, AY908986) [20]. A pool of DNA sequences showed no similarities with well-known sequences in data bank. The most abundant toxin was a lipocalin of 21 kDa, and analysis of its N-terminal sequence shows 100% homology with Lopap (GenPept accession number: AAW88441). The Lopap whole sequence (accounting for 1.6% of the total clones) was identified in this cDNA library (accession number: AY908986).

Functional categories	No. of clusters	No. of clones	Clones/ clusters	% of Total	% of Hits
General Metabolism	72	94	1.30	6.1	7.0
Transcriptional and translational	165	462	2.80	30.7	37.0
Processing and sorting	10	13	1.30	0.8	1.0
Degradation	9	20	2.22	1.3	2.0
Structural functions	47	243	5.17	16.2	19.0
Cell regulation	26	82	3.15	5.4	6.0
Other functions	138	244	1.77	16.2	19.0
Conserved unknown proteins	42	120	2.86	8.0	9.0
TOTAL	509	1278	2.51	85.0	100.0

Table 2. Major transcripts present in the *Lonomia obliqua* bristle extract identified by EST-based strategy. Adapted from EST data-bank of NCBI deposited by Reis and colleagues in 2004 [19, 20].

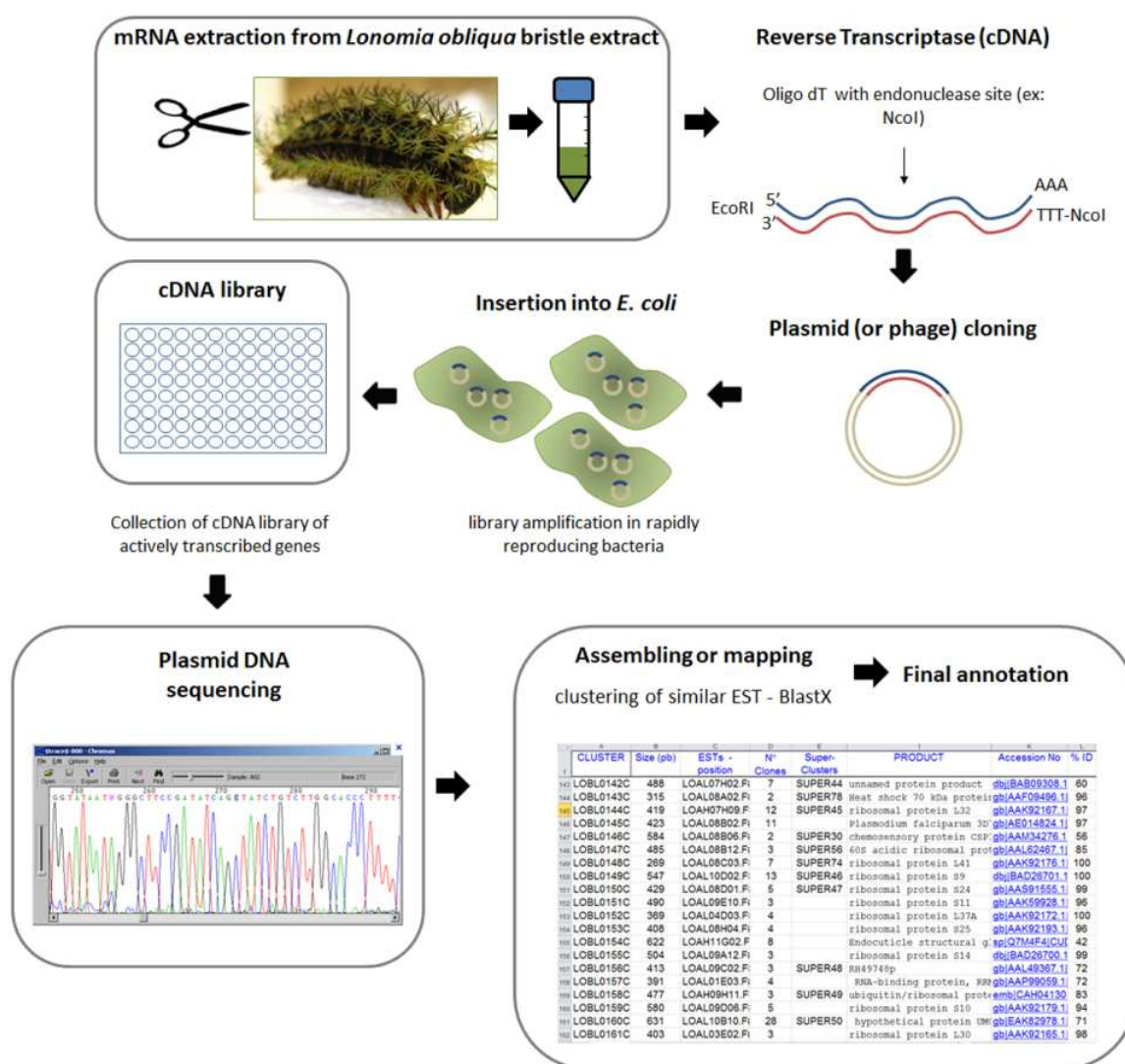


Figure 4. Schematic view of a transcriptomic approach based on EST-strategy. The strategy of construction of the cDNA library starts by the extraction of total RNA from a sample (ex. *L. obliqua* bristle extract). After purification of the mRNAs with an oligo (dT)-cellulose column, the cDNAs are synthesized (reverse transcription) by using synthetic oligo-nucleotides containing a restriction sites (in figure: NcoI and EcoRI for sense and antisense primers, respectively). The cDNA obtained can be inserted into a vector (plasmids or phages), generating a cDNA library. The library can be perpetuated by transforming the clones (plasmids) or infecting themselves (in the cases of phages) in *E. coli*. Based on DNA sequence information, bioinformatics tools predict the amino acid sequence of the corresponding gene products and their similarity to known genes. The redundant EST data sets are organized and integrated into cluster [54, 55].

Other cDNA libraries were constructed from bristles and integument [43]. The transcripts of those libraries revealed the presence of sequences related to trypsin-like enzymes, blood coagulation factors, prophenoloxidase cascade activators, cysteine proteases, phospholipase A2, serpins, cystatins, antibacterial proteins, lipocalins, and others (GenBank accession number: AY829732–AY829859) [43]. Sequences deposited independently in gene banks from both cDNA libraries are complementary. Apart from new venom component precursors, both

libraries describe gene products related to cellular processes important for venom production, including high protein synthesis, tuned post-translational processing and trafficking. Those important projects contributed significantly to the characterization of this venom, which showed to be a rich source of proteins and active principles. Further studies about the biological and pharmacological properties of these molecules are necessary to understand its involvement in the envenoming process. Recently, the next-generation of sequencing methods - for example, pyrosequencing - have improved and increased the sequencing reducing time and cost compared to the traditional Sanger method [47, 56].

2.2. Microarray analysis

The identification of genes expressed in cells of a tissue is a basic step to provide essential information about gene function and tissue physiology. The gene expression analysis through the microarray technology (cDNA arrays) has become a powerful tool for rapid analysis of the functional effects of toxins on cells and tissues [57]. The main application of cDNA arrays is to compare the expression of known genes in different physiological situations, for example, tissues in normal and pathological conditions [58]. Thus, analyses of array data contribute to a better understanding of complex gene expression patterns related to physiology and metabolism, unveiling networks or pathways previously unknown.

A study of the effects of *L. obliqua* bristle extract on the gene expression profile of cultured human fibroblasts showed that many genes are up- and down-regulated, especially those related to the inflammatory processes such as IL-8, IL-6, CXCL1 and CCL2 [44]. Other changes in the expression pattern of some genes, such as prostaglandin-endoperoxide synthase 2, urokinase-type plasminogen activator receptor and tissue factor, were also observed, which could contribute to the pathological effects of lonomism. The authors suggest that the clinical manifestations may be a result of the direct action of *L. obliqua* venom on the host cells allied to an indirect effect caused by alteration in the gene expression pattern in host tissues.

2.3. Immunoproteome of *Lonomia obliqua* bristle extract

The identification of antigens eliciting an immune response by applying proteomic technologies can be defined as *Immunoproteomics*. Some usual immunoproteomics approaches are shown in Figure 5. Here, the perspective for its application regards the improvement of serum therapy by the selection of antigens for toxin-specific immunization of horses. Furthermore, some applications correlate the identification of antigens with certain diseases, such as infectious, autoimmune or cancer, providing diagnostic and monitoring informations. In this way, these methodologies are good choices in developing clinical applications and also to discover biomarkers. [59].

In classical gel-based strategy, the isolation and identification of proteins/antigens comprises a combination of bidimensional electrophoresis, immunoblotting and mass spectrometry. The aim of bidimensional electrophoresis is to isolate proteins based on their charge and mass [60, 61]. The first step is isoelectric focusing (IEF), where proteins migrate to reach their isoelectric point in an immobilized pH gradient gel under high voltage. All proteins are given negative

charge by addition of SDS detergent. This step also includes denaturation of proteins by reduction and alkylation. The second step is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), where smaller proteins migrate faster through the gel to the anode than larger ones. Detection of proteins can be performed by gel staining or immunoblotting.

Immunoblotting involves the transfer of proteins from gel to a nitrocellulose or PVDF membrane in an electric field [62]. The immobilized proteins in the membrane are subsequently incubated with antibodies that have affinity for the proteins of interest. Detection is carried out by enzyme-labelled secondary antibodies against the constant region of the primary IgG antibody, followed by the addition of a chemilluminent substrate. The substrate reaction can be visualized by fluorescence.

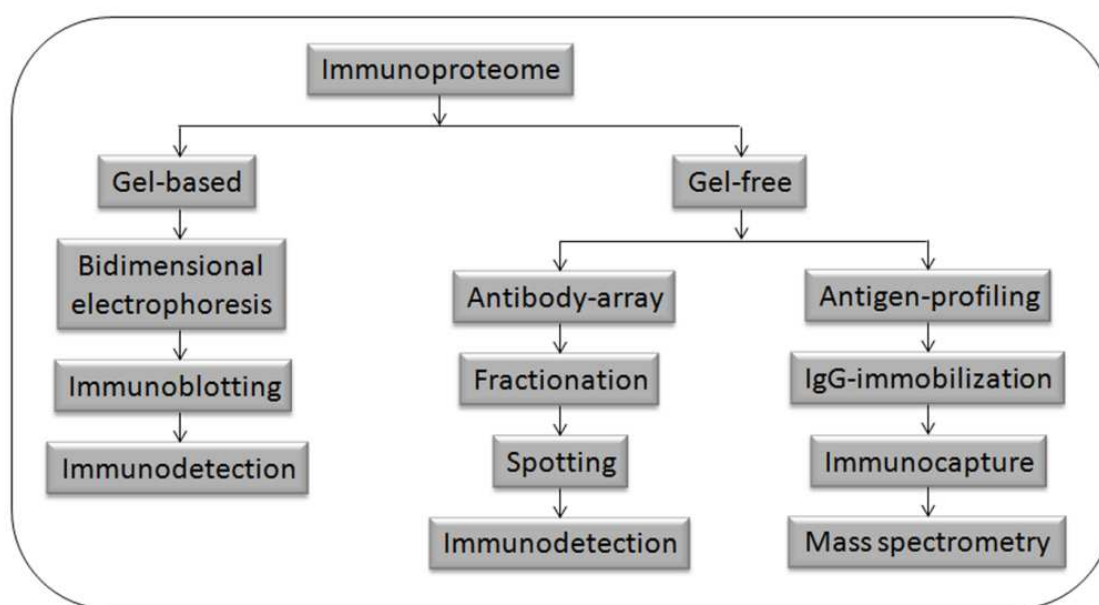


Figure 5. General approaches in Immunoproteomics. **Gel-based approach:** Bidimensional electrophoresis is based on protein separation on their pI and molecular mass. Then, the proteins are transferred from gel and immobilized on membrane (Western blotting). Antigens will be detected after serum incubation, followed by addition of secondary labelled antibodies and their substrates. **Gel-free approaches:** Antigen array: Proteins are fractionated (pI, hydrophobicity, etc) and spotted in a solid support. After that, antigenic fractions can be detected using patient serum and secondary labelled antibodies. Antigen profiling: Immunocapture is based on immobilization of patient immunoglobulins G, which are directly used to capture and isolate antigenic proteins from a complex mixture of proteins. Captured antigens are profiled by mass spectrometry (modified from 66).

Following this, the immunogenic proteins are removed from the gel and enzymatically digested for further mass spectrometry analyses [63]. Trypsin is generally used, cleaving an amide bond on the C-terminal side of lysine and arginine residues, which will be protonated and analyzed in positive-ion mode. Addition of diluted acid (0.1% formic acid or 1% trifluoroacetic acid) to the sample contributes to the ionization process.

The ionization methods that are most often used for peptides and proteins are Matrix Assisted Laser Desorption Ionization (MALDI) and Electrospray Ionization (ESI). Peptides and proteins

can be identified by Peptide mass fingerprinting (PMF) or *de novo* sequencing [63]. PMF is based on their fragmentation pattern, considering that identical peptide maps have identical amino acid sequences. For *de novo* sequencing analyses, the precursor ion is selected for fragmentation and the product ions are evaluated by mass differences between successive peaks in the spectrum, which are related to the individual mass of their residues.

The identification of immunogenic compounds from *L. obliqua*'s bristle extract was performed on gel-based approach using the polyclonal horse anti-Lonomic hyperimmune serum and anti-Lopap specific rabbit serum produced by the Butantan Institute [9]. Bidimensional electrophoresis of bristle extract revealed 157 silver stained spots, under non-reducing conditions (without DTT and iodoacetamide addition), providing an overview protein mapping (Figure 6A). However, 153 spots were immunodetected using anti-Lonomic serum (Figure 6B) and 30 spots detected using anti-Lopap serum (Figure 6C). Abundant proteins from 24 selected colloidal Coomassie Blue gel spots, corresponding to immunogenic proteins, were digested with trypsin and analysed by tandem mass spectrometry. The identification searches were carried out using the *L. obliqua* bristle EST databank. Lipocalins (spots 05, 09, 10, 14, 15, 16, 18, 24), cuticle protein (spots 05, 06, 07, 08, 11, 12, 13) and serpins (spot 21) were amongst the proteins identified (Figure 6A) [9]. Lipocalins can play a role in homeostasis and inflammation, as a defense mechanism in haematophagous arthropods. Lopap, characterized as a lipocalin protein member, and its all isoforms were highly represented as immunogenic proteins, revealed by the specific anti-Lopap serum (Figure 6C). The bristle' cDNA libraries also confirm the high abundance of lipocalins. As previously described [9, 19], these proteins have important role in envenoming. The cuticle proteins identified can be related to the inflammatory response caused by macerated spicule proteins. The serpin protein may also be involved in the defense mechanism.

Besides the biochemical and pharmacological tests, the quality control of serum and vaccine production can be monitored by proteomic technologies [64], such as chromatographic analyses, bidimensional electrophoresis and immunoblotting, once they are able to detect protein degradation and also confirm the presence of specific antibodies. However, immunotherapy can be more effective if a better characterization of venom composition is performed, improving immunization procedures, increasing its specificity and reducing side effects. The new generation of high affinity antibodies against low abundant immunogenic toxins can be evaluated by an antivenomic approach [64, 65].

A novel approach is the investigation of post-translational modifications (PTM) that affect antigen recognition, given that many peptides presented to T cells by the major histocompatibility complex are post-translationally modified [66]. Glycosylation and phosphorylation are important PTMs of proteins, playing crucial roles in several biological processes, including cell recognition and signalling pathway [67, 68]. Some potential targets for cancer therapy are based on glycosylated and phosphorylated epitopes discoveries [59].

Otherwise, phosphorylated proteins are usually enriched by immunoprecipitation (mainly for phosphotyrosine peptides) or by chromatographic procedures, such as Strong Cation exchange (SCX), Hydrophilic Interaction Liquid Chromatography (HILIC), Immobilized Metal Affinity Chromatography (IMAC) or Metal Oxide Affinity Chromatography (MOAC).

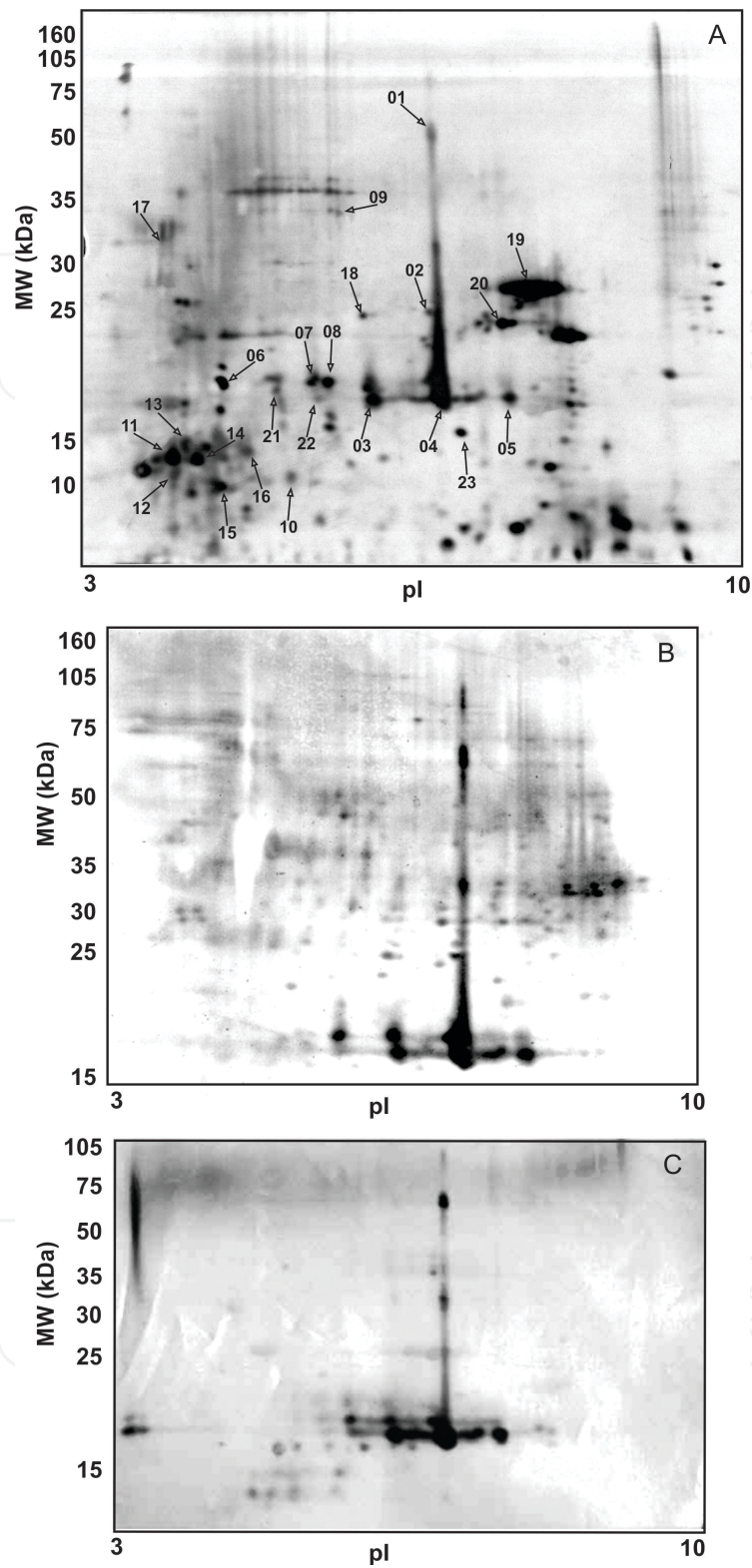


Figure 6. Bidimensional electrophoresis and immunoblotting from *Lonomia obliqua*'s bristle extract. (A): silver stained bidimensional gel (100 μ g of protein applied) under non-reducing condition. Panels (B) and (C): PVDF immunoblotted 2D gels incubated with anti-Lonomic horse serum diluted 1:500 (B) or with anti-Lopap rabbit serum diluted 1:250 (C) [9].

Different metals may be used (iron, zirconium, gallium, etc) and peptides eluted by acidic or basic conditions, releasing mono-phosphorylated and multi-phosphorylated peptides, respectively [67].

The simultaneous screening of thousands of proteins from complex samples in a fast and sensitive manner can be performed using protein arrays. Amongst the different protein microarray applications are biomarker discovery, protein interaction studies, enzyme-substrate profiling, immunological profiling and vaccine development. As our interest is in the immune response, an antibody microarray can be used for identification of antigens that react specifically with the antibodies spotted on a solid support, with the complex formed then detected by fluorescence [59].

A large number of not yet identified proteins are considered as unknowns, but higher probabilities of identifications are reached when different methodologies are applied for analysis of complex samples. The combination of several proteomic techniques described here could improve the detection of immunogenic compounds and create new perspectives for effective immunotherapies.

3. *Lonomia obliqua* toxins

The *L. obliqua* caterpillar venom contain non-protein and protein components [21]. The procoagulant proteins present in the venom cause hemostatic disturbances mainly mediated by thrombin formation, the key enzyme of blood coagulation. Physiologically, thrombin generation from prothrombin occurs by assembling of the prothrombinase complex, which consists of factor Xa (catalytic factor), factor Va (non-enzymatic cofactor), calcium and a phospholipid membrane surface. Despite figured as a cascade of subsequent activation of coagulation factors, blood coagulation is currently conceived in a cell based-model [69]. In Figure 7 a simplified cascade model of hemostasis is illustrated, showing the known interactions of the *L. obliqua* venom and toxins.

Several molecules and activities were reported in bristles or hemolymph (Table 1). Some of them are related to the pathophysiology of the envenoming others to the development process of the animal such as regulation of the cell cycle [16]. Donato and colleagues [70] identified in the bristle extract a direct factor X activator which is calcium-independent, and a prothrombin activator. The prothrombin and factor X activators were later isolated and named Lopap and Losac, respectively [22, 24]. Interestingly, both molecules are no longer similar with any well-known procoagulant molecule from human or any other species.

3.1. Lopap: Functional characterization, recombinant production and bioinformatics analysis

Lopap (*Lonomia obliqua* Prothrombin Activator Protease) was purified from the bristle extract as a 69-kDa protein through gel filtration followed by reverse-phase chromatography. The purified protein was subjected to trypsin hydrolysis and partial amino acid sequences of N-terminal and internal fragments were obtained through Edman degradation [24].

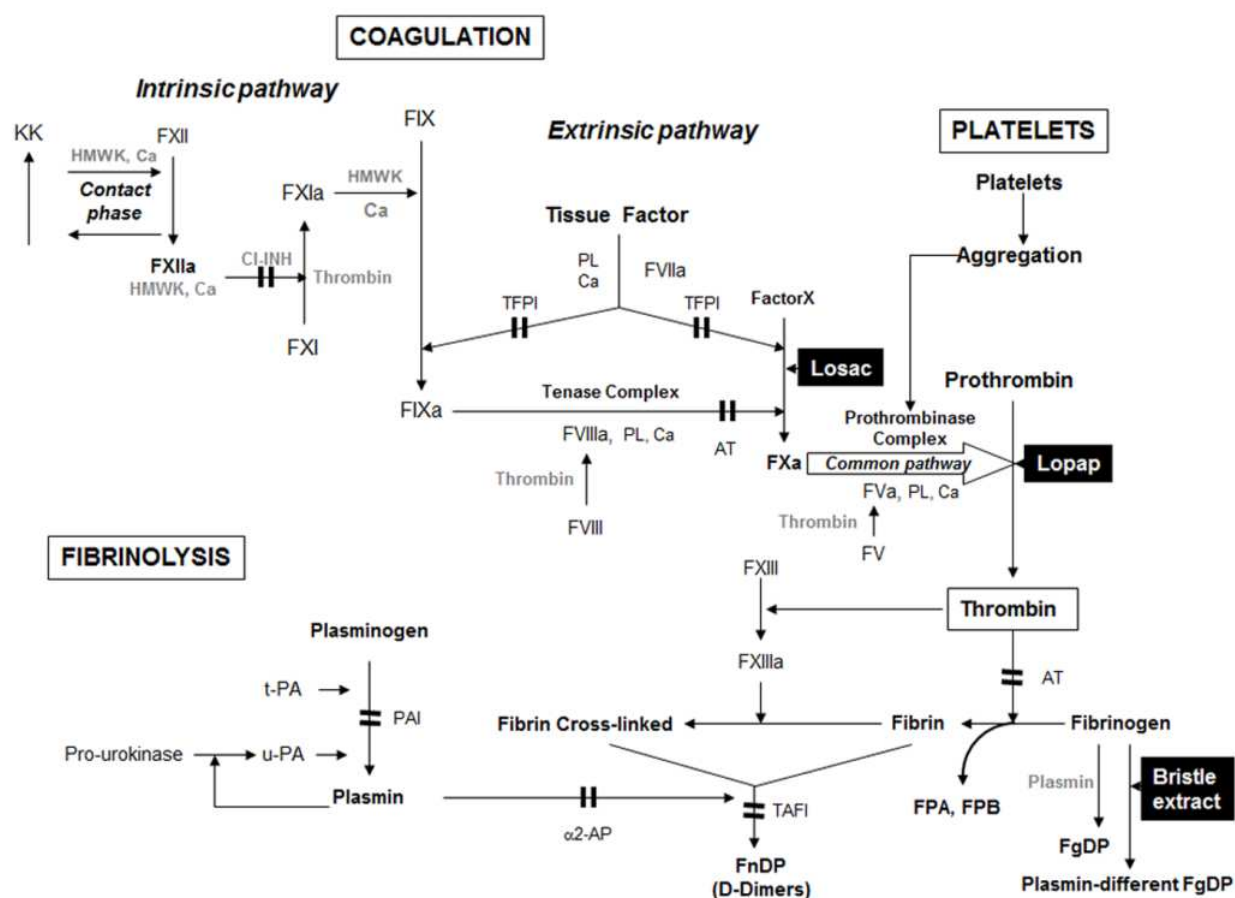


Figure 7. Schematic overview of hemostasis. Dark double-bars indicate where inhibitors act. HMWK = high molecular-weight-kininogen. PK = Prekallikrein. KK = Kallikrein. CI-INH = CI-inhibitor. TFPI = tissue factor pathway inhibitor. PL = phospholipids. Ca = calcium ions. AT = antithrombin. FDA = Fibrinopeptide A. FDB = Fibrinopeptide B. TAFI = Thrombin-activatable fibrinolysis inhibitor. FnDP = Fibrin degradation products. FgDP = Fibrinogen degradation products. α_2 -AP = α_2 -antiplasmin. Known interactions of the *L. obliqua* venom are indicated in the black boxes. Losac = *L. obliqua* Stuart factor activator. Lopap = *L. obliqua* prothrombin activator protease.

Interestingly, the deduced amino acid sequence of Lopap showed no similarity with other prothrombin activators or serine proteases, but was similar to lipocalin family members, either from insects or mammals [71]. Lopap sequence alignment with other lipocalins is shown in Figure 8. Members of lipocalin family usually share only about 30% of similarity in amino acid

sequence, despite showing conserved secondary and tertiary structures. Furthermore, these proteins have in primary structure three characteristic conserved motifs [72].

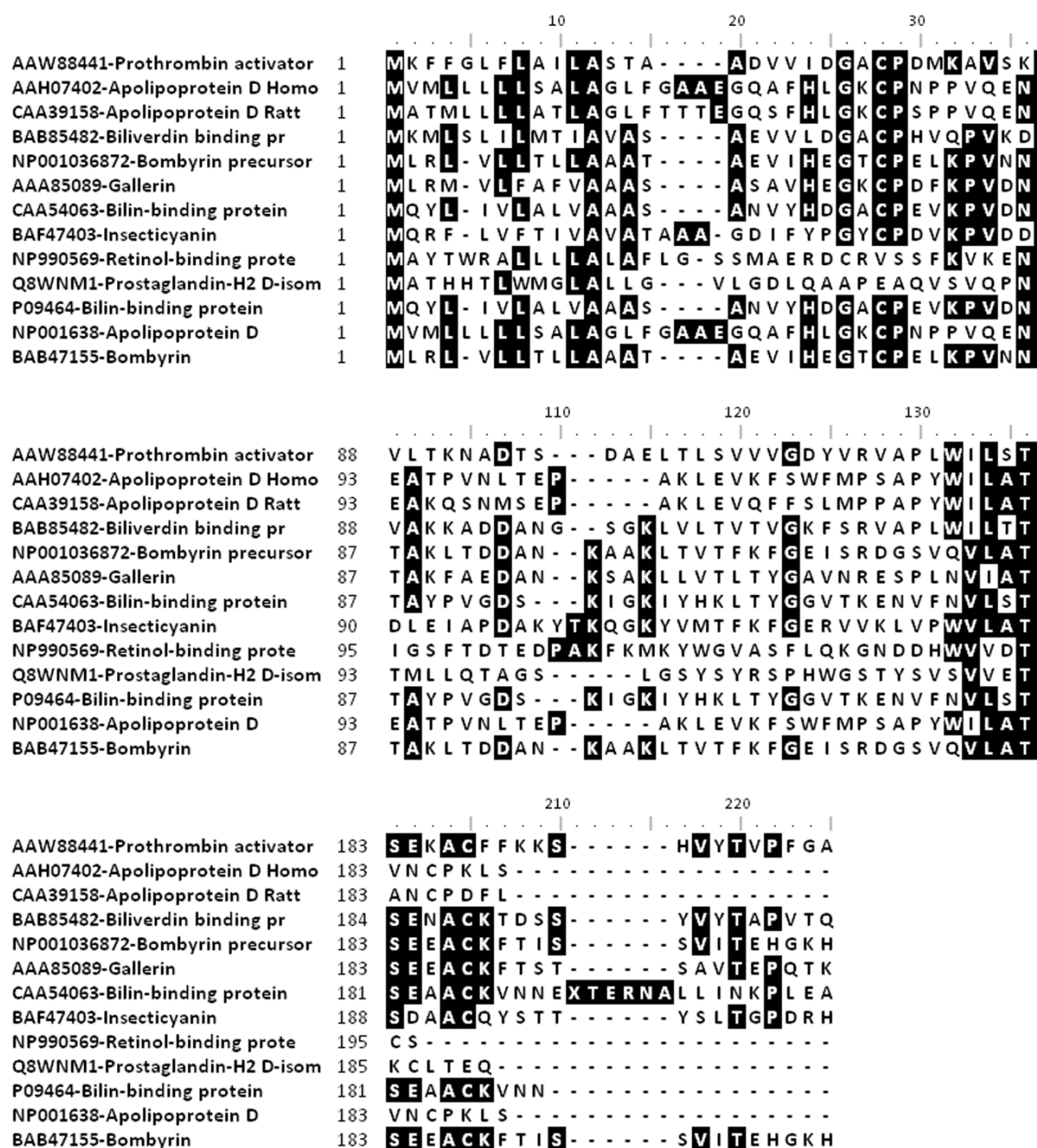


Figure 8. Amino acid sequence alignment of Lopap and other lipocalins. Sequences were accessed from protein data bank at NCBI and aligned using BioEdit [80].

Lopap's tridimensional structure obtained by molecular modeling has the characteristic fold of lipocalins, consisting in an eight stranded antiparallel β -barrel (Figure 9) with a hydrophobic pocket for binding of hydrophobic ligands. A serine protease catalytic triad was also predicted

[19]. Lopap is the first lipocalin described that displays proteolytic activity. On the other hand, through a peptide mapping approach based on lipocalin conserved motifs found in the Lopap's primary structure, a synthetic peptide was obtained (Figure 10), which has been proposed as a sequence signature among lipocalins, sharing a common role in cell protection and development process [73]. Other lipocalins that have been described with antiapoptotic activity share similar sequences, which have similar conformations in their tridimensional structures [73].

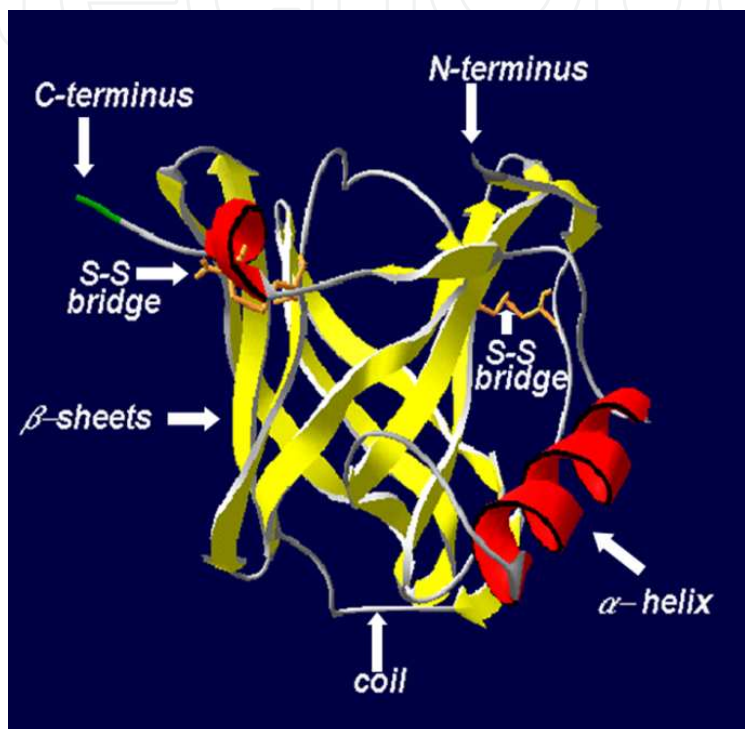


Figure 9. Model of the tridimensional structure of Lopap [81].

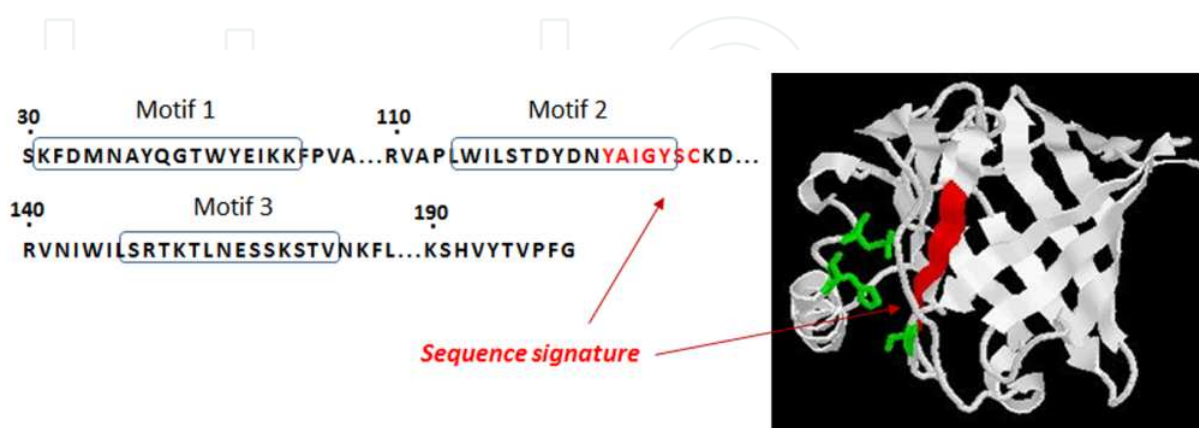


Figure 10. Lipocalin sequence signature highlighted among lipocalin conserved motifs identified in Lopap sequence [19] and in the model of tridimensional structure. Residues predicted in the catalytic triad are shown in green [25].

Lopap shows specific proteolytic activity towards prothrombin. It displays serine protease-like activity and activates human prothrombin through hydrolysis of Arg²⁸⁴-Thr²⁸⁵ and Arg³²⁰-Ile³²¹ peptide bonds, generating active thrombin, without formation of the intermediate meizothrombin [24]. This mechanism is similar to prothrombin activation by FXa in absence of the prothrombinase complex (Figure 11), previously described [74]. This is the unique prothrombin activation mechanism described for an exogenous serine protease, which is independent of prothrombinase complex components. All other exogenous prothrombin activators (metalloproteases and serine proteases) currently described from snake venoms fit into four groups, sharing similar mechanisms of action [75].

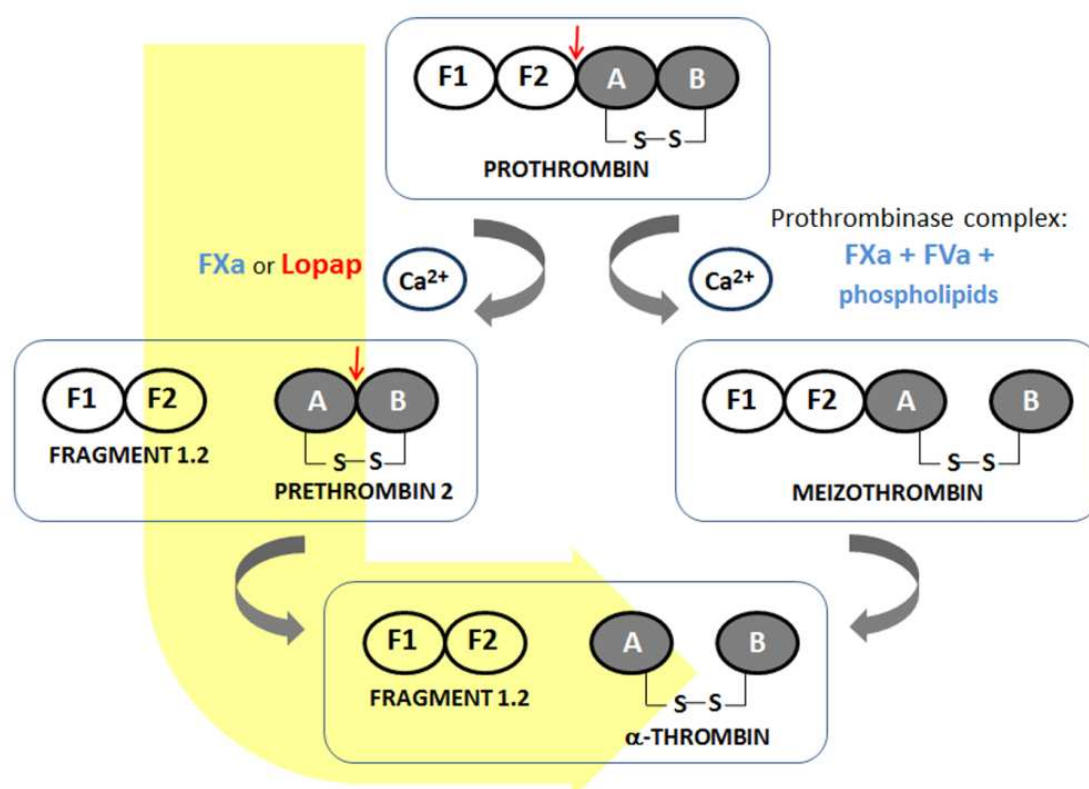


Figure 11. Prothrombin activation mechanisms indicating the Lopap hydrolysis sites and its generated products.

When administered *in vivo*, Lopap induces blood clotting into microvessels, resulting in fibrinogen consumption and blood incoagulability [76]. These effects resemble the consumption coagulopathy triggered by the whole venom, indicating the involvement of this prothrombin activator as a key toxin in envenoming [26]. In addition, Lopap is able to modulate endothelial cell responses promoting cell survival, IL-8, nitric oxide (NO) and PGI₂ release, expression of the cell adhesion molecules ICAM-1 and E-selectin, but not VCAM-1 and PCAM-1 [45, 77, 78]. Lopap also displays cytoprotective activity in neutrophils and do not modify expressions of L-selectin and β2 integrin. Secretion of the proinflammatory cytokines IL-6 and TNF-α is not changed by Lopap treatment in both cell cultures [78]. Lopap seems to

have no effect on modulation of coagulation and fibrinolysis through endothelial cell response, since it does not modify von Willebrand factor (vWF) and tissue plasminogen activator (t-PA) release or tissue factor procoagulant activity on endothelial cell surface [45, 77].

The Lopap-derived peptide obtained through chemical synthesis (Survivalin) reproduces the Lopap's modulation on endothelial cells and neutrophils cell, triggering antiapoptotic activity [73]. Survivalin also induces fibroblast responses, decreasing caspase-3 and increasing Bcl-2, Ki-67, IL-1 β and the receptors for IL-8 and IL-6. Enhanced production of extracellular matrix proteins, such as collagen, fibronectin, tenascin and laminin is also induced by Survivalin in fibroblast culture [79].

3.2. Losac: Functional characterization

Losac is the first factor X activator purified from a lepidopter secretion. It was obtained from caterpillar's bristle extract as a single polypeptide chain protein of about 45 kDa [22]. Some years later, from a cDNA library of *L. obliqua* bristle transcripts [19], the specific clone encoding for Losac was identified and the recombinant protein produced in bacteria system (for details, see section 3.3). Studies using the native or recombinant form of Losac (rLosac) revealed specificity toward factor X [20, 22, 28]. Moreover, Losac had no effect on fibrin or fibrinogen, indicating its specificity for blood coagulation activation, and it was recognized by the antilonomic serum produced in Butantan Institute. Thus, it is plausible that this protein participates in the consumption coagulopathy observed in patients.

Biochemical characterization of Losac has shown that, although its sequence did not show an equivalent among other factor X activators, Losac possess a similar mechanism of action than RVV-X, a factor X activator purified from Russell's viper venom *Daboia russelli* [28, 82, 83]. Like RVV-X, factor X activation by Losac can be accelerated in the presence of calcium and phospholipids, two important cofactor in the assembling of blood coagulation complexes [69]. In spite of this, Losac can activate factor X independently of these cofactors. Moreover, both activators require a stable conformation of factor X and the presence of the Gla-domain of factor X for an appropriated activity. Interestingly, the cleavage fragments of factor X generated by both activators were quite similar. Although there are strong functional similarities, the major difference is in the structure of both activators. Apparently, Losac activates factor X through a serine protease-like activity, while RVV-X has a typical metalloproteinase structure [20, 28, 84].

A model proposed by Morita [83] and crystallographic studies of RVV-X [85] support the hypothesis that it primarily recognizes the calcium-bound conformation of Gla-domain in factor X through an exosite formed by the light chains, followed by the catalytic conversion of factor X to factor Xa. Despite the structural differences between Losac and RVV-X, it remains possible that they share a similar mechanisms for recognition of factor X involving calcium ions, phospholipids and the Gla-domain of factor X followed by its proteolytic conversion to active factor X.

Besides its role in coagulation [22, 28], Losac is also capable of inducing proliferation and inhibiting endothelial cell death while stimulating the release of NO, a known molecule with

antiapoptotic activity [86, 87], and t-PA, a component of fibrinolytic-pathway involved in matrix remodeling [88]. The authors suggest that the cell proliferation and cell viability activities elicited by Losac are probably related to the NO liberation [22], since NO was also described as an endothelial survival factor, inhibiting apoptosis [86, 87]. Moreover, it was also observed that the production/expression of some important molecules involved in inflammation and coagulation systems such as ICAM-1, PGI₂, DAF, IL-8, vWF and tissue factor were not affected by Losac.

It has been show that hemolymph from some insects can increase cell longevity by inhibiting apoptosis [89, 90]. The increase of *Spodoptera frugiperda* Sf-9 cell growth in almost 3-fold was reported after supplementation with *L. obliqua* hemolymph [91]. This effect was attributed to the presence of three factors with different activities: a potential antiapoptotic factor, a growth-promoting factor, and an enzyme that hydrolyzes sucrose. Furthermore, an antiapoptotic protein of 51 kDa was purified from *L. obliqua* hemolymph [36]. This protein was able to prevent apoptosis in Sf-9 cell culture induced by nutrient deprivation and by Actinomycin D. Later reports [37, 38] described in the hemolymph a potent antiviral activity against human virus.

3.3. Molecular cloning and heterologous expression of Losac

The production of Losac in a recombinant form was important due to the disadvantages of purifying Losac from bristle extract: the use of many caterpillars to prepare the bristle extract and the low yield of native Losac (0.3%) [22]. Cloning and production scheme to obtain rLosac is shown in Figure 12 [28].

Nucleotide and deduced amino acid sequences were compared with data banks in order to identify similar genes and their products. The analysis revealed a high similarity with members of the immunoglobulin-like superfamily of cell adhesion molecules (IgCAMs), especially with neural CAMs (NCAMs) [28]. Members of this group have diverse functions but none was associated with proteolytic activities [94]. Multiple comparison of the deduced amino acid sequence revealed different degrees of identity with IgCAMs: 26% of identity with L1-NCAM from humans, 34% with the protein neuroglian from *Drosophila melanogaster*; and 47-76% with hemolins from lepidoptera [95-98]. Although no structural data was reported for Losac, a tertiary structure model was built through homology modeling based on crystal structure of *Hyalophora cecropia* hemolin (HcHemolin, Protein Data Bank code 1BIH). Both proteins share 76% of sequence identity [28] and the same multi-domain structure (four Ig-like domains: D1 to D4) and conserved motifs as shown in Figures 13 and 14. Both structures are composed of β -strands, arranged in a globular shape resembling a horseshoe (Figure 14A), akin to hemolin [97], axonin [99], and the four N-terminal Ig domains of neurofascin [100]. Because Losac shares its main sequence features with hemolins, it can be perfectly classified as one of them. It was demonstrated that Losac activate factor X in a similar way than RVV-X [28]. Nevertheless, unlike Losac, no hemolins or cell adhesion molecules were associated with proteolytic activities.

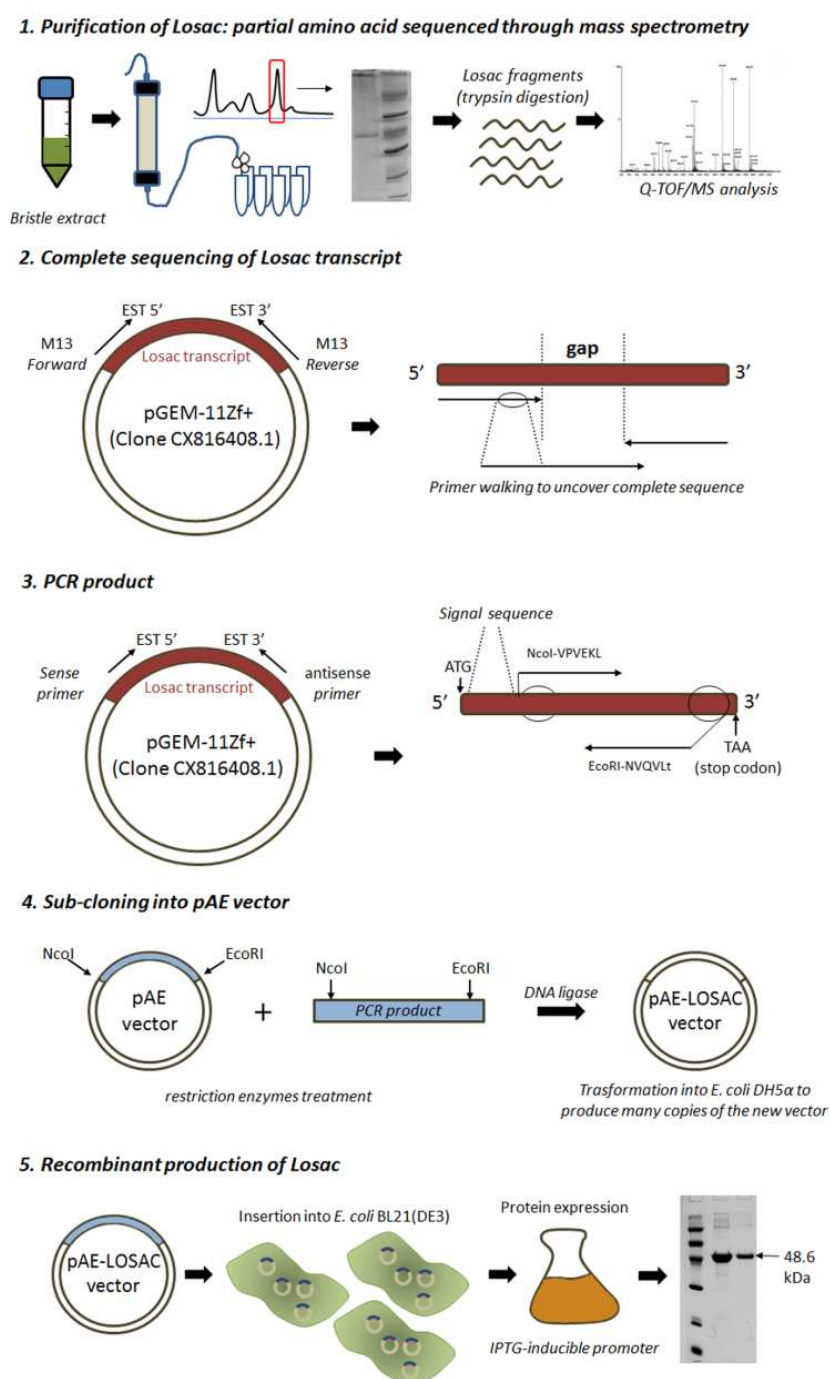


Figure 12. 1)After purification from bristle extract [22], Losac was submitted to a tryptic digestion to obtain partial amino acid sequences of internal fragments. Those sequences were obtained by mass spectrometry and used to screen the cDNA library to identify the transcript encoding the protein. The cDNA library was previously constructed with *L. obliqua* bristle mRNAs that were converted to cDNA and cloned into pGEM-11Zf+ plasmid [19, 20]. **2)** From this cDNA library, a transcript corresponding to the clone LOAH12B08 (GenBank™ accession number CX816408.1), matching the tryptic peptide sequences, was identified. However, this transcript was partially sequenced and the complete sequence was achieved through the *primer walking* strategy using a specific primer designed from an internal sequence of the transcript allowing uncover the complete sequence of Losac gene [92]. The nucleotide sequence has been deposited in GenBank™ (DQ479435), and the deduced protein has been deposited in the NCBI protein sequence database (ABF21073). **3)** The cDNA that encodes mature Losac was amplified by PCR using a sense and an

antisense primer designed according to the deduced N- and C-terminal sequences of the mature protein carrying *Nco*I or *Eco*RI restriction sites, respectively. **4)** The cDNA corresponding to mature Losac was sub-cloned into the pAE vector [93]. The PCR product and the pAE vector were restricted with *Nco*I and *Eco*RI, purified, and ligated with T4 DNA ligase and used to transform *E. coli* DH5α cells. **5)** The resulting pAE-Losac plasmid was used to transform *E. coli* BL21(DE3) cells. The recombinant protein was expressed in fusion to a minimal N-terminal His6-tag as a 48.6 kDa protein: Protein was recovered in inclusion bodies after cell lysis and subjected to refolding and purification after solubilization in urea.

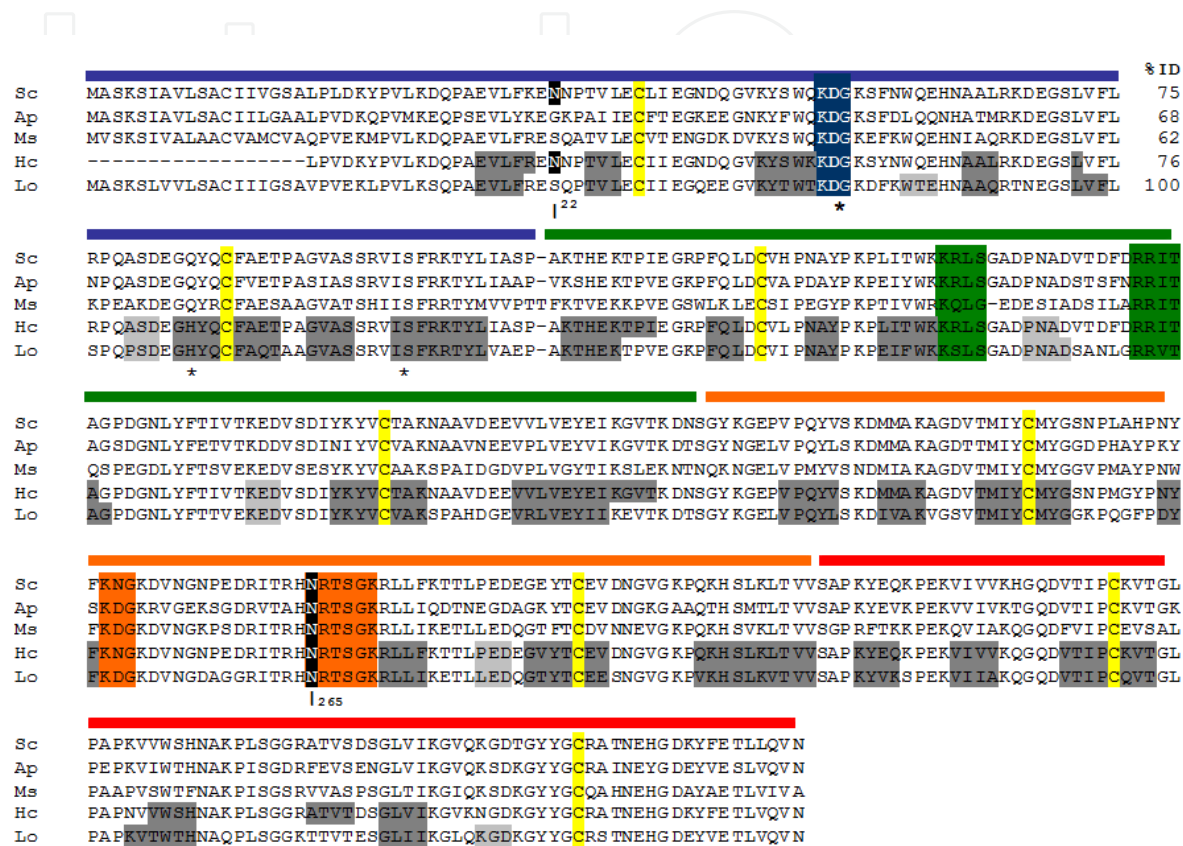


Figure 13. Multiple amino acid sequence alignment of Losac with other hemolin proteins. Sequence identity is shown for all proteins related to Losac. The structures encompass four constant-type immunoglobulin domains (clg) (D1–D4). The bars above the sequences correspond to domains 1 (D1, blue), 2 (D2, green), 3 (D3, orange) and 4 (D4, red). Sequence identity is shown for all hemolins related to Losac (%ID). The α -helices and β -sheets observed in *Hyalophora cecropia* hemolin (Protein Data Bank code 1BIH) and Losac are shaded in light and dark gray, respectively. Previously it was predicted that hemolins contain conserved regions and motifs (28, 36). Conserved motifs are shown inside boxes according to the domain they belong. The eight cysteines (in yellow) form four intra-chain disulfide bridges. Losac conserves the well-known motifs involved in cell-adhesion mechanism (KDG motif in D1 and D3), as well as the highly conserved *N*-glycosylation site in D3 (Asn²⁶⁵, in black). An additional *N*-glycosylation site (Asn²²) is found in *H. cecropia* and *S. c. ricini* hemolins (D1). The LPS-binding site (NRTS motif: Asn²⁶⁵, Arg²⁶⁶, Thr²⁶⁷ and Ser²⁶⁸) in D3 (orange). In D2 are located the KRLS cAMP/cGMP-dependent protein kinase phosphorylation site and the RRIT motif (green boxes). Positions of the residues forming the putative catalytic site are evidenced with an asterisk. Hemolin sequences (with abbreviation and GenBank™ accession numbers in brackets) are from: *Samia cynthia ricini* (Sc, BAE07175), *Antheraea pernyi* (Ap, AAS99343), *Manduca sexta* (Ms, AAC46915), Losac (Lo, ABF21073) and *H. cecropia* (Hc, AAB34817).

At this stage we can only speculate about the mechanism of action of Losac. One possibility is to evaluate structural features that might contribute to the Losac-induced factor X activation. Thus, a search for serine protease active-site was undertaken based on the *Catalytic Site Atlas* (CSA) program analysis. Three possible catalytic residues, Asp(D)⁴⁰, His(H)⁷² and Ser(S)⁹⁰, were

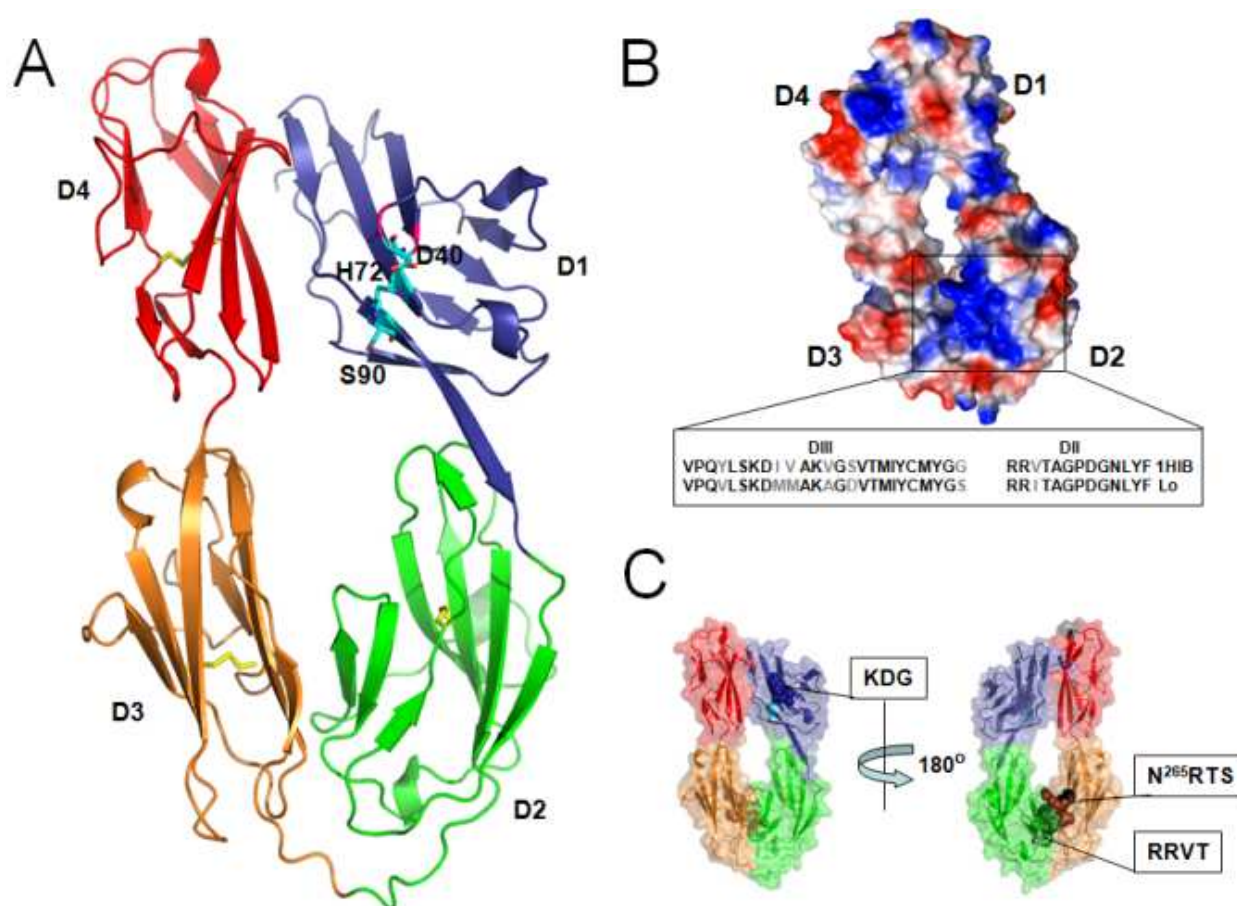


Figure 14. Three-dimensional structure model of Losac. **(A)** Cartoon view of the predicted model of Losac protein built from the structural coordinates of *H. cecropia* hemolin (PDB code 1BIH) using Modeller 9v1 [102]. Each domain (D1-D4) comprises α -helices and 7 strands arranged in 2 antiparallel β -sheets that are linked together by a disulfide bridge (shown in yellow stick). The residues of the putative catalytic triad predicted in D1 by the program *Catalytic Site Atlas* (<http://www.ebi.ac.uk/thornton-srv/databases/CSA/>) program, are indicated as stick cyan color by the one letter code followed by the residue number, D⁴⁰, H⁷², S⁹⁰. **(B)** Electrostatic potential surface of Losac showing inside the box the conserved phosphate-binding site at the D2-D3 interface domains, exactly as was demonstrated for HcHemolin [97]. **(C)** Surface view of Losac model evidencing the conserved adhesive motif KDG (deep blue) and the LPS-binding motif RRVT (green) and NRTS (chocolate), which contains the glycosylation site in N²⁶⁵. All the figures were produced in Pymol v1.5 (<http://www.pymol.org/>).

located on D1 (Figure 13 and 14A) and could fit to such activity. Theoretical analysis (Figure 13 and 14) and observed results - total inhibition by PMSF as described in [28] - seem to suggest the presence of a serine protease-like active-site in Losac which would be responsible for the proteolytic activation of factor X [20, 28, 84]. Some molecular techniques, such as Site-directed mutagenesis [101], could be applied in an effort to understand the structural requirements for ligand binding and selectivity and identification of active site residues.

Functionally, hemolins were first associated with the insect immune system because of their over-expression after bacterial infection [103]. Due to their adhesion properties, some hemolins have been involved in the cell adhesion mechanisms. In the last two decades independent studies demonstrated that hemolins are multifunctional molecules involved in a diverse range

of cell interaction [104-110]. The high identity among Losac and hemolins suggests that Losac could also assume some of these functions in *L. obliqua*. The adhesive properties of Losac probably are relevant to understanding the human umbilical vein endothelial cell responses observed in previous studies.

4. Biomedical applications

Studies on *L. obliqua* toxins with a molecular approach have applications beyond the pathophysiology and therapeutic perspectives of envenoming. As procoagulant proteins, Lopap and Losac can be useful as tools for developing clotting assays and diagnostic kits. Exogenous factor X activators, such as recombinant Losac, has also the potential to be used for detection of factor X deficiency and lupus anticoagulant [111]. In the case of Lopap, an exogenous prothrombin activator, two patents were applied to use this compound in diagnostic kits for detection of dysprothrombinemias using the native form purified from the venom as well as the recombinant form produced in bacteria. This prothrombin activator has also the potential to be used in clotting time assays, prothrombin assays, and to monitor patients anticoagulated with hirudin. A recent study suggests that exogenous procoagulant proteins could also be considered for therapeutic use to manage bleeding complications caused by anticoagulation therapy. Treatment with Lopap was able to reduce the bleeding time of rabbits anticoagulated with low molecular weight heparin, through direct prothrombin activation, bypassing factor Xa inhibition [112]. Patent information about those applications can be consulted in Table 3.

Modulation of cell responses triggered by *Lonomia* toxins can have valuable therapeutic and biotechnological applications. Promoting cell survival can be useful to improve cell culture technologies and vaccine productions, and for treatment of degenerative diseases. In addition, the effects of Lopap on extracellular matrix remodeling can be valuable to develop wound healing formulations and to regeneration issues (Table 3). For this approaches, design and synthesis of short peptides derived from Lopap amino acid sequence is an interesting task to minimize toxic and side effects and for production of this molecules for proofs of concepts, pre-clinical and clinical tests (Table 3). Isolating specific domains and sequences can also help to understand the multifunctional properties of the studied proteins and direct structure-function insights.

Unveiling the mechanisms of action and structure-function relationship of these multifunctional molecules may pointing out these molecules as promising candidates to development of new therapeutic drugs, reagents in diagnostic kits for coagulation dysfunctions, and biotechnological applications.

5. Concluding remarks

Nature has been finding ways to gift living beings with functions that are advantageous, regarding natural selection, mainly by evolutionary process. Among all the lepidopterans of

WIPO patent application	Publication date	Patent	Institutions involved
WO/2003/070746	08.28.2003	Purification and characterization of a prothrombin activator from the bristle of <i>Lonomia obliqua</i> : to be used in diagnosis kits for detecting plasma prothrombin in hemorrhagic state patients	Instituto Butantan (Brazil); Fapesp (Brazil) and Biolab Sanus Farmacêutica Ltda (Brazil)
WO/2006/021062	02.03.2006	Process for obtaining the recombinant prothrombin activating protease (rLopap) in monomeric form; the recombinant prothrombin activating protease (rLopap) as well as its amino acid sequence; the use of this protease as a defibrinogenase agent and the diagnosis kit for dysprothrombinemias	Instituto Butantan (Brazil); Fapesp (Brazil) and Biolab Sanus Farmacêutica Ltda (Brazil)
WO/2007/028223	03.15.2007	Lopap-based pharmaceutical compositions and uses thereof: it refers to the use of Lopap as modulators of cell death and degeneration caused by wounds, aging and external agents	Instituto Butantan (Brazil); Fapesp (Brazil) and Biolab Sanus Farmacêutica Ltda (Brazil)
WO/2009093189	07.30.2009	Peptides, compositions, and uses thereof: it refers to the uses of Lopap-derived peptides for regenerating tissues and wound repair	Instituto Butantan (Brazil); Fapesp (Brazil) and Biolab Sanus Farmacêutica Ltda (Brazil)

Table 3. International patents associated to Lopap and peptides derived from its amino acid sequence. Information was obtained from World Intellectual Property Organization (WIPO).

medical interest in the world, *Lonomia* sp. caterpillars (family: Saturniidae) is the only genus that causes dramatic damages in human blood coagulation [16, 113]. This feature is reflected in the diversity of toxins produced by the caterpillar and their unusual enzymatic properties.

Application of molecular approaches in the study of *L. obliqua* toxins has been a valuable strategy in understanding the biological means of these molecules for the source organism itself and the dynamic pathways in envenoming syndrome. On the other hand, this approach reveals these toxins as interesting tools for therapeutic and biotechnological applications. The best examples are Lopap (a prothromin activator lipocalin) and Losac (the only hemolin with proteolytic activity). If, in one hand, the molecular basis of target recognition and proteolysis of factor X and prothrombin by Losac and Lopap, respectively, needs to be further investigated, on the other hand, efforts need to be focused on understanding the pro-survival activity of both molecules.

Integrating transcriptomic, proteomic and microarray analysis will provide a wealth of valuable information about venom composition. Molecular cloning and expression of recombinant toxins from *L. obliqua* opens new perspectives in the identification and characterization of macromolecular fine structure of toxins and its implications for toxic activity as well as new action mechanisms and target cell binding that should be an area of rapid development. The next several years will likely see some very significant advances in this field and, in the future, those approaches will permit the identification of molecular mechanisms at a new level.

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