Exploring New Molecules and Activities from *Lonomia obliqua* Caterpillars

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Key Words

Lonomia obliqua · Procoagulant proteins · Expressed sequence tag · Two-dimensional map

Abstract

Skin contact with *Lonomia* caterpillar bristles causes a consumptive coagulopathy. From a cDNA library we cloned and expressed a prothrombin activator (rLopap) in active form, and from the bristles extract we characterized a FX activator (Losac). Several clones were sequenced and analyzed by expressed sequence tags. A database of about 1,270 sequences was constructed and deposited in NCBI. Both the native protein from the venom (Lopap) and the recombinant form (r-Lopap) promoted prothrombin hydrolysis, generating prethrombin-2, F1.2 and thrombin. Losac is a single-chain (43 kDa) protein that cleaves the FX heavy chain producing FX α . In HUVECs rLopap and Losac are able to modulate cell survival by preventing apoptosis. rLopap increases NO and PGI₂ concentration and Losac induces t-PA expression. Finally, to identify the venom proteins related to human envenomation, a 2D electrophoresis map is being performed as an attempt to find the major toxins recognized by the anti-*Lonomia* venom.

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Financial support: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), FINEP, and CNPq.

Introduction

In recent decades, a severe hemorrhagic syndrome produced by contact with bristles of *Lonomia obliqua* caterpillars has become epidemic in southern Brazil. Fatal cases due to renal complications and cerebral hemorrhages have been reported [1, 2]. Hemorrhagic syndrome seems to be the most important clinical complication produced in people who experienced contact with these bristles [1]. Recently, it was reported that the consumptive coagulopathy in patients envenomed by contacting this caterpillar is a consequence of severe depletions of coagulation factors, as well as secondary fibrinolysis activation [3]. In vitro studies showed that crude bristles extracted from *L. obliqua* induce clot formations by triggering activation of both prothrombin and Factor X [1, 4]. Thus, our studies [3, 5] support previous findings that the venom is mainly procoagulant and that the activation of fibrinolysis seems to be secondary. In this report, we review the studies conducted up to now concerning the venom of this caterpillar.

Studies Related with the *L. obliqua* Human Envenoming

In vitro studies on the *L. obliqua* crude bristles extract (LOCBE) have demonstrated that this venom induces clot formations by triggering activation of both prothrombin and Factor X [4], and it is unable to activate plas-

minogen in plasmin of purified systems, indicating that LOCBE does not present t-PA- or u-PA-like activities or direct lysis of fibrin [5]. It was also shown that LOCBE neither induces FXIII degradation nor possesses the ability to form a complex with FXIII. In patients, a significant thrombin production with intense consumption of fibrinogen and high D-dimer production indicate consumption coagulopathy and secondary fibrinolysis [3].

Some in vitro studies with LOCBE showed that *L. obliqua* bristles secretions possess fibrinogenolytic activity [5, 6]. Pinto et al. [7] described a 35-kDa fibrinogenolytic protein named Lonofibrase purified from *L. obliqua* secretion. This enzyme cleaves preferentially the A α chain of fibrinogen, presenting smaller efficiency by B β chain. Fritzen et al. [5] suggested that the direct fibrinogenolytic activity of the venom seems to be irrelevant for the *L. obliqua* hemorrhagic syndrome. These authors demonstrated that the fibrinogenolytic activity hardly occurs with high concentrations of the bristles extract or after a long-time incubation with the fibrinogen molecule, inducing proteolytic activity on the A α chain of fibrinogen and generating products different from those induced by the plasmin. Fritzen et al. [5] concluded that the role of this activity in the envenomation could be irrelevant because when the plasma clot is formed in vitro by the procoagulant activity of the venom, no clot lyses was observed before a 24-hour incubation. It indicates that LOCBE did not contain enzymes capable of degrading cross-linked fibrin, reinforcing previous findings which stated that the *L. obliqua* venom mainly exerts procoagulant activity causing a consumption coagulopathy leading to a hemorrhagic state induced by fibrinogen depletion and secondary fibrinolysis [1, 4, 5]. Corroborating with this hypothesis, in experimental thrombosis studies, Prezoto et al. [8] have shown that while the streptokinase infusion in rats induced local thrombolysis, LOCBE promoted fibrinogen depletion and decreased thrombus size without inducing thrombolysis. In addition, it was also demonstrated that the infusion of Lopap, a prothrombin activator isolated from LOCBE, triggered in mice a condition similar to that of human poisoning [9], indicating that procoagulant activities are the major biological effects of the bristles extract. Duarte et al. [2] reported more evidence that fibrinolytic enzymes present in the *Lonomia obliqua* venom probably are not directly responsible for the hemorrhagic syndrome. They treated animals envenomed by LOCBE with anti-fibrinolytic drugs such as aprotinin and aminocaproic acid associated with whole blood, fresh-frozen plasma or cryoprecipitates. This treatment

failed at reversing the clinical manifestations of the envenoming and indeed, instead of reversing them, it really exacerbates them.

Procoagulant Toxins in the Bristles of *L. obliqua*

Lonomia obliqua Prothrombin Activator Protein (Lopap)

Up to the present, the prothrombin activator named Lopap is the most studied protein of the *L. obliqua* bristles extract. Lopap is a 69-kDa (fig. 1a) tetrameric protein that possesses a prothrombin activator activity independent of the prothrombinase cofactors (Factor Va, phospholipids), although calcium ions increase this activity [10]. Serine protease inhibitors, such as PMSF, inhibit its activity [10]. Lopap does not possess factor X activating activity, fibrinogenolytic activity, or plasminogen activating activity [10]. In vivo studies support the hypothesis that Lopap contributes to the syndrome observed in patients after the contact with *L. obliqua* most probably through thrombin generation resulting in a consumptive coagulopathy [9].

The fragmentation of prothrombin by Lopap (fig. 1b) is similar to that induced by the factor Xa in absence of prothrombinase components (fig. 2), forming prethrombin 2, fragments 1 + 2 and thrombin, although meizothrombin is not formed [10].

Besides its procoagulant activity, Lopap exerts action on cell responses [11, 12]. In human umbilical vascular endothelial cells (HUVECs) Lopap stimulates the increase of IL-8, ICAM-1 and E-selectin but not of VCAM-1 [11]. Lopap also increases the secretion of molecules such as nitric oxide (NO) and prostacyclin (PGI₂), both potent vasodilators and inhibitors of platelet activation. A previous in vivo study [10] showed that the infusion of a high concentration of Lopap promotes neutrophil and monocyte migration in pulmonary microcirculation vessels. Probably, the over-expression of IL-8, ICAM-1 and E-selectin by Lopap [11] could be related with the recruitment of leukocytes at sites of envenomation. Another interesting effect of Lopap on HUVECs is the significant increase in the cell viability and the inhibitory effect on cellular death [12]. This effect is probably related to NO liberation, since NO is an endothelial survival factor, inhibiting apoptosis [13].

The analysis of the native Lopap sequence [9] shows that the N-terminal portion (46 residues) presents 55% sequence identity with Bombyrin from *Bombyx mori* and 51% sequence identity with insecticyanin from the hemo-

Fig. 1. a SDS-PAGE (10%) of 20 μ g of purified Lopap (lane 1) and standard proteins (lane 2). **b** SDS-PAGE (10%) of prothrombin (72 kDa) hydrolysis by Lopap (30 nM) under reducing conditions. Activation of prothrombin resulted in fragments of molecular masses of 52, 36, 32, 27, and 16 kDa representing the F1/F2 activation peptide, prethrombin 2, thrombin B-chain, fragment 1 (F1), and fragment 2 (F2), respectively [10].

Fig. 2. Prothrombin activation. Prothrombin can be activated by prothrombinase complex and by Factor Xa alone. Lopap, a prothrombin activator from *L. obliqua*, is also able to activate prothrombin generating prothrombin 2, fragments 1 + 2 and thrombin.

Fig. 3. A schematic representation of factor X activation by Losac. Gla, EGF1 and EGF2 represent Gla domain, first growth-factor domain and second growth-factor domain. AP represents the activation peptide. S, D, and H in the dark boxes of the heavy chain represent the serine protease catalytic triad. A, C, R, S and I represent Ala, Cys, Arg, Ser and Ile amino acids. The numbers below the boxes represent the position of the amino acid in the molecule. The cleavage of Arg52-Ile53 peptide bond results in the formation of FXα.

lymph of *Manduca sexta*, both members of the lipocalin protein family. A recombinant monomeric form (rLopap) of 21 kDa, which maintains the prothrombin activating activity of the native protein, was produced in *E. coli* cells [14]. The rLopap complete sequence contains conserved domains characteristics of the lipocalins, confirming its homology with members of the lipocalin family of proteins.

Lonomia obliqua Stuart-Factor Activator (Losac)

Losac is the first factor X activator purified from a lepidopteran secretion. It was purified from LOCBE, resulting in a single polypeptide chain protein of about 43 kDa. This activator is able to activate factor X in a concentration-dependent manner. Losac neither activates other coagulation factors such as prothrombin, nor presents amidolytic activity on S-2222, S-2765 and S-2238 chromogenic substrates [15]. Losac cleaves human factor X, producing similar fragments compared to those produced by RVV-X [15, 16]. This result suggests that Losac is likely to activate human factor X by cleaving the Arg₅₂-Ile₅₃ peptide bond in the heavy chain, producing factor Xα (fig. 3). Factor X cleavage by Losac was totally inhibited when Losac was previously treated with 5 mM PMSF, indicating that it is a serine protease. Studies on endothelial cells (HUVECs) showed that Losac also exerts

proliferative and anti-apoptotic activities. Only the concentrations of NO, a molecule known for having anti-apoptotic activity in endothelial cells [13], and of t-PA, a fibrinolytic protein also involved in matrix remodeling [17], were enhanced.

Expressed Genes in Lonomia obliqua Bristles

EST-based strategies are commonly used for identifying large number of genes in species of interest [18]. In the case of *L. obliqua*, we used this approach to identify and characterize the major transcripts present in the venom [14]. Sequences from 1,270 independent clones were assembled into 702 clusters of distinct genes (and corresponding proteins) (table 1) as lipocalins, hemolins, serine proteases, serpins, tumor suppressors and other proteins were identified and deposited in GenBank in 2004. The Lopap whole sequence (accounting for 1.6% of the total clones) is identified in GenBank as the access number AY908986.

Recently, Veiga et al. [19] published a catalog for the transcripts from *L. obliqua* structures, where a lipocalin of 19 kDa is the most abundant transcript. However, the authors did not observe that the sequence of this lipocalin corresponds to the Lopap sequence. From this cDNA library 960 independent clones from the bristle were sequenced.

Table 1. Important proteins expressed by *Lonomia obliqua*

Product	Cluster	Clones	Total clones, %	Sequence completeness
Proteases				
Lopap	4	24	1.6	FF
Coagulation factor-like	3	3	0.1	FP
Proclotting enzyme precursor	1	1	<0.1	P
Trypsin-like protease	2	4	0.2	FP
Serine proteases	4	6	0.4	FP
Phospholipase A2	2	2	0.1	FP
Matrix metalloproteinase 1	1	1	<0.1	P
Cathepsin B-like cysteine proteinase	2	2	0.1	FP
Cathepsin L-like protease	2	2	0.1	P
Inhibitors				
Thrombin inhibitor	1	1	<0.1	P
Serpins	3	3	0.2	FF
Kunitz-like protease inhibitor	1	1	<0.1	P
Tumor suppression				
QM protein	1	10	0.7	FF
Serine/threonine protein phospholipase 2	1	2	0.1	FP
Smu-1 suppressor of mec-8 and unc-52 homolog	1	1	<0.1	P
Tumor suppressing sub transferable candidate 1	1	1	<0.1	P
Other proteins				
Kininogen precursor	1	22	1.5	FF
Cellular retinoic acid-binding protein	1	2	0.1	P
Heat shock-like proteins	9	19	1.3	FF
Anti-bacterial peptides	2	2	0.1	FF
Anti-fungal peptide gallerimycin	1	1	<0.1	P

P = Partially sequenced clusters without ATG initiation codon; FP = partially sequenced clusters without stop codon; FF = fully sequenced clones.

Lilla et al. [20] published a protein with FXa-like activity with about 27 kDa and the sequence of this protein also corresponds to Lopap. All this results have demonstrated that Lopap seems to be one of the major proteins in the *L. obliqua* venom.

Sequences information deposited independently [14, 19] in GenBank from both cDNA libraries are complementary. On the other hand, the exact toxins involved in the envenomation are not clear at the moment.

Conclusions

In conclusion, *L. obliqua* venom induces in patients a special form of disseminated intravascular coagulation with consumption coagulopathy, depletion of certain coagulation factors and inhibitors, as well as secondary fibrinolysis. This is different from the disseminated intravascular coagulation observed in other clinical situations,

such as trauma, neoplasia or sepsis. Treatment of patients is largely based on anti-lonomia venom (SALon) administration, which is an effective way to reverse hemostatic disturbances and bleeding. Anti-fibrinolytic agents are not recommended in *L. obliqua* envenomation once this venom presents main pro-coagulant activity as a major property.

Characterization of the transcripts presents in the venom showed several kinds of components that will distinctly participate in the envenoming. To clarify this point, our group is working on two-dimensional protein maps, and mass spectrometry studies to find the major toxins recognized by a specific anti-Lonomia venom produced by Butantan Institute.

On the other hand, because Lopap and Losac exert activities on coagulation factors we are working to characterize their catalytic sites. Finally, to better understand the activities both molecules possess on cell cultures, we are studying their specific mechanism of action.

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