Losac, the First Hemolin that Exhibits Procogulant Activity through Selective Factor X Proteolytic Activation*

Envenoming by the contact of human skin with *Lonomia obliqua* caterpillars promotes a hemorrhagic syndrome characterized by a consumptive coagulopathy. Losac (*Lonomia obliqua* Stuart factor activator) is a component of the bristle of *L. obliqua* that is probably partially responsible for the observed syndrome because it activates factor X and is recognized by an effective antilomonic serum. Here we unveil the proteolytic activity of Losac and demonstrate the feasibility of its recombinant production. On the other hand, Losac has no homology to known proteases, but it can be inhibited by PMSF, a serine protease inhibitor. Instead, it shows closer homology to members of the hemolin family of proteins, a group of cell adhesion molecules. The recombinant protein (rLosac) shortened the coagulation time of normal and deficient plasmas, whereas it was ineffective in factor X-deficient plasma unless reconstituted with this protein. rLosac was able to activate factor X in a dose- and time-dependent manner but not γ-carboxyglutamic acid domainless factor X. Moreover, phospholipids and calcium ions increased rLosac activity. Also, rLosac had no effect on fibrin or fibrinogen, indicating its specificity for blood coagulation activation. Linear double reciprocal plots indicate that rLosac follows a Michaelis-Menten kinetics. Cleavage of factor X by rLosac resulted in fragments that are compatible with those generated by RVV-X (a well known factor X activator). Together, our results validate Losac as the first protein from the hemolin family exhibiting procogulant activity through selective proteolysis on coagulation factor X.

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Lon. obliqua caterpillar envenomings have medical importance in South/Southern Brazil because they can lead to hemorrhagic syndrome, the most important clinical complication in people who experience contact with its bristles (1, 2). The pathophysiological process involved in the hemorrhagic syndrome is not completely known. However, several studies have indicated that this effect is mediated mainly by thrombin formation due to procogulant proteins present in the venom followed by the activation of fibrinolysis secondary to these events (1, 2). Two procogulant proteins were reported: Lopap, a prothrombin activator (3), and Losac, a factor X activator (4, 5). Many studies indicate that Lopap, the most studied molecule from *L. obliqua*, could contribute to the pathophysiological mechanism of the envenomation through a complex mechanism affecting both coagulation and endothelial function (2). In the case of Losac, little is known about its precise role in the pathophysiology of *L. obliqua* envenoming. However, it was reported that Losac is capable of inducing proliferation and inhibiting endothelial cell death while stimulating the release of nitric oxide and tissue plasminogen activator (5).

Factor X, or Stuart factor, is a vitamin K-dependent factor present in blood as a two-chain glycoprotein. Its active form participates in the coagulation process integrating the prothrombinase complex to produce thrombin and consequently to develop the fibrin clot (6). Under physiological hemostasis, it is activated by the tissue factor-factor VIIa complex during the initial phase of coagulation (7). This activation can be sustained by the factor IXa-factor VIIIa complex (tenase complex). Both complexes require Ca$^{2+}$ and phospholipids, and the activation results from cleavage of the Arg$^{22}$-Ile$^{53}$ peptide bond in the heavy chain of factor X with consequent loss of the 52-residue activation peptide (8). Apart from the physiological factor X activators, other activators have been described in exogenous sources, such as fungus (9), bacteria (10), and plants (11) and under pathological conditions, such as in malignant tissues, where the activator was named cancer procogulant protein (12). Especially in snake venoms, many factor X activators were studied, most of them being Ca$^{2+}$-dependent, but only a limited number have been isolated and characterized (13).

In this work, we report the cloning, heterologous expression, and characterization of the recombinant Losac (rLosac). In order to support the hypothesis that rLosac is a procogulant protein and based on experiments using deficient plasmas, we demonstrate that rLosac could induce blood coagulation through selective factor X proteolytic activation. Interestingly, Losac has no homology to known proteases. Instead, it shows high similarity with hemolin, a cell

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2 The abbreviations used are: rLosac, recombinant Losac; IPTG, isopropyl β-D-thiogalactopyranoside; aPTT, activated partial thromboplastin time; Gla-domain, γ-carboxyglutamic acid domain; PC/PS, phosphatidylycholine/phosphatidylserine vesicles.
adhesion molecule from *Hyalophora cecropia* (Lepidoptera order) (14). Hemolin is a bacteria-inducible immunoglobulin-like protein whose role in insect immunity has been better studied. In the last 2 decades, independent studies have demonstrated that hemolin proteins are multifunctional molecules involved in a diverse range of cell interaction and are able to (a) bind lipopolysaccharides and be up-regulated in hemolymph during infections (15–17); (b) be up-regulated during metamorphosis (18, 19); (c) promote opsonin-like effects, such as increased cell adhesion and phagocytosis (20, 21); (d) agglutinate bacterial cells (17, 22); (e) bind to hemocytes and inhibit their aggregation (15, 20, 22, 23); and (f) regulate embryonic development (24, 25).

Taken together, our results contribute to amplify the scope of the biological function of hemolins and allow us to place them with catalytic antibodies as members of the immunoglobulin superfamily that exhibit proteolytic function, naturally occurring in their conserved scaffold. As far as we know, it is the first reported factor X activator from this taxonomical order and the first hemolin exhibiting enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Chemicals, and Proteins**—The *E. coli* DH5-α and BL21(DE3) strains, restriction enzymes NcoI and EcoRI, and T4 DNA ligase were purchased from Invitrogen; isopropyl β-d-thiogalactopyranoside (IPTG) and the Spectra Multicolor Broad Range Protein Ladder were acquired from Fermentas (St. Leon-Roth, Germany); thrombin, streptokinase, RVV-X, trypsin, EDTA, E-64, β-mercaptoethanol, ampicillin, l-α-phosphatidylcholine, and l-α-phosphatidylethanolamine were obtained from Sigma-Aldrich; human fibrinogen, pronase, thrombomin, factor Xa, and factor X were purchased from Merck/Cabiochem; human GlA-domainless factor X was obtained from Thrombinerecovery.com (Bedford, MA); chromogenicsubstrate S-2765 (N-p-nitroanilide) was acquired from Chromogenix (Mölndal, Sweden); PMSF was purchased from Panreac (Barcelona, Spain); dithiothreitol and β-mercaptoethanol were from PAA Laboratories (Cleveland, OH); primers and reverse universal primers and by primer walking using the nucleotide primer 5'-GAGATCTTCTGTAGAGAA-GAGCC-3' (designed from an internal sequence from the transcript), using the ABI 377 automated DNA sequencer and the ABI prism Big Dye Terminator kit (Applied Biosystems), following the instructions of the manufacturer. Nucleotide and deduced amino acid sequences were compared with the GenBank nucleotide and protein databank using the BLASTN and BLASTX programs at NCBI in order to identify similar genes and their products.

**Molecular Cloning and Expression of rLosac**—The cDNA that encodes mature Losac was amplified by PCR using a sense (5'-GGTTCCATGGAATCCGTAACACACT-3') and an antisense (5'-GTTGAATTTCTGAGACGTG-C-3') primer designed according to the deduced N- and C-terminal sequences of the mature protein carrying Ncol or EcoRI restriction sites, respectively. The PCR product and the pAE vector (27) were restricted with Ncol and EcoRI, purified, and ligated with T4 DNA ligase. The sequences of the insert and frame were confirmed by DNA sequencing.

Competent *E. coli* BL21(DE3) strains were transformed with 5 ng of pAE-Losac. Transformed cells were grown at 37 °C in 2× YT medium, supplemented with ampicillin (100 μg/ml) to a cell density of *D* _<sub>600</sub> = 0.4–0.6. The protein expression was induced by 0.5 mM IPTG, and incubation was continued for 3 h at 37 °C. Cells were harvested by centrifugation at 3200 × g at 4 °C for 12 min and suspended in lysis buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, and 1% Triton X-100), and then the cells were ruptured by sonication on ice (Viba Cell, Sonics & Materials Inc.). The lysate was centrifuged at 20,198 × g at 4 °C for 15 min. The pellet (inclusion bodies) was dissolved in a solubilization buffer (100 mM Tris-HCl, pH 8.0, containing 0.5 mM NaCl, 8 mM urea, and 10 mM β-mercaptoethanol) and incubated overnight.
A Hemolin with Procoagulant Activity

Refolding and Purification of rLosac—Inclusion bodies were submitted to refolding by dilution of the protein into a refolding buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 50 mM CaCl₂, 5 mM imidazole, and 5 mM β-mercaptoethanol) to reach a final concentration of 2 mM urea. After filtration (AP20 prefilters), the solution was loaded onto an Ni²⁺-NTA His-Bind column previously equilibrated with the refolding buffer. Nonspecifically bonded molecules were washed with buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) containing 5 mM imidazole. Bound proteins were eluted with buffer B (buffer A containing 150 mM imidazole). Fractions of 1 ml were pooled and applied into a Hitrap™ column at a flow rate of 0.5 ml/min using a Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.5). All operations were conducted at 4°C. All samples were further analyzed through SDS-PAGE and Western blot.

Sequence Analysis and Homology Modeling—The deduced amino acid sequence of Losac was aligned with selected sequences by ClustalW version 1.8 (24) using (a) protein weight matrix = gonnets, (b) gap opening = 10, and (c) gap extension = 0.5. The sequence similarities were analyzed according to the BLOSUM62 matrix (28) using EMBO pairwise alignment algorithms (29). Signal peptide and glycosylation prediction was performed using SignalP3.0 and the NetOglyc and NetNglyc servers (available at the Center for Biological Sequence Analysis Web site). A tertiary structure model of Losac was built through homology modeling driven by satisfaction of spatial restraints implemented in Modeler9v1 (30) using the crystal structure of H. cecropia hemolin as template (Protein Data Bank code 1BIH) (31).

Clotting Assays—Clotting times were carried out in a Quik-Timer coagulometer (Drake, São Paulo, Brazil). For plasma recalcification time, 50 μl of human citrated plasma (0.38% final citrate concentration) was mixed in a coagulometer cuvette for 2 min at 37°C with 50 μl of rLosac or Tris-buffered saline. After that, 25 μl of warmed 25 mM CaCl₂ was added, and the time to clot formation was measured. For the activated partial thromboplastin time (aPTT) assay, 25 μl of plasma and 25 μl of rLosac (or Tris-buffered saline) were incubated in a cuvette at 37°C for 1 min, followed by the addition of 50 μl of DIACELIN®, incubation at 37°C for 3 min, and the addition of 50 μl of 25 mM CaCl₂ to initiate coagulation.

Chromogenic Assays—Factor X activation was estimated according to Alvarez Flores et al. (5) by using human factor X or a Gla-domainless factor X.

Steady-state Kinetics of Factor X Activation—Different concentrations of factor X were incubated at 37°C with 5 mM rLosac in Tris-buffered saline, containing 10 mM CaCl₂ and 10 μg/ml PC/PS (final volume of the reaction solution, 195 μl). After 30 min, 5 μl of 8 mM S-2765 were added, and the amidolytic activity of factor Xa produced in the reaction was monitored at 405 nm in a SpectraMax 190 spectrophotometer (Molecular Devices). The factor Xa concentration produced by rLosac was calculated by using as a standard the amidolytic activity of purified factor Xa, as described previously (32). The initial velocity of factor Xa production at various factor X concentrations was determined by the best fit line for each factor Xa concentration at the five incubation periods (30, 60, 90, 120, or 150 s). The values of the Michaelis constant (Kₘ) and the maximum velocity (Vₘₐₓ) in the Michaelis-Menten equation were obtained using three different plots, [S]₀/[V] versus [S]₀, 1/[V] versus 1/[S]₀, and ν versus ν/[S]₀ (ν and [S]₀ denote the catalytic rate and the initial substrate concentration, respectively), where the best fit values were determined by the method of least squares with Taylor expansion, described by Sakoda and Hiromi (33).

Fibrinolytic Activity Assay on Fibrin Plate—A fibrin-agarose gel (34) was prepared in Petri dishes by mixing 2.5 ml of human fibrinogen (2 mg/ml clotting protein) and a preheated solution of 2% agarose, both in 0.1 M sodium barbital buffer, pH 7.75. Then 6 μl of thrombin (200 IU/ml) and 50 μl of CaCl₂ (1 mM) were added. On this solidified fibrin plate, wells of equal diameter were punched, and samples were added and kept at 37°C for 24 h. The diameter of the halo around the well was measured to calculate the functional activity of rLosac.

Analysis of Fibrinogenolytic Activity—Briefly, 2.5 μg of human fibrinogen (1 mg/ml) was incubated for 60 min with 70 nM rLosac (fibrinogen/rLosac molar ratio of 1:10) at 37°C in 50 mM Tris-HCl buffer, pH 8.0, at a final volume of 15 μl. The reaction was stopped by adding 15 μl of 5 mM Tris-HCl buffer, pH 6.8, containing 10% glycerol, 10% β-mercaptoethanol, 2% SDS, and 0.05% bromphenol blue. Samples were heated at 100°C and analyzed through SDS-PAGE.

SDS-PAGE and Western Blot—Protein samples were analyzed by their molecular masses on 12.5% (w/v) SDS-polyacrylamide gels and stained with Coo massie Brilliant Blue R250. For Western blot, gels were electroblotted to a PVDF membrane for 17 h at 4°C and 80 mA constant current in transference buffer (192 mM glycine and 15% methanol). The proteins were detected with the appropriate antibodies using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate. The mouse His₆ monoclonal antibody (Clontech) was used at a dilution of 1:500 (1 μg/ml). The antilobiqua bristle extract from Instituto Butantan was used at a dilution of 1:500. For factor X cleavage product analysis, a goat factor X (C-20) polyclonal unconjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a dilution of 1:200 (1 μg/ml). Secondary antibodies were as follows: phosphatase-labeled affinity-purified antibody to mouse IgG (KPL, Gaithersburg, MD) at a dilution of 1:1000 (0.1 μg/ml); a rabbit anti-horse IgG-alkaline phosphatase (Sigma-Aldrich) at a dilution of 1:4000; and a rabbit anti-goat IgG-alkaline phosphatase (Santa Cruz Biotechnology, Inc.) at a dilution of 1:500 (0.8 μg/ml).

RESULTS

Partial Amino Acid Sequencing of Native Losac—The rLosac internal peptides obtained after digestion by trypsin were analyzed through mass spectrometry on Q-TOF MS. The analysis allows the sequencing of 12 tryptic fragments shown in Table 1. The peptide sequences obtained correspond to 32% of the entire protein, assuming the molecular mass of 45 kDa as described (5).
Identification of the cDNA — Each peptide sequence was used to search parental proteins at NCBI using BLAST-P. The analysis shows that the internal peptide sequences matched with proteins known as hemolin from different species of lepidopters. BLAST searches against expressed sequence tag data corresponding to the previously constructed cDNA library (3, 4) revealed the existence of a partial transcript (accession number CX816408) whose deduced amino acid sequence matches several tryptic peptides. The complete sequencing of this transcript revealed an open reading frame.
A Hemolin with Procoagulant Activity

(ORF) of 1239 bp encoding 413 amino acid residues. A signal peptide was found in the N-terminal portion (Fig. 1), and the cleavage site was predicted between positions 18 and 19 (GSA↓VP). The deduced full sequence of Losac indicates that the mature protein is made up of 395 amino acids resulting in a 43.4-kDa protein with a theoretical pI of 7.64 (JUFO program; available on the World Wide Web).

Sequence Comparisons—Deduced amino acid sequence revealed variable percentages of identity with cell adhesion molecule members of the immunoglobulin superfamily: 26% identity with L1-NCAM (human neural cell adhesion molecule L1), which is involved in development of the nervous system and neurite outgrowth in humans (35); 34% identity with the four first domains of Neuroglian from Drosophila melanogaster (36), an ortholog of L1-NCAM; and between 47 and 76% identity with several members of the hemolin family (31). With the latter, rLosac shares the same multidomain structure (D1–D4, bars above sequences) and conserved motifs (Fig. 2A). Previously, it was predicted that hemolin contains conserved regions and motifs (31, 37): the LPS-binding site (Asn265, Arg266, Thr267, and Ser268), a conserved PKC site and a cAMP/cGMP kinase site, all conserved in the sequences shown. Moreover, all of the hemolins present eight cysteines, and, as previously described for H. cecropia hemolin (31), they form four intrachain disulfide bridges (Fig. 2A, small arrows). A predicted N-glycosylation site in Asn265 (D1) was found. Two predicted KDG conserved motives are found in D1 and D3, respectively. Besides the KDG motif, in domain 3, we also found the following predicted motifs: (a) SGK protein kinase C phosphorylation; (b) RRIT; and (c) KRLS cAMP/cGMP-dependent protein kinase phosphorylation (all three of them conserved in most hemolins).

rLosac three-dimensional structure was modeled from the structural coordinates of the H. cecropia hemolin (Fig. 2B). The four immunoglobulin-like (Ig) domains (D1–D4) form a horseshoe shape, akin to hemolin (31), axonin (38), and the four N-terminal Ig domains of neurofascin (39).

Cloning and Production of rLosac—From the original clone pGEM-11Zf(+)(accession number DQ479435), the cDNA fragment coding the mature Losac was amplified by PCR and subcloned in the T7-based promoter pAE plasmid. The pAE-Losac construct was confirmed through sequencing. The final construct encompasses the total 416-residue and encodes an N-terminally fused sequence containing a His6 tag plus 14 amino acid residues derived from alternative vector restriction sites. Expression of the rLosac monomer was clearly visible in the insoluble fraction of IPTG-induced E. coli BL21 (DE3) cells transformed with pAE-Losac (Fig. 3A). It was observed that rLosac migrates with a slightly higher apparent molecular mass when compared with the predicted molecular mass (about 48.6 kDa versus about 46 kDa). By using a specific anti-His tag antibody, a Western blot analysis confirmed that Losac was expressed in the cells as inclusion bodies (Fig. 3B), and it is also possible to observe a basal expression of Losac in...
non-induced cultures. The protein in the inclusion bodies was solubilized by urea. Solubilized inclusion bodies were submitted to refolding in a fast dilution procedure and further purified by Ni^{2+}-chelating affinity chromatography, resulting in 1.5 mg of purified monomers/liter of cell culture (Fig. 3C).

**Effect of rLosac on Human Coagulation**—To determine if rLosac modulates thrombus formation or dissolution, we made some in vitro assays. rLosac showed procoagulant activity in a dose concentration manner (2–30 nM) because a decrease in normal plasma recalcification time was observed (Fig. 4A). On the other hand, a higher concentration of rLosac (70 nM) did not exhibit fibrinolytic activity, evaluated through the fibrin-plate method using streptokinase as a control. Even after 24 h of incubation at 37 °C, no lysis area was formed by rLosac (Fig. 4B). Through SDS-PAGE, it was further found that rLosac is not capable of cleaving fibrinogen chains (Fig. 4C). In order to check if rLosac is recognized by an antilonomous horse hyperimmune serum (used for the treatment of coagulation disturbances caused by Lonomia envenomation), we subjected rLosac in non-reducing conditions to Western blot analysis. As seen in Fig. 4D, several components present in L. obliqua bristle extract as well as rLosac were clearly recognized for the antilonomous serum.

**rLosac Induces Procoagulant Activity through the Activation of Factor X**—To characterize the mechanism for reducing coagulation (Fig. 4A), the effect of rLosac was also examined for aPTT and recalcification time by using normal and deficient plasmas in a factor in the intrinsic and extrinsic pathways. rLosac shortened aPTTs of plasmas deficient in factor XII, XI, IX, or VIII (Table 2). However, rLosac did not affect the aPTT of factor X-deficient plasma, unless it was reconstituted with the missing factor (Table 2). These results suggest that rLosac shortened aPTT through factor X activation. On the other hand, rLosac also reduced the recalcification time of factor VII-deficient plasma, whereas there was no effect on the recalcification time of factor X-deficient plasma unless it was reconstituted with factor X (Table 2). These data again suggest that rLosac induces procoagulant activity through factor X activation.
**A Hemolin with Procoagulant Activity**

**Activation of Factor X by rLosac**—To investigate the mechanism of factor X activation by rLosac, we incubated rLosac and factor X and measured the factor Xa activity produced through the chromogenic assay. rLosac generated factor Xa in a dose- and time-dependent manner and performed in the absence of Ca$^{2+}$ ions (Fig. 5A). Coagulation reactions are largely accelerated by two important cofactors, calcium and phospholipid (7). Thus, we also investigated the effect of both cofactors on factor X activation by rLosac. Calcium ions increased ~3.5-fold (corresponding to 20 mM Ca$^{2+}$) the activity of rLosac on factor X (Fig. 5B). The presence of phospholipid as the sole cofactor only had an effect on factor X activation by rLosac at low concentrations (~3.1-fold increase corresponding to 10 µg/ml PC/PS) (Fig. 5C). With added Ca$^{2+}$ and phospholipids, the activity of rLosac was increased ~8.9-fold (Fig. 5D). These data indicate that rLosac is able to activate factor X in the absence of coagulation cofactors, and the presence of both cofactors increases its activity.

**Kinetics of Factor X Activation by rLosac**—To investigate if rLosac possesses an enzymatic behavior, we studied the kinetics of factor X activation by rLosac. Thus, the values of $K_m$ and $k_{cat}$ were determined for the interaction of factor X with rLosac at a phospholipid concentration of 10 µg/ml in the presence of 10 mM CaCl$_2$. The values of $K_m$ and $k_{cat}$ were 188 nM and 3.57 × 10$^{-3}$/s (Fig. 6), and the catalytic efficiency ($k_{cat}/K_m$) was 1.89 × 10$^4$ M$^{-1}$ s$^{-1}$. The kinetic constants of rLosac for factor X conversion were compared with those of factor VIIa complexed with tissue factor (40), factor IX complexed with factor VIIIa (41), or RVV-XCP (41). The $K_m$ value of rLosac was higher than those of physiological factor X activators but lower than the $K_m$ value of RVV-XCP (Table 3). The $k_{cat}$ value of rLosac was lower than that of any of the other factor X activators. The $k_{cat}/K_m$ value was much lower than the values of physiological factor X activators and RVV-XCP. These data suggest that rLosac is a less potent factor X activator than the physiological factor X activators and RVV-XCP.

**Mechanism of Factor X Activator by rLosac**—To investigate the cleavage pattern of rLosac on factor X, changes in Losac-treated factor X electrophoretic mobility were followed (Fig.

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**TABLE 2**

Effect of rLosac on clotting times of plasmas deficient in a factor in the intrinsic and extrinsic pathway

| Plasmas deficient in a factor in the intrinsic pathway$^a$ | aPTT (mean ± S.D.) (n = 3) |
|----------------------------------------------------------|-----------------------------|
| Control                                                  | rLosac (45 nM)$^b$          |
| Normal                                                   | 57.7 ± 0.2                  | 45.7 ± 0.6                 |
| Factor VIII-deficient                                    | 135.7 ± 1.0                 | 75.7 ± 2.0                 |
| Factor IX-deficient                                      | 131.0 ± 3.9                 | 82.6 ± 5.0                 |
| Factor XI-deficient                                      | 185.8 ± 2.9                 | 86.5 ± 3.0                 |
| Factor XII-deficient                                     | 472.9 ± 34.8                | 120.5 ± 3.0                |
| Factor X-deficient                                       | 209.2 ± 4.4                 | 222.9 ± 1.0                |
| Factor X-deficient reconstituted$^c$                     | 53.4 ± 0.5                  | 43.6 ± 1.2                 |

| Plasmas deficient in a factor in the extrinsic pathway$^a$ | Recalcification time (mean ± S.D.) (n = 3) |
|----------------------------------------------------------|---------------------------------------------|
| Control                                                  | rLosac (20 nM)$^b$                         |
| Normal                                                   | 293.0 ± 18.7                               | 141.0 ± 0.7                  |
| Factor VII-deficient                                     | 359.7 ± 12.3                               | 170.1 ± 0.7                 |
| Factor X-deficient                                       | >20 min                                    | >20 min                     |
| Factor X-deficient reconstituted$^c$                     | 366.1 ± 10.5                               | 176.1 ± 0.3                 |

$^a$ Plasmas in the assay were diluted 2-fold with Tris-buffered saline (control) or rLosac.
$^b$ Concentration in the plasma.
$^c$ Factor X-deficient plasma reconstituted with 9 µg/ml factor X.

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**FIGURE 5. rLosac-induced factor X activation.** A, dose-dependent activation. Different concentrations of rLosac were incubated during 20 min at 37 °C with 0.3 µM factor X in 25 mM Tris-HCl, pH 8.0, to a final volume of 195 µl. Then 5 µl of S-2765 were added, and the substrate hydrolysis was followed for 30 min at 405 nm at 37 °C by recording the liberation of p-nitroanilide by the active factor X formed in the reaction. rLosac concentrations were as follows: 0 nM (solid line), 2 nM (dashed line), 5 nM ( ), 10 nM ( ), 20 nM ( ), 30 nM ( ), 40 nM ( ), 80 nM ( ). B–D, effect of cofactors in rLosac-induced factor X activation. A chromogenic assay was performed by incubation for 20 min at 37 °C of 5 nM rLosac, 0.3 µM factor X, and 25 mM Tris-HCl, pH 8.0, in the presence of different concentrations of CaCl$_2$ (B), PC/PS (C), or both (D). Values were expressed as a percentage of the values obtained at 405 nm.

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**TABLE 2**

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|----------------------------------------------------------|-----------------------------|
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| Factor VIII-deficient                                    | 135.7 ± 1.0                 | 75.7 ± 2.0                 |
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| Plasmas deficient in a factor in the extrinsic pathway$^a$ | Recalcification time (mean ± S.D.) (n = 3) |
|----------------------------------------------------------|---------------------------------------------|
| Control                                                  | rLosac (20 nM)$^b$                         |
| Normal                                                   | 293.0 ± 18.7                               | 141.0 ± 0.7                  |
| Factor VII-deficient                                     | 359.7 ± 12.3                               | 170.1 ± 0.7                 |
| Factor X-deficient                                       | >20 min                                    | >20 min                     |
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$^a$ Plasmas in the assay were diluted 2-fold with Tris-buffered saline (control) or rLosac.
$^b$ Concentration in the plasma.
$^c$ Factor X-deficient plasma reconstituted with 9 µg/ml factor X.
lead to a hemorrhagic syndrome (1, 2). Many studies have been carried out to understand the pathophysiological mechanism of the envenoming by *L. obliqua* (2). All of these studies point to a fibrinolysis activation secondary to the action of procoagulant toxins at different levels of the coagulation cascade. In the present work, we have provided experimental evidence that Losac, a hemolin family member, is able to induce coagulation through factor X activation by selective proteolytic cleavage.

Aiming to shed light on the structure of Losac and to facilitate future functional studies, the region coding for its deduced mature protein was cloned in fusion to an N-terminal His tag for purification and detection convenience. The analysis of the sequence revealed that Losac shares different degrees of similarity with cell adhesion molecule members of the immunoglobulin superfamily involved in the development of the nervous system and critical for neurite outgrowth: hemolins from lepidopters (47–76%) (31, 37), neuroglian (34%) from *D. melanogaster* (36), and L1-NCAM (26%) from humans (35).

Hemolins are exclusively expressed on lepidopters (43, 44). Their structures encompass four immunoglobulin-like domains linked together by a disulfide bond. Functionally, they were first associated with the insect immune system because of their overexpression after bacterial infection (44). Due to its adhesion properties, some hemolins have been involved in the cell adhesion mechanism (37). Some studies reveal that hemolins are present in neural tissues in several phases of embryonic development (24), and their expression correlates with hormones that regulate moulting and metamorphosis of lepidopters (19, 45). It seems clear that hemolins are multifunctional proteins with roles in defense (cellular and humoral) and in development (22). The high identity among Losac, hemolins, and neural cell adhesion molecules 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 (5). Because Losac shares its main sequence features with hemolins, it can be perfectly classified as one of them. Nevertheless, unlike Losac, no hemolins or cell adhesion molecules were associated with proteolytic activities.

As an initial step toward the functional characterization of Losac and its possible role in *L. obliqua* envenomation, we obtained its recombinant form (rLosac) (Fig. 3). Our results reveal that rLosac possesses procoagulant activity toward human plasma (Fig. 4A). According to our results, rLosac triggers blood coagulation through the activation of factor X because aPTT and recalcification time of several deficient plasmas in a factor of the intrinsic and extrinsic pathways were shortened except for factor X-deficient plasma (Table 2). This was corroborated by the normalization of clotting times after reconstitution of factor X-deficient plasma with purified factor. Moreover, clot dissolution is not likely to be induced by rLosac because neither fibrinogen nor fibrin were affected, even when employing higher rLosac concentrations than those used in the procoagulant test (Fig. 4, B and C). On the other hand, rLosac was clearly recognized by the antilionic
serum (Fig. 4D), suggesting that Losac is one of the immunogenic components contained in the venom of L. obliqua. Therefore, it is likely that Losac performs an important role during the envenomation process.

Although its uncovered sequence did not show an equivalent among factor X activators, the mechanism of factor X activation by rLosac was also studied. The factor X activation activity of rLosac is clearly calcium-independent (Fig. 5A) because the addition of CaCl₂ to the reaction increases rLosac activity (Fig. 5B). Most factor X activators from snake venom, including RVV-X, critically depend upon the presence of Ca²⁺ (13). Physiologically, calcium ions induce conformational changes in the Gla-domain of factor X that enhances its binding to procoagulant (negatively charged) membranes (46). In the case of rLosac, low concentrations of phospholipids (5 and 10 μg/ml) induce an increase of factor Xa production, whereas higher concentrations did not (Fig. 5C). In the presence of both cofactors, Ca²⁺ and phospholipids, factor X activation by rLosac was accelerated ~8.9-fold (Fig. 5D). Previously, it was shown that hemolin from Manduca sexta has two binding sites for LPS, one that interacts with the lipidic portion and one that interacts with the outer core carbohydrates of LPS (17). Moreover, it was shown that another hemolin, H. cecropia hemolin, is a calcium-binding protein (47). It was demonstrated that calcium ions are important to cell adhesion properties of this hemolin, playing a role in hemocyte adhesiveness. All of this evidence suggests a probable binding between rLosac, Ca²⁺, phospholipids, and factor X. Because phospholipids and calcium ions are important blood coagulation cofactors (7), the stimulatory effect on rLosac-induced factor X activation can occur in vivo.

In the presence of both cofactors, kinetics analysis was done. Linear double reciprocal plots indicate that rLosac follows a Michaelis-Menten kinetics (Fig. 6). The $K_m$ value (190 nM) of rLosac (Table 3) was similar to the factor X concentration in human plasma (around 10 μg/ml, 170 nM). Even taking into consideration the fact that the $k_{cat}/K_m$ value of rLosac was lower than those values obtained by physiological factor X activators and RVV-X (Table 3), this does not rule out the possibility that Losac-induced factor X activation can occur in plasma of patients affected by L. obliqua venom.

It is known that the activation of factor X by the metalloprotease RVV-X or by physiological coagulation complexes involves the cleavage of the Arg25–Ile29 peptide bond and formation of factor Xa (13). In order to understand the factor X activation by rLosac, we analyzed the cleavage pattern of fac-
tor X. Native and recombinant Losac were able to cleave human factor X, generating fragments similar to the ones generated by RVV-X (Fig. 7, B and C). That cleavage pattern was totally inhibited by PMSF, suggesting that a serine protease-like activity in rLosac would be responsible for the proteolytic activation of factor X. Moreover, we show that rLosac activity was significantly diminished (but not abolished) when we used Gla-domainless factor X (Fig. 7). Thus, complete factor X was a much better substrate than Gla-domainless factor X, indicating either a direct role of the Gla-domain in interacting with rLosac or a role for the domain in altering the conformation of the factor X.

The mechanism of recognition and cleavage of factor X by RVV-X has been well studied. RVV-X is a snake venom metalloprotease of the P-IV class (48). This protein shows three domains in the heavy chain (a metalloprotease, a disintegrin-like, and a cysteine-rich domain) and two light chains sharing sequence similarity with C-type lectin proteins (49). A model proposed by Morita (50) and crystallographic studies of the molecule of RVV-X (51) support the hypothesis that RVV-X primarily recognizes the calcium-bound conformation of the Gla-domain in factor X through an exosite formed by the light chains, followed by the catalytic conversion of factor X to factor Xa. Thus, it is totally explained why Gla-domainless factor X and non-carboxylated factor X are poor substrates for RVV-X (52).

All of these observations allow us to infer that, despite the structural differences between rLosac and RVV-X, it remains possible that they share a similar mechanism involving Ca$^{2+}$ and phospholipids for recognition of factor X Gla-domain followed by its proteolytic conversion to active factor X. If this plausible hypothesis is confirmed, it would constitute an astonishing example of functional convergence involving very divergent molecules and organisms toward the self-preservation of the latter by generation of toxins from physiological proteins.

Cases of convergent molecular evolution toward specific enzymatic mechanisms have been reported before, even for proteins previously believed to be non-enzymatic. The discovery of catalytic antibodies, also known as abzymes, with several previously unexpected functions constitutes an example of such convergence (53–60). Focusing on the findings reported here, special attention should be given to proteolytic antibodies. The fact that their activities are frequently blocked by inhibitors of well characterized enzymes, such as serine and metallopeptidase inhibitors, seems to indicate that these intriguing proteins should harbor sequences that allow them to fold up into conformational regions that resemble active sites of well characterized enzymes. In fact, serine protease-like active sites were ascribed to abzymes: ECL2B-2, i41SL1–2, VIPase (56), and 17E8. In 1994, Zhou et al. (61) solved the crystal structure of the complex involving 17E8 and HEP (phenyl[1-(n-succinylamino)pentyl]phosphonate) (Protein Data Bank code 1EAP) (61, 62).

Our results reveal an interesting enzymatic function for Losac, a hemolin for which effects on cells were already demonstrated (5). The molecular details of all of these important and distinctive processes remain, however, non-elicudated.

The molecular basis of target recognition and proteolysis of factor X by rLosac needs to be further investigated.

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