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Insularinase A, a prothrombin activator from *Bothrops insularis* venom, is a metalloprotease derived from a gene encoding protease and disintegrin domains

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Abstract

The first low-molecular-mass metalloprotease presenting prothrombin activating activity was purified from *Bothrops insularis* venom and named insularinase A. It is a single-chain protease with a molecular mass of 22 639 Da. cDNA sequence analysis revealed that the disintegrin domain of the precursor protein is post-translationally processed, producing the mature insularinase A. Analysis of its deduced amino acid sequence showed a high similarity with several fibrin(ogen)olytic metalloproteases and only a moderate similarity with prothrombin activators. However, SDS-PAGE of prothrombin after activation by insularinase A showed fragment patterns similar to those generated by group A prothrombin activators, which convert prothrombin into meizothrombin independently of the prothrombinase complex. In addition, insularinase A activates factor X and hydrolyses fibrinogen and fibrin. Chelating agents fully inhibit all insularinase A activities. Insularinase A induced neither detachment nor apoptosis of human endothelial cells and was also not able to trigger an endothelial proinflammatory cell response. Nitric oxide and prostacyclin levels released by endothelial cells were significantly increased after treatment with insularinase A. Our results show that, although its primary structure is related to class P-I fibrin(ogen)olytic metalloproteases, insularinase A is functionally similar to group A prothrombin activators.

Keywords: *Bothrops insularis*; coagulation; endothelial cell; metalloprotease; prothrombin activator; snake venom.

Introduction

The human blood coagulation system comprises a series of linked glycoproteins that upon activation induce the generation of downstream enzymes ultimately forming fibrin. Thrombin plays a central role in blood coagulation and therefore the rate of thrombin generation and the total amount of thrombin formed provide a good estimate of the potential coagulation activity (Spronk et al., 2003). Prothrombin activators are present in the venom of a large number of different snake species and can be classified into four different groups based on their structural and functional properties (Kini et al., 2001). Group A corresponds to metalloproteases with actions on prothrombin independent of any plasma or exogenous cofactors. Group B is composed of Ca^{+2}-dependent metalloproteases. These groups are widely distributed in venoms of many kinds of viper, e.g., genera *Echis* and *Bothrops*, and are presumably the most toxic, since they are resistant to the natural coagulation inhibitors (serpins) present in mammalian plasma, such as antithrombin-III (Rosing and Tans, 1992). Group C activators are serine proteases that require Ca^{+2} ions and negatively charged phospholipids for maximal activity. Group D comprises serine proteases with structures and functions homologous to blood coagulation factors and activity strongly stimulated by Ca^{+2} ions, factor Va and negatively charged phospholipids (Kini et al., 2001). The prothrombin-activating metalloproteases already purified from the venoms of *B. erythromelas*, *B. asper*, *B. neuwiedi* and *B. atrox* convert prothrombin into meizothrombin, independently of the prothrombinase complex components, having been classified as group A prothrombin activators. All of them are single-chain proteases of high molecular mass (60–78 kDa), classified as P-III metalloproteases (Govers-Riemslag et al., 1987; Hofmann and Bon, 1987; Loria et al., 2003; Silva et al., 2003).

Snake venom metalloproteinases (SVMPs) are members of the reprolysin family (MEROPS family M12 subfamily adamalysin) of zinc-containing metalloproteinases and are grouped into four classes (P-I to P-IV) according to their domain structure (Bjarnason and Fox, 1995). The P-I class presents only the metalloprotease domain. In the P-II class, the protease domain is followed by a disintegrin domain. Class P-III enzymes are composed of protease and disintegrin-like and cysteine-rich domains. The P-IV class has a lectin-like domain in addition to the previously described domains (Jia et al., 1996; Gutiérrez and

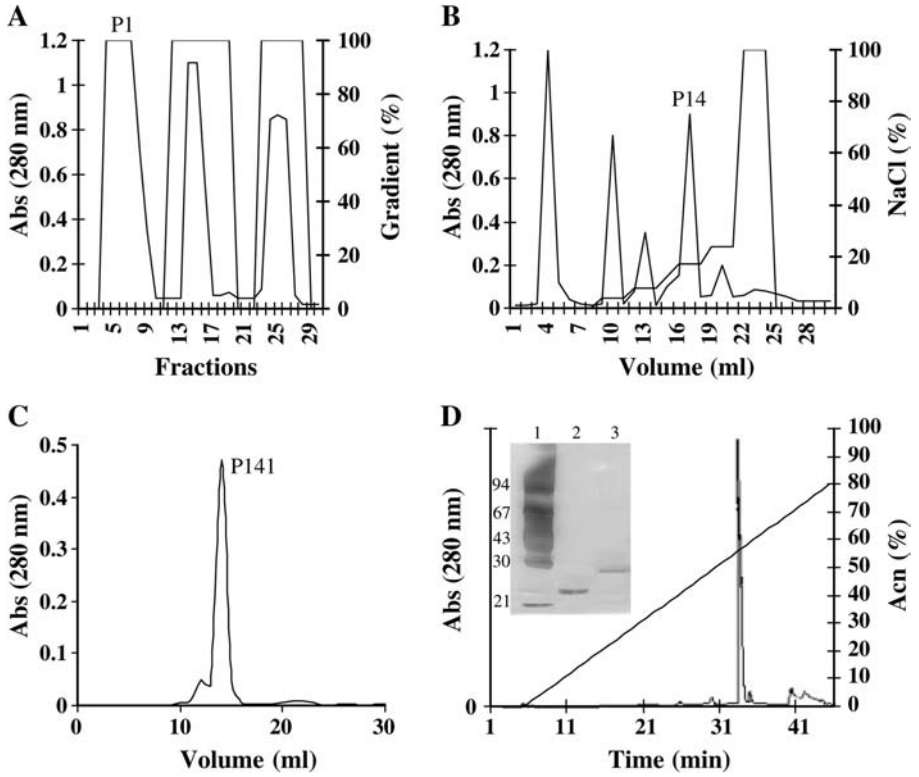

Figure 1 Purification of insularinase A.
(A) Affinity chromatography of *B. insularis* venom (50 mg) on a benzamidine Sepharose 6B column equilibrated with 0.02 M phosphate buffer, pH 7.8 and eluted with 0.02 M phosphate buffer, pH 7.8, and the same buffer containing 0.5 M NaCl and 0.02 M glycine buffer, pH 3.2. (B) Ion-exchange chromatography of P1 on a Resource S column equilibrated with 0.05 M MES buffer, pH 6.0 and eluted with a gradient of 0–1 M NaCl. (C) Gel filtration chromatography of P14 on Superdex 200 column equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. (D) Reversed-phase HPLC of insularinase A (100 μg) on a C8 column equilibrated with solvent A (0.1% TFA in water) and eluted with a 0–80% gradient of solvent B (acetonitrile/solvent A, 9:1 v/v). Inset: SDS/PAGE of insularinase A (10 μg) under non-reducing (lane 2) and reducing conditions (lane 3). Lane 1, molecular mass markers (phosphorylase *b*, 94 kDa; albumin, 67 kDa; ovoalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21 kDa).

Rucavado, 2000). In general, P-II metalloproteases are proteolytically processed by autocatalysis or by other proteases, generating disintegrins and P-I metalloproteases. Although the exact site of proteolysis is variable, this processing always occurs within the spacer region, located between the protease and the disintegrin domains. Atrolysin E and HR2a hemorrhagic enzymes isolated from *Crotalus atrox* and *Trimeresurus flavoviridis* venoms, respectively, are examples of P-I metalloproteases produced as a result of the processing of its P-II metalloprotease precursors (Shimokawa et al., 1997; Yamada et al., 1999). Interestingly, mature P-II metalloproteases are rarely found in snake venoms, and the first findings have been published relatively recently (Nikai et al., 2000; Chen et al., 2003).

Bothrops insularis (jararaca ilhoa) is a dangerous viperid snake restricted to Queimada Grande Island, located off the São Paulo State coast (Brazil). In contrast to other bothropic snakes, its diet consists of birds and some invertebrates as a result of the absence of mammals on this island. Consequently, *B. insularis* venom may present some specific toxins selected to better capture its prey (Duarte et al., 1995). Recently, Junqueira-de-Azevedo and Ho (2002) reported the analysis of expressed sequence tags (ESTs) generated from the cDNA library for *B. insularis* venom glands. According to the authors, the metalloproteases are the most diversified and expressed group of proteins in this venom.

In this report, we describe the isolation, cDNA sequence, primary structure and biological specificity of the first P-I SVMP with prothrombin activating activity purified from *B. insularis* venom, named insularinase A. This metalloprotease is generated upon cleavage of its precursor, a class P-II metalloprotease.

Results

Purification of insularinase A

Prothrombin and factor X activating activities and lysis of fibrin plate were obtained in the unbound peak (P1) from the benzamidine Sepharose 6B column (Figure 1A). P1 was then fractionated on a Resource-S chromatography column into a major peak eluting just before the end of the gradient, and four other peaks (Figure 1B). Pool P14 containing prothrombin activating activity was submitted to a further chromatography on a Superdex 200 column, revealing the major peak P141 (Figure 1C). The purity of P141 was demonstrated by RP-HPLC on a C8 column, resulting in a homogeneous peak eluted at a retention time of 34 min. The purified active protein (P141) was then named insularinase A (Figure 1D).

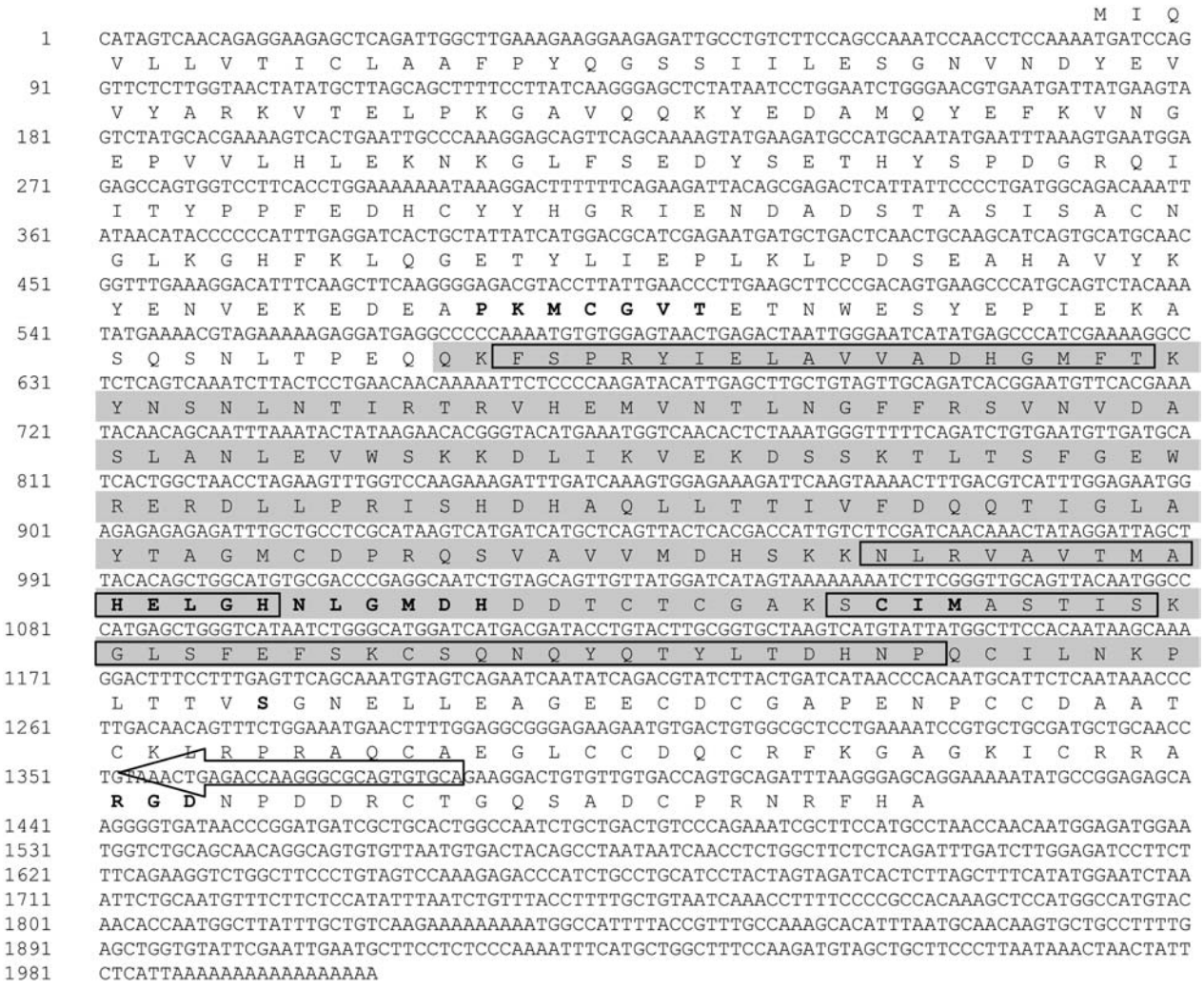

Figure 2 cDNA and deduced amino acid sequences of insularinase A.
The arrow indicates the region where the primer Insularin_3R was designed. The deduced sequence of insularinase A is shaded. Boxes indicate the amino acid sequences obtained by Edman degradation after insularinase A digestion with Lys-C endoproteinase. PKMCGVT, HEXXHXXGXXH and CIM sequences, Ser405 and the RGD motif are indicated in bold.

Molecular mass and glycosylation analysis

Analysis by SDS-PAGE revealed that insularinase A is a non-glycosylated protein (data not shown) composed of one single chain with molecular mass of 23 000 and 27 000 Da under non-reducing and reducing conditions, respectively (Figure 1D, inset). When analysed by MALDI-TOF MS, the molecular mass of the purified protein was 22 639 Da, similar to the value found by SDS-PAGE under non-reducing conditions.

Partial sequencing

Direct sequencing of native insularinase A was unsuccessful, probably due to N-terminal blockage, a common characteristic of snake venom metalloproteases (Gomis-Rüth et al., 1994). To obtain partial internal sequence information, reduced and alkylated insularinase A was digested with Lys-C endoproteinase and 10 major peptides were isolated by RP-HPLC. Five peptides were submitted to Edman degradation and the sequences obtained were CSQNQYQTYLTDHNP, NLRVAVTMAHELGH, FSPRYIELAVVADHGMFT, GLSFEFSK and SCIMASTIS.

Cloning of the cDNA

The sequence of the first peptide obtained (CSQNQYQTYLTDHNP) was used to screen a set of clusters of ESTs from *B. insularis* venom glands (Junqueira-de-Azevedo and Ho, 2002). Among the 33 different metalloprotease sequences described on this database, only one (BITM08A) presented a segment of total identity with this peptide. This fragment corresponded to the C-terminal region of the metalloprotease domain of a P-II precursor, which also codes for an RGD disintegrin (Figure 2). Since this cluster corresponded to a partial sequence, a primer was designed based on this EST and used to amplify the remaining 5′ segment of the molecule from the pooled library. The amplified fragment was cloned, completely sequenced and perfectly assembled with the original fragment, deriving the complete insularinase A cDNA sequence shown in Figure 2. All other peptide sequences obtained by Edman sequencing, totalling 64 amino acid residues, matched the translated cDNA sequence as

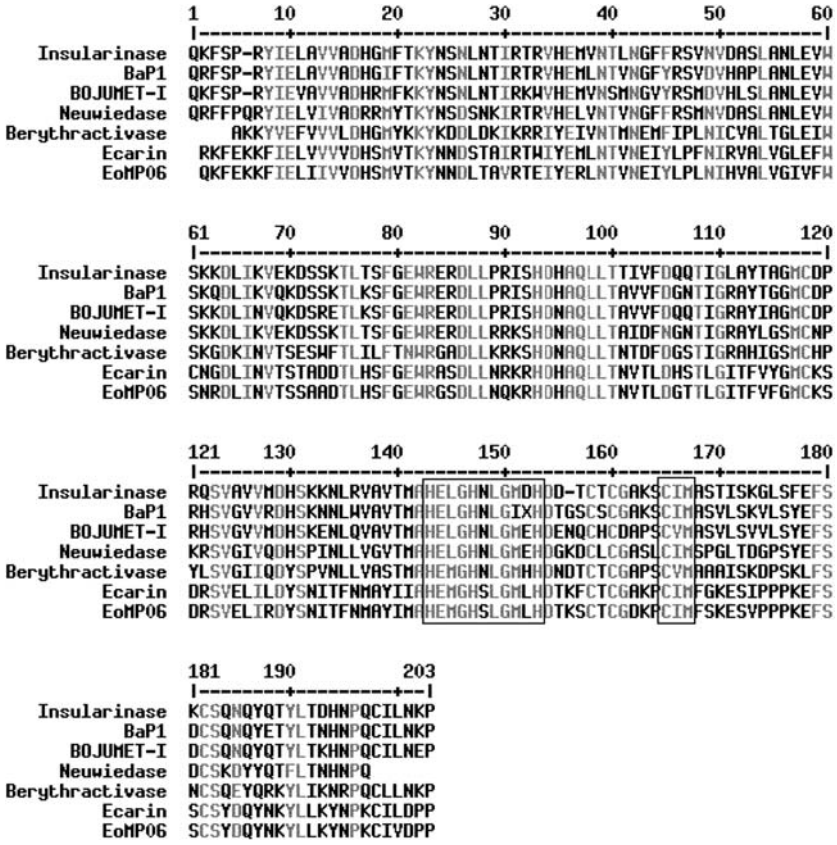

Figure 3 Comparison of the deduced amino acid sequence.
Insularinase A sequence was aligned with other members of the metalloprotease family. BaP1 (Watanabe et al., 2003), BOJUMET-I (Kashima et al., 2004) and neuwiedase (Rodrigues et al., 2000) are fibrin(ogen)olytic P-I SVMPs. Berythractivase (Silva et al., 2003), ecarin (Nishida et al., 1995) and EoMP06 (Hasson et al., 2003) are prothrombin activators P-III SVMPs. Residues in grey indicate high consensus. Boxes indicate the putative zinc ligands and active site.

shown in Figure 2, confirming that this was indeed the cDNA precursor of insularinase A.

The isolated cDNA clone consists of 1986 bp and presents a signal peptide, an N-terminal pro-domain containing the highly conserved PKMCGVT segment, a central protease domain with HEXXHXXGXXH and CIM sequences and a C-terminal disintegrin possessing the RGD triad, coding for a typical P-II metalloprotease (Figure 2).

Similar to other PII metatolloproteases, the insularinase A precursor has a serine residue at position 405 (Figure 2), indicating that this pre-pro-metalloprotease is further processed, resulting in mature insularinase A and disintegrin. Located between the protease and the disintegrin domains, residue Ser405 is highly conserved in the processed enzymes. On other hand, this residue is replaced by Cys405 in all members of the known metalloprotease/disintegrin/cysteine-rich proteins that are not further processed in metalloprotease and disintegrin domains (Selistre et al., 1997).

Mature insularinase A is composed of 203 amino acid residues, including six cysteine residues (Figure 3), which is in accordance with the molecular masses determined either by MALDI-TOF (22 639 Da) or by SDS-PAGE under non-reducing conditions (23 kDa). Based on its amino acid sequence, an isoelectric point of 7.8 was calculated. Potential sites for N-glycosylation have not been identified in the insularinase A sequence.

When compared with other SVMPs, its sequence presented high similarity with BaP1 (82%), a haemorrhagic and fibrin(ogen)olytic P-I metalloprotease from *Bothrops asper* venom (Watanabe et al., 2003), with BOJUMET-I (78%), a P-I metalloproteases from *Bothrops jararacussu* venom (Kashima et al., 2004), and with neuwiedase (70%), a non-haemorrhagic and fibrin(ogen)olytic P-I metalloprotease from *Bothrops neuwiedi* venom (Rodrigues et al., 2000). On the other hand, the similarity between insularinase A and prothrombin activators was lower: 52% with berythractivase from *Bothrops erythromelas* (Silva et al., 2003), 48% with ecarin precursor from *Echis carinatus* venom (Nishida et al., 1995) and 46% with EoMP06 isolated from *Echis ocellatus* venom (Hasson et al., 2003), all of them P-III metalloproteases corresponding to group A prothrombin activators.

Prothrombin activation

Prothrombin was rapidly activated by insularinase A in a dose-dependent manner (Figure 4A), independently of the presence of phospholipids and similarly under conditions with or without exogenous Ca^{2+} (data not shown). The prothrombin activation by insularinase A was completely abolished by the metal chelator EDTA (10 mM) and by the metalloprotease inhibitor 1,10-phenanthroline (10 mM), but not by the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (5 mM) (Figure 4A). The

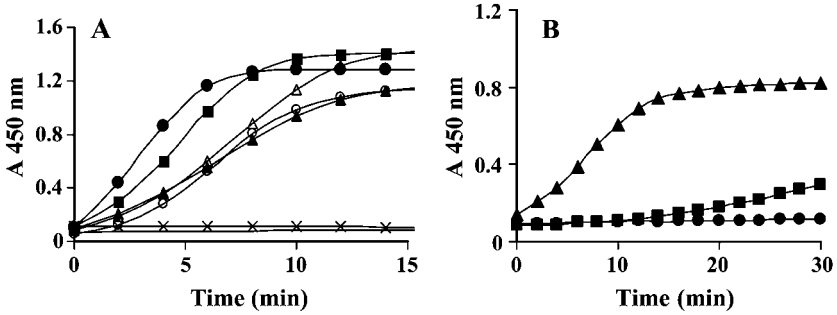

Figure 4 Prothrombin activation.
(A) Incubation of 4.5–36 nM insularinase A (0.01–0.08 μg) pre-incubated or not with inhibitors with 90 nM prothrombin and chromogenic substrate S-2238 (500 μM) in 0.05 M Tris-HCl, pH 7.4 to a final volume of 100 μl, at 37°C for 15 min: (▲) 4.5 nM, (Δ) 9 nM, (■) 18 nM and (●) 36 nM insularinase A; (○) 4.5 nM insularinase A+PMSF (5 mM); (×) 4.5 nM insularinase A+EDTA (10 mM) and 4.5 nM insularinase A+1,10-phenanthroline (10 mM). Final concentrations of PMSF, EDTA and 1,10-phenanthroline in the final reaction volume (100 μl) were 0.05, 0.10 and 0.10 mM, respectively. (B) Incubation of 4.5 nM factor Xa with 90 nM prothrombin and 500 μM S-2238 in 0.05 M Tris-HCl, pH 7.4, at 37°C for 30 min, in the presence (▲) or absence (■) of the prothrombinase components. — (A) and ● (B): prothrombin+S-2238. Values correspond to final concentrations.

catalytic efficiency of insularinase A (4.5 nM) was comparable to factor Xa (4.5 nM) in the prothrombinase complex (Figure 4B). Insularinase A and factor Xa were not able to directly hydrolyse S-2238 (data not shown).

Analysis of prothrombin fragments

The activation of prothrombin by insularinase A is shown in Figure 5. Under reducing conditions, the main hydrolysis products presented molecular masses of 55 and 32 kDa (Figure 5A), probably corresponding to a F1/F2/A fragment and the thrombin B-chain, respectively, which points to the generation of meizothrombin. Under non-reducing conditions, beyond the 36-kDa band, corresponding to the thrombin A-B chain, a fragment of 52 kDa was detected (Figure 5B), apparently corresponding to the F1/F2 fragment resulting from the autocatalytic action of meizothrombin generated.

Activity on human plasma

Insularinase A clotted human citrated plasma with an MCD value (defined as the concentration of protein inducing plasma coagulation in 60 s) of 0.3 μM.

Factor X activation

Insularinase A was less effective in activating factor X and this activity was dependent on pre-incubation of higher concentrations of the purified protein with factor X (Figure 6A). The activation of factor X by insularinase A was completely abolished by EDTA (10 mM) or 1,10-phenanthroline (10 mM) (Figure 6B). This metalloprotease was not able to directly hydrolyse the chromogenic substrate S-2765 (data not shown).

Fibrinolytic and fibrinogenolytic activities

Insularinase A has lower fibrinolytic and fibrinogenolytic activities when compared to prothrombin activation. Lysis areas on fibrin plates and the degradation of fibrinogen were obtained with higher concentrations of the enzyme and after a longer incubation period.

The purified protein (20 μg) induced a lysis area of 480 mm² after 18-h incubation. The lysis was completely inhibited by 1,10-phenanthroline, while no inhibition was observed even when high concentrations of PMSF (10 mM) were used. The protein did not clot fibrinogen, indicating that it is devoid of thrombin-like activity. The fibrinogenolytic activity of insularinase A was directed toward the Aα- and Bβ-chains of the fibrinogen molecule, without affecting the γ-chains. The Aα-chain was

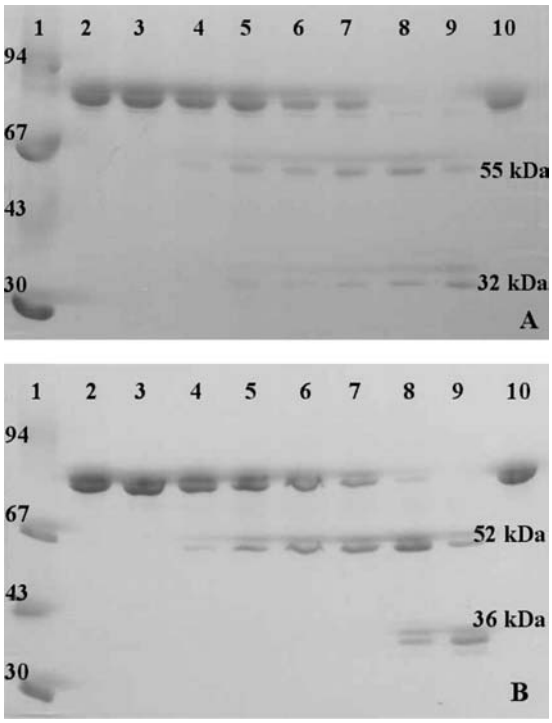

Figure 5 Hydrolysis of prothrombin by insularinase A.
SDS-PAGE (10%) of prothrombin after incubation at 37°C with insularinase A for 0, 5, 10, 15, 20, 30 and 60 min (lanes 3–9, respectively) at a protease/prothrombin molar ratio of 1:20, under reducing (A) and non-reducing (B) conditions. Lane 1, molecular-mass markers (phosphorylase *b*, 94 kDa; albumin, 67 kDa; ovoalbumin, 43 kDa; carbonic anhydrase, 30 kDa). Lanes 2 and 10, prothrombin incubated for 0 and 24 h, respectively.

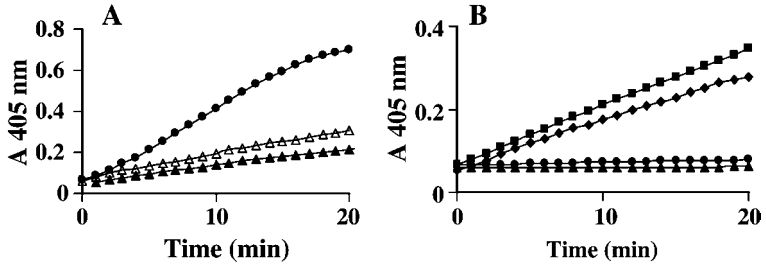

Figure 6 Factor X activation by insularinase A.
(A) Incubation of 0.3–2.25 μM insularinase A (0.675–5.0 μg), preincubated with 0.7 μM factor X for 15 min, with chromogenic substrate S-2765 (500 μM) in 0.05 M Tris-HCl, pH 7.4 at 37°C for 20 min: (▲) 0.3 μM; (Δ) 1.15 μM; (●) 2.25 μM. (B) Insularinase A (2.25 μM) was pre-incubated with 10 mM EDTA, 10 mM 1,10-phenanthroline or 5 mM PMSF at 37°C for 30 min. Mixtures of protein/inhibitor were incubated with prothrombin and substrates as described above. Final concentrations of PMSF, EDTA and 1,10-phenanthroline in the reaction were 0.05, 0.10 and 0.10 mM, respectively: (■) 2.25 μM insularinase A; (♦) insularinase A+PMSF; (●) insularinase A+EDTA; (▲) insularinase A+1,10-phenanthroline. Values correspond to final concentrations.

completely digested after 30 min, while the Bβ-chain was fully degraded after 2 h (Figure 7).

HUVEC studies

Under our assay conditions, insularinase A was not able to induce either detachment or apoptosis of human umbilical cord vein endothelial cells (HUVECs) cultured in medium supplemented with either 1% or 10% FBS. In addition, the protease neither induced cell proliferation nor promoted an increase in the endothelial cell growth factor (ECGF) effect. After analysing cell adhesion molecules by flow cytometry, we demonstrated that insularinase A did not increase the expression levels of E-selectin, ICAM-1 or DAF. On the other hand, insularinase A significantly increased the liberation of NO and PGI_2 by HUVECs, but not of t-PA. The effect of 0.22 μM insularinase A on nitric oxide release was similar to effect of thrombin and did not increase further when higher concentrations of insularinase were used (Figure 8). Contrary to nitric oxide, after 1 h of incubation with insularinase (0.22 μM) the concentrations of 6-keto-prostaglandin F1α on HUVEC supernatants were only weakly increased compared to thrombin (Figure 9). 6-Keto-prostaglandin F1α levels on 24 h supernatants revealed significantly increased PGI_2 levels only for thrombin-pretreated HUVECs (data not shown).

Hemorrhagic activity

In contrast with *B. insularis* venom (10 μg), insularinase A (10–100 μg) did not show hemorrhagic activity when injected into the dorsal skin of mice (data not shown).

Discussion

Prothrombin-activating metalloproteases already purified from snake venoms are single- or double-chain proteases with high molecular masses, belonging to class

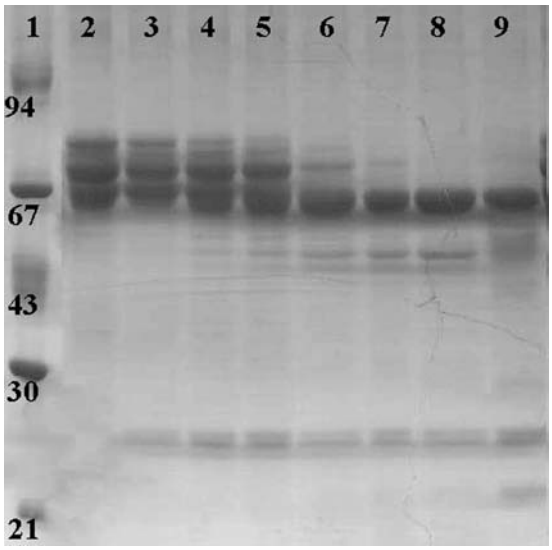

Figure 7 Bovine fibrinogen degradation by insularinase A.
SDS-PAGE (10%) of fibrinogen after incubation at 37°C with insularinase A for 0, 5, 10, 30, 60, 120 min and 24 h (lanes 3–9, respectively) at a protease/fibrinogen molar ratio of 1:6.5, under reducing conditions. Lane 1, molecular mass markers (phosphorylase *b*, 94 kDa; albumin, 67 kDa; ovoalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21 kDa). Lane 2, fibrinogen incubated for 24 h.

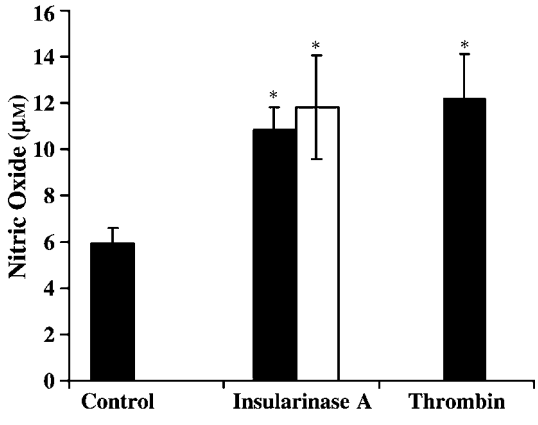

Figure 8 Effect of insularinase A on nitric oxide release by HUVECs.
HUVECs treated with 0.22 or 1 μM insularinase A (5 or 23 μg/ml) for 1 h at 37°C. Nitrite and nitrate in the supernatants were reduced with a VCl_3-saturated solution in 1 M HCl at 90°C, and the nitric oxide generated was measured by NO-ozone chemiluminescence in the gaseous phase. Results are the mean values±SEM of four independent experiments, performed in triplicate. $p<0.005$ by Student's *t*-test.

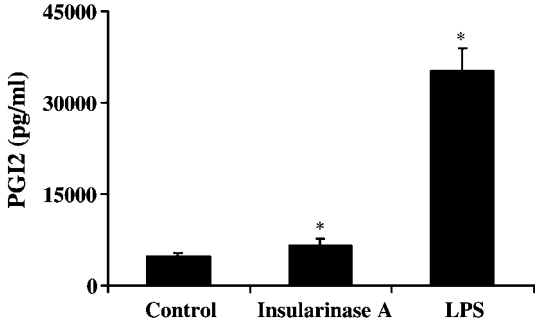

Figure 9 Effect of insularinase A on prostacyclin production by HUVECs.

HUVECs were treated with 0.22 μM insularinase A (5 μg/ml) or 5 U/ml thrombin for 1 h at 37°C. 6-Keto-prostaglandin F1α levels were measured in culture supernatants using commercial ELISA kits. Results are the mean values±SEM of four independent experiments, performed in triplicate. $p<0.005$ by Student's *t*-test.

P-III (Govers-Riemslag et al., 1987; Hofmann and Bon, 1987; Loria et al., 2003; Silva et al., 2003) or class P-IV (Yamada et al., 1996; Gao et al., 2002). In the present study, we have described the first low-molecular-mass metalloprotease with prothrombin activating activity, named insularinase A. The enzyme was isolated from *Bothrops insularis* venom as a non-glycosylated single-chain protein of approximately 22 600 Da, classified as a P-I metalloprotease (Bjarnason and Fox, 1995). Insularinase A activates prothrombin in a Ca^{+2}-independent manner, generating meizothrombin as an intermediate product. Thus, this enzyme resembles other class A prothrombin activators, such as ecarin from the venom of *E. carinatus* (Nishida et al., 1995), basparin A from *B. asper* venom (Loria et al., 2003) and berythractivase from *B. erythromelas* (Silva et al., 2003).

In addition to insularinase A, prothrombin activation by berythractivase was also tested indirectly using S-2238 substrate (Silva et al., 2003). However, in contrast to insularinase A, a higher dose of berythractivase (60 mM) and a lower concentration of prothrombin (44 nM) were used, suggesting that this P-III class metalloprotease is less powerful in activating prothrombin compared to insularinase A. Similarly, the serine protease lopap, isolated from bristle extracts of the caterpillar *Lonomia obliqua* (Reis et al., 2001), also showed lower activity in activating prothrombin compared to insularinase A.

Interestingly, prothrombin activation by insularinase A was higher than that of factor Xa alone and comparable to activation by factor Xa in the prothrombinase complex. Factor Xa is a vitamin K-dependent serine protease that forms a high-affinity complex with other components of the prothrombinase complex (factor Va, negatively charged phospholipid vesicles and calcium) to activate prothrombin to thrombin during the blood coagulation process. These results demonstrate the high catalytic efficiency of insularinase A.

At higher concentrations and for longer incubation times, insularinase A was also capable of degrading fibrinogen and fibrin. Despite its fibrin(ogen)olytic activity, insularinase A produced rapid and consistent human plasma clots, indicating that this metalloprotease is preferentially a procoagulant molecule. The clots formed were stable for hours, indicating that its fibrinogenolytic activity is less efficient in the presence of cross-linked fibrin. Corroborating these data, fibrin lysis areas (on fibrin plates) were only observed after 18 h incubation. Since insularinase A does not show thrombin-like activity and is only able to activate factor X at high concentrations, its clotting activity is probably the result of its capacity to activate prothrombin. Insularinase A was fully inhibited by 1,10-phenanthroline, indicating that the various biological properties of this enzyme are dependent on its catalytic activity.

Recently, a potent procoagulant P-III metalloprotease isolated from a bothropic venom was described by Loria et al. (2003). Basparin A, isolated from *Bothrops asper* venom, is also a group A prothrombin activator; however, it exhibits higher clotting potential on human plasma compared to insularinase A. As for other class P-III snake venom metalloproteases (Kamiguti et al., 1994; Estevao-Costa et al., 2000; Escalante et al., 2003), the clotting activity of basparin A is not inhibited by α_2-macroglobulin (Loria et al., 2003), and this may be associated with the presence of additional domains in the structure of these enzymes, which might hamper interaction with the inhibitor (Kamiguti et al., 1994). In contrast, class P-I metalloproteases are strongly inhibited by α_2-macroglobulin (Souza et al., 2001; Escalante et al., 2004; Tseng et al., 2004), thus explaining the high minimum coagulant dose obtained for insularinase A.

Mature insularinase A is found in venom as a P-I metalloprotease. However, the analysis of its cDNA sequence showed that, in addition to pro- and protease domains, insularinase A precursor has a disintegrin domain containing the Arg-Gly-Asp (RGD) sequence, which is a typical signature of P-II metalloprotease precursors. It is well known that class P-I metalloproteases and disintegrins are derived from common precursors that are post-translationally processed (Kini, 1995). Similarly to insularinase A, the disintegrin domains present in the precursors of atrolysin E and HR2a are lacking in the mature forms of these proteases (Shimokawa et al., 1997; Yamada et al., 1999). Studies using a recombinant pro-atrolysin E suggest that metalloproteases from *C. atrox* venom, including atrolysin A and active atrolysin E, but not pro-atrolysin E itself, can activate and process this precursor. The scheme of activation shows that the first cleavage by metalloproteases removes the pro-domain, followed by processing of the disintegrin domain (Shimokawa et al., 1997). Residue Ser405, located between the protease and the disintegrin domains, is highly conserved in the proteolytically processed metalloproteases (class P-II) and is replaced by Cys405 in proteins in which the disintegrin domains are not further processed (Selistre et al., 1997). Indeed, the only two class P-II metalloproteases already isolated from snake venom, jerdonitin and bilitoxin-1 from *Trimeresurus jerdonii* and *Agkistrodon bilineatus* venoms, respectively, have an additional cysteine residue (Nikai et al., 2000; Chen et al., 2003). Cys405 is known to form an intrachain disulfide bond with another cysteine residue located in the disintegrin domain, thereby avoiding separation of the metalloprotease and the disintegrin domains (Siigur et al., 1996). The insularinase

A precursor, as well as all other processed P-II metalloproteases, has a serine residue in the 405 position.

Analysis of the deduced amino acid sequence of insularinase A showed only moderate similarity to prothrombin activators. Despite this, the fragments generated after prothrombin activation by berythractivase, ecarin and EoMP06 are similar to those formed by insularinase A (Nishida et al., 1995; Hasson et al., 2003; Silva et al., 2003).

Different to classical prothrombin activators, insularinase A exhibits high similarity with several haemorrhagic and non-haemorrhagic fibrin(ogen)olytic metalloproteases. The highest homology was found with BaP1 from *Bothrops asper* venom (82% identity). Despite this high sequence identity, insularinase A and BaP1 exhibit distinct effects on blood coagulation and on endothelial cells. BaP1 is a fibrinogenolytic P-I metalloprotease with weak haemorrhagic activity and devoid of procoagulant activity. In addition, BaP1 induces detachment of endothelial cells by proteolysis of the matrix components, resulting in anoikis independent of the Bcl-2 family members Bax and Bcl-xL and associated with caspase-8 activation and $cFLIP_L$ up-regulation (Gutierrez et al., 1995; Rucavado et al., 1995; Diaz et al., 2005). Anoikis is a subtype of apoptosis induced by disruption of cell-matrix interactions. Cell-cell and cell-matrix contacts are necessary for the maintenance of anchorage-dependent cell survival (Mallat and Tedgui, 2000). In contrast to BaP1, insularinase A is preferentially procoagulant and is not able to induce either detachment or apoptosis of endothelial cells.

In general, cell adhesion molecules are known to help recruitment, adhesion and transendothelial migration of leukocytes into tissues (Smith et al., 1989; Springer, 1994; van de Stolpe and van der Saag, 1996). Insularinase A did not increase surface expression of ICAM-1, E-selectin or DAF, molecules playing an important role in inflammatory responses.

In vivo, a powerful triad composed of prostacyclin (PGI_2), nitric oxide (NO) and tissue plasminogen activator (t-PA) is secreted by endothelial cells, regulating thrombo-resistance and vascular tone. Alliance between them occurs at the level of protection against the deposition of thrombi over the vascular wall. Activation of fibrinolysis by t-PA through the generation of plasmin is complemented with the inhibition of platelet aggregation by PGI_2 and by inhibition of the release of plasminogen activator inhibitor-1 (PAI-1) from platelets by NO. On the other hand, endothelial regulation of vascular tone is exclusively NO-dependent (Gryglewski, 1995).

Our studies demonstrated that insularinase A significantly increases the NO released by endothelial cells similarly to thrombin. Since NO is a relevant regulator of the vascular tone, it is possible to suggest its involvement in the hypotension state occurring in severe human envenomations by *Bothrops* snake venoms (Rosenfeld, 1971).

On the other hand, t-PA release was not modified by insularinase A and only a slight increase in PGI_2 release by endothelial cells was observed. Taking into account that PGI_2 is a potent platelet activation inhibitor, its increased release could be involved with the inhibition of aggregation *in vivo*.

The ability of SVMPs to induce haemorrhage has been associated with their capacity to hydrolyse extracellular matrix (ECM) proteins (Bjarnason and Fox, 1994; Hati et al., 1999) and large variations in the haemorrhagic potency of SVMPs are observed. Phylogenetic analyses based on 30 complete sequences of P-I metalloproteases revealed that they may be classified into three functional subtypes: highly haemorrhagic acidic enzymes, moderately haemorrhagic basic enzymes, and non-haemorrhagic neutral enzymes (Tsai et al., 2000). In agreement with this classification, insularinase A, with a neutral pI, was devoid of haemorrhagic activity when tested in the dorsal skin of mice. It's important to mention that the haemorrhagic activity of insularinase A could not be assayed by intravital microscopy because, as a result of its high procoagulant activity, thrombus formation was observed in small vessels even when using low doses of the protease (data not shown).

In summary, we have isolated and characterised insularinase A and cloned the respective gene of the first prothrombin activator belonging to the P-I metalloprotease class. Despite the moderate similarity to ecarin and berythractivase, insularinase A is a classic group A prothrombin activator. Moreover, insularinase A also shows other secondary activities, all of which are dependent on its catalytic activity. However, structural elements involved in modulating the interaction of insularinase A with its substrates remain unknown.

Materials and methods

Reagents

Bothrops insularis crude venom was obtained from Butantan Institute, SP, Brazil. Benzamidine Sepharose 6B, Resource-S and Superdex 200 columns and dithiothreitol (DTT) were from Amersham Biosciences (Uppsala, Sweden). 1,10-Phenanthroline monohydrate was purchased from Merck (Darmstadt, Germany) and PMSF from Acros Organics (Morris Plains, NJ, USA). Chromogenic substrates H-D-Phe-PIP-Arg-pNA (S-2238) and *N*-α-Z-D-Arg-Gly-Arg-pNA (S-2765) were from Chromogenix (Milan, Italy). EDTA, L-glutamine, 2-mercaptoethanol, human factor II, human factor X, factor Xa, factor Va, bovine fibrinogen (90% clottable), human thrombin, endothelial cell growth factor (ECGF) and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal antibodies PE-conjugated-anti-CD54, CD55 and CD62E were obtained from PharMingen (BD Biosciences, San Diego, CA, USA). Endothelial cell growth supplement from bovine neural tissue was purchased from Calbiochem (La Jolla, CA, USA). Foetal bovine serum (FBS), trypsin, RPMI 1640 medium, HAM F 12 medium, penicillin and streptomycin were purchased from Cultilab (Campinas, SP, Brazil). EIA kit 6-keto-prostaglandin F1α was obtained from Cayman Chemical Company (Ann Arbor, MI, USA) and the T-PA ELISA kit was purchased from Oncogene Research Products (San Diego, CA, USA). Sequencing grade endoproteinase Lys-C was from Boehringer Mannheim (Mannheim, Germany). All other chemicals used were of the highest purity commercially available.

Purification of insularinase A

Chromatography assays were performed using the FPLC system from Amersham Biosciences. Lyophilised crude venom samples dissolved in 2 ml of 0.02 M sodium phosphate buffer, pH 7.8 were fractionated on a benzamidine Sepharose 6B affin-

ity column, pre-equilibrated with the same buffer at a flow rate of 2 ml/min. Proteins were eluted in three steps using 0.02 M phosphate buffer, pH 7.8, and the same buffer containing 0.5 M NaCl and 0.02 M glycine buffer, pH 3.2. The fraction unbound to the column was concentrated, dialysed against 0.05 M 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer, pH 6.0, and subsequently submitted to a Resource S column equilibrated with the same buffer. Elution was carried out isocratically with the equilibration buffer, followed by a linear NaCl gradient from 0 to 1 M in the same buffer at a flow rate of 3 ml/min. Eluted fractions were tested for their ability to activate prothrombin and the more active pool was submitted to a Superdex 200 column, previously equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, at a flow rate of 0.5 ml/min. Finally, the purified active protein was chromatographed on an RP-HPLC C_8 column (Beckman, Fullerton, CA, USA) at a flow rate of 1 ml/min. Elution was performed with an increasing gradient from 0% solvent A [0.1% trifluoroacetic acid (TFA) in water] to 80% solvent B (0.1% TFA in 90% acetonitrile). Protein contents were determined either by the method of Markwell et al. (1978) or by measuring the absorbance at 280 nm.

Molecular mass and glycosylation analysis

The insularinase A molecular mass was determined by matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS) on a Voyager DE-PRO instrument (Perseptive Biosystems, Framingham, MA, USA). The matrix used was 3,5-dimethoxy-4-hydroxycinnamic acid. Insularinase A under reducing and no- reducing conditions was also analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% separating and 4% stacking gels (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R250 for mass molecular analysis and with periodic acid-Schiff (PAS) stain according to Segrest and Jackson (1972) for detection of glycosylation.

Amino acid sequence

Native insularinase (10 μg) was desalted through a ProSorb cartridge (Perkin Elmer, Foster City, CA, USA) and directly submitted to N-terminal sequencing. Another aliquot of insularinase A (100 μg) was dissolved in 40 μl of 0.5 M Tris-HCl buffer, pH 8.3, containing 6 M guanidine HCl and 1 mM EDTA, and incubated for 30 min at 50°C. A 20-fold molar excess of DTT, assuming seven cysteine residues in insularinase A, was added to the reaction mixture for 1 h at 50°C. This assumption was based on the Cys contents of the two metalloproteases from *Bothrops insularis* venom for which the cDNAs were completely sequenced (Junqueira-de-Azevedo and Ho, 2002). Alkylation of free thiol groups was performed after incubation with a 1.5-fold molar excess of iodoacetamide for 1 h at room temperature in the dark. All incubations were performed under nitrogen. Excess reagents were removed by RP-HPLC on a Vydac C_{18} column (The Separations Group, Hesperia, CA, USA) using a flow rate of 0.2 ml/min. The absorbance was monitored at 215 nm. The solvent system used was 0.1% TFA in water (solvent A) and 0.09% TFA in acetonitrile (solvent B). The gradient was 5% solvent B for 15 min, 75% B at 50 min and 75% B for 10 min. Reduced and alkylated insularinase A was collected, speed vac dried, further dissolved in 50 μl of 0.025 M Tris-HCl buffer, pH 8.5 containing 1 mM EDTA, and digested with endoproteinase Lys-C at an enzyme/substrate molar ratio of 1:50 (w/w) at 37°C for 18 h. The peptides obtained by Lys-C digestion were isolated by RP-HPLC on a C_{18} column as described above. The gradient program started with 5% solvent B for 5 min and was increased to 45% B at 55 min and to 75% B at 60 min. Peptide peaks were collected, speed vac concentrated to 20 μl and Edman sequenced on a PSQ-23A protein sequencer (Shimadzu, Kyoto, Japan), according to the manufacturer's instructions.

cDNA cloning

The sequenced peptide fragments were used to screen a database of expressed sequence tags (ESTs) from *B. insularis* venom (Junqueira-de-Azevedo and Ho, 2002). From the matched EST, a specific reverse primer, Insularin_3R (5′-ACA CTG CGC CCT TGG TCT C-3′), was designed and used together with T7 primer to amplify by PCR the full-length fragment of this metalloprotease from the same cDNA library used for EST sequencing. The fragment of the expected size was cloned in the TOPO system (Life Technologies) and completely sequenced using M13F and M13R universal primers. The nucleotide sequence of insularinase A has been deposited in the GenBank database under the accession number AY736107.

Prothrombin activation

Activation of prothrombin was indirectly determined using the thrombin-specific substrate S-2238. The reaction was carried out in microtitre plates containing 0.05 M Tris-HCl buffer, pH 7.4, 90 nM prothrombin (factor II), 4.5–36 nM insularinase A (0.01–0.08 μg) or 4.5 nM factor Xa and 500 μM S-2238 in a final volume of 100 μl. To assays in the presence of the prothrombinase complex, 2.5 μM factor Va, 300 μM phospholipids and 10 mM $CaCl_2$ were added to the buffer. Substrate cleavage by the thrombin generated was monitored by measuring the formation of *p*-nitroaniline for 30 min at 405 nm, using a Spectra Max 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Assays were also performed after preincubation of insularinase A with 10 mM 1,10-phenanthroline, 10 mM EDTA or 5 mM PMSF for 30 min at 37°C. Values correspond to final concentrations.

Analysis of prothrombin fragments

The proteolytic activity of insularinase A upon prothrombin was tested in 0.05 M Tris-HCl buffer, pH 7.4, at a protease/prothrombin molar ratio of 1:20 at 37°C. After 0, 5, 10, 15, 20, 30 and 60 min of incubation, aliquots of the mixture were removed and analysed under reducing and non-reducing conditions by 10% SDS-PAGE. Fragments were analysed after staining the gel with Coomassie brilliant blue R250.

Activity on human plasma

To test procoagulant activity, 0.1–0.5 μM insularinase A (0.225–1.125 μg) was incubated at 37°C with 100 μl of human citrated plasma and the clotting time was measured. The minimum coagulant dose (MCD) was defined as the amount of protein that induced plasma coagulation in 60 s.

Factor X activation

FX activation was carried out in a microtitre plate after preincubation at 37°C for 15 min of FX (0.7 μM) with 0.3–2.25 μM insularinase A (0.675–5.0 μg) in 100 μl of 0.05 mM Tris-HCl, pH 7.4, followed by the addition of 400 μM S-2765. The rate of substrate cleavage by FXa formed was monitored by measuring the formation of *p*-nitroaniline for 30 min at 405 nm, using a Spectra Max 190 spectrophotometer. Assays were also performed after preincubation of insularinase A with 10 mM 1,10-phenanthroline, 10 mM EDTA or 5 mM PMSF for 30 min at 37°C. Values correspond to final concentrations.

Fibrinolytic activity

Fibrinolytic activity was measured by the fibrin-plate method, as described by Jespersen and Astrup (1983). Briefly, a fibrin-agarose gel was prepared by mixing human fibrinogen (2 mg/ml of clotting protein) with a preheated solution of 2% agarose in 0.1 M sodium barbital buffer, pH 7.75, and 20 U/ml thrombin. Samples of 20 μg insularinase A alone or preincubated at 37°C for 10 min with the protease inhibitors PMSF (10 mM) or 1,10-phenanthroline (10 mM) were applied to wells of the solidified gel and incubated at 37°C for 18 h. The lysed area was then measured.

Fibrinogenolytic activity

Bovine fibrinogen solution (2 mg/ml clotting protein), diluted in 0.05 M Tris-HCl buffer, pH 7.3, containing 5 mM $CaCl_2$ and 0.2 M NaCl, was preincubated at 37°C with insularinase A at a 1:6.5 molar ratio (insularinase A/fibrinogen). Aliquots of 30 μl were removed at 0, 5, 10, 30, 60, 120 min and 24 h and subjected to 10% SDS-PAGE under reducing conditions. Fibrinogen fragments were analysed after staining the gel with Coomassie Brilliant Blue R250.

HUVEC studies

Cell cultures Endothelial cells were obtained from human umbilical-cord veins (HUVECs) by collagenase digestion as described by Jaffe et al. (1973) and cultured on gelatin-coated plastic dishes in RPMI 1640 medium supplemented with 1% or 10% (v/v) FBS, 45 μg/ml heparin, 25 μg/ml endothelial supplement growth factor, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 7 μg/ml polymyxin B, 50 μM mercaptoethanol and 0.75% (v/v) mouse brain extract. Cells were maintained at 37°C in a humidified 5% CO_2 incubator. For the assays, cells were trypsinised and cultivated on plastic culture plates precoated with gelatin at 4°C overnight.

Morphological changes and apoptosis HUVECs in a confluent monolayer were incubated with 0.22 or 2.2 μM insularinase A (5 or 50 μg/ml) for 24 or 48 h at 37°C. After treatment, morphological changes were analysed by inverse microscopy (Leitz Diaplan, Wetzlar, Germany). Apoptosis was analysed by fluorescence microscopy after treatment of adherent and non-adherent cells with acridine orange (50 μg/ml) and ethidium bromide (50 μg/ml). The cell cycle was analysed by flow cytometry. Briefly, after 24 h incubation with 0.22 μM insularinase A (5 μg/ml), cells were harvested and fixed in 70% ethanol. Before analysis by flow cytometry, cells were washed with phosphate-buffered saline (PBS), treated with 8 g/l RNase, 1.2 g/l spermine and stained with 50 mg/l propidium iodide for at least 30 min. DNA content was determined by FACScan flow cytometry (Coulter, Scalibur, Mijdrecht, The Netherlands).

Expression of cell adhesion molecules After treatment of HUVECs with 5 or 50 μg/ml insularinase A (0.22 or 2.2 μM) or 2 μg/ml lipopolysaccharide (positive control) for 1 h, medium was removed and the cells were further cultured for 4 h for expression of E-selectin (CD62E) or 24 h for expression of intercellular adhesion molecule-1 (ICAM-1:CD54) and decay accelerating factor (DAF:CD55) in RPMI containing 10% FBS. Cells were harvested by treatment with 0.25% trypsin/0.02% EDTA solution, followed by centrifugation at 400 *g* for 10 min at 4°C. The pellets were resuspended in 50 μl of PBS/FBS (10%) containing saturating concentrations of PE-conjugated anti-CD62E, -CD54, -CD55, or equivalent concentrations of an isotypic control IgGI. After incubation for 40 min at 4°C, cells were fixed with 1% paraformaldehyde and analysed by flow cytometry in a FACScan cytofluorimeter (Becton Dickinson, Mountain View, CA, USA). Appropriate settings for forward and side scatter gates were used to examine 10 000 cells per experiment.

Determination of nitric oxide release HUVECs were initially treated with 0.22 or 1 μM insularinase A (5 or 23 μg/ml) or 5 U/ml thrombin (positive control) for 1 h; supernatants were then collected and centrifuged at 400 *g* for 10 min at 4°C. Nitric oxide was measured by reduction of its final products, nitrite and nitrate, with a VCl_3-saturated solution in 1 M HCl at 90°C. The nitric oxide generated was determined by NO-ozone chemiluminescence using a NOA™ 280 NO analyser (Sievers, Boulder, CO, USA) and the concentration was calculated from a $NaNO_3$ standard curve using Bag software 2.2 (Sievers).

Prostacyclin (PGI_2) and tissue-type plasminogen activator (t-PA) assays After treatment of HUVECs with 0.22 μM insularinase A (5 μg/ml) or 5 U/ml thrombin (positive control) for 1 h, medium was removed and the cells were cultured for 24 h in RPMI containing 10% FBS. Supernatants removed after 1 and 24 h were centrifuged for 10 min, 400 *g* at 4°C, to exclude the interference of cellular debris. t-PA and 6-keto-prostaglandin F1α (PGI_2 stable metabolite) levels were measured using commercial ELISA kits.

Haemorrhagic activity

Hemorrhagic activity was determined by the method of Kondo et al. (1960) as modified by Gutierrez et al. (1995). Insularinase A (10–100 μg) in 0.15 M NaCl was injected intradermally into the shaved dorsal skin of adult male Swiss mice (18–22 g). Control mice received 0.15 M NaCl (negative control) alone or 10 μg of *B. insularis* venom (positive control) in 0.15 M NaCl. After 2 h, the animals were sacrificed, the dorsal skin was removed and the hemorrhagic lesions were measured.

Statistics

All results are expressed as the mean±SEM. Statistical analysis was performed using Student's *t*-test for paired data. Differences with a p value <0.005 were considered statistically significant.

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