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Short communication

Cotiarinase is a novel prothrombin activator from the venom of *Bothrops cotiara*

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ABSTRACT

Snake venom serine proteinases (SVSPs) may affect hemostatic pathways by specifically activating components involved in coagulation, fibrinolysis and platelet aggregation or by unspecific proteolytic degradation. In this study, we purified and characterized an SVSP from *Bothrops cotiara* venom, named cotiarinase, which generated thrombin upon incubation with prothrombin. Cotiarinase was isolated by a two-step procedure including gel-filtration and cation-exchange chromatographies and showed a single protein band with a molecular mass of 29 kDa by SDS-polyacrylamide gel electrophoresis under reducing conditions. Identification of cotiarinase by mass spectrometric analysis revealed peptides that matched sequences of viperid SVSPs. Cotiarinase did not show fibrinogen-clotting, platelet-aggregating, fibrinogenolytic and factor X activating activities. Upon incubation with prothrombin the generation of thrombin was detected using the peptide substrate D-Phe-Pip-Arg-*p*NA. Moreover, mass spectrometric identification of prothrombin fragments generated by cotiarinase in the absence of co-factors (phospholipids, factor Va, factor Xa and Ca^{2+} ions), indicated the limited proteolysis of this protein to release prothrombin 1, fragment 1 and thrombin. Cotiarinase is a novel SVSP that acts on prothrombin to release active thrombin that does not match any group of the current classification of snake venom prothrombin activators.

Snake venom serine proteinases (SVSPs) are among the major components of South American viperid toxin repertoire and are implicated in an array of hemostatic disturbances upon envenomation [1–3]. SVSPs are classified in the clan PA, subclan PA(S), family S1 (chymotrypsin), subfamily A of proteolytic enzymes. They are considered as trypsin-like enzymes and cleave peptide bonds following Arg or Lys at the P1 position. However, despite the high degree of sequence identity among themselves and the similarity of primary specificity with trypsin, the stringent macromolecular substrate specificity of SVSPs contrasts with the less specific activity of trypsin. Several exogenous prothrombin activators are found in snake venoms and the characterization of these proteins is important for our understanding of structure-function relationships in the mammalian prothrombinase complex. Group A and B venom prothrombin activators are metalloproteinases whereas group C and D prothrombin activators are serine proteinases [4].

Bothrops cotiara [5] is an endemic and threatened viperid species restricted to pine forests (*Araucaria angustifolia*) of Southern Brazil and, for this reason, there are few reports on the composition of *B. cotiara* venom. Tashima and colleagues [6] have explored the venom proteome of *B. cotiara* and its close related taxa *Bothrops fonsecai*, and reported the identification of molecular taxonomic markers of these species. This study also pointed out the SVSPs as the second most expressed toxin class in the *B. cotiara* venom. In another study, we explored the subproteomes of heparin-binding toxins of various *Bothrops* venoms and detected the presence of a basic SVSP of ~30 kDa in *B. cotiara* venom that showed high-affinity for heparin [7]. By affinity chromatography to heparin the yield of isolation of this SVSP was rather low and the presence of traces of metalloproteinases in the same preparation was detected by mass spectrometry. Therefore we decided to establish a different protocol for the purification of this SVSP and here we report a two-step chromatographic procedure for the isolation of the enzyme, named cotiarinase, from *B. cotiara* venom. Venom was obtained

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from snakes bred in captivity at the Herpetology Laboratory of Instituto Butantan (Sao Paulo, Brazil) and centrifuged for 30 min at 2000× *g*, at 4 °C, to remove any scales or mucus, lyophilized and stored at −20 °C until use. Protein concentration was determined using the Bradford reagent (Sigma) and bovine serum albumin (Sigma) as a standard. The isolation procedure started with a sample of 60 mg venom that was dissolved in 1.4 mL 0.25 M ammonium bicarbonate, 0.001 M $CaCl_2$, and submitted to size-exclusion chromatography using a Superdex 75 column (GE Healthcare) coupled to an ÄKTA-FPLC system (GE Healthcare) at a flow rate of 0.1 mL/min (Fig. 1A). The subset of proteins present in the third chromatographic peak ranged from 30 to 14 kDa and was pooled, dialyzed against buffer A (sodium phosphate 0.05 M, pH 7.0) and submitted to cation-exchange chromatography on a Mono S HR 5/5 column (GE Healthcare) at a flow rate of 1 mL/min and bound proteins were eluted with a gradient of 0–500 mM of NaCl in buffer A within 40 min (Fig. 1B). The purified enzyme was detected in fractions eluted with 180–300 mM NaCl yielding 0.1 mg protein. Cotiarinase behaved as a single chain protein under reducing and non-reducing conditions by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) analysis (Fig. 1C). Reduced cotiarinase showed a molecular mass of ∼29 kDa whereas its non-reduced form showed a mobility corresponding to ∼20 kDa likely due to the fact that in general disulfide bonds make proteins more compact, thus they may run faster on a SDS-polyacrylamide gel. Western blot analysis using a polyclonal anti-SVSP antibody (anti-moojeni serine proteinases 1 and 2) [8] revealed a single protein band at ∼29 kDa (Fig. 1C). For the determination of the molecular mass of native cotiarinase a sample of 600 ng protein in 0.1% formic acid was submitted to RP-HPLC (C18 column; 100 μm i.d. × 100 mm; Waters) coupled with nanoelectrospray MS on a Q-ToF-Ultima mass spectrometer (Waters) at a flow rate of 600 nL/min. The gradient was 0–80% acetonitrile in 0.1% formic acid over 20 min while spray voltage was set at 3.1 kV and the instrument was operated in full scan (MS1) mode only (in the *m*/*z* range of 200–2000). Deconvolution of the MS1 protein spectrum using the MaxEnt 1 module (MassLynx; Waters) yielded an isotope-averaged molecular mass of 29,044 Da which is similar to the value obtained by SDS–PAGE (Fig. 1D). For protein identification, cotiarinase (Fig. 1C, lane 1) was submitted to *in gel* trypsin digestion [9] and the resulting peptide mixture (4.5 μL) was analyzed by LC-MS/MS as described elsewhere [10]. Automated database (NCBI NR database restricted to the taxa Serpentes) searching of tryptic peptides resulted in the sequences DIMLIR, FLVALYTSR, TLCAGILEGGK, IMGWGTISPTK, WDKDIMLIR and VSYPDVPHCANINLLDYEVCR, all of which displayed identity with SVSPs of viperid venoms (Supplemental Table 1). Taken together, these results strongly support the characterization of cotiarinase as a novel, basic SVSP of *B. cotiara* venom. Interestingly, in contrast to many SVSPs, cotiarinase did not display fibrinogenolytic activity when it was incubated with human fibrinogen (Calbiochem) at 1:10 enzyme-to-substrate ratio (w/w) (Supplemental Fig. 1). We also incubated

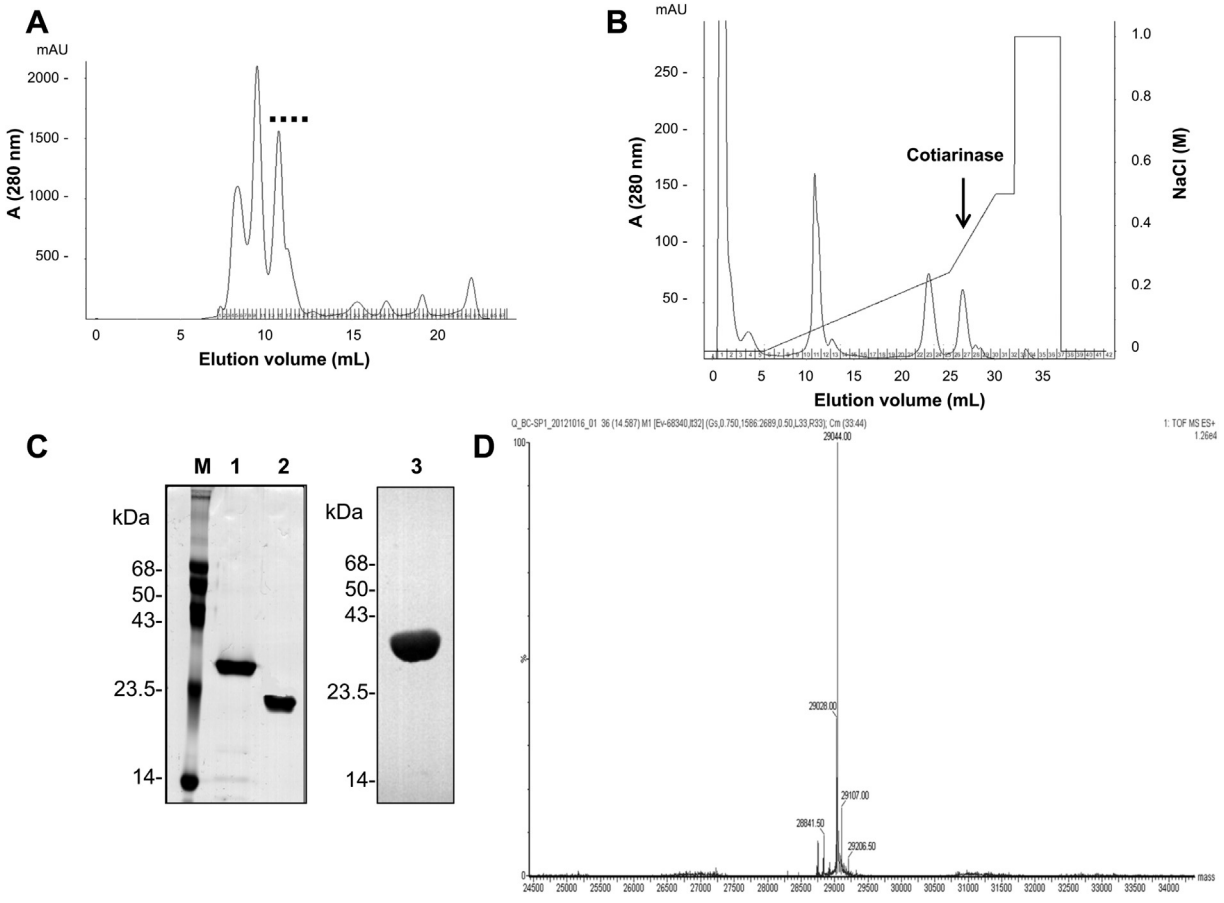

Fig. 1. Isolation of cotiarinase from *B. cotiara* venom. (A) Venom chromatography on Superdex 75 column. (B) The protein peak marked with the dashed line in (A) was submitted to chromatography on a Mono S column and eluted with a NaCl gradient. (C) SDS–PAGE profile of (1) reduced and (2) non-reduced purified cotiarinase stained with silver (2 μg), and Western blot analysis (25 μg) using and anti-serine proteinase antibody [8]. (D) Molecular mass determination of native cotiarinase (600 ng) by electrospray-ionization (ESI) mass spectrometry using a Quadrupole Time-of-Flight mass spectrometer (Q-ToF).

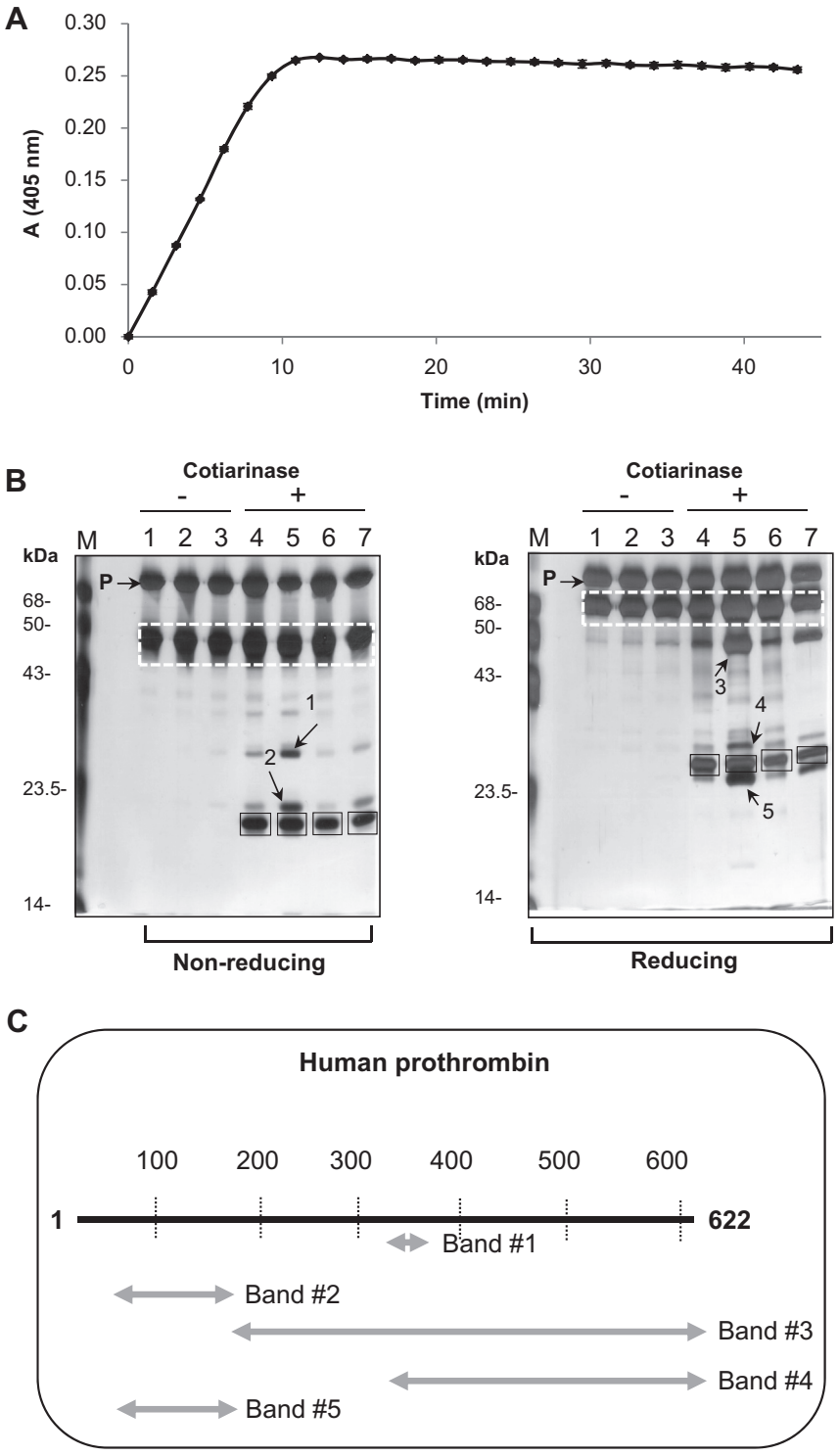

Fig. 2. Activity of cotiarinase on human prothrombin. (A) Incubation of cotiarinase (500 ng) with prothrombin (500 nM) in the presence of thrombin peptide substrate D-Phe-Pip-Arg-*p*NA (500 μM) at 37 °C and hydrolysis monitoring at 405 nm. Data are given as mean ± S.D. ($n = 4$). (B) SDS–PAGE profile of the cleavage of prothrombin by cotiarinase. Prothrombin incubated without cotiarinase for 0 h, 1 h and 4 h (lanes 1, 2 and 3, respectively) or with cotiarinase at a 1:5 enzyme-to-substrate ratio (w/w) for 1 h and 4 h (lanes 4 and 5, respectively) or at a 1:10 enzyme-to-substrate ratio (w/w) for 1 h and 4 h (lanes 6 and 7, respectively). Molecular mass markers (lane M). Rectangular black outlines indicate cotiarinase. White dashed outlines indicate the bovine serum albumin band. Letter P indicates the human prothrombin band. Arrows indicate bands that were excised for *in gel* trypsin digestion and mass spectrometric identification. (C) Identification of prothrombin cleavage products. Bands indicated in (B) were identified as pieces of prothrombin by mass spectrometry. Double-ended arrows indicate the regions to which the identified peptides belong.

cotiarinase with plasminogen (Calbiochem) and with the recombinant N-terminal exodomain of thrombin receptor PAR1 [11] at a 1:10 enzyme-to-substrate ratio (w/w), however no proteolytic activity was detected (Supplemental Fig. 1).

Basic SVSPs are much less abundant in viperid venoms than acidic ones and an interesting feature of some basic SVSPs is their ability to directly induce platelet aggregation in platelet rich plasma (PRP) [8,12–14]. Unexpectedly, cotiarinase was not able to promote

platelet aggregation using human PRP even at the concentration of 0.8 μM (data not shown) which is ~20 times higher than that at which PA-BJ, from *Bothrops jararaca*, and MSP1, from *Bothrops moojeni*, activate platelets [8,12]. Likewise, upon incubation with PRP, cotiarinase did not show any coagulant activity. On the other hand, as a typical feature of SVSPs, cotiarinase displayed amidolytic activity as detected by the hydrolysis of the chromogenic substrates D-Phe-Pip-Arg-pNA (6.7 pmol min^{-1} mg^{-1}) and D-Val-Leu-Lys-pNA (2.5 pmol min^{-1} mg^{-1}) at pH 8.0, 37 °C; however, the rate of hydrolysis of these peptides by cotiarinase was rather low in comparison to other SVSPs isolated from *Bothrops* venoms [12,15,16].

We next evaluated the ability of cotiarinase to activate prothrombin (Factor II) using a colorimetric assay. Thus, 500 ng of cotiarinase in 100 μL 20 mM Tris–HCl, 5 mM $CaCl_2$, pH8.3 reaction buffer were incubated with the chromogenic substrate D-Phe-Pip-Arg-*p*NA (500 μM, final concentration; Chromogenix) and prothrombin (500 nM, final concentration; Hyphen Biomed) at 37 °C. Under the same reaction conditions we tested the ability of cotiarinase to activate Factor X (500 nM, final concentration; Hyphen Biomed) using the chromogenic substrate D-Arg-Gly-Arg-*p*NA·(500 μM, final concentration; Chromogenix) but no enzymatic activity upon this protein was detected (not shown). Generation of active thrombin by cotiarinase upon incubation with prothrombin was mainly verified within the first 10 min, when the reaction reached its maximum of hydrolysis of D-Phe-Pip-Arg-pNA (Fig. 2A). To further evaluate the activity of cotiarinase on prothrombin, we performed another incubation of the protein with cotiarinase at 1:5 and 1:10 enzyme-to-substrate ratios (w/w) in the above described reaction buffer to which 1 mg/mL bovine serum albumin was added in order to stabilize prothrombin, for 1 h or 4 h, at 37 °C. Cotiarinase was able to cleave prothrombin and reaction products were analyzed by SDS–PAGE. Analysis of products under non-reducing conditions revealed two main protein bands (Fig. 2B) that were submitted to *in gel* trypsin digestion as described by [9] and LC-MS/MS analysis. The prothrombin identified peptides (SwissProt accession number P00734) matched to regions corresponding to thrombin (band #1) and fragment 1 (band #2) (Supplemental Fig. 2 and Table 2). Likewise, the identification of the three major hydrolysis products observed under reducing conditions (Fig. 2B) revealed fragments corresponding to prothrombin 1 (band #3), thrombin (band #4) and fragment 1 (band #5) (Supplemental Fig. 2 and Table 2). The positions of the identified peptides in the sequence of prothrombin (Fig. 2C) suggest that it underwent limited proteolysis by cotiarinase at two main sites that resulted in the generation of thrombin, as detected by the colorimetric assay (Fig. 2A). Incubation of cotiarinase for 30 min at room temperature with 5 mM phenylmethanesulfonyl fluoride (PMSF) abolished its ability to activate prothrombin indicating the involvement of the active site serine residue for this activity (Supplemental Fig. 3).

Activation of human prothrombin involves the cleavage of the activation peptide fragments by the prothrombinase complex (phospholipids, factor Va, factor Xa and Ca^{2+} ions) releasing the light and heavy chains of active thrombin [17].

SVSPs classified in groups C and D of venom prothrombin activators require as co-factors Ca^{2+} ions plus phospholipids, or, Ca^{2+} ions plus phospholipids plus factor Va, respectively, for prothrombin activation [4]. Moreover, group C and D venom prothrombin activators are structurally and functionally similar to mammalian coagulation factors and contain non-catalytic domains [4]. From *B. cotiara* venom, Senis and colleagues reported the isolation of cotiaractivase, a ~22 kDa metalloproteinase that generated active thrombin from prothrombin in a Ca^{2+}-dependent manner [18]. The present study shows that *B. cotiara* venom also contains a SVSP that activates prothrombin in the absence of the typical co-factors required for the specific cleavage of prothrombin, therefore, it does not match any group of the current classification of snake venom prothrombin activators [4]. Since SVSPs escape inhibition by plasma inhibitors, our findings identify cotiarinase as a unique agent capable of directly activating prothrombin and provide a rational foundation for the exploration of its potential therapeutic application in pathological conditions where the controlled activation of prothrombin is desirable.

Deshimaru and colleagues observed that the SVSPs genes form a multigene family, and showed that non-synonymous nucleotide substitutions have accumulated at a high rate in the mature protein-coding regions to cause amino acid changes that diversified their enzymatic activities [19]. Cotiarinase is another example of the diversification of SVSP primary structure giving rise to an enzyme showing a rather narrow substrate recognition and biological activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2013.04.006.

References

[1] S.M. Serrano, The long road of research on snake venom serine proteinases, Toxicon 62 (2013) 19–26.
[2] S.M. Serrano, R.C. Maroun, Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved, Toxicon 45 (2005) 1115–1132.
[3] R.M. Kini, Serine proteases affecting blood coagulation and fibrinolysis from snake venoms, Pathophysiol. Haemost. Thromb. 34 (2005) 200–204.
[4] R.M. Kini, The intriguing world of prothrombin activators from snake venom, Toxicon 45 (2005) 1133–1145.
[5] A.M. Fenwick, R.L. Gutberlet Jr., J.A. Evans, C.L. Parkinson, Morphological and molecular evidence for phylogeny and classification of South American pitvipers, genera *Bothrops, Bothriopsis* and *Bothrocophias* (Serpentes:Viperidae), Zool. J. Lin. Soc. 156 (2009) 617–640.
[6] A.K. Tashima, L. Sanz, A.C.M. Camargo, S.M.T. Serrano, J.J. Calvete, Snake venomics of the Brazilian pitvipers *Bothrops cotiara* and *Bothrops fonsecai*. Identification of taxonomic markers, J. Proteomics 71 (2008) 473–485.
[7] A.F.P. Leme, E.S. Kitano, M.F. Furtado, R.H. Valente, A.C.M. Camargo, P.L. Ho, J.W. Fox, S.M.T. Serrano, Analysis of the subproteomes of proteinases and heparin-binding toxins of eight Bothrops venoms, Proteomics 9 (2009) 733–745.
[8] S.M. Serrano, M.F. Matos, F.R. Mandelbaum, C.A. Sampaio, Basic proteinases from *Bothrops moojeni* (caissaca) venom–I. Isolation and activity of two serine proteinases, MSP 1 and MSP 2, on synthetic substrates and on platelet aggregation, Toxicon 31 (1993) 471–481.
[9] S.L. Hanna, N.E. Sherman, M.T. Kinter, J.B. Goldberg, Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry, Microbiology 146 (2000) 2495–2508.
[10] A. Zelanis, A.K. Tashima, M.M. Rocha, M.F. Furtado, A.C. Camargo, P.L. Ho, S.M. Serrano, Analysis of the ontogenetic variation in the venom proteome/peptidome of *Bothrops jararaca* reveals different strategies to deal with prey, J. Proteome Res. 9 (2010) 2278–2291.
[11] A. Kuliopulos, L. Covic, S.K. Seeley, P.J. Sheridan, J. Helin, C.E. Costello, Plasmin Desensitization of the PAR1 Thrombin Receptor: Kinetics, sites of truncation, and implications for thrombolytic therapy, Biochemistry 38 (1999) 4572–4585.
[12] S.M. Serrano, R. Mentele, C.A. Sampaio, E. Fink, Purification, characterization, and amino acid sequence of a serine proteinase, PA-BJ, with platelet-aggregating activity from the venom of *Bothrops jararaca*, Biochemistry 34 (1995) 7186–7193.
[13] N. Marrakchi, R.B. Zingali, H. Karoui, C. Bon, M. el Ayeb, Cerastocytin, a new thrombin-like platelet activator from the venom of the Tunisian viper *Cerastes cerastes*, Biochim. Biophys. Acta. 1244 (1995) 147–156.

[14] S. Niewiarowski, E.P. Kirby, T.M. Brudzynski, K. Stocker, Thrombocytin, a serine protease from *Bothrops atrox* venom. 2. Interaction with platelets and plasma-clotting factors, Biochemistry 18 (1979) 3570–3577.
[15] S.M.T. Serrano, Y. Hagiwara, N. Murayama, S. Higuchi, R. Mentele, C.A.M. Sampaio, A.C.M. Camargo, E. Fink, Purification and characterization of a kinin-releasing and fibrinogen-clotting serine proteinase (KN-BJ) from the venom of *Bothrops jararaca*, and molecular cloning and sequence analysis of its cDNA, Eur. J. Biochem. 251 (1998) 845–853.
[16] S.M. Serrano, C.A. Sampaio, R. Mentele, A.C. Camargo, E. Fink, A novel fibrinogen-clotting enzyme, TL-BJ, from the venom of the snake *Bothrops jararaca*: purification and characterization, Thromb. Haemost 83 (2000) 438–444.
[17] C.T. Esmon, C.M. Jackson, The conversion of prothrombin to thrombin. IV. The function of the fragment 2 region during activation in the presence of factor V, J. Biol. Chem. 249 (1974) 7791–7797.
[18] Y.A. Senis, P.Y. Kim, G.L. Fuller, A. García, S. Prabhakar, M.C. Wilkinson, H. Brittan, N. Zitzmann, R. Wait, D.A. Warrell, S.P. Watson, A.S. Kamiguti, R.D. Theakston, M.E. Nesheim, G.D. Laing, Isolation and characterization of cotiaractivase, a novel low molecular weight prothrombin activator from the venom of *Bothrops cotiara*, Biochim. Biophys. Acta 1764 (2006) 863–871.
[19] M. Deshimaru, T. Ogawa, K. Nakashima, I. Nobuhisa, T. Chijiwa, Y. Shimohigashi, Y. Fukumaki, M. Niwa, I. Yamashina, S. Hattori, M. Ohno, Accelerated evolution of crotalinae snake venom gland serine proteases, FEBS. Lett. 397 (1996) 83–88.