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Snake venom serine proteinases specificity mapping by proteomic identification of cleavage sites

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ABSTRACT

Many snake venom toxins are serine proteases but their specific in vivo targets are mostly unknown. Various act on components of the coagulation cascade, and fibrinolytic and kallikrein–kinin systems to trigger various pathological effects observed in the envenomation. Despite showing high similarity in terms of primary structure snake venom serine proteinases (SVSPs) show exquisite specificity towards macromolecular substrates. Therefore, the characterization of their peptide bond specificity is important for understanding the active site preference associated with effective proteolysis as well as for the design of peptide substrates and inhibitors. *Bothrops jararaca* contains various SVSPs among which Bothrops protease A is a specific fibrinogenolytic agent and PA-BJ is a platelet-activating enzyme. In this study we used proteome derived peptide libraries in the Proteomic Identification of protease Cleavage Sites (PICS) approach to explore the peptide bond specificity of Bothrops protease A and PA-BJ in order to determine their individual peptide cleavage sequences. A total of 371 cleavage sites (208 for Bothrops protease A and 163 for PA-BJ) were detected and both proteinases displayed a clear preference for arginine at the P1 position. Moreover, the analysis of the specificity profiles of Bothrops protease A and PA-BJ revealed subtle differences in the preferences along P6–P6′, despite a common yet unusual preference for Pro at P2. Taken

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together, these results map the subsite specificity of both SVSPs and shed light in the functional differences between these proteinases.

Biological significance
Proteolysis is key to various pathological effects observed upon envenomation by viperid snakes. The use of the Proteomic Identification of protease Cleavage Sites (PICS) approach for the easy mapping of proteinase subsite preferences at both the prime- and non-prime sides concurrently gives rise to a fresh understanding of the interaction of the snake venom serine proteinases with peptide and macromolecular substrates and indicates that their hydrolytic activity is influenced by the amino acid sequences adjacent to the scissile bond.

1. Introduction

Snake venoms, especially those of the Viperidae family, contain a variety of proteolytic enzymes especially metalloproteinases and serine proteinases, which are structurally similar to their mammalian counterparts and which trigger various pathological effects observed in the envenomation process. Snake venom serine proteinases (SVSPs) form a group of extensively studied toxins whose main known targets are the components of the hemostatic system [1]. SVSPs are classified in the clan PA, subclan PA(S), family S1 (chymotrypsin), subfamily A of proteolytic enzymes (http://merops.sanger.ac.uk/). A comparison of the exon/intron organization of the proteinase regions of serine proteinase genes, and of the organization of the gene encoding the SVSP batroxobin, indicated that the SVSPs evolved from glandular kallikrein [2], and underwent an accelerated evolution process that provided amino acid substitutions in the mature protein region to generate other functions [3]. The structure of SVSPs resembles those of chymotrypsin-like enzymes, in which the active site cleft is located at the junction of the two six stranded beta-barrels [4]. The catalytic mechanism of SVSPs includes the catalytic triad His^{57}-Asp^{102}-Ser^{195} (chymotrypsinogen numbering), in which the reactive serine residue plays a role in the formation of a transient acyl–enzyme complex which is stabilized by the presence of histidine and aspartic acid residues within the active site [5]. Although they show a high degree of mutual primary structure identity (~65%), SVSPs are quite specific toward macromolecular substrates and in many aspects they are functionally similar to endogenous enzymes of the hemostatic system as they interfere with the maintenance and regulation of the blood coagulation cascade by proteolytically cleaving specific bonds and activating proteins involved in blood coagulation, fibrinolysis, platelet aggregation and the kallikrein–kinin system [6,7].

The venom glands of the Brazilian species *Bothrops jararaca* undergoes a significant rearrangement upon newborn to adult transition in which the abundance of transcripts encoding SVSPs changes from 3.3% to 8.1% and the sub-proteomes of SVSPs show components of variable molecular masses and isoelectric points depending on the extension of their main post-translational modification, i.e. *N*-glycosylation [8,9]. *Bothrops* protease A is an acidic, thermostable SVSP isolated from the venom of *B. jararaca* [10–13]. Bothrops protease A is a potent fibrinogenolytic agent in vitro, as it readily degraded human and rat fibrinogen at a very low enzyme concentration. Indeed, in vivo, injection of 0.75 nmoles of Bothrops protease A in rats completely avoided thrombus formation induced by stasis in the vena cava or by endothelium injury in the jugular vein [14]. The highly specific activity of Bothrops protease A upon fibrinogen and the failure of plasma serine proteinase inhibitors to impair its activity are likely due to the steric hindrance imposed by its carbohydrate moieties [14].

Early studies revealed the esterase and amidase activities of Bothrops protease A on synthetic arginine substrates as well as its proteolytic activity on the B-chain of oxidized insulin where it cleaved at Arg^{22}↓Gly^{23}, Phe^{25}↓Tyr^{26} and Tyr^{26}↓Thr^{27} [11]. The platelet-aggregating enzyme PA-BJ is a basic SVSP of 30 kDa isolated from *B. jararaca* venom that resembles thrombin as it causes platelet aggregation both in platelet-rich plasma and washed platelet suspensions [15]. The G-protein coupled receptors Protease Activated Receptors (PAR) 1 and PAR4 mediate the effect of PA-BJ on platelets. PA-BJ cleaves the recombinant soluble N-terminal domain of PAR1 at Arg^{41}↓Ser^{42} and at Arg^{46}↓Asn^{47}, resulting in the inactivation of the tethered ligand. PA-BJ also causes calcium mobilization in fibroblasts transfected with PAR4 and desensitizes these cells to the action of thrombin [16]. Platelet aggregation induced by PA-BJ is inhibited by heparin and by the monoclonal antibody IIaR, which recognizes the peptide R^{35}NATLDPRSFLLRR^{46} containing thrombin's cleavage site of PAR1, but hirudin and thrombomodulin do not inhibit it [16]. Even though Bothrops protease A and PA-BJ show ~60% amino acid sequence identity, they differ strongly in terms of molecular mass, isoelectric point and macromolecular substrate specificity. As for the substrate specificity, they act as trypsin-like enzymes and cleave peptide bonds following Arg or Lys at P1 position. However, the hydrolytic activity of SVSPs toward peptide substrates is also strongly influenced by the peptide moiety adjacent to the scissile bond [1].

Although a number of SVSPs have been characterized so far, little is known about their cleavage site specificity and the residues surrounding the scissile bond. Active site specificity provides insights into the mechanisms that underlie their selectivity toward distinct macromolecular substrates. This knowledge is also crucial for the rational design of molecular tools such as selective inhibitors that offer potential as new drugs for treatment of the acute phase after snake bite injury or specific activity based probes for molecular pathological investigations. However, most of the studies on SVSPs were based on the use of a few chromogenic/fluorogenic substrates, which in part mimic the short range interactions occurring between a few enzyme subsites and the substrate upon the formation of the enzyme–substrate complex. Although this is a relatively straight-forward and simple strategy for the evaluation of the selectivity of proteinases, it does not allow