Biochimica et Biophysica Acta 1804 (2010) 1937–1942

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Autolysis at the disintegrin domain of patagonfibrase, a metalloproteinase from *Philodryas patagoniensis* (Patagonia Green Racer; Dipsadidae) venom

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ARTICLE INFO

Article history:
Received 11 April 2010
Received in revised form 27 May 2010
Accepted 1 June 2010
Available online 9 June 2010

Keywords:
Autodegradation
Colubridae
Metalloproteinase

ABSTRACT

Patagonfibrase is a 57.5-kDa hemorrhagic metalloproteinase isolated from the venom of *Philodryas patagoniensis* (Patagonia Green Racer), a South American rear-fanged snake. Herein we demonstrate that patagonfibrase undergoes autolysis at its pH optimum (7.5) and at 37 °C, primarily producing a ~32.6 kDa fragment composed of disintegrin-like and cysteine-rich domains, as identified by mass spectrometry and N-terminal sequencing. The autolysis site for production of this fragment is similar to that observed for metalloproteinases from front-fanged Viperidae snake venoms. In the presence of Ca^{2+}, patagonfibrase was only partially autolysed, giving rise mainly to one fragment of ~52.2 kDa. In addition, calcium markedly enhanced the azocaseinolytic activity of patagonfibrase. Our findings contribute to the understanding of the structural and mechanistic bases of this family of metalloenzymes that are widely distributed among snake venoms, demonstrating that important post-translational modifications such as proteolysis can also contribute to the diversity and complexity of proteins found in rear-fanged snake venoms.

1. Introduction

Snake venoms are complex mixtures of proteins, peptides and small organic molecules with a variety of potent enzymatic and ligand-based biological activities [1]. Among the enzyme-based toxins, an important class includes the snake venom metalloproteinases (SVMPs) which act synergistically with many other toxins to induce a complex series of local and systemic pathophysiological effects upon envenomation [2]. SVMPs are members of the reprolysin subfamily of the M12 family of metalloproteinases [3], and they can be divided into three classes (P-I to P-III) that minimally share homologous (P-I) proteinase domains; classes P-II and P-III also contain a disintegrin domain. In the class P-III metalloproteinases, there is an additional cysteine-rich carboxyl terminal domain [4,5]. This third class of SVMPs is particularly interesting, in terms of the diversity of structural features and biological activities associated with this class [3], and most P-III SVMPs produce severe hemorrhagic effects.

Some P-III metalloproteinases isolated from venoms of front-fanged viperid snakes are known to undergo autolysis under several conditions, giving rise mainly to an approximately 30-kDa stable fragment composed of a disintegrin-like domain and a cysteine-rich domain [4,6–10]. Based on this autodegradation property, these metalloproteinases are classified in the P-IIIb subfamily [4,5]. The proteolytically processed form, although lacking the metalloproteinase domain, is a potent inhibitor of collagen-stimulated platelet aggregation [7]. Further, P-III SVMPs are major components of rear-fanged snake venoms and account for several of the main toxic effects observed following envenomation [11,12]. However, to date, there are few studies on the primary structure of P-III SVMPs found in rear-fanged snake venoms. Moreover, there are no published studies on their autocatalytic processing, either *in vivo* or *in vitro*.

Patagonfibrase is a 57.5-kDa metalloproteinase isolated from the venom of *Philodryas patagoniensis* (Patagonia Green Racer), a South American rear-fanged snake which is now considered a member of the family Dipsadidae [13]. This enzyme, among other activities, induces hemorrhage and inhibits platelet aggregation [14]. It is the only metalloproteinase isolated to date in native form from a rear-fanged snake venom, and it exhibits biochemical features and biological activities similar to those of P-III metalloproteinases from Viperidae venoms [14]. In this study, we evaluated whether patagonfibrase undergoes autolysis under varying experimental conditions, and using mass spectrometric analysis and N-terminal sequencing of the native

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doi:10.1016/j.bbapap.2010.06.005

enzyme and its main autoproteolytic fragment, we demonstrated that it is subjected to specific autoproteolytic cleavage at the initiation of the disintegrin domain.

2. Materials and methods

2.1. Purification of patagonfibrase

Patagonfibrase from *P. patagoniensis* venom was purified using a previously described procedure [14].

2.2. Autolysis assay of patagonfibrase

Patagonfibrase (0.5 mg/mL) was incubated at 4 °C or 37 °C in 50 mM Tris–HCl buffer, pH 7.5 (its optimum pH, [14]), for different time intervals (0, 5, 15, 30, 60 and 120 min; 18 h), or under other conditions such as in the absence or presence of Na_2EDTA, 1,10-phenanthroline, $CaCl_2$. The enzyme without pre-incubation was used as a control. The reaction was terminated by the addition of SDS-PAGE sample buffer, with or without 2-mercaptoethanol. Autolysis was visualized by SDS-PAGE (12% running gel), following the method of Laemmli [15] and then silver stained [16].

2.3. Residual proteolytic activity of patagonfibrase

Patagonfibrase (30 μg/mL) was incubated as described above and the residual protease activity was measured at 37 °C using azocasein as a substrate [14]. The enzyme without pre-incubation was used as a control.

2.4. N-terminal sequence

Samples of patagonfibrase and its main autoproteolytic fragment were subjected to Edman degradation analysis on either a Shimadzu protein/peptide sequencer (model PPSQ-21) (Instituto Butantan) or an Applied BioSystems Procise sequencer (University of Nebraska Omaha Protein Structure Core Facility) to determine N-terminal amino acid sequences.

2.5. LC-MS/MS analysis

Protein bands were excised and in-gel chymotrypsin and trypsin digestion was performed according to Hanna et al. [17]. An aliquot (4.5 μl) of the peptide mixture was separated by C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-ToF Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. The gradient was 0–80% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in the "top three" mode, in which one MS spectrum is acquired followed by MS/MS of the top three most intense peaks detected. The resulting spectra were processed using Mascot Distiller 2.2.1.0, 2008, Matrix Science (MassLynx V4.1) and searched against the *Philodryas olfersii* cluster database (7710 sequences 2009.09.04) [11] and/or non-redundant protein database restricted to snakes (NCBInr 2009.07.20, 17420 sequences) using the search engine MASCOT v.2.0 (Matrix Science Ltd.), with carbamidomethylation as a fixed modification, oxidation of methionine as a variable modification, one chymotrypsin or trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions.

2.6. Statistical analysis

The results of azocaseinolytic activity were expressed as mean ± standard deviation (SD). Differences between groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's test. Statistical analyses were performed using the software InfoStat/Professional, version 1.1. A value of $P<0.05$ indicated statistical significance.

3. Results and discussion

Patagonfibrase, a metalloproteinase found in *P. patagoniensis* venom [14], underwent autolysis *in vitro* at 37 °C and its optimum pH (Fig. 1A), but not at 4 °C even after incubation for 18 h (data not shown). In the absence of Ca^{2+} ions, the electrophoretic migration pattern of samples (under reducing conditions) showed a progressive decreasing intensity of the patagonfibrase band (57.5 kDa) and an increase in the staining density of bands below 45 kDa over incubation (Fig. 1A). The main degradation product appeared as a

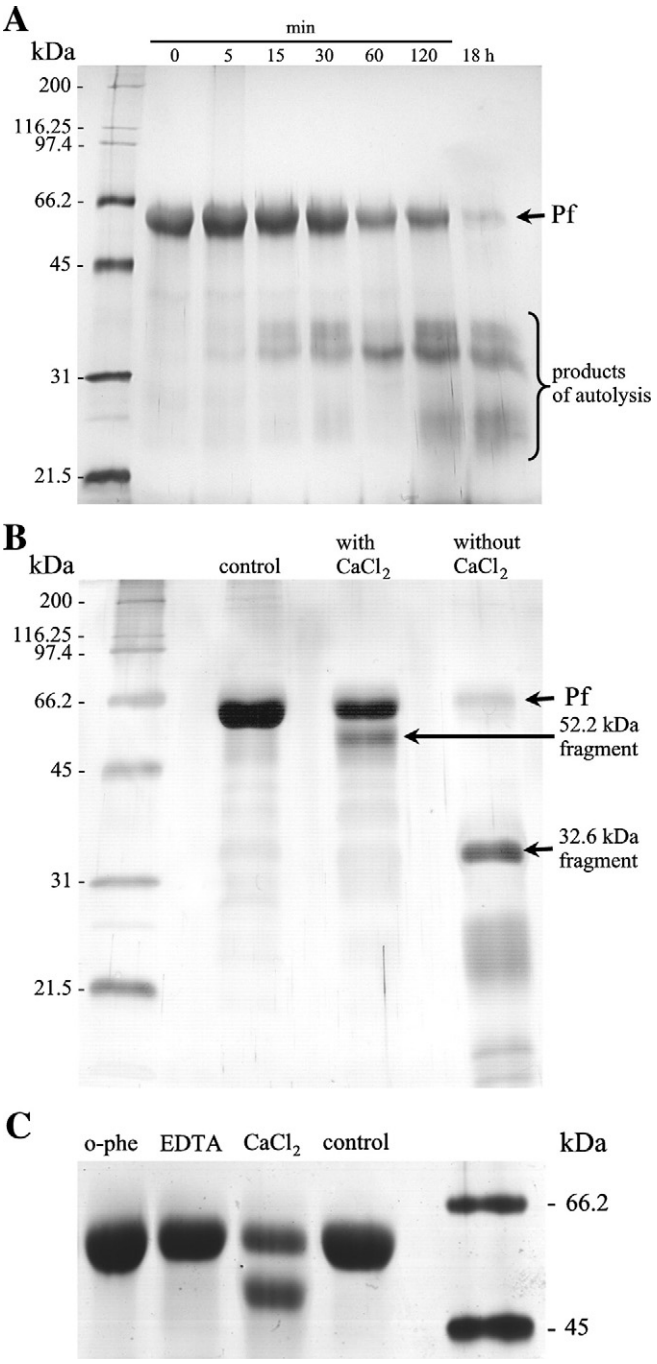

Fig. 1. SDS-PAGE analysis of the autolysis of patagonfibrase (Pf, 2 μg) at 37 °C at pH 7.5. Samples were run under reducing conditions. (A) Patagonfibrase was incubated for different time intervals (0–18 h) in the absence of calcium ions. (B) Patagonfibrase was incubated for 18 h in the presence or absence of 1 mM $CaCl_2$. (C) Patagonfibrase was incubated for 18 h in the presence of 1 mM $CaCl_2$, 1 mM Na_2EDTA or 1 mM 1,10-phenanthroline. Gels were silver-stained.

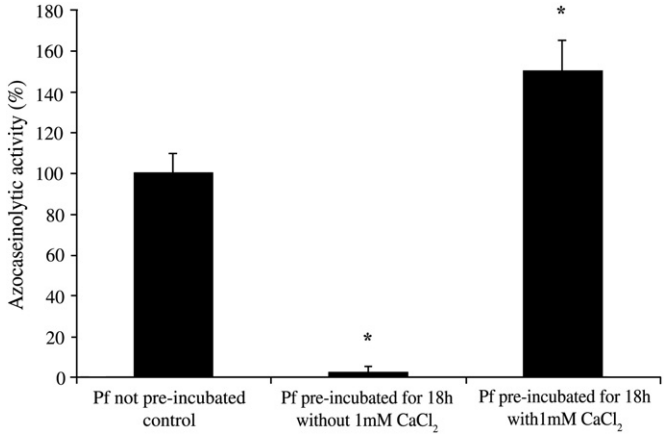

Fig. 2. Residual azocaseinolytic activity of patagonfibrase (Pf, 30 μg/mL) after pre-incubation at 37 °C for 18 h in the presence or absence of 1 mM $CaCl_2$. Asterisks indicate statistically significant differences in comparison with control ($P<0.01$).

band of ~32.6 kDa (Fig. 1B), whose molecular mass is compatible with disintegrin-like/cysteine-rich domains [18]. This fragment is not observed when non-reduced samples were submitted to SDS-PAGE (data not shown), indicating that it is still connected by disulfide bonds to the parent structure. Effective inhibitors of metalloproteinases, either 1 mM Na_2EDTA or 1 mM 1,10-phenanthroline, significantly abrogated autoproteolysis (Fig. 1C), suggesting that divalent-ion chelators promoted a structural modification of patagonfibrase, so that it lost its autoproteolytic activity. Consistent with observations from other studies on P-III type SVMPs [4,9,10,19], patagonfibrase was almost totally autolysed and failed to hydrolyze azocasein after 18 h of incubation (Fig. 2). Thus, based on its mass, sequence and autoproteolytic processing, patagonfibrase is hypothesized to belong to the P-IIIb subclass of SVMPs [4,5].

Autodegradation of metalloproteinases is markedly suppressed by the addition of Ca^{2+} ions [9,20]. Similarly, in the presence of 1 mM $CaCl_2$, patagonfibrase was only partially autolysed, even after 18 h of incubation, giving rise mainly to one fragment of ~52.2 kDa under reducing (Fig. 1B and C) or non-reducing conditions (data not shown). These findings indicate that the 52.2-kDa fragment was excised/completely removed from the parent structure. In addition, preincubation of patagonfibrase at 37 °C for 18 h in the presence of 1 mM $CaCl_2$ caused an approximately 50% increase in its azocaseinolytic activity (Fig. 2), which is in accordance with a previous report [14]. Calcium ions are important for structural integrity of SVMPs found in venoms from different species of snakes [7,21]. Calcium ions in the SVMP multidomain

Table 1
Identification of proteins obtained from Pf and its 32.6-kDa autoproteolytic fragment by LC-MS/MS analysis.

Source	Matched protein class GenBank accession/Cluster number Organism	Peptide sequence, m/z[a], z[b]
In-gel chymotrypsin digestion of Pf	Metalloprotease POLF0061C *Philodryas olfersii* (Serpentes: Dipsadidae*)	FIAVDNGMF, 507.2510, +2 ITSIDFDGPTVGLGY, 777.8716, +2 CTQGNGMPCIVRY, 778.3221, +2 NGKCPTLESQCIAL, 795.8447, +2 QMTSKICNISSPYL, 829.3574, +2 QLANSKMAPF, 561.7834, +2
	Metalloprotease POLF0059C *Philodryas olfersii* (Serpentes: Dipsadidae*)	GECCEQCRF, 623.2043, +2 STDIVAPPVCGNNF, 745.8280, +2 RMHCGRYLERY, 778.8613,+2
	Metalloprotease POLF1164S *Philodryas olfersii* (Serpentes: Dipsadidae*)	QVSLQHPQW, 561.7834, +2
	Metalloprotease POLF0006C *Philodryas olfersii* (Serpentes: Dipsadidae*)	QIWSGSF, 412.7318, +2KAARPY, 353.2421, +2
	Metalloprotease POLF0125C *Philodryas olfersii* (Serpentes: Dipsadidae*)	KKNIKPI, 420.7474, +2
	Group III snake venom metalloproteinase gi|83523634 *Echis ocellatus* (Serpentes: Viperidae)	GECCEQCRF, 623.2043, +2
In-gel chymotrypsin digestion of the 32.6-kDa autoproteolytic fragment	Metalloprotease POLF0059C *Philodryas olfersii* (Serpentes: Dipsadidae*)	QITPKLQSSL, 557.8043, +2 GECCEQCRF, 623.2283, +2
	Metalloprotease POLF1164S *Philodryas olfersii* (Serpentes: Dipsadidae*)	QVSLQHPQW, 561.7692, +2
	Group III snake venom metalloproteinase gi|83523634 *Echis ocellatus* (Serpentes: Viperidae)	GECCEQCRF, 623.2283, +2
In-gel trypsin digestion of Pf	Full-Hemorrhagic metalloproteinase-disintegrin kaouthiagin gi|32469675 *Naja kaouthia* (Serpentes: Elapidae)	QTVLLPR, 413.7634, +2
	Metalloproteinase precursor gi|118151738 *Demansia vestigiata* (Serpentes: Elapidae)	KTVLLPR, 413.7634, +2
In-gel trypsin digestion of the 32.6-kDa autoproteolytic fragment	Metalloprotease POLF0061C *Philodryas olfersii* (Serpentes: Dipsadidae*)	LFCTQGNGMPCIVR, 826.8716, +2 YFPNNPDDGMVEHGTK, 607.5903, +3 DGHPCQNNQGYCYNGK, 637.9294, +3 YFPNNPDDGMVEHGTKCGDGK, 585.2657, +4 APKHDCDMAENCNGQSAECPADRPQR, 754.3209, +4

* former Colubridae.
[a] m/z = mass to charge ratio.
[b] z = charge.

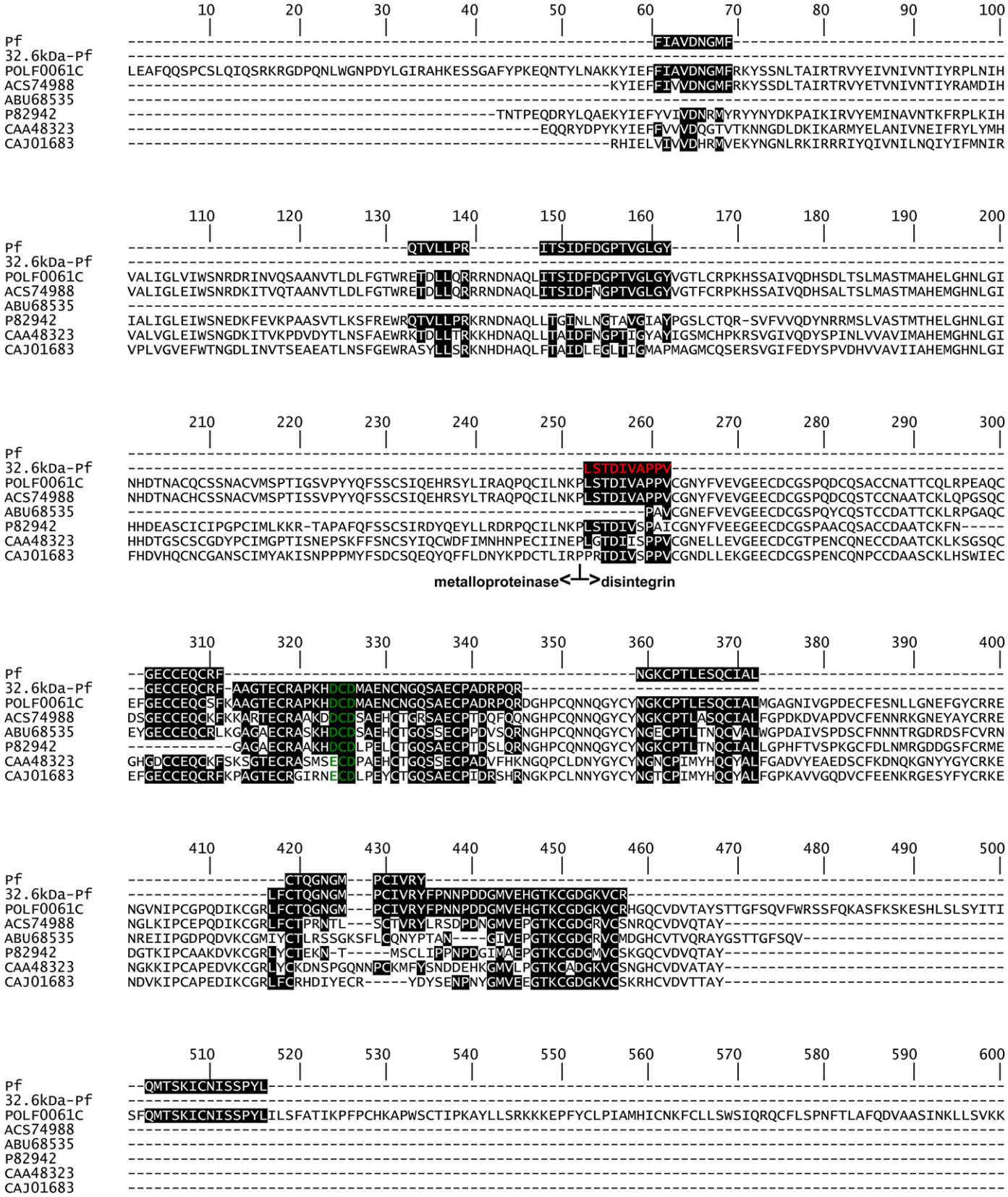

Fig. 3. Sequence alignment of the N-terminus of the 32.6-kDa autoproteolytic fragment of patagonfibrase (red), and peptides sequenced by LC-MS/MS (see Table 1) from patagonfibrase and its 32.6-kDa autoproteolytic fragment, with other P-III SVMPs. Protein sequences were aligned using the program ClustalW [34]. The numbers indicate the amino acid residues of a metalloprotease from *P. olfersii*, POLF0061C [11]. The other SVMPs were referenced by their GenBank accession numbers: ACS74988, from *P. olfersii* (Dipsadidae); ABU68535, from *Thrasops jacksoni* (Colubridae); P82942, kaouthiagin, from *Naja kaouthia* (Elapidae); CAA48323, jararhagin, from *Bothrops jararaca* (Viperidae); and CAJ01683, from *Echis ocellatus* (Viperidae). Identical residues are boxed in black. The boundary between the metalloproteinase and disintegrin domains of SVMPs is indicated, and disintegrin-like sequences are shown in green.

parent structure play an important role in stabilizing and tightening the segment connecting the proteolytic domain with the succeeding disintegrin domain [19,22]. As shown here, patagonfibrase structure is partially stabilized by Ca^{2+}, since there is a proteolysis product at 52.2 kDa in the presence of this ion. This fragment likely represents patagonfibrase processed to release a portion of the amino-terminal region of the metalloproteinase domain, as already suggested for a 43-kDa proteolysis product of jararhagin [7]. In addition, Ca^{2+} might play a role for patagonfibrase similar to that it plays for acutolysin D, since the presence of 1 mM Ca^{2+} in incubation medium markedly enhanced the azocaseinolytic activity of patagonfibrase. In the proteolytic assay, casein–which is usually in a higher concentration than that of the enzyme–likely binds Ca^{2+} [21], resulting in the dissociation of Ca^{2+} ions from patagonfibrase. This dissociation can be inhibited by the presence of increased concentrations of free Ca^{2+} in the medium, explaining the increase in proteolytic activity of patagonfibrase compared to the control.

Silver-stained bands of patagonfibrase and its 32.6-kDa autoproteolytic fragment were excised from SDS-PAGE, digested with either chymotrypsin or trypsin and analyzed by LC-MS/MS. After analysis using the Mascot search engine, different 13 chymotryptic and 2 tryptic peptide fragments from patagonfibrase, and 3 chymotryptic and 5 tryptic peptide fragments from the 32.6-kDa autoproteolytic fragment were identified (Table 1). The data indicate that these fragments show homology with several P-III SVMPs, and in particular, patagonfibrase shares a high degree of identity primary structure with metalloproteinases from *P. olfersii* venom [11]. In addition, 5 distinct tryptic peptides obtained from the 32.6-kDa autoproteolytic fragment showed identity with the disintegrin-like/cysteine-rich domains [11,23] of metalloproteinases from rear-fanged snake venoms (Fig. 3). A BLAST search with these sequences as queries revealed high similarity to other SVMPs, primarily of type III, from viperid, elapid and "colubrid" venoms (Fig. 3).

To determine the NH_2-terminal amino acid sequence, patagonfibrase and its 32.6-kDa autoproteolytic fragment were blotted to PDVF membrane and subjected to protein sequencing. Attempts to obtain NH_2-terminal sequence information from purified patagonfibrase were unsuccessful. Thus, patagonfibrase likely had its N-terminus blocked, like other viperid SVMPs, which commonly have a cyclized glutamate residue [24] in their first amino acid residue. The NH_2-terminal sequence of the 32.6-kDa fragment of patagonfibrase was highly homologous with the region at the boundary between the metalloproteinase and disintegrin domains of P-III SVMPs, and it showed complete identity with this region from the *P. olfersii* SVMPs (Fig. 3).

At present, no processed proteinase domain from a PIIIb-SVMP has been isolated from viperid crude venoms, whereas several disintegrin-like/cysteine-rich domain products have been isolated from these venoms [4,25]; examples of this include jararhagin C, alternagin C, and catrocollastatin C found in the venoms of *B. jararaca*, *B. alternatus* and *Crotalus atrox*, respectively [26–28]. Furthermore, Moura-da-Silva et al. [7] have shown that during the biosynthesis of jararhagin in the venom gland, at least three forms are present: one form which is rapidly processed to give rise to jararhagin-C, one which is resistant to processing in the venom and autolysis *in vitro*, and one minor form which is susceptible to autolysis under conditions that promote destabilization of its structure. As shown in this work, the entire structure of patagonfibrase undergoes autolysis *in vitro* in the absence of Ca^{2+}. In the presence of this ion, only a fraction of patagonfibrase undergoes autolysis, but it does not produce a disintegrin-like/cysteine-rich domain. Therefore, the existence of patagonfibrase isoforms in *P. patagoniensis* venom is also possible, and a previous mass spectrometry analysis indicated proteins with masses similar to patagonfibrase [29]. However, the similarity (or lack thereof) of these isoforms to those from jararhagin is currently unknown. In addition, whether patagonfibrase can also undergo proteolytic processing *in vivo* to produce an unstable proteinase domain and a stable disintegrin-like/cysteine-rich domain is still unclear. In contrast with observed autolysis of purified venom components, the stored and secreted venom is exceptionally stable under many different conditions [30]. In viperids, numerous mechanisms within the gland, including acidification, low affinity inhibition via peptide inhibitors and inhibition via citrate [31–33], act to stabilize and inhibit metalloproteinases and other venom enzymes. At present, it is unknown whether rear-fanged snake venom glands also possess similar stabilizing mechanisms.

In conclusion, this work demonstrates for the first time the autoproteolytic processing of a rear-fanged SVMP, contributing to a better understanding of the structural and mechanistic bases of this class of enzymes that are widely distributed among snake venoms. Accordingly, autolysis likely contributes to a significant amount of both the structural and functional complexity of *P. patagoniensis* venom. Understanding this complexity and its generation will allow us to understand better the action of venom constituents and help to develop more efficacious therapeutic interventions following envenomation.

Acknowledgments

We thank Dr. Laura Rey for her assistance with snakes. We are indebted to Dr. Inácio L.M. Junqueira-de-Azevedo, from Instituto Butantan, for sharing the EST sequences from his *Philodryas olfersii* cDNA library with us. Financial support was provided by FAPESP (08/02996-8) and Fundação Butantan from Brazil, and CONICET (PIP 114-200801-00088) from Argentina.

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