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Purification and characterization of a cysteine-rich secretory protein from *Philodryas patagoniensis* snake venom

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ABSTRACT

Cysteine-rich secretory proteins (CRiSPs) are widespread in reptile venoms, but most have functions that remain unknown. In the present study we describe the purification and characterization of a CRiSP (patagonin) from the venom of the rear-fanged snake *Philodryas patagoniensis*, and demonstrate its biological activity. Patagonin is a single-chain protein, exhibiting a molecular mass of 24,858.6 Da, whose NH_2-terminal and MS/MS-derived sequences are nearly identical to other snake venom CRiSPs. The purified protein hydrolyzed neither azocasein nor fibrinogen, and it could induce no edema, hemorrhage or inhibition of platelet adhesion and aggregation. In addition, patagonin did not inhibit contractions of rat aortic smooth muscle induced by high K^+. However, it caused muscular damage to murine gastrocnemius muscle, an action that has not been previously described for any snake venom CRiSPs. Thus, patagonin will be important for studies of the structure-function and evolutionary relationships of this family of proteins that are widely distributed among snake venoms.

1. Introduction

Cysteine-rich secretory proteins (CRiSPs) encompass a growing family of proteins commonly found in venoms of lizards and snakes (Mackessy, 2002; Yamazaki and Morita, 2004), and they are related to proteins present in the mammalian male reproductive tract. They are secreted single-chain proteins with molecular masses of about 20–30 kDa, and exhibit homologous amino acid sequences containing 16 highly conserved cysteine residues, which form 8 disulfide bonds (Guo et al., 2005). Remarkably, 10 of these cysteines are clustered in the C-terminal third of the polypeptide chain, the cysteine-rich domain and the "hinge" region. However, the NH_2-terminus sequence is more highly conserved as compared to other regions of these proteins (Yamazaki and Morita, 2004; Osipov et al., 2005).

The first discovered CRiSP was the acidic epididymal glycoprotein in rats in 1981 (Kierszenbaum et al., 1981), and since then many CRiSPs have been sequenced and characterized. Recent studies have revealed that CRiSPs are widespread in snake venoms from all five continents, including those of front-fanged and rear-fanged snake families (Yamazaki et al., 2003; Vidal et al., 2007), suggesting that their inclusion into the venom proteome occurred early in snake evolution (Fry et al., 2003; Fry and Wuster, 2004). However, biological activities have not yet been demonstrated for most of these proteins.

Several snake venom CRiSPs – such as ablomin from *Gloydius blomhoffi* (Viperidae), triflin from *Protobothrops flavoviridis* (Viperidae), and latisemin from *Laticauda semifasciata* (Elapidae) – inhibit depolarization-induced contraction of rat tail arterial smooth muscle, similar to L-type Ca^{2+} channel blockers (Yamazaki et al., 2002b). Others, such as pseudechetoxin and pseudecin (from the venoms of the elapid snakes *Pseudechis australis* and *P. porphyriacus*, respectively), are cyclic nucleotide-gated ion channel-blocking toxins (Brown et al., 1999; Yamazaki et al., 2002a). In addition, piscivorin, ophanin and catrin-2, from the venoms of *Agkistrodon piscivorus piscivorus* (Viperidae), *Ophiophagus hannah* (Elapidae) and *Crotalus atrox* (Viperidae), respectively, have also been reported to present a small but significant inhibition of smooth muscle contraction evoked by high K^+ (Yamazaki et al., 2003). Recently, natrin from *Naja atra* (Elapidae) venom has been demonstrated to block ion channel currents of the high-conductance calcium-activated and voltage-gated potassium channels (BK_{Ca} and Kv1.3) (Wang et al., 2005; Wang et al., 2006). However, the functions and even the molecular targets of most snake venom CRiSPs, particularly those from rear-fanged snakes, remain to be determined. Tigrin, from the venom of *Rhabdophis tigrinus tigrinus*, is the only CRiSP isolated in native form from a rear-fanged snake venom (Yamazaki et al., 2002b), but its biological activity is still unknown. In the present study we describe the purification, biochemical characterization and biological activity on

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mammalian skeletal muscle of a CRiSP from the venom of *Philodryas patagoniensis* (patagonin), an opisthoglyphous snake widespread in South America.

2. Materials and methods

2.1. Materials

Philodryas patagoniensis venom solution was prepared as previously described (Peichoto et al., 2007). BCA (bicinchoninic acid) protein assay kit, BSA (bovine serum albumin), collagen, bovine thrombin, ADP (adenosine 5'-diphosphate), ristocetin, divalent cation ionophore A23187, azocasein, azocoll and α-cyano-4-hydroxycinnamic acid were obtained from Sigma-Aldrich Chemical. Molecular mass markers and sequencing grade side-chain protected porcine trypsin were obtained from Bio-Rad and Promega, respectively. All other chemicals were of analytical reagent grade or better.

2.2. Animals

In order to determine hemorrhagic and edematogenic activities of patagonin, male Swiss mice (*Mus musculus*) (18–22 g) were used. To determine the effect of patagonin on smooth muscle contractions induced by high K^+, male Wistar rats (200–250 g) were used. These animals were supplied by the Animal House of Butantan Institute, Brazil. To determine local and systemic damage induced by patagonin, male CF-1 mice (18–20 g), supplied by the Animal House of Faculty of Veterinary Sciences, Northeastern National University, Argentina, were used. All experiments followed the ethical standards for animal experiments in toxinological research recommended by the International Society of Toxinology (Meier et al., 1993), and they were approved by the Ethical Committee for the Use of Animals at Butantan Institute (process 530/08).

2.3. Purification of patagonin

Venom was fractionated on a Mono-Q column as described previously (Peichoto et al., 2007). Basic fractions were pooled, dialyzed, and brought to 1 M $(NH_4)_2SO_4$ prior to being applied to an HiTrap Phenyl HP column (1 mL) (GE Healthcare, Sweden) equilibrated with 50 mM Tris–HCl, pH 7.4, 1 M $(NH_4)_2SO_4$, using an FPLC system. After washing the column, bound proteins were eluted with a decreasing linear gradient of 1 to 0 M $(NH_4)_2SO_4$. Protein concentration was monitored by measuring the absorbance at 280 nm. One mL fractions were collected, and SDS-PAGE was performed on selected fractions.

2.4. Protein quantification

Protein concentration of samples was determined by BCA protein assay, using BSA as standard (Smith et al., 1985).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoresed on 12% polyacrylamide slab gels following the method of Laemmli (1970) and then silver stained (Blum et al., 1987).

2.6. Mass spectrometry

In order to determine the molecular mass of patagonin, approximately 0.5 μg protein in 50% acetonitrile was spotted onto sinapinic acid matrix and allowed to dry. Mass spectra were obtained using an Applied Biosystems Voyager DE Pro MALDI-TOF mass spectrometer, operating in delayed extraction and linear mode (Mackessy et al., 2006).

For protein identification, protein bands of interest were excised from a silver-stained SDS-PAGE gel, destained and subjected to reduction with DTT, alkylation with iodoacetamide, and then in-gel digestion with sequencing grade side-chain protected porcine trypsin using a ProGest digestor (Genomic Solutions), following the manufacturer's instructions. The tryptic peptide mixtures were dried in a Speed-Vac and redissolved in 5 μL of 70% acetonitrile and 0.1% TFA (trifluoroacetic acid). Digests (0.65 μL) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA, dried, and analyzed with a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer. Singly-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were sequenced by collision-induced dissociation tandem mass spectrometry. Spectra were interpreted using the on-line form of the MASCOT program at http://www.matrixscience.com.

2.7. NH2-terminal sequence analysis

The NH_2-terminal amino acid sequence of patagonin was determined by automated Edman degradation using an Applied BioSystems Procise sequencer at the Protein Structure Core Facility, University of Nebraska Omaha Medical Center (USA).

Homologous protein sequences were searched using the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al., 1997). Multiple sequence alignment was carried out using the ExPASy proteomics tools (http://www.expasy.org/tools/), in FASTA format (Pearson and Lipman, 1988). Amino acid sequences were aligned using the T-Coffee software (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi) (Notredame et al., 2000).

2.8. Protease activities

Potential cleavage of bovine fibrinogen (Biggs, 1976) by patagonin was determined by SDS-PAGE using 12% polyacrylamide gels (Peichoto et al., 2007). Azocasein (Wang and Huang, 2002) and azocoll (Váchová and Moravcová, 1993) assays were also used to determine proteolytic activity of patagonin.

2.9. Biological activities

The hemorrhagic (Peichoto et al., 2007) and edema-inducing (Lomonte et al., 1993) activities of patagonin were evaluated as described elsewhere. The effect of patagonin on the aggregation of washed human platelets or PRP (platelet-rich plasma) was tested as described previously (Santoro et al., 1999), using a Chrono-log lumiaggregometer (model 560VS). Platelet adhesion-inhibiting activity was measured by a microtiter-plate assay (Bellavite et al., 1994). The local and systemic effects induced by patagonin in mice were determined according to a method previously reported (Peichoto et al., 2007). Rat aortic smooth muscle contraction experiments were performed using a modification of a previously described method (Hooker et al., 1977). Briefly, animals were killed by decapitation. Thoracic aorta was rapidly dissected and placed in Krebs-bicarbonate solution (129.87 mM NaCl, 4.69 mM KCl, 1.56 mM $CaCl_2$, 1.17 mM KH_2PO_4, 1.17 mM $MgSO_4$, 14.87 mM $NaHCO_3$, 5.5 mM glucose, pH 7.4). Adhering perivascular tissue was carefully removed, a 4 mm ring was cut and the endothelium was removed with a wooden stick. The vessel was mounted onto two thin stainless steel holders in a 5 mL organ bath containing Krebs-bicarbonate solution, at 37 °C, continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2. A resting tension of 2 g was applied on the ring. A depolarizing solution, made by the replacement of NaCl with equimolar KCl (60 mM, final concentration, high K^+ solution), was used to induce smooth muscle contraction. The isometric tension was recorded with

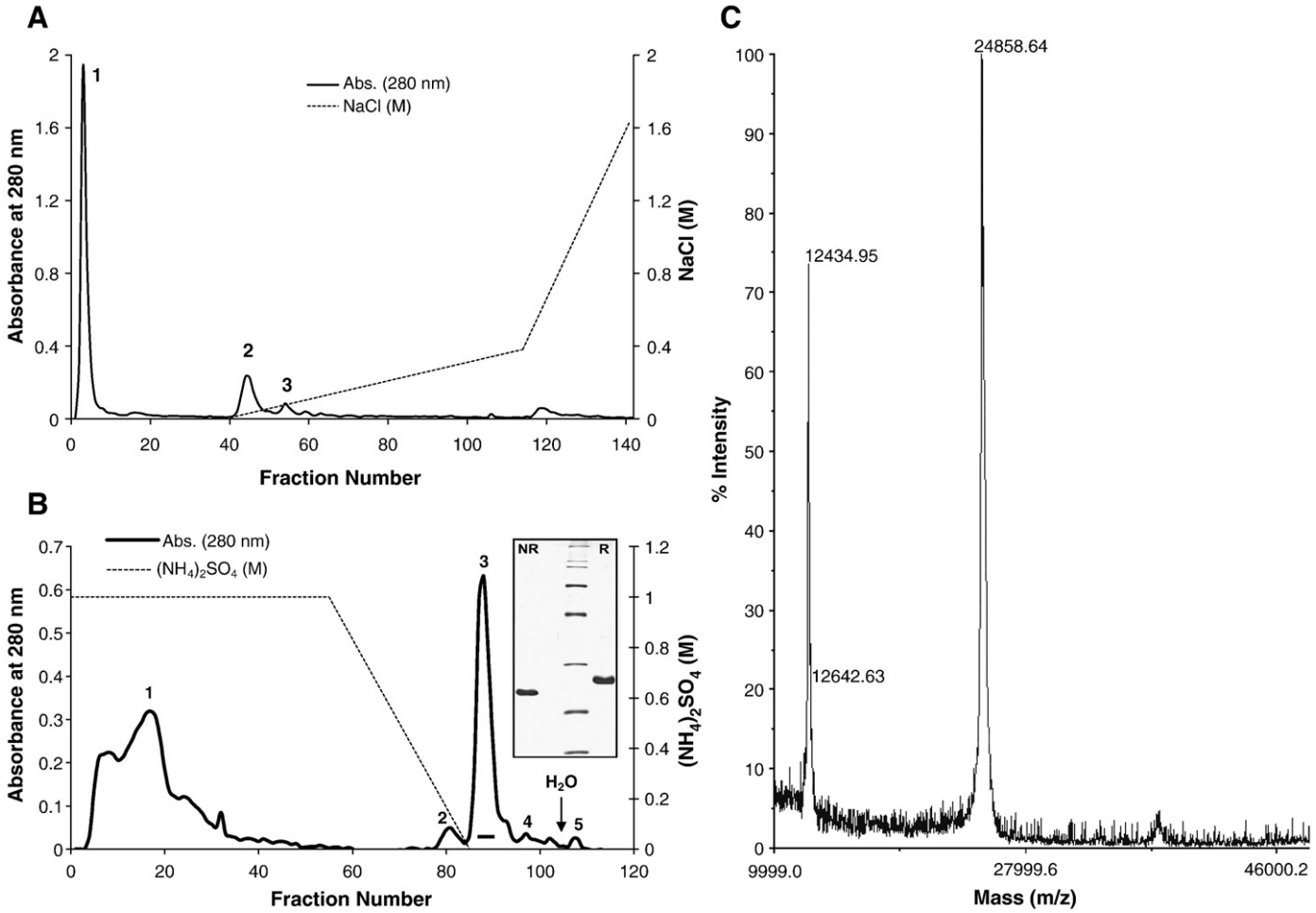

Fig. 1. Purification of patagonin from *P. patagoniensis* venom. A. Anion-exchange chromatography of *P. patagoniensis* venom on a Mono-Q column. B. Hydrophobic interaction chromatography on a HiTrap Phenyl HP column of the basic pool (peak 1) from the Mono-Q step. Fractions (1 mL) were collected and pooled as indicated by the horizontal bar. Insert shows SDS-PAGE of the purified protein (1.5 μg) on 12% gel. NR: non-reducing conditions; R: reducing conditions. Relative molecular mass of marker proteins (from top to bottom): 200,000; 116,250; 97,400; 66,200; 45,000; 31,000; 21,500 and 14,400 Da. Gel was silver stained. C. MALDI-TOF mass spectrum of intact patagonin. The mass of this protein is 24,858.6 Da, and the peak at 12,434.9 represents the doubly-charged ion.

a force transductor (Ampère, Brazil) connected to a recording system (ECB, Brazil).

2.10. Statistical analysis

Where appropriate, the results 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

3.1. Purification of patagonin

When *P. patagoniensis* venom was fractionated on a Mono-Q column (Peichoto et al., 2007), patagonin was eluted in peak 1 (Fig. 1-A). These fractions were pooled, desalted, brought to 1 M $(NH_4)_2SO_4$, filtered and chromatographed on a HiTrap Phenyl HP column (Fig. 1-B). Peak 3 from this chromatographic step was homogeneous by SDS-PAGE (insert, Fig. 1-B), and the protein present in this peak was further investigated. The relative molecular mass of patagonin determined by SDS-PAGE under non-reducing and reducing conditions was 25.3 and 27.4 kDa, respectively, demonstrating that it exists as a single polypeptide chain protein. MALDI-TOF mass spectral analyses of the intact

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Patagonin (Dipsadidae):          VDFDSESPRRPEIQ-
CRiSP-PHI1 (Dipsadidae):        LVDFNSESPRRPEIQ-
CRiSP-PHI2 (Dipsadidae):        LVDFNSESPRRPEIQ-
CRiSP-TRI1 (Dipsadidae):        NVDFNSESPRRPEKQ-
HG26 (Dipsadidae):               QDFNSEPPRKPEIQ-
HT26 (Dipsadidae):              YVDFNSQSPRRPEIQ-
CRiSP-LIO1 (Dipsadidae):        RIDIDSQSTRRPEIQ-
Tigrin (Natricidae):            NVDFNSESPRNPGKQ-
CRiSP-DIS1 (Colubridae):        NVDFNSESPRIKAKQ-
Ablomin (Viperidae):            NVDFDSESPRKPEIQ-
Triflin (Viperidae):            NVDFDSESPRKPEIQ-
Ophanin (Elapidae):             NVDFNSESTRRQKKQ-
Pseudechetoxin (Elapidae):      TADFASESSNKKNYQ-
VAR-11 (Varanidae):           MTSLDLDDLMTTNPEIQ-
Helothermine (Helodermatidae):  EASPKLPGLMTSNPDQQ-
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Fig. 2. NH_2-terminal sequence alignment of patagonin and representative venom CRiSPs of snakes and lizards: CRiSP-PHI1 (AAZ75603) and CRiSP-PHI2 (AAZ75604) from *Philodryas olfersii*, CRiSP-TRI1 (AAZ75607) from *Trimorphodon biscutatus* (Fry et al., 2006), HG26 from *Hydrodynastes gigas*, HT26 from *Hypsiglena torquata* (Hill and Mackessy, 2000; Mackessy, 2002), CRiSP-LIO1 (AAZ75601) from *Liophis poecilogyrus* (Fry et al., 2006), tigrin (Q8JGT9) from *Rhabdophis tigrinus tigrinus* (Yamazaki et al., 2002b), CRiSP-DIS1 (AAZ75595) from *Dispholidus typus* (Fry et al., 2006), ablomin (Q8JI40) from *Gloydius blomhoffi*, triflin (Q8JI39) from *Protobothrops flavoviridis* (Yamazaki et al., 2002b), ophanin (Q7ZT98) from *Ophiophagus hannah* (Yamazaki et al., 2003), pseudechetoxin (Q8AVA4) from *Pseudechis australis* (Brown et al., 1999), VAR-11 (Q2XXP1) from *Varanus varius* (Fry et al., 2006) and helothermine (Q91055) from *Heloderma horridum horridum* (Morrissette et al., 1995). The alphanumeric codes in parentheses refer to the Universal Protein Resource (UniProt) Accession Number. Identical residues are in shaded blocks.

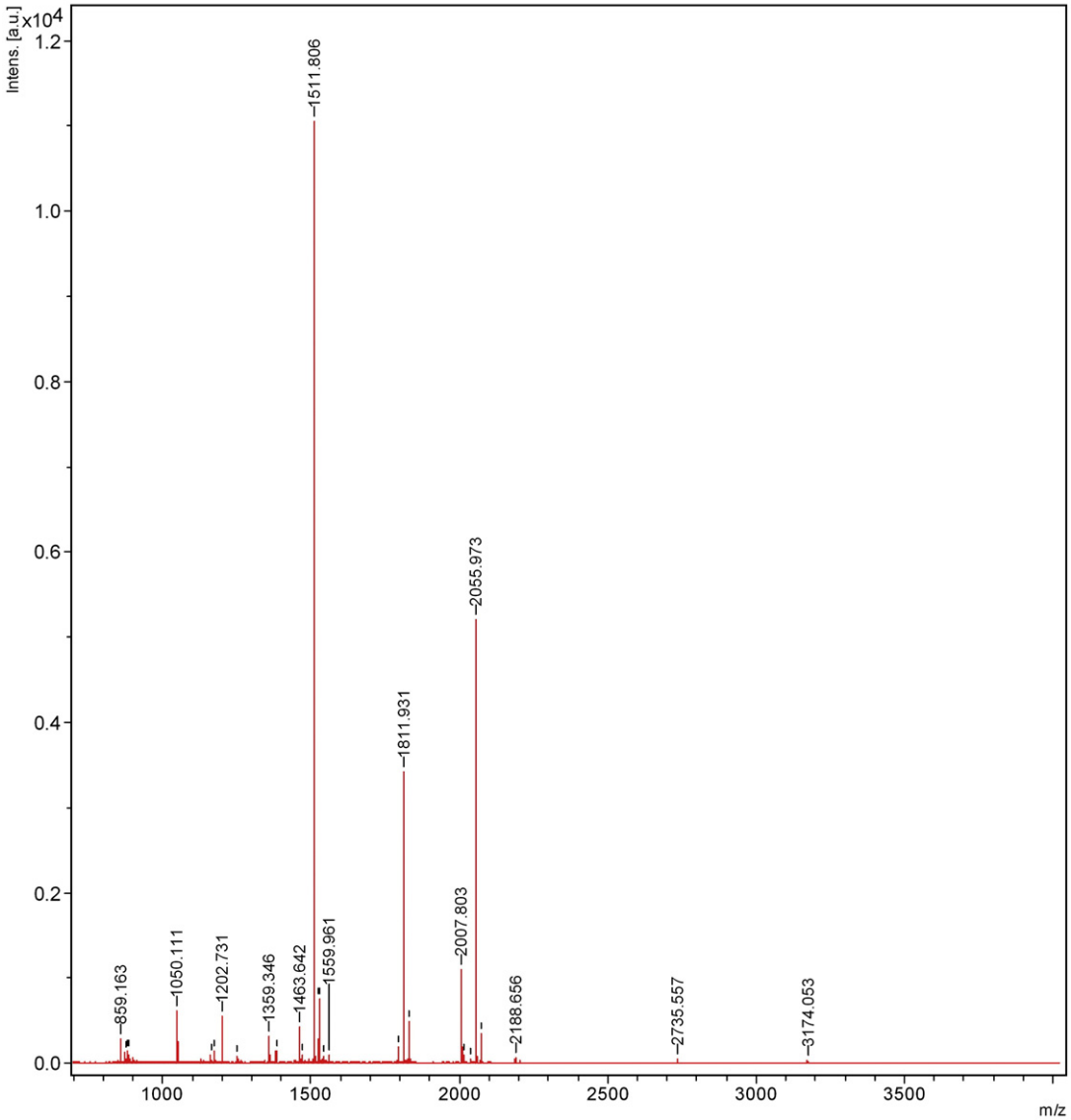

Fig. 3. MALDI-TOF mass spectrum of patagonin in-gel digested with trypsin. MS/MS CID sequence of peptide 1511.8 matched internal sequence of both known *P. olfersii* CRiSPs, confirming identity of patagonin as a venom CRiSP.

protein yielded a molecular mass of 24,858.6 Da (Fig. 1-C). The peaks of 12,434.9 and 12,642.6 Da correspond to doubly-charged ($z = 2$) cationic forms.

The NH_2-terminal 14-amino acid sequence VDFDSESPRRPEIQ- (UniProt Accession Number P85099) showed great similarity to the NH_2-termini of several snake venom CRiSP sequences (Fig. 2). SDS-PAGE-

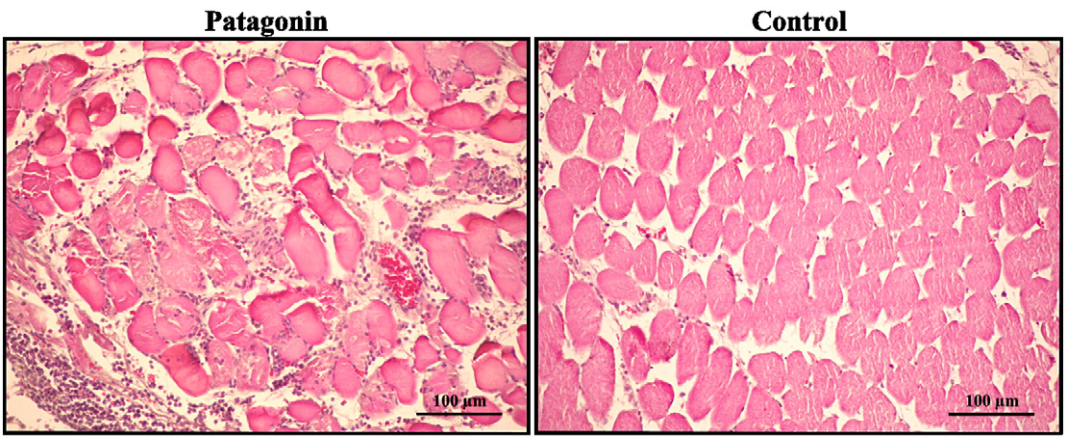

Fig. 4. Histopathological changes induced by 87 µg of patagonin in mouse gastrocnemius muscle 12 h after i.m. injection. Note cell necrosis, edema and inflammatory infiltrate of polymorphonuclears induced by patagonin. Sections were stained with hematoxylin-eosin.

separated protein bands were excised, in-gel digested with trypsin and the resulting peptides were analyzed by MALDI-TOF peptide mass fingerprinting followed by MALDI-TOF/TOF. The MALDI-TOF mass spectrum of the digested protein is shown in Fig. 3. The MS/MS spectrum of the fragmented singly-charged peptide ion ($m/z = 1511.806$) was matched by MASCOT to an internal sequence within the PR-1 (pathogenesis-related proteins of group 1) domain, MEWYAEAAANAER, from CRiSP-PHI1 and CRiSP-PHI2 of *Philodryas olfersii* (Ching et al., 2006; Fry et al., 2006). All of these results confirmed that a CRiSP from *P. patagoniensis* snake venom had been purified.

3.2. Patagonin activities

The purified protein, up to a final concentration of 400 μg/mL, hydrolyzed neither azocasein nor fibrinogen. When incubated with azocoll, patagonin (554 μg/mL, final concentration) did not degrade this substrate. It did not induce edema or hemorrhage, even at a dose of 20 μg. When added to washed human platelet suspensions or PRP, patagonin at concentrations up to 100 nM (final concentration) neither induced platelet aggregation directly nor inhibited platelet aggregation induced by ADP, collagen, convulxin, thrombin, ristocetin or the divalent cation ionophore A23187. In addition, collagen-induced platelet adhesion was not inhibited by patagonin.

When we examined the effects of patagonin on endothelium-denuded rat thoracic aortic rings, it showed a different behavior from that exhibited by some viperid and elapid CRiSPs. Patagonin, up to a final concentration of 2 μM, did not affect the basal tension of the denuded thoracic aortic rings in normal Krebs-bicarbonate solution. In addition, it did not affect the contractions of rat aortic smooth muscle induced by a 60 mM K^+ solution.

When injected intramuscularly, both doses of patagonin (43 and 87 μg) caused muscular damage to murine gastrocnemius muscle, an activity that has not been previously described for snake venom CRiSPs; more extensive damage was noted with the higher dose. Groups of necrotic muscle fibers were observed after twelve hours, with evident disorganization of myofibrillar material (Fig. 4). Concomitantly, edema and an inflammatory reaction characterized by the presence of a polymorphonuclear infiltrate were also noticed. However, hemorrhage was not observed. In agreement with these observations, significant increases in serum CK activity were recorded after i.m. injection of patagonin (43 μg: 138 ± 11 U/L; 87 μg: 178 ± 16 U/L; control: 53 ± 4 U/L; $n = 4$; $p < 0.05$). Systemic alterations were also investigated after i.m. injection of patagonin in mice, but no histological change was observed in cerebellum, cerebrum, heart, kidney, liver and lung tissues.

4. Discussion

CRiSPs are very broadly distributed among reptile venoms (Yamazaki and Morita, 2004; Osipov et al., 2005), suggesting that they are functionally important components in venoms. However, most venom CRiSPs have unknown activities. To date, tigrin (from *Rhabdophis tigrinus tigrinus* venom) has been the sole CRISP toxin from a rear-fanged snake that has been characterized (Yamazaki et al., 2002b); however, its mode of action still remains unclear. We have purified herein to homogeneity a CRiSP from *P. patagoniensis* opisthoglyphous snake venom and studied its biological functions.

CRiSP members are highly conserved in different snake venoms (Jin et al., 2003). Patagonin showed an NH_2-terminal amino acid sequence nearly identical to two other CRiSP isoforms from *P. olfersii* (Ching et al., 2006; Fry et al., 2006), several other CRiSPs from rear-fanged snake venoms (Hill and Mackessy, 2000; Fry et al., 2006) and viper toxins of the same gene family (Yamazaki et al., 2002b; Sanz et al., 2008). In addition, its MS/MS-derived sequence was identical to both CRiSPs from *P. olfersii* (Ching et al., 2006; Fry et al., 2006). Moreover, CRiSPs from *Vipera berus* and *Vipera raddei* venoms, whose MS/MS-derived sequences also share great similarity with patagonin, have been recently reported (Sanz et al., 2008; Ramazanova et al., 2009). Therefore, our current results reinforce the conclusion that CRiSPs are common and highly conserved proteins in snake venoms (Fry et al., 2003).

Despite a remarkable degree of sequence conservation within the CRiSP family, several of its members have been found to interact with different target proteins, i.e. cyclic nucleotide-gated ion channels as well as L-type Ca^{2+} and K^+-channels (BK_{Ca} and Kv1.3) (Brown et al., 1999; Yamazaki et al., 2002a; Yamazaki et al., 2002b; Wang et al., 2005; Wang et al., 2006). Most of the few snake venom CRiSPs whose functions are known show effects on smooth muscle contraction (Yamazaki et al., 2002b; Yamazaki et al., 2003; Wang et al., 2005). However, patagonin and other CRiSPs – e.g. tigrin, NA-CRVP1, NA-CRVP2 and TJ-CRVP – showed no activity toward smooth muscle (Yamazaki et al., 2002b; Jin et al., 2003).

Although several snake venom CRiSPs have not been tested for skeletal myotoxicity, NA-CVRP1, NA-CRVP2 and TJ-CVRP were found to be devoid of this biological activity (Jin et al., 2003). Patagonin, a CRiSP isolated from a rear-fanged snake venom, shows skeletal myotoxic activity, and to the best of our knowledge, it is the first CRiSP with such activity. As binding to ion channels is speculated to be a general property of CRiSPs (Fry et al., 2008), we suggest that patagonin induces muscle necrosis by this mechanism, and this hypothesis is currently being investigated. A native CRiSP from the venom of *Philodryas* species had not been isolated yet, although nucleotide sequences have been determined for several other CRiSPs from rear-fanged snakes, including the related *P. olfersii*. The mechanism of action of patagonin is now being further analyzed, representing a critical step towards the understanding of the role of this family of proteins in snake venoms, particularly among the rear-fanged snake family, whose venoms often have a high CRiSP content (Mackessy, 2002).

In conclusion, this is the first report on the isolation and characterization of biological activity of a CRiSP from the venom of an opisthoglyphous snake. Venom CRiSPs have similar primary structures, particularly the highly conserved cysteines participating in disulfide formation. CRiSPs, like numerous snake venom toxin families (e.g., the three finger toxins; (Doley et al., 2008)), likely have a highly conserved molecular scaffold but a variety of different pharmacological activities. Considering that patagonin exhibited an activity (myotoxicity) that has not been reported previously, it will be an important contributor to studies of the structure-function and evolutionary relationships of this family of widely distributed venom proteins.

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