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Proteolytic, edematogenic and myotoxic activities of a hemorrhagic metalloproteinase isolated from *Bothrops alternatus* venom

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Abstract

A hemorrhagic metalloproteinase has been isolated from *Bothrops alternatus* venom from specimens that inhabit the north-east region of Argentina. The present study aimed at evaluating the proteolytic, hemorrhagic, edematogenic and myotoxic activities of the purified metalloproteinase, in order to consider its participation on the phatophysiology of the intoxication by *Bothrops alternatus* venom. The hemorrhagic metalloproteinase was isolated by a combination of DEAE-Cellulose chromatography and gel filtration on Sephadex G-75. The enzyme showed a molecular mass around 55 kDa, it exhibited a hemorrhagic activity with a minimal hemorrhagic dose of 1.9 μg, almost two fold minor than the whole venom (3.6 μg). The enzyme showed a weak proteolytic activity on casein (18.72 U/mg enzyme), similar to the one exhibited by the whole venom (20 U/mg venom). Besides, the ability to degrade casein could be detected by SDS-PAGE; β-casein was the fraction that showed the higher degradation, followed by αs_1-casein and κ-casein degradation. The hemorrhagic metalloproteinase rapidly hydrolysed the Aα-chain of fibrinogen, followed by Bβ-chain degradation and leaving the γ-chain unaffected. Proteolytic activities were inhibited by EDTA whereas they were not inhibited by benzamidine and PMSF. The metalloproteinase showed several polypeptides chains after autocatalytic processing, including a chain of 28 kDa, it could be the processed disintegrin-like and cysteine-rich domains. The isolated enzyme exhibited myotoxic activity with high CK levels at 6 h, due to local ischemia resulting of its hemorrhagic activity, and a significant edema-inducing effect (MED = 1.3 μg), corroborated both results by the histological observations of samples of gastrocnemius muscle. These findings showed that this hemorrhagic metalloproteinase, possesses high edematogenic and myotoxic activities and, in despite of exhibiting a weak proteolytic activity, it is able to degrade fibrinogen. So, this enzyme would contribute markedly to the phatophysiology of the bothropic envenomation.
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Keywords: Hemorrhagin; Metalloproteinase; Snake venom; *Bothrops alternatus*

1. Introduction

The majority of snakebites in the north-east region of Argentina are caused by *Bothrops alternatus* snake that belongs to Viperidae family, its venom induces a complex series of local effects including hemorrhage, myonecrosis and edema, in addition to systemic effects such as hemorrhage, coagulation disorders, cardiovascular shock and acute renal failure. These effects are induced by a variety of venom components, such as phospholipases A_2 (Nisenbom et al., 1986), thrombin-like enzymes (Smolka et al., 1998) and metalloproteinases, among others

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(Acosta de Pérez et al., 1996a,b; 1998; Teibler et al., 1999). It is known that these last ones are the main compounds responsible for bleeding and the prominent local and systemic effects that result from *Bothrops* snake bites (Kamiguti et al., 1996; Gutiérrez and Rucavado, 2000).

Metalloproteinases comprise a large group of zinc-dependent proteinases of varying domain composition named snake venoms metalloproteinases (SVMPs), which are members of the Reprolysin subfamily that is part of the 'metzincin' family (Bjarnason and Fox, 1994; Hooper, 1994). They were divided into four classes (P-I–P-IV) according to their molecular mass and domain organization (Bjarnason and Fox, 1994; Jia et al., 1996), P-I class proteins have only the metalloproteinase domain (with molecular mass about 24 kDa); P-II class has a metalloproteinase followed by a disintegrin-like domain (medium size proteins); P-III class, comprises metalloproteinase, disintegrin-like and high-cysteine domains (about 55 kDa); and P-IV class (95 kDa), presents, besides the three described domains, an additional lectin-like domain (Gutiérrez and Rucavado, 2000; Matsui et al., 2000).

These proteases induce hemorrhage, local myonecrosis, skin damage and inflammation. In addition, they degrade extracellular matrix components and impair the regeneration of affected skeletal muscle (Gutiérrez and Rucavado, 2000). Some of metalloproteases also affect platelet function, through their non-catalytic disintegrin-like and cysteine-rich domains, which explains, at least in part, their hemorrhage-enhancing effect (Kamiguti et al., 1996; Moura-da-Silva et al., 1999; Jia et al., 2000). Besides to these effects they degrade blood clotting factors, precluding a normal hemostatic response after microvessel damage (Gutiérrez and Rucavado, 2000).

The direct fibrin(ogen)olytic metalloproteinases degrade preferably Aα-chain of fibrinogen followed by Bβ-chain degradation. The Aα-chain specificity is typical of the majority of these enzymes (Markland, 1998). These proteinases have been purified from the venoms of *Bothrops jararaca* (Maruyama et al., 1992), *Bothrops moojeni* (Serrano et al., 1993), *Bothrops neuwiedi* (Rodrigues et al., 2000), *Lachesis muta muta* (Estevão-Costa et al., 2000) and *Bothrops jararacussu* (Mazzi et al., 2004). The primary and three-dimensional structures of several metalloproteinases have been determined, showing that they are structurally related (Matsui et al., 2000).

Souza et al. (2000) have isolated a hemorrhagin metalloproteinase/disintegrin and its disintegrin-like and cysteine-rich domains from *B. alternatus* venom, obtained from specimens that inhabit in Brazil, called alternagin and alternagin-C, respectively. They demonstrated that the disintegrin-like and cysteine-rich domains of this hemorrhagin is a potent inhibitor of the collagen binding to $\alpha_2\beta_1$ integrin, contributing to the understanding of the structure-function relationship of disintegrins as well as the molecular basis of cell adhesion. However, biological and biochemical characterizations of alternagin related with intoxication were not investigated until the present.

In this work a hemorrhagic metalloproteinase, probably alternagin, was isolated and its proteolytic, edematogenic and myotoxic activities were studied in order to consider its participation on the phatophysiology of the intoxication by *Bothrops alternatus* venom, from specimens that inhabit the north-east region of Argentina.

2. Material and methods

2.1. Materials

Bothrops alternatus venom was purchased from Serpentarium of the local zoo, Corrientes, Argentina. Crude venom was lyophilized; after that, it was kept frozen at -20 °C. When required, the venom was diluted with phosphate buffered saline solution, pH 7.2. The small amount of insoluble material was centrifuged and the clear supernatant was applied for studies. DEAE-Cellulose, Sephadex G-75, Human fibrinogen type I and weight markers were purchased from Sigma Chemical Co. Bovine casein was purchased from Fluka Biochemika. All others reagents were of analytical grade.

2.2. Metalloproteinase isolation

B. alternatus crude venom (40 mg) was fractionated by ion exchange chromatography on a DEAE-Cellulose column (1×5 cm) equilibrated with 0.01 M, pH 7.2, phosphate buffer. The column was washed with the equilibration buffer (4 ml/tube) followed by stepswise changes in elution buffers 0.1, 0.2 and 0.3 M NaCl, where fractions of 5, 1 and 1 ml/tube, respectively, were collected at a flow rate of 1 ml/min and then were tested for proteolytic and hemorrhagic activities. Selected fractions were further separated on a gel filtration on Sephadex G-75 column (1×50 cm). Elution was carried out using the same buffer and fractions of 0.75 ml/tube were collected at a flow rate of 10 ml/h. Two purifications steps were performed at 4 °C. The fractions displaying high hemorrhagic activity were pooled and submitted to characterization.

2.3. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE)

In order to assay the isolated enzyme for purity, electrophoresis was performed on 12% polyacrylamide slab gels following the method of Laemmli (1970). Samples were pretreated in reducing conditions (SDS plus β-mercaptoethanol) at 100 °C for 5 min. Runs were of 1 h with 40 mA, and bromophenol blue was used as a tracking dye. Gels were stained with Coomassie brilliant blue R-250. Standards molecular mass markers were bovine albumin (66 kDa), egg albumin (45 kDa),

glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.20 kDa).

2.4. Proteolytic activity on casein

This was tested using casein from bovine milk as substrate, according to Friedrich and Tu (1971) with Lomonte and Gutiérrez (1983) modification. One millilitre of enzyme solution, ranging from 0.014 to 1.800 mg/ml, was incubated with 2.0 ml of a 1% casein solution for 30 min at 37 °C. Then, 4.0 ml of 5% trichloroacetic acid was added and sample left at room temperature for 30 min. The tubes were centrifuged and the absorbance of the clear supernatant was determined by spectrophotometric measurement at 280 nm using a Cam Spec M330 spectrophotometer. Control solution was applied, in which the enzyme solution was omitted. The caseinolytic activity, expressed as units/mg, is defined as the percentual change in absorbance yielded by 1 mg of enzyme incubated with casein at 37 °C during 30 min.

2.5. Hemorrhagic activity assay

The hemorrhagic activity was evaluated by the skin method of Kondo et al. (1960) with some modifications. Groups of four mice (CF1, 18–22 g) were shaved on the back and then intradermicaly (i.d) injected with different doses of enzyme (1 a 10 μg), in 100 μl of phosphate buffer saline (PBS). Skins were removed 2 h after and the diameters of the hemorrhagic spots were measured on the inside surfaces. The Minimum Hemorrhagic Dose (MHD) was defined as the amount (μg) of venom producing a hemorrhagic spot of 10 mm in diameter. For inhibition studies, 2 MHD of hemorrhagin isolated was incubated with equal volumes of 10 mM Na_2EDTA (metalloproteinases inhibitor) for 1 h at 37 °C.

2.6. Specific cleavage on casein

Specific cleavage on casein by isolated metalloproteinase was shown by electrophoresis on 12% polyacrylamide gels according to the method of Laemmli (1970) with some modifications. One hundred microliters of casein (previously boiled at 100 °C for 20 min and filtered) at a concentration of 5 mg/ml in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 mM $CaCl_2$ and 0.02% NaN_3 were incubated at 37 °C with 100 μl of isolated metalloproteinase at a concentration of 250 μg/ml in the buffer mentioned above, corresponding to a ratio of 20:1 (w/w). At various time intervals, aliquots of 30 μl were withdrawn from the digestion mixture. The samples were denatured and reduced by boiling for 5 min with 30 μl of denaturing solution (5% urea, 4% SDS and 1% 2-mercaptoethanol in 35 mM phosphate buffered saline solution, pH 7.0) before the run on SDS-polyacrylamide gel. For inhibition studies, isolated metalloprotease was incubated in presence of 20 mM Na_2EDTA, 2 mM PMSF (phenylmethylsulfonyl fluoride) and 20 mM Benzamidine, for 1 h at 37 °C before the incubation with casein.

2.7. Proteolytic activity on fibrinogen

Specific cleavage of fibrinogen by isolated metalloproteinase was shown by electrophoresis on 12% polyacrylamide gels according to the method of Laemmli (1970) with some modifications. One hundred microliters of fibrinogen (Human fibrinogen type I) at a concentration of 5 mg/ml in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 mM $CaCl_2$ and 0.02% NaN_3 were incubated at 37 °C with 100 μl of isolated metalloproteinase at a concentration of 250 μg/ml in the buffer mentioned above, corresponding to a ratio of 20:1 (w/w). At various time intervals, aliquots of 30 μl were withdrawn from the digestion mixture. The samples were denatured and reduced by boiling for 5 min with 30 μl of denaturing solution (5% urea, 4% SDS and 1% 2-mercaptoethanol in 35 mM phosphate buffered saline solution, pH 7.0) before the run on SDS-polyacrylamide gel. For inhibition studies, isolated metalloproteinase was incubated in presence of 20 mM Na_2EDTA, 2 mM PMSF and 20 mM Benzamidine, for 1 h at 37 °C before the incubation with human fibrinogen (Assakura et al., 1994).

2.8. Autocatalytic processing assay

In order to assay the autocatalytic processing of the isolated metalloproteinase, one millilitre of enzyme solution (1 mg/ml in 0.01 M, pH 7.2, phosphate buffer) was incubated at 37 °C. At various time intervals, aliquots of 20 μl were withdrawn from the digestion mixture. The samples were denatured and reduced by boiling for 5 min with 30 μl of denaturing solution (5% urea, 4% SDS and 1% 2-mercaptoethanol in 35 mM phosphate buffered saline solution, pH 7.0) before the run on SDS-polyacrylamide gel.

2.9. Edema-forming activity

The method of Yamakawa et al. (1976) was used. Five groups of four mice (CF1, 18-22 g) were injected s.c. in the right foot pad with 50 μl of solutions containing different amounts of hemorrhagin (from 0.05 to 10 μg), whereas the left foot pad was injected with 50 μl of phosphate buffered saline solution (pH 7.2). Mice were anesthetized with i.p. injection of cloral hydrate and killed by cervical dislocation 1 h after injection and both feet were cut off and weighed individually. Edema was expressed as the percentage increase in weight of the right foot compared to that of the left one. The minimum edema dose (MED) was defined as the least quantity of enzyme causing 30% increase in the weight, compared to the control.

2.10. Myotoxic activity

Groups of 4 mice (CF1, 18–22 g) were injected i.m. in the right gastrocnemius with 30 μg of hemorrhagin dissolved in 0.1 ml of phosphate buffered saline solution, pH 7.2. Four mice were used as control samples, receiving each of them 0.1 ml of phosphate buffered saline solution (pH 7.2). After 1, 3, 6, 12 and 24 h of hemorrhagin injection, mice were anesthetized with cloral hydrate i.p. 300 mg/kg to collect blood samples without using anticoagulant. Serum was obtained to analyze the activity of creatine kinase (CK) with the kinetic method U. V. (Randox) based on the measurement of the creatinine formed in the reaction ADP/phosphocreatine. Creatine kinase activity was expressed in units per liter. In order to have a histological assessment of myotoxicity, mice were sacrificed by cervical dislocation 1, 3, 6, 12 and 24 h after enzyme injection and samples of injected muscle were taken and fixed with Bouin solution for 24–48 h. Thereafter, the muscle was dehydrated in a graded alcohol series and embedded in paraffin. Sections 5 μm thick were stained with Haematoxylin and Eosin (H & E). Control muscles were processed in an identical manner.

Necrosis was classified according to the method of Homma and Tu (1971), based on the morphology of the necrotic fibers. The myolytic type was characterized by fibrilar material, alterning with clear areas. In the coagulative necrosis type, the fibers acquired a hyaline appearance and its distribution was homogeneous.

2.11. Statistical analysis

All experiments were repeated at least four times. The MHD and MED were estimated by linear regression, adjusted to the minimum square method. The results of the enzymatic determination of the serum CK levels were expressed as the mean ± SD. The significance of differences between means was assessed by ANOVA followed by Dunnet's test for multiple comparisons among groups. P values less than 0.05 were considered to be statistically significant.

3. Results

In this work, a hemorrhagic metalloproteinase from *Bothrops alternatus* venom was purified for two steps, namely, a combination of ion-exchange chromatography and gel filtration. The fractionation of the crude venom on DEAE-Cellulose showed five major peaks (Fig. 1(A)), all of them showed proteolytic activity on casein and hemorrhagic activity. Then the fraction (IV) was resolved into two fraction (IVa and IVb) using a Sephadex G-75 column (Fig. 1(B)). The second fraction (IVb) did not exhibit hemorrhagic activity while the first fraction (IVa) induced hemorrhage in mice and showed a single band on SDS-PAGE, as determinated under reducing (Fig. 2) and non reducing conditions (not shown). The molecular mass of the isolated enzyme was around 55 kDa and it was estimated by interpolation from a linear logarithmic plot of relative molecular mass versus distance of migration.

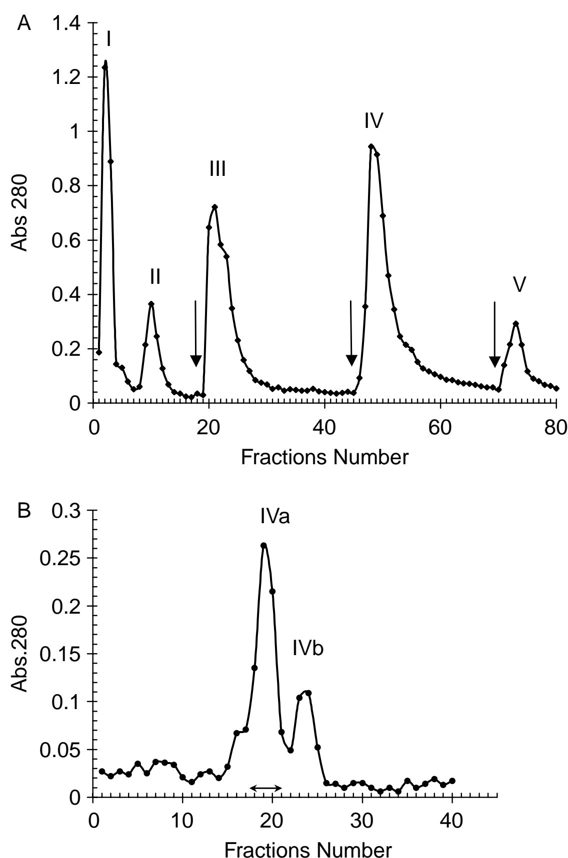

Fig. 1. Isolation of the hemorrhagin. (A) Ion exchange chromatography of *B. alternatus* venom. Forty milligram on a 1.0×5.0 cm column of DEAE-Cellulose previously equilibrated with 0.01 M, pH 7.2, phosphate buffer and eluted with an increasing concentration by stepswise elution with 0.1, 0.2 and 0.3 M NaCl. Arrows indicate stepswise changes in elution buffers. All the peaks showed proteolytic and hemorrhagic activities. (B) Chromatography of the pooled hemorrhagic fraction (IV) on column of Sephadex G-75 (1× 50 cm) previously equilibrated with 0.01 M, pH 7.2, phosphate buffer. Double arrow indicates fractions that showed hemorrhagic activity.

Proteolytic activity was assayed using solutions of different enzymes concentration, values of absorbance changes (ΔAbs280) obtained were plotted versus logarithm of venom concentration (each point represented the mean of four determinations); the obtained linear relationship ($r=0.9873$) let us determine the proteolytic activity. The hemorrhagic metalloproteinase showed a weak value (18.72 U/mg enzyme) when tested on bovine casein.

The hemorrhagic halos were evaluated 2 h after enzyme injection and were proportional to the amount of enzyme

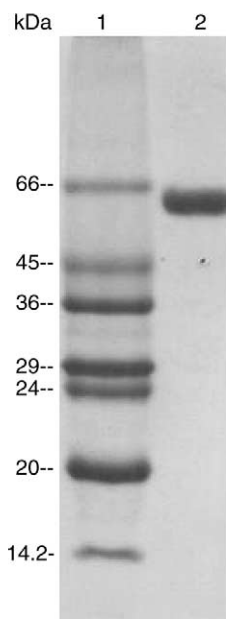

Fig. 2. SDS-PAGE at 12% (w/v). Molecular Mass Estimation. Lane 1, molecular mass markers: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.20 kDa). Lane 2, IV fraction (3 μg) after denaturation with SDS and reduction with 2-mercaptoethanol.

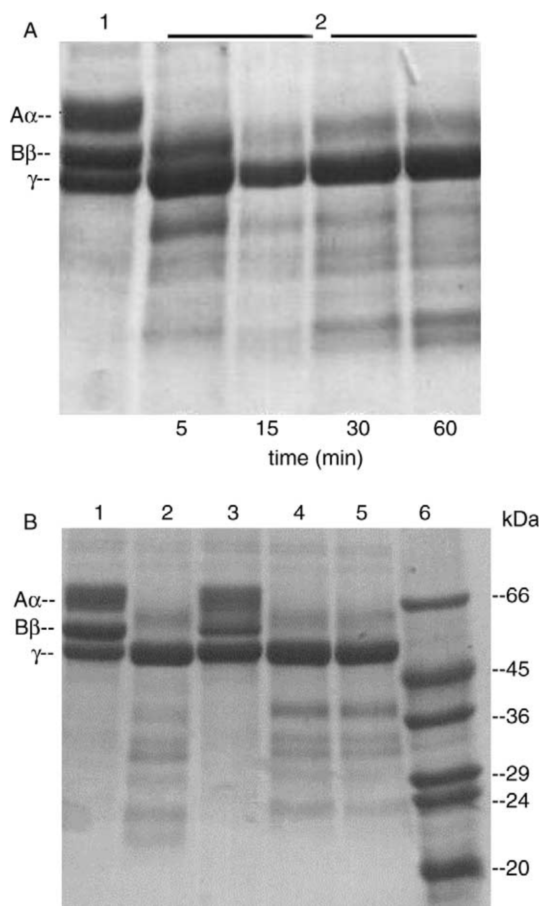

Fig. 3. (A) SDS-PAGE analysis of human fibrinogen after digestion by the isolated hemorrhagin in a 12% gel under reducing conditions. Lane 1, human fibrinogen control incubated at 37 °C for 1 h without hemorrhagin. The Aα- (63 kDa), Bβ- (56 kDa), and γ- (47 kDa) chains of fibrinogen are indicated on the left. Lanes 2, human fibrinogen samples after incubation at 37 °C with the hemorrhagin at a ratio 20:1 (w/w) for 5, 15, 30 and 60 min, respectively. The gel was stained with Coomassie Brilliant Blue R-250. (B) Effect of inhibitors on the digestion of fibrinogen by the isolated hemorrhagin in a 12% gel under reducing conditions. Lane 1, human fibrinogen control incubated at 37 °C for 1 h without hemorrhagin. Lane 2, human fibrinogen incubated at 37 °C for 60 min with hemorrhagin. Lane 3, human fibrinogen incubated at 37 °C for 60 min with hemorrhagin + Na_2EDTA. Lane 4, human fibrinogen incubated at 37 °C for 60 min with hemorrhagin + PMSF. Lane 5, human fibrinogen incubated at 37 °C for 60 min with hemorrhagin + Benzamidine. Lane 6, molecular mass markers. The gel was stained with Coomassie Brilliant Blue R-250.

injected; the obtained linear relationship ($r = 0.9955$) let us determine the MHD. The isolated metalloproteinase exhibited a hemorrhagic activity with a minimal hemorrhagic dose of 1.9 μg. The hemorrhagic activity of isolated hemorrhagin was totally abolished after incubation with a chelating agent (Na_2EDTA 10 mM, final concentration).

The isolated enzyme (0.25 mg/ml) hydrolysed fibrinogen (Fig. 3(A)). It rapidly digested the Aα-chain of fibrinogen, at 5 min, followed by Bβ-chain degradation, within 1 h, and leaving the γ-chain unaffected. Fibrinogenolytic activity was completely abolished by Na_2EDTA, a metalloproteinases inhibitor, whereas it was not inhibited by PMSF and benzamidine, both serineproteinases inhibitors (Fig. 3(B)).

The enzyme hydrolysed bovine casein at 37 ° C; β-CN was the fraction that showed the higher degradation at 5 min, followed by αs_1-CN and γ-CN degradation within 30 min (Fig. 4(A)). The degradative patterns sustained αs_1-CN and γ-CN bands throughout the assayed time, while no β-CN bands were found after the 30 min at 37 ° C. There was greater protein degradation bovine casein, which was evidenced by the higher numbers of low-molecular-mass protein fractions below the κ-casein band. Caseinolytic activity was completely abolished by Na_2EDTA, whereas it was not inhibited by PMSF and benzamidine (Fig. 4(B)).

The analysis of electrophoretic migration patterns of the hemorrhagin pre-incubated at pH 7.2 and at 37 ° C during 18 h showed a progressive disappearance of this proteinase band and an increase in bands ranging from 14 to 45 kDa (Fig. 5). After 18 h of hydrolysis the principal degradation product was a band of 28 kDa, a molecular mass compatible with its disintegrin-like and cysteine-rich domains, named alternagin-C by Souza et al. (2000).

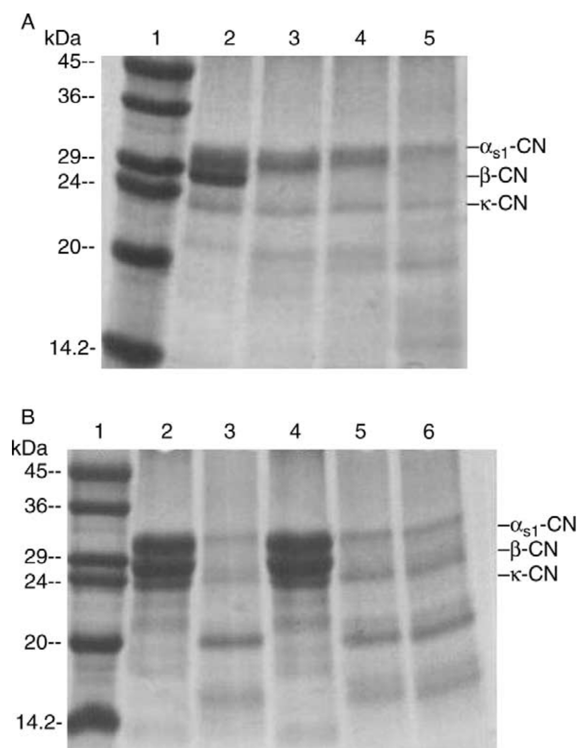

Fig. 4. (A) SDS-PAGE analysis of bovine casein after digestion by the isolated hemorrhagin in a 12% gel under reducing conditions. Lane 1, molecular mass markers. Lane 2, bovine casein control incubated at 37 °C for 30 min without the hemorrhagin. Lanes 3–5, bovine casein samples after incubation at 37 °C with the hemorrhagin for 5, 15 and 30 min, respectively. The gel was stained with Coomassie Brilliant Blue R-250. (B) Effect of inhibitors on the digestion of bovine casein by the isolated hemorrhagin in a 12% gel under reducing conditions. Lane 1, molecular mass markers. Lane 2, bovine casein control incubated at 37 °C for 30 min without the hemorrhagin. Lane 3, bovine casein incubated at 37 °C for 30 min with the hemorrhagin. Lane 4, bovine casein incubated at 37 °C for 30 min with the hemorrhagin + Na_2EDTA. Lane 5, bovine casein incubated at 37 °C for 30 min with the hemorrhagin + PMSF. Lane 6, bovine casein incubated at 37 °C for 30 min with the hemorrhagin + Benzamidine. The gel was stained with Coomassie Brilliant Blue R-250.

The isolated metalloproteinase exhibited intense edematogenic activity when tested by the foot-pad assay (four mice per dose were used, $n=4$). The percentage increases in weight of the right foot compared to that of the left were evaluated 1 h after enzyme injection and were proportional to the amount of enzyme injected; the obtained linear relationship ($r=0.9839$) let us determine the MED. Compared to mouse foot pads injected with phosphate buffered saline solution, a 30% increase in the weight was produced by injecting 1.3 μg of the hemorrhagin.

After i.m. injection isolated hemorrhagin induced a late increase in serum creatine kinase (Fig. 6), which is a specific marker for muscle damage. At a dose of 30 μg/animal,

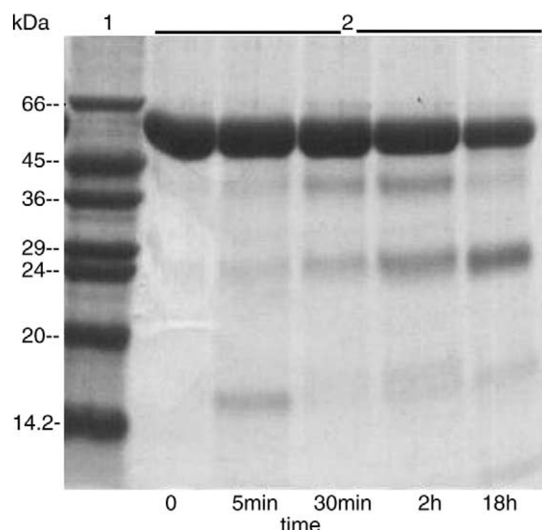

Fig. 5. SDS-PAGE analysis of autocatalytic processing. Lane 1, molecular mass markers. Lanes 2, 20 μg of the hemorrhagin incubated at pH 7.2 for 0, 5, 30 min, 2 and 18 h, respectively.

the CK levels, maximum levels were observed at 6 h, and decreased afterward. CK levels after 24 h were not significantly different from the control value (236.75 ± 50.31 IU/l). The histological observations of samples of gastrocnemius muscle obtained 1, 3, 6, 12 and 24 h after injection corroborated the myotoxicity and edemaforming activity. The control sample retained a normal appearance (not shown), whereas those samples of gastrocnemius muscle injected with 30 μg of enzyme showed prominent damage characterized by myonecrosis, hemorrhage, edema and leukocyte infiltration. Sixty minutes after injection,

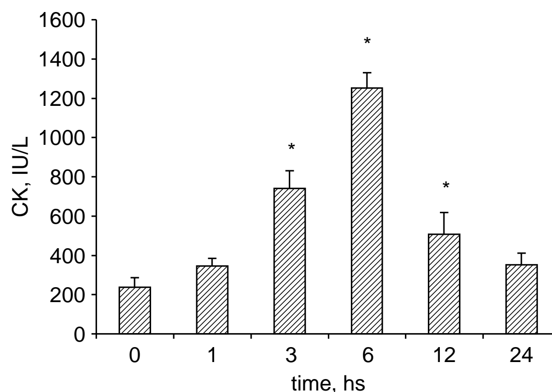

Fig. 6. Changes in serum CK levels of mice after i.m. injection of 30 μg of the isolated hemorrhagin. At various time intervals mice were bled and serum CK levels determined. The CK activity was expressed in international units/L. Results are presented as means ± SD of four isolated experiments. Control mice injected with phosphate buffered saline solution had serum CK activity of 236.75 ± 50.31 IU/L. $*P<0.05$ when compared to values of control mice.

moderate inflammatory infiltrate composed mainly of polymorphonuclear leucocytes (particularly neutrophils) and interfibrilar hemorrhage were observed. Three hours later, the histological sections of gastrocnemius muscle showed a prominent inflammatory infiltrate, interfibrilar hemorrhage and edema, and a number of necrotic muscle cells (Fig. 7(A)). The results obtained 6 h after injection were similar to those recorded 3 h later. However, at this time the myonecrosis reached its highest intensity, observing several necrotic muscle cells characterized by a conspicuous disorganization of the myofibrillar material and by the presence of abundant inflammatory cells within the cellular space. Both, myolysis and coagulation type necrosis were observed (Fig. 7(B)). Twelve hours later, was observed a decrease of interstitial hemorrhage and of the edema, leucocytes and necrotic muscle cells were abundant (Fig. 7(C)). At 24 h after injection, necrotic muscle fibers and infiltrations of polymorphonuclear leucocytes were only observed. The liberation of CK observed in assays in vivo, at different time intervals studied, was coincident with the results obtained from these preparations.

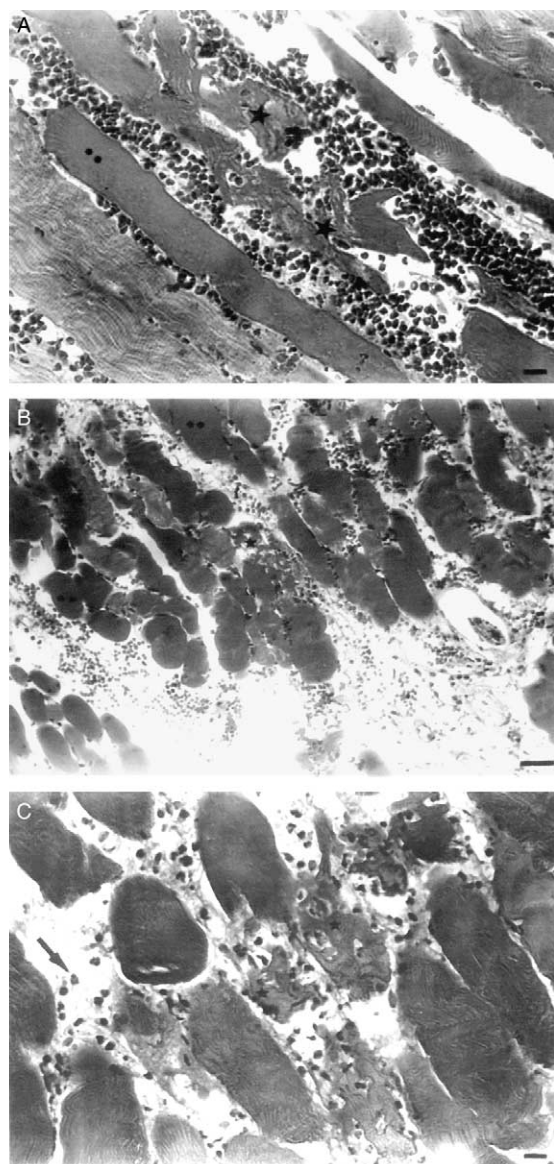

Fig. 7. Paraffin histological section of mouse gastrocnemius muscle. (A) Three hours after i.m. injection of 30 μg of the isolated hemorrhagin. Note the presence of several necrotic muscle cells with evident disorganization of myofibrillar material, myolysis (stars) and coagulation type necrosis (asterisks) are showed. Prominent polymorphonuclear inflammatory infiltrate within and outside the cellular space, and edema indicated by the increased spacing between muscle cells are observed. Extravasated erythrocytes are located in the interstitial space. Bar = 10 μm. (B) Six hours after i.m. injection of 30 μg the isolated hemorrhagin. Note intense myolysis (stars) and coagulation type necrosis (asterisks) with important interfibrilar hemorrhage and edema. Bar = 30 μm. (C) Twelve hours after i.m. injection of 30 μg the isolated hemorrhagin. It is observed a decrease of interstitial hemorrhage and of the edema, leucocytes (arrow) and necrotic muscle cells (stars) are abundant. Bar = 10 μm.

4. Discussion

In this work, a hemorrhagic metalloprotease, was isolated from *Bothrops alternatus* venom from the northeast region of Argentina by a combination of ion-exchange chromatography and gel filtration. This enzyme exhibited a hemorrhagic activity with a minimal hemorrhagic dose of 1.9 μg, almost two fold minor than the whole venom (3.6 μg) (Acosta de Pérez et al., 1996a). This activity was totally abolished after incubation with a chelating agent (Na_2EDTA), corroborating the metal-dependency of this effect. It is widely accepted that hemorrhagic metalloproteinases induce bleeding due to proteolytic degradation of extracellular matrix components in addition to degeneration and rupture of endothelial cells in capillary blood vessels (Gutiérrez and Rucavado, 2000), so this enzyme participates in the vascular damage exhibited by whole venom.

The hemorrhagic metalloproteinase exhibited proteolytic activity about various substrates. The electrophoretic pattern of the autoproteolytic processing showed several polypeptides including a chain of 28 kDa, that it could be the processed disintegrin-like and cysteine-rich domains (alternagin-C), as it was described by Souza et al. (2000). This property is correlated with the enzymatic action of venom proteinases exhibited upon its own constituents (Sousa et al., 2001; Moura-da-Silva et al., 2003). Besides, the isolated enzyme showed a weak proteolytic activity on casein, similar to the one exhibited by the whole venom (20 U/mg venom). Although the ability to degrade casein could be detected by SDS-PAGE, a non conventional technique for venom caseinolytic studies, but fully used in food analysis and other studies (Park and Jin, 1998; Moussaoui et al., 2003). On the other hand, the hemorrhagin

hydrolysed both, Aα- and Bβ-chains of fibrinogen, like hemorrhagic toxins found in viperid venoms (Markland, 1998). Both caseinolytic and fibrinogenolytic activities exhibited by the hemorrhagin were completely abolished when the enzyme was preincubated with a metal chelating agent, whereas it was not inhibited by serineproteinases inhibitors. These results corroborate that the isolated hemorrhagin is a metal-depending proteinase.

The edema-forming activity exhibited by the hemorrhagin was higher than the whole venom was (MED: 4.0 μg, Acosta de Pérez et al., 1998). The histological observations of samples of gastrocnemius muscle obtained 1, 3, 6, 12 and 24 h after injection corroborated edema-forming activity and important myotoxicity. This latter begins to be evident 3 h after injection, reaches its highest intensity 6 h after injection. Such alterations are similar to phospholipase A_2 enzyme induces in muscular tissue (Gutiérrez and Lomonte, 1995; Acosta de Pérez et al., 1998). However, in these cases the intensities of the damage and the period at which they are evident, are different from those obtained for the enzyme isolated in this work. So it suggests that widely different mechanisms are involved in the phatophysiology action of phospholipase A_2 and metalloproteinases from bothropic venoms. It is probably that the isolated hemorrhagin induces myonecrosis due to local ischemia resulting of its hemorrhagic and edematogenic action, detected in histological sections through the accumulation of fluid, blood red and leucocytes in the injured area, such as it was described by Gutiérrez et al. (1995).

According to the results obtained in this work, it is probable that the isolated enzyme is alternagin, the hemorrhagic metaloprotease isolated from specimens of *Bothrops alternatus* snake venom from Brazil, by Souza et al. (2000). However, they have only studied inhibitory effects of this protein and its processed disintegrin-like and cysteine-rich domains (alternagin-C) on cell adhesion, in order to may aid in the understanding of the structure-function relationship of disintegrins as well as de molecular basis of cell adhesion.

In conclusion our findings demonstrate that the hemorrhagic metalloproteinase presents in the *Bothrops alternatus* venom from the north-east region of Argentina, probably alternagin, possesses high edematogenic and myotoxic activities and, in despite of exhibiting a weak proteolytic activity, it is able to degrade fibrinogen. So, this enzyme would contribute markedly to the phatophysiology of the bothropic envenomation.

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