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Inhibitory properties of the anti-bothropic complex from *Didelphis albiventris* serum on toxic and pharmacological actions of metalloproteases and myotoxins from *Bothrops asper* venom

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Abstract

Anti-bothropic complex (ABC) was isolated from the serum of the South American opossum (*Didelphis albiventris*) by single-step affinity chromatography using a Sepharose-immobilized metalloprotease (BaP1) from *Bothrops asper* as the binding protein. Biochemical characterization of ABC showed the presence of two glycosylated subunits of 43 and 45 kDa, respectively, with an isoelectric point < 4. The two subunits were separated by ion-exchange HPLC. The N-terminal sequences of both subunits (LKAMDPTPXLWIETESP, where X is Arg-9 and Pro-9, respectively) showed a high degree of identity with other serum inhibitors isolated from different marsupials. Functional studies pointed out that ABC inhibits the hemorrhagic and proteolytic activities on fibrin, fibrinogen, and casein induced by the metalloproteases BaP1 and BaH4 isolated from *B. asper* venom. In addition to the anti-hemorrhagic and anti-proteolytic activities, ABC also showed anti-myotoxic, anti-lethal, and anti-edematogenic effects against myotoxic phospholipases A_2 isolated from the same venom. Moreover, it had inhibitory effects on the phospholipase A_2 activity of the crude venom as well as the isolated venom phospholipases A_2.

Keywords: Anti-bothropic complex; Anti-ophidian activity; *Didelphis albiventris;* Metalloproteases; Myotoxic phospholipases A_2; *Bothrops asper;* Snake venom

1. Introduction

Bothrops (family Viperidae, subfamily Crotalinae) snake venoms are known to produce local tissue damage such as hemorrhage, myonecrosis, and edema owing to the action of various toxins [1,2]. Local hemorrhage induced by Viperidae snake venoms is produced by zinc metalloproteases with high substrate specificity, probably acting on connective tissue and basement membrane components of the capillary vessels and leading to extravasation [3]. Depending on their domain structure (metalloprotease, desintegrin-like, cysteine-rich, and lectin-like), these SVMPs have been classified into four classes: P-I to P-IV [4].

Bothrops asper is medically the most important venomous snake in Central America [5]. Its venom induces prominent hemorrhage [6] and myonecrosis [2]. Five hemorrhagic components (BaH1, BH2, BH3, BaP1, and BaH4) and four myotoxins (Basp-I, Basp-II, Basp-III, and Basp-IV) have been isolated from this venom [2,7–10]. BaP1 and BaH4, *B. asper* SVMPs used in this study, are examples of P-I and P-III hemorrhagic toxins, respectively. Among the

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Abbreviations: SVMPs, snake venom metalloproteases; ABC, anti-bothropic complex from *Didelphis albiventris* serum; DA43, 43-kDa subunit of anti-bothropic complex from *D. albiventris* serum; DA45, 45-kDa subunit of anti-bothropic complex from *D. albiventris* serum; MHD, dose able to induce a hemorrhagic lesion of 10 mm diameter; BaP1, basic hemorrhagic metalloprotease from *Bothrops asper* venom; BaH4, acidic hemorrhagic metalloprotease from *B. asper* venom; Basp-I, myotoxin I from *B. asper* venom; Basp-II, myotoxin II from *B. asper* venom; and Basp-III, myotoxin III from *B. asper* venom.

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myotoxins isolated from *B. asper* venom, Basp-I and Basp-III Asp-49 are phospholipases A_2 (Asp-49-PLA_2, with detected phospholipase A_2 activity), whereas Basp-II is a Lys-49 PLA_2-like myotoxin (with no phospholipase A_2 activity on artificial substrates).

Intravenous administration of antivenoms constitutes the main way to treat snakebite envenomations [11]. However, neutralization of local effects is achieved only partially [12]. Consequently, there is need to develop alternative approaches that may be applied, complementing antivenom therapy in the neutralization of venom-induced local effects.

Some animals present natural resistance toward the effects of snake venoms and, in many cases, this resistance can be explained by the presence of neutralizing factors in their blood sera [13–15]. Specifically, the resistance to hemorrhagic venoms has been attributed to serum proteins that have some common features: they are acidic glycoproteins with molecular masses ranging from 52 to 90 kDa and highly stable to variations in pH and temperature.

From *Didelphis sp* serum (common Central and South American opossum), an ABC was isolated, composed of two acidic glycoprotein subunits with estimated M_r values of 45,000–48,000, respectively [16–20]. ABC is a venom metalloprotease inhibitor that shows, in addition to its anti-hemorrhagic activity, anti-myonecrotic [20–22], anti-lethal [17,18], anti-edematogenic [18,20,23], and anti-hyperalgesic [18,24] properties.

In this study, we investigated the inhibitory properties (anti-hemorrhagic, anti-proteolytic, anti-lethal, anti-myotoxic, anti-edematogenic, and anti-phospholipase A_2) of ABC from *Didelphis albiventris* serum on toxic and pharmacological actions of the metalloproteases (BaP1 and BaH4) and myotoxins (Basp-I, -II, and -III) isolated from *B. asper* snake venom. A new procedure for the purification of ABC by affinity chromatography is described, based on its high specificity for BaP1. In addition, the N-terminal sequence (first 14 amino acid residues) of both subunits is reported.

2. Materials and methods

2.1. Reagents

Fibrinogen from bovine plasma was from the Sigma Chemical Co. CNBr-activated Sepharose 4B was purchased from Pharmacia. *D. albiventris* specimens were caught in the outskirts of Ribeirão Preto City, under a license of the Brazilian Environmental Institute (IBAMA). Purified metalloproteases and myotoxins were isolated from *B. asper* venom as previously described [8,10,25].

2.2. Purification of anti-bothropic complex

Purified metalloprotease BaP1 was coupled to 10 mL of swollen CNBr-activated Sepharose 4B, as described by the manufacturer. The matrix was packed in a column and equilibrated with 0.1 M sodium phosphate buffer (PB), pH 7.2. Lyophilized serum from *D. albiventris* (500 mg) was dissolved in 10 mL PB and passed through the Sepharose 4B-metalloprotease column at a flow rate of 2 mL/min. The effluent was recirculated for 1 hr, followed by extensive washing with PB. Absorbance of the effluent solution was recorded at $\lambda = 280$ nm on an ISCO UV detector system. Bound material was eluted with 0.1 M Gly–HCl buffer, pH 3.0, and the peak was collected in a reservoir containing 2 mL of 0.5 M Tris–HCl, pH 8.0. Buffer was usually removed from the inhibitor preparations by ultrafiltration through an Amicon YM30 membrane, followed by lyophilization.

2.3. Isolation of DA43 and DA45

The subunits were separated further by ion-exchange HPLC on a Shimadzu anionic column (2.0 × 25.0 cm) with a linear 0 to 1.0 M gradient of sodium chloride in 100 mM sodium acetate buffer, pH 4.0, at a flow rate of 1.0 mL/min for 70 min. The elution profile was monitored by the absorbance at 280 nm, and the protein concentration was determined by the micro-biuret method [26]. All steps of the purification procedure were carried out at room temperature (~ 25°).

2.4. Biochemical characterization of the anti-bothropic complex

SDS–PAGE was run as previously described [27]. Isoelectric focusing was carried out according to Vesterberg [28]. Buffalyte, pH range 3.0 to 9.0, was used to generate the pH gradient. Determination of the amino acid composition was performed using the phenylthiocarbamyl derivative method [29] after acid hydrolysis with 6 N HCl containing 1% phenol at 110° for 4 or 22 hr. A mixture containing 2.5 nmol of each amino acid (Standard H, Pierce Chemical Co.) was freshly derivatized, and 4% (100 pmol) was used as the standard. Amino acid sequencing was performed with a model 491 Procise sequencer (Perkin-Elmer-Applied Biosystem Division) using either a gas-phase or pulse liquid system with on-line identification of phenylthiohydantoin derivatives. Subunits (DA43 and DA45) were freed of any possible contaminating material by SDS–PAGE and blotted onto a PVDF membrane. Glycoproteins were visualized using periodic acid Schiff reagent.

2.5. Hemorrhagic activity

The method of Kondo *et al.* [30] was used to quantitate hemorrhagic activity. Swiss-Webster mice were injected intradermally with doses of 2–20 μg of either crude *B. asper* venom or isolated venom metalloproteases, dissolved in 0.1 mL PBS. Control mice received PBS alone. Three hours later, mice were killed, and the diameter of the hemorrhage spot in the skin was measured.

2.6. Proteolytic activity on fibrin

The method described by Rodrigues *et al.* [31] was followed with 0.2% (w/v) human fibrinogen in 5 mM phosphate buffer containing 0.15 M NaCl, pH 7.4. The solutions were pipetted into Petri dishes, and 100 U bovine thrombin in 1 mL of the same buffer was added. The mixture was kept standing until clotting was complete (1 hr at room temperature). Concentrations of 2–40 μg of the metalloproteases in a 15 μL volume were then added and incubated for 24 hr at 37°. The haloes of lysis were measured and compared with those of a control in which the enzyme solution was replaced by buffer.

2.7. Proteolytic activity on fibrinogen

The method of Rodrigues *et al.* [31] was used with some modifications. Samples of 50 μL of bovine fibrinogen (1 mg/mL of PBS) were incubated with concentrations of 2.0 μg of enzyme at 37° for 2 hr. The reaction was stopped with 25 μL of 0.05 M Tris–HCl buffer, pH 8.8, containing 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 2% (w/v) SDS, and 0.05% (w/v) bromophenol blue. The samples were then analyzed by 13.5% (w/v) SDS–PAGE. The effect of ABC on the fibrinogenolytic activity was assayed after preincubation of 2 μg enzyme with different amounts of ABC.

2.8. Proteolytic activity on casein

Proteolytic activity was measured by the method of Rodrigues *et al.* [31], using casein as the substrate. Briefly, 0.5 mL of crude venom or BaP1 solution (20 μg/mL) was incubated with 1.5 mL of 1% (w/v) casein in 0.1 M Tris–HCl buffer (pH 8.0) for 15 min at 37°. The reaction was stopped by the addition of 3 mL of 5% (v/v) trichloroacetic acid (TCA), and the mixture was left standing for 30 min at room temperature before centrifugation (1500 *g*) for 5 min at 25°. Proteolytic activity was estimated by recording the absorbance of the clear supernatant at 280 nm. Proteolytic activity was also assayed in the presence of ABC, preincubating 20 μg of the enzyme with different amounts of ABC for 30 min at 37°. One unit of caseinolytic activity was defined as the amount of enzyme that produces an increase in absorbance of 0.001 units/min.

2.9. Myotoxic activity

The assay for determination of plasma creatine kinase (CK) activity was carried out using the CK-UV kinetic kit from Sigma. Toxins (1 μg/μL) were injected in 18–22 g male Swiss mice (50 μL; i.m.) (N = 6). Animals used as negative controls were injected with PBS. After 3 hr, a blood sample was collected from the tail in heparin-coated capillary tubes and centrifuged at 450 *g* for 10 min at 25° for plasma separation [32,33]. CK activity was determined using 4 μL plasma according to the instructions of the manufacturer. Enzyme activity was expressed in U/L, one unit being defined as the amount of enzyme that produces 1 μmol of NADH/min under these assay conditions.

2.10. Edema-inducing activity

Groups of six male Swiss mice (18–22 g) were injected in the subplantar region with 50 μL of myotoxins (50 μg). At different time intervals, paw edema was measured using a low pressure spring caliper (Mitutoyo) [32,33]. Zero time points were subtracted from all of the values, and the differences were expressed as the percent increment (median percent ± SD).

2.11. Lethal dose 50%

Lethality induced by *B. asper* venom and isolated myotoxins was evaluated by i.p. injection of 2 ld_{50} in male Swiss mice (18–22 g; N = 6). Deaths occurring within 48 hr were recorded.

2.12. Phospholipase a_2 activity

Phospholipase A_2 (doses of 50 μg) activity of isolated venom phospholipases A_2 was assayed as previously described [34].

2.13. Inhibition studies of biological activities

Doses of crude *B. asper* venom or isolated venom metalloproteases or phospholipase A_2 myotoxins were selected after dose–response studies for each particular effect. Inhibition by ABC was evaluated after incubation at different ABC:toxin (w/w) ratios (4:1, 2:1, 1:1, or 0.5:1) for 30 min at 37°. Biological and enzymatic activities were expressed as a percentage, 100% corresponding to the activity of venom, metalloproteases, or myotoxins incubated without the inhibitor.

2.14. Statistical analysis

Results are presented as means ± SD obtained with the indicated number of animals. The statistical significance of differences between groups was evaluated using Student's unpaired *t*-test. A P value < 0.05 was considered to indicate significance.

3. Results

3.1. Isolation and biochemical characterization of ABC

Resolution of ABC from *D. albiventris* serum into its two subunits (43 and 45 kDa, respectively) by ion-exchange HPLC is shown in Fig. 1. The N-terminal sequence for both subunits was: LKAMDPTPXLWIETESP, where X is Arg-9 in DA43 and Pro-9 in DA45 (Table 1). Amino acid analysis

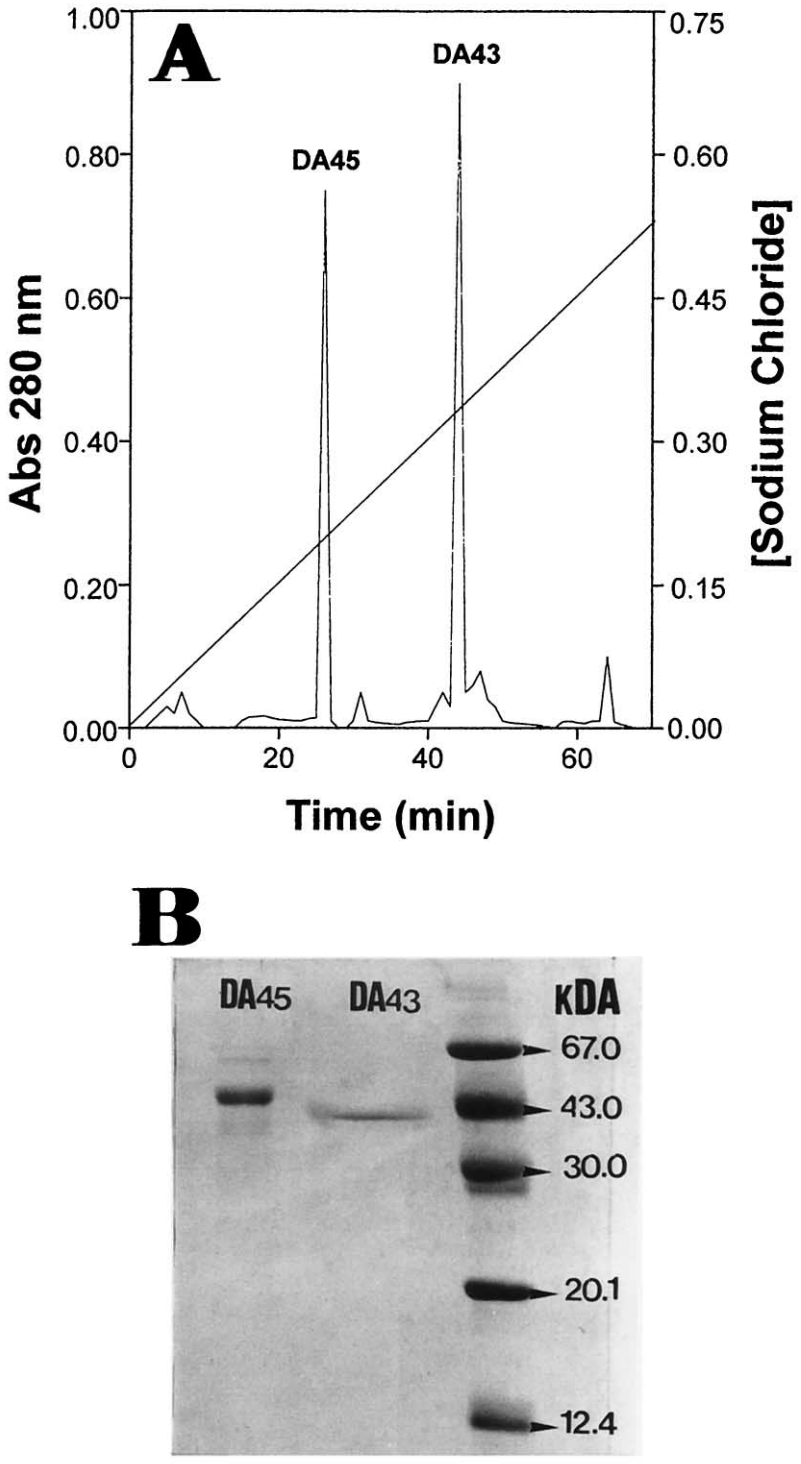

Fig. 1. Ion exchange HPLC and SDS–PAGE analysis of the anti-hemorrhagic complex (ABC) from *D. albiventris* serum. (A) Ion exchange HPLC on a Shimadzu anionic column (2.0 × 25.0 cm) of affinity-purified ABC yielded two protein fractions (DA43 and DA45). Elution was initiated with 100 mM sodium acetate buffer, pH 4.0, followed by a linear gradient (0 to 1.0 M) of sodium chloride. Fractions (0.5 mL) were collected at a flow rate of 1.0 mL/min. (B) SDS–PAGE (13.5%) of DA45 (lane 1), DA43 (lane 2), and molecular mass standards (lane 3).

showed 286 residues for DA43 and 279 for DA45 (Table 2). The presence of carbohydrates was evidenced by means of the Schiff reagent, and the isoelectric point was < 4 for both subunits (results not shown).

3.2. Inhibition of biological and enzymatic activities by ABC

The hemorrhagic activity induced by crude *B. asper* venom (MHD $\cong$ 2.5 μg) or the isolated venom metallopro-

Table 1
Comparison of the N-terminal amino acid sequences of DA43 and DA45 with other anti-hemorrhagic factors from *Didelphis* species serum

Protein	Source	N-terminal sequence[a]	Similarity (%)	Reference
DA43	*D. albiventris*	LKAMDPTPRLWIETESP	100.0	This work
DA45	*D. albiventris*	LKAMDPTPPLWIETESP	93.7	This work
DM40	*D. marsupialis*	LKAMDPTPRLWIKTESPS	93.7	[18]
DM43	*D. marsupialis*	LKAMDPTPPLWIKTEXP	87.5	[18]
DA2-II	*D. albiventris*	LKAMDTTPPLKIKKEPVK	62.5	[19]
Oprin	*D. virginiana*	LKAMDTTPRLWIETESPS	93.7	[35]

[a] Conserved residues among the proteins are enclosed in a box.

teases, BaP1 (MHD ≅ 20 μg) and BaH4 (MHD ≅ 2.0 μg), was inhibited approximately 50, 70, and 58%, respectively, by ABC, at a venom:inhibitor ratio of 1:1 (w/w) (Fig. 2A). The proteolytic activities of *B. asper* venom and the metalloproteases when assayed on fibrin (Fig. 2B), fibrinogen, and casein (results not shown) were also inhibited by ABC. Inhibition of the hemorrhagic and proteolytic activities was shown to be dose dependent. ABC alone did not induce any proteolysis on these substrates.

In addition to the anti-hemorrhagic and anti-proteolytic properties of ABC, inhibitory effects were also observed on the myotoxicity, edema-inducing, lethality, and phospholipase A_2 activities of the crude *B. asper* venom and its myotoxins: Basp-I (Asp-49 myotoxin), Basp-II (Lys-49 myotoxin), and Basp-III (Asp-49 myotoxin).

Table 2
Amino acid composition of DA43 and DA45 from *D. albiventris* serum

Amino Acid	DA43 (mol/mol)	DA45 (mol/mol)
Asp	24.68 (25)[a]	23.78 (24)[a]
Thr	21.45 (21)	19.71 (20)
Ser	21.09 (21)	20.82 (21)
Glu	27.69 (28)	28.05 (28)
Pro	27.08 (27)	25.05 (25)
Gly	21.03 (21)	19.78 (20)
Ala	14.21 (14)	14.77 (15)
½ Cys	6.02 (6)	5.86 (6)
Val	21.91 (22)	19.84 (20)
Met	3.12 (3)	3.68 (4)
Ile	10.84 (11)	10.86 (11)
Leu	27.05 (27)	26.13 (26)
Tyr	7.12 (7)	6.89 (7)
Phe	14.09 (14)	12.86 (13)
Lys	11.87 (12)	11.74 (12)
His	1.89 (2)	2.77 (3)
Arg	15.09 (15)	13.81 (14)
Trp	10.27 (10)	8.98 (9)
Total residues	(286)	(279)
Molecular mass of the proteic portion	31,460	30,690
Total molecular weight[b]	43,000	45,000
% Calculated carbohydrate	26.83	31.80

[a] Assumed integer values are in parentheses.
[b] SDS-PAGE.

ABC was also efficient in neutralizing the myotoxicity induced by *B. asper* venom and myotoxins I and II, when present at a 1:4 (w/w) ratio (Fig. 3A). Edema induced 30 min after injection of *B. asper* venom, as well as myotoxins I and II, was inhibited by ABC up to 58, 78, and 88%, respectively, at a 1:8 venom:ABC (w/w) ratio (Fig. 3B). Assays for the detection of the inhibition of myotoxic activity without previous incubation of ABC with *B. asper* venom were also carried out. A partial reduction (25–30%) of these venom activities was observed when venom and inhibitor were injected simultaneously, but only 5–10% reduction was observed when the inhibitor was injected 5 min after the venom (results not shown). Lethality induced by 2 ID_{50} of *B. asper* venom (≅ 150 μg/mice) was partially inhibited by ABC, at a venom:inhibitor ratio of 1:4 (w/w), while a higher anti-lethal effect was observed against the isolated venom myotoxins (2 ID_{50} ≅ 300 μg/mice), using the same ponderal ratio (Fig. 4A). On the other hand, phospholipase A_2 activities of the crude *B. asper* venom and venom myotoxins Basp-I and Basp-III were inhibited 20–35% by ABC, at a 1:2 venom:ABC (w/w) ratio (Fig. 4B).

4. Discussion

In the last few years, the number of anti-toxin factors isolated from the blood of snakes and mammals, which neutralize specific snake venom toxins, grew significantly. Anti-hemorrhagins [36], anti-neurotoxins [37], and anti-phospholipases A_2 [38–40] are some examples. Snake venom metalloprotease inhibitors were isolated from the sera of different mammals and snakes [14], including *D. virginiana*, *D. marsupialis*, *D. albiventris*, *Trimeresurus flavoviridis*, and *B. asper*. According to structural homology studies, these proteins can be classified in at least two different groups: a supergene family of immunoglobulins (anti-hemorrhagins from *D. virginiana*) and a superfamily of cystatins (from *T. flavoviridis*) [35,41]. In spite of that, structural and functional studies are still needed to afford a better understanding of the action mechanisms of these

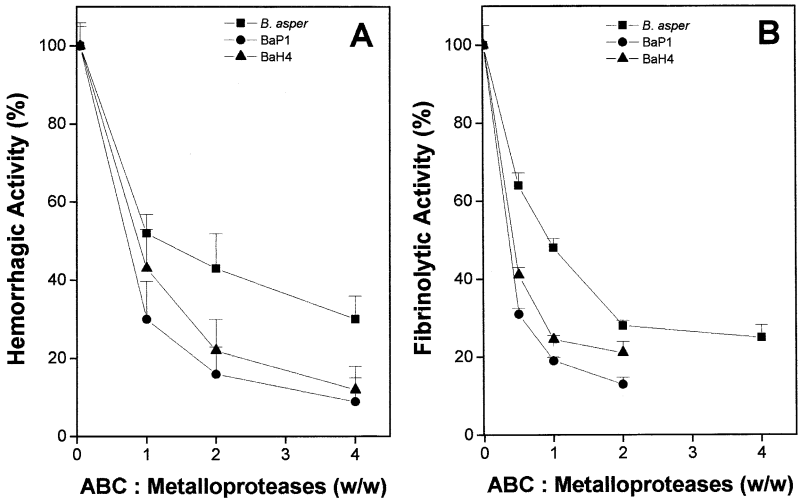

Fig. 2. Inhibition of hemorrhagic (A) and fibrinolytic (B) activities of crude *B. asper* venom or its component metalloproteases (BaP1 and BaH4) by ABC. A constant amount of crude venom or metalloprotease (2 MHD and 1–20 μg for hemorrhagic and fibrinolytic activities, respectively) was incubated for 30 min at 37° with either PBS or various concentrations of ABC (w/w). Hemorrhagic and fibrinolytic activities were expressed as a percentage, 100% corresponding to the activity of the crude venom or its metalloproteases incubated without the inhibitor. One hundred percent hemorrhagic activity = 19.5 ± 1.3 mm for crude *B. asper* venom and 20.8 ± 1.1 and 20.3 ± 1.6 mm for BaP1 and BaH4, respectively. One hundred percent fibrinolytic activity = 20.5 ± 0.5 mm for crude *B. asper* venom and 20.0 ± 0.5 and 11.0 ± 0.5 mm for BaP1 and BaH4, respectively. Each point represents the mean ± SD (N = 6).

natural inhibitors. Consequently, the search for alternative treatments against snakebite accidents is a relevant task.

ABC was isolated previously from the sera of *D. albiventris* by ion-exchange chromatography on DEAE-Sephacel [20]. We report now on its purification by affinity chromatography using the metalloprotease BaP1, isolated from *B. asper* venom, coupled to a Sepharose 4B gel, thus exploiting the specificity of the ABC for this protease. This inhibitor is an acidic glycoprotein complex comprised of two subunits (43 and 45 kDa), with a high degree of identity in their N-terminal amino acid sequence (first 17 residues). When compared with other natural inhibitors isolated from opossums, some invariable residues are found in the N-terminal region, such as Pro-8, Leu-10, Trp-11, and Glu-15. Recently it was demonstrated that the N-terminal amino acid sequence of the anti-bothropic factor (ABF) isolated from the sera of *D. marsupialis*, in addition, contains a unique amino acid substitution at position 9. The DA43 subunit shows an amino acid composition very similar to that of DA45 and to other serum inhibitors [18,35] in which

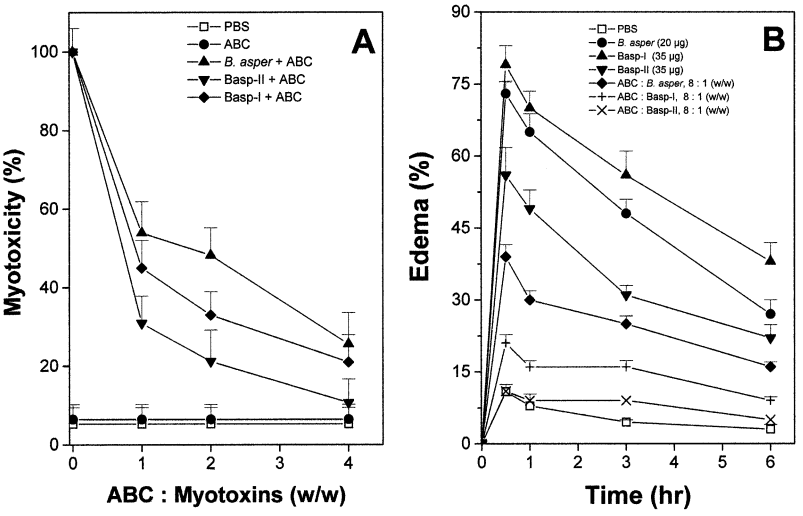

Fig. 3. Inhibition of myotoxic (A) and edema-inducing (B) activities of crude *B. asper* venom or its component myotoxins (Basp-I and -II) by ABC. For evaluation of myotoxic activity, a constant amount of crude venom (25 μg) or myotoxins (50 μg) was incubated for 30 min at 37° with PBS or various concentrations of ABC (w/w). Myotoxicity and edema-inducing activity were expressed as a percentage, 100% corresponding to the activity of venom or myotoxins incubated without the inhibitor. One hundred percent myotoxicity = 2498.45 ± 283.12 U/L for *B. asper* venom, and 1678.97 ± 201.69 and 1054.23 ± 178.55 U/L for Basp-I and Basp-II, respectively. One hundred percent edema-inducing activity = 2.28 ± 0.17 mm for *B. asper* venom, and 2.10 ± 0.10 and 1.56 ± 0.12 mm for Basp-I and Basp-II, respectively. Each point represents the mean ± SD (N = 6).

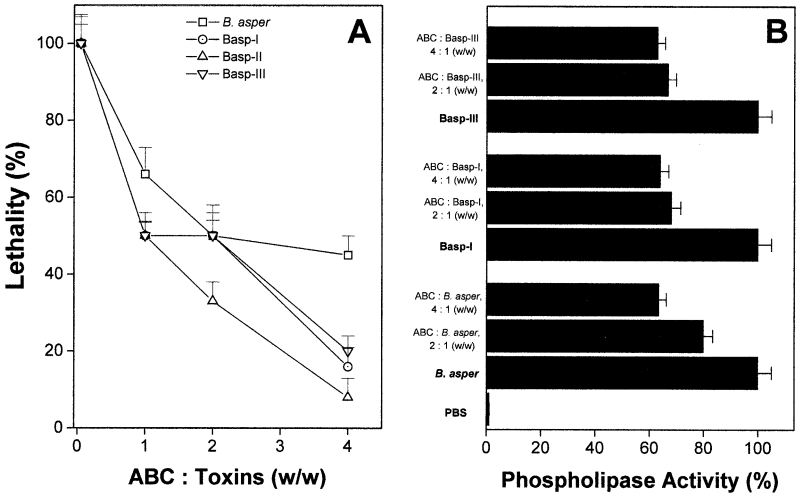

Fig. 4. Inhibition of lethal (A) and phospholipase A_2 (B) activities of crude *B. asper* venom or its component myotoxins (Basp-I, -II, and -III) by ABC. A constant amount of crude venom or myotoxins (2 ld_{50} and 50 μg for lethality and PLA_2 activity, respectively) was incubated for 30 min at 37° with PBS or various concentrations of ABC (w/w). Lethality and phospholipase A_2 activity were expressed as a percentage, 100% corresponding to the activity of venom or myotoxins incubated without the inhibitor. One hundred percent lethality = 7.65 ± 1.21 μg/g for *B. asper* venom, and 13.87 ± 1.05, 16.61 ± 1.33, and 15.03 ± 1.45 μg/g for Basp-I, Basp-II, and Basp-III, respectively. One hundred percent phospholipase A_2 activity = 69.4 ± 2.5 U/mg for *B. asper* venom, and 49.7 ± 1.8 and 53.1 ± 2.1 U/mg for Basp-I and Basp-III, respectively. Each point represents the mean ± SD (N = 6).

nearly 48% of the amino acid residues were hydrophobic. ABC isolated in this work is relatively thermostable [0–45°] and pH stable between 3.0 and 9.0 (results not shown). The above described properties of ABC (isoelectric point, carbohydrate presence, temperature, and pH stability) are common to other natural inhibitors isolated from mammals and snakes [15].

Snake venom metalloproteases induce hemorrhage, myonecrosis, dermal necrosis, and edema. The hemorrhagic and proteolytic activities against casein, fibrinogen, and fibrin induced by the crude *B. asper* venom and its component metalloproteases were inhibited by ABC, which alone did not induce any toxic, enzymatic, or biologic activity on the assayed models. ABC also presents a higher specificity for metalloprotease BaP1, inhibiting the hemorrhage and proteolysis induced by this low molecular weight protease more than those caused by whole *B. asper* venom and BaH4. These findings are in accordance with the results found for other metalloproteases of low molecular weight isolated from the venom of *B. neuwiedi* (Trento EP and Soares AM, unpublished results).

In addition to its primary hydrolytic function, snake venom phospholipases A_2 induce several toxic and pharmacological effects, such as myotoxicity, neurotoxicity, edema, inhibition of blood platelet aggregation, hypotension, and anticoagulation [1,2]. *B. asper* venom contains basic phospholipase A_2 myotoxins (~ 14 kDa) that are involved with the local tissue damage. These toxins belong to the group II phospholipases A_2, but only myotoxins I and III have enzymatic activity. Myotoxin II (Basp-II) and its catalytically inactive isoform Basp-IV have a Lys-49 residue substituting for the typical aspartic acid (Asp-49), which is required for the binding of Ca^{2+}, essential for enzymatic activity. Besides the anti-hemorrhagic and anti-proteolytic properties of ABC, this serum inhibitor showed anti-lethal, anti-myotoxic, and anti-edematogenic properties. These results suggest that the ability of ABC to inhibit a wide spectrum of pharmacological and toxic effects could be a consequence of the primary inactivation of myotoxins and metalloproteases present in *B. asper* venom.

Recently, Neves-Ferreira *et al.* [18] have investigated the interaction between the inhibitors DM40 and DM43, isolated from *D. marsupialis* serum, and the metalloprotease jararaghin, from *B. jararaca* venom. Both DMs formed stable and soluble complexes with jararaghin and inhibited its hemorrhagic and enzymatic activities on fluorogenic substrates. These inhibitors were not hydrolyzed by this protease. As observed in our study, the same action mechanism of inhibition probably occurs with ABC from *D. albiventris*, leading to the production of a soluble inactive complex between ABC and the toxin (metalloprotease or myotoxin). This hypothesis is apparently the most acceptable for the inhibitors of low M_r SVMPs, since other authors have also shown the formation of complexes between the inhibitors and toxins either by chromatographic or electrophoretic techniques [17,18,35].

In addition, this inhibitory mechanism may involve, besides the acidic character of the inhibitor and low M_r proteins [13], participation of carbohydrates to form this stable complex, since, after deglycosylation, both DMs from *D. marsupialis* became insoluble [18].

Lethality induced by the crude *B. asper* venom was inhibited almost 50% by ABC at a toxin:inhibitor ratio of 1:2 (w/w), while the individual venom myotoxins were inhibited about 80–95% by ABC at a ratio of 1:4 (w/w). Similar results were observed when a DM43 subunit, iso-

lated from *D. marsupialis*, was incubated with crude venom from *B. jararaca* [18]. ABC was efficient in neutralizing lethal, myotoxic, and edematogenic effects induced by myotoxins I and II of *B. asper*. The anti-myotoxic and anti-edematogenic effects of ABC have a higher specificity for the catalytically inactive Lys-49 Basp-II myotoxin than the enzymatically active Asp-49 Basp-I myotoxin. The phospholipase A_2 activity of the Asp-49 myotoxins was reduced to a lesser extent than that of the Lys-49 myotoxins, showing that ABC is not a good inhibitor of phospholipases A_2. Similar results regarding phospholipase activity were obtained when ABC was incubated with venoms of *B. moojeni*, *B. jararacussu*, and *B. pirajai* [20].

Since phospholipases A_2 from snake venoms participate in different pathophysiological effects, studies on the isolation and characterization of natural inhibitors of phospholipases A_2 from snake venoms, animals, and plants have been actively pursued and noticed in the last few years. Borges *et al.* [42] showed recently that the extract from *Casearia sylvestris* was efficient in neutralizing the phospholipase A_2 and myotoxic activities of crude venoms and phospholipases A_2 isolated from different species of snakes.

To further elucidate the inhibitory specificity of ABC from *D. albiventris* against myotoxins and metalloproteases isolated from snake venoms, a deeper understanding of its structure and function is necessary. Furthermore, the potential use of these inhibitors to complement anti-venom as an alternative treatment of snakebite envenomations needs to be evaluated in future studies.

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