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Purification of a fragment obtained by autolysis of a PIIIb-SVMP from *Bothrops alternatus* venom

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Introduction

Snake venoms comprise a complex pool of proteins, peptides, organic and inorganic compounds which display a variety of biological activities. Viperid venoms may contain several hundred proteins [1], most of which are proteinases, such as serine- and metalloproteinases. *Bothrops alternatus* is a pitviper widespread in South America that represents one of the most important species associated to snakebites in Argentina. Snake Venom Metalloproteinases (SVMPs) represent 43.1% of the identified components in this venom [2] and play an important role in envenomation, causing relevant local effects such as hemorrhage, edema and myotoxicity as well as systemic bleeding [3, 4].

SVMPs can be divided into three classes (PI to PIII) according to the extension of several structural domains [5, 6]. PI enzymes comprise only the metalloproteinase domain, PII enzymes contain a disintegrin domain after the metalloproteinase domain, and PIII enzymes contain an additional cysteine-rich carboxyl terminal domain. PIII class is the most remarkable of the SVMPs groups in terms of its contribution to venom complexity. PIII-SVMPs are further divided into subclasses based on their distinct post-translational modifications: PIIIa, a subclass refractory to proteolytic processing; PIIIb, which undergoes autoproteolysis to release a 30 kDa fragment with a disintegrin-like/cystein-rich domain (DC domain); and PIIIc, which forms a dimeric structure. Formally called PIV, the heterotrimeric class of SVMPs that contain an additional snake C-type lectin-like (snaclec) domain [7] is included in the PIII group as PIIId subclass, as no P-IV mRNA transcript has been found to date [6].

Disintegrins represent a family of polypeptides present in venoms that selectively block the function of integrin receptors [8]. Disintegrins and disintegrin-like domains are released in venom by proteolytic processing of PII and PIII metalloproteinases respectively [9], and represent potent inhibitors of integrin–ligand interaction. The members of the PII class are characterized by the presence of a disintegrin domain, which contain an adhesive tripeptide motif such as RGD, VGD, or KGD, among others [10]. The best characterized are the RGD-containing disintegrins. They are non-enzymatic peptides which inhibit platelet aggregation and interact with adhesion molecules in particular integrins in a dose-dependent manner [11]. Another group of integrin–ligand interaction inhibitors comprise the disintegrin-like domains, which are released by proteolytic processing of PIII-SVMPs, non-RGD molecules containing disintegrin-like and cysteine-rich domains [12, 13]. In this group, the RGD motif is replaced by an X-E/DCD disulfide-bonded cysteinyl sequence in that region [14].

Baltergin is a PIIIb-SVMP isolated from *B. alternatus* venom able to undergo autolysis *in vitro*, giving rise mainly to a~30kDa stable fragment [3]. Several disintegrin-like/cystein-rich fragments obtained by *in vitro* processing of SVMPs have been described [15-20]. Proteolytically processed fragments, lacking the metalloproteinase domain, are relatively non-toxic compounds with potential properties related to disintegrin activity, such as inhibition of cell adhesion and platelet aggregation [21, 22]. Additionally, other processed forms have been isolated directly from snake venom. For example, processed disintegrin-like/cystein-rich domains and jararhagin-C were obtained from *B. atrox* [20] and from *B. jararaca* [16] venoms, respectively. A 28 kDa disintegrin-like/cystein-rich domain, named alternagin-C, was directly isolated from *B. alternatus* venom [22]. This protein inhibits integrin-mediated cell adhesion and selectively interacts with the major collagen I receptor, the $\alpha_2\beta_1$-integrin, inhibiting collagen binding [22, 23]. Although there are several works which report pharmacological effects of processed disintegrin-like domains, their production from native protein autolysis, subsequent purification and characterization have not been described so far. In this work,

autolysis conditions of baltergin were adjusted in order to carry out the purification of a disintegrin-like/cystein-rich fragment and its effect on cell adhesion was studied.

2. Materials and methods

2.1. Venom

B. alternatus venom was purchased from the serpentarium of the local zoo, Corrientes, Argentina. The pools were desiccated and stored at -20 °C. When required, the venom was diluted with phosphate buffered saline solution, pH 7.0. The small amount of insoluble material was centrifuged and the clear supernatant was used for baltergin purification.

2.2. Baltergin isolation

Baltergin was isolated from *B. alternatus* crude venom (40 mg) as described previously [3]. In order to assay the purity of the isolated enzyme, electrophoresis was performed on 12% acrylamide slab gels [24].

2.3. Autocatalytic processing assay of baltergin

The enzyme was incubated at 37 ºC (pH 7.5) at different times (5 and 30 min, 2, 4, 6, 18, 24, 48 and 96 h). Baltergin was also incubated for 18 h at different pHs (3.0, 5.0, 7.0, 8.0 and 9.0) and temperatures (4, 25, 37 and 45 °C). At the extreme pH values (3 and 9), autoproteolytic activity was also tested at 4 °C. Immediately after incubation, autolysis was stopped by addition of sample buffer with 2-ME and aliquots were analysed by SDS–PAGE (10% or 12% of acrylamide).

2.4. Residual proteolytic activity

Enzyme (0.57 mg/mL) was incubated at different pHs and temperatures for 18 h; the residual proteolytic activity was assayed using azocasein as substrate (Sigma-Aldrich, USA), according to a method previously described [25]. The enzyme without pre-incubation was used as a control.

2.5. N-terminal sequence

After electrophoresis, the 32 kDa band was electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, USA). The protein band was subjected to N-terminal amino acid sequence analysis employing a PPSQ-33A (*Shimadzu*) analyser, following manufacturer's instructions.

2.6. Purification of the 32 kDa fragment

Baltergin was incubated for 96 h at 37 °C in 0.01 M phosphate buffer, pH 7.5. Separation of proteolytic fragments was performed by exclusion chromatography using an ÄKTA Explorer 10 (Pharmacia). Sample solution was applied to a Superdex 75 column (HiLoad® 16/600 Superdex 75 pg) and eluted with 0.01 M phosphate buffer, pH 7.5. Eluted fractions were then analysed by 12% SDS-PAGE.

A comparative assay was performed with *B. alternatus* venom, baltergin and baltergin-DC (1 mg/mL) in order to check the proteolytic activity using azocasein as substrate (Sigma-Aldrich, USA), according to the method described above.

2.7. Cell culture

C2C12 (CRL-1772, ATCC) is a subclone murine skeletal muscle cell line derived from mouse myoblast cells obtained from normal adult C3H leg muscle. This cell line rapidly differentiates and produces extensive contracting myotubes that express characteristic muscle proteins [26]. Cells were grown in 25 cm^2 flasks using Dulbecco's minimum essential medium (DMEM – Gibco, Argentina), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA), Penicillin-Streptomycin (Invitrogen), in a humidified atmosphere with 5% CO_2 at 37 °C.

2.8. Adhesion assay

For adhesion assay, C2C12 cells (2 x10^4/well) were preincubated for 30 min at room temperature with baltergin or 32 kDa fragment suspension (0.45 - 1.80 μM in DMEM - 5% FBS) and then added to 96-well plate previously coated with FBS:PBS (1:4) for 1 h at 37 °C followed by blocking with 1% heat-denaturated BSA (Sigma-1 μg/μL) in PBS. Cells preincubated only with culture medium were used as control group. After 1.5 h, non-adherent cells were removed by careful aspiration and washed with PBS. The unattached cells were stained with Trypan blue dye to assess viability. Adherent cells were fixed with methanol:glacial acetic acid (3:1 ratio), and stained with 0.5% crystal violet in 20% (v/v) methanol. After dye release with ethanol:glacial acetic acid (3:1 ratio), adherent cells were microscopically observed and recorded photographically. The optical density of the released dye solution was read at 620 nm and the percentage of cell adhesion was determined relative to those obtained for the control assays, which were arbitrarily set as 100%. All assays were carried out in triplicate.

2.9. Statistical analysis

Data represent the mean ± standard deviation (SD) of at three or four replications. Groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's test. A value of $P < 0.05$ indicated statistical significance.

3. Results

3.1. Autocatalytic processing assay

The time course analysis of the electrophoretic profile of baltergin incubated at pH 7.5 and at 37 ºC showed a progressive disappearance of proteinase band (55 kDa) and an increase in bands ranging from 5 to 45 kDa (Fig. 1.A). After 96 h of incubation the main degradation product was a band of 32 kDa, a molecular mass compatible with the disintegrin-like and cysteine-rich domain, named baltergin-DC (DC: disintegrin-like/cystein-rich).

Regarding pH and temperature conditions, electrophoretic patterns showed an increase in the intensity of the 32 kDa band mainly at 37 ºC and at the optimal pH of 7.0 – 8.0 (Fig. 1.B-C). When baltergin autolysis was assayed at 25 ºC, 45 ºC or at pH 9.0, an additional band of 45 kDa was observed. Appreciable degradation did not take place at 4 ºC (pH 7.0) even after incubation of 18 h. Similarly, baltergin band remained unchanged at pH 3.0.

Fig. 1 Analysis of baltergin autolysis. **A**. Baltergin was incubated at different times (0 and 30 min, 2, 18, 4, 6, 18, 24, 48 and 96 h) at 37 ºC (pH 7.5), aliquots were run under reducing conditions in 12% SDS-PAGE. Gel was stained with Coomassie Blue. Molecular weight markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), anhydrase carbonic (29 kDa), soybean trypsin inhibitor (20.1 kDa), cytochrome C (12.4 kDa). **B**. Baltergin was incubated at different pHs (3.0, 5.0, 7.0, 8.0, 9.0) at 37ºC for 18 h. **C**. Baltergin was incubated at different temperatures (4, 25, 37, 45 ºC) at pH 7.0 for 18 h. In B-C: control is baltergin without incubation; samples were run under reducing conditions in 10% SDS-PAGE and were silver and Coomassie Blue stained, respectively. Molecular weight markers: phophorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), anhydrase carbonic (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

3.2. Residual proteolytic activity

Autolysis of baltergin was also evaluated by measuring residual azocaseinolytic activity after 18 h of incubation, varying conditions of temperature and pH. Residual proteolytic activity decreased with increasing temperature up to 45 °C, at this temperature the activity was almost suppressed (Fig. 2.A-B). Very low residual activity was observed at pH 8.0 as expected from SDS-PAGE patterns. Such alkaline conditions do promote autolysis *in vitro*. However, the activity was completely abolished at extreme pH, 3.0, after 18 h of incubation at 37 ºC. Interestingly, when incubation was carried out at 4 ºC under the same pH conditions, the residual activity was partially retained at pH 9.0 but not at pH 3.0 (data not shown). Although alkaline pH promotes autolysis, a combination of extreme pH and high temperature leads to protein denaturation.

Fig. 2 Residual proteolytic activity of baltergin (0.57 mg/mL) after 18 h of incubation. **A**. Baltergin was incubated at different pHs (3.0, 5.0, 7.0, 8.0, 9.0) at 37 ºC. **B**. Baltergin was incubated at different temperatures (4, 25, 37, 45 ºC) at pH 7.0. Control sample is activity of baltergin without incubation. Asterisks indicate statistically significant differences in comparison with control.

3.3. N-terminal sequence of 32 kDa fragment

Autolysis fragments were separated by SDS-PAGE under reducing conditions and were transferred to a PVDF membrane. The band of 32 kDa (baltergin-DC) was cut and the sequence was determined. A 36-residue peptide derived from the N-terminal sequence, which begins with the residue isoleucine (I), showed identity with others disintegrin-like/cysteine-rich domains from viperid species [16, 22]. Fig. 3 shows sequence alignment of the N-terminal of baltergin-DC and two ECD-containing disintegrin-like proteins.

Fig. 3 Sequence alignment of the N-terminal of the main fragment obtained by autolysis of baltergin (baltergin-DC) with other two ECD-containing disintegrin-like proteins, jararhagin-C, AAB30855, from *B. jararaca*, and

alternagin-C, P0C6R9, from *B. alternatus*. Protein sequences were aligned using the program *Clustal O* (1.2.4) multiple sequence alignment.

3.4. Purification of autocatalytic fragment

In order to characterize the main autolysis product, baltergin-DC, their purification was carried out. For this, a gel filtration chromatography was applied. The fractionation of the autolysis mixture on Superdex 75 column showed two main peaks (Fig. 4), peak 1 corresponding to residual baltergin band, 45 kDa band and autolysis fragments with low molecular weight (probably non-covalently associated) and peak 2 baltergin-DC, respectively (Fig. 4, insert). Baltergin-DC did not show azocaseinolytic activity (Fig. 5).

Fig. 4 Purification of baltergin-DC on Superdex 75 column. Fractions 1 and 2 correspond to baltergin and baltergin-DC, respectively. Insert shows 12% SDS-PAGE profile of eluted fractions. Molecular weight markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), anhydrase carbonic (29 kDa), soybean trypsin inhibitor (20.1 kDa), cytochrome C (12.4 kDa).

Fig. 5 Proteolytic activity of baltergin-DC. Proteolytic activity of *B. alternatus* venom, baltergin and baltergin-DC was determined using the standard azocaseinolytic assay. Asterisks indicate statistically significant differences in comparison with venom.

3.5. Adhesion assay

Both proteins (baltergin and baltergin-DC) were effective in inhibiting the binding of C2C12 cells to plates coated with FBS:PBS. The ability to inhibit cell adhesion was dose-dependent. After exposition to 1.8 μM of protein, percentage of cell adhesion was 49% and 62% for baltergin-DC and baltergin, respectively (Fig. 6), indicating that the inhibitory effect of baltergin-DC was higher than the effect caused by intact enzyme. Trypan blue staining showed that cell viability of non adherent C2C12 cells was preserved after baltergin / baltergin-DC incubation.

Fig. 6 Inhibition of cell adhesion. Effect of baltergin and baltergin-DC on C2C12 adhesion to FBS components. Each bar represents mean ± SD of triplicate assays. Asterisks indicate statistically significant differences in comparison with control. Crosses indicate statistically significant differences in comparison between baltergin and baltergin-DC. Insert: C2C12 cells (2×10^4 cells/well) exposed to medium alone (control), + baltergin (0.90 μM) or + baltergin-DC (0.90 μM). C2C12 cells were stained with crystal violet and observed under phase contrast microscopy (X40).

4. Discussion

The high molecular weight metalloproteinases from snake venoms, as represented by the class PIII toxins, are known to undergo autolysis under several conditions, giving rise to an unstable proteinase domain and a stable disintegrin domain [6]. Previously, we have isolated a metalloproteinase named baltergin from *B. alternatus* venom and

this protein was assumed to be a PIIIb-SVMP. Baltergin is able to undergo autolysis *in vitro*, releasing mainly a stable fragment of ~30kDa [3]. In the present study, autolysis conditions of baltergin were adjusted in order to improve obtention of the stable fragment. After autoproteolytic process, we purified a 32 kDa fragment (named baltergin-DC) by gel filtration chromatography and studied its effect on cell adhesion.

The electrophoretic analysis of baltergin autolysis products at different times showed a progressive disappearance of the proteinase band and an increase in bands with molecular weights ranging from 5 to 45 kDa, with predominance of a 32 kDa band (baltergin-DC) at long incubation times. The autolysis process was also assayed at different temperatures and pH values. The disappearance of the native protein band and visualization of baltergin-DC by SDS-PAGE was maximal at 37ºC and at a pH range of 7.0 – 8.0. At these conditions the residual proteolytic activity on azocasein markedly decreased due to that of intact baltergin had undergone autolysis. For jararhagin, a very studied model of PIII-SVMP found in *B. jararaca* venom, the major fragment obtained after autolysis of the native protein was jararhagin-C (28 kDa), mainly under conditions of basic pH and in presence of 2-ME [16]. Moura-da-Silva et al. (2003) proposed that fragments from within the metalloproteinase domain must be of rather small size since they are not observed in 12% gels.

When baltergin autolysis was assayed at 25 ºC, 45 ºC or at pH 9.0, a band of 45 kDa was observed. This protein is likely an intermediate product, which disappears when the autolysis is carried out at optimal temperature and pH values and long incubation times, giving rise to peptides of small size. The 45 kDa fragment is similar to those reported by autolysis of jararhagin [16] and represents a PIII-SVMP lacking a portion of the amino-terminal of the metalloproteinase domain. On the other hand, autolysis of brevilysin H6 (a PIII-SVMP isolated from *Gloydius halis brevicaudus* venom) at 37 ºC for 6 or 24 h releases both a 45 and 29 kDa (p29K) fragments [19]. However, when incubation time was increased at 60 h, only a p29K and several small peptides fragments were generated.

Baltergin-DC is a disintegrin-like/cystein-rich fragment obtained by *in vitro* processing of a PIII-SVMP from *B. alternatus* venom. Its amino-terminal structure begins with IISPPVCGNELLEVGEECDCGTPENCQNECCDAATC-. This sequence shows a high degree of homology to other DC proteins isolated directly from venom or obtained by autolysis of large SVMPs [16, 18, 22]. By analogy, it is appropriate to assume that this N-terminal primary structure, which derives from C-terminal region of baltergin, consists of the disintegrin-like and the cysteine-rich domains.

In order to assay the disintegrin activity of the fragment obtained, a cell adhesion test was carried out. Disintegrins and disintegrin-like proteins are capable of binding to integrins and interfering with integrin function through the RGD- and ECD-integrin recognition motifs, respectively. Integrins mainly mediate the interactions of cells with extracellular matrix (ECM) components and play critical roles in promoting cell survival, proliferation, and migration [27]. For our study, C2C12 myoblast culture was selected as cellular model. Plates were coated with fetal bovine serum (FBS), which is rich in ECM proteins such as fibronectin. This protein is important for cell adhesion to substrates in spite of its quite low concentration in the serum (0.2 g/L) [28] and constitutes a major extracellular ligand capable of recognizing RGD-dependent and non-RGD-dependent integrins [29]. Baltergin and baltergin-DC were both able to inhibit C2C12 adhesion to FBS coated plates. After exposition to 1.8 µM of baltergin or baltergin-DC, percentage of cells that remained adhered was 62 and 49%, respectively. Intact PIII-SVMPs are able to digest extracellular matrix proteins through their metalloproteinase domain [30]. However, the importance of the disintegrin-like domain for adhesion inhibition, rather than the proteolytic activity of the catalytic domain, was previously

demonstrated using jararhagin inactivated with 10 mM 1,10-phenanthroline [31]. Thereby, it is probable that both baltergin and baltergin-DC are able to interact with surface integrins impairing C2C12 adhesion to coated plate. Support this proposal the fact that the inhibitory effect of baltergin-DC (a processed form of baltergin, free of proteolytic activity) was stronger than that exhibited by baltergin. Previous work, Souza and collaborators [22] demonstrated that the disintegrin domain is the responsible for the collagen inhibition of α2β1 integrin in K562 transfected cells, using alternagin and alternagin-C isolated from *B. alternatus* venom. In this study authors stated that the IC50 values for both proteins (134 and 100 nM for alternagin and alternagin-C, respectively) were not significantly different. Considering that our study was in another cell line, probably, changes in the shape of the disintegrin-like domain that are triggered when baltergin undergoes autolysis, lead to additional molecular interactions resulting in a more stabilized integrin-disintegrin binding when baltergin-DC interacts with integrins present on C2C12 cells.

Alternagin-C is a disintegrin-like protein purified directly from *B. alternatus* venom which have a ECD motif [22].This protein interacts with the major collagen I receptor, the α2β1-integrin, inhibiting collagen-I mediated adhesion of K562-α2β1-transfected cells and also inhibits the adhesion of a mouse fibroblast cell line to collagen I [22, 23]. On the other hand, an RGD-disintegrin, *DisBa-01*, isolated from a cDNA library made with RNAs from the venom gland of *B. alternatus* was previously studied [32]. The 11,637 Da recombinant monomeric form of *DisBa-01* is a potent inhibitor of $\alpha_v\beta_3$-dependent adherence mechanisms involved in neo-vascularization and tumor metastasis processes. Thereby, up to date, three molecules that are able to interact with integrins were obtained from *B. alternatus* venom: two disintegrin-like proteins (autolyzed and isolated forms) and a recombinant RGD-disintegrin, demonstrating the complexity of the components present in this secretion.

Previously, the concept of metalloproteinase isoforms based on disulfide bond patterns of jararhagin was suggested [16]. One form is an unstable molecule which is rapidly processed in the venom to produce DC fragment. A second isoform probably with a different disulfide bond pattern gives rise to a DC-fragment *in vivo* and the last isoform is refractory to proteolysis *in vivo* and autolysis *in vitro*. On the other hand, Ölher et al. (2010) investigated the proteome of *B. alternatus* venom from Brazil. They found that almost 50% of the components identified in this proteome are metalloproteinases, which exclusively belong to P-III class. Two groups of SVMPs were identified: one group with metalloproteinases ranging 70 – 100 kDa of molecular masses and a group homologous to jararhagin, with 55 – 60 kDa proteins. It is probably that baltergin-DC and alternagin-C are isoforms or come from the same native protein, but this suggestion needs further investigation.

The role of cystein-rich domain in these processed molecules is also associated with integrin interaction [33]. Kamiguti et al., 2003 observed that synthetic peptide based on the cysteine-rich domain of atrolysin A and jararhagin contains two sequences that inhibit the interaction of α2β1 integrin-expressing K562 cells and platelets with collagen. Effect of cystein-rich domain in interaction with C2C12 surface integrins still remains unstudied. Finally, disintegrins and disintegrin-like proteins are also potent inhibitors of platelet aggregation [21, 22] and are effective to strongly inhibit adhesion, migration, invasion of tumor cells and tumor growth [34, 35]. Action of baltergin-DC in platelet aggregation and on tumor cells is the next step in our investigation.

5. Conclusions

This is the report about the purification of a processed disintegrin-like/cystein-rich fragment obtained by autolysis of baltergin, a PIIIb-SVMP from *B. alternatus* venom. These studies highlight the complexity of autolysis process of baltergin and the activity associated with baltergin-DC, which is able to inhibit cell adhesion probably by binding to membrane integrins. Baltergin-DC, lacking of proteolytic action, becomes an attractive molecule for future studies of blocking integrin–ligand interactions.

Disclosure of conflict of interest

We have no conflicts of interest to declare.

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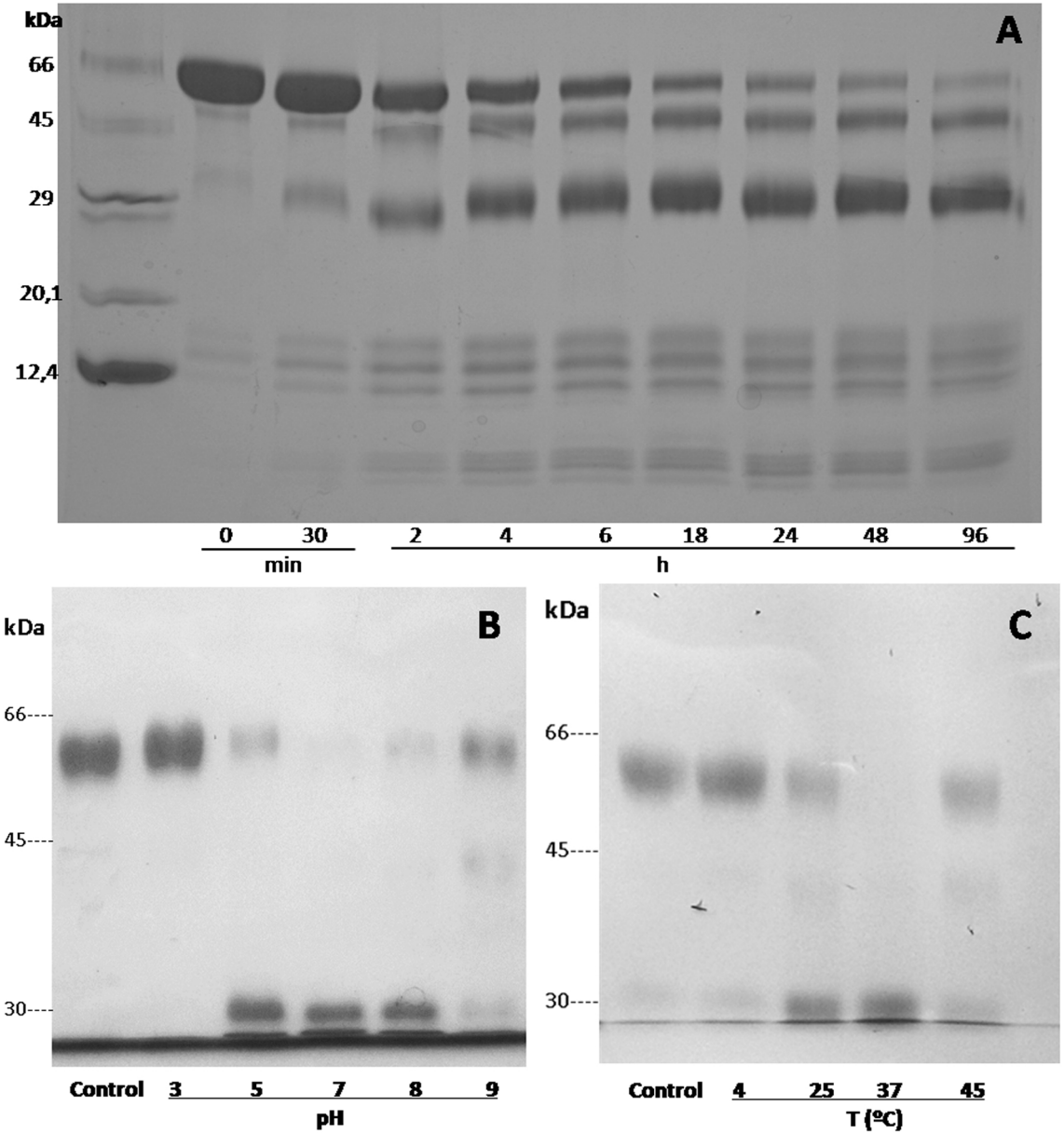
Highlights

- Baltergin is a PIIIb-SVMP from Bothrops alternatus venom able to undergo autolysis.
- Autolysis conditions were adjusted to obtain a disintegrin-like fragment.
- Effect of purified baltergin-DC on cell adhesion was studied.
- Baltergin and baltergin-DC were both able to inhibit C2C12 adhesion to coated plate.
- Baltergin-DC devoid of catalytic activity becomes a striking blocking-integrin molecule.


A
kDa
66
45
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20,1
12,4
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30
min
2
4
6
18
24
48
96
h
B
kDa
66----
45----
30----
Control
3
5
7
8
9
pH
C
kDa
66----
45----
30----
Control
4
25
37
45
T (ºC)

Figure 1

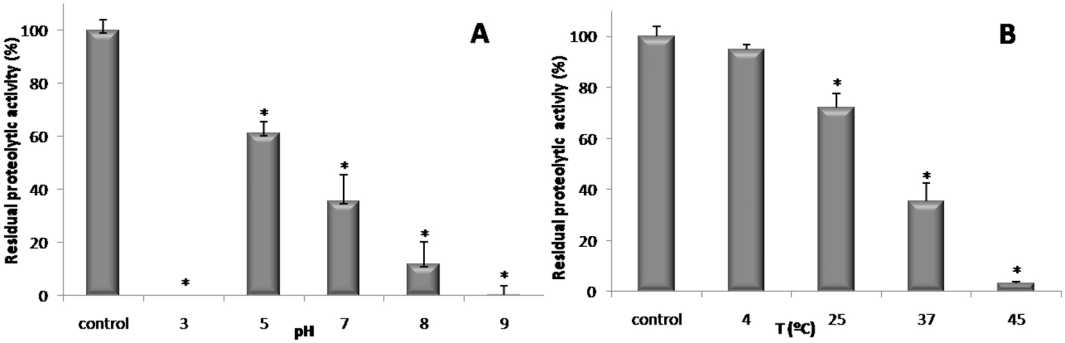
A
Residual proteolytic activity (%)
100
80
60
40
20
0
control
3
5
7
8
9
pH
B
Residual proteolytic activity (%)
100
80
60
40
20
0
control
4
25
37
45
T (ºC)

Figure 2

```
alternagin-C      IISPPVCGNELLEVGEECDCGTPENCQNXCCDAATCKLKSGSQCGHXDCCEQCKFTKSGT  60
jararhagin-C      IISPPVCGNELLEVGEECDCGTPENCQNECCDAATCKLKSGSQCGHGDCCEQCKFSKSGT  60
baltergin-DC      IISPPVCGNELLEVGEECDCGTPENCQNECCDAATC----------------------  36
                  ***************************** *******

alternagin-C      ECRASMSECDPAEHCTGQSXXCXXDVFHKNGQPCLDNYGYCYNGNCPIMYHAQCYALFGA  120
jararhagin-C      ECRASMSECDPAEHCTGQSSECPADVFHKNGQPCLDNYGYCYNGNCPIMYH-QCYALFGA  119
baltergin-DC      ------------------------------------------------------------  36

alternagin-C      DVYEAEDSCFKDNQKGNYYGYCRKENXXXXXCXXXDVKCGRLYCKDNSPKQNNPCKMFYS  180
jararhagin-C      DVYEAEDSCFKDNQKGNYYGYCRKENGKKIPCAPEDVKCGRLYCKDNSPGQNNPCKMFYS  179
baltergin-DC      ------------------------------------------------------------  36

alternagin-C      NDDEHKGNVLPGTKCE----------------    196
jararhagin-C      NDDEHKGMVLPGTKCADGKVCSNGHCVDVATAY   212
baltergin-DC      --------------------------------    36
```

Figure 3

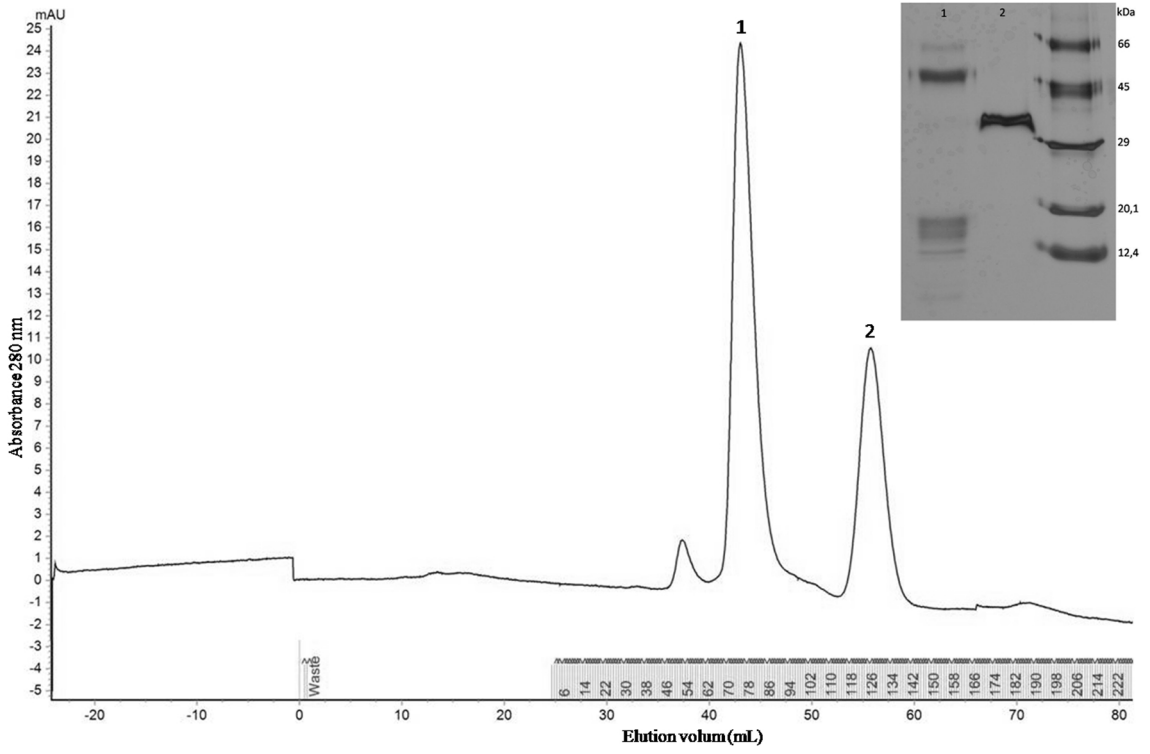
mAU
Absorbance 280 nm
Elution volum (mL)
Waste
1
2
kDa
66
45
29
20,1
12,4

Figure 4

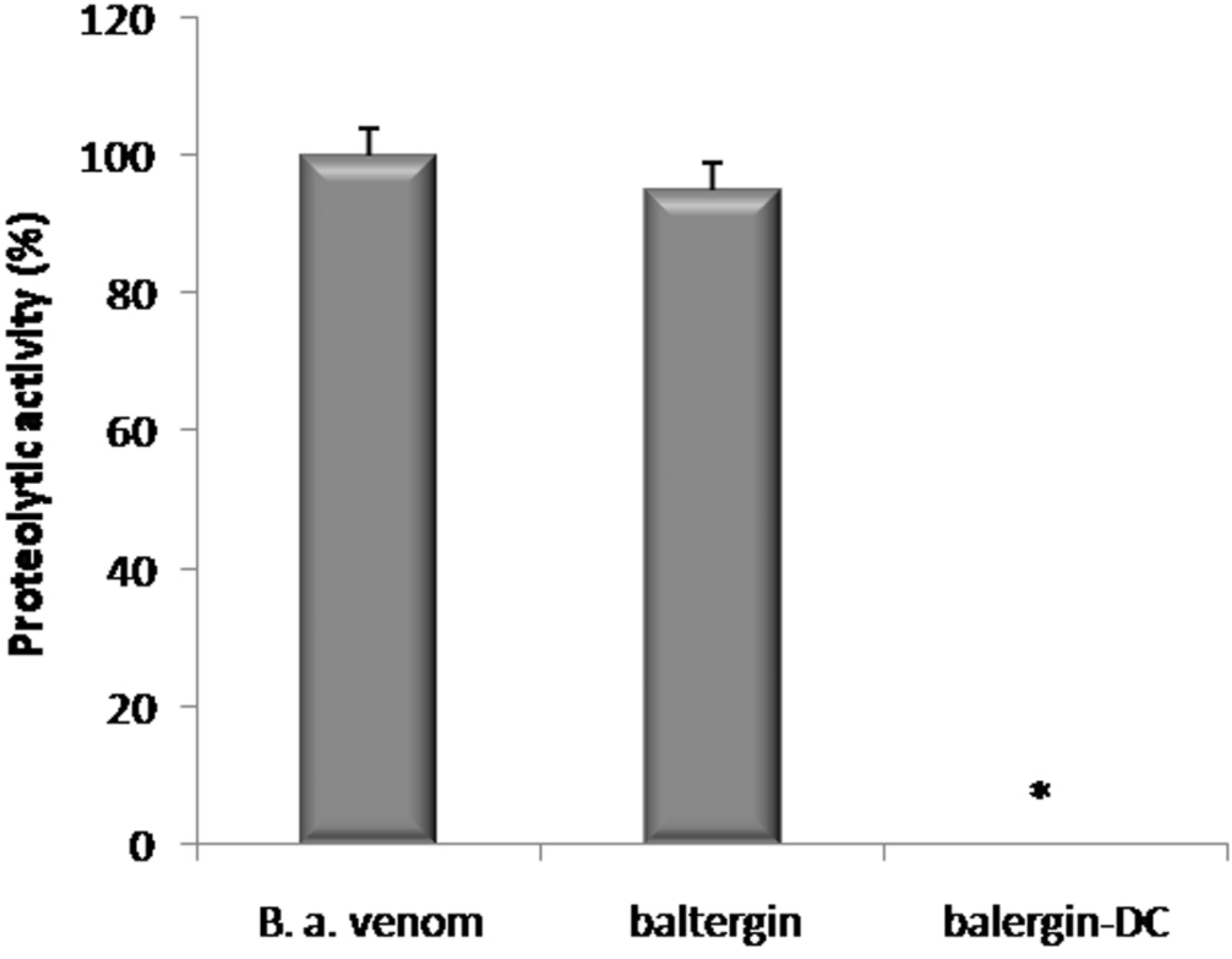
120
100
80
60
40
20
0
Proteolytic activity (%)
*
B. a. venom
baltergin
balergin-DC

Figure 5

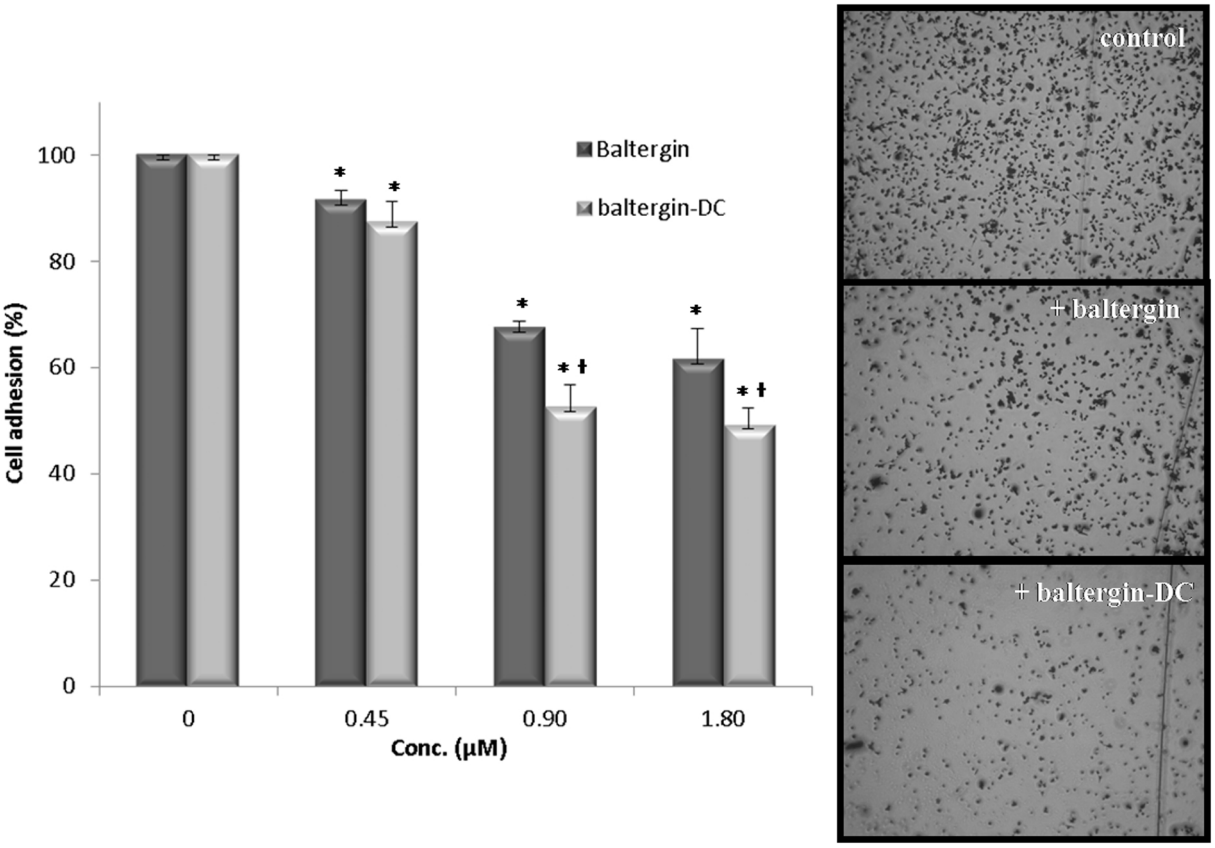
Cell adhesion (%)
100
80
60
40
20
0
Baltergin
baltergin-DC
*
*
*
*
*
*
†
*
†
0
0.45
0.90
1.80
Conc. (μM)
control
+ baltergin
+ baltergin-DC

Figure 6