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IMMUNE RESPONSE AGAINST BOTHROPS DIPORUS VENOM PRE-TREATED WITH NA_2EDTA FORMULATED WITH A NANOSTRUCTURED (COA-ASC16) AND CPG-ODN SEQUENCES

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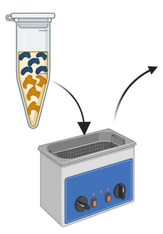
Introduction and Objetives

In South America, most snakebites are caused by genus Bothrops. The *B. diporus* venom is composed mainly of metalloproteinases (SVMPs) responsible for local effects such as hemorrhage, edema, myotoxicity and systemic bleeding. The only treatment for snakebite is antivenom, produced by immunizing animals with snake venom using Freund's adjuvant, which causes local damage at the injection site and affects the welfare of producer animals. Previous works demonstrated that CpG-ODN/Coa-ASC16 adjuvant causes very few local reactions when used with other antigens. **Taking into account these antecedents, in the present work, *B. diporus* venom was treated with Na_2EDTA and used as inmmunogen in combination with CpG-ODN/Coa-ASC16 adjuvant.**

Materials and Methods

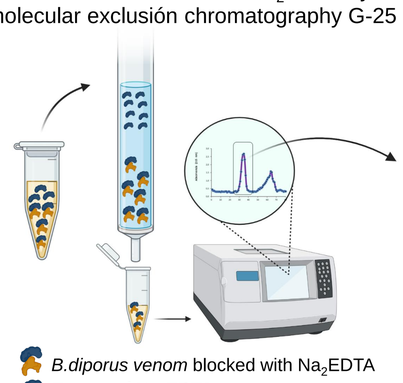
Pre-treatmen of *B.diporus* venom with Na_2EDTA

I: Pre-incubation of *B.diporus* venom with Na_2EDTA (blocking)



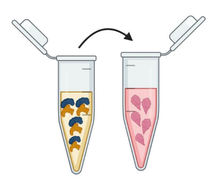
B.Diporus venom (1,8 mg/mL)
Na_2EDTA (200 mM)

II: Elimination of excess Na_2EDTA by molecular exclusión chromatography G-25



B.diporus venom blocked with Na_2EDTA
Excess of Na_2EDTA

III: Measurement of proteolytic activity of *B.diporus* venom blocked with Na_2EDTA **(BdV/EDTA)**



B.diporus venom blocked with Na_2EDTA (BdV/EDTA)
Azocaseín

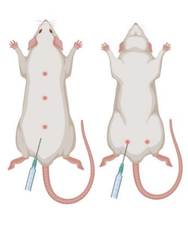
Immunization protocol

I: Immunization protocol

Days	BdV/Na2EDTA (µg)	BdV (µg)
0	7	7
15	15	15
30	30	30
45	final bleeding	

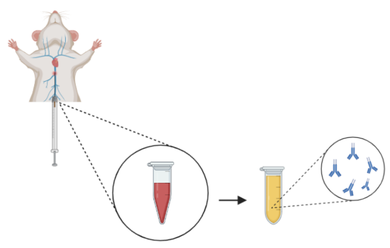
BdV/Na2EDTA or BdV was emulsified with CpG-ODN/Coa-ASC16 adjuvant

II: Inoculation



subcutaneous inoculation

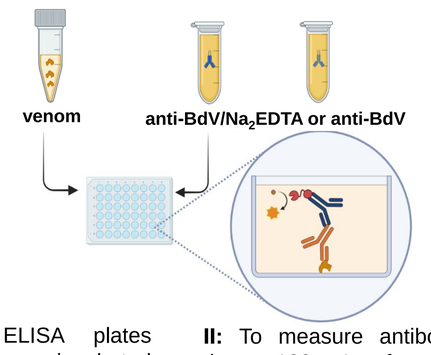
III: Obtaining hyperimmune serum



final bleeding — serum anti-BdV/Na_2EDTA or anti-BdV

Immunoassays

ELISA

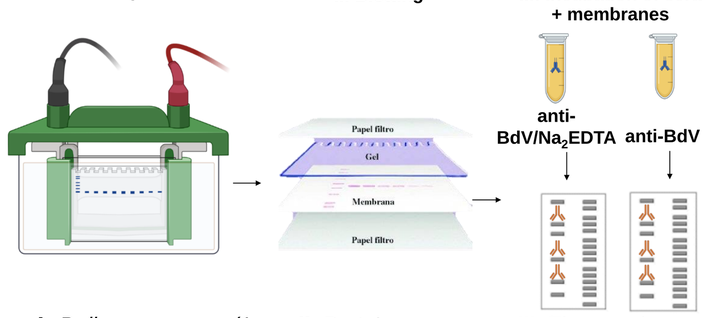


I: ELISA plates were incubated with *B.diporus* venom (100 µL) overnight at 4 °C.

II: To measure antibody titers, 100 µL of serial dilutions of serum (anti-BdV/Na_2EDTA or anti-BdV) were added to the plates and incubated for 1 h at 37 °C.

Immunoblotting

I: Electrophoresis — **II: Blotting** — **III: Incubation of sera + membranes**

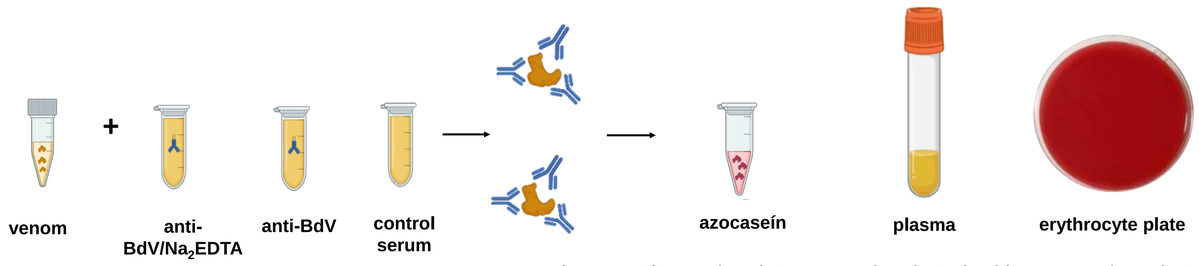


I: *B.diporus* venom (1 mg/mL) was separated by polyacrylamide gel electrophoresis at 100 V for 1 h.

II: Proteins were electrophoretically transferred to nitrocellulose membranes.

III: The membranes were incubated overnight, with anti-BdV/Na_2EDTA or anti-BdV.

Neutralization test



I: *B. diporus* venom was mixed in equal parts with anti-BdV/Na_2EDTA; anti-BdV or control serum. The mixtures were then incubated at 37 °C for 30 min

II: Subsequently, each mixture was incubated with azocasein substrate, plasma and erythrocyte plate to assess the neutralizing capacity of the sera against the proteolytic, coagulation and indirect hemolytic activity respectively of Bothropic venom.

Results

Molecular exclusion chromatography

The fig. 1.A. corresponds to the profile of the venom without inhibitor, which presents a single peak, with proteolytic activity (BdV) (Fig.1.C) while the elution of the venom blocked with Na_2EDTA generates two groups of fractions, peak I corresponding to elution of BdV/Na_2EDTA (without proteolytic activity) and peak II elution of excess Na_2EDTA (1.B). Thus, it is verified that the Na_2EDTA that complexes the SVMPs remain united even after the chromatographic separation.

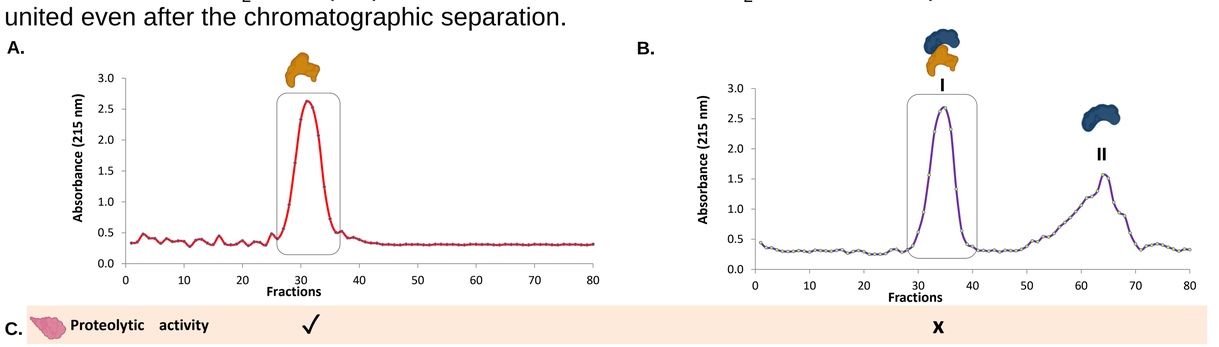


Figure 1. Elution profile. **(A)** Venom. **(B)** Venom trated with Na_2EDTA . **C.** Proteolytic activity of the fractions.

Immunoassays

The enzyme-linked immunosorbent assay results indicated that anti-BdV/Na_2EDTA and anti-BdV exhibited similar antibody titers (~2.56 x 10^4) against Bothropic venom (Fig. 2). Western Blot analysis revealed that the anti-BdV/Na_2EDTA serum recognized the main venom proteins (15-150 kDa) similarly to the anti-BdV serum (Fig. 3.B).Finally, both experimental sera (anti-BdV/Na_2EDTA or anti-BdV) displayed neutralizing abilities against the proteolytic and indirect hemolytic (summarized in table 1). Regarding the coagulant activity tested in vitro, the results obtained indicate that anti-BdV/Na_2EDTA lengthened 15.5 seconds compared to anti-BdV.

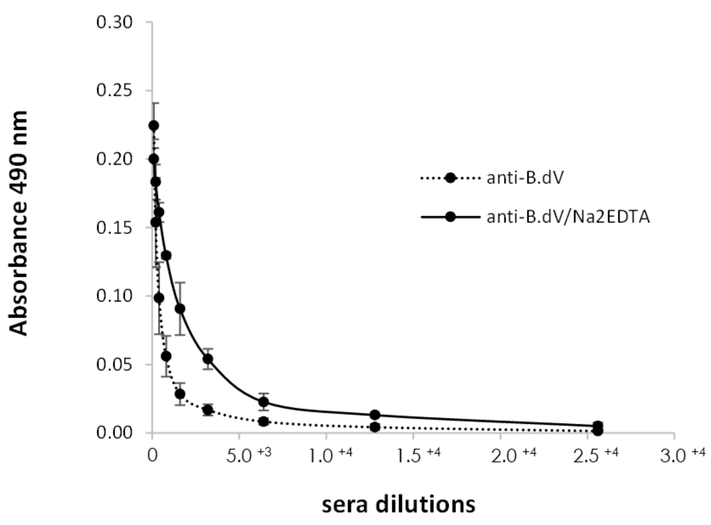


Figure 2. Final antibody titer against *B.diporus* venom measured by ELISA. Specific antibody titer IgG in sera of mouse immunized with B.dV/Na_2EDTA or B.dV respectively on 14 days after the last immunization. The points represent the value of the logarithm of the titer obtained by ELISA ± SD.

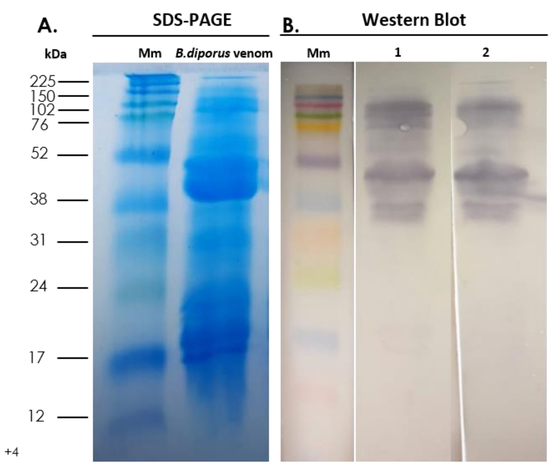


Figure 3. The reactivity of antisera produced by the different protocols against *B. diporus* venom was measured by immunoblotting. A: Venom was resolved in 12% SDS-PAGE, then transferred on two nitrocellulose membranes. **B:** The membrane 1 and 2 were incubated with anti-anti-B.dV/Na_2EDTA and B.dV respectively. From left to right: molecular markers (Mm), *B. diporus* venom. Strips 1 and 2 show the proteins recognized by the anti-B.dV and anti-B.dV/Na_2EDTA IgG respectively.

Table 1. % Neutralization of the Proteolytic and Indirect hemolytic activities of *B.diporus* venom by the experimental sera.

Enzyme or activity/Mixture	Venom + anti-BdV/Na_2EDTA	Venom + anti-BdV	Venom + control serum
Proteolytic (%)	14	21	0
Indirect hemolytic (%)	50	41.7	0

Conclusion

These findings suggest that Na_2EDTA does not impair protein immunogenicity, and BdV/Na_2EDTA together with CpG-ODN/Coa-ASC16 adjuvant was an appropriate immunogen since the animals immunized with it showed an adequate immune response to *B.diporus* venom similar to that of animals immunized with venom without an inhibitor.

Acknowledgments

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