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Article

Action of Bromelain and Ficin on horse anti *Bothrops sp* venom Antibodies

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The treatment with hyperimmune sera constitute the only specific and effective therapy available against snakebite envenomation, most common in developing countries. Serum quality is an important factor on patient recovery time and in the incidence of death and permanent disability. To date, most sera consist of pepsin digested IgG antibodies harvested from hyperimmune animals. The use of animal derived enzymes, such as pepsin, to digest IgG, constitute a source of adventitious agents and contaminants, such as porcine circovirus. The present study aims to evaluate the use of the plant derived enzymes bromelain and ficin, as an alternative to pepsin. To this purpose, horse serum immunized against *Bothrops* venoms was purified with caprylic acid and digested with bromelain or ficin. SDS-PAGE results evidence the formation of $F(ab)'_2$ fragments and suggest that a digestion time superior to 8 hours may be required to completely digest the antibodies with bromelain or ficin. $F(ab)'_2$ fragments obtained by digestion with either bromelain or ficin digestion preserved the ability to recognize *Bothrops sp.* venom in western blotting assays. Therefore, both enzymes are suitable for use in large-scale production, minimizing contamination risks and increasing safety and efficiency of serotherapy treatments.

Keywords: Bromelain. Ficin. Enzyme. $F(ab)'_2$ fragments. Hyperimmune serum.

INTRODUCTION

Snakebite envenoming is a neglected tropical disease that affects 1.8 to 2.7 million people worldwide each year, causing 81,000 to 138,000 deaths and 400,000 cases of permanent disability (WHO, 2019). In Brazil, 471,801 cases of snake envenomation were reported between 2000 and 2017, an average of 27,753 per year (Brasil, 2017), with a total of 1,892 deaths (Brasil, 2019). Most accidents were caused by snakes of the genera *Bothrops* and *Bothrocophias* (Brasil, 2017).

To date, the only specific therapy available against snakebite envenomation involves the use of hyperimmune sera (Mathew *et al*., 2020; Silva de Oliveira *et al*., 2020; Taherian *et al*., 2018). Although antivenom sera are featured in the WHO List of Essential Medicines (WHO, 2019), there is currently a crisis regarding the availability, accessibility, and affordability of safe and effective antivenom on a global basis, most notably in sub-Saharan Africa (Gutierrez, 2018; Squaiella-Baptistao *et al.,* 2018).

Enzyme-cleaved antibodies from equine or ovine sera are widely used in the treatment of snakebite poisoning. The cleavage of IgG antibodies to harvest $F(ab)'_2$ or F(ab) fragments only is performed to minimize adverse events such as acute anaphylactic reactions and serum sickness induced mainly by Fc fragments (Jones, Landon, 2002; Kordzangene *et al*., 2018). This process is usually achieved using pepsin, an animal derived enzyme of the peptidase family, which generates $F(ab)'_2$ fragments.

Even though digestion of the serum antibodies with pepsin contributes to increased serum potency and safety,

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clinical reports suggest a range of product safety profiles. Early reactions are reported with a range of incidence from 0 to 37% and delayed serum sickness from 0 to 19% (Boyer *et al*., 2013; Chippaux *et al*., 2007; Schaeffer *et al*., 2012). Overall, hyperimmune sera are of good quality. But the adverse events do occur and might be due to the presence of eventual contaminants introduced during the production processes or clinical administration (Boyer *et al*., 2013).

Pepsin is generally harvested from pig stomach mucosa, an animal source, which may lead to contamination with porcine circovirus or other adventitious agents (Lamoyi, Nisonoff, 1983; Altun, Cetinus, 2007). Antivenoms are mostly used in developing countries and geographically remote clinics. Therefore, sera that are not processed to higher safety standards may pose enough risks to prohibit their use outside monitored hospital units where resuscitative equipment is readily available (Boyer *et al*., 2013).

Alternative sources of antibody digesting enzymes, such as plants, that generate the same digestion pattern as pepsin, may contribute to produce a safer serum, potentially free of adventitious agents. In this work, bromelain and ficin, extracted from pineapple and fig, respectively, and which generate $F(ab)'_2$ fragments, were assessed for their applicability as an IgG digesting agent for safer serum production.

MATERIAL AND METHODS

Venom and horse hyperimmune serum

Bothrops sp. venom and Anti-*bothrops sp.* horse hyperimmune anti-serum were kindly provided by Instituto Butantan (Brazil). The serum samples were obtained from horses previously immunized with a venom pool of snakes from the genus *Bothrops* following Instituto Butantan's internal immunization protocols.

Purification by caprylic acid

The first purification step was performed according to the methodology described by dos Santos and colleagues (1989). The serum was heated to 56°C for 15 minutes and centrifuged at 900g for 10 minutes. After pH adjustment to 5.0 with 0.5M Acetic acid (Synth, Labsynth Products for Laboratories BRA Ltda., Diadema, Brazil), Caprylic acid (Synth, Labsynth Products for Laboratories BRA Ltda., Diadema, Brazil) was added dropwise until a final concentration of 8.7% (v/v). The mixture remained under stirring for 30 min on a magnetic stirrer. After centrifugation (11,000 g, 15 min), the supernatant was removed and filtered through a 0.45 µm membrane (Merck Millipore, Billerica, MA, USA) and the pH was adjusted to 7.0 with 0.1N NaOH, (Synth, Labsynth Products for Laboratories BRA Ltda., Diadema, Brazil). Lastly, samples were diafiltered in molecular filtration tubes (50 kDa cutoff) (Merck Millipore, Billerica, MA, USA) and stored (2ºC to 8ºC) until further use.

Digestion of anti-Bothropic serum with enzymes Ficin or Bromelain

The total protein content of the previously purified serum was adjusted to 500 µg/mL with deionized water. Bromelain (EC 3.4.22.32) or ficin (EC 3.4.22.3) (Sigma-Aldrich, St. Louis, MO, USA) was resuspended according to supplier instructions and they were used in the proportion of 1:50 and 1:30 E/S respectively (MARIANI *et al*, 1991).

Both mixtures were diluted to the final volume of 5.0 mL with Tris-HCl buffer pH 7.0 (Synth, Labsynth Products for Laboratories BRA Ltda., Diadema, Brazil). The resulting solutions containing approximately 2.5 mg of total protein were kept at 37 °C for 8 h. For digestion with ficin, L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) was also added to a final concentration of 4 mM.

At the end of the process, enzyme inhibitor was added (Protease Inhibitor Cocktails – (Sigma-Aldrich, St. Louis, MO, USA) in the proportion 1:10 (v/v). After diafiltration (cutoff 50 kDa - Merck Millipore, Billerica, MA, USA) the mixtures were frozen (20 ºC or less) until the purification of the immunoglobulin fragments.

Purification of immunoglobulin fragments

The purification of immunoglobulin fragments was performed using a Superdex 200 10/300 GL column (GE

Healthcare USA Inc., Pittsburgh, PA, USA) attached to an Akta Purifier 100 system (GE Healthcare USA Inc., Pittsburgh, PA, USA). Fractions were eluted with 50 mM ammonium bicarbonate (Synth, Labsynth Products for Laboratories BRA Ltda., Diadema, Brazil) pH 7.0 at a constant flow rate of 0.45 mL/min.

The samples containing 400 µg of total protein each were manually injected in a maximum volume of 400 µL. Fractions containing the proteins of interest (purified immunoglobulin fractions) were identified by SDS-PAGE (10 % gels stained with Coomassie blue) and lyophilized.

SDS-PAGE and Immunoblotting assay

The immunoglobulin fragments were characterized by SDS-PAGE using a 10 % polyacrylamide gel stained with Coomassie blue or Silver. For immunoblotting, the samples were electrophoresed using a 10 % SDS-PAGE gel and electrotransferred to Hybond N nitrocellulose membranes (GE Healthcare USA Inc., Pittsburgh, PA, USA) using a 160 mM glycine, 25 mM Tris, and 20% (v/v) methanol. The procedure occurred at 90 V for 30 min using the Bio-Rad semi-wet transfer system (Bio-Rad Laboratories USA Inc., Hercules, CA, USA). The membranes were stained with a 0.1 % Ponceau red solution (Bio-Rad Laboratories USA Inc.) in 10 % acetic acid and then incubated from 16 to 18 hours at 4 °C with a blocking solution (5 % skimmed milk (Molico®, Nestlé S.A., Vevey, VD, Switzerland)). The membranes were then incubated for 1 h at room temperature (RT) with *Bothrops* venom at a dilution of 0.05 µg/mL in PBS-T (0.05% Tween 20 (v/v), Invitrogen, Life Technologies Corporation USA Inc.). After washing 3 times (10 minutes each) with PBS-T, the membranes were incubated with rabbit antibothropic serum (1/5000, overnight, RT) (Sigma-Aldrich, St. Louis, MO, USA). The material was again washed 3 times with PBS-T for 10 minutes each time and the membranes we incubated with peroxidase-conjugated anti-rabbit IgG antibody (Sigma-Aldrich, St. Louis, MO, USA) (1/5000, 1 h, RT). Finally, after washing again 3 times with PBS-T, the membrane material was detected using the SuperSignal kit (Thermo Fisher).

RESULTS

The study started with the purification of the sample, collected from horses immunized with a mixture of Bothrops snake venoms and kindly provided by the Instituto Butantan. During the purification process, it was possible to observe changes regarding the sample's physical characteristics (appearance and color) (Figure 1a – steps 1 to 4). Thermal coagulation was followed by the addition of caprylic acid to induce the precipitation of non-immunoglobulin proteins. As a result, a limpid sample was obtained. The purification efficiency was verified by 10 % SDS-PAGE electrophoresis (Figure 1b). The resulting gel evinced that most of the impurities were removed. A characteristic band around 160kDa, corresponding to the molecular mass of IgG, was observed.

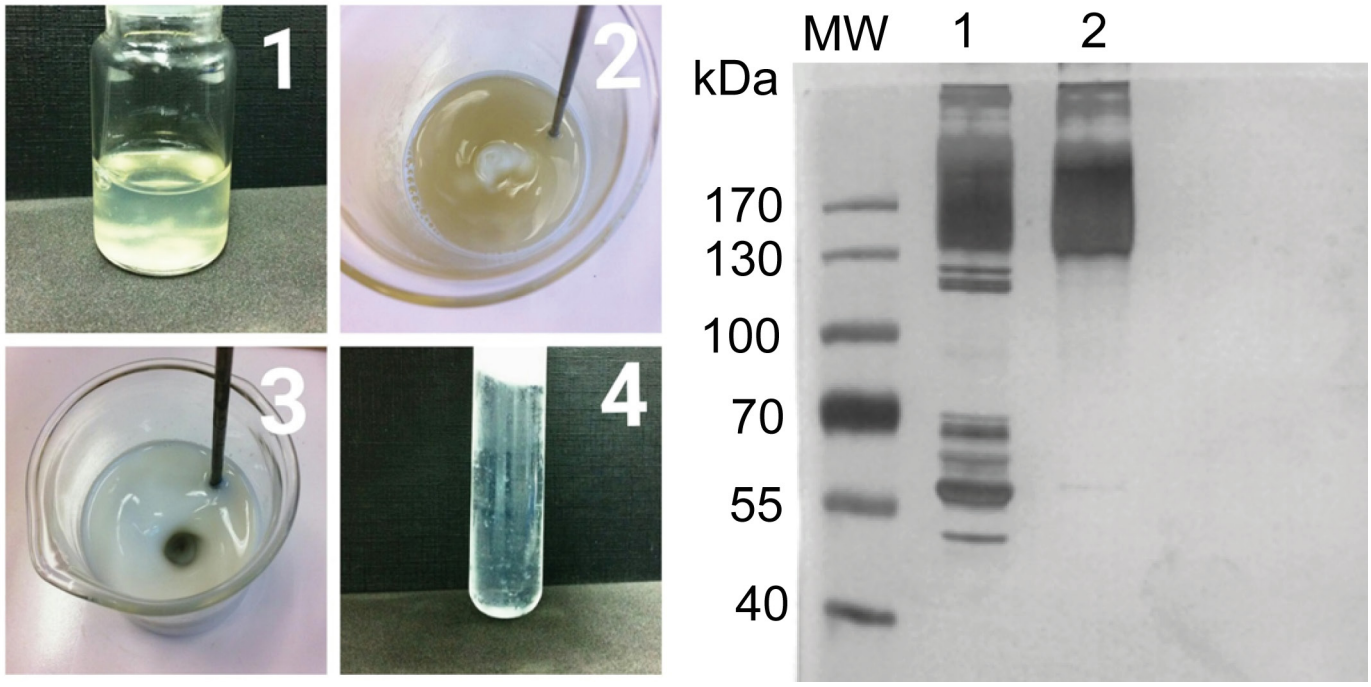

FIGURE 1 - Purification of anti-bothropic serum using caprylic acid. **(A)** Steps of the purification process are highlighted from 1 to 4. Where 1) represents the sample before the purification process, 2) thermocoagulation step, 3) precipitation of impurities using caprylic acid at a final concentration of 8.5 % and 4) represents the sample at the end of the process. **(B)** 10 % SDS-PAGE under reducing conditions of anti-bothropic serum, silver-stained. Samples were applied at 10 µg of protein content before and after purification steps to analyze the relative purity.

Sample digestions were performed using Bromelain or Ficin under the conditions described in Methods. The SDS-PAGE analysis shows that the digestion of both antibody samples was not yet complete after 8 hours of incubation, as a band of 160 kDa corresponding to undigested IgG, remained, together with a 110 kDa band, corresponding to digested IgG. The observed band with a molecular mass of 110 kDa corresponds to the expected IgG $F(ab)'_2$ fragment's size and the band of approximately 50kDa corresponds to the Fc fragments (Figure 2).

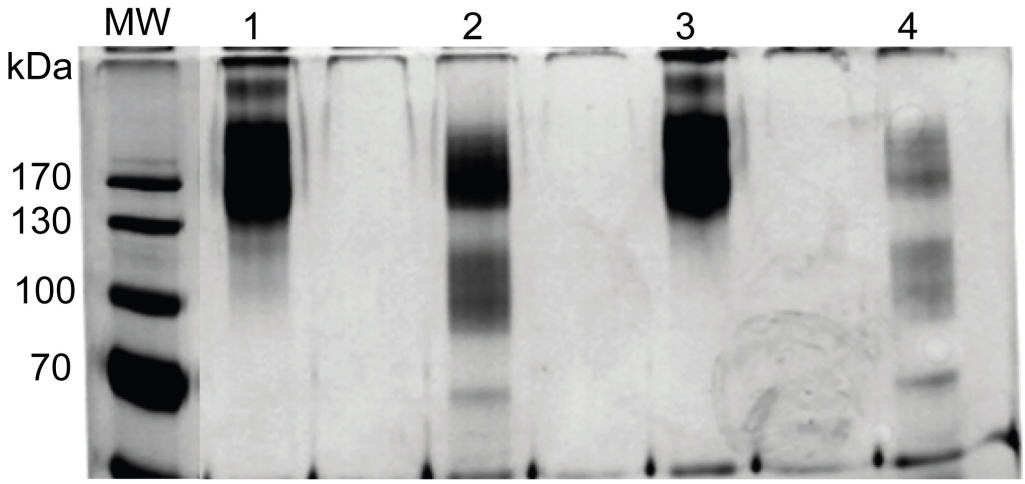

FIGURE 2 - Generation of F(ab)'$_2$ fragments obtained from anti-bothropic antibodies using bromelain or ficin. 10 % SDS-PAGE, under reducing conditions, of F(ab)'$_2$ fragments obtained by bromelain digestion (lanes 1 before digestion and 2 after digestion) and ficin digestion (lanes 3 before digestion and 4 after digestion) of anti-bothropic serum. Samples were applied at 10 µg of protein content per line to analyze the digestion process.

Due to the partial efficiency of the digestion process, molecular exclusion chromatography was performed to isolate the F(ab)'$_2$ fragments. The sample was separated into two distinct fractions (Figure 3a and 3b). Through SDS-PAGE analysis, we confirmed the presence of a single IgG fragment with a molecular mass of approximately 110 kDa in one of the separated samples.

Both bromelain and ficin digested samples recognized the venom in the immunoblotting assay (Figure 3c).

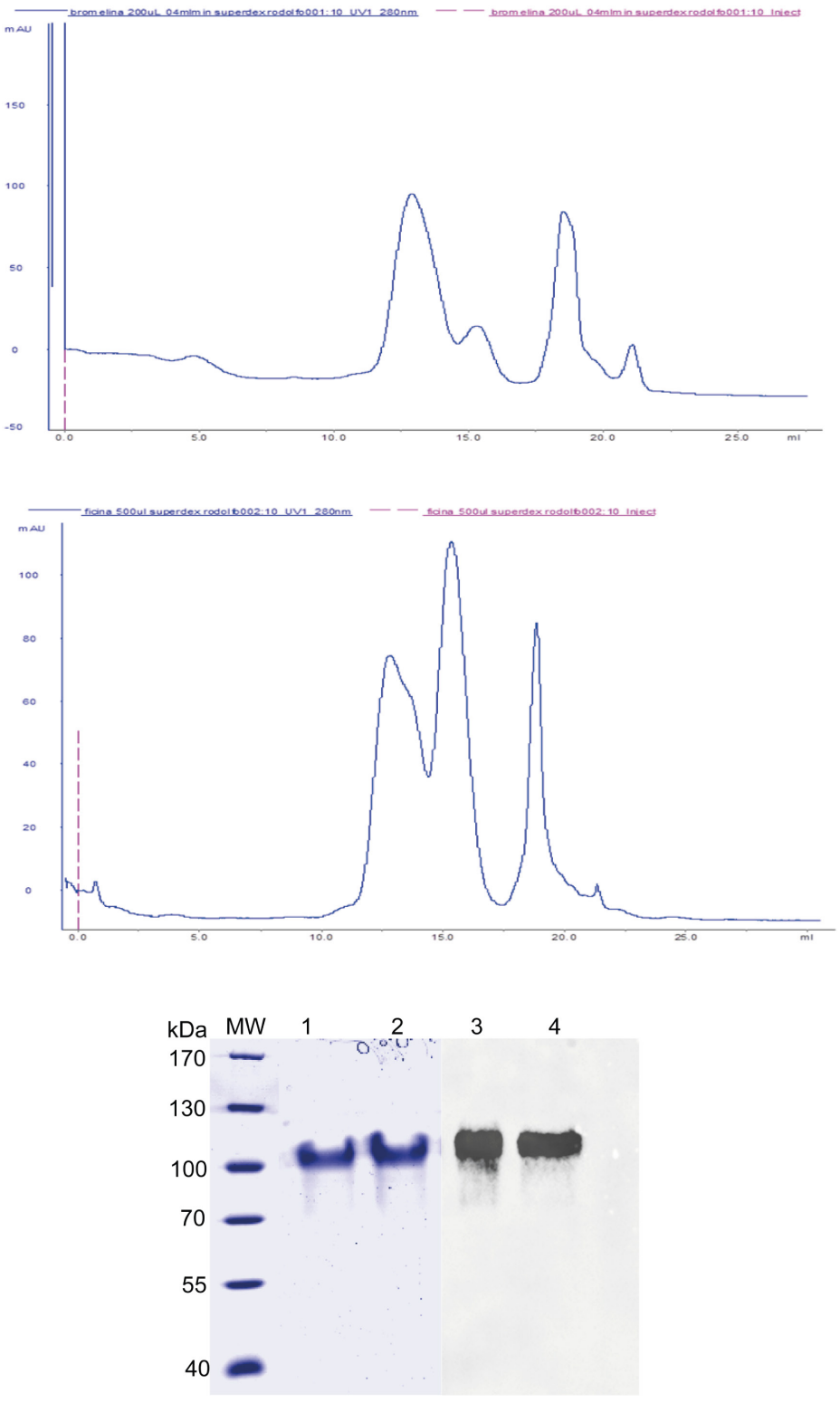

FIGURE 3 - Purification of anti-bothropic serum fragments by gel filtration chromatography and analysis of immunoreactivity by western blotting. **(A)** Bromelain and **(B)** Ficin FPLC gel filtration chromatography werre performed according to "Materials and Methods", under a constant flow of ammonium bicarbonate 50 mM and monitored at 280 nm. Fractions were collected manually for SDS-PAGE analyses. **(C)** 10 % SDS-PAGE analysis under reducing conditions (on left side) of purified $F(ab)'_2$ fragments, gel stained with Coomassie blue, was performed applying 2 µg of protein per lane and Western blotting analysis was performed using the same fractions and concentration (on the right side). Bromelain and ficin generated $F(ab)'_2$ fragments are represented in line 1 and line 2, respectively.

DISCUSSION

Serotherapy is currently the only proven effective treatment for accidents involving venomous animals and toxins. This therapy consists of injecting the patients with a serum made of concentrated antibodies produced by a different organism, often a horse, in response to repeated inoculations of small doses of snake venom. These antibodies bind to the venom's toxins and prevent their activities in the victim's organism (Pucca *et al*., 2019). In Brazil alone, more than twenty thousand cases of accidents with snakes of the *Bothops* genus were reported in 2018 (Brasil, 2019).

Several industrial processes of hyperimmune sera production have been described (WHO, 2016). In agreement with the "Expert Committee on Biological Standardization" published by the World Health Organization (WHO, 2016), both the ammonium sulfate precipitation and the caprylic acid precipitation methods are recognized and have been well described (WHO, 2016). We opted for the caprylic acid precipitation technique standardized by Mariani and colleagues (1991). We consider it a fast, efficient and better yield method since it promotes the precipitation of serum non-immunoglobulin proteins resulting in sera with a high purity degree (Mariani *et al*., 1991). Our purification process resulted in a single predominant band with a molecular mass of approximately 160 kDa, as expected for immunoglobulins and as observed by SDS-PAGE (Figure 1b).

The current process of hyperimmune sera production - including the antibothropic serum - involves the antibodies enzymatic digestion step, which aims to generate $F(ab)'_2$ fragments and completely degrade the Fc portion, which is responsible for activating the complement system and is the cause of several allergic reactions, and eventually leading to anaphylactic shock (Lamoyi, Nisonoff, 1983). For the enzymatic digestion, the enzyme pepsin is commonly used. However, pepsin is obtained mainly through extraction from fresh pig stomach mucosa, thus classified as an animal input (Lamoyi, Nisonoff, 1983; Altun, Cetinus, 2007).

An important issue caused by this approach is the possibility of introducing contaminants into the serum, such as porcine circovirus or other adventitious agents (Gilliland *et al*., 2012; Petricciani *et al*., 2014; Burnouf *et al*., 2004). Another problem already identified with the use of pepsin is that immunoreactive antibody fragments are not always produced, reducing the effitiency of serotherapy. Corroborating these informations, Milenic and cols. (1989) made tests involving the enzymes bromelain and pepsin to treat the monoclonal antibody B72.3 (an IgG1 that was generated by immunizing BALB/c mice with a membrane-enriched fraction of a human breast tumor metastasis to the liver). While samples digested with pepsin had an immunoreactivity lower than 25%, the samples digested with bromelain maintained an immunoreactivity index of approximately 100% compared to the control (undigested antibody). These tests show that, in addition to being possible to generate F(ab)'2 fragments with bromelain, the immunoreactivity of the resulting antibody fragments is highly preserved. Additionally, the enzymes Bromelain and Ficin, both derived from plants, not only preserve the immunoreactivity of the $F(ab)'_2$ fragments, but theydo not carry animal derived biological contaminants (dos Santos *et al*., 1989).

After the digestion process with either ficin or bromelain, some undigested antibody remained, as observed by SDS-Page (Figure 2). This indicates that the digestion process should be optimized, extending the reaction time, for example.. Nevertheless, $F(ab)'_2$ fragments from both bromelain and ficin treatments preserved the ability to recognize *Bothrops sp.* venom, as shown by western blotting assay.

Therefore, both enzymes may be suitable for large scale production and further studies are needed to optimize immunoglobulin digestion and determine conditions for upscaling. The substitution of bromelain or ficin for pepsin in anti-bothropic serum production, as well as in the production of other hyperimmune sera, will minimize contamination risks, thus increasing serotherapy safety and potentially serum efficiency.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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