



Research paper

Biochemical and molecular characterization of the hyaluronidase from *Bothrops atrox* Peruvian snake venom

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ABSTRACT

Snake venoms are a rich source of enzymes such as metalloproteinases, serine proteinases phospholipases A2 and myotoxins, that have been well characterized structurally and functionally. However, hyaluronidases (E.C.3.2.1.35) have not been studied extensively. In this study, we describe the biochemical and molecular features of a hyaluronidase (Hyal-Ba) isolated from the venom of the Peruvian snake *Bothrops atrox*. Hyal-Ba was purified by a combination of ion-exchange and gel filtration chromatography. Purified Hyal-Ba is a 69-kDa (SDS-PAGE) monomeric glycoprotein with an N-terminal amino acid sequence sharing high identity with homologous snake venom hyaluronidases. Detected associated carbohydrates were hexoses (16.38%), hexosamines (2.7%) and sialic acid (0.69%). Hyal-Ba selectively hydrolyzed only hyaluronic acid (HA; specific activity = 437.5 U/mg) but it did not hydrolyze chondroitin sulfate or heparin. The optimal pH and temperature for maximum activity were 6.0 and 40 °C, respectively, and its Km was 0.31 μM. Its activity was inhibited by EDTA, iodoacetate, 2-mercaptopropanoic acid, TLCK and dexamethasone. Na⁺ and K⁺ (0.2 M) positively affect hyaluronidase activity; while Mg²⁺, Br²⁺, Ba²⁺, Cu²⁺, Zn²⁺, and Cd²⁺ reduced catalytic activity. Hyal-Ba potentiates the hemorrhagic and hemolytic activity of whole venom, but decreased subplantar edema caused by an L-amino acid oxidase (LAAO). The Hyal-Ba cDNA sequence (2020 bp) encodes 449 amino acid residues, including the catalytic site residues (Glu135, Asp133, Tyr206, Tyr253 and Trp328) and three functional motifs for N-linked glycosylation, which are conserved with other snake hyaluronidases. Spatial modeling of Hyal-Ba displayed a TIM-Barrel (α/β) fold and an EGF-like domain in the C-terminal portion. The phylogenetic analysis of Hyal-Ba with other homologous Hyals showed the monophyly of viperids. Further, Hyal-Ba studies may extend our knowledge of *B. atrox* toxinology and provides insight to improve the neutralizing strategies of therapeutic antivenoms.

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1. Introduction

Abbreviations: Hyal, hyaluronidase; HA, hyaluronic acid; Ba, *Bothrops atrox*; UDF, Di-Ferrante units; ECM, extracellular matrix; TFA, Trifluoroacetic acid; CTAB, Cetyl Trimethyl Ammonium Bromide; EDTA, Ethylene diamine tetraacetic acid; PMSF, Phenylmethanesulfonyl fluoride; MHD, minimum hemorrhagic dose; hBRC, human blood red cells.

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Snakebite envenomation constitutes a serious health issue, justifying its recent classification into category A of the neglected tropical diseases by WHO [1]. Annually, hundreds of thousands to several million ophidian accidents occur worldwide, and are mainly reported in impoverished tropical and sub-tropical rural

areas of developing Asian, African and South American countries [2].

Peru, is a country with a large venomous ophidian fauna (33 formally reported species) [3], and snakebite envenomation represent a relevant public health issue [3,4]. The pitviper *Bothrops atrox* is by far the main causative agent of the vast majority (~87.6%) of reported snakebites in the low lands east of the Andes and in tropical rain forests up to 1200 m [4]. As happen with the majority of viperid snakes, envenoming by *B. atrox* is characterized by a complex pathophysiology including local tissue damage (hemorrhage, erythema, edema, inflammation, necrosis and ECM degradation) as well as systemic hemostatic dysfunctions resulting in multi-organ bleeding, renal alterations and coagulopathies, with consumption of fibrinogen and other blood coagulation factors [4,5]. Thereby, the severity of the envenomation symptoms correlates with venom features, such as amount, composition and systemic distribution via the bloodstream and appropriate therapeutic intervention by using specific antivenoms [4–6].

Despite the recognized medical relevance of accidents caused by *B. atrox* in Peru, only recently for the last few years, studies to elucidate the venom composition and its pharmacological properties have received more attention, including proteomic elucidation [6,7] as well as the identification and detailed characterization of important compounds/toxins. Among them are metalloproteinases which induce hemorrhage and coagulopathies [8], L-amino acid oxidase (L-AAO) [9] that triggers autophagy, apoptosis and necrosis in human keratinocytes.

Hyaluronidase (Hyal) enzyme (E.C. 3.2.1.35), commonly known as “spreading factor”, is another venom component with limited in vitro and *in vivo* studies [10]. Although the Hyal is claimed to be non-toxic, it degrades hyaluronic acid (HA) of connective tissue, thereby facilitating the diffusion of toxic components of the venom throughout the victim's organism and blood vessels, thus accelerating the envenoming process [10–12]. Thus, the synergy of its action with other venom component may cause a fast and more severe collapse of the prey physiology. Therefore, its mode of action is similar in many venomous snakes [12]. Due to the role in venom induced toxic effects by its spreading property of venom toxins throughout the victim's body, Hyal has received special attention regarding the efficacy of therapeutic antivenoms [10].

The main limitations for studying Hyals are related to the complex isolation process due to their high instability, rapid degradation and low concentration in the venom [13,14]. Despite these disadvantages, several snake venom Hyals have been characterized from the venoms of *Naja naja* [15], *Agkistrodon contortrix contortrix* [16], *Cerastes cerastes* [17], *Daboia russelli* [18], *Crotalus durissus terrificus* [12] and *Lachesis muta rhombata* [19]. From *Bothrops* genus, homologous Hyals have been previously reported from *B. moojeni* [20] and *B. pauloensis* [14]. Our studies highlight the fact that the earlier administration of specific antivenom, in conjunction with the rapid spread of venom toxins promoted by Hyal are key aspects to take in consideration for better management of snakebites.

The present work reports the structural and functional characterization of a hyaluronidase (Hyal-Ba) from the venom of the Peruvian pitviper *B. atrox*, which was previously identified in our laboratory [21]. Furthermore, we describe the cDNA and predicted the amino acid sequence of Hyal-Ba. We believe that our results provide important information on the structure-function relationship of Hyal-Ba, emphasizing the importance of the spreading effect of the enzyme on snakebite envenomation. This, may assist understanding of pathophysiological processes involving Hyal with the aim to improve the anti-venom therapy caused by *B. atrox* or other pitviper species.

2. Materials and methods

2.1. Venom and reagents

Bothrops atrox venom was obtained from specimens captured in several localities of Perú. Table 1, shows the localities where the snakes were captured, (Alto Marañoñ (3), Pucallpa (5) Tingo María (1), Junín (2) y La Merced (1)), and the hyaluronidase specific activity. *B. atrox* specimens were kept in captivity at the Serpentarium “Oswaldo Meneses”, located in the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos. Snakes were milked by applying mechanical pressure on the venom glands. The venoms were collected in free-DNAse and RNAse sterile tubes. Anti-bothropic polyvalent serum (Batch: 01000376) was obtained from the Instituto Nacional de Salud (INS), Lima, Peru. One neutralizing dose was considered as 10 mL of antivenom that neutralizes 25 mg of venom. Hyaluronic acid (HA), chondroitin sulfate and heparin were obtained from the Sigma® Chemical Co. Other reagents were of analytical grade acquired from Roche®, Merck® and Invitrogen®.

All *in vivo* experiments were in accordance with the ethical recommendations of the International Guiding Principles for Biomedical Research Involving Animals developed by the Council of International Organizations of Medical Sciences (CIOMS).

2.2. Enzymatic activity

Hyaluronidase activity was measured according to the turbidimetric method [22] adjusted to 96-well microplates. Briefly, 5 µl of the sample (venom or fraction) were added to the reaction mixture (containing 2 µl of HA [0.5 mg/ml] and 25 µl of 0.05 M ammonium acetate buffer, pH 5.0 and 0.15 M NaCl) and incubated at 37 °C for 15 min. The reaction was stopped by adding 200 µl of 2.5% Cetyl Trimethyl Ammonium Bromide (CTAB), in a 2% aqueous NaOH solution. Assays were monitored by absorbance at 400 nm against a blank of acetate buffer (250 µl) and CTAB (500 µl). Activity was expressed in Di-Ferrante units (DFU), where one unit was defined as the amount of enzyme required to reduce 50% of initial turbidity of the reaction mixture.

2.3. Enzyme stability

Prior to enzyme purification, stability of Hyal activity, was assayed at different time points (0–60 h) by using two buffers at different pH values (0.1 M ammonium acetate, pH 4.0–6.0 and 0.1 M Tris-HCl, pH 7.0–8.0), and incubated at 20 °C in the presence of 0.15 M or 0.3 M of NaCl. Assays were performed in triplicate.

Table 1
Geographic variability of hyaluronidase activity from *Bothrops atrox* venom.

Locality	Specimen ID	Specific Activity (DFU/mg) ^a (mean ± S.D.)
Pucallpa	1	11.60 ± 1.32
	2	10.43 ± 0.92
	3	8.55 ± 1.78
	4	10.75 ± 2.27
	5	11.71 ± 1.67
Alto Marañoñ	1	12.97 ± 1.45
	2	13.09 ± 2.23
	3	13.41 ± 2.41
Junín	1	15.72 ± 1.44
	2	10.48 ± 2.02
Tingo María	1	12.24 ± 1.89
La Merced	1	12.75 ± 1.43

^a Di Ferrante turbidimetric method.

2.4. Enzyme purification

Hyal-Ba was purified by three chromatographic steps using ion-exchange and two size exclusion chromatography. First step, 300 mg of *B. atrox* venom in 0.05 M ammonium acetate buffer, pH 5.0 was centrifuged at 4000 rpm for 15 min to remove insoluble materials. The venom solution was loaded to a DEAE Sephadex A-50 (1.2 × 40 cm) column, equilibrated with 0.05 M ammonium acetate buffer pH 5.0. Samples were eluted with NaCl (0–0.6 M) linear gradient. One ml fractions were collected at a flow rate of 14 ml/h. Fractions with hyaluronidase activity were pooled using AMICON concentrated tubes. In a second step, hyaluronidase-containing samples were applied to a Sephadex G-100 (1 × 40 cm) column, equilibrated and eluted with the same buffer, and 1 ml fractions were collected at a flow rate of 10 ml/h. Finally, SDS-PAGE in a 10% polyacrylamide separation gel showed minor contaminants in the active fraction pool. Therefore, it was applied to a Sephadex G-75 (1 × 30 cm) column, equilibrated and eluted with the same buffer, and 1 ml fractions were collected at a flow rate of 10 ml/h. Homogeneity of the active fraction was confirmed by RP-HPLC Vydac C₁₈ (22 mm × 25 cm) column. Sample was eluted with a linear gradient from 0 to 70% (acetonitrile, ACN, 0.1% of Trifluoroacetic acid, TFA) for 70 min at a flow rate of 1 ml/min. Absorbances were registered at 280 nm. The N-terminal amino acid sequence of purified protein was determined by Edman degradation method [23] using a protein sequencer Shimadzu Automatic System (PSSQ-21A).

2.5. Molecular mass, carbohydrate detection and zymography

Hyal-Ba molecular mass (Mr) was determined by SDS-PAGE (10% gel) under reducing (4% 2-mercaptoethanol) or non-reducing conditions according to Ref. [24], and Coomassie blue G-250 stained. The presence of carbohydrate in Hyal-Ba (hexoses, hexosamines and sialic acid) was determined as previously described in Ref. [25]. Zymography assay using gel containing hyaluronan [26] was also used to detect hyaluronidase activity of Hyal-Ba. The gel was stained with Alcian Blue (Sigma Chemical Co.).

2.6. Biochemical parameters

Optimal conditions for Hyal-Ba activity were identified as mentioned in the enzymatic activity section, using 0.1 M buffers (ammonium acetate [pH 4.0–10.0], and Tris-HCl [pH 7–8], at temperature intervals (4–60 °C). Additionally, the effect of ions K⁺, Na⁺, Ca²⁺ and Mg²⁺ were assessed on Hyal-Ba activity at 0.01 M, 0.05 M, 0.1 M and 0.15 M, respectively. Assays were performed in triplicates and the optimal conditions were taken for kinetic studies.

2.7. Kinetic studies

Hyal-Ba (1.0 µg/ml) activity on HA (0–100 µg/ml) was performed according to Poh et al. [27]. The velocity of the reaction was expressed as micrograms of substrate hydrolyzed per milliliter per minute. Km and Vmax were calculated using the GraphPad (Software Inc., San Diego, USA). Additionally, the specificity of the purified Hyal-Ba was assayed using several native substrates (chondroitin sulfate A, B, C, and heparin, 5–50 µg/ml, each one).

2.8. Enzymatic inhibition

Several enzyme inhibitors including EDTA, iodoacetate, PMSF, 2-mercaptoethanol and dexamethasone were tested on Hyal-Ba

activity. The purified enzyme (1 µg) was pre-incubated with the inhibitors (5 mM each) in 50 mM ammonium acetate buffer, pH 6.0, for 10 min at 37 °C and the mixture Hyal-Ba/inhibitors was further tested for residual Hyal-Ba activity as described above.

2.9. Biological characterization

2.9.1. Toxicity

Toxicity was assessed by intraperitoneal injection (i.p.) of samples into mice (male BalbC strain, 18–22 g, INS). Groups of five mice were injected with 0.1 ml of physiological saline containing 5 or 20 µg of Hyal-Ba, *B. atrox* venom (20 µg) or 0.9% NaCl (negative control). Mice were monitored every 12 h and clinical signs were recorded. After 48 h mice were euthanized and carcasses were analyzed for cutaneous or visceral damages.

2.9.2. Hemorrhagic activity

Five groups of four Balb/c mice were used to test hemorrhagic activity of Hyal-Ba according to Kondo et al. [28]. Briefly, the first group was injected with 1 minimum hemorrhagic dose (MHD) equivalent to 1.2 µg of *B. atrox* venom [29]. The second group was injected with 1 heat-inactivated MHD venom at 60 °C. The third group was injected with 1.2 µg of Hyal-Ba. The fourth group received a mixture of crude venom with Hyal-Ba (1.2 µg each, 1 MHD), and the fifth group was injected a mixture of 1 heat inactivated MHD with 1.2 µg of Hyal-Ba. After 2 h injection, mice were sacrificed and the presence of hemorrhagic spots in the inner side of skin was measured.

2.9.3. Edema induction

Edema-forming activity of Hyal-Ba was evaluated in male Balb/C mice (20–22 g) according to Ref. [12]. Briefly, 0.1 ml of purified L-AAO (15 µg) from the same *B. atrox* venom with/or without Hyal-Ba (1.4 µg) were injected into the subplantar area of the back right paw. The volume of the paw was measured using a paquimeter before and after toxin injections at different time intervals (n = 5). As negative control, an equal volume of saline solution was injected into the contralateral paw.

2.9.4. In-vitro hemolytic activity

In-vitro hemolytic activity of Hyal-Ba was evaluated according [30]. Briefly, an overlaid mixture containing agarose (1.5%), 3% of human blood red cells suspension (hBRC), egg yolk emulsion (3%) and 10 mg/mL HA (diluted in 0.1 M phosphate buffer, pH 7.0 containing 0.15 M NaCl) was prepared. Then, 5 mL of the heated overlaid mixture was loaded into 12 mm plate dishes. Once it was solidified, four holes of 4 mm diameter were punched-out, and each hole was filled with 15 µg of *B. atrox* venom, 15 µg of Hyal-Ba and 15 µg of Hyal-Ba plus 15 µg of venom or 15 µg of heated venom (at 60 °C). As negative control (0.85% NaCl) was used. Plates were incubated for 12 h at room temperature at 80% relative humidity and after incubation, the hemolysis area was measured.

2.10. Assays with antithrombotic polyvalent serum

An agar-gel immunodiffusion assay (AGID) was performed to test the neutralizing effect of commercial antithrombotic polyvalent serum with Hyal-Ba. Briefly, 0.15 M Phosphate Buffered Saline (PBS) pH 7.0 was used to prepare 1% agarose gels in 4 inch × 2 inch glass slides. Later, 20 µl of antithrombotic polyvalent serum were loaded in a central well against *B. atrox* venom and Hyal-Ba (10 µl each). Gels were incubated at 10 °C for 48 h. After incubation, the agarose gels were washed twice with a washing solution (5% trisodium citrate and 0.9% NaCl) and the precipitation bands were stained with Coomassie blue. Neutralizing capacity of therapeutic antivenom on

Hyal-Ba activity was determined after 30 min incubation (37°C) of antivenom neutralizing doses (half, one and two) in the presence of purified native Hyal-Ba ($20\ \mu\text{g}/\mu\text{l}$). Then, antivenom/Hyal-Ba mixtures were tested for enzymatic activity as mentioned above.

2.11. Molecular characterization

2.11.1. Synthesis and sequencing of cDNA

cDNA was obtained from *B. atrox* fresh venom as described

previously [31]. Two external primers HD0: 5'-ACCTCGTCGTCTCTGCCTGGCCTACA-3' and HD11: 5'-CCACACAATACTTTAA-TATTTGGG-3' were designed on the basis of the highly conserved cDNA sequences encoding Hyal from *Protobothrops mucrosquamatus* (XM_015822281.1), *Ovophis okinavensis* (AB851978.1), *Protobothrops flavoviridis* (AB851937.1) and *Bothrops neuwiedi pauloensis* (FJ654998.1). Primers were synthesized by Invitrogen. Hyal-Ba gene was amplified using a Master Mix Platinum® Taq DNA Polymerase kit (Invitrogen) according to the manufacturer's instructions and the

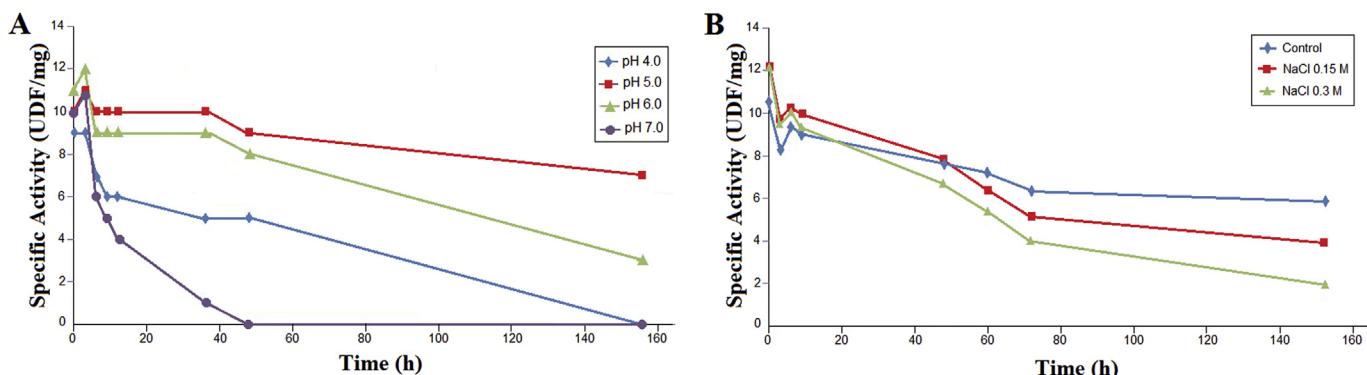


Fig. 1. Stability assay of hyaluronidase activity from *Bothrops atrox* venom. Prior to the purification of Hyal-Ba, samples of crude venom ($1\ \text{mg}/\text{mL}$) were evaluated by Di Ferrante turbidimetric method from 0 to 160 h after being incubated at 20°C under different pH (A) and NaCl (B) conditions. The values are presented as mean \pm SE ($n = 3$) and represent three independent experiments.

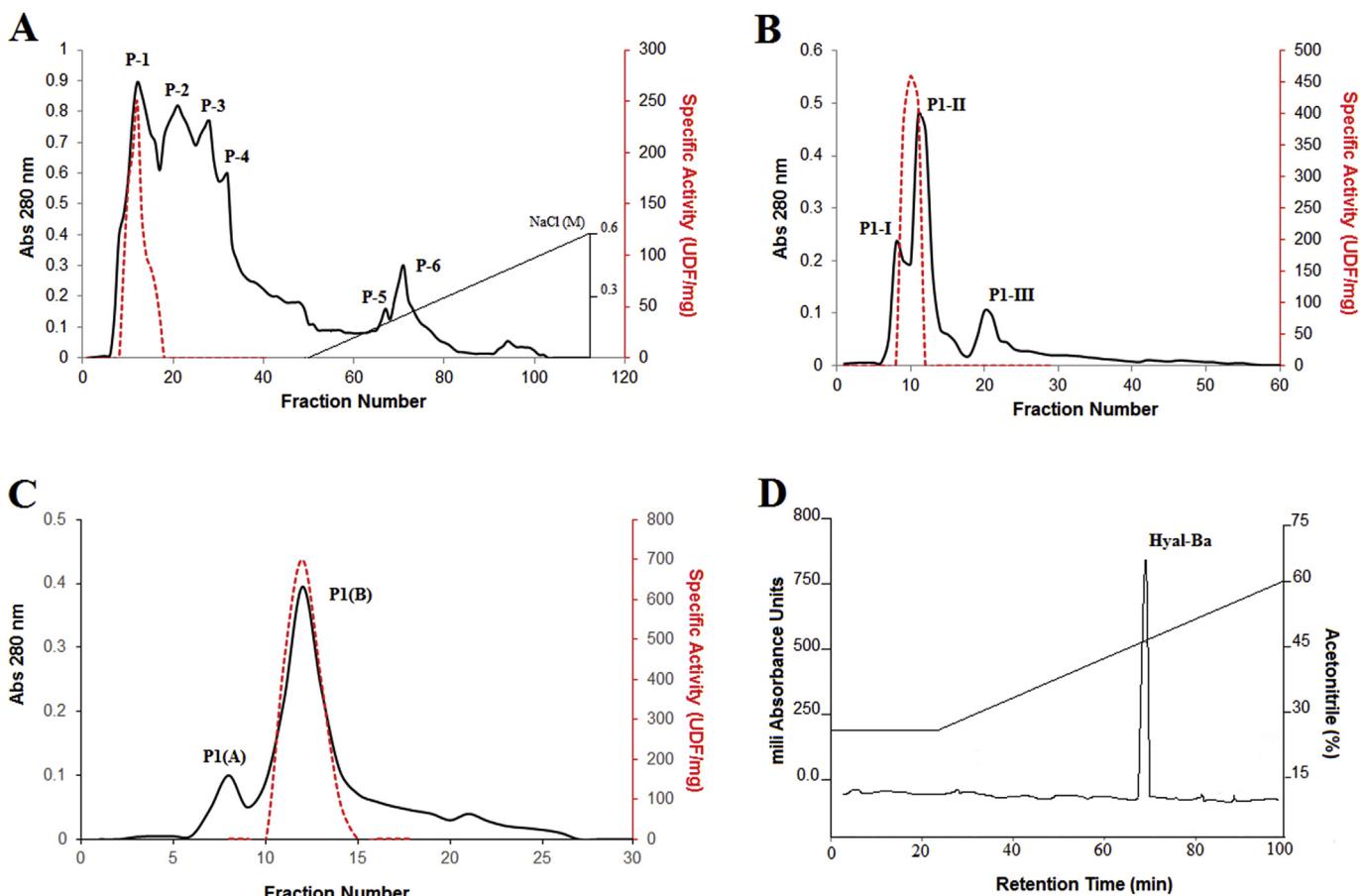


Fig. 2. Purification profile of Hyal-Ba from Peruvian *Bothrops atrox* venom. Black lines represent the amount of proteins measured at 280 nm. Red dotted lines represent the specific Hyal-Ba activity. A) Ion-exchange chromatography of crude venom (300 mg) on DEAE Sephadex A-50 column equilibrated with 0.05 M ammonium acetate buffer (pH 5.0) and eluted with a linear gradient of NaCl (0–0.6 M). B) Size exclusion chromatography on Sephadex G-100 column. P-1 fractions from the previous step (3.2 mg) containing hyaluronidase activity were applied to a size exclusion G-100 column. The column was equilibrated and eluted with 0.05 M ammonium acetate buffer (pH 5.0). C) Gel filtration chromatography on Sephadex G-75 of fractions (P1-I and P1-II) from previous step (2.5 mg). The elution was under the same conditions that in B. D) Reverse-phase HPLC analysis of purified protein using Vydac C₁₈ (822 mm × 25 cm) column equilibrated with TFA 0.1%. Under an acetonitrile gradient (0–60%).

Table 2Purification scheme of Hyal-Ba from Peruvian *Bothrops atrox* venom.

Steps	Total protein (mg) ^a	Protein recovery (%)	Total activity	Specific Activity ^b (U/mg)	Purification (fold)	Recovery (% initial activity)
Crude Venom	300	100	3600	12	1	100
DEAE A-50	13.08	4.3	2070	158.3	13	57.5
Sephadex G-100	3.219	1.1	1352	420	35	37.5
Sephadex G-75	2.58	0.86	11129	437.5	36	29.55

^a Total protein quantified by Lowry method.^b One unit (U) of enzymatic activity is defined as the amount of enzyme that reduce 50% turbidity caused by 100 µg of HA substrate of the control turbidity.

purified amplicons were sequenced by using two internal primers (HD5: 5'-TGAAACCATATTGAGGTCAAGT-3' and HDR7: 5'-CACATCTTCCACGT TTCTTGCA-3') on the ABI 3730 XL automated sequencer (Macrogen, Inc, South Korea). Hyal-Ba cDNA sequence was translated to protein employing the Translate tool server (<https://web.expasy.org/translate/>), and an *in silico* analysis was performed with a ProtParam tool server (<https://web.expasy.org/protparam/>).

2.11.2. Multiple alignment and phylogenetic analysis

Hyaluronidases amino acid sequences were retrieved from Cazy, Pfam, Expasy, Uniprot and NCBI databases (Hyaluronidases identifiers EC/3.2.1.35, PF01630, GH56 and IPR001329). Amino acid sequences were pre-aligned using the CLUSTAL X multiple sequence alignment program [32] and the representation was generated using ESPript server (ESPript - <http://escript.ibcp.fr>). Statistical and phylogenetic analyses were carried out in R by using the Pvclust package [33].

2.11.3. Structural modeling

Hyal-Ba three-dimensional structure prediction was made using comparative homology modeling [34]. A BLASTp search was performed to identify appropriated Hyal-Ba homologue templates. Only three template structures were considered for modeling: 1FCU (*Apis mellifera*), 2TAM (*Vespa vulgaris*) and 2PE4 (*Homo sapiens sapiens*). Modeller v 9.16 [34] was used to generate Hyal-Ba model in.pdb format. The best model was selected based on the least discrete optimized protein energy (DOPE) and modeller objective function (MOF) scores. The protein structure was refined using 3Drefine server (<http://sysbio.rnet.missouri.edu/3Drefine/>). Root Mean Square Deviation (RMSD) was calculated using the program VMD v 1.9.2 [35]. Validation analysis was performed using SAVES Sever (<https://services.mbi.ucla.edu/SAVES/>) which includes PROCHECK WHAT_CHECK, ERRAT, VERIFY_3D and Ramachandran counter map calculations. Finally, the structure visualization was generated by using the Pymol software [36].

2.12. Statistical analysis

The enzymatic activity data were expressed as mean of three independent experiments ± standard deviation (S.D.). Significant differences between treatments were estimated using ANOVA and the Student's T-test implemented in the software GraphPad Prism 6.07 (GraphPad Software Inc. USA). *P*-values less than 0.01 (*p* < 0.01) were considered statistically significant.

3. Results

3.1. Enzymatic stability

Hyaluronidase activity of *B. atrox* venom was more stable at pH 5.0 for 40 h, with a slight decrease after 160 h incubation (Fig. 1A). In addition, NaCl (0.15 M) enhanced its activity within the first 20 h, but slightly decreased at 24 h - (Fig. 1B). Geographic variation in hyaluronidase activity of *B. atrox* venom samples was not detected,

but individual differences were identified (Table 1). Venom samples from Alto Marañon locality were used to purify Hyal-Ba.

3.2. Purification of Hyal-Ba

Hyal-Ba was purified to homogeneity using successive chromatography on DEAE Sephadex A-50, Sephadex G-100 and Sephadex G-75 columns as shown in Fig. 2, and summarized in Table 2. First, the venom (300 mg) was separated in 6 peaks (P1 to P6) by DEAE Sephadex A-50 column at pH 5 using a gradient of NaCl as eluent (Fig. 2A). Hyal activity was concentrated in P1 (13 mg) which eluted before any NaCl gradient has started. This fraction was dialyzed against distilled water and lyophilized. For the second step, the active P1 pool was applied to a Sephadex G-100 column, and three main peaks (P1-I to P1-III) were obtained (Fig. 2B). Hyal activity was detected between P1-I and P2-II, and thus, the corresponding fractions were pooled (3.2 mg). Third step, active material from the preceding step was applied to a Sephadex G-75 column (Fig. 2C), by this procedure, 2.58 mg of active enzyme was obtained (36-fold purification), that was termed Hyal-Ba, and it represents 0.86% of total protein in the venom (Table 2). Homogeneity of the purified enzyme was analyzed by RP-HPLC (Fig. 2D) and SDS-PAGE (12% gel) that shows a single protein peak and a band of approximately 69 kDa, respectively (Fig. 3).

The molecular mass (Mr) of the isolated enzyme under both, reducing and non-reducing conditions, as well as by zymography assay was approximately 69 kDa (Fig. 3A and B). Moreover, the first 21 N-terminal amino acids of the purified enzyme (MQAKAPMYPNEPFLVFWNAPT) were identical to its corresponding cDNA

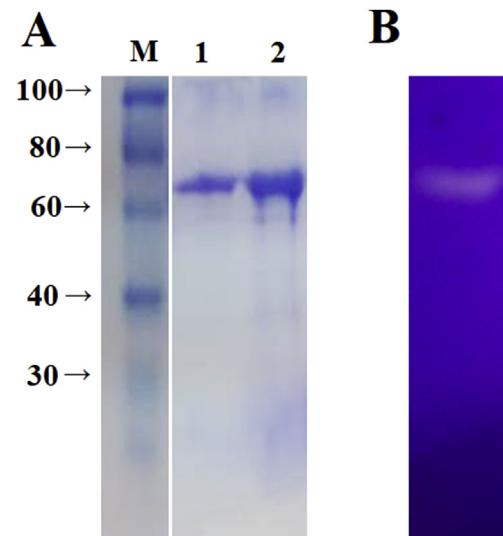


Fig. 3. Homogeneity analysis of Hyal-Ba. A) SDS-PAGE profile (12%) stained with Coomassie Blue. M: Molecular markers, Line 1: Under reducing conditions and 2) non reducing conditions. B) Zymography analysis in gel containing hyaluronic acid (0.5 mg/mL) stained with Acian Blue.

deduced sequence (Fig. 4). The content of hexoses and hexosamines were 16.38% and 2.7%, respectively. Finally, the sialic acid contents accounts for 0.69% of the enzyme composition.

3.3. Enzymatic and biochemical properties

The purified Hyal-Ba hydrolyzed HA (specific activity = 437.5 U/mg) but it did not hydrolyze neither chondroitin sulfate (A, B, and C), nor heparin (data not shown). Optimal enzymatic activity was found at pH 6.0 and at 40 °C (Fig. 5). Its activity was greatly diminished below pH 4.0 and above pH 7.0 (Fig. 5A). Likewise, temperatures below 25 °C and above 45 °C reduced its enzymatic activity ($p < 0.001$) (Fig. 5B). Most of the Hyal-Ba catalytic activity was maintained using a NaCl solution at pH 6.0 for 96 h at 38–40 °C (Fig. 5C), while its activity was abolished at pH 5 after 48 h. In the

presence of 0.2 M of Na^+ and K^+ its activity was enhanced. By contrast, Mg^{2+} , Br^{2+} , Ba^{2+} , Cu^{2+} , Zn^{2+} , and Cd^{2+} cations reduced Hyal-Ba catalytic effect (Fig. 5D). K_m value was 0.31 μM (21.3 $\mu\text{g/ml}$) and the V_{max} was 1.9 μM (13.6 $\mu\text{g/min}$). Furthermore, Hyal-Ba activity was inhibited by EDTA, iodoacetate, 2-mercaptoethanol, TLCK and dexamethasone (Fig. 6A). However, PMSF the specific inhibitor of serine proteases had no effect on the enzyme activity. On the other hand, commercial antithrombotic serum efficiently neutralize its enzymatic activity (Fig. 6B).

3.4. Spreading activity of Hyal-Ba

Hyal-Ba (1.2 µg) did not induce any toxic effect in BalbC mice after i.p. injection, although the same dose of 1.2 µg of *B. atrox* venom injected in a comparable group of mice caused the

Fig. 4. Nucleotide sequence and inferred amino acid sequence of Hyal-Ba. The right and left numeral of the figure refer to nucleotides and amino acid position in the sequence respectively. Signal peptide is underline. The catalytic residue (E_{135}) and positional ($D_{133}, Y_{206}, Y_{253}, W_{328}$) are black and grey highlighted respectively. ORF termination by TAA codon is indicated by (–). Direct N-terminal sequence of the mature protein determined by Edman degradation is in bold. Putative N-Glycosylation sites are marked with boxes and highlighted according to the score levels: blue high; brown: low.

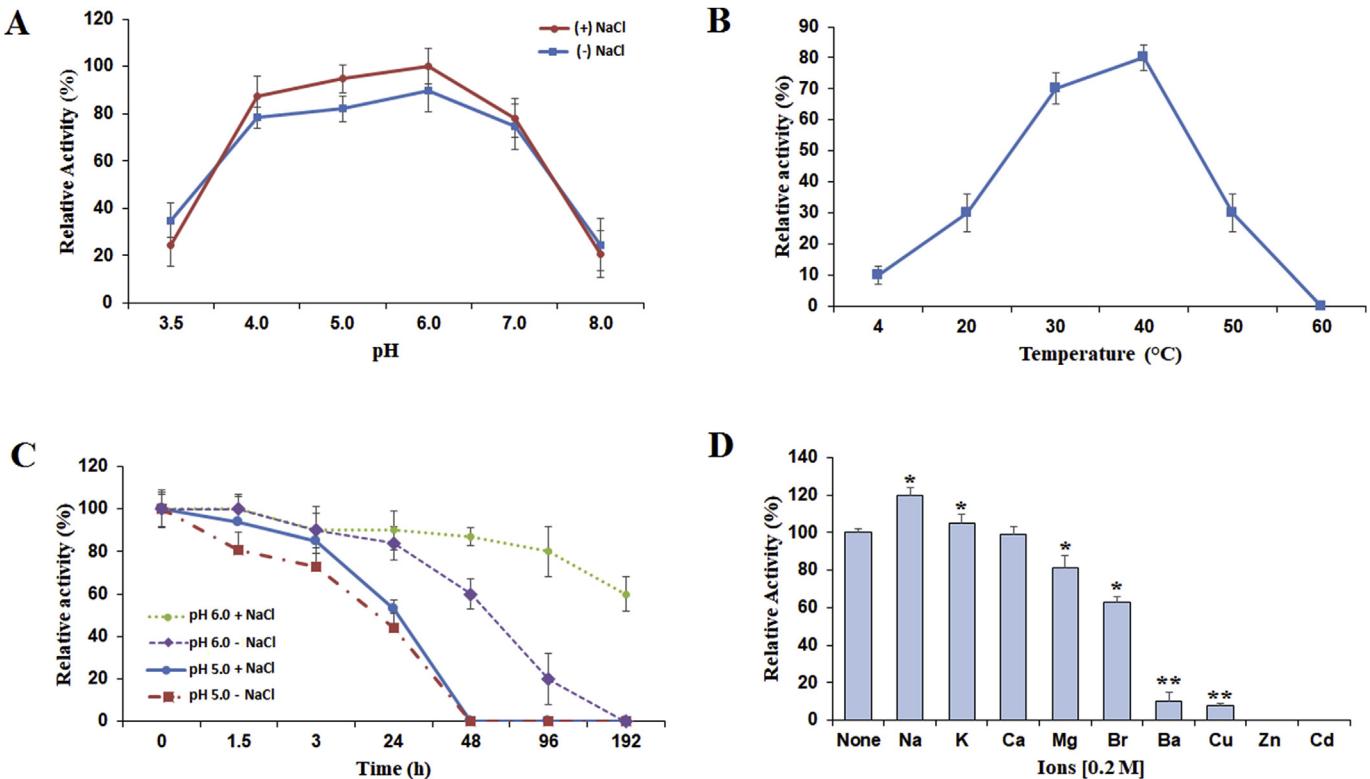


Fig. 5. Biochemical characterization of Hyal-Ba. Effect of pH (A), temperature (B), incubation period (C) and cations (D) in the Hyal-Ba activity. The enzymatic activity was measured using hyaluronic acid as substrate. The relative activity of Hyal-Ba determined under standard conditions was 100%. Data are expressed as the mean \pm S.D. ($n = 3$), and represent three independent experiments. ** $p < 0.001$ and * $p < 0.01$ compared to control in absence of ions.

characteristic pathophysiological disorders. Hence, they were likely caused by venom components other than Hyal-Ba. (Fig. 7A). Subcutaneous injection of (1.2 μ g) *B. atrox* venom induced extravascular bleeding on the underside of the mouse skin (hemorrhage of 15 mm diameter), this hemorrhagic spot increased by 86.7% with the addition of 1.2 μ g Hyal-Ba (diameter of 28 mm) (Fig. 7B). On the other side, venom after being heated to 60 °C resulted in a reduction of hemorrhagic spot (4.7 mm), and no significant difference ($p > 0.01$) was detected even after addition of Hyal-Ba. Furthermore, Hyal-Ba did not cause hemorrhagic activity by itself. Finally, the hemorrhagic activity was completely inhibited by one dose of the therapeutic antivenom serum (Fig. 7B). Moreover, mice injected with purified L-AAO from the same *B. atrox* venom exhibited the highest edema formation (90% paw thickness) at 50 min post-injection (Fig. 7C). Interestingly, limited edema formation, even below the control group, was induced by injection of Hyal-Ba. In addition, Hyal-Ba reduced the edema formation after being mixed with L-AAO ($p < 0.01$). All treatment groups showed significant differences as compared to the control group ($p < 0.005$) (Fig. 7C). Also, crude *B. atrox* venom possessed hemolytic activity (halo diameter 16 ± 2 mm). This was slightly enhanced ($p < 0.01$) after the addition of Hyal-Ba (halo diameter 21 ± 3 mm) (Fig. 7D). In contrast, the hemolytic effect was absent when *B. atrox* venom was heated to 60 °C, even after the addition of Hyal-Ba (data not shown).

3.5. Hyal-Ba nucleotide sequence and in silico studies

A full length cDNA sequence (2020 pb) of Hyal-Ba (GenBank accession: KY499627) (Fig. 4) was obtained and its deduced amino acid sequence encoded a protein of 449 aa carrying a signal peptide of 20 aa (GenBank accession: AUF71538). Mature Hyal-Ba is a

50.10 kDa glycoprotein with a theoretical isoelectric point (pI) of 9.19. In silico analysis of the Hyal-Ba amino acids revealed six potential N-glycosylation sites (Asn67, Asn103, Asn111, Asn153, Asn357 and Asn401), but only Asn111, Asn153 and Asn357 residues had the highest of presumptive glycosylation motifs according to the program NetNglyc. A multiple sequence alignment of Hyal (Fig. 8) identified a highly conserved catalytic residue (Glu135) and structurally relevant amino acids (Asp133, Tyr206, Tyr253 and Trp328). The presence of cysteines (Cys47, Cys211, Cys227, Cys340, Cys365, Cys370, Cys376, Cys427, Cys429 and Cys438) are highly conserved amongst snake species due to their structural relevance. Furthermore, Hyal-Ba exhibits unique mutations (Asn284, Phe290, and Asp319) relating to other Hyal sequences (Fig. 8).

Phylogenetic analysis of Hyal-Ba and other Hyals isolated from *Viperidae* venoms supported a monophyletic clade containing two subclades (Crotalinae and Viperinae) (Fig. 9). In addition, *Bothrops* Hyals formed a robust subgroup and their few mutations are shown in Table 3. Finally, the monophyly of Hyals reported for Elapidae species was not evidenced.

The structural modeling of Hyal-Ba was predicted using a human Hyal (2PE4) as a template (40% identity and 91% sequence cover). The structure corresponded to a TIM barrel (α/β) (Position: 36–356 residues) and an EGF-like domain (Position: 358–440 residues) (Fig. 10A). The structural modeling displayed a quality resolution of 91.72% and 89.35% by using the PROCHECK and VERIFY3D software's respectively. A Ramachandran plot showed that 95.3% of amino acids were located in a favorable position with 4% in the allowed region and 0.7% in low confidence region. The PROSA analysis indicated a Z-score of -10.21. The predicted N-glycosylation sites were distant from the catalytic site and possible 5 sulfide bridges were also present in the structure (Fig. 10B). The molecular surface site is shown in Fig. 10C. The RMSD and carbon backbone

superposition with the template was 0.68 Å (Fig. 10D).

4. Discussion

This paper reports the purification (36-fold) and its molecular and biological characterization of a hyaluronidase (Hyal-Ba) from the venom of the Peruvian *Bothrops atrox* snake. Hyals are endoglycosidases which primarily degrade hyaluronan, a glycosaminoglycan (GAG) of ECM [15,16], and are widely distributed in nature, including in snake venoms. Unlike to other toxic compounds found in snake venoms e.g. SVMPs, PLA₂s, L-amino acid oxidases, myotoxins amongst others, Hyals are apparently the least studied proteins, because they have been known as spreading factors and non-toxic enzymes [15–18]. Thus, these enzymes have received much attention due to the fact that Hyals appear to play a significant role in venom-caused local and systemic effects [10]. They are involved in several pathophysiological processes, such as inflammation, angiogenesis, wound healing and systemic distribution of venom compounds [11,15,16].

Hyal-Ba has been characterized as a 69-kDa monomeric glycoprotein whose molecular mass falls within the range of 33–110 kDa described for other homologous Hyals from animal venoms such as scorpions, bees and snakes [12,37]. This data contrast with the value of 110 kDa determined for a Hyal previously isolated from the same *B. atrox* venom [21]. The presence of Hyal isoforms of high or low molecular mass are commonly found in snake venoms as well

as in other sources including in humans [38,39]. Regarding this issue, individual and geographical variations might cause a selective gene expression of different Hyal isoforms in animal venoms [39–43]. Furthermore, several studies have reported genetic variation in the gene expression of other components of snake venom [13,44]. Native Hyal-Ba maintains its functional activity when kept at room temperature and between pH 5.0 to pH 6.0 for 5 days. This contrasts with a Hyal isolated from *Naja naja* venom that was stable for 15 days at 37 °C [37], while other Hyals maintains their activity for a long time under freezing conditions [12,40]. Unlike, Hyal-Ba loss more than 50% of its activity at 50 °C, probably due to the loss of its structural conformation, which is a common feature of proteins like Hyals. These data are in accordance to those described for Hyals of *Bungarus caeruleus* and *Akigistodon blomhoffii ussurensis* [41,42].

Furthermore, Hyals have been grouped according to their pH-optimum of activity into acid (active between pH 3 and 4) or neutral active enzymes (active between pH 5 and 6) [12]. Based on its biochemical properties, Hyal-Ba which exhibited maximum hydrolysis of HA at pH 6.0 belongs to the group of neutral active enzymes like to CdtHya1, isolated from the venom of South American rattlesnake [12]. These results are closely related to those reported in the process of envenoming, where some components of the venom of snakes such as SVMPs, LAAOs, phospholipases, among others, induce an inflammatory response and whose exudates cause an additional decrease in pH (between 6.0 and 6.9) in the subcutaneous tissue which has already an acid pH [12]. Consequently, those conditions might promote an optimum acid environment for Hyal-Ba to exert its maximum diffusin-supportive activity on other critical venom components into the prey or humans.

Hyal-Ba contains 13% of carbohydrate associated with a high percentage of hexosamines, while the Hyals from *Lachesis muta* and *Apis mellifera* had 18% and 7% of carbohydrates, respectively [43,45]. N-glycosylation is the most common posttranslational modification of the Hyals and is involved in their catalytic activity [46]. Our results suggest five Hyal-Ba potential glycosylation sites, which are highly conserved in other snake venom Hyals (Fig. 8). Of these residues, Asn99 and Asn350 are conserved in human Hyals. Interestingly, Asn350 is an invariant N-glycosylation motif for mammalian Hyals [47]. As previously reported for other Hyals, Na⁺ is a significant enhancer of Hyal-Ba activity whereas Mg²⁺, Br²⁺, Ba²⁺, Cu²⁺ and Zn²⁺ are important inhibitors. However, there is a heterogeneous effect with other ions such as K⁺ which stimulate Hyal-Ba and CdtHya1 enzymatic activity [12] but inhibited the enzymes from *L. muta*, *B. caeruleus* and CcHasell [19,41,48]. Ca²⁺ does not affect Hyal-Ba as well as the Hyals from *A. c. contortrix* and *B. caeruleus* [16,41], but is a weakly inhibitor of CcHasell and CdtHya1. Furthermore, Zn²⁺ is a potent inhibitor for Hyal-Ba and CcHasell, but not for the Hyal of *B. caeruleus* [41]. Like other homologous enzymes, Hyal-Ba was not affected by PMSF, a specific inhibitor of serine proteinases. In contrast, 2-mercaptoethanol considerably inhibited the enzymatic activity suggesting that disulfide bonds have a crucial role in the Hyal-Ba structure stabilization. Inhibition caused by EDTA suggests that Hyal-Ba activity is metal-dependent. Overall, the effects of several inhibitors found in this study were similar to those obtained for CcHasell [48], but not to Hyals isolated from the venoms of stonefish and scorpion *Palamneus gravimanous* [49]. Interestingly, TLCK and iodoacetate showed a significant inhibition of Hyal-Ba in a similar way to the reported for a Hyal from *L. m. muta* [43] but unlike to CcHasell. In spite of this evidence, there is not a clear understanding of the action of these inhibitors, but they might interact with critical histidine and cysteine residues. Moreover, the anti-inflammatory agent dexamethasone inhibited Hyal-Ba activity in a similar way

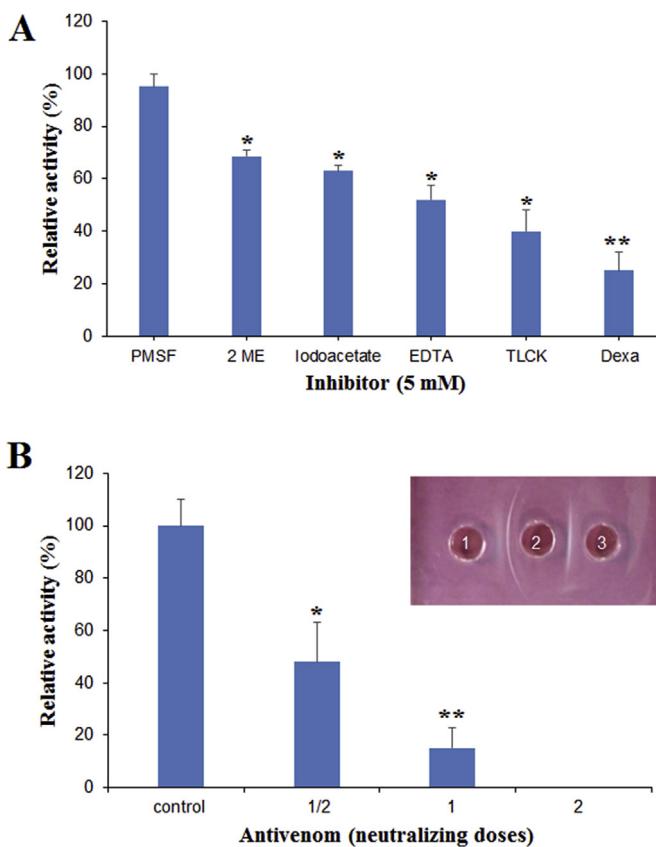


Fig. 6. Inhibition and neutralization of Hyal-Ba activity. A) Effect of inhibitors (at 5 mM) upon Hyal-Ba activity using hyaluronic acid as substrate. B) Recognition and neutralization of Hyal-Ba by antivenom polyvalent serum (INS) using 0.5, 1 and 2 neutralizing dose (1 dose = 10 ml of antivenom per 25 mg of venom). Insert: Immunodiffusion probe, (1) Crude venom, (2) Antivenom polyvalent serum and (3) purified enzyme. The relative activity of Hyal-Ba determined under standard conditions is 100%. Data are expressed as the mean \pm S.D. ($n = 3$), and represent three independent experiments. ** $p < 0.001$ and * $p < 0.01$ compared to control.

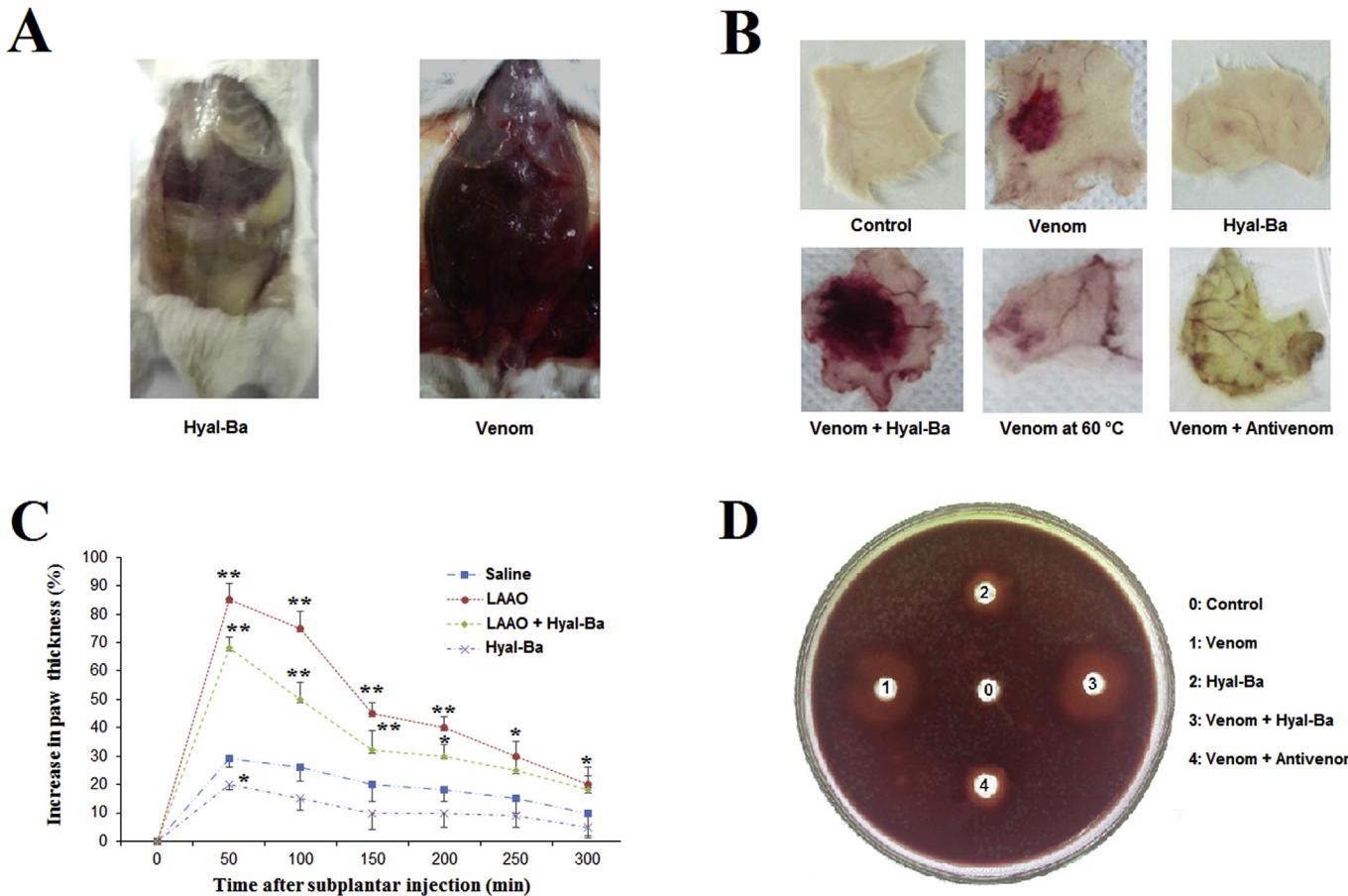


Fig. 7. Spreading property of Hyal-Ba. A) Evaluation of visceral damage in Balb C mice due to *B. atrox* venom. B) Hemorrhagic activity, addition of Hyal-Ba (1.2 µg) increased the hemorrhagic spot ($28 \text{ mm}^2 \pm 3.0$) compared with crude venom alone ($15 \text{ mm}^2 \pm 2.0$). C) Edema activity, Hyal-Ba (1.2 µg) reduce the paw edema produce by LAAO from *B. atrox* (15 µg) and by saline solution (control). D) Hemolytic activity in plate, 5 µg of the purified enzyme increase the hemolysis ($\varnothing = 21 \text{ mm} \pm 3 \text{ mm}$) compared with 15 µg of crude venom ($\varnothing = 16 \text{ mm} \pm 2 \text{ mm}$). Antibotropis serum neutralized the hemorrhagic and hemolytic activities (B and D). The quantitative probes were made in triplicate and mean is showed plus S.D. ** $p < 0.001$ and * $p < 0.01$ compared to control.

as NNH1 from *N. naja* [50,51], mainly due to interference with hyaluronic acid substrate and thus reducing its availability for the Hyal. To our knowledge degradation products of hyaluronic acid promote an inflammatory response, because its degradation alters the structural integrity of ECM during snakebites. Therefore, Hyals merits prompt consideration and much more studies before beginning clinical trials that may contribute to new therapeutic strategies for better management of snakebite [15,50].

Hyals cleave hyaluronic acid from the connective tissue of the prey or victim and allow the diffusion of toxins from the venom [15], thereby they have been known as “spreading factor” for a long time. In this way, hyaluronidases play an essential role as mediators of venom toxicity, both at the local and systemic level [52]. Our results demonstrated that Hyal-Ba acts as a spreading factor because it enhanced the hemorrhagic activity caused by SVMPs and decrease edema. Similar results were reported with CcHasell from *C. cerastes* [48] and CdHya1 isolated from the venom of South American rattlesnake [12]. Moreover, Hyal-Ba increased the hemolytic halo of the crude venom in the blood-agar plate assay. Interestingly, all in vitro activities were efficiently neutralized by antihotropic polyvalent serum. Therefore, the neutralization of Hyal-Ba could contribute to ameliorate the local tissue damage and the systemic distribution of lethal toxins of *B. atrox* venom, as had been reported for other Hyals [46,48,53].

This work, described the cDNA and predicted amino acid

sequence of Hyal-Ba which is consistent with Hyals found in other animal venoms. The primary structure of Hyal-Ba shares high sequence homology with other Hyals, e.g. BATXHYAL1, from the Brazilian *B. atrox* (Accession: JAV01892) and BmooHyal-1 from *B. moojeni* (Accession: ATU85542). Six different residues were present in Hyal-Ba related to BATXHYAL1 in the positions 150 (K→E), 167 (Q→R), 169 (F→Y), 192 (H→Q), 200 (P→T) and 232 (I→V); however, each change was kept within the same group of amino acids. Moreover, the conserved residues are primarily involved in protein tertiary structure. Cysteines and disulfide bonds are highly conserved in the protein backbone. According to Harrison et al. [54], the predicted disulfide bonds are C₂₇–C₃₂₀, C₁₉₁–C₂₀₇, C₃₄₅–C₃₅₀, C₃₅₆–C₄₀₉. Conservation of cysteines among Hyals from diverse organisms suggests that they have similar tertiary structures [46,54]. The binding site cleft defined that the catalytic residues (Asp129, Glu131 and Tyr247) are conserved related to hHyal-1 (Fig. 8). Furthermore, Tyr202, which is important for interaction with hyaluronic acid and other substrates, is also conserved in Hyal-Ba [47]. Additionally, motif B(X)₇B, related to binding to hyaluronic acid [55] and which was reported in a Hyal from stonefish [56], is present in four regions inside the predicted primary structure of Hyal-Ba (R149 to R157, R169 to R177, K356 to K364 and K364 to K372). The two last regions are consecutive and conserved in all reported Hyals from snake venoms. These results reinforce the specific affinity of Hyal-Ba for hyaluronic acid. The

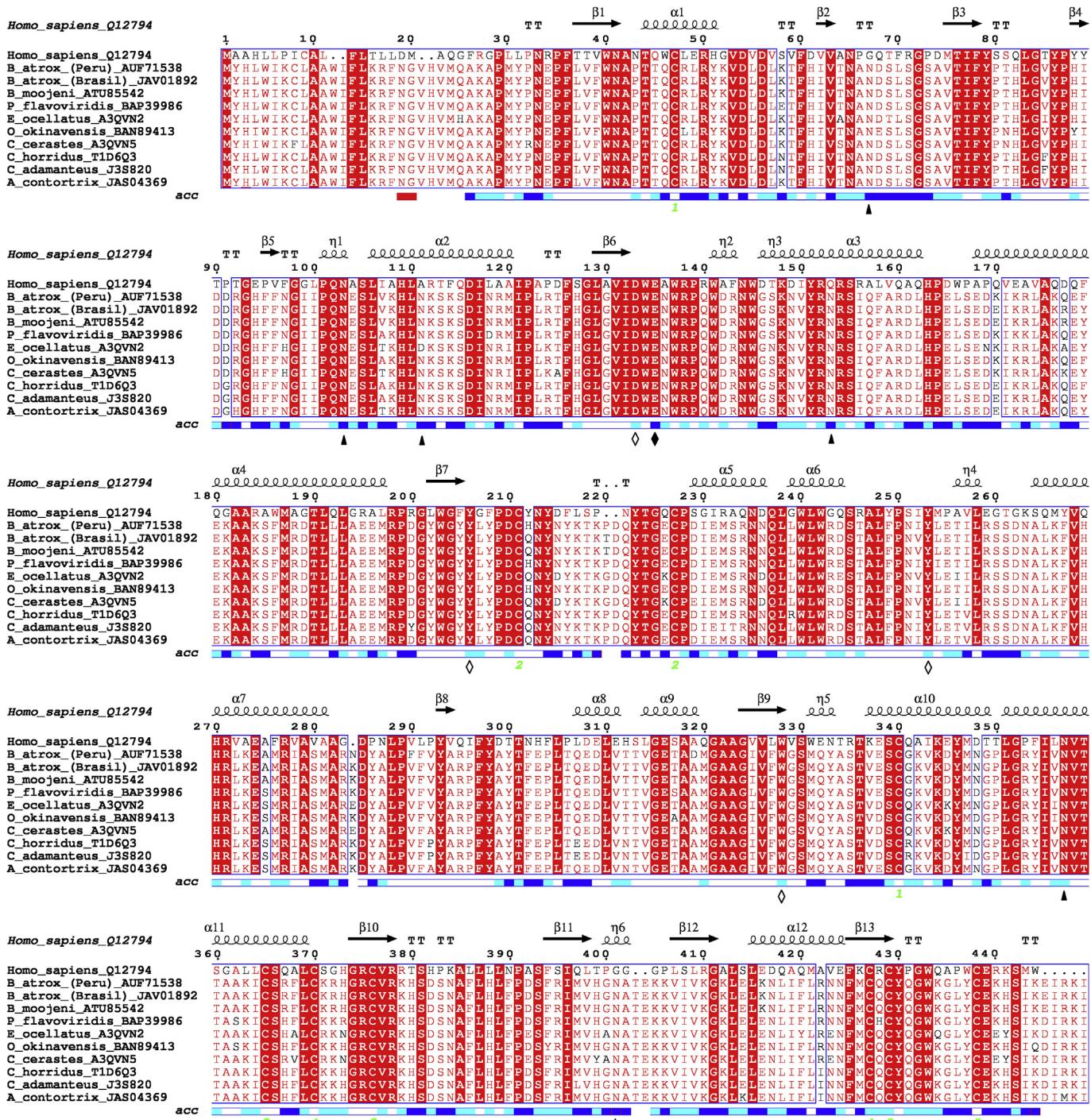


Fig. 8. Multiple alignment of Hyal-Ba with other snake venom and human hyaluronidase. The red colored residues show the conserved regions throughout. The residues were numbers in alignment to human hyaluronidase and its secondary structural elements. The catalytic residue E₁₃₅ is in (◆). The residues D₁₃₃, Y₂₀₆, Y₂₅₃ and W₃₂₈ essential for the positioning of the carbonyl group of the substrate catalysis are in (◊) plus the residue Tyr₂₀₂ (■), responsible for specificity for hyaluronic acid (Bordon et al., 2002). The asparagines for N glycosylation motif Asn-aaX-Ser/Thr are marked with (▼). Cys -Cys bonds are showed with green number. Surface accessibility is depicted for modeled regions with white, cyan and blue color for buried, intermediate, accessible residues and incomputable residues respectively. Alignment and figure were generated by Clustal W 8.0 program and ESPript server, respectively.

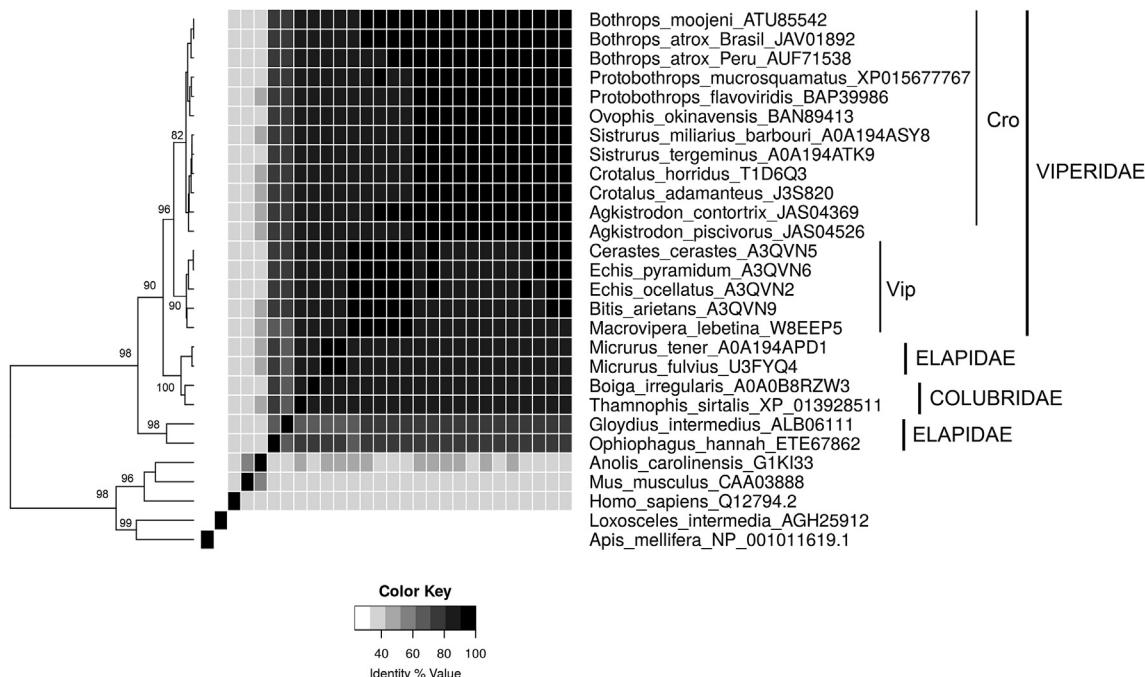


Fig. 9. Identity Matrix Heatmap and Neighbor-Joining Tree for venom hyaluronidases. Each block in the heat map represents the sequence identity of a species when compared to all others in this tree. The alignment was generated with Clustal Omega. The color key goes from cero identity (white) to 100% (black). The cladogram is located to the left of the figure and the species names at the right. The figure was produced with the package gplots at the software R.

Table 3
Singular mutation of hyaluronidase from clade Bothrops.

Position	Clade Bothrops	Others Clades
275	Ala	Ser
367	Arg	His
416	Lys	Glu
445	Glu	Asp

superposition of the modeled structure of Hyal-Ba with the reported structure of the Human Hyal 1 (PDB: 2PE4) shown structural similarity (Fig. 10 D), despite the fact that there are only 171 residues in conserved position (145 in the catalytic domain and 26 in the EGF-like domain). The major difference in other residues might be related to the variability of enzyme activity between these proteins. In Hyal-Ba, the EGF-like domain is distant from the active site (Fig. 10A and B), and thus it is not likely to be involved in substrate recognition. EGF domains are involved in cell adhesions, cell-cell communication, blood coagulations and they are thought to mediate protein-protein interactions associated with regulations of cellular growth and development [47]. However, the exact role of EGF-like domains in Hyals from snake venoms is yet to be determined [57].

The phylogenetic analysis of the selected homologous sequences to Hyal-Ba was in agreement with the results reported by Barathi et al. [57], and evidenced a remarkable Hyals monophyly of the viperids. This clade included an evident separation between the Hyals from crotaline (pitviper) and viperine (true viper) snakes. Likewise, four mutations in positions 103, 365, 407, 436 (Table 3), resolve the *Bothrops* clade as monophyletic. The phylogenetic tree within the viperids indicates that the analyzed Hyals have a remarkable correlation with the speciation of these snakes, thus demonstrating the orthologous character of these enzyme genes in the viperids. Surprisingly, Hyals monophyly was not resolved for the elapids and colubrids, indicating that the Hyals of the *Micrurus*

species are more related to the colubrids than to the Ophiophagus. On the other hand, it seems that the evolutionary history of the Hyals from snakes has followed a different genetic path to the Hyals from mammals and arthropods. The monophyly of the Hyals from snakes can be supported by amino acid changes of the same family. The bioinformatic analysis of the primary structure of snake Hyals report a molecular mass between 49 and 50 kDa and a pI between 8.87 and 9.23, which turn out to be higher to their mammalian and arthropod Hyals counterparts (39 kDa – 44 kDa and pI 8.6–8.7) analyzed in this work.

According to the biochemical characterization, deduced amino acid sequence and phylogenetic analysis, Hyal-Ba is a glycoside hydrolase enzyme (Family 56 as described by the carbohydrate-active enzymes database at www.cazy.org) with specific hyaluronidase activity (E.C. 3.2.1.35). This group comprises venom enzymes and biologically active peptides (around 220 proteins) and their sequences which had a high degree of similarity to PH-20 sperm protein involved in cellular adhesion. Within this group, Hyals can be considered as relevant biological tools with emphasis in the medical field since some of them are widely used in orthopedic surgery, ophthalmology, internal medicine, oncology, dermatology and gynecology. In addition, clinical studies have suggested topical or systemic application of Hyals as adjuvants and may increase the therapeutic index of anticancer drugs [17,58]. More detailed investigations of Hyal-Ba in this field, would propose the value of this enzyme as a potential biotechnological tool.

5. Conclusion

Hyals are important compounds during the envenoming process caused by viper snakes and despite its great value available information on it is still limited. A Hyal from the Peruvian *Bothrops atrox* venom (Hyal-Ba) was purified and characterized at the biochemical and molecular level. Hyal-Ba was considered as a spreading factor that promotes distribution of lethal toxins from

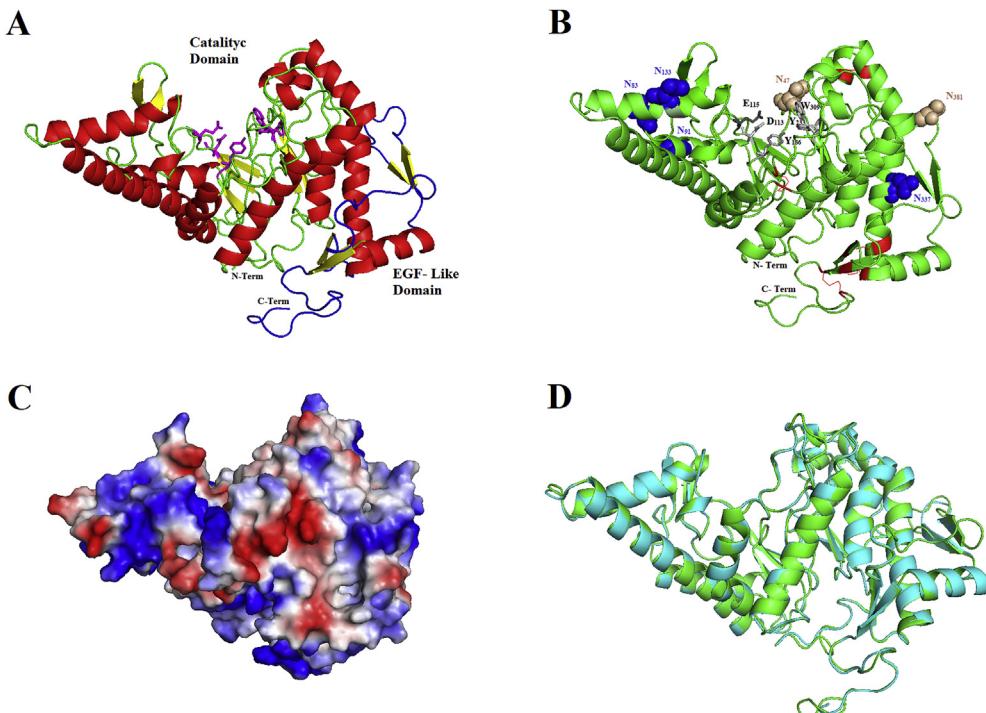


Fig. 10. Theoretical Hyal-Ba model. **A)** The representation shows the secondary structural features and the catalytic (green) and EGF-Like (blue) domains. Functional residues belong to catalytic domain are in magenta stick representation. Helix and β -sheets are in red and yellow respectively. **B)** Principal residues in Hyal-Ba, disulfide bridges are highlighted in red, the predicted N-glycosylation sites ($N_{47}, N_{83}, N_{91}, N_{133}, N_{337}$ and N_{381}) are in blue (high score) and light brown (low score) spheres. The functional residues ($E_{115}, D_{113}, Y_{186}, Y_{233}$ and W_{309}) are highlighted in dark and light grey according to Fig. 9. **C)** Molecular surface electrostatic potential of Hyal-Ba. Negative and positive potentials are in red and blue respectively. **D)** Superposition of Alpha C of Hyal Ba (green) with the structure of Human Hyaluronidase 1 (PDB: 2PE4).

B. atrox venom and thus, contribute to pathophysiological effects of snake bite victims. For these reasons, neutralization of Hyals could play a relevant role in the efficient management of envenomations caused by *Bothrops* snakes.

Conflict of interest

The authors declare no conflict of interest.

Author contribution

Study design: D.E.V.R., J.D.,
Experiment conduction: E.G.K., J.D., D.E.V.R., E.R., G.S., F.L.
Data analysis: D.E.V.R., E.F.S., J.D., C.H.O., A.Y., P.M.P.
Manuscript writing and revision: D.E.V.R., E.F.S., P.M.P., E.G., A.Y.

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