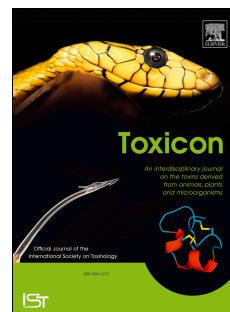


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Biochemical, biological and molecular characterization of an L-Amino acid oxidase (LAAO) purified from *Bothrops pictus* Peruvian snake venom

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Abstract

An L-amino acid oxidase from Peruvian *Bothrops pictus* (*Bpic*-LAAO) snake venom was purified using a combination of size-exclusion and ion-exchange chromatography. *Bpic*-LAAO is an homodimeric glycosylated flavoprotein with molecular mass of ~65 kDa under reducing conditions and ~132 kDa in its native form as analyzed by SDS-PAGE and gel filtration chromatography, respectively. N-terminal amino acid sequencing showed highly conserved residues in a glutamine-rich motif related to binding substrate. The enzyme exhibited optimal activity towards L-Leu at pH 8.5, and like other reported SV-LAAOs, it is stable until 55 °C. Kinetic studies showed that the cations Ca²⁺, Mg²⁺ and Mn²⁺ did not alter *Bpic*-LAAO activity; however, Zn²⁺ is an inhibitor. Some reagents such as β-mercaptoethanol, glutathione and iodoacetate had inhibitory effect on *Bpic*-LAAO activity, but PMSF, EDTA and glutamic acid did not affect its activity. Regarding the biological activities of *Bpic*-LAAO, this enzyme induced edema in mice (MED = 7.8 µg), and inhibited human platelet aggregation induced by ADP in a dose-dependent manner and showed antibacterial activity on Gram (+) and Gram (-) bacteria. *Bpic*-LAAO cDNA of 1494 bp codified a mature protein with 487 amino acid residues comprising a signal peptide of 11 amino acids. Finally, the phylogenetic tree obtained with other sequences of LAAOs, evidenced its similarity to other homologous enzymes, showing two well-established monophyletic groups in Viperidae and Elapidae families. *Bpic*-LAAO is evolitively close related to LAAOs from *B. jararacussu*, *B. moojeni* and *B. atrox*, and together with the LAAO from *B. pauloensis*, form a well-defined cluster of the *Bothrops* genus.

Keywords: L-amino acid oxidase, *Bothrops pictus*, *Bpic*-LAAO.

Abbreviations: *Bpic*-LAAO, *Bothrops pictus*-L-amino acid oxidase; CFU, colony-forming units; FAD, flavin adenine dinucleotide; LAAO, L-amino acid oxidase; PNGase F, peptide *N*-glucosidase; RP-HPLC, reverse-phase high performance liquid chromatography; svLAAO, snake venom L-amino acid oxidase; OVA, ovalbumin

1.- Introduction

Peru has a rich biodiversity as a result of its topographic heterogeneity and consequent ecosystem variety. This country is the second in Latin America in snake diversity and, according to Espinoza and Icochea (1995), from the 33 different venomous snake species that inhabit Peruvian lands, 9 are endemic and 24 are spread over neighbor countries (Guerra-Duarte et al., 2015). In Peru, ophidism is an important cause of morbidity and mortality (Martinez-Vargas, 2004). The majority of snakebites are reported in rural forest regions, east of the Andes, since Peruvian Amazon represents approx. 58% of the territory. In these regions, the main species responsible for snakebites is *Bothrops atrox*, causing approximately 80% of the accidents (Schneider et al., 2016). In areas within coast regions and the Andes Mountains we can also find other endemic medically relevant species including *B. pictus* (Tschudi, 1845), which is known as Macanche, Sancarranca, Víbora, Jergon de la Costa. This snake is distributed along the Pacific coast of Peru and the occidental western slopes of the Andes, from the Department of La Libertad south into the Department of Arequipa, at altitudes from sea level to approx. 1800 m. This species also inhabits some of the interior drier valleys and mountains, including the northern region of the metropolitan area of Lima city, where this snake is the main responsible for human accidents (Maguiña et al., 1998).

Bothrops (lanceheads) snakes are medically relevant poisonous snakes found in tropical and sub-tropical regions of Central and South America, where they are responsible for the vast majority of human snakebite envenomings. *Bothrops* venoms induce a complex pathophysiological picture that involves drastic local and systemic effects, such as edema, hemorrhage, inflammation, nephrotoxicity, intravascular coagulopathy and necrosis, thereby causing deaths and morbidity of surviving victims (White, 2005; Warrell, 2004; Albuquerque et al., 2013). The main components found in these venoms are proteins/toxins with or without enzymatic activity, including metalloproteinases (SVMPs), serine proteinases (SVSPs), phospholipases A₂ (PLA₂s), L-amino acid oxidases (LAAOs), hyaluronidases, disintegrins, C-type lectin related proteins among others (Sanchez et al., 2007; Calvete, 2010). Moreover, proteomic characterization of the Peruvian pitvipers *B. atrox*, *B. barnetti* and *B. pictus* indicated that these venoms contain 21-22 proteins, which belong to 7 groups of protein families. SVMPs (P-I and P-III classes), PLA₂s, SVSPs, LAAOs are abundant and three minor protein families are also present: disintegrins, C-type lectin-related proteins, cysteine-rich secretory protein (CRISP), each representing less than 4% of total proteins (Kohlhoff, et al., 2012). The main difference in the composition of these venoms was the absence of LAAO

in the white sample venom of *B. pictus* used by Kohlhoff *et al.* LAAO, is responsible for the yellow color in snake venoms, due the presence of flavin (mainly FAD) cofactor. In addition, the lack of expression of LAAO proteins in some samples of *B. pictus* venom may be due to the lack of LAAO gene expression among the founder population used for that research. As far as we known, interpopulation, geographic and individual venom variation has been documented in the literature and appears to be a general feature of animal venoms (Guercio, et al., 2006; Alape-Giron et al., 2008; Calvete et al., 2011).

We report, the isolation of an L-Amino acid oxidase from *B. pictus* venom, termed *Bpic-LAAO* and characterized its main molecular and biochemical properties including its effect on platelet function and antibacterial activity. Moreover, the primary structure of *Bpic-LAAO* was deduced from its cDNA obtained from fresh venom as a template and primers synthetized based on partial amino acid sequences. *Bpic-LAAO* can provide important data to better understand the bothropic envenomations mechanism and might have biotechnological importance as a model for therapeutic strategies.

2. Material and Methods

2.1. Reagents and venom

Bothrops pictus venom was collected from 4 adult specimens (3 males and 1 female) from Pachacamac (altitude 70 m), south of Lima city, Peru and maintained at Oswaldo Meneses serpentarium, Natural History Museum, Universidad Nacional Mayor de San Marcos (UNMSM). The venom showed a yellow color plausibly suggesting the presence of LAAO. Antibotropis polyvalent serum (Batch: 01000376) was obtained from Instituto Nacional de Salud (INS), Lima, Peru. Bacterial strains: *Staphylococcus aureus* (ATCC 95923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 25922) and *Vibrio cholerae* (INS Inaba serotype) was obtained from Laboratorio de Microbiología y Biotecnología Microbiana, Facultad de Ciencias Biológicas-UNMSM. Other reagents were of analytical grade and purchased from Sigma Chem Co, Merck, Roche and Invitrogen®. Protein content in whole venom and isolated fractions were determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.2. *B. pictus* LAAO (*Bpic-LAAO*) purification

Three hundred milligrams of *B. pictus* venom were dissolved in 100 µL of ammonium acetate buffer (0.05M; pH 6.0) and homogenized until complete dissolution, followed by

centrifugation (4000 g for 20 min). The supernatant was recovered and applied onto a molecular exclusion column of Sephadex G-100 (40 x 1.2 cm), previously equilibrated with the same buffer, at a flow rate of 17 mL/h. Fractions containing *Bpic*-LAAO activity were collected and concentrated to 1.2 mL using an Ultracell Centrifugal Filter Unit (Amicon). This material (29.4 mg) was then applied to a CM Sephadex C-50 column (28 x 1.1 cm) equilibrated with the above buffer and eluted with a linear salt gradient of 0.1-1 M NaCl at flow rate of 17 mL/h. Fractions containing *Bpic*-LAAO activity were concentrated as previously mentioned and stored at 4 °C. In order to check its purity, the purified enzyme was submitted to a RP-HPLC chromatography on an analytical C-18 Vydac column (4.6 x 250 mm).

2.3. Molecular mass, carbohydrate determination and amino acid sequence of *Bpic*-LAAO

The homogeneity of purified *Bpic*-LAAO was assessed by SDS-PAGE on 10% polyacrylamide gel as described by Laemmli (1970) under reducing (4% β-mercaptoethanol) and non-reducing conditions. Relative molecular mass (Mr) of the enzyme was calculated by comparison with protein markers containing: BSA (66 kDa), OVA (45 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa). Gels were stained with Coomassie brilliant blue G-250. *Bpic*-LAAO Mr was also calculated by molecular exclusion as described by Laemmli (1964), using a Sephadex S-200 column (42.4 x 1.1 cm) equilibrated with Tris-HCl buffer (50 mM, pH 7.5 containing 0.15M NaCl). Protein calibration mixture consisted of alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa).

For carbohydrate characterization *Bpic*-LAAO was treated with PNGase F. Briefly, 40 µg of *Bpic*-LAAO were dissolved in 50 µL of denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) and the solution was boiled for 5 min. After addition of 50 µL of reaction buffer containing 50 mM Tris-HCl, pH 8.0, 2.5 µL of detergent solution (IGEPAL 15%, Roche) and 2 units of recombinant PNGase F, the sample was incubated for 24h at 37 °C. The reaction was stopped by boiling for 5 min. Native and deglycosylated enzymes were analyzed by SDS-PAGE (10%) after reduction.

The presence of hexoses and hexosamines was determined by acidic hydrolyses as described by Winzler (1955) with minor modifications. Briefly, 0.4 mL of purified enzyme (0.520 mg/mL) was hydrolyzed with 3N HCl at 100 °C for 4 h and neutralized with 3N NaOH; the volume was adjusted with distilled H₂O to 0.5 mL. Then, 0.5 mL of acetylacetone was added, mixed and boiled for 15 min. Then, 2 mL and 0.5 mL of 95% ethanol and Ehrlich

reagent, respectively were added. The mixture was incubated for 30 min at 20 °C and its absorbance was registered at 530 nm. These values were transformed to mg by comparison to a standard of hexosamine (0.5 mg/mL). They were expressed as percentage of hexosamine compared to the total amount of protein used. On the other hand, the presence of sialic acid was determined as described by Warren (1959). Briefly, 0.1 mL of purified enzyme (0.520 mg/mL) was incubated at 80°C for 1 h in 0.1N H₂SO₄ after the final volume had been adjusted with H₂O to 0.2 mL. Thereafter, 0.1 mL of periodate solution was added, homogenized by stirring, rested by 20 min at 20 °C, followed by adding 1 mL of arsenite solution and 2 mL of acid 2-tiobarbituric. The mixture was stirred until the yellow color disappeared. The tubes were immersed in boiling water for 15 min and cooled down. Samples of 1.5 mL were taken and mixed with 1.5mL of cyclohexanone (1:1), stirred twice and centrifuged at 300 rpm for 10 min. The absorbance of the supernatant was measured at 549 nm. The values obtained were converted to mg by comparison with the sialic acid standard and expressed in percentage with respect to the total amount of protein used.

The N-terminal amino acid sequence of *Bpic*-LAAO was determined in a Shimadzu protein sequencer Automatic System (PPSQ-21A), by Edman degradation method (Edman and Begg, 1967).

2.4. *Bpic*-LAAO biochemical characterization

2.4.1. Enzymatic activity

LAAO activity was determined spectrophotometrically following the procedure described in Worthington Enzyme Manual (1993), using L-leucine as substrate, with minor modifications. The reaction mixture (300 µL) of 0.2 M Tris-HCl buffer, pH 7.5, containing 0.1% of L-leucine, 0.0065% of *o*-dianisidine, 0.001% of horseradish peroxidase and a known amount of the whole venom, fractions containing LAAO or purified *Bpic*-LAAO. The mixture was incubated at 37°C for 5 min. The initial rate of reaction was measured as an increase in absorbance at 436 nm. The molar absorption coefficient was $8.31 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. One unit (U) of activity was defined as the amount of enzyme that oxidized 1 µL of L-Leu per min at 37 °C. Specific activity was expressed as U per mg.

2.4.2. Stability assays

Effects of temperature and pH on LAAO activity was evaluated at pH values range from 4 to 10 and at temperatures from 30 to 100 °C, using L-leucine as substrate. The following buffers (200 mM) were used in the reactions: ammonium acetate (pH 5.0-6.5) and

Tris-HCl (pH 7.0-10.0). To assess the temperature stability, *Bpic*-LAAO was incubated at temperatures between 30 and 100 °C. The enzyme was used at the same concentration (0.2 mM) in all assays. Tris-HCl buffer (50 mM, pH 7.4) was used as solvent for optimum temperature assays.

2.4.3. Ion metal effect

Effects of divalent cations on *Bpic*-LAAO activity was investigated using MgCl₂, MnCl₂, CaCl₂ and ZnCl₂ respectively at concentrations of 0.75, 1.5, 3 or 6 M. Enzymatic activity was evaluated using 30 µL (1 µg) of *Bpic*-LAAO (for each probe), previously incubated with the respective divalent ion at 37 °C for 15 min. LAAO activity assay was performed as described above.

2.4.4. Effect of some reagents on enzymatic activity

Efficacy of several reagents on the purified enzyme were examined. The purified enzyme (1 µg) was pre-incubated with 30 µL of glutathione, PMSF, β-mercaptoethanol, EDTA, glutamic acid or acetate iodine (at concentrations of 2.5, 5.0 a 10.0 mM each). Mixtures were subjected to LAAO activity assay as described above.

2.5. *Bpic*-LAAO biological characterization

Animal care was in accordance with ethical recommendations of the International Guiding Principles for Biomedical Research Involving Animals of the Council of International Organizations of Medical Sciences (CIOMS).

2.5.1. Platelet aggregation

Platelet aggregation assays were performed in citrated human platelet-rich plasma (PRP). Blood was collected from healthy volunteers who had not taken any medication for at least 2 weeks prior to assays. Platelet concentration used in each assay was adjusted to 300 x 10⁶ platelets/ml in a final volume of 0.3 mL and its aggregation was measured as described by Sakurai et al (2001), using a Qualiterm digital aggregometer with AgrePic software in the presence or absence of *Bpic*-LAAO using ADP (2 µg/ml, final concentration) as agonist. Effect of H₂O₂ towards ADP-induced platelet aggregation was also investigated. Various concentrations of H₂O₂ were added 30s before the addition of ADP. Further, it was analyzed the effect of catalase on ADP induced platelet aggregation in the presence of *Bpic*-LAAO.

2.5.2. Hemolytic activity

Hemolytic activity was determined as described by Condrea et al. (1964) with slight modifications. Briefly, the reaction mixture contained 0.8 mL of buffered saline, 0.1 mL washed human erythrocytes and 0.1 mL of *Bpic*-LAAO (45 µg). This mixture was incubated at 37 °C for 2 h and the reaction was stopped by addition of a solution (2 mL) containing 0.9% sodium chloride and 0.2M EDTA, pH 7.3. Degree of hemolysis was assayed by determining the amount of hemoglobin released from erythrocytes into the suspending medium. Therefore, the mixture was centrifuged at 500 g for 5 min and the amount of hemoglobin was measured spectrophotometrically at 540 nm. Results were expressed in percentage of the total hemoglobin present in the original erythrocyte suspension.

2.5.3. Edema-inducing activity

Edema-inducing effect was assessed as described by Yamakawa et al. (1976). The paws of mice were injected with different doses of *Bpic*-LAAO (2.5, 5, 10, 20, 30, 45 µg in 50 µL), four mice in each group. As a negative control, an equal volume of saline solution was injected into the contralateral paw of the mouse. After 3 h, the thickness of the swollen paws was measured with a Vernier caliper. The minimum edema-forming dose (MED) was defined as *Bpic*-LAAO dose inducing 30% increase in the thickness of the swollen paws.

2.5.5 Antimicrobial assay

Antimicrobial susceptibility testing was performed as described by Grove and Randall (1955). For the agar diffusion assay, *S. aureus* (ATCC 95923), *E. faecalis* (ATCC 29212), *P. aeruginosa* (ATCC 9027), *E. coli* (ATCC 25922) and *V. cholerae* (INS Inaba serotype) were grown in Mueller-Hinton (MH) agar plates and suspended in 5 mL of sterile MH broth. The inoculum was adjusted for each organism to yield a cell concentration of 5×10^6 CFU/mL (colony-forming units). Bacterial inoculums were spread with a sterile cotton swab on the surface of MH agar. After 5 min wells were made in the agar (4 mm in diameter) using a sterile punch. Ten microliters of varying amount (3, 6 and 12 µg/µL) of the whole venom or purified *Bpic*-LAAO were applied in these wells. Ten microliters of tetracycline (2.5 µg/µL) were used as positive control, and PBS (pH 7.4) as a negative control. After incubation at 35 °C for 24 h, the zone of inhibition diameter was recorded in millimeters. Results are means ± SD (n=3). The effect of 0.01% catalase (10 and 20 µL) upon antibacterial activity of *Bpic*-LAAO was assayed with *Bpic*-LAAO's sensitive bacteria.

2.6. Molecular characterization

2.6.1 Synthesis and sequencing of cDNA

cDNA was obtained from *B. pictus* fresh venom as described by Vivas-Ruiz et al., (2013). Two primers, F: 5'-ATGAATGTCTTCTTATGTCTC-3'; R: 5'CTCAGAACGACGATTCACATC-3', were designed on the basis of the highly conserved cDNA sequences encoding LAAO from *Bothrops moojeni* (AY398692.1), *Bothrops neuwiedi* (EU870608.1), *Bothrops jararacussu* (AY398691.1), *Lachesis muta* (JX171244.1) and *Agkistrodon blomhoffi* (AB072392.1), and synthesized (Invitrogen Custom Primers). For extraction and purification of mRNA, High Pure RNA Isolation kit (ROCHE®), and for synthesis of cDNA, Transcriptor First Strand cDNA Synthesis kit (Sigma) were used, in accordance with the manufacturer's instructions. To amplify *Bpic*-LAAO gene, Master Mix Platinum® Taq DNA Polymerase kit (Invitrogen) was used according to the manufacturer's instructions. Sequencing of the amplification products was performed on an ABI 3730 XL automated sequencer (Macrogen, Inc, South Korea). Both cDNA and deduced protein sequences were compared with other sequences deposited in GenBank and SwissProt.

2.6.2. Multiple alignment and phylogenetic analyses

Multiple alignment of LAAO sequences were performed using CLUSTAL X (Thompson et al., 1997). The phylogenetic tree was constructed using the MEGA program v. 6.0 according to the Neighbor-Joining method. Phylogenetic distance was calculated using Kimura method two parameters and performing 1000 replicates (bootstraps).

2.6.3. Modeling

Bpic-LAAO amino acid sequence was inserted in a structure model of a LAAO isolated from the *Bothrops jararacussu* (PDB ID: 4E0V_B) with MODELLER program v. 9.2. (Sali and Blundell, 1993). *Bpic*-LAAO model was built with MODELLER and SWISS MODEL. RMS deviations were calculated using the program VMD v 1.9.2 (Humphrey et al., 1996). Stereochemistry of the predicted model was checked by PROCHECK (Laskowski et al., 1993). Structure visualizations were generated by the program PyMOL (Delano, 2002).

3. Results

3.1. *Bpic*-LAAO purification

Bpic-LAAO was purified by two chromatographic steps on Sephadex G-100 and CM Sephadex C-50. Three major peaks (P1-P3) were obtained from the venom (300 mg protein) on Sepadex G-100 (Fig. 1A). As observed, LAAO activity was found in peak 1. Further separation of peak 1 (19.4 mg) on a CM Sephadex C-50 column resulted in four peaks (P1-I - P1-IV) (Fig. 1B) and LAAO activity was concentrated in peak P1-I, containing 3.8 mg protein. Homogeneity of this peak was analyzed by reverse phase HPLC, immunodiffusion and SDS-PAGE (Fig. 1C and 2A). Results of purification are summarized in Table 1.

SDS-PAGE of *Bpic*-LAAO showed a single band of approx. 65 and 58 kDa, under both reducing and no reducing conditions (Fig. 2A). A Mr of 132 kDa was determined by size exclusion chromatography (Fig. 2B). These results suggest that *Bpic*-LAAO can be composed by two subunits. On the other hand, the Mr of *Bpic*-LAAO was reduced to approx. 47 kDa after PNGase F treatment. Sugars accounted for approximately 18% of its total mass as determined by SDS-PAGE (Fig. 2C). The content of hexose amines, hexoses and sialic acid in the glycoconjugates linked to protein was 15.6%, 2.42% and 0.73% respectively.

N-terminal amino acid sequence of the S-pyridyl-ethylated *Bpic*-LAAO was determined up to the 20th residue as being ADDRNPLEECFRETDYEEFLE, which is identical to the corresponded deduced sequence from the cDNA (Fig. 3). Comparison of this sequence with those from *Bothrops* and *Lachesis* venom enzymes, revealed a high homology among SV-LAAOs (95-100%).

3.2. Functional characterization

Relative *Bpic*-LAAO activity with L-Leu as substrate as a function of temperature was measured between 30 and 100 °C. The optimal temperature of *Bpic*-LAAO activity was found between 35 and 45 °C (Fig.4A) and above of 50 °C, its activity decreased rapidly. On the other hand, the oxidase activity of *Bpic*-LAAO as a function of pH (Fig.4B), showed that the enzyme maintained its full activity between pH 7.0-10, and its activity decreases dramatically below pH 7.0 and above 10.0.

In order to check the effects of several divalent cations and other reagents on *Bpic*-LAAO, the enzymatic activity was measured at different concentrations (0.75 to 6.0 mM) of Ca²⁺, Mg²⁺, Mn²⁺ and Zn²⁺ and using the reagents, glutathione, PMSF, β-mercaptoethanol, EDTA, glutamic acid and acetate iodine (2.5, 5 a 10 mM each). Results showed that *Bpic*-LAAO activity was affected only by Zn²⁺ in a dose-dependent manner (Fig. 4C). Similar data has been previously reported for other LAAOs from *L. muta*, *B. brazili* and *B. atrox* (Cisneros et al., 2006, Solis et al., 1999 and Lazo et al., 2007). These results needed to be confirmed by

other structural studies as some LAAOs contains Zn^{2+} as part of its structure but, it is not crucial for its activity (Georgieva et al., 2011, Ullah et al., 2012, Sun et al., 2010). In addition, *Bpic*-LAAO activity was strongly inhibited by β -mercaptoethanol and glutathione and in minor degree by PMSF, acetate iodine and glutamic acid, but, was not affected by EDTA (Fig. 4D).

Bpic-LAAO (140 μ g/mL) inhibited platelet aggregation in human PRP induced by ADP in a dose-dependent manner (Fig 5A). The amount of enzyme that inhibited 50% platelet aggregation was 3.2 μ g/mL. Furthermore, the addition of increasing concentrations of catalase to platelets reverted the inhibition of aggregation by *Bpic*-LAAO in a concentration-dependent manner (Fig 5 B).

Bpic-LAAO showed edematogenic activity in paw pads of mice (not showed). Increments in paw pads thickness obtained with 2.5, 5, 10, 15 and 20 μ g of *Bpic*-LAAO were of 5.8, 21.8, 31.7, 51.8 and 89% respectively. The minimum edema dose (MED) was 7.8 μ g. *Bpic*-LAAO had no hemolytic activity (data not shown).

Bpic-LAAO presented antibacterial activity (Table 2). The growth of the bacteria *P. aeruginosa*, *V. cholerae*, *S. aureus*, *E. faecalis* and *E. coli* was concentration-dependently suppressed. This effect was more pronounced on *E. faecalis* (maximum inhibition zones measured an average of 20 mm at the maximum *Bpic*-LAAO concentration). The bactericidal effect of *Bpic*-LAAO (12 μ g/mL) was also inhibited by catalase

3.3. Molecular characterization of *Bpic*-LAAO

A cDNA of 1494 bp was obtained, codifying a mature protein with 487 amino acid residues and a fragment of 11-amino acid signal peptide identical to other SV-LAAOs. *Bpic*-LAAO has a Mr of approx. 55.28 kDa, and theoretical isoelectric point (pI) of 5.75. Using the glycosylation prediction software NeTNGlyc (<http://www.cbs.dtu.dk/services/NetNGlyc/>), two potential N-glycosylation sites (Asn-X-Thr/Ser) were located at amino acid residues 172 and 192 respectively (Fig. 3).

The complete amino acid sequence of *Bpic*-LAAO predicted from the cDNA nucleotide sequence (GenBank accession: KJ094993.1) was aligned with those of other SV-LAAOs and was found to be closely homologous with them (Fig. 6). The sequence of *Bpic*-LAAO exhibits the highest identity with other homologous proteins from *Bothrops* snake venoms, especially with the LAAOs of *B. jararacussu* and *B. moojenii* (95% and 97% respectively). Furthermore, the enzyme contains six cysteine residues in conserved sites. The

proposed active site key residues Arg 90, His 223, Arg 322 and Gly 464 are conserved like in all the Viperidae SV-LAAOs (Fig. 6).

3.4. 3D model of *Bpic*-LAAO and phylogenetic tree of SV-LAAOs

The theoretical 3D model of *Bpic*-LAAO (Fig. 7) was generated by knowledge-based homology modeling (program MODELLER v.9.2) using the crystallographic structure of LAAO from *B. jararacussu* (PDB-ID: 4E0V_B) as a template showing a root mean square deviation of 0.3. The generated model showed good stereochemistry quality with nearly 95% of its amino acid residues in the core and additionally allowed regions of Ramachandran plot. Furthermore, all residues had negative potential energies and the Z-score of the model was -10.84.

The phylogenetic tree generated by the program MEGA v.6.0 showed clearly that SV-LAAOs from two adjacent clades comprising elapid and viperid SV-LAAOs (Fig.8). However, the LAAO from *Ophiophagus hannah* was grouped in a separated branch with two LAAOs from *Gallus gallus* and *Meleagris gallopavo*, respectively. *Bpic*-LAAO form a robust group with other LAAOs from *Bothrops* genera.

4. Discussion

An L-amino acid-oxidizing enzyme was purified from the venom of the Peruvian snake *Bothrops pictus*, in a two-step procedure. The activity yield of *Bpic*-LAAO was 27.8% with a 22-fold increase in the specific activity and the enzyme represents 1.2% of total protein content in *B. pictus* venom. It was detected as unique peak showing LAAO activity in all steps, excluding the presence of isoforms as reported in the venoms from *B. alternatus* and *B. jararaca* (Stabeli et al., 2004; Ciscotto et al., 2009).

Bpic-LAAO has a Mr of 65 kDa as estimated by SDS-PAGE and ~132 kDa as measured from its elution profile in size exclusion chromatography. *Bpic*-LAAO is an homodimeric protein with non-covalent bonds between the subunits, as found in other SV-LAAOs (Ullah et al., 2012). Recent reports indicated that dimerization is mediated by Coulomb forces between SBD (high content in negative amino acids) and FBD (high content in positive amino acids) (Ullah et al., 2012). PNGase F treatment and analysis of associated carbohydrates demonstrated that *Bpic*-LAAO is a glycoprotein containing 18.73% carbohydrates. To our knowledge, glycosylation in SV-LAAOs contributes to microheterogeneity and in some cases, could play an important role on their biological,

enzymatic and antibacterial activities (Hayes and Wellner 1969, Moustafa et al., 2006; Fitzpatrick, 2004; Geyer et al., 2001). However, it is not a general rule since in some species of *Bothrops* genus, deglycosylation did not affect their enzymatic activity (Guo et al., 2012; Rodrigues et al., 2009; Ciscotto et al., 2009; Izidoro et al., 2006). The intrinsic roles of carbohydrates in SV-LAAOs could have been related to their physicochemical properties such as solubility, charge stability (Geyer et al., 2001) and enzyme protection from venom proteolysis (Dos Santos et al., 1993). The identity of *Bpic*-LAAO was confirmed by N-terminal sequencing, which was congruent with the sequence obtained from the cDNA analysis. The N-terminal 20 amino acid residues were determined to be ADDRNPLEECFRETDYEEFLE. This sequence is very similar to other SV-LAAOs, and belongs to motifs which are highly conserved in these enzymes, *e.g.* the presence of a glutamine-rich motif which is related to substrate binding (Guo et al., 2012).

In relation to its functional properties, *Bpic*-LAAO was stable until 55 °C, quickly losing its activity in temperatures above 60 °C (Fig. 4A), similarly to data reported for LAAO from *B. brazili* (Solis et al., 1999). This behavior could be attributed to the lack of covalent bonds in the quaternary structure. The purified enzyme exhibited optimal hydrolysis of L-leucine between pH 7.0 and 9.5, reaching maximum values at pH 8.5, which corroborates the results for other SV-LAAOs. On this context, buffers with pH values above 9.0 or below 7.0 could cause changes in SV-LAAOs as well as in peroxidase, thereby interfering with the assay (Bregge-Silva et al., 2012). The inhibitory effect of β-mercaptoethanol could be explained by the reduction of disulfide bonds that affects the conformational structure of the enzyme. In the case of glutathione, this tripeptide showed a considerable inhibition as it induced reduction of flavin (Mannervick et al., 1980). The weak inhibition effect of iodoacetate may be due to its interference with sulfhydryl groups of cysteine near to active site. Previous studies with LAAO from *Vipera aspis* suggested that iodoacetate acted as a reversible inhibitor (Zeller et al., 1965).

Furthermore, it has been reported a wide variety of biological effects of SV-LAAOs including apoptosis, cytotoxicity, induction or inhibition of platelet aggregation, edema, hemolysis and inhibition of coagulation among others (Teixeira et al., 2016). *Bpic*-LAAO induced edema in mice (MED = 7.8 µg). This result is similar to that obtained for other SV-LAAOs, *e.g.* the enzyme of *O. hannah* (MED = 6.8 µg) (Tan and Choy, 1994), *T. mucrosquamatus* (MED = 8 µg) (Wei et al., 2002), *E. macmahoni* (MED = 4.8 µg) (Ali et al., 2000). Possibly, edema induction may be due to the generation of H₂O₂ and subsequent inflammatory response mediated by the release of autacoids or eicosanoids (prostaglandin,

serotonin, kinin) and metabolism of cyclooxygenases (Zamuner and Teixiera, 2002). Our studies of *Bpic*-LAAO on platelet function indicate that the enzyme inhibited platelet aggregation of PRP induced by ADP in a dose-dependent manner ($IC_{50} = 3.2 \mu\text{g/mL}$) compared with the enzyme of *B. leucurus* using washed platelets ($IC_{50} = 0.21 \mu\text{gM}$) (Naumann et al., 2011). To our knowledge, the effects of SV-LAAO on platelet function is still controversial since some of these enzymes inhibited aggregation while others promoted aggregation (Izidoro et al., 2014; Guo et al., 2012). Mainly, these effects are supposed to be mediated by H_2O_2 that is released in the oxidation process because catalase the H_2O_2 scavenger blocked the effects (Sun et al., 2010). A possible mechanism for the inhibition of platelet aggregation may be due to the interference of peroxide in the process of release between activated-platelet integrin (GPIIb/IIIa) and fibrinogen (Takatsuka et al., 2011, Samel et al., 2008), but, this point needs to be elucidated. Finally, it was showed that crude venom as well as *Bpic*-LAAO exhibited antibacterial effect on both Gram (+) and Gram (-) bacteria. This activity was also in a dose-dependent manner and was blocked by catalase, which highlighted the crucial role of H_2O_2 in the antimicrobial activity (Toyama et al., 2006; Wei et al., 2002). Ehara et al. (2002) pointed out that SV-LAAOs are able to bind to the surfaces of bacteria and release high levels of H_2O_2 in the surrounding area and thus inhibiting bacterial growth.

Multiple sequence alignment of *Bpic*-LAAO with other SV-LAAOs, including the enzyme from the Peruvian *B. atrox* reported by our group (Genbank: ALL27300), (Fig. 6) showed remarkable conservation of structural characteristics of SV-LAAOs including 6 cysteine residues with only 13 unique mutations with respect at consensus (Table N° 3). *Bpic*-LAAO exhibited residues of functional importance such as His223 and Arg323 which play a role on the enzymatic reaction (Moustafa et al., 2006), Lys326 involved in binding to the FAD cofactor (Pawelek et al., 2000). The residue Asn172 is usually glycosylated and contributes to cytotoxicity by mediating the binding of the enzyme to the cell surface and accumulation of H_2O_2 (Geyer et al., 2001).

The superposition of modeled structure of *Bpic*-LAAO with LAAO from *B. jararacussu* (PDB: 4E0V) showed a high structural similarity between both proteins (Fig 7C), however, there are 18 discrepancies in some amino acid positions: 9 in SBD, 6 in FBD and 3 in HD. These mutations could be connected with the variability of enzyme activity between these proteins despite their structural similarities. A similar observation was reported for LAAOs from *B. moojeni* and *C. rhodostoma* (França et al., 2007). Finally, the phylogenetic tree obtained with homologue sequences of *Bpic*-LAAO is similar to other reported studies

(Rodrigues et al., 2009, Jin et al., 2007), showing two well-established monophyletic groups: Viperidae and Elapidae. *Bpic-LAAO* is evolutively closely related to LAAOs from *B. jararacussu*, *B. moojeni* and *B. atrox*. Together with LAAO from *B. pauloensis* they form a well-defined cluster of *Bothrops* genus inside monophyletic group of Viperids. The topology of the tree obtained with these sequences agrees with the evolution of these species, showing these codifying genes are orthologs (Rodrigues et al., 2009).

In conclusion, our findings showed that LAAO from the venom of *B. pictus*, an endemic Peruvian pit viper snake, displays important pharmacological and antimicrobial properties. Furthermore, this protein may be involved in some pharmacological effect produced by the whole venom. Probably, the generated H₂O₂ is not the only factor involved in its biological activities, e.g. effect on platelet function. Also, this report reinforces the idea of genetic variation in gene expression of proteins in venom glands. This situation could be more marked in *B. pictus* for which interpopulation variation was observed.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Figure Legends

Fig. 1. Purification profile of *Bpic*-LAAO from *Bothrops pictus* venom. **A)** Gel filtration chromatography of crude venom (300 mg) on Sephadex G-100 column. The column was equilibrated and eluted with 0.05M ammonium acetate buffer (pH 6.0). Fractions 14-19 (indicated by dotted line), showed L-amino acid oxidase activity. **B)** Ion-exchange chromatography on CM-Sephadex C- 50. Fraction from the previous step (29.4 mg) containing LAAO activity was applied to a cation exchange column, equilibrated with 0.05M ammonium acetate buffer (pH 6.0), and eluted with a linear gradient of NaCl (0-1.0 M). L-AAO eluted at P1-I (indicated by dotted line). **C)** Reverse-phase HPLC analysis of purified protein. **C)** Immunodiffusion cross-reactivity (inserted) of: crude venom (1) and *Bpic*-LAAO (3) with antbotropic serum (2, INS).

Fig. 2. **A)** SDS-PAGE analysis of *Bpic*-LAAO. Lane 1: molecular mass markers. From top to bottom: bovine serum albumin, 66 kDa; Ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa and lysozyme, 14.3 kDa; lane 2: *Bpic*-LAAO under reducing and lane 3: *Bpic*-LAAO under non-reducing conditions. **B)** Molecular mass determination by size exclusion chromatography on Sephacryl S-200 column. Protein standards: 1, alcohol dehydrogenase (150 kDa); 2, bovine serum albumin (66 kDa) and 3, carbonic anhydrase (29 kDa). **C)** Carbohydrate determination of purified enzyme; line 1, *Bpic*-LAAO after treatment with PNGase F, line 2, native *Bpic*-LAAO and line 3, molecular mass markers (used in A).

Fig. 3. cDNA sequence and deduced amino acid sequence of *Bpic*-LAAO (GenBank accession: KJO94993 and AHN91985 respectively). The predicted amino acid sequence for *Bpic*-LAAO fragment of the signal peptide is underlined. Direct N-terminal sequence of the mature protein determined by Edman degradation is in bold. The two potential N-glycosylation sites are in gray.

Fig. 4. Biochemical characterization of *Bpic*-LAAO. Effects of temperature (**A**), pH (**B**), divalent cations (**C**) and some reagents (**D**) on the enzymatic activity. The enzymatic activity was measured using L-leucine as substrate. The relative activity of *Bpic*-LAAO determined under standard condition is 100%. Data are expressed as the mean \pm S.D. (n=3).

Fig. 5. Effect of purified enzyme on platelet aggregation. **A)** Dose-dependent inhibition of ADP-induced platelet aggregation by *Bpic*-LAAO. **B)** Effect of catalase upon inhibition of ADP-induced platelet aggregation

Fig. 6. Amino acid sequence comparison of *Bpic*-LAAO with snake venoms LAAOs using CLUSTAL X Program. Sequences were from the following sources: Ba-LAAO (ALL27300), from *B. atrox* Bp-LAAO from *B. pauloensis* (B5AR80), BmooLAAO from *B. moojeni* (Q6TGQ8), BjussuLAAO-I from *B. jararacussu* (Q6TGQ9) and LmLAAO from *L. muta* (J7H670). The highly conserved glutaminic-rich motif at N-terminal is in cyan. The conserved cysteine positions are in red. Putative N-linked glycosylation site are in yellow box. The amino acids that participate in the active site are in grey (according to Pawelek et al., 2000) FBD: FAD-Binding domain, SBD: Substrate binding domain, HD: helical domain. (*) positions fully conserved. (.) Strongly similar residues.

Fig. 7. **A** and **B**, Three-dimensional structure model for *Bpic*-LAAO. FAD-binding domain (yellow), the substrate-binding domain (magenta) and the α -helical domain (green). FAD molecule is represented in light blue and the glycosylation sites (Asn172 and Asn 192) are found in orange. **C**, Superposition of three-dimensional structures of LAAO from *B. pictus* (green) and LAAO from *B. jararacussu* (cyan).

Fig. 8. “Tree”. Evolutionary relationships of LAAOs. The evolutionary history was inferred using the Neighbor-joining method. Phylogenetic analyses were conducted using MEGA 6. Evolutionary distances were computed using the JTT matrix-based method. The numbers indicate the MEGA 6 bootstrap test (2000 replicates). GenBank code are: *Bothrops pictus* (this work, black triangle, AHN91985) *Bothrops jararacussu* (Q6TGQ9), *Bothrops pauloensis* (ACG55578), *Bothrops moojeni* (Q6TGQ8), *Calloselasma rhodostoma* (P81382), *Calloselasma rhodostoma* (2IID_A), *Crotalus adamanteus* (O93364), *Crotalus atrox* (P56742), *Echis ocellatus* (CAQ72894), *Gloydius blomhoffi* (Q90W54), *Gloydius halys* (Q6STF1), *Sistrurus catenatus edwardsi* (ABG26996), *Trimeresurus stejnegeri* (AAQ16182), *Viridovipera stejnegeri* (Q6WP39), *Bungarus fasciatus* (ABN72540), *Bungarus multicinctus* (ABN72539), *Demansia vestigiata* (A6MFL0) *Notechis scutatus scutatus* (Q4JHE2), *Ophiophagus hannah* (ABN72538), *Oxyurana scutellata* (Q4JHE3), *Pseudechis australis* (Q4JHE1) *Gallus gallus* (NP_001092821) *Meleagris gallopavo* (ACA64754), *Monodelphis domestica* (XP_001381664) and *Mus musculus* (NP_598653).

Table 1.

Purification scheme of *Bpic*-LAAO from *B. pictus* venom.

Step	Total protein (mg)	Total activity	*Specific activity (U/mg)	Purification (fold)	Recovery (% initial activity)
Crude venom	300	57.9	0.193	1	100
Sephadex G-100	19.4	38.15	1.967	10	65.4
CM-Sephadex C-50	3.8	16.15	4.25	22	37

*One unit (U) of enzymatic activity is defined as the amount of enzyme that hydrolyzes 1 µL L- Leu/min. Specific activity is expressed as U/mg protein.

Table 2

Antimicrobial activity of *Bpic*-LAAO.

Bacteria	Bpic-LAAO (0.3 µg/µL)	Bpic-LAAO (0.6 µg/µL)	Bpic1-LAAO (1.2 µg/µL)	PBS	Tetracycline (2.5 µg/µL)
<i>S. aureus</i>	12 ± 1 mm	15 ± 2 mm	19 ± 2 mm	ND	16 ± 1 mm
<i>E. faecalis</i>	16 ± 2 mm	17 ± 1 mm	20 ± 1 mm	ND	18 ± 2 mm
<i>P. aeruginosa</i>	12 ± 1 mm	17 ± 1 mm	19 ± 2 mm	ND	15 ± 1 mm
<i>V. cholerae</i>	10 ± 2 mm	12 ± 1 mm	18 ± 1 mm	ND	19 ± 1 mm
<i>E. coli</i>	ND	9 ± 2 mm	15 ± 2 mm	ND	17 ± 2 mm

The values are presented as mean ± SD (n = 3) and represent a LAAO inhibition zone (mm) determined by disk-diffusion method after 24 h at 35 °C.

ND= Not determined

Table 3

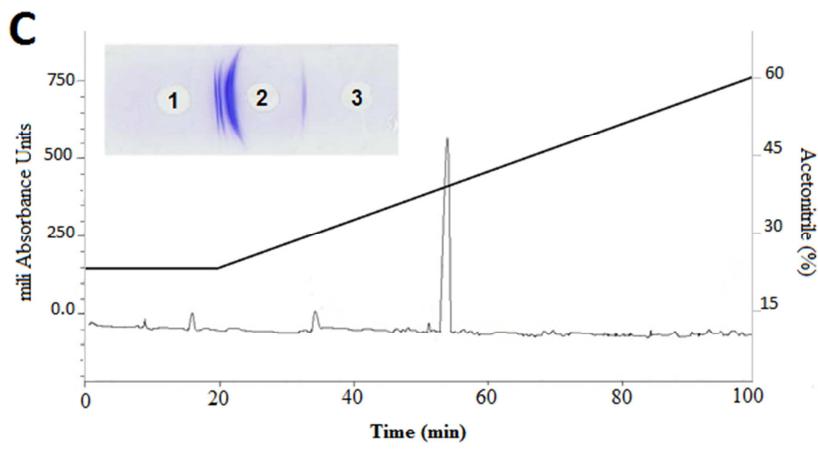
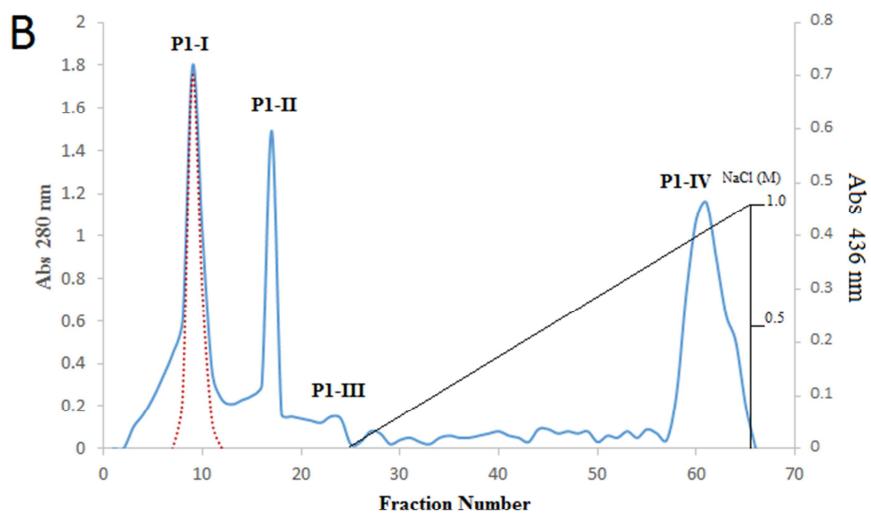
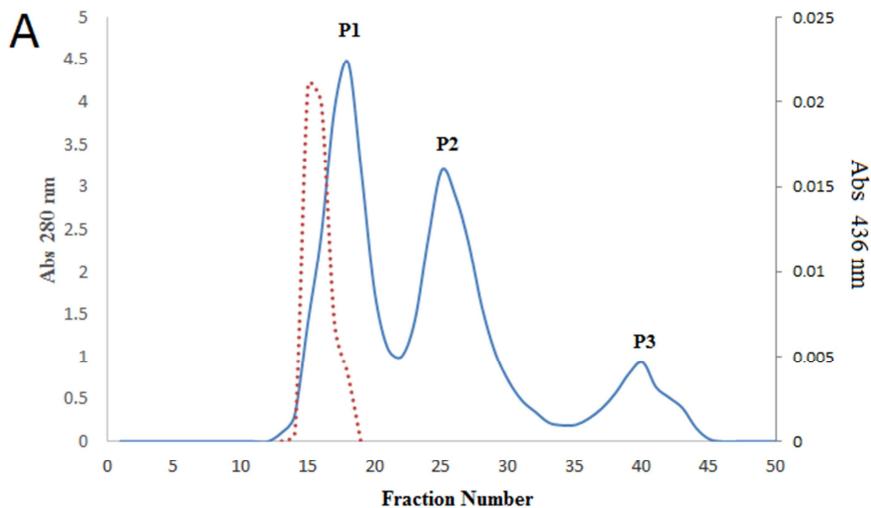
Single amino acid residue present in *Bpic*-LAAO

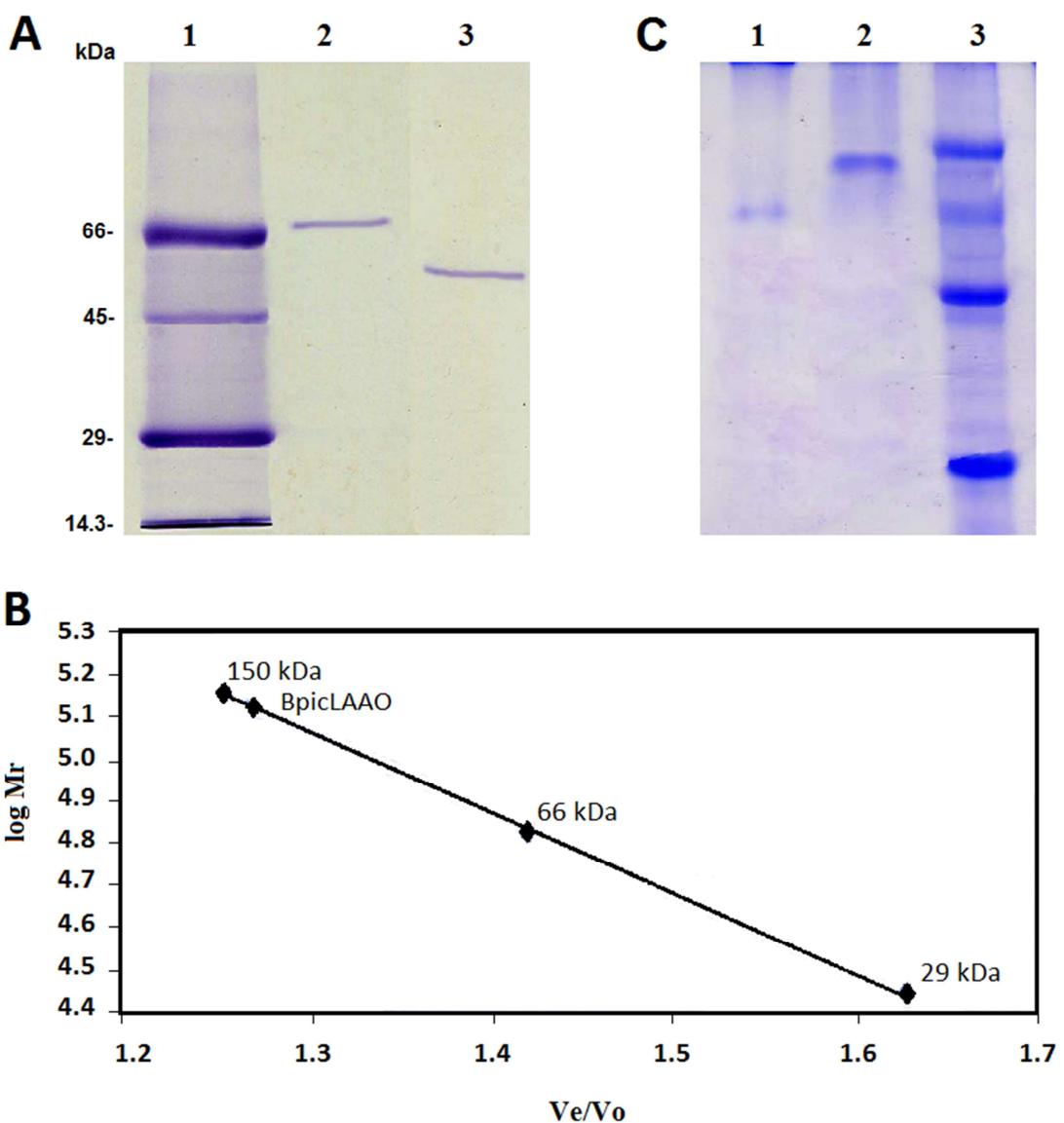
Region	Position	Bpic-LAAO	LAAO Consensus ⁺
SBD	103	K	R
	108	R	Q
	123	L	I
	124	Q	K
	127	K	R
	131	R	G
	424	N	K
	437	H	Q
FBD	260	E	K

	276	Q	E/A
	303	T*	K
	317	L	R
HD	159	R	Q

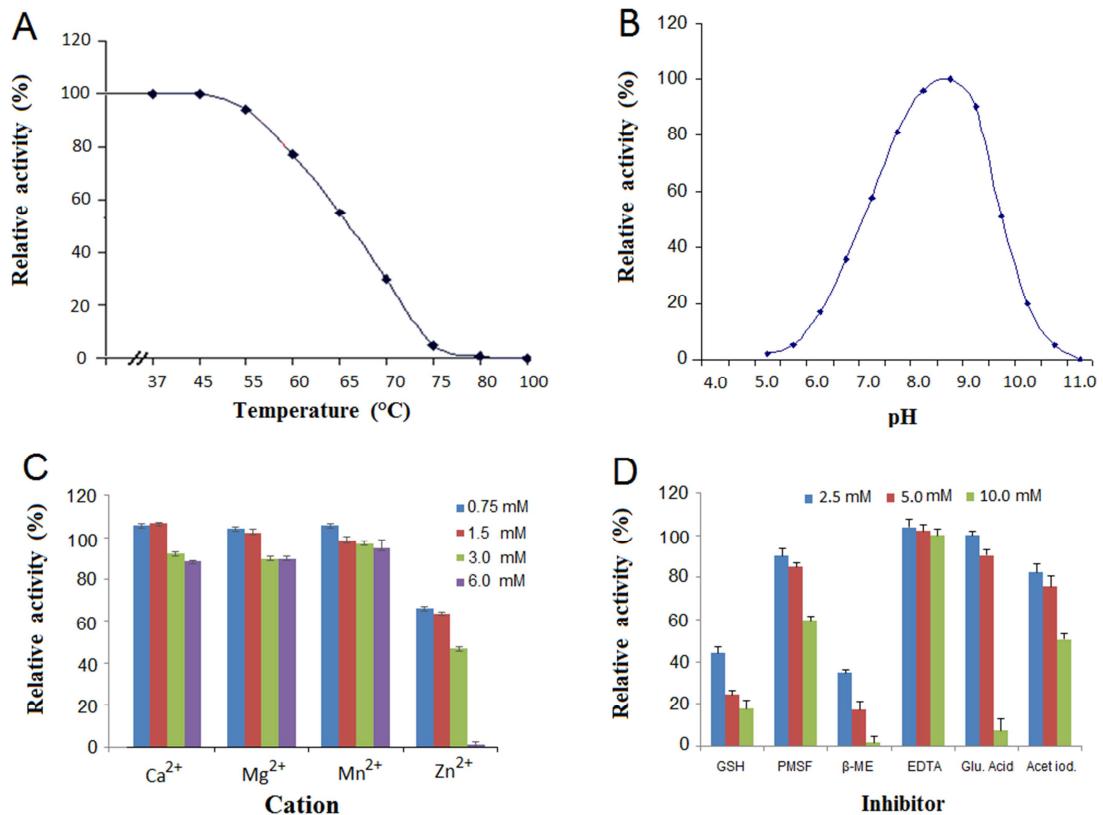
*Present also in LAAO from *Lachesis muta* venom.

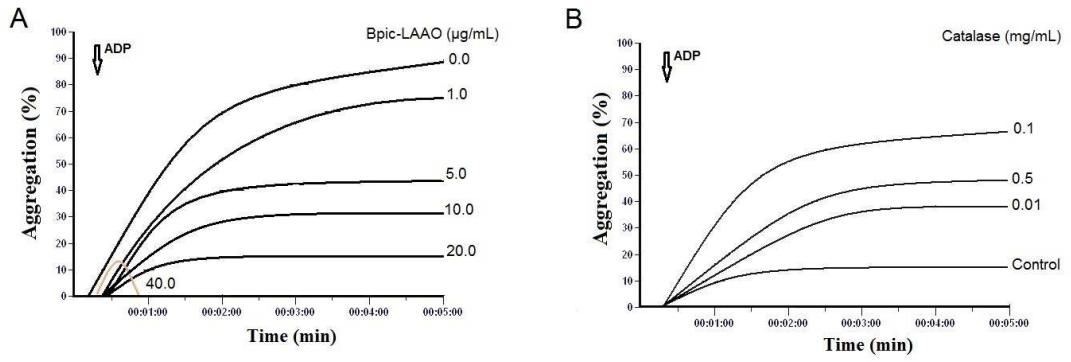
+Respect to amino acid alignment showed in the Figure 6.



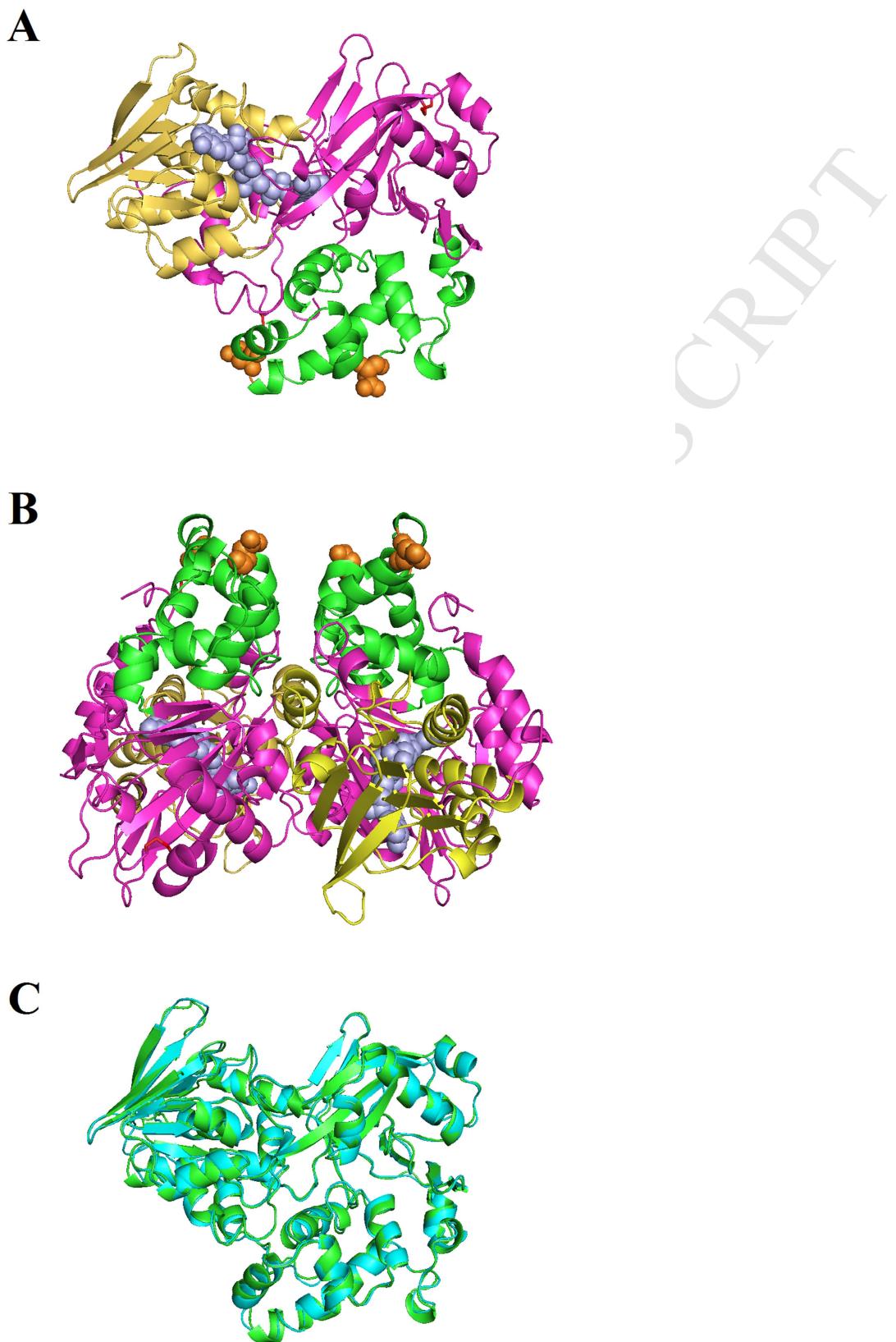


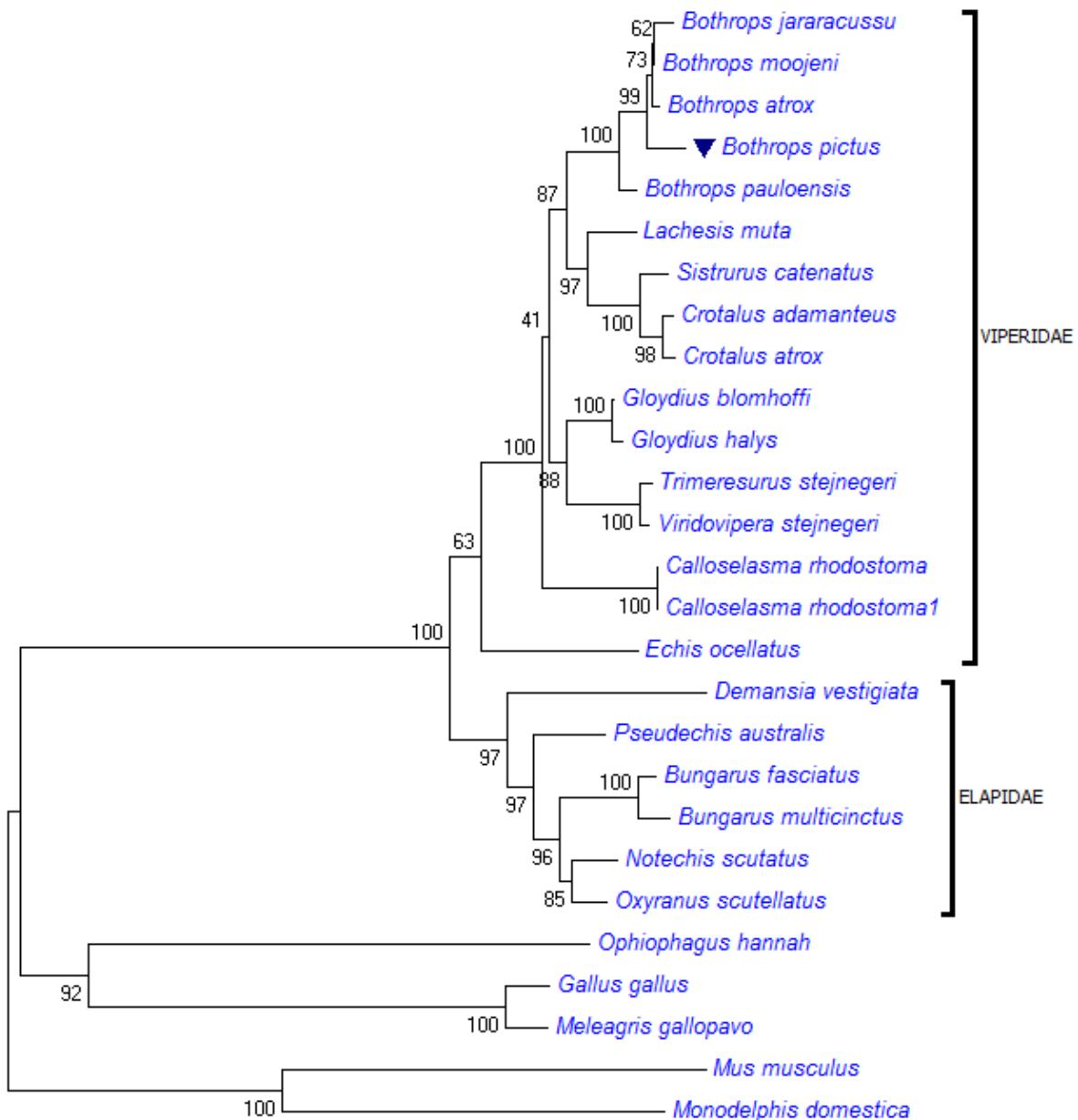
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 A D D R N P L E E
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 C F R E T D Y E E F L E
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 1081 cttccatcccgattcatctactaccctaaaccataacttccataatggagttgggttatt
 L P S R F I Y Y P N H N F P N G V G V I
 1141 atagcctatggc当地tggatgtatgccaattacttcaagcttgc当地tggactgt
 I A Y G I G D D A N Y F Q A L D F E D C
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 G D I V I N D L S L I H Q L P K E E I Q
 1261 gccatctgtcgccctcaatgatccatggggatgggatggataattatgc当地atgggtgg
 A I C R P S M I Q R W S L D N Y A M G G
 1321 ataaccaccttgc当地tccctaccacttcaacattttagtgc当地accatggggatggggatgg
 I T T F T P Y H F Q H F S E A L T A P V
 1381 gacagaatctacttgc当地ggggatgtatcagccaaatgc当地atgggtggattgacagcaca
 D R I Y F A G E Y T A Q A H G W I D S T
 1441 attaagtgc当地gggtggatgtatcagcaacatggggatggggatggggatggggatggggatgg
 I K S G L R A A T D V N R A S E N K





		SBD	FBD	SBD
<u>Bpic-LAAO</u>	1 ADDRNPLEECFRETDYREFLEIAKNGLSTTSNPKRVVIVGAGMSGLSAAYVIANAGHQVTYLEASERAGGRVKTYRNEKEGWYANLGPMRLPEKHRI			
Ba-LAAO	1 ADDRNPLEECFRETDYREFLEIAKNGLSTTSNPKRVVIVGAGMSGLSAAYVIANAGHQVTYLEASERAGGRVKTYRNEKEGWYANLGPMRLPEKHRI			
<u>BmooLAAO-I</u>	1 ADDRNPLEECFRETDYREFLEIAKNGLSTTSNPKRVVIVGAGMSGLSAAYVIANAGHQVTYLEASERAGGRVKTYRNEKEGWYANLGPMRLPEKHRI			
<u>Bp-LAAO</u>	1 ADDGNPLEECFRETDYREFLEIAKNGLSATSNPKHVVIVGAGMSGLSAAYVIANAGHQVTYLEASERAGGRVKTYRNDKEGWYANLGPMRLPEKHRI			
<u>BjussuLAAO-I</u>	1 ADDRNPLEECFRETDYREFLEIAKNGLSTTSNPKRVVIVGAGMSGLSAAYVIANAGHQVTYLEASERAGGRVKTYRNEKEGWYANLGPMRLPEKHRI			
<u>ImLAAO</u>	1 ADDRNPLEECFRETDYREFLEIAKNGLRATSNPKHVVIVGAGMSGLSAAYVLAEGHQVTYLEASERAGGRVKTYRNDKEGWYANLGPMRLPEKHRI			
<u>consensus</u>	1 **** * ***** ***** ***** ***** . ***** . ***** ***** ***** . ***** . ***** . ***** ***** ***** *****			
<u>Bpic-LAAO</u>	101 YIKKFDLRLNEFSQENENAWYFLQNICKRREVNKDPGVLEYPVKPSEVGKSAGQLYEESLRKAVEELRTTNCSYMLNKYDTYSTKEYLLKEGNLSPGAV			
Ba-LAAO	101 YIKKFDLQLNEFSQENENAWYFIKNIRKRVGEVNKDGPVLEYPVKPSEVGKSAGQLYEESLRKAVEELRTTNCSYMLNKYDTYSTKEYLLKEGNLSPGAV			
<u>BmooLAAO-I</u>	101 YIKKFDLQLNEFSQENENAWYFIKNIRKRVGEVNKDGPVLEYPVKPSEVGKSAGQLYEESLRKAVEELRTTNCSYMLNKYDTYSTKEYLLKEGNLSPGAV			
<u>Bp-LAAO</u>	101 YIKKFGLQLNEFSQENENAWYFIKNIRKRVGEVNKDGPVLDYFVKPSEVGKSAGQLYEESLRKAVEELRTTNCSYMLNKYDTYSTKEYLLKEGNLSPGAV			
<u>BjussuLAAO-I</u>	101 YIKKFGLQLNEFSQENENAWYFIKNIRKRVGEVNKDGPVLDYFVKPSEVGKSAGQLYEESLRKAVEELRTTNCSYMLNKYDTYSTKEYLLKEGNLSPGAV			
<u>ImLAAO</u>	101 YIKKFGLQLNEFSQENENAWYFIKNIRKRVGEVNKDGPVLDYFVKPSEVGKSAGQLYEESLRKAVEELRTTNCSYMLNKYDTYSTKEYLLKEGNLSPGAV			
<u>consensus</u>	101 **** * ***** ***** ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . *****			
<u>Bpic-LAAO</u>	201 DMIGDLNEDSGYYVSFIESLKHDDIFAYEKRFDIEVGGMDKLPTSMYQAIQEKVHLNARVIEIQQDVKEVTVTYQTSQETLTSVTADYVIVCTTSRAAR			
Ba-LAAO	201 DMIGDLNEDSGYYVSFIESLKHDDIFAYEKRFDIEVGGMDKLPTSMYQAIQEKVHLNARVIKIQDVKEVTVTYQTSEKETLTSVTADYVIVCTTSRAAR			
<u>BmooLAAO-I</u>	201 DMIGDLNEDSGYYVSFIESLKHDDIFAYEKRFDIEVGGMDKLPTSMYQAIQEKVHLNARVIKIQDVKEVTVTYQTSEKETLTSVTADYVIVCTTSRAAR			
<u>Bp-LAAO</u>	201 DMIGDLNEDSGYYVSFIESLKHDDIFAYEKRFDIEVGGMDKLPTSMYQAIQEKVHLNARVIRKIQDVKEVTVTYQTSEKETLTSVTADYVIVCTTSRAAR			
<u>BjussuLAAO-I</u>	201 DMIGDLNEDSGYYVSFIESLKHDDIFAYEKRFDIEVGGMDKLPTSMYQAIQEKVHLNARVIRKIQDVKEVTVTYQTSEKETLTSVTADYVIVCTTSRAAR			
<u>ImLAAO</u>	201 DMIGDLNEDSGYYVSFIESLKHDDIFGYEKRFDEIVGMDKLPTSMYQAIKEKVRFNARVIRKIQDNDREVTVTYQTSANEMSPVTADYVIVCTSRATR			
<u>consensus</u>	201 **** * ***** ***** ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . *****			
<u>Bpic-LAAO</u>	301 RITFEPPLPPPKAHALLSVHYRSGTKIFLTCTKKFWEDDGIGHGKSTTDLPSRFIYYYPNHNFPNGVGVIIAYIGGDANYFQALDFEDCGDIVINDLSLI			
Ba-LAAO	301 RIKFEPPLPPPKAHALLSVHYRSGTKIFLTCTKKFWEDDGIGHGKSTTDLPSRFIYYYPNHNFPNGVGVIIAYIGGDANYFQALDFEDCGDIVINDLSLI			
<u>BmooLAAO-I</u>	301 RIKFEPPLPPPKAHALLSVHYRSGTKIFLTCTKKFWEDDGIGHGKSTTDLPSRFIYYYPNHNFPNGVGVIIAYIGGDANYFQALDFEDCGDIVINDLSLI			
<u>Bp-LAAO</u>	301 RIKFEPPLPPPKAHALLSVHYRSGTKIFLTCTKKFWEDDGIGHGKSTTDLPSRFIYYYPNHNFPNGVGVIIAYIGGDANYFQALDFKDCGDIVINDLSLI			
<u>BjussuLAAO-I</u>	301 RIKFEPPLPPPKAHALLSVHYRSGTKIFLTCTKKFWEDDGIGHGKSTTDLPSRFIYYYPNHNFPNGVGVIIAYIGGDANYFQALDFKDCGDIVINDLSLI			
<u>ImLAAO</u>	301 RITFEPPLPPPKAHALLSVHYRSGTKIFLTCTKKFWEDDGIGHGKSTTDLPSRFIYYYPNHNFTSGVGVIIAYIGGDANFFQALDFKDCGDIVINDLSLI			
<u>consensus</u>	301 **** * ***** ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . *****			
<u>Bpic-LAAO</u>	401 HQLPKEEIQAICRPSMIQRWSDLNDYAMGGITTFTPYHFQHSEALTAPVDRIFTYFAGEYTAQAHGWIDSTIKSLGRAATDVNRASENK-----			
Ba-LAAO	401 HQLPKEEIHAICRPSMIQRWSLDKYAMGGITTFTPYFQHFSEALTAPVDRIFTYFAGEYTAQAHGWIDSTIKSLGRAARDVNRASEIK-----			
<u>BmooLAAO-I</u>	401 HQLPKEEIQAICRPSMIQRWSLDKYAMGGITTFTPYFQHFSEALTAPVDRIFTYFAGEYTAQAHGWIDSTIKW-----			
<u>Bp-LAAO</u>	401 HQLPKEEIQAICRPSMIQRWSLDKYAMGGITTFTPYFQHFSEALTAPVDRIFTYFAGEYTAQAHGWIDSTIKSLTAARDVNRASE-----			
<u>BjussuLAAO-I</u>	401 HQLPKEEIQAICRPSMIQRWSLDKYAMGGITTFTPYFQHFSEALTAPVDRIFTYFAGEYTAQAHGWIASTIKSGPEGL-DVNRASE-----			
<u>ImLAAO</u>	401 HQLPKDIDQTCYPSMIQRWSLDKYAMGGITTFTPYFQHFSEALTAPFKRIFTYFAGEYTAQFHGWIDSTIKSLTAARDVNRASENPGISIHLSDNEL			
<u>consensus</u>	401 **** * . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . *****			





Highlights

- We present a structure-function relationships of an L-amino acid oxidase, *Bpic*-LAAO, from *Bothrops pictus*.
- *Bpic*-LAAO is a dimeric glycoprotein and its sequence was deduced from its cDNA
- *Bpic*-LAAO is a toxic compound of the venom and reduced the growth of *P. aeruginosa*, *V. cholerae*, *S. aureus*, *E. faecalis* and *E. coli*.