Toxicon 139 (2017) 74–86

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

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

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ARTICLE INFO

Article history:
Received 9 May 2017
Received in revised form
18 September 2017
Accepted 8 October 2017
Available online 9 October 2017

Keywords:
L-amino acid oxidase
Bothrops pictus
Bpic-LAAO

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 a 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 Ca^{2+}, Mg^{2+} and Mn^{2+} did not alter *Bpic*-LAAO activity; however, Zn^{2+} 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 evolutively 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.

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 Icochéa (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 (Martínez-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., 2014). In areas

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.

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https://doi.org/10.1016/j.toxicon.2017.10.001
0041-0101/

within coast regions and the Andes Mountains we can also find other endemic medically relevant species including *B. pictus*, which is known as 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; 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 A_2 (PLA_2s), 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), PLA_2s, 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 (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. Antibotropic 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 × 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 × 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 × 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 (1970), using a Sephacryl S-200 column (42.4 × 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 24 h 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 3 N HCl at 100 °C for 4 h and neutralized with 3 N NaOH; the volume was adjusted with distilled H_2O 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