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Isolation and biochemical, functional and structural characterization of a novel L-amino acid oxidase from *Lachesis muta* snake venom

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The aim of this study was the isolation of the LAAO from *Lachesis muta* venom (LmLAAO) and its biochemical, functional and structural characterization. Two different purification protocols were developed and both provided highly homogeneous and active LmLAAO. It is a homodimeric enzyme with molar mass around 120 kDa under non-reducing conditions, 60 kDa under reducing conditions in SDS-PAGE and 60852 Da by mass spectrometry. Forty amino acid residues were directly sequenced from LmLAAO and its complete cDNA was identified and characterized from an Expressed Sequence Tags data bank obtained from a venom gland. A model based on sequence homology was manually built in order to predict its three-dimensional structure. LmLAAO showed a catalytic preference for hydrophobic amino acids (K_m of 0.97 mmol/L with Leu). A mild myonecrosis was observed histologically in mice after injection of 100 μg of LmLAAO and confirmed by a 15-fold increase in CK activity. LmLAAO induced cytotoxicity on AGS cell line (gastric adenocarcinoma, IC_{50}: 22.7 μg/mL) and on MCF-7 cell line (breast adenocarcinoma, IC_{50}:1.41 μg/mL). It presents antiparasitic activity on *Leishmania brasiliensis* (IC_{50}: 2.22 μg/mL), but *Trypanosoma cruzi* was resistant to LmLAAO. In conclusion, LmLAAO showed low systemic toxicity but important *in vitro* pharmacological actions.

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1. Introduction

Snake venoms of the genus *Lachesis* comprise a complex mixture of pharmacologically active substances, such as metalloproteases (Rucavado et al., 1999), phospholipases A_2 (Ferreira et al., 2009), serine proteases (Magalhães et al., 1997) and other important enzymes. The venom of *Lachesis muta*, from Brazil (Campbell & Lamar, 1989), contains L-amino acid oxidase (LAAO; EC 1.4.3.2), but its functional and structural characterization has not been performed (Sanchez and Magalhães, 1991). This venom induces tissue damage, nausea, vomiting, sweating, bradycardia, hypotension, shock, and, in severe cases, death due to neurotoxic, hemorrhagic and coagulant activities of this complex

Abbreviations: LmLAAO, L-amino acid oxidase from *Lachesis muta* venom; svLAAO, snake venom LAAO.

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mixture of pharmacologically active substances (Jorge et al., 1997).

LAAOs are homodimeric flavoenzymes that catalyze the stereospecific oxidative deamination of L-amino acids by reduction of cofactor FAD. This reaction generates an intermediate imino acid which produces ammonia and the corresponding α-keto acid. In a parallel reaction, the reoxidation of cofactor FAD by molecular oxygen generates hydrogen peroxide (Massey and Curti, 1967; Curti et al., 1992; Sun et al., 2010). According to Du and Clemetson (2002), snake venom LAAOs (svLAAO) have 110–150 kDa when determined by gel filtration, or 50–70 kDa as judged by electrophoresis on polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE). To exert their activity, LAAOs may be organized as dimers, therefore with molar mass between 110 and 150 kDa. Pawelek et al. (2000) showed that *Calloselasma rhodostoma* LAAO is a homodimer of 55 kDa monomers. Furthermore, svLAAOs may be acidic or basic proteins, showing isoelectric points ranging from 4.4 to 8.5 (Ahn et al., 1997; Curti et al., 1992; Du and Clemetson, 2002). Some svLAAO crystal structures have been determined (Moustafa et al., 2006; Zhang et al., 2004) revealing a functional dimer in which each monomer consists of a FAD-binding domain, a substrate-binding domain and a helical domain that is involved in protein dimerization.

Concerning enzymatic properties, different svLAAOs have shown a preference for hydrophobic L-amino acids. This catalytic profile has been observed with LAAOs from *Naja naja oxiana* (Samel et al., 2008), *Bothrops pirajai* (Izidoro et al., 2006) and *C. rhodostoma* (Ande et al., 2008).

Several studies were performed with svLAAO in order to determine its activities *in vivo*. Wei et al. (2009) showed the induction of paw edema in mice after injection of 5 μg of *Bungarus fasciatus* LAAO. Besides edema, they have been shown to induce hemorrhage (Zhong et al., 2009) and systemic effects such as renal toxicity (Boer-Lima et al., 1999). Unexpectedly, despite its toxicity *in vivo*, LAAO does not cause lethality after injection of 120 μg/30 g in Swiss-Wistar mice (Ali et al., 2000). *In vitro* studies with svLAAOs have shown antibacterial (Sun et al., 2010; Ciscotto et al., 2009), leishmanicidal (Rodrigues et al., 2009) and trypanocidal activities (Franca et al., 2007), toxicity upon cancer cell lines (Alves et al., 2008) and both induction and/or inhibition of platelet aggregation (Alves et al., 2008; Li et al., 1994; Sakurai et al., 2001; Sun et al., 2010; Zhong et al., 2009). It has been shown that these effects are correlated with the production of H_2O_2.

Currently, many compounds from snake venoms have been the basis for therapeutic agents (Barros et al., 2009; Lewis and Garcia, 2003) and svLAAOs emerge as an important tool for possible pharmacological applications. Although many svLAAOs have been isolated and studied, this is the first report on the LAAO from *L. muta* venom. The aim of this work was to isolate this enzyme and perform its biochemical, structural and functional characterization. Two different purification protocols were developed and allowed the isolation of pure and active enzyme. Its primary structure was obtained by cloning and sequencing of its cDNA, and a model based on sequence homology was manually built in order to predict its three-dimensional structure. Additionally, LmLAAO has been kinetically characterized and both *in vivo* and *in vitro* assays were used to determine its pharmacological properties in different biological systems.

2. Material and methods

2.1. Venom

L. muta venom was obtained from the Serpentarium Bosque da Saúde, Americana city, state of São Paulo, Brasil (IBAMA Register: 647.998). All chemicals used were of analytical grade.

2.2. Purification protocol 1

2.2.1. Gel filtration on Sephacryl S-100®

Crude venom from *L. muta* (20 mg) was dissolved in 500 μL of 20 mM Tris–HCl buffer plus NaCl 0.15 M (pH 7.0) and centrifuged at 3000×*g* for 10 min to remove insoluble material. The supernatant was applied to a Sephacryl S-100® (Hiprep 16/60, GE Healthcare) column pre-equilibrated with 20 mM Tris–HCl plus 0.15 M NaCl buffer, pH 7.0 and eluted at a flow rate of 0.5 mL/min. The fractions were monitored at 280 nm and tested for LAAO activity.

2.2.2. Ion exchange in Mono Q®

Fractions with LAAO activity were collected and immediately applied on a Mono Q® 5/50GE Healthcare column pre-equilibrated with 20 mM Tris–HCl buffer, pH 7.0 and eluted with a stepwise gradient of 20 mM Tris–HCl plus NaCl 1 M buffer, pH 7.0, at a flow rate of 1 mL/min. The fractions were also monitored at 280 nm and tested for LAAO activity.

2.3. Purification protocol 2

2.3.1. Gel filtration on Sephacryl S-200®

Crude venom from *L. muta* (200 mg) was dissolved in 3 mL of 20 mM Tris–HCl buffer plus 0.15 M NaCl, pH 7.0, and centrifuged at 3000×*g* for 10 min to remove insoluble material. The supernatant was applied to a Sephacryl S-200® (GE Healthcare) column pre-equilibrated with 20 mM Tris–HCl plus 0.15 M NaCl buffer, pH 7.0, and eluted at a flow rate of 0.5 mL/min. The fractions were monitored at 280 nm and tested for LAAO activity.

2.3.2. Hydrophobic interaction on Phenyl-Sepharose®

The fraction with LAAO activity collected from Sephacryl S-200® was submitted to hydrophobic interaction chromatography on Phenyl-Sepharose® resin equilibrated with 20 mM Tris–HCl, 1.5 M NaCl. The chromatography was performed on gradient steps with 20 mM Tris–HCl, pH 8.0, and decreasing concentrations of NaCl, ranging from 1.5 to 0 M, and finished with deionized water. The flow rate was maintained at 1 mL/min. The fractions were monitored at 280 nm and tested for LAAO activity.

2.3.3. Low pressure liquid chromatography on Affi-Gel Blue®

The fraction with LAAO activity eluted from the hydrophobic interaction chromatography on Phenyl-Sepharose®

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