Current Research Journal of Biological Sciences 3(1): 6-11, 2011
ISSN: 2041-0778

Received: June 26, 2010 Accepted: July 31, 2010 Published: January 15, 2011

Isolation and Purification of L-amino Acid Oxidase from Indian Cobra *Naja naja*

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Abstract: The enzyme L-Amino Acid Oxidase (LAAO) is widely distributed in snake venoms, contributes a reasonable toxicity to the venoms. However LAAO from Indian cobra (*Naja naja*) snake venom has not yet isolated previously. In the present study LAAO from Indian cobra (*Naja naja*) snake venom was purified by sequential steps of cation exchange chromatography (CM-Sephadex C-25; CM-52 cellulose), followed by sephadex G-100 gel filtration chromatography. The purified enzyme was named as Indian Cobra *Naja naja* L-Amino Acid Oxidase (ICN-LAAO). This ICN-LAAO is a monomer and its molecular mass has been found to be 61.91 kDa determined by MALDI-TOF and 12% SDS-PAGE. The enzyme has an isoelcetric point of 8.12 and a pH optimum of 8.5. It shows remarkable thermal stability and partial inactivation by freezing. The enzyme may contribute in the development of severe hematological disorder due to cobra venom envenomation.

Key words: Indian cobra, L-amino acid oxidase, *Naja naja*, purification

INTRODUCTION

Snake venoms are a mixture of simple and complex substances, such as biologically active peptides and proteins, but their biochemical characteristics change according to the snake species studied (Markland, 1998). The enzyme L-amino acid oxidase (LAOs, EC 1.4.3.2) is one of the major components of snake venoms, which possess numerous biological functions and thought to contribute to the toxicity of the venom (Samel *et al*., 2006). From the class of oxidoreductases, the enzyme L- amino acid oxidase has become an attractive object in the last few years, for the studies of pharmacology, structural biology and enzymology (Du and Clemetson, 2002). In the earlier works, the effect of LAAO is reduced to its main function, catalysis of the oxidative deamination of L-amino acids to form the corresponding α-keto acids and ammonia accompanied with the reduction of FAD. Simultaneously, hydrogen peroxide is liberated that has been a major contributor in the toxic effects of LAAO (Du and Clemetson, 2002; Samel *et al*., 2006). During oxidative stress, H_2O_2 at low levels is believed to have a role in the activation of transcription of some genes which could prevent cell death (Wiese *et al*., 1995). However, an increase in H_2O_2 concentration induces a growth arrest and apoptotic death of cells (Wiese *et al*., 1995; Suzuki *et al*., 1997) which could explain the LAAO effect on the induction of apoptosis. The reported effects of LAAOs on platelets are quite controversial. LAAO from *Echis colorata* inhibits ADP-induced platelet aggregation. LAAOs from *Agkistrodon halys blomhoffii*, *Naja naja kaouthia* and king cobra inhibit agonist-induced or shear stress-induced platelet aggregation (Takatsuka *et al*., 2001; Jin *et al*., 2007). Numerous LAAOs have been isolated from different venoms (Samel *et al*., 2006; Du and Clemetson, 2002; Wei *et al*., 2003; Stabeli *et al*., 2004; Toyama *et al*., 2006; Izidoro *et al*., 2006). As stated above, generally LAAOs from different sources differ in their substrate specificities, structure stability and biological activities (Du and Clemetson, 2002). In this study we report for the first time the enzyme L-amino acid oxidase isolated and purified from the Indian cobra *Naja naja*.

MATERIALS AND METHODS

The present study was carried out between May 2009 to December 2009 at VIT University, Vellore, Tamilnadu, India. The lyophilized crude venom of *Naja naja* was purchased from Irula Snake Catchers' Industrial Co-operative Society Ltd., India 969. CM-Sephadex C-25 was purchased from GE Health care Bioscience Ltd., UK. CM-52 cellulose, Sephadex G100, and O-Dianisidine were obtained from Sigma. L-amino acid substrates, and peroxidase, were obtained from SRL India. All other reagents were analytical grade.

Isolation of L-amino acid oxidase by cation exchange chromatography: The lyophilized crude venom (200 mg) was dissolved in 1 mL of sodium acetate buffer (50 mM sodium acetate, pH 5.8) and centrifuged at 3000 g for 5 min. The supernatant was loaded on to the CM-sepahdex C-25 column (1.8x14.5 cm^2). Unbound proteins were

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washed out with equilibrated buffer (50 mM sodium acetate, pH 5.8). Bounded molecules were eluted with same buffer and followed by elution with gradients of 0.1-1.0 M of NaCl at a flow rate of 60 mL/h. The protein eluted was monitored at 280 nm using Amersham bioscience double beam spectrophotometer. Active fractions of ICN-LAAO were collected, pooled, and desalted by Amicon Ultra fitration 10 kDa membrane (Millipore USA) for further experiments.

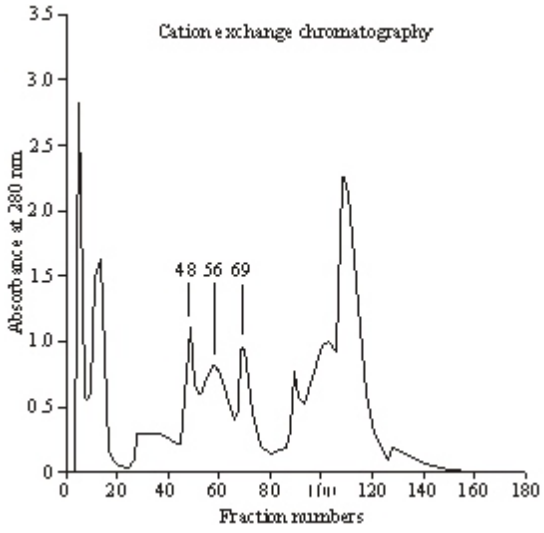
Purification of L-amino acid oxidase by Cation exchange chromatography CM-52 cellulose: Seven milligrams of concentrated ICN-LAAO from CM-Sephadex C-25 was loaded on to the CM-52 cellulose column (1.8x6 cm^2). Unbound proteins were washed out with 50 mM sodium acetate buffer, pH 5.8 and bounded molecules were eluted with same initial buffer followed by elution using different gradients (0.1 M, 0.5 M) of NaCl at a flow rate 45 mL/h. The protein elution was monitored at 280 nm using Amersham bioscience double beam spectrophotometer. Active fractions were collected, pooled, and desalted using Amicon ultra filtration 10 kDa membrane (Millipore USA) for further experiments.

Gel filtration chromatography Sephadex G-100: Active LAAO of 6.6 mg in concentration, obtained from CM sephadex C-52 column was dissolved in 1 mL of 50 mM sodium acetate buffer at pH 5.8 and it was applied to gel filtration on sephadex G-100 column (6x1.8 cm^2) in 0.2 M ammonium acetate buffer. Elution was carried out at the flow rate 1 mL/1min.

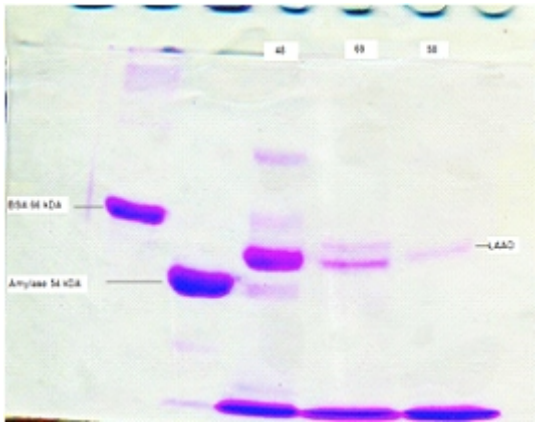
Protein estimation: Protein estimation was carried out for the pooled, concentrated fraction mixture obtained from gel filtration chromatography, using Lowry's method (Lowry *et al*., 1951).

LAAO enzyme activity and substrate specificity: LAAO activity was determined spectrophotometrically according to the method modified from (Bergmeyer *et al*., 1983). The reaction mixture (1 mL) contained 0.1 M Tris-HCl buffer, pH 8.5, 1 mM L-leucine as a substrate, 0.26 mM O-dianisidine, 20 μg horseradish peroxidase (7 U) and the known amount of the eluted fractions or purified LAAO as well as the whole venom. One unit of enzyme activity was defined as the oxidation of 1 μmol of L-leucine per minute.

Determination of molecular mass by SDS-PAGE: SDS-PAGE was carried out using 12% gel at pH 8.3 according to the method of (Laemmli, 1970) to determine the approximate molecular mass of a purified LAAO enzyme. Molecular mass standards were bovine serum albumin-66.2 kDa, amylase-55 kDa and lysozyme-14.4 kDa. Gels



(a)

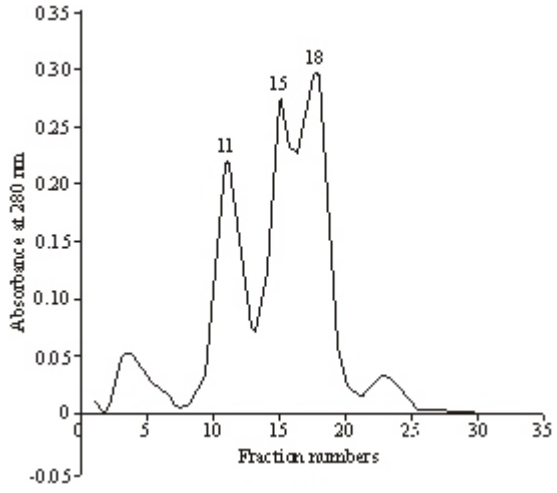


(b)

Fig. 1: (a) 100 mg of venom loaded on to CM-Sephadex C-25 column. Dimension of coulmn packing, 0.9 x 14.5cm; flow rate 2 ml/min; fraction volume 2 ml, at room temperature. Elution was carried out stepwise with 0.1 to 1M NaCl gradient; (b) SDS-PAGE and molecular weight determination of LAAO by Cation exchange chromatography. 12% SDS-PAGE: lane 1 (BSA), lane 2 (Amylase), Lane 3 (F.no 48), lane 4 (69), and lane5 (F.no 58)

were stained for proteins with Coomassie Brilliant Blue R-250.

After desalting, the recovered peptide sample was subjected to matrix-assisted laser desorption/ionization-time of flight (MALDITOF) mass spectrometric analysis. MALDI-TOF mass spectra for mass fingerprinting and MALDI-TOF/TOF mass spectra for identification by fragment ion analysis were acquired using the Ultraflex TOF/TOF instrument. Protein identification with the



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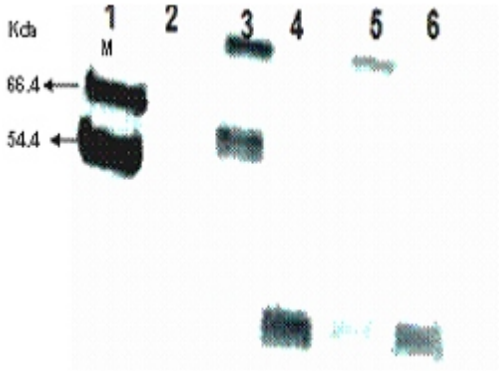


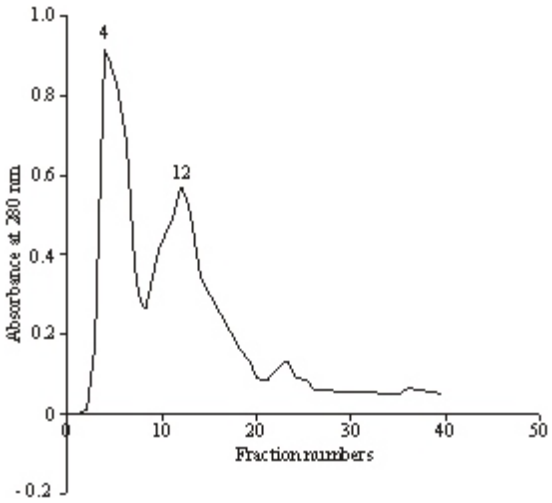
(b)

Fig. 2: (a) Step two: purification of LAAO by CM-Cellulose-52 column chromatography. Dimension of coulmn packing, 0.9 x 5 cm; flow rate 2 mL/min; fraction volume 2 mL, at room temperature; (b) Molecular weight determination of LAAO by 12% SDS-PAGE: lane - (BSA (66.4 kDa & Amylase 54.4 kDa), lane-2 (F.no 11), Lane-3 (LAAO from f.no 15), lane-4 (16), lane-5 (F.no 17), and Lane-6 (F.no18)

generated data was performed using Mascot® Peptide Mass Fingerprint and MS/MS Ion Search programs.

RESULTS

Isolation and Purification of LAAO from Indian cobra Naja naja: The LAAO from Indian cobra *Naja naja* was isolated and purified in three steps. In the first step LAAO was isolated from the crude venom using CM-sephadex C-25 column chromatography. A total of 160 fractions of 2 mL each were collected at 4ºC out of which fraction numbers 48, 58 and 69 were found to have active LAAO.



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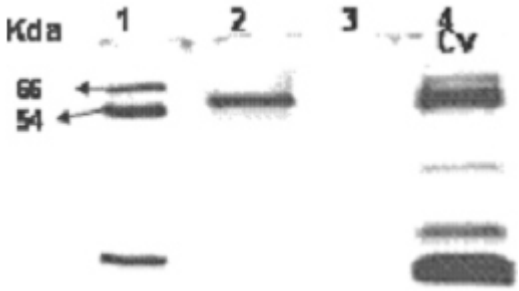

(b)

Fig. 3: (a) Purified LAAO eluted on gel filtration Chromatography. Peak 12 represents the pure LAAO enzyme; (b) Molecular weight determination of LAAO by 12% SDS-PAGE: lane 1 (BSA 66.4 kDa, Amylase 54.4 kDa), lane 2 (F.no 12), Lane 4 (crude venom)

Fig 1a represents the major peaks obtained by spectroscopy analysis of the collected fractions from the column at 280 nm and Fig. 1b represents the SDS-PAGE analysis in 12% gel with the active fractions along with the crude venom. Bovine serum albumin was used as molecular standard. From the PAGE analysis it is observed that the peaks had more than one compound of proteins.

In the second step, the concentrated active LAAO fractions of 48, 58 and 69 were mainly eluted with 50 mM sodium acetate buffer followed by elution with different NaCl gradient buffers at pH 5.8 using cation exchange chromatography on CM-52 cellulose column. Around 30 fractions were collected from which fraction numbers 11, 15 and 18 were taken for the LAAO analysis (Fig. 2a) and SDS-PAGE analysis (Fig. 2b). Notably, CM-52 cellulose purified preparation showed reduced bands compared to CM-sephadex bands.

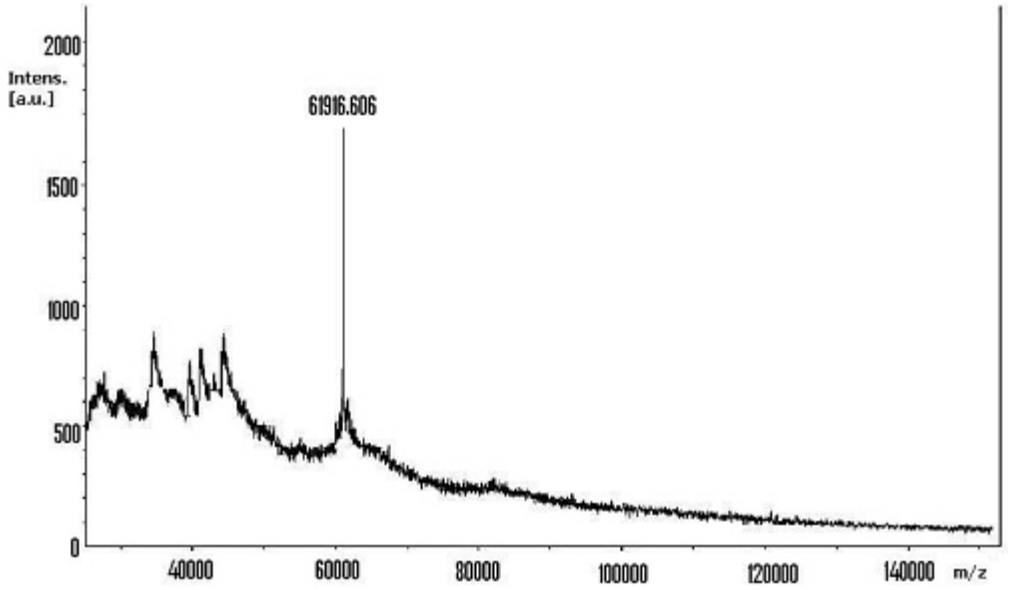

Fig. 4: The purity and mass of IC-LAAO was determined by MALDITOF analysis. The arrow indicates the LAAO protein peak showing mwt 61.91 Kda

Finally the gel filtration chromatography has performed in which, the purified and concentrated fractions of 11, 15 and 18 of CM-52 cellulose preparation were eluted with 50 mM sodium acetate buffer, pH 5.8 on sephadex G-100 column (Fig. 3a). Overall, 40 fractions were collected out of which peak 12 was found to have LAAO activity and this fraction showed a single band in 12% SDS-PAGE analysis (Fig. 3b). One microgram of purified LAAO produced 1.522 μg H_2O_2/min upon oxidation of L-lucien. The molecular mass results for the purified LAAO from Indian cobra *Naja naja* was determined as 61.96 kDa by MALDITOF (Fig. 4).

DISCUSSION

LAAOs are flavo-enzymes that are major components of many snake venoms. Some of these activities are known to date, but still poorly understood with regard to snake venoms, though they are postulated to be toxins (Du and Clemetson, 2002). Over the last 10-15 years, LAAOs have become an interesting object for pharmacological studies showing apoptotic, cytotoxic, platelet aggregation and other physiological effects (Sun *et al*., 2003). Mainly, these effects are supposed to be mediated by hydrogen peroxide that is liberated in the oxidation process (Torii *et al*., 1997). In the present study crude snake venom was loaded on CM-Sephadex C-25 column in order to separate ICN-LAAO from other acidic proteins and most basic proteins, such as neurotoxins (Lu and Lo, 1981), cytotoxins (Tsai *et al*., 2007) and phospholipase A2 (Lu and Lo, 1981; Tsai *et al*., 2007; Xu *et al*., 2007). The next step was followed by CM-cellulose C-52 column chromatography and gel filtration chromatography. The monomer structure with an apparent molecular weight 61.96 kDa (Fig. 4) reveals that ICN-LAAO is similar to LAAOs from the elapid venom *Naja naja kaouthia* (Tan and Swaminathan, 1992) as well as for the *C. rhodostoma* LAAO but slight different in molecular weight of LAAO isolated from *Agkistrodon halys pallas* and *Eristocophis macmahoni* venom. Using a two step chromatographic procedure (Wei *et al*., 2009) has reported the monomer structure of LAAO from *Bungarus fasciatus* snake venom with a molecular weight of 70 kDa. (Pawelek *et al*., 2000) presented the X-ray structure of *C. rhodostoma* LAAO that indicates that this enzyme is functionally a dimer. Each subunit comprises a FAD-binding domain, a substrate-binding domain and a helical domain. Usually the oxidizing activity is determined using L-Leu as substrate. In the case of elapid snake venoms, hydrophobic amino acids (incl. L-Leu) are the best substrates for LAAO. The substrate specificity of *Naja naja kaouthia* LAAO is in good accordance with other studied snake venom LAAOs.

CONCLUSION

Overall the present study reports the isolation and purification procedure for the enzyme LAAO from Indian cobra for the first time and further it has been named as ICN-LAAO (Indian cobra *Naja naja*-L-Amino Acid

Oxidase).However, further investigations on the related molecular and functional correlation of this enzyme would provide valuable information on the therapeutic drug development. ICN-LAAOs are therefore interesting enzymes, not only for a better understanding of the envenomation mechanism, but also as models for potentially novel therapeutic agents.

ACKNOWLEDGMENT

This work was supported by School of Bioscience and Technology, VIT University, Tamilnadu, India.

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