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Original Article

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF L-AMINO ACID OXIDASE FROM WESTERN REGION INDIAN COBRA (*NAJA NAJA*) VENOM

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ABSTRACT

Objective: Purification and biochemical characterization of LAAO from western region Indian Cobra (*Naja naja*) venom.

Methods: LAAO was purified from Indian cobra (*Naja naja*) venom using sequential chromatography on Sephadex G-75 gel filtration followed by Ion exchange on CM-Sephadex C-25 column. Biochemical characterization *viz*., pH, Temperature, Km and Vmax were determined. Molecular weight of LAAO was determined by electrophoresis. Inhibition of LAAO from cold water extracts of *Curcuma zedoria, Curcuma ceasia, Curcuma aromatic, Curcuma longa, Curcuma amad*a, *Cucumis sativus* and *Benincasa hispida* was done.

Results: Purified LAAO showed the single band on non reducing SDS-PAGE with approximate molecular weight of 65 kDa. Further biochemical characterization revealed that, LAAO from *Naja naja* (western region) has an optimum pH of 7.0 and is stable at room Temperature upto 37 °C and showed an optimum enzyme concentration of 4µg/ml and Km 134.1µM and Vmax is 21.87 U/min. Cold water extract of *Curcuma ceasia, Curcuma aromatic* and *Benincasa hispida* showed comparatively significant inhibition of LAAO.

Conclusion: LAAO has promising therapeutic prospects because of its effects on various biological functions. Variation in snake species and their geographical distribution also contributes to the venom properties such as composition, toxicity level, pharmacological and biological activities. The significant difference in protein profiling leads to substantial lethality among different geographical regions. Thus in this study Indian Cobra (*Naja naja*) venom from the western region of India was subjected to purification of LAAO and biochemical characterization.

Keywords: Indian Cobra (*Naja naja*), Venom, L amino acid oxidase-LAAO.

INTRODUCTION

Snake bite is a neglected topic in Indian health services. Farmers and laborers in rural areas are majorly affected from snake bites which threaten their lives with time-limiting medical emergency. About 45,000 deaths per annum throughout the world occur due to snake bites amongst them India stands highest with approximately 11,000 numbers of deaths. WHO has also included snake envenomation as "Neglected Tropical Diseases" in April 2009 [1]. The four venomous snakes that are held responsible for most of the snake envenomation causing fatalities in India are called "Big Four": Cobra (*Naja naja*), Common Krait (*Bungarus Cearuleus*), Russell's viper (*Daboia russelli*) and Saw-scaled viper (*Echis Carinatus*). Indian Cobra (*Naja naja*) is found abundantly in an Indian subcontinent, belonging to *Elapidea* family was first described by Carl Linnaeus. Snake venoms are the amplest sources of complex cocktails of active proteins, peptides, organic and inorganic materials which have various pharmacological effects on victims or preys [2]. This venom is usually delivered through specialized pointed teeth such as hollow fangs and assists the snake in the immobilization of their prey as well as for the defense mechanism. The pathophysiology of snake venom comprises of systemic toxicities like neurotoxicity, cardiotoxicity, hypersensitive effects so on and local toxicities like edema, hemorrhage, myonecrosis, platelet aggregation and inflammation. Added to this, some of the reports suggest that toxicity of these enzymes acting either independently or with other toxins contribute to the overall toxic effects of the venoms [3, 4].

LAAO is widely distributed across diverse phyla from bacteria to mammals and venomous snakes. LAAO occurs in many organisms namely *Cyanobacteria* [5], *Proteus* [6], *Corynebacterium*[7], *Alysia californica* [8], *Trichoderma viride* [9] and *Sebastes schlegeli* [10]. Most prominently in venomous snakes families such as *Crotalidae, Elapidae* and *Viperidae*. LAAO (EC 1.4.3.2) from venom is a homodimeric flavo enzyme from the class of oxidoreductases, catalyzes oxidative deamination of L-amino acid to α-keto acids in a stereo specific mode along with the production of ammonia and hydrogen peroxide *via* an imino acid intermediate [11, 12]. LAAO is non-covalently bound to coenzyme FAD or FMN as a prosthetic group, which in-turn imparts yellow coloration to the venom [6]. LAAO is the only FAD-dependent oxidase found in snake venom and its toxicity, possibly through the generation of hydrogen peroxide. Low levels of H_2O_2 prevent cell death, but increase in H_2O_2 levels induces growth arrest and apoptotic cell death [13]. Platelets are another potential and target of blood borne snake venom components. However, the earlier reported effects of snake venom LAAO on platelets functions are still controversial. LAAOs from *Bothrops pirajai* and from *Ersitocophis macmohani* induce platelet aggregation triggered by several agonists dose-dependently in the range of 0.1-2.5 µM [14]. Just like its effect on platelet aggregation, induction of cell death by LAAO appears to involve both in the generation of H_2O_2 and the molecular interaction of the glycan moiety of the enzyme with structures at the cell surface [15]. LAAO finds numerous applications as a catalyst in biotransformation, amperometric biosensors and for production of α-keto acids [16]. *Pseudoalteromonas tunicata* expresses a protein that has an important role in biofilm development and cell dispersal [17]. Snake venom may open the doors for the new era of research in medicine.

Snake venom variability due to geographical distribution of the snake species is reported for varied biochemical, toxicological properties such as venom composition, lethality, immunological functions and pharmacology [18]. In other study, the diversity in Indian Cobra (*Naja naja*) venoms from different geographical locations of India *viz.,* eastern, western and southern region venom showed varied proteomic profiles along with the chemical composition of the venoms within inter family and intra species [19]. Venom variability from many species of snakes from various geographical distributions has been extensively studied in many laboratories, to check its biochemical characteristics. Western venom is more myotoxic than the other two regional venoms [20]. In the present study, purification and characterization of LAAO from western region (Mumbai) Indian Cobra (*Naja naja*) venom was studied.

MATERIALS AND METHODS

Lyophilized *Naja naja* venom was obtained from the Haffkine Institute, Mumbai, India. *o*-dianisidne hydrochloride, horseradish peroxidase, Sephadex G-75, CM-Sephadex C-25 were purchased from Sigma–Aldrich, India. Analytical grade HCl were obtained from Fisher Scientific. Tris (hydroxymethyl) aminomethane, Fatty acid free Bovine Serum Albumin (BSA) fraction V, Sodium hydroxide, Calcium chloride dihydrate and Sodium chloride were purchased from Merck. Sodium acetate and Glycine were purchased from SRL Chemicals. Ultracel YM-3 centricons was purchased from Millipore. Milli-Q water was used throughout the experiments. All chemicals were of analytical grade.

Sephadex G-75 column chromatography

150 mg of crude lyophilized venom were dissolved in 1 ml of 50 mM sodium acetate buffer, pH was adjusted to 7 and centrifuged at 3000 rpm for 5 min. The amount of protein in supernatant was estimated by Bradford's method [21]. The supernatant was loaded onto the pre equilibrated Sephadex G-75 column (1.0×150 cm) with 50 mM sodium acetate buffer, pH 5. The elution was carried out with the same buffer at a flow rate of 15 ml/h with fraction volume of 1.5 ml at room temperature and fractions were collected and monitored at 280 nm using spectrophotometer. Each peak fraction was checked for the LAAO activity. Fractions were pooled and stored at-4 °C for further experiments.

CM-Sephadex C-25 column chromatography

LAAO active peak fraction 1, obtained from Sephadex G-75 was loaded on to the CM-sephadex C-25 column (0.5x45 cm) pre equilibrated with 50 mM sodium phosphate, pH 7 binding buffer. Unbound proteins were washed with binding buffer. Bound molecules were eluted with the same buffer, followed by elution with gradients of 0.1-1.0 M of NaCl at a flow rate of 15 ml/h. The protein eluted was monitored at 280 nm. Each major peak fraction was checked for LAAO activity. Subsequently the fractions were pooled, concentrated and passed through Ultracel YM-3 centricons centrifuged at 4 °C. Desalted supernatant was stored at-4 °C for further experiments.

LAAO enzyme activity

Determination of LAAO activity was carried out spectrophotometrically using method from Bergmeyer [22], Briefly, a total reaction mixture of 1 ml, containing 0.1M Tris buffer pH 7, 1 mM L-Leu, 0.26 mM *o*-dianisidine, 10µg of horseradish peroxidase were added and incubated for 5 min at 37ºC. Activity was initiated by adding crude venom or purified LAAO. Reaction mixture was incubated for 30 min at 37ºC. One unit of LAAO activity was defined as the oxidation of 1 µmol of L-Leu/min. In this assay, the oxidation deamination of L-Leu produced hydrogen peroxide, which was reduced in the presence of horseradish peroxidase by *o*-dianisidine to produce a colored oxidized product.

LAAO substrate concentration

Briefly, a total reaction mixture of 1 ml, containing substrate L-amino acid (L-Leu), 50 mM Sodium acetate buffer pH 7, 0.26 mM o-dianisidine, 10µg of horseradish peroxidase was added and incubated for 5 min at 37ºC. Activity was initiated by adding eluted fractions or purified LAAO as well as the whole venom. Reaction mixture was incubated with different concentration of L-Leu (0-250µM) for 30 min at 37ºC. Activity was measured as described earlier.

Effect of pH and temperature on LAAO enzyme activity

Briefly, a total reaction mixture of 1 ml containing different buffers ranging from pH 2.0-9.0 was used to determine the pH-dependence on enzyme activity. 50 mM Sodium acetate buffer (pH 4.0-6.0), Tris-HCl buffer (pH 7-8) and glycine-NaOH buffer (pH 9-13) were added to respective reaction mixture containing 1 mM L-Leu, 0.26 mM *o*-dianisidine, 10µg horseradish peroxidase and incubated for 5 min at 37ºC. Activity was initiated by adding LAAO eluted fractions from CM-Sephadex C-25 column and enzyme activity was calculated as described earlier. Similarly, the reaction mixture 1 ml was incubated at various temperatures for 30 min; activity was initiated by adding eluted LAAO fractions. Activity was measured as described earlier.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is known as a potential medium for separating biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility [23]. SDS-PAGE (12.5%) was performed for crude *Naja naja* venom, according to the method of Laemmli [24] under non-reducing conditions. The gel was stained with 0.25% Coomassie brilliant blue R-250. Bovine serum albumin 66.2 kDa was used as standard.

Native PAGE was carried out for active peak fraction from G-75 and CM-Sephadex C-25 were loaded on 12.5 % polyacrylamide gel using 1.5M KOH-Acetic acid buffer pH 4.3 in separating gel without SDS. Stacking gel was prepared with 0.5M KOH-acetic acid buffer pH 6.8. Samples were added in sample buffer containing 50% glycerol and few traces of malachite green as indicator. Separation was carried out on the tank buffer containing 300 mM β-alanine in 0.14M acetic acid pH 4.3. After electrophoresis, the gels were stained with 0.25 % Coomassie brilliant blue R-250.

Inhibition of LAAO

Inhibition of LAAO activity was carried out by modifying the spectrophotometric method from Bergmeyer [22], Briefly, a total reaction mixture of 1 ml, containing 0.1M Tris buffer pH 7,1 mM L-Leu, 0.26 mM *o*-dianisidine, 10 µg of horseradish peroxidase were added and incubated for 5 min at 37ºC. Cold water extracts of *Curcuma zedoria, Curcuma ceasia, Curcuma aromatic, Curcuma longa, Curcuma amad*a [25], *Cucumis sativus* [26] and *Benincasa hispida* were used for inhibition studies. Activity was initiated by pre-incubated 5µg (w/v) of lyophilized cold water extracts with LAAO. Reaction mixture was incubated for 30 min at 37ºC. Percentage of inhibition were calculated and expressed according to Keerthy *et al.,* [27].

RESULTS AND DISCUSSION

Snake venom LAAO has promising therapeutic prospects because of its effects on various biological functions such as platelet aggregation, cell apoptosis and cytotoxicity. It is also believed to show anti-microbial, anti-leishmaniasis, anti-tumor and anti-HIV activity. Snake venom L amino acid oxidase is also being explored for its potential in cancer therapy.

The LAAO from Indian cobra *Naja naja* was isolated and purified. In the first step LAAO was isolated from the crude venom using Sephadex G-75. Three major peaks were resolved and LAAO activity was detected in the first peak (fig. 1). Peak 1 fraction showed the band at 65kDa in 10% SDS-PAGE analysis (fig. 2a). Fig. 2b represents the native acidic gel, with active fraction, which was carried out using 10% gel at pH 4.3 which confirms that LAAO is a basic protein with the approximate molecular weight of 65 kDa. Where as in southern region of *Naja naja* LAAO molecular weight was found to be 61.96 kDa [28].

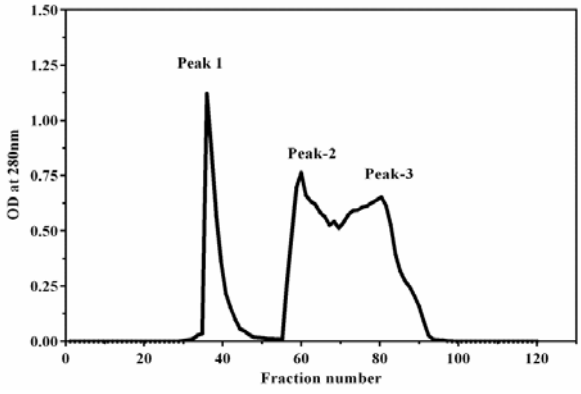

Fig. 1: Sephadex G-75 elution profile of Western region *Naja naja* venom. The column was pre-equilibrated with 50 mM sodium acetate buffer. 140 mg of *Naja naja* venom was dissolved in 1 ml (w/v) of equilibrated buffer and loaded on Sephadex G-75

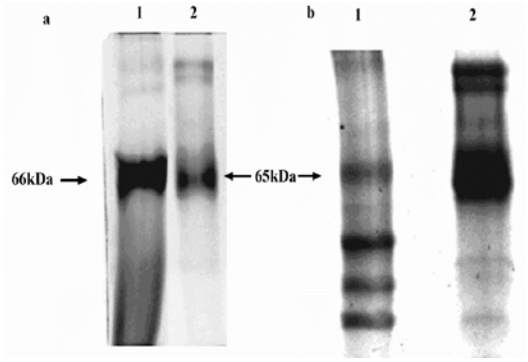

Fig. 2: Electrophoretic pattern of Peak 1 eluted from Sephadex G-75 a. SDS-PAGE (Non Reducing) of LAAO at 10 % gel. Lane: 1-BSA (25µg), Lane: 2-PI (20µg). b. Acidic-PAGE of LAAO at 10 % gel. Lane: 1-*N. naja* crude venom (10 µg), Lane 2-PI (10 µg)

In the second step, Peak 1 from G-75 fraction was lyophilized (60 mg) and loaded on Cation exchange chromatography on CM Sephadex C-25. Fractions were eluted with 50 mM sodium phosphate buffer pH 7, containing varied ionic strength (0.1-1.0 M NaCl). The column was eluted with 15 ml/hr in a stepwise gradient (fig. 3). The fractions were collected and protein elution was monitored at 280 nm in a shimadzu spectrophotometer. Three peaks were resolved, Peak 3 from CM-Sephadex C-25 eluted at 200 mM, showed LAAO activity. Inset fig. 3a represents the SDS-PAGE analysis in 12.5% gel of Peak-3 fractions of CM-Sephadex C-25.

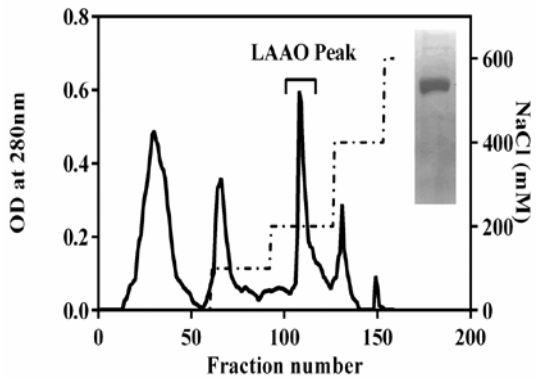

Fig. 3: CM-Sephadex C-25 ion exchange chromatography of LAAO peak from Sephadex G 75 column. The column of 0.5x45 cm pre-equilibrated with 50 mM sodium acetate buffer. Inset: Electrophoretic pattern of LAAO peak 3 (25 µg) from CM Sephadex C-25 column. SDS-PAGE (Non Reducing) of LAAO at 12.5% gel

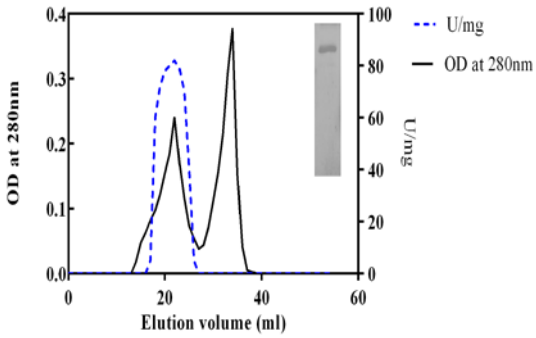

Fig. 4: Re-chromatography of *Naja naja* venom on Sephadex G75 column. The column of 0.5x25 cm pre-equilibrated with 50 mM sodium acetate buffer. Inset: Electrophoretic pattern of Peak 1 (20 µg) from Sephadex G-75 column under 12.5% non-reducing SDS-PAGE

In final step, the active LAAO fraction from CM-Sephadex C-25 column was Re-chromatography on Sephadex G75 column (0.5x25 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 7, with 40 mg of *Naja naja* venom dissolved in 1 ml of equilibrated buffer (fig. 4). Peak 1 re-chromatographic fraction showed LAAO activity. Electrophoretic mobility of the same fraction under non reducing condition showed a single sharp band (Inset fig. 4). Purification summary is described in table 1.

Table1: Summary of purification of LAAO from *Naja naja* venom

Sample	Total Protein (mg)	[a]Total Enzyme Activity 1U	Specific Activity (U/mg)	% Yield
Crude	140	2490 U	17	100
Sephadex G-75	85	1829 U	21	60.7
CM-Sephadex C-25	42	1367 U	32	30
Re-chromatography Sephadex G-75	10.5	850 U	81	6.5

Total enzyme activity was estimated in the pooled peaks, [a] 1 U=1 µmol of H_2O_2/min

One microgram of purified LAAO resulted in producing 6.861 µg H_2O_2/min upon oxidation of L-Leu. The effect of various enzyme concentrations was checked using LAAO assay. Biochemical characterization of LAAO was carried out and it was found that the optimum enzyme concentration was 4 µg, as shown in fig. 5a. Km was 134.1µM and Vmax was found to be 21.87 U/min and substrate concentration was 150µM as in fig. 5b. The optimum pH for L-amino acid oxidase activity is 7, while the optimum temperature is 37 ° C, as shown in the fig. 5c and 5d respectively. The purified enzyme was found to be stable at–4 °C for two months and partial inactivation by freezing. The enzyme activity remained unaffected between 30 and 40 °C. Freezing and thawing did not affect the enzyme activity as long as it was stored at a pH 6.5-7.5.

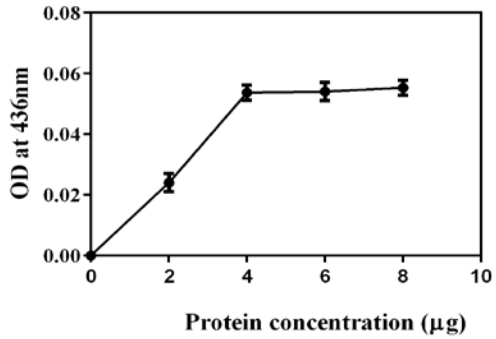

Fig. 5a: Effect of Enzyme concentration. Different concentration (0-8µg) of LAAO was incubated with the reaction mixture for 30 min at 37 °C and activity was observed at 436 nm. Values represent the arithmetic mean and SD (n=3)

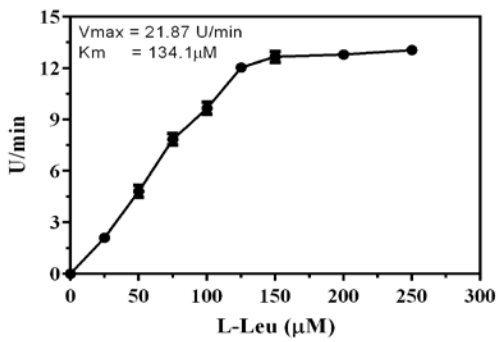

Fig. 5b: Effect of substrate concentration on different concentration (0-200µM) of L-Leu was incubated with the reaction mixture for 30 min at 37ºC. Values represent the arithmetic mean and SD (n=3)

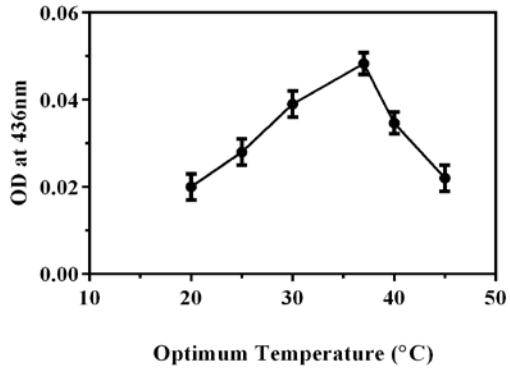

Fig. 5c: Effect of Temperature on LAAO activity. Reaction mixture was incubated at different temperatures for 30 min, activity was observed at 436 nm. Values represent the arithmetic mean and SD (n=3)

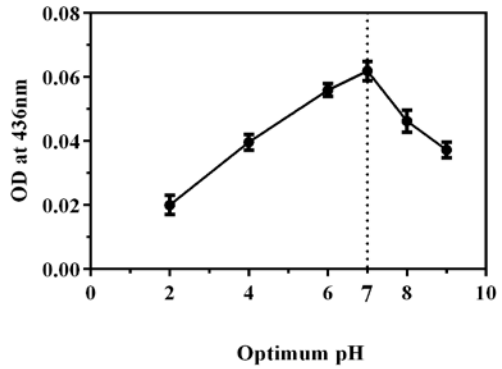

Fig. 5d: pH Effects on LAAO activity. Reaction mixture was incubated with buffers ranging in pH from 2.0-9.0 for 30 min and absorbance was read at 436 nm. Values represent the arithmetic mean and SD (n=3)

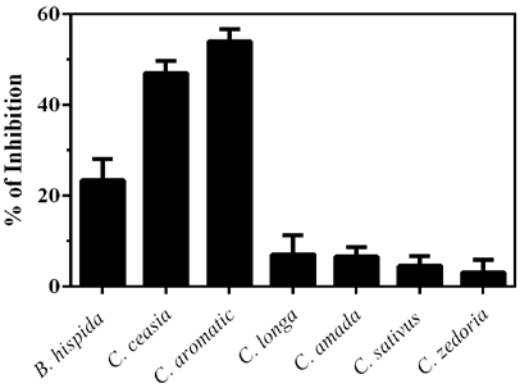

Fig. 6: Inhibition study of LAAO using cold extracts of *Benincasa hispida, Curcuma ceasia, C. zedoria, C. aromatic, C. longa, C. amada*, and *Cucumis sativus.* Values represent the arithmetic mean and SD (n=3)

Curcuma ceasia, Curcuma aromatic and *Benincasa hispida* showed comparatively high inhibition of LAAO as shown in the fig. 6, Percentage of inhibition were observed because of the presence of flavonoids, polyphenols and alkaloids [25]. Further studies would enhance the active components responsible for inhibition of LAAO from Indian cobra *Naja naja.*

CONCLUSION

LAAOs are useful enzymes due to their biotechnological potential as a target for therapeutic drugs and medicine. Overall the present study reports the purification and biochemical characterization of the enzyme LAAO from western region Indian cobra *Naja naja* venom. Geographical variation is one of the major reasons that affect the toxicity of the venom samples for the high mortality rate seen in India. Results confirm that, geographical variation in protein differ from region to region as there is differences in molecular weights of LAAO from western and eastern regions of India. Plant extracts could be alternative therapy for snake bite. These extracts provide satisfactory remedy for venom induced toxicities. Thus, it is important for the researchers, clinicians and importantly governing bodies to focus on the issues related to snakebite management in the subcontinent. However, further work is needed for the study of pharmacological potency of LAAO from Indian Cobra *Naja naja* venom and exploring specific and safe generics of lead molecule(s) towards inhibiting snake venom L-amino acid oxidase.

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CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Mohapatra B, Warrell, David A, Suraweera, Wilson, Bhatia, *et al*. Snakebite mortality in india: a nationally representative mortality survey. PLoS Neglected Trop Dis 2011;5:e10-8.
2. Narendra Sharath Chandra JN, Ponnappa KC, Sadashiva CT, Priya BS, Nanda BL, Veerabasappa T Gowda, *et al*. Chemistry and structural evaluation of different phospholipase A2 inhibitors in arachidonic acid pathway mediated inflammation and snake venom toxicity. Curr Top Med Chem 2007;7:787-800.
3. Kumar JR, Basavarajappa BS, Arancio O, Aranha I, Gangadhara NS, Yajurvedi HN, *et al*. Isolation and characterization of "Reprotoxin", a novel protein complex from daboia russelii snake venom. Biochimie 2008;90:1545-59.
4. Kasturi S, T Veerabasappa Gowda. Purification and characterization of a major phospholipase A2 from Russell's viper (*Vipera russelli*) venom. Toxicon 1989;27:229-37.
5. Du, Xiao-Yan, Clemetson, Kenneth J. Snake venom L-amino acid oxidases. Toxicon 2002;40:659-65.
6. Huang CL. Venoms: chemistry and molecular biology. J Pharm Sci 1978;67:893-3.
7. Coudert, Michele, Vandecasteele, Jean-Paul. Characterization and physiological function of a soluble L-amino acid oxidase in Corynebacterium. Arch Microbiol 1975;102:151-3.
8. Yang H, Johnson PM, Ko KC, Kamio M, Germann MW, Derby CD, *et al*. Cloning, characterization and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica*. J Exp Biol 2005;208:3609-22.
9. Kusakabe H, Kodama K, Kuninaka AA. New antitumor enzyme, L-lysine alpha-oxidase from *Trichoderma viridea*. Purification and enzymological properties. J Biol Chem 1998;255:976-81.
10. Kitani Y, Tsukamoto C, Zhang G. Identification of an antibacterial protein as L-amino acid oxidase in the skin mucus of rockfish *Sebastes schlegeli*. FEBS J 2007;274:125-36.
11. Massey, Vincent, Curti B. On the reaction mechanism of crotalus adamanteus L-Amino acid oxidase. J Biol Chem 1967;242:1259-64.
12. Raghu Ram Achar, Venkatesh BK, Sharanappa P, Priya BS, S Nanjunda Swamy. Evidence for peroxidase activity in *Caralluma umbellata*. Appl Biochem Biotechnol 2014;173:1955-62.
13. Wiese AG, Pacifici RE, Davies KJA. Transient adaptation to oxidative stress in mammalian cells. Arch Biochem Biophys 1995;318:231-40.

14. Naumann GB, Liliana F Silva, Luciana Silva. Cytotoxicity and inhibition of platelet aggregation caused by an LAAO from *Bothrops leucurus* venom. Biochim Biophys Acta 2011;1810:683-94.
15. Stábeli, Rodrigo G. Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from *Bothrops alternatus* snake venom. Bioorg Med Chem 2004;12:2881-6.
16. Sarkar, Priyabrata, Tothill, Ibtisam E, Setford, Steven J, *et al*. Screen-printed amperometric biosensors for the rapid measurement of L-and D-amino acids. Analyst 1999;124:865-70.
17. Ande, Sudharsana Rao K, Phaneeswara Rao D, Sigrid M, Michael M, Peter G, *et al*. Mechanisms of cell death induction by L-amino acid oxidase, a major component of ophidian venom. Apoptosis 2006;11:1439-51.
18. Shashidharamurthy R, Jagadeesha DK, Girish KS, Kemparaju K. Variation in biochemical and pharmacological properties of Indian cobra *(Naja naja naja)* venom due to geographical distribution. Mol Cell Biochem 2002;229:93-101.
19. Mukherjee AK, Maity CR. The composition of *Naja naja* venom samples from three districts of west bengal, India. Comp Biochem Physiol Part A: Mol Integr Physiol 1998;119:621-7.
20. Shashidharamurthy R, Kemparaju K. Region-specific neutralization of Indian cobra (*Naja naja*) venom by polyclonal antibody raised against the eastern regional venom: a comparative study of the venoms from three different geographical distributions. Int Immunopharmacol 2007;7:61-9.
21. Bradford, Marion M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
22. Berger R. Methods of enzymatic analysis (3rd Edition). Volume II "Samples, Reagents, Assessment of Results". Weinheim/Deerfield Beach, Florida/Basel: Verlag Chemie 1983. 539 S., 37 Abb, 21 Tab., 215 DM (wenn alle Bände), 245 DM (Einzelband). Acta Biotechnol 1984;4:332-2.
23. Davis, Baruch J. Disc electrophoresis-II method and application to human serum proteins. Ann N Y Acad Sci 1964;121:404-27.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
25. Shivalingu BR, Vivek HK, Zohara N, Priya BS, S Nanjunda Swamy. Comparative analysis of procoagulant and fibrinogenolytic activity of crude protease fractions of turmeric species. J Ethnopharmacol 2015;172:261–4.
26. Zohara Nafeesa, Shivalingu BR, Vivek HK, Priya BS, S Nanjunda Swamy. Exploring a new serine protease from *Cucumis sativus* L. Appl Biochem Biotechnol Part A 2015;175:2787-94.
27. Keerthy HK, Garg M, Mohan Chakrabhavi D, Madan, Vikas, Kanojia D, *et al*. Synthesis and characterization of novel 2-amino-chromene-nitriles that target Bcl-2 in acute myeloid leukemia cell lines. PLoS One 2014;9:e107-18.
28. Dineshkumar P, Muthuvelan B. Isolation and purification of l-amino acid oxidase from indian cobra *Naja naja*. Curr Res J Biol Sci 2011;3:6-11.