Isolation of an L-Amino Acid Oxidase from *Agkistrodon contortrix contortrix* (Southern copperhead) Venom

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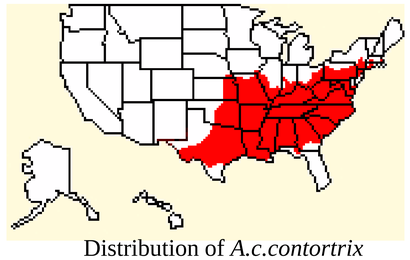
A.c.contortrix

Snake venom is a source of biological study for its ability to induce apoptosis, platelet aggregation, hemorrhage, and edema. L-amino acid oxidases (LAAOs) are components of snake venom that can induce apoptosis, and they are under extensive review for cancer therapy. These LAAOs act on L-amino acid substrates to generate an excess amount of hydrogen peroxide placing the cell under severe oxidative stress, leading to the induction of apoptosis via the intrinsic pathway.

Partial isolation of an LAAO from *Agkistrodon contortrix contortrix* snake venom was accomplished with a combination of size exclusion and ion exchange chromatographies. Preliminary data for the purified LAAO will be presented as well as ideas focusing toward the effects of LAAO coupled with heat shock therapy on cells in vitro.



Distribution of *A.c.contortrix*

INTRODUCTION

Apoptosis has been defined as programmed cell death, and when viable it is an organism's defense to unwanted cell proliferation. A multicellular organism can induce apoptosis and still maintain tissue integrity and function as damaged and unwanted cells are eliminated (Raffray and Cohen, 1997).

There are two known signaling pathways, the extrinsic and intrinsic pathways. Through the binding of cell proteins known as ligands to pro-apoptotic cell surface death receptors, apoptosis can be induced by the extrinsic pathway. Fas ligand is a type I membrane protein that belongs to the tumor necrosis factor family (TNF), and the binding of Fas ligand with its receptor FasR induces apoptosis (Ashkenazi and Dixit, 1998). The intrinsic pathway may be triggered by DNA damage from severe oxidative stress, like that brought on the cell from the toxins of snake venom. This pathway, known as the mitochondria-mediated pathway, involves the release of pro-apoptotic proteins that activate caspase enzymes from the mitochondria which kill the cell (Kroemer and Reed, 2000).

Apoptosis induction is an important mechanism of anticancer agents, and today many apoptosis-inducing factors have been found from animal toxins (Sun et al., 2004). Snake venom contains many toxins which consist of proteins, enzymes, and coagulants. Snake venom L-amino acid oxidase (SV-LAAO) is a flavoenzyme that acts upon an L-amino acid substrate to produce an α-ketoacid, ammonia, and hydrogen peroxide. SV-LAAO is a large protein, usually homodimeric with a molecular mass around 110-150 kDa when measured by gel filtration under non-denaturing conditions, but its mass is detected from 50-70 kDa when assayed by SDS/PAGE both under reducing and non-reducing conditions (Du and Clemetson, 2002). Most of the biological effects of LAAOs are believed to be due to a secondary effect of H_2O_2 which is produced in the enzymatic reaction (Li, Yu, and Lian, 1994).

METHODS

Venom

Crude *A. c. contortrix* venom was supplied by Sigma.

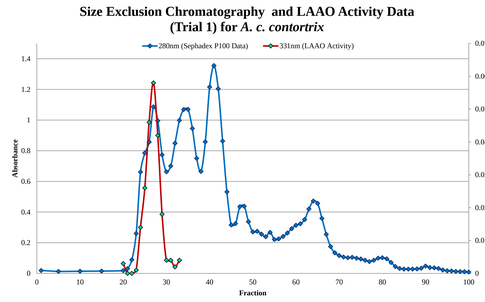
Isolation of Venom Component Exhibiting L-Amino Acid Oxidase Activity

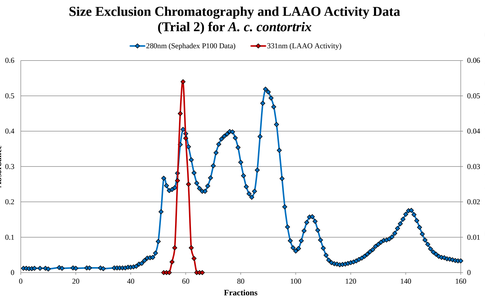
Crude venom (130 mg,) from *A. c. contortrix* was fractionated by size-exclusion chromatography on a Sephadex P100 column (60 cm x 3 cm), equilibrated with 10 mM HEPES and 100 mM NaCl. Elution was carried out using the same buffer at a flow rate of 0.8 mL/min for the first trial. A second trial only 83 mg of crude venom was fractionated at a flow rate of 0.4 mL/min. Isolation was further resolved with HiTrap Q anion exchange. The fractions showing most activity from size exclusion were dialyzed and then the venom components were lyophilized. The isolated protein wasdiluted to 1 mg/mL and fractionated on a HiTrapQ column. Gradient elution was carried out starting with15 mM Tris, pH 8.25 and increasing linearly to Tris buffer with 0.5 M NaCl. Factions were assayed for LAAO activity.

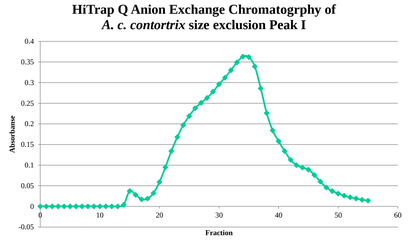
L-Amino Acid Oxidase Assay

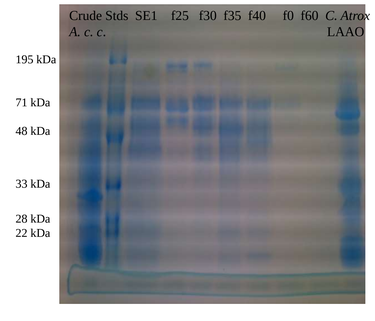
LAAO activity towards the substrate L-kynurenine was determined using the method of Weisbach, Robertson, Witkop, and Wenfriend (1961). In triplicate, reconstituted venom (25.0 uL) were mixed with 625.0 uL of 100 mM HEPES buffer, pH 8.0, containing 100 mM NaCl. Blanks were prepared using 650.0 uL of buffer alone. Both samples and blanks were vortexed for 5 sec and placed in an ice bath at 2˚C for 5 min prior to addition of 75.0 uL of L-kynurenine salt (1.04 mg/mL in HEPES buffer). Tubes were then vortexed prior to the incubation for 30 min at 37˚C. At the end of the 30 min, sample tubes were chilled on ice as 750.0 uL of 10% trichloroacetic acid were added and the sample vortexed. Absorbance was measured at 331 nm.

RESULTS









DISCUSSION

In this study LAAO was partially isolated from *Agkistrodon contortrix contortrix* venom by means of size-exclusion chromatography. The size-exclusion method provided an incomplete resolution of the enzyme; therefore, anion exchange chromatography was used in an attempt to better isolate the LAAO. The results from anion exchange were examined for LAAO activity and the results were inconclusive. We assumed the enzyme was still on the column. Using SDS-PAGE we sought to compare size exclusion fractions, anion exchange fractions, and a known sample of *Crotalus atrox* LAAO with a standard. The results clarified that the size-exclusion and anion exchange chromatographies were insufficient for complete isolation of the LAAO from *A. contortrix contortrix*.

We know that SV-LAAO is an apoptosis-inducing factor (Suhr and Kim, 1996; Zhang and Cui 2007), and now plan to use cation exchange chromatography to better isolate the enzyme in order to observe its apoptotic effects in vitro. Heat shock-induced apoptosis is currently being used in clinical trials alone and in conjunction with radiation and chemotherapeutic agents. In the future investigation we plan to combine heat shock therapy with the enzymatic effects of SV-LAAOs and observe the cells in vitro at temperatures from 38-45ºC. We intend to measure the cytotoxic effect by MTT method, and discriminate apoptosis stages by phosphatidyl serine exposure to characterize the induction of apoptosis across different concentrations of LAAO and temperatures on the A549 human lung carcinoma cell line (Zhang and Cui, 2007).

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