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Research paper

Biochemical, functional and structural characterization of Akbu-LAAO: A novel snake venom L-amino acid oxidase from *Agkistrodon blomhoffii ussurensis*

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ABSTRACT

An L-amino acid oxidase (Akbu-LAAO) was isolated from the venom of *Agkistrodon blomhoffii ussurensis* snake using DEAE Sephadex A-50 ion-exchange, Sephadex G-75 gel filtration, and high performance liquid chromatographies. The homogeneity and molecular mass of Akbu-LAAO were analyzed by SDS-PAGE and MALDI-TOF spectrometry. The sequences of ten peptides from Akbu-LAAO were established by HPLC-nESI-MS/MS analysis. Protein sequence alignment indicated that i) that Akbu-LAAO is a new snake venom LAAO, and ii) Akbu-LAAO shares homology with several LAAOs from the venoms of *Calloselasma rhodost*, *Agkistrodon halys*, *Daboia russellii siamensis*, and *Trimeresurus stejnegeri*. Akbu-LAAO is a homodimer with a molecular mass of ~124.4 kDa. It reacts optimally with its enzymatic substrate, Leu, at pH 4.7 with a K_m of 2.1 mM. ICP-AES measurements showed that Akbu-LAAO contains four Zn^{2+} per dimer that are unessential for the hydrolytic activity of the enzyme. The emission fluorescence intensity of Akbu-LAAO decreases by 61% on removal of Zn^{2+} indicating that the zinc probably helps maintain the structural integrity of the enzyme. The addition of exogenous metal ions, including Mg^{2+}, Mn^{2+}, Ca^{2+}, Ce^{3+}, Nd^{3+}, Co^{2+} and Tb^{3+}, increases the L-Leu hydrolytic activity of the enzyme. Akbu-LAAO shows apparent anti-aggregation effects on human and rabbit platelets. It exhibits a strong bacteriostasis effect on *Staphylococcus aureus*, eighteen fold that of cephalosporin C under the same conditions. Taken together, the biochemical, proteomic, structural and functional characterizations reveal that Akbu-LAAO is a novel LAAO with promise for biotechnological and medical applications.

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1. Introduction

L-Amino acid oxidases (LAAOs, EC 1.4.3.2) are flavoenzymes catalyzing the stereospecific oxidative deamination of a wide range of L-amino acids to form corresponding α-keto acids, H_2O_2 and ammonia via an imino acid intermediate. LAAOs are widely distributed in the venomous snake families Viperidae, Crotalidae and Elapidae. Snake venom LAAOs (SV-LAAOs) are usually homodimeric FAD-(Flavin Adenine Dinucleotide) or FMN-(Flavin Mononucleotide) binding glycoproteins with a molecular mass in the range 110–150 kDa. They are found in high concentrations in snake venoms, are responsible for their yellow color, and contribute to venomic toxicity via the oxidative stress arising from the production of H_2O_2 [1–7]. LAAOs isolated from snake venoms are the best characterized members of this enzyme family and have been shown to vary widely in terms of molecular mass, substrate specificity, interaction with platelets, induction of hemorrhage and apoptosis, and antibacterial and antiparasitic activities [1,7–9].

Until the late 1990s, studies of SV-LAAOs mainly focused on their enzymatic and physicochemical properties [7]. Recently, other activities including induction or inhibition of platelet aggregation, anticoagulant activity, stimulation of oedema formation, hemorrhage, and antibacterial, antimicrobial, antiviral, cytotoxic and antiproliferative effects on tumor cells, leishmanicidal functions, and anti-HIV activity have been reported [1,5,6,8–16].

Abbreviations: A-50, DEAE Sephadex A-50; ACN, acetonitrile; Akbu, *Agkistrodon blomhoffii ussurensis* snake venom; nESI-MS/MS, nano-electrospray ionization-tandem mass spectrometry; FA, formic acid; FAD, flavin adenine dinucleotide; G-75, Sephadex G-75; HPLC, high performance liquid chromatography; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; MALDI-TOF MS, matrix-assisted laser adsorption time of flight mass spectrometry; PLA_2, phospholipase A_2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; UV, ultra-violet visible spectroscopy.

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The *Agkistrodon blomhoffii ussurensis* snake is widespread throughout Northeast China, Japan, Korea, and Russia. Our laboratory has previously characterized the biochemical, structural and functional properties of a homogenous plasminogen activator [17], a serine protease [18], a fibrinolytic enzyme [19] and a phospholipase A_2 [20] from the venom of Akbu. Now, we report the purification and characterization of a new LAAO from Akbu with promise for biotechnological and medical applications.

2. Materials and methods

2.1. Materials

Lyophilized snake venom was a generous gift from Liuhe Pharmaceutical Factory, Jilin, China. DEAE Sephadex A-50 (A50), Sephadex G-75 (G-75), acrylamide, ammonium persulfate, N,N′-methylenebisacrylamide, 1,4-dithiothreitol, iodoacetamide, bromophenol blue, and ammonium bicarbonate were from GE Healthcare (USA). Formic acid (FA), sodium dodecyl sulfate (SDS), and glycine were from Sigma–Aldrich (USA). HPLC-grade water and acetonitrile (ACN) were from Brodick Johnson Inc. Modified trypsin (sequencing grade) was from Promega (Madison, WI, USA). All other chemicals were analytical grade from commercial sources.

2.2. Purification

The first two steps of the purification, A-50 ion-exchange and G-75 gel filtration chromatography, were the same as used for the purification of Akbu-PLA_2 [20]. The protein containing fractions were loaded onto a G-75 column and the protein was eluted with 0.02 M Tris–HCl (pH 7.5) containing 0.15 M NaCl. The final step of the purification utilized HPLC and a C4 reverse phase column. Mobile phase A consisted of HPLC-grade water with 0.1% FA while mobile phase B was 90% ACN with 0.1% FA. The polypeptide was eluted at a flow rate of 500 μL/min with a linear gradient of 0–90% B over 70 min. The UV absorbance of the eluate was monitored at 280 nm. All isolation processes were carried out at 4 °C.

2.3. Purity and molecular mass analysis

SDS-PAGE was performed using a 10% separation gel to check the purity and molecular mass of Akbu-LAAO according to the method of Laemmli [21,22]. A more accurate molecular mass of Akbu-LAAO was obtained using a MALDI orthogonal time of flight mass spectrometer (MALDI-TOF). Experimental methods were as previously reported [20,23]. The concentration of Akbu-LAAO was 3 μM, sinapinic acid (10 mg/mL in 70% ACN containing 0.1% TFA) served as the matrix, and the mass spectrum was obtained from an accumulation of 200 single shots. The error in the molecular mass is less than 500 ppm.

2.4. In-gel tryptic digestion

Akbu-LAAO was digested in-gel with trypsin in order to increase the signal to noise ratio for mass spectrometry. The gel slice containing Akbu-LAAO was carefully excised, twice destained for 10 min with 50% ACN in 25 mM ammonium bicarbonate, reduced with 10 mM DTT at 56 °C for 45 min, and alkylated with 55 mM iodoacetamide in the dark at room temperature for 45 min. The gel slices were thoroughly washed with 100% ACN in 25 mM ammonium bicarbonate, rehydrated in 10 μL modified trypsin solution for 10 min at 4 °C, overlaid with 25 mM ammonium bicarbonate, and digested overnight at 37 °C. Tryptic peptides were then extracted from the gel with 50% ACN containing 1% FA [17–20,24].

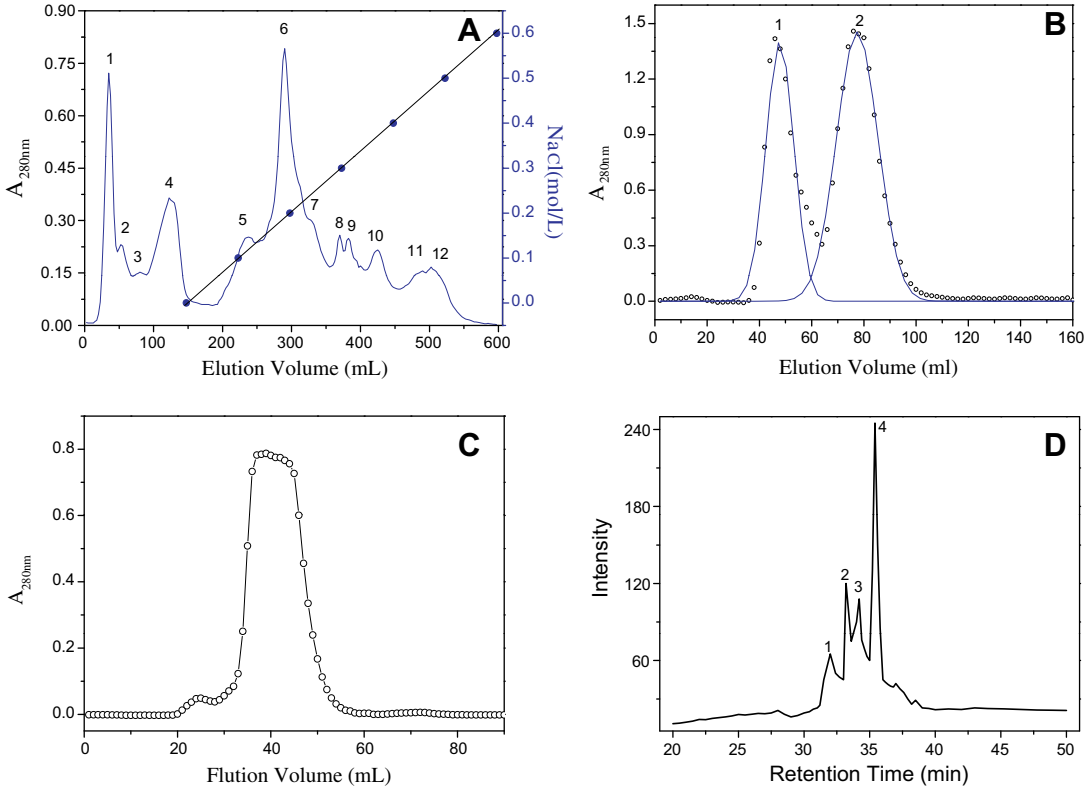

Fig. 1. (A) A50 chromatography of Akbu-LAAO. Active fractions were in peak 6. (B) First G-75 (1.6 × 96 cm) gel filtration chromatography of Akbu-LAAO. Protein was eluted with 0.02 M Tris–HCl (pH 7.50) containing 0.15 M NaCl at a flow rate of 0.3 mL/min. (C) Second G-75 (1.6 × 96 cm) chromatography of Akbu-LAAO. Protein was eluted with 0.02 M Tris–HCl (pH 7.50) containing 0.15 M NaCl at a flow rate of 0.3 mL/min. (D) HPLC chromatography of Akbu-LAAO. The active fractions were in peak 4.

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