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ARTICLE

Heterodimeric L-amino acid oxidase enzymes from Egyptian *Cerastes cerastes* venom: Purification, biochemical characterization and partial amino acid sequencing

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Abstract Two L-amino acid oxidase enzyme isoforms, Cc-LAAOI and Cc-LAAOII were purified to apparent homogeneity from *Cerastes cerastes* venom in a sequential two-step chromatographic protocol including; gel filtration and anion exchange chromatography. The native molecular weights of the isoforms were 115 kDa as determined by gel filtration on calibrated Sephacryl S-200 column, while the monomeric molecular weights of the enzymes were, 60, 56 kDa and 60, 53 kDa for LAAOI and LAAOII, respectively. The tryptic peptides of the two isoforms share high sequence homology with other snake venom L-amino acid oxidases. The optimal pH and temperature values of Cc-LAAOI and Cc-LAAOII were 7.8, 50 °C and 7, 60 °C, respectively. The two isoenzymes were thermally stable up to 70 °C. The K_m and V_{max} values were 0.67 mM, 0.135 μmol/min for LAAOI and 0.82 mM, 0.087 μmol/min for LAAOII. Both isoenzymes displayed high catalytic preference to long-chain, hydrophobic and aromatic amino acids. The Mn^{2+} ion markedly increased the LAAO activity for both purified isoforms, while Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} and Ba^{2+} ions showed a non-significant increase in the enzymatic activity of both isoforms. Furthermore, Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and AL^{3+} ions markedly inhibited the LAAOI and LAAOII activities. L-Cysteine and reduced glutathione completely inhibited the LAAO activity of both isoenzymes, whereas, β-mercaptoethanol, O-phenanthroline and PMSF completely inhibited the enzymatic activity of LAAOII. Furthermore, iodoacitic acid inhibited the enzymatic activity of LAAOII by 46% and had no effect on the LAAOI activity.

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1. Introduction

L-Amino acid oxidases (LAAOs) are widely distributed in snake venoms, which contribute to the toxicity of venoms. Snake venoms are recognized as useful sources of bioactive substances showing a wide range of pharmacological activities. Snake venoms cause a variety of different biological effects as they are a mixture of simple and complex substances, such as biologically active peptides and proteins but their biochemical characteristic change according to the snake species studied. This complex cocktail of both toxic and non-toxic components includes several peptides and enzymes, such as L-amino acid oxidases (LAAO, EC 1.4.3.2) which may represent 1–9% of total venom proteins [1]. As suggested by the name of the family, snake venom LAAOs (sv-LAAOs) are flavoenzymes which function to catalyze the stereospecific oxidative deamination of an L-amino acid to give rise to an alpha-keto acid, ammonia and hydrogen peroxide. Studies have indicated that most sv-LAAOs prefer hydrophobic L-amino acids as substrates [2]. Most sv-LAAOs are reported as being homodimers with subunit molecular weights of around 50–70 kDa; under non reducing conditions. Natives v-LAAO appears to be around 120 kDa [1,3]. The interaction of the subunits is via non covalent interactions. Further, sv-LAAO can be found as acidic, neutral and basic forms of the protein [1]. X-ray crystallographic studies confirm that sv-LAAO is a functional dimer with each dimer having three domains: FAD-binding; substrate-binding; and a helical domain [4]. A variety of related biological activities have been reported for isolated sv-LAAOs including cytotoxic, apoptotic, platelet aggregation effects, edema and bactericidal and anti-parasitic activities [5,6]. All of these activities are considered to be due to the L-amino oxidase activity of the proteins resulting in the production of hydrogen peroxide with oxygen free radical resulting in oxidative stress. The role of sv-LAAOs in venom-induced pathology is less clear [1]. Certainly given the activities noted above one could suggest some role in the complex nexus of venom toxin activities although that has not adequately been demonstrated. One could also suggest sv-LAAO in venom stored in venom glands could play a role in the maintenance and stabilization of the venom and venom gland by virtue of its anti-bacterial properties however this is speculation. *Cerastes cerastes* (*C. cerastes*) is a venomous viper species found in arid North Africa and Arabian Peninsula. The venom of *C. cerastes* contains a variety of proteins and enzymes that disturb hemostasis [7]. In the present study we reported the purification, characterization and partial sequencing of LAAO enzyme(s) from *C. cerastes* venom, as a step toward possible application in biotechnological and medical purposes.

2. Materials and methods

2.1. Materials

C. cerastes venom was milked from several adult snakes collected from their Egyptian natural habitat (Giza). The venom was lyophilized and stored at −20 °C. Samples were thawed and centrifuged before use. L-Leucine, O-phenylenediamine (OPD), horseradish peroxidase (HRP) and Tris Base were obtained from Sigma chemicals Co., hydrogen peroxide from Fluka, and Sephacryl S-2oo was from Pharmacia. BLUelf Prestained Protein Ladder was produced by Gene Direx. DEAE-Sepharose resin and gel filtration molecular weight markers kit (for molecular weights 12,000–200,000 Da) were from Sigma. All other chemicals and reagents were of analytical grade. The buffers were prepared according to Gomorie [8] and Blanchard [9] and the final pH was checked by pH meter (Hanna, pH 211 Microprocessor pH meter).

2.2. Purification of LAAO enzyme from C. cerastes venom

Pooled *C. cerastes* venom (100 mg) dissolved in 1 ml of 50 mM Tris–HCl, pH 8 buffer was loaded on Sephacryl S-200 column (1.6 × 90 cm). The buffer was used for equilibration of the column and elution of the loaded samples. Fractions of 4 ml were collected at a flow rate of 24 ml/h using a fraction collector (Pharmacia LKB, Sweden). The active fractions showing LAAO activity were pooled and directly applied on DEAE-Sepharose column (1.6 × 15 cm) equilibrated with the same buffer. The unbound proteins were washed with the equilibration buffer, while the bound proteins were eluted using 0–0.3 M NaCl at a flow rate of 36 ml/h. 3 ml fractions were collected and the LAAO activity was determined according to the Kishimoto and Takahashi method [10]. The fractions showing LAAO activity were pooled and stored at 4 °C and designed as Cc-LAAOI and Cc-LAAOII. Absorbance of elutes was monitored at 280 nm. All chromatographic processes were carried out at room temperature and the enzyme fractions were stored at 4 °C.

2.3. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis under non-denaturing conditions was performed in 10% (w/v) acrylamide slab gel according to the method of Davis [11] using a Tris–glycine, pH 8.3 buffer. Protein bands were located by staining with Coomassie brilliant blue.

2.4. Molecular weight determination

Molecular weight (MW) was determined by: (1) Gel filtration technique using Sephacryl S-200 according to Oberg and Philipson [12]. The column (1.6 × 90 cm) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and b-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume. (2) 12% SDS–PAGE; Electrophoretic analysis was performed in the Mini-Protean II Dual-Slab Cell (Bio Rad, USA). Preparation of gels, samples, and electrophoresis was performed according to the conditions described by Laemmli [13]. BLUelf Prestained Protein Ladder was used as a standard protein marker with 13 major bands resolved in polyacrylamide gel.

2.5. L-Amino acid oxidase (LAAO) activity

The assay was conducted in a 96-well microplate in duplicate as described by Kishimoto and Takahashi [10]. Briefly, 10 µl of the crude venom/enzyme solution was incubated with 90 µl of the reaction mixture containing 5 mM L-leucine as substrate, 0.81 U/ml horseradish peroxidase (HRP) and

2 mM o-phenylenediamine (OPD; as a substrate for peroxidase) in 50 mM Tris–HCl, pH 8.0 buffer at 37 °C. After 1 h, the reaction was terminated by adding 20 µl of 2 M H_2SO_4. The amount of the product (H_2O_2) was estimated by measuring the absorbency at 490 nm and the specific enzymatic LAAO activity of the venom was determined using a calibration curve of known concentrations of freshly prepared hydrogen peroxide as a standard. One unit of LAAO activity was defined as the amount of enzyme required to produce 1 µmol of H_2O_2 per minute under the described conditions.

2.6. Tryptic digestion and peptide analysis using nano LC-MS/MS

Analysis of Gel Bands was carried out at the Proteomic Facility, SBBS, Durham University as follow: Spots of diameter 1.5 mm were excised from selected gel bands and transferred to a microtitre plate for trypsin digestion using a Pro Gest Investigator™ automated workstation (Genomic Solutions). Samples were digested with the standard Pro Gest long trypsin protocol supplied with the instrument, using iodoacetamide for cysteine derivatization. Final eluates were dried in a vacuum centrifuge and residues resuspended in 0.2% formic acid before dilution to 2% ACN, 0.1% formic acid. Peptides were then analyzed by nano-LC MS/MS using an Ultimate 3000 LC system (Dionex) linked to a Q-TOF mass spectrometer (QStar Pulsar i, AB SCIEX) via a nanospray source (Protana) with a PicoTip silica emitter (New Objective). Samples were loaded via a Zorbax300SB-C18 5 × 0.3 mm column (Agilent) and peptides resolved using an Acclaim® PepMap 100 C18 column 75 µm × 15 cm (Thermo Scientific). A linear gradient of 4–32% ACN in 0.1% formic acid over 47 min at a flow rate of 300 nL/min was run and the remaining peptides were then eluted with 72% ACN, 0.1% formic acid. MS and MS/MS data were acquired using Analyst QS software version 1.1, switching between 1 s survey and 3 × 3 s product ion scans during peptide elution. Ions with charge state 2+ to 4+ and TIC > 10 counts were selected for fragmentation.

2.7. Protein identification and data analysis

MS/MS data-files were processed with Protein Pilot software version 2.0.1 (AB SCIEX), using the incorporated Paragon search algorithm for protein identification from SwissProt (Uniprot September 2013). Search parameters were: sample type, ID; cys alkylation, iodoacetamide; digestion, trypsin; instrument QSTAR ESI; and a protein detection threshold score of 1.3 (95% confidence) as the minimum for protein identification to be included in the output. Data were additionally processed at the remote Mascot Server (http://www.matrixscience.com/) using MS/MS ion searches against SwissProt (current release) with Peptide tolerance 50 ppm, MS/MS tolerance 0.6 Da and oxidized methionine and carbamidomethyl (C) set as variable modifications. The sequence of CC-LAAO isoforms were submitted to the BLAST server to search for homology in the database of non redundant sequences with default parameters.

2.8. Effect of pH and temperature on Cc-LAAO activity and stability

The enzyme-coupled microplate standard assay was used to determine the optimum pH and temperature, Cc-LAAOI and Cc-LAAOII activities were measured individually at different pHs, using 50 mM citrate acetate buffer, sodium phosphate buffer, Tris–HCl buffer over the pH ranges (3–4), (4–6) and (6–9), respectively, and temperatures (4–70 °C) using L-leucine as substrate and a final volume of 10 µl of the enzymes. The pH stability was determined by pre-incubating separately 10 µl of the enzyme (s) with different pHs at 37 °C for 1 h. The enzymatic activity was measured under standard assay conditions. Thermal stability was determined by pre-incubating the enzymes individually for 1 h at different temperatures (4–70 °C), followed by cooling in ice bath prior to substrate addition. The LAAO activity was measured under standard assay conditions as mentioned above according to Kishimoto and Takahashi [10].

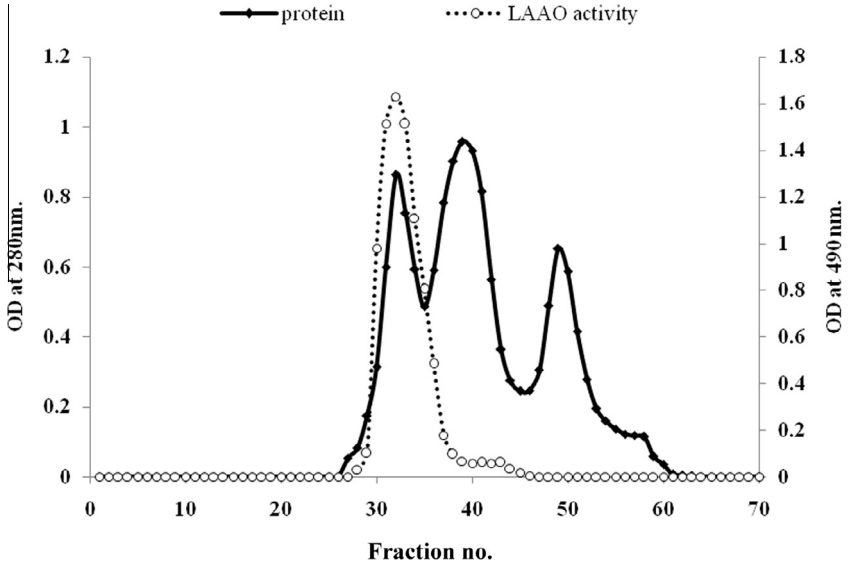

Figure 1A Gel filtration of 100 mg of *C. cerastes* on Sephacryl S-200 column (1.6 × 90 cm) equilibrated with 0.05 M Tris–HCl buffer, pH 8. Elution was performed with the same buffer at a flow rate of 24 ml/h and 4 ml fractions were collected.

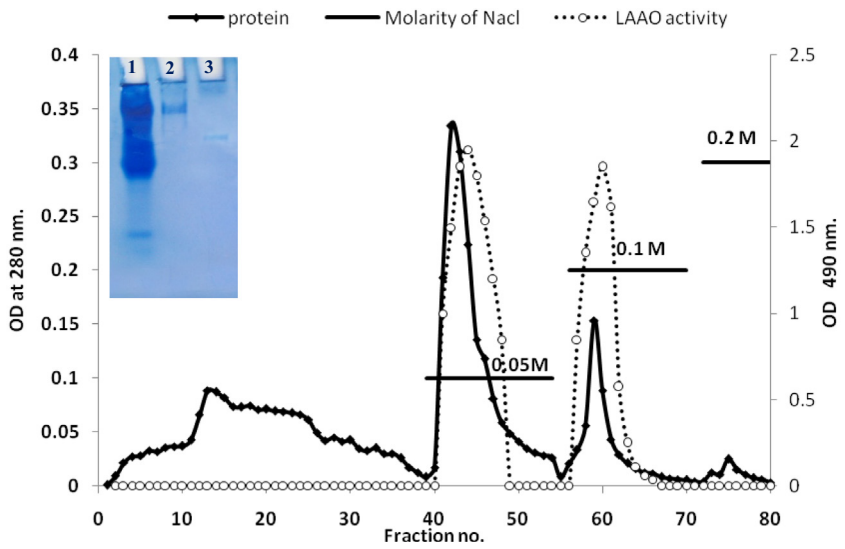

Figure 1B Ion exchange chromatography of 15 mg of the active fractions, from Sephacryl S-100 column, on DEAE-Sepharose column (1 × 12 cm) equilibrated with 0.05 M Tris–HCl buffer, pH 8. Adsorbed proteins were eluted with 0–0.3 M NaCl in the equilibration buffer at a flow rate of 36 ml/h, 3 ml fractions were collected. Insert: 10% Native-PAGE of *C. cerastes* venom (1), purified Cc-LAAOI (2) and Cc-LAAOII (3).

2.9. Substrate specificity

LAAO activity was tested against 17 different L-amino acids as substrates. To determine the substrate specificity for Cc-LAAOI and Cc-LAAOII, L-Leu was replaced with other L-amino acids (L-Gly, L-Ala, L-Val, L-Ile, L-Met, L-Pro, L-Phe, L-Tyr, L-Trp, L-Ser, L-Thr, L-Cys, L-Asn, L-Gln, L-Asp, and L-Arg) under standard assay conditions. The final volumes of both isoenzymes in the reaction mixture were 10 µl containing 0.5 µg protein.

2.10. Effect of metal ions and inhibitors

Cc-LAAOI and Cc-LAAOII were pre-incubated for 1 h at 37 °C with 10 mM of different metal ions or 5 mM of the different inhibitors individually at the final volume of 10 µl as indicated prior to substrate addition, the LAAO activity was measured under standard assay conditions as mentioned above and the remaining activity was determined.

2.11. Effect of different concentrations of L-leucine

The assay was performed at different concentrations of L-leucine (0–7.5 mM) and LAAO enzymes at a final volume of 10 µl containing 0.05 µg protein. LAAO activity was measured under standard assay conditions as mentioned above.

2.12. Protein determination

Protein content in venom samples was determined according to the Bradford method using bovine serum albumin (BSA) as a standard [14].

3. Results

3.1. Purification of LAAO enzyme from *C. cerastes* venom

Two LAAO enzyme isoforms were isolated from *C. cerastes* venom using a combination of gel filtration and ion exchange chromatography. In the first step, the *C. cerastes* venom (100 mg protein) was dissolved in 50 mM Tris–HCl, pH 8 buffer and applied on an equilibrated Sephacryl S-200 gel filtration column. The venom was resolved into three main protein peaks, LAAO activity was detected at the first peak (fractions 28–34) (Fig. 1A and Table 1). The active fractions, designated as Seph-LAAO, were pooled and applied directly on DEAE-Sepharose ion exchange chromatography, from which two LAAO enzyme isoforms were eluted with 0.05 and 0.1 M NaCl, in 50 mM Tris–HCl, pH 8 buffers

Table 1 Purification scheme of L-amino acid oxidase isoforms isolated from *C. cerastes* venom.

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	% Recovery		Purification fold
				Protein	Activity	
C. cerastes venom	100	250,000	2500	100	100	1
Cc Seph-LAAO	15	100,000	6666.7	15	40	2.7
Cc-LAAOI	5.5	55,000	10,000	5.5	22	4
Cc-LAAOII	2.5	41667.5	16,667	2.5	16.7	6.7

(Fig. 1B). These isoforms were designated as Cc-LAAOI and Cc-LAAOII. As compared with the specific activity in the crude venom, the purified Cc-LAAOI and Cc-LAAOII isoforms showed 5.5, 2.5% protein and 22, 16.7% LAAO enzymatic activity, respectively.

3.2. Biochemical characterization of Cc-LAAOI and Cc-LAAOII

3.2.1. Purity and molecular mass

The two isoforms showed one band in native PAGE under non-reducing conditions (Fig. 1B). In reducing SDS–PAGE the purified LAAO isoforms showed two bands corresponding to apparent molecular masses of 60, 56 kDa for Cc-LAAOI and 60, 53 kDa for Cc-LAAOII, respectively (Fig. 2A). In addition, on gel filtration, the native purified LAAO enzyme isoforms eluted corresponding to a molecular mass of about 115 kDa (Fig. 2B).

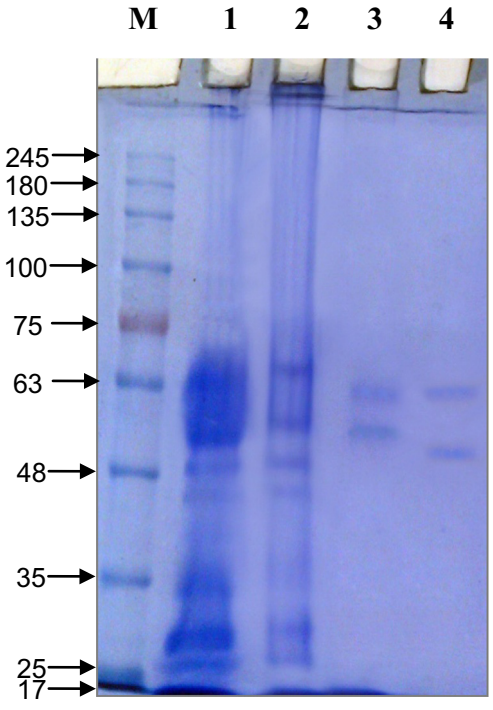

Figure 2A 12% SDS–PAGE of about 20 μg of *C. cerastes* crude venom (1), Cc.Seph-LAAO (2), Cc-LAAOI (3), Cc-LAAOII (4) and MW markers (M) under reducing conditions.

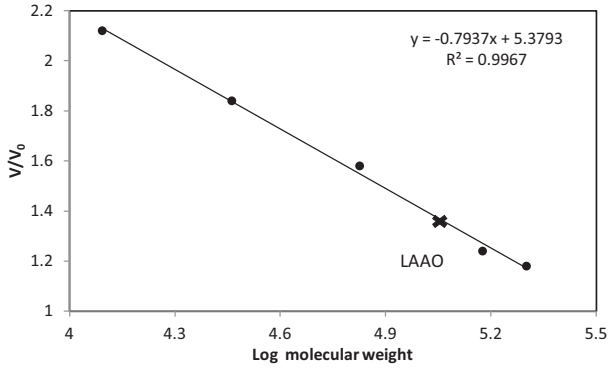

Figure 2B Molecular mass determination of native Cc-LAAO by gel filtration chromatography on Sephacryl S-200. Standard proteins from 1 to 5 are cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and b-amylase (200,000), respectively. Dextran blue (2,000,000) was used to determine the void volume.

3.2.2. Sequence analysis

The *C. cerastes* LAAO enzyme isoforms were separated by 12% SDS–PAGE, the subunits of the isoforms were separated, designated as 1, 2 for LAAOI and 3, 4 for LAAOII, then sequenced as mentioned above. The sequences of twelve tryptic peptides from these subunits detected by LC-MS/MS are given (Table 2). Further, the comparison of the determined internal sequences from the tryptic peptides with the BLASTP database confirmed the strong similarity of this enzyme to the other snake venom LAAOs (Fig. 3).

3.2.3. Effect of pH

In order to check the optimal pH of the enzymatic activity, Cc-LAAOI and Cc-LAAOII activities were measured individually at a broad pH range of the incubation buffer. The results showed that the optimum pHs were 7.8 and 7 for Cc-LAAOI and Cc-LAAOII, respectively (Fig. 4A). In addition, the pH stabilities of the Cc-LAAOI and Cc-LAAOII were examined. Both enzymes maintained their full activity at a wide range of pH from 4 to 9 (Fig. 4B).

3.2.4. Effect of temperature

Optimum temperatures were 50 °C and 60 °C for Cc-LAAOI and Cc-LAAOII, respectively (Fig. 5A). To study the thermal stabilities of the two isoforms, they were incubated in the buffers individually at different temperatures for 1 h prior to substrate addition and the enzymatic activities were measured under standard conditions. The two isoforms were thermally stable, since no loss of enzymatic activities were recorded up to 40 °C for Cc-LAAOI and 50 °C for Cc-LAAOII, whereas 50% loss of the enzymatic activities were recorded at 60 °C for Cc-LAAOI and 65 °C for Cc-LAAOII, respectively. On the other hand, a complete loss of activities was detected above 70 °C for both isoforms (Fig. 5B).

Table 2 Amino acid sequence of the tryptic peptide fragments of *C. cerastes* LAAO isoforms.

Peptide number	Amino acid sequence	Presence in LAAO subunit
01	ADDKNPLEECFR	2
02	EADYEEFLEIAK	1,3,4
03	VTVLEASER	4
04	NDKEDWYANLGPMRLPEK	1,2,4
05	LNEFVQETENGWYFIK	2,4
06	YPVKPSEEGK	1,2,3,4
07	SAGQLYEESLRK	1
08	SAGQLYQESLGKA	2,4
09	HDDIFAYEKRFDEIVDGMDK	1,2,4
10	LPTSMYQAIQE	2,4
11	RINFKPPLPPKK	4
12	YAMGAITTFTPYQFQHFSEALTAPVGR	2

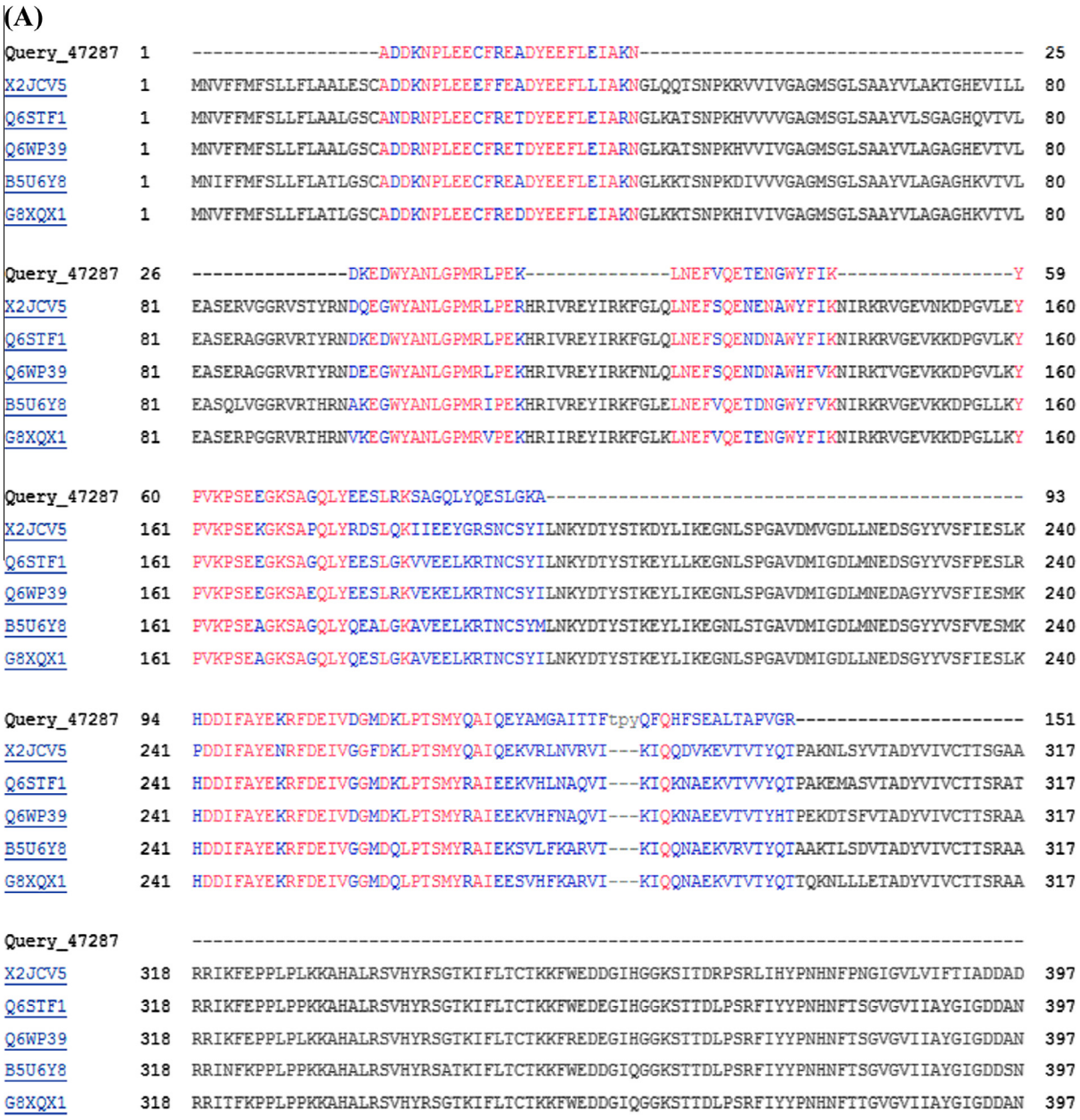

Figure 3 Alignment of partial sequences determined from tryptic fragments of CC-LAAOI, Query_47287 (A) and CC-LAAOII, Query_53437 (B) with LAAO of five different snake venoms (Accession numbers: X2JCV5, *C. cerastes*, Q6STF1, *G. halya*, Q6WP39, *T. stejnegeri*, B5U6Y8, *E. ocellatus*, and G8XQX1, *D. russelii*).

3.2.5. Substrate specificity

The purified Cc-LAAO isoforms present broad substrate specificity when tested using a wide range of amino acids as substrates. It revealed high specificity for L-Arg, L-Met, L-Phe, L-Leu, L-Tyr, L-Ile and L-Trp (Fig. 6). On the other hand, they showed low affinity for L-Val, L-Ala, L-Asn, L-Gln and no specificity toward L-Pro, L-Ser, L-Thr, L-Cys, L-Gly and L-Asp (Fig. 6).

3.2.6. Effect of metals

The Mn^{2+} ions markedly increased the enzymatic LAAO activities for the purified enzyme isoforms, while Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} and Ba^{2+} ions showed a non-significant increase in the enzymatic activity of both isoforms. Furthermore, Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and AL^{3+} ions completely inhibited LAAOI and LAAOII activities (Fig. 7).

3.2.7. Effect of inhibitors

The purified Cc-LAAO isoforms were pre-incubated with 5 mM of different inhibitors for 1 h at 37 °C prior to substrate addition and the enzymatic activities were determined under standard assay conditions. L-Cysteine and reduced glutathione completely inhibited the LAAO activities of the purified enzyme isoforms, in addition, O-phenanthroline, β-mercaptoethanol and PMSF completely inhibited the enzymatic activity of LAAOII (Fig. 8). Furthermore, iodoacitic acid inhibited the enzymatic activity of LAAOII by 46% and had no effect on the LAAOI activity (Fig. 8).

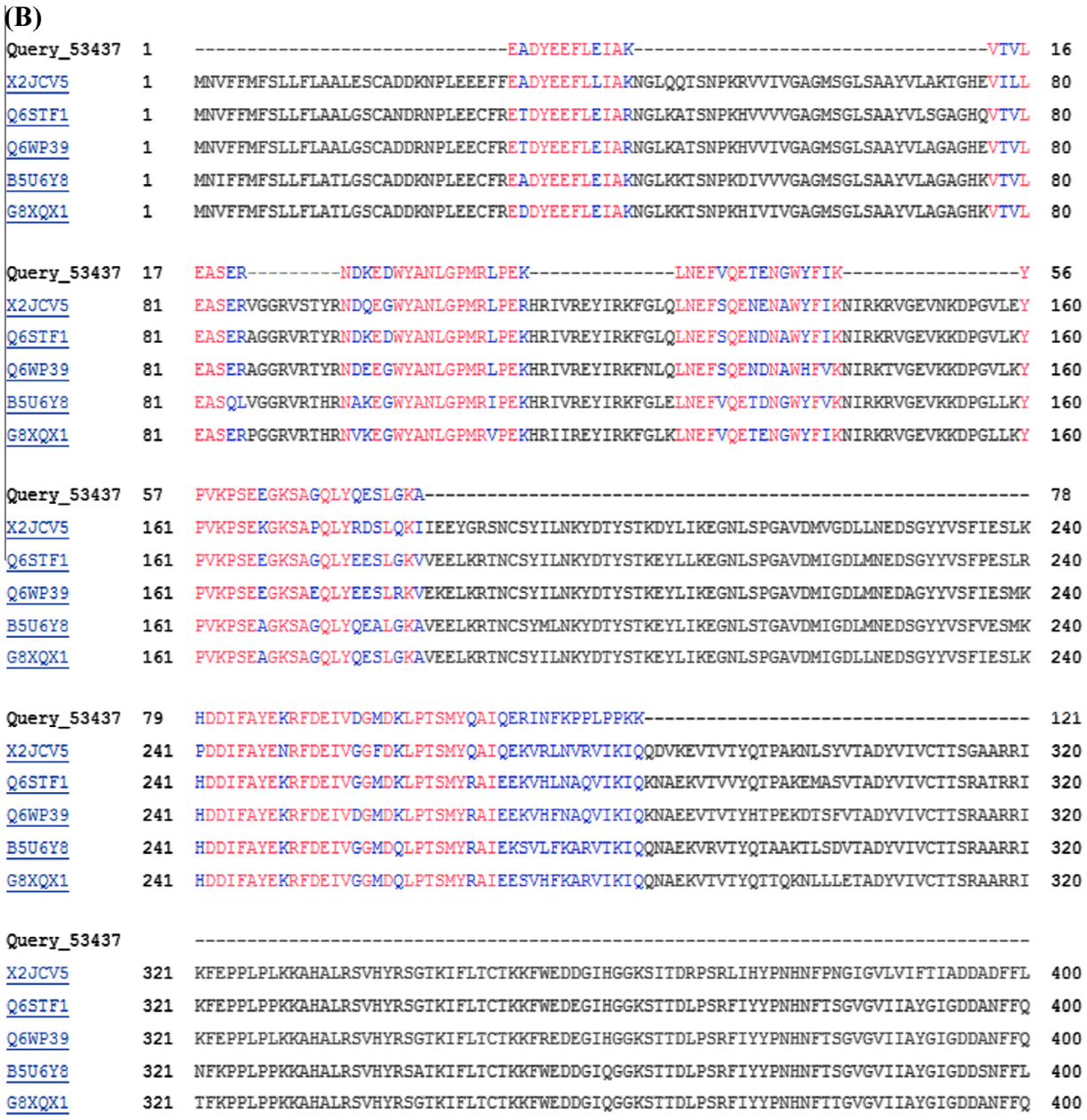

Fig. 3 (*continued*)

3.2.8. Enzyme kinetics

The determination of K_m and V_{max} for the substrate L-leucine was performed by the Lineweaver–Burk plot. The K_m and V_{max} values were found to be 0.67 mM, 0.135 µmol/min for LAAOI (Fig. 9A) and 0.82 mM, 0.087 µmol/min for LAAOII (Fig. 9B), respectively.

4. Discussion

LAAOs have objects of great interest for pharmacological, molecular biology, and structural studies. In the present work, we isolated and characterized two heterodimeric LAAO isoforms from the Egyptian horned viper *C. cerastes,* the richest source of LAAO enzyme among all vipers inhabiting the Egyptian environment (data not shown). SV-LAAOs are usually homodimeric FAD- or FMN binding glycoproteins with molecular masses ranged 110–150 kDa and pIs ranged 4.4–8.5 [1,15]. While *Bungarus fasciatus* LAAO (BF-LAAO) was reported as a monomer [16]. In this work, two LAAO enzyme isoforms with different elution properties from anion exchange chromatography, were isolated from *C. cerastes* venom in a two-step procedure (Fig. 1). LAAO isoforms are found existing in the snake venoms of *Bothrops alternatus* (Balt-LAAO) [17], *Pseudechis australis*, *Vipera berus berus* [15], *Bothrops jararaca* [18] and *Agkistrodon blomhoffii ussurensis* (Akbu-LAAO) [19], this phenomenon might be caused by protein glycosylation or protein synthesis from different genes. Interestingly, acidic, neutral and basic forms of SV-LAAOs coexist in the same type of snake venom. SV-LAAOs with different *pIs* might have different pharmacological properties [20]. The purified LAAO enzyme isoforms isolated in this study seems

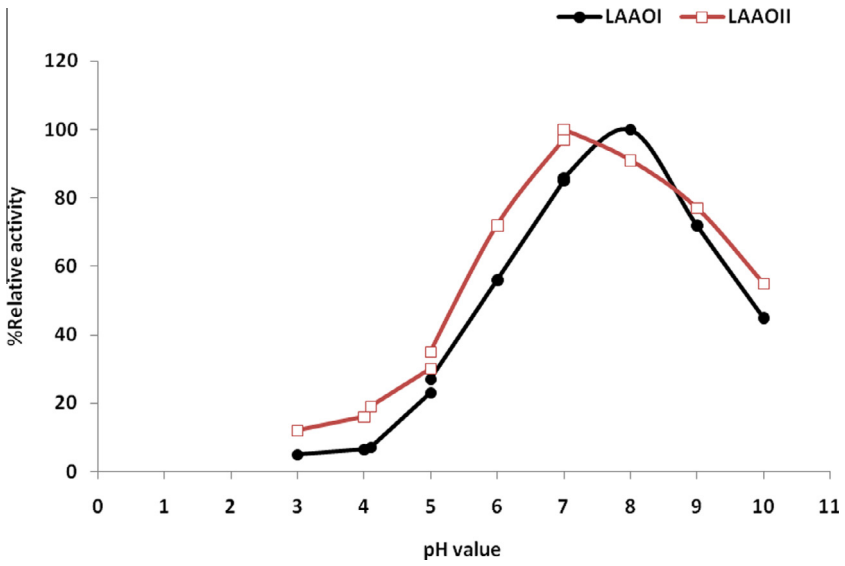

Figure 4A Optimal pH values of the purified Cc-LAAOI and Cc-LAAOII using the standard assay, each point represent the average of two experiments.

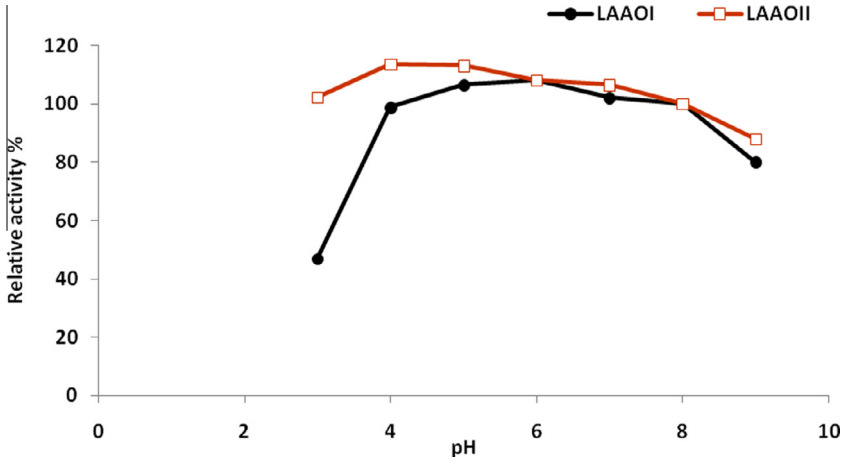

Figure 4B pH stability of the purified Cc-LAAOI and Cc-LAAOII using the standard assay, each point represent the average of two experiments.

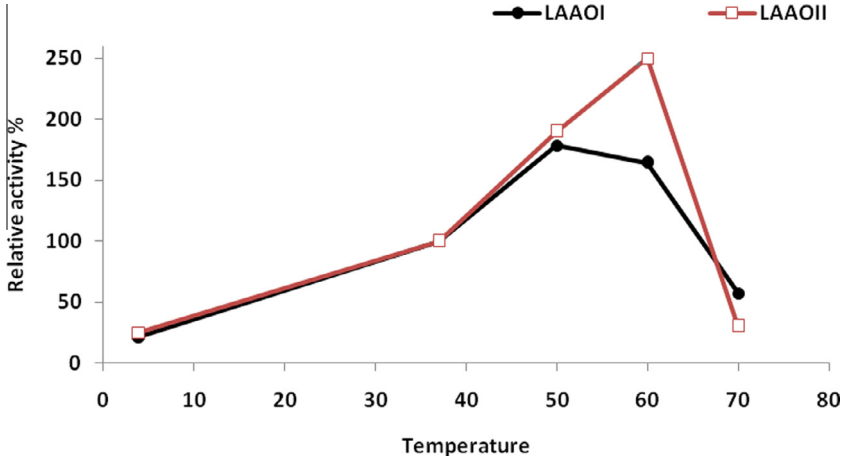

Figure 5A Optimal temperature values of the purified Cc-LAAOI and Cc-LAAOII using the standard assay, each point represent the average of two experiments.

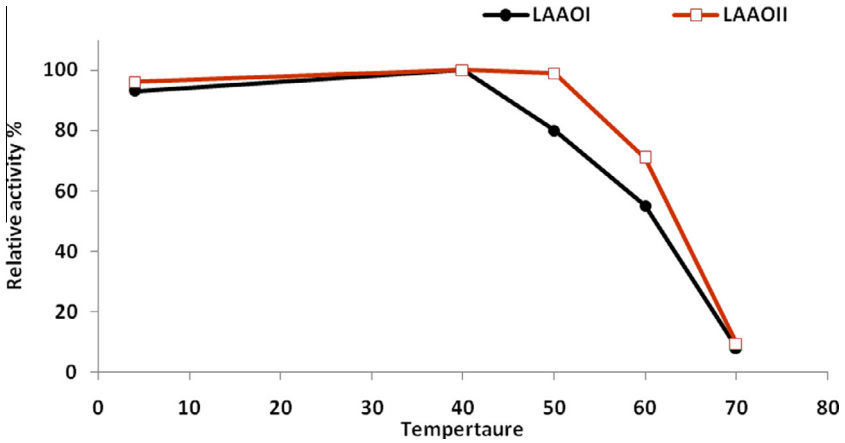

Figure 5B Thermal stability of the purified Cc-LAAOI and Cc-LAAOII using the standard assay, each point represent the average of two experiments.

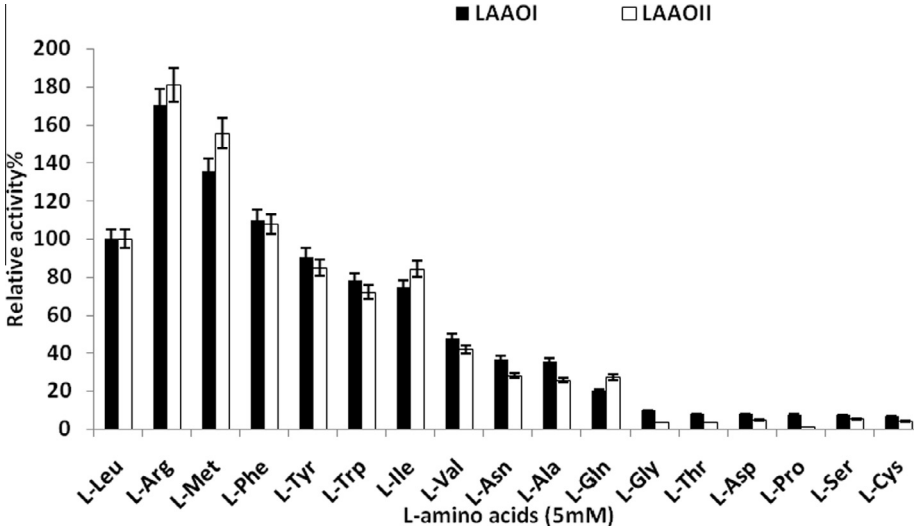

Figure 6 Substrate specificity of the purified Cc-LAAOI and Cc-LAAOII. The reaction mixture consisted of 1 μg LAAO, 2 mM o-phenylenediamine (OPD), 0.81 U/mL horseradish peroxidase and 5 mM L-amino acid as the substrate in 50 mM Tris–HCl buffer, pH 8. Data represent mean ± SD of three independent experiments.

to be noncovalently linked heterodimeric glycoproteins unlike other snake venom LAAOs which are in general, homodimeric glycoproteins [16,17,21–23], they have molecular masses of 60, 56 kDa for LAAOI and 60, 53 kDa for LAAOII as measured by reducing electrophoresis. This result is also unlike that of Abdelkafi-Koubaa et al. [24], who isolated a homodimeric single form of glycosylated flavoprotein LAAO from *C. cerastes* venom collected from Tunis, with a molecular mass around 58 kDa under reducing conditions and about 115 kDa in its native form. The heterogenicity obtained in our results may reflect the polymorphism of the *C. cerastes* LAAO enzymes among the donor snakes. The phenomena of polymorphism was previously reported in *C. cerastes* by Fahmi et al. [7], who stated that "Compared to the previously reported venom composition of the same species from Tunisia, the venom of *C. cerastes* from Morocco exhibits remarkable compositional variation. It contains lower amounts of C-type lectin-like molecules and serine proteinases, and is devoid of L-amino acid oxidase. On the other hand, the venom from Morocco *C. cerastes* expresses higher amounts of dimeric disintegrins, and contains a PI-SVMP and a CRISP molecule not found in the venom from Tunisia [25]. Venoms represent trophic adaptations, and different venom formulations have evolved in different taxa for the same purpose: rapid immobilization of prey [26]. Intraspecific venom variation represents a well documented phenomenon, which is particularly evident among species that have a wide distribution range [27], supporting the concept that these species should be considered as a group of metapopulations. From a medical standpoint, intraspecific geographic variability may have an impact in the clinical picture of envenomation treatment [28,29].

Peptide sequence characterization of *C. cerastes* LAAO enzyme isoforms were carried out by Nano LC-MS/MS as mentioned above and twelve peptides were identified (Table 2). Sequence comparisons of *C. cerastes* LAAO enzyme isoforms with the other sv-LAAOs indicated that the peptide fragments of these isoforms were found to be closest to the corresponding fragments of *C. cerastes*, X2JCV5, *G. halya*, Q6STF1, *T. stej-*

negeri, Q6WP39, *E. ocellatus*, B5U6Y8, and *D. russelii*, G8XQX1. This finding further confirmed the strong similarity of this enzyme to the other snake venom LAAOs.

The results in the present study revealed that both Cc-LAAO enzyme isoforms maintained their full activity at a wide range of pH from 4 to 9 and the optimum pHs were 7.8 and 7 for Cc-LAAOI and Cc-LAAOII, respectively. These results are similar to that of Abdelkafi-Koubaa et al. [24]. Similar to all the reported venom LAAOs, the temperatures higher than 50 °C result in a gradual decrease in activity caused by disruptions in hydrophobic interactions and hydrogen bonds between the different subunits of the enzyme [24,30].

Similar to previously characterized sv-LAAOs, Cc-LAAO isoforms oxidized the hydrophobic and aromatic amino acids (L-Met, L-Phe, L-Leu, L-Tyr, L-Trp and L-Ile) and did not oxidize the other amino acids such as L-Pro, L-Ser, L-Thr, L-Cys, L-Gly and L-Asp, showing that the catalytic site has a conserved structure among snake species [18,24,31–33]. In addition to the above mentioned hydrophobic and aromatic amino acids, Cc-LAAO isoforms oxidized L-Arg, a basic positively charged amino acid, which is similar to *O. hannah* LAAO enzymes [34] and *Vipera berus berus* LAAO enzymes [15]. These catalytic differences may be due to variations in the side chain binding sites for substrate specificity of LAAOs

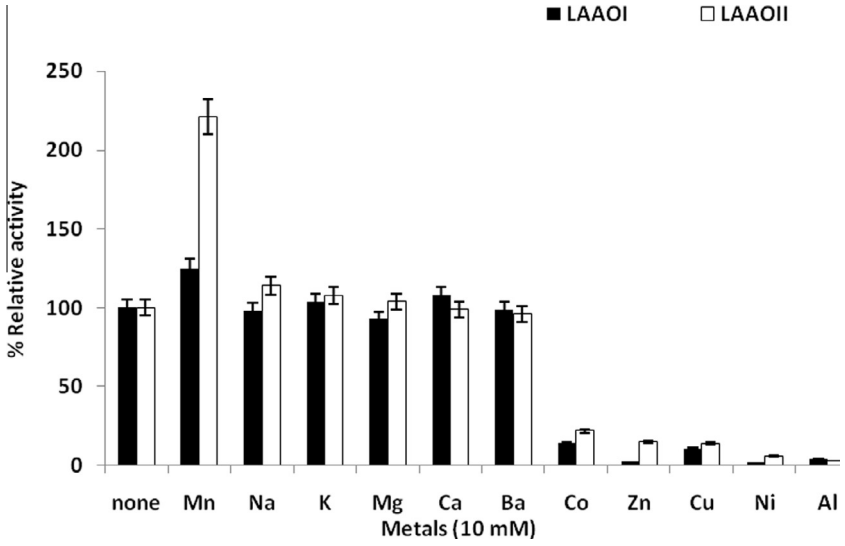

Figure 7 Effect of different metal ions (10 mM) on the purified Cc-LAAOI and Cc-LAAOII activity. Data represent mean ± SD of three independent experiments.

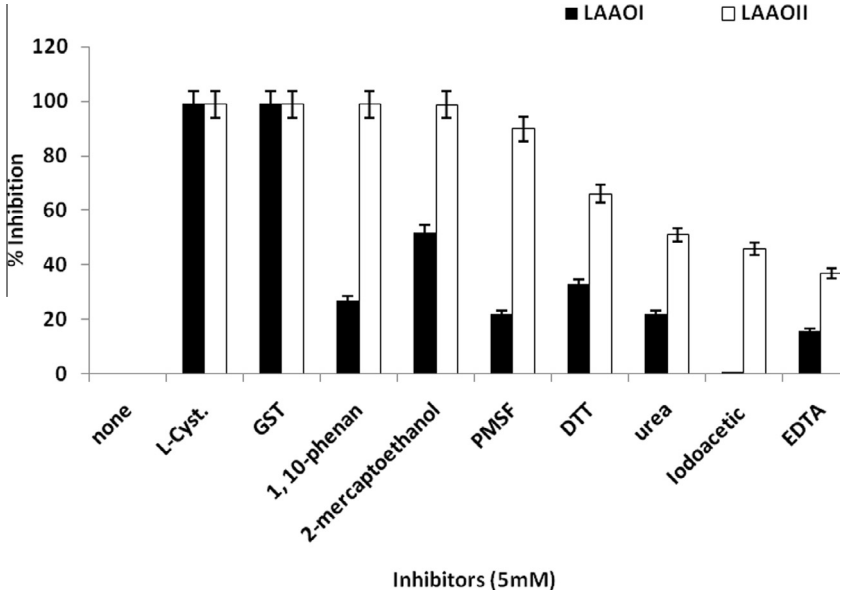

Figure 8 Effect of various inhibitors (5 mM) on the purified Cc-LAAOI and Cc-LAAOII activity. Data represent mean ± SD of three independent experiments.

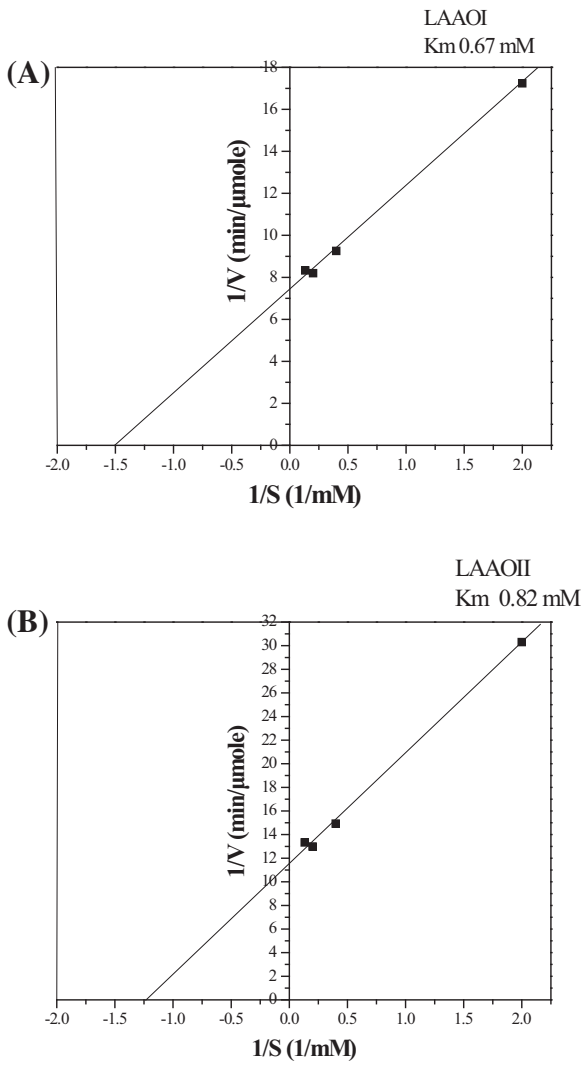

Figure 9 Lineweaver–Burk plot for Cc-LAAOI (A) and Cc-LAAOII (B) at different concentrations of L-Leucine as the substrate under standard conditions. Each point represents the average of two experiments.

[16]. Most sv-LAAOs have an amino acid side chain binding site comprised of 3–4 hydrophobic subsites (a, b, c, d), while *O. hannah* LAAO contains an additional amino binding subsite for the ε-amino group of L-Lys. Aromatic amino acids are commonly the best substrates for sv-LAAOs as their cyclic structure can be incorporated perfectly at the side chain binding site of sv-LAAOs [34–36]. Concordantly, the hydrophobic portions of amino acids can be inserted perfectly into the hydrophobic pockets within the sv-LAAO enzyme active sites, which gives a better catalytic specificity of sv-LAAOs toward L-hydrophobic amino acids [37].

The activity of the *C. cerastes* LAAO was inhibited in the presence of the lower concentrations of inhibitors such as reduced glutathione, L-cystiene, 2-mercaptoethanol, O-phenanthroline, as well as PMSF and EDTA. The enzymatic inactivation may be due to the reduction of LAAO cofactors NAD or FAD. Different ions can activate or inhibit the specific activity of some LAAOs. The addition of Mn^{2+} increases the L-Leu hydrolytic activity of the enzyme. This may be related to the stabilization of the structural integrity of the enzyme [19].

Concerning the kinetics parameters, the low value of Michaelis–Menten constant for the amino acid L-leucine of *C. cerastes* LAAOI as determined from the Lineweaver–Burk plot confirms the high catalytic preference of the *C. cerastes* LAAO compared to *Daboia russelii, Crotalus durissus cumanensis*, *Naja naja kaouthia*. This catalytic preference might be explained by the presence of amino acids that promote a non-polar environment in the catalytic site [32].

5. Conclusion

In this work, two LAAO isoforms which share a high degree of homology with *Viperidae* sv-LAAOs, have been isolated from the Egyptian *C. cerastes* snake venom. The two isoenzymes seem to be non-covalently linked to heterodimeric glycoproteins with slightly different MW subunits. Both isoenzymes displayed high catalytic preference to long-chain, hydrophobic and aromatic amino acids with L-Arg exhibiting the highest specificity. The biochemical and structural studies indicate that Cc-LAAOI and Cc-LAAOII are novel LAAOs that might be promising pharmacological agents and could be modulated by some specific ions &/or chemical compounds to be included in the design of new drugs.

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