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EXPERIMENTAL ARTICLES

Purification and Characterization of L-Amino Acid Oxidase from the Solid-State Grown Cultures of *Aspergillus oryzae* ASH[1]

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Abstract—L-Amino acid oxidase (L-AAO) was purified from the solid state-grown cultures of *A. oryzae* ASH (JX006239.1) by fractional salting out, followed by ion exchange and gel filtration chromatography, to its molecular homogeneity, displaying 3.38-fold purification in comparison with the crude enzyme. SDS-PAGE revealed the enzyme to be a homo-dimer with ~55-kDa subunits, with approximate molecular weight on native PAGE of 105–110 kDa. Two absorption maxima, at 280 nm and 341 nm, for the apoproteinic and FMN prosthetic group of the enzyme, respectively, were observed, with no detected surface glycosyl residues. The enzyme had maximum activity at pH 7.8–8.0, with ionic structural stability within pH range 7.2–7.6 and pH precipitation point (pI) 4.1–5.0. L-AAO exhibited the highest activity at 55°C, with plausible thermal stability below 40°C. The enzyme had $T_{1/2}$ values of 21.2, 8.3, 3.6, 3.1, 2.6 h at 30, 35, 40, 50, 60°C with Tm 61.3°C. Kinetically, *A. oryzae* L-AAO displayed a broad oxidative activity for tested amino acids as substrates. However, the enzyme had a higher affinity towards basic amino acid L-lysine (K_m 3.3 mM, K_{cat} 0.04 s^{-1}) followed by aromatic amino acids L-tyrosine (K_m 5.3 mM, K_{cat} 0.036 s^{-1}) and L-phenylalanine (K_m 6.6 mM), with 1ow affinity for the S-amino acid L-methionine (K_m 15.6 mM). The higher specificity of *A. oryzae* L-AAO to L-lysine as substrate seems to be a unique property comparing to this enzyme from other microbes. The enzyme was significantly inhibited by hydroxylamine and SDS, with slight inhibition by EDTA. The enzyme had a little effect on AST and ALT, with no effect on platelet aggregation and blood hemolysis in vivo with an obvious cytotoxic effect towards HepG2 (IC_{50} 832.2 μg/mL) and MCF-7 (IC_{50}, 370.6 μg/mL) tumor cells in vitro.

Keywords: *Aspergillus oryzae*, L-amino acid oxidase, biochemical properties, cytotoxic activity

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L-Amino acid oxidase (L-AAO, EC 1.4.3.2) is a flavo-enzyme catalyzing the oxidative deamination of L-amino acids to the corresponding α-keto acids, ammonia and hydrogen peroxide as follows:

$$R{-}CH(NH_2){-}COOH + H_2O + O_2 \xrightarrow{\text{L-AAO}} R{-}CO{-}COOH + H_2O_2 + NH_3.$$

L-AAOs received much attention because of their broad applications as antibacterial, antiviral, antifungal and antiprotozoal agents [1–3]. Moreover, the enzyme has potential anticancer activity towards various types of tumors [4, 5]. Chemically, L-AAO with the flavin prosthetic group is the main component of snake and insect venoms [6] and is responsible for their yellow color. The cytotoxic effect of L-AAOs may be ascribed to the released hydrogen peroxide inducing mitochondrial caspases and cytochrome *c* causing apoptosis, platelet aggregation with anticoagulant activity [7–9].

L-AAOs from various prokaryotes and snakes were extensively characterized [10–15], contrary to rather few studies on L-AAOs from micro eukaryotes. Catalytically, L-AAOs from bacterial species have broad substrate specificity affecting various L-amino acids [16], with less affinity to neutral (leucine/methionine) and basic (L-lysine and L-arginine) and higher specificity to aromatic amino acids (L-phenylalanine, tyrosine and tryptophan) [10, 17]. However, the antigenic properties of bacterial therapeutic enzymes for their stereo-tertiary structure, in vivo, was approved for various therapeutic enzymes, compared to the corresponding enzymes from eukaryotes as reviewed by El-Sayed [18]. Thus, the search for new sources for L-AAO with unique biochemical properties seems to be well-justified.

In comparison with the numerous publications on prokaryotic L-AAO, this enzyme from eukaryotes, especially from fungi, received less attention. Only few studies on fungal genera *Trichoderma* and *Aspergillus* were reported as L-AAO producers [5, 19, 20]. In our preliminary studies we focused on the screening for L-AAO production from varies fungal genera, and *Aspergillus oryzae* was selected as the potential enzyme producer [21].

[1] The article is published in the original.

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The objective of the present work was to purify L-AAO from *A. oryzae* using rice bran as substrate under solid–state fermentation, in order to assess the biochemical properties of this enzyme, for further therapeutic exploitation. The cytotoxic effect of this enzyme in vivo and in vitro against various standard cell lines was evaluated.

MATERIALS AND METHODS

Materials. Nessler's reagent, horseradish peroxidase, guaiacol, L-lysine, L-cysteine, L-methionine, L-phenylalanine, and L-arginine were purchased from Sigma-Aldrich Co. DEAE-cellulose and Sephadex G_{200} were purchased from Pharmacia Biotechnology Co. (Sweden). Rice bran was obtained from local Egyptian markets. All other chemicals were of analytical grade.

Fungal strain and growth conditions. *Aspergillus oryzae* was selected as an L-AAO producer from our preliminary screening studies [21]. In addition to the morphological identification, the isolate was molecularly identified based on the sequence of 18S rRNA–28S rRNA (flanking the sequence of ITS1, 5.8S rRNA, and ITS2) according to our previous studies [22, 23]. Depending on the DNA sequence analysis, the isolate was identified as *A. oryzae* ASH with accession no. JX006239.1. *A. oryzae* was grown in a 1000-mL Erlenmeyer conical flask with 40 mL of the salt solution containing glucose (1%), L-lysine (80 mM), KH_2PO_4 (0.1%), KCl (0.05%), and $MgSO_4 \cdot 7H_2O$ (0.05%), in accordance with our nutritional optimization studies [21], and 20 g rice bran. A total of 200 g of rice bran as solid substrate for growth of the fungal isolate was prepared. After autoclaving, the medium waslihoculated with 2 mL of the fungal spore suspension of 5 days age, and incubated for 8 days at 30°C. After incubation of the fungal cultures under optimum conditions, the enzyme was extracted with potassium phosphate buffer (pH 7.5) [24].

Purification of L-AAO from the solid fermented cultures of *A. oryzae*. L-AAO was purified from the solid cultures of rice bran-grown *A. oryzae* by fractional slating out, followed by ion exchange and gel filtration chromatography, according to our protocols [23, 25, 26]. The activity and protein contents of the pooled fractions were determined as described below. The most active fractions were checked for their homogeneity by SDS-PAGE, collected, and lyophilized prior to further enzymatic studies.

L-Amino acid oxidase assay. The activity of L-AAO was determined based on the amount of the released hydrogen peroxide according to [27] with slight modifications by El-Sayed et al. [21]. The reaction mixture contained 10 mM L-lysine, 0.2 mM guaiacol, 5 U horseradish peroxidase and 200 μL of the enzyme preparation in 100 mM potassium phosphate buffer (pH 7.0), in a total volume 1 mL. After incubation of the reaction mixture for 30 min at 30°C, the activity was stopped by freezing at 10 min at –20°C, and the absorbance of the tetraguaiacol produced was measured at 436 nm. One unit of L-AAO was defined by as amount of the enzyme which releases 1 μmol of H_2O_2 per min, under standard assay conditions. The enzyme activity was calculated according to the formula: Activity of L-AAO (U/mL) = A_{436} nm/min × 4/tetraguaiacol coefficient (25.5). The molar extinction coefficient was determined by preparing 1 M solution of guaiacol in potassium phosphate buffer (100 mM), supplemented with 0.1 M H_2O_2, then the developed color was assessed at A_{436}. The guaiacol molar extinction coefficient was expressed as (A_{436} oxidized – A_{436} reduced)/guaiacol initial concentration. The concentration of the L-AAO protein was determined according to Lowry et al. [28], using bovine serum albumin as a standard.

SDS-PAGE and native-PAGE analysis. The homogeneity and molecular mass of the purified *A. oryzae* L-AAO were assessed using 10% denaturing PAGE [29]. The protein samples (50 μL) were boiled in dissociation buffer (50 mM Tris–HCl, pH 6.8; 1.0% glycerol; 2% SDS; 5% 2-mercoptoethanol and 0.1% bromophenol blue) and loaded onto the gel. After gel running, staining and de-staining, the molecular mass of the revealed protein bands was determined using the standard protein marker (Broad-way dual prestained protein marker ranged from 7 to 240 kDa).

The native PAGE for the purified enzyme was conducted using the same protocol [29], without SDS in the resolving gel and dissociation buffer.

Absorption spectrum of *A. oryzae* L-AAO. The absorption spectrum of the purified *A. oryzae* L-AAO was scanned using a UV/Vis spectrophotometer (UV-1600, Shimadzu, Japan) in the range of 200 to 700 nm, using 100 mM potassium phosphate buffer (pH 7.0) as the blank. FAD (10 mM) was disso1ved in the same buffer.

Determination of the FAD content of L-AAO. The FAD moiety of L-AAO was quantitatively determined by acid hydrolysis assay [4]. Briefly, enzyme solution (98.4 U/mL) was mixed with of 25% TCA (0.2 mL) for 30 min at 30°C. After centrifugation at 10000 rpm for 10 min, the supernatant was neutralized by 1 M K_2HPO_4. Then the developed color of the FAD/flavin moieties was measured at 450 nm, to the molar absorption coefficient of FAD (ε; 11.300).

Degree of glycosylation of *A. oryzae* L-AAO. The intrinsic carbohydrate content of the purified *A. oryzae* L-AAO was determined by glucose GOD-PAP kit [30]. The reaction mixture contained 500 μL of the enzyme sample, glucose oxidase (3 Ukat), horseradish peroxidase (0.01 Ukat), 4-aminophenazone (0.7 mM) and phenol (11 mM) in potassium phosphate buffer. The developed red color of 4-benzoquinone-monoimino-phenazone was measured at 505 nm.

Biochemical properties of *A. oryzae* L-AAO. The optimum pH for enzyme activity was assayed in pH

Table 1. Overall purification profile of L-AAO from solid cultures of *A. oryzae*

Purification step	Total protein, mg	Total activity, U	Specific activity, U/mg	Purification fold	Yield
Crude enzyme	2170	71625	3.3	1	100
Ammonium sulfate Precipitates, 20–75%	2760	20160	7.4	2.25	28.2
DEAE-cellulose	1356.8	12441.6	9.2	2.78	17.4
Sephadex G_{200}	568.9	6325	11.2	3.38	8.84

range (3.0–10.4), using 50 mM citrate–phosphate (pH 3.0–6.5), 50 mM potassium phosphate (pH 5.2–8.0), and 50 mM glycine–NaOH buffers (pH 8.0–10.4).

The pH stability was checked by preincubation of the enzyme without the substrate at various pH values (5.2–10.0) for 2 h at 4°C, then the enzymatic residual activity was determined as mentioned above.

The optimum temperature and thermal stability for the purified *A. oryzae* L-AAO were determined as described by El-Sayed [25]. The thermal catalytic parameters as thermal inactivation rate (K_r), half-life time ($T_{1/2}$) and half-life temperature (T_m, °C) were calculated according to El-Sayed and Shindia [26].

The effect of various inhibitors/activators on activity of *A. oryzae* L-AAO was determined by pre-incubation of the enzyme with each compound for 1 h at 4°C, then measuring the residual enzyme activity by the standard assay. The affinity of the purified enzyme for oxidation of various L-amino acids as lysine, alanine, asparagine, arginine, glutamine, methionine, glycine, cysteine, phenylalanine, and tyrosine, as well as glutathione was assessed. In addition, the oxidative-proteolytic activity of the purified enzyme was determined for bovine serum albumin and casein. The kinetic parameters as K_m, V_{max} and K_{cat} for the most oxidized amino acids were evaluated.

Cytotoxicity of A. oryzae L-AAO

Transaminases activity. The cytotoxic effect of the purified enzyme was determined using male Swiss Albino mice (6–8 week old) injected by a single dose of the enzyme (0.5 mL, 98.8 U/mL). The blood was collected in tubes containing EDTA every 8 h during 2 days and the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the sera were assessed and compared to the negative (without enzyme) and positive (zero time) controls. The activity of ALT and AST where determined by double assay methods using lactate dehydrogenase and malate dehydrogenase, respectively, based on NAD^+ releases [31] according to the manufacturer's recommendations (Roche Diagnostics GmbH, Sandhofer Strasse, Mannheim).

Platelet aggregation. The impact of L-AAO on induction of the human plasma platelet aggregation was assessed according to Wei et al. [32]. The platelet-rich plasma (350 μL) was incubated with 50 μL of L-AAO for 20 min at room temperature, then the aggregations of the platelets was measured with a blood Lumi-aggregometer.

Hemolytic activity. The hemolytic activity of the purified *A. oryzae* L-AAO was assessed using human blood agar [33].

Antitumor activity of *A. oryzae* L-AAO in vitro. The cytotoxic effect of the purified enzyme to human hepatocarcinoma (HepG2) and breast adenocarcinoma (MCF-7) cells was assessed using an MTT assay [34]. The MTT assay was based on the ability of active mitochondrial dehydrogenase of living tumor cells cleave the tetrazolium ring of the yellow MTT, forming dark blue insoluble formazan crystals. After solubilization of the crystals, the number of viable cells was expressed by the development of dark blue color of soluble formazan at 570 nm using an ELISA reader. The relative cell viability was calculated by the A_{570} of sample/A_{570} of control ×100.

Statistical analysis. All the experiments of enzyme characterization were conducted in triplicates. The reported data are the means ± SD.

RESULTS

Purification of *A. oryzae* L-AAO. L-AAO was purified from the solid-state cultures of *A. oryzae* grown on rice bran under solid-state fermentation (SSF) [21]. After incubation of the cultures, the crude enzyme was exctracted, fractionally precipitated by salting out (20–75% saturation), followed by ion exchange (DEAE-cellulose) and gel filtration (sephadex G_{200} column) chromatographic procedures. Upon salting out the enzyme activity increased 2.25 times, with about 28.2% yield. Subsequently, using a DEAE-cellulose column, the enzyme was maximally eluted by 100 mM potassium phosphate buffer (pH 7.0) with 100 mM NaCl. Molecular homogeneity of the active fractions was confirmed by SDS-PAGE, and the homogenous fractions were substantially loaded to the column of sephadex G_{200}. The colorimetric activity and molecular homogeneity of the collected fractions were assessed. From the overall purification profile (Table 1), the specific activity of L-AAO was increased 3.4-fold with 8.8% yield, compared to the crude enzyme.

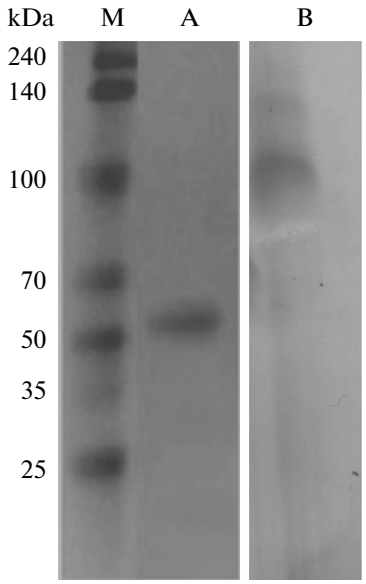

Fig. 1. SDS-PAGE (A) and Native-PAGE (B) of the purified *A. oryzae* L-AAO. The denaturing and native PAGE was conducted as according to Laemmli (1970) as described in materials and methods. (M) Broad-way Dual Pre-stained Protein Marker was used.

Native PAGE and SDS-PAGE analysis. The homogeneity and molecular mass of purified *A. oryzae* L-AAO were tested using the profiles of native and SDS-PAGE (Figs. 1A, 1B). Under non-denaturing conditions (Native-PAGE) the enzyme displayed a distinct band of approximate molecular weight 105110 kDa. From the SDS-PAGE profile (Fig. 1), a single homogenous band of about 55 kDa appeared at the last purification step, ensuring the efficacy of our designed protocol for enzyme purification from the solid-state fungal cultures. From the native and SDS-PAGE profiles, it could be deduced that the enzyme had two identical subunits of homodimeric identity.

Glycosyl contents of *A. oryzae* L-AAO. There was no detectable surface structural glycosyl moiety of the purified enzyme according to glucose oxidase/horseradish peroxidase assay Kit, ensuring the enzyme purity from additional natural glycol-proteins.

Table 2. Biochemical and molecular properties of the purified *A. oryzae* L-AAO

Optimum pH	7.8–8.0
pH stability	7.2–7.6
Optimum temperature, °C	55
Molecular mass on native-PAGE	105–110 kDa
Molecular mass under SDS-PAGE	55 kDa
Number of subunits	2
Absorption maxima, nm	280, 341
Precipitation pH, pI	4.1–5.0
Surface glycosyl	Not detected

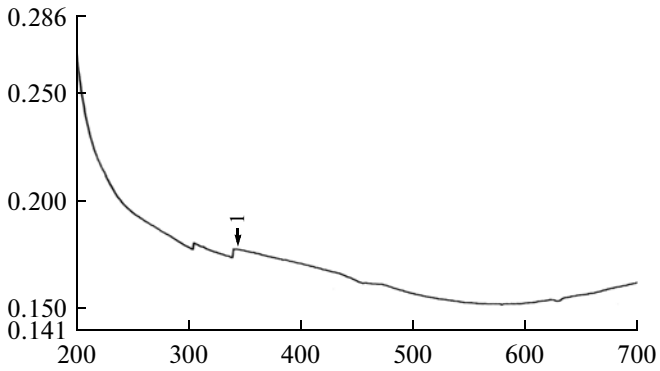

Fig. 2. Absorption spectra of *A. oryzae* L-AAO. The spectra of purified enzyme was scanned UV/Vis spectrophotometer in range of 200 to 700 nm, using potassium phosphate buffer as blank baseline.

Absorption spectra of *A. oryzae* L-AAO. The absorption spectra of the purified *A. oryzae* holo-L-AAO were scanned by a UV/Vis scanning spectrophotometer within the range from 200 nm to 700 nm, in potassium phosphate buffer (pH 7.0). According to the absorption spectra (Fig. 2), the holoenzyme displayed the maxima at 280 and 341 nm. Upon dialysis of the holoenzyme against the buffer containing hydroxylamine (20 μM), a single peak appeared at 280 nm for apo-L-AAO, with disappearance of the peak at 341 nm (data not shown).

Biochemical Properties of A. oryzae L-AAO

Optimum pH and pH stability. The biochemical properties of the purified L-AAO from *A. oryzae* are summarized in Table 2.

The optimum pH for activity of L-AAO was assessed using L-lysine as the substrate under standard assay conditions. The highest enzyme activity (70.0 U/mL) was observed at pH 7.8–8.0, with a slight decrease in its activity at pH 5 and 9.6. Actually, the enzyme activity decreased by about 50% of its initial activity at the optimum at acidic pH (3.0) and by less than 3% at alkaline one (pH 10.4).

The pH stability of L-AAO was assessed using L-lysine as substrate at pH range 3.0 to 10.0. The enzyme exhibited a broad pH stability (6.0–9.5), with the highest stability from pH 7.2–7.6, in potassium phosphate buffer. However, at acidic conditions (pH 3–4), the rate of enzyme inactivation was relatively higher than at alkaline ones (pH 9.0–10), confirming, the basic identity of the enzyme. Practically, the dramatic negative effect on the enzyme activity at acidic pH might be assumed to pH being close to intrinsic pI (4.1–5.0), dissociation of FAD moieties, or denaturation of the enzyme subunits. By preincubation at pH 7.2 for 2 h at 4°C, the enzyme activity was

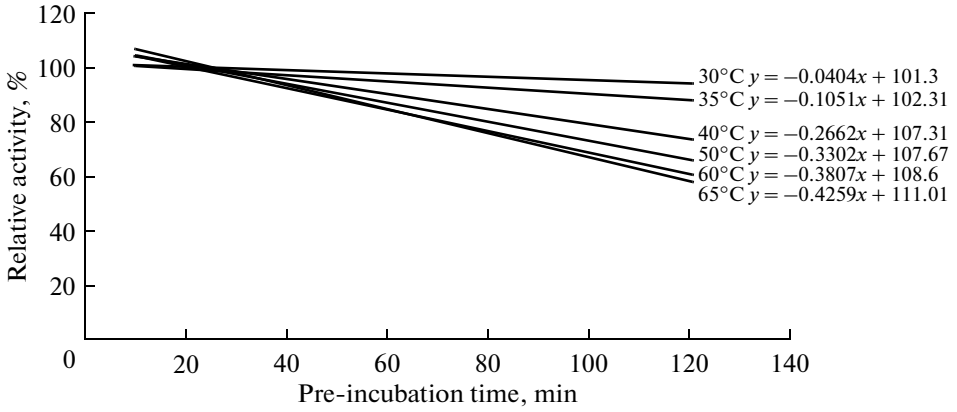

Fig. 3. Thermal stability of *A. oryzae* L-AAO. The enzyme was pre-incubated at different temperatures (30–65°C), then the residual oxidative activity was measured using lysine as substrate as in Materials and Methods.

increased by about 2.5%, suggesting the stabilization of the tertiary structure and/or proper reorientation of the surface active sites.

The precipitation of *A. oryzae* L-AAO by incubation at different pH was assessed as adopted by El-Sayed [25]. The maximum precipitation of the enzyme was measured at pH range from 4.1–5.0, revealing the neutral identity of their constitutional amino acids. Thus, the isoelectric point (pI) of the enzyme was ranging from 4.1 to 5.0, being an advantage for its therapeutic utility, in vivo.

Optimum temperature and thermal stability. The impact of different temperatures (10–65°C) on the activity of *A. oryzae* L-AAO was assessed. The maximum enzyme activity was observed at 55°C, with a slight decrease in its activity (by ~15%) at 65°C. Practically, the enzyme activity was substantially increased with the reaction temperature, revealing an acquisition of activation energy for the substrate binding to the enzyme active sites.

From the profile of thermal stability (Fig. 3) it can be seen that *A. oryzae* L-AAO has a plausible catalytic stability below 40°C, with substantial inactivation at high temperature. The thermal inactivation parameters for *A. oryzae* were summarized in Table 3. The enzyme half-life time ($T_{1/2}$) was 21.2, 8.3, 3.6, 2.6, and 2.4 h, at 30, 35, 40, 50, 60, and 65°C, respectively, with half-life temperature (T_m) 61.3°C. At 35°C, the enzyme had intrinsic stabilizing potency about 3.46 times higher than at 65°C. Practically, the thermal enzyme denaturation rate (K_t) was 0.404×10^{-3}, 1.07×10^{-3}, 2.6×10^{-3}, 3.39×10^{-3}, 3.94×10^{-3} and 4.36×10^{-3} s^{-1} at 30, 35, 40, 50, 60, and 23°C, respectively.

Substrate specificity of *A. oryzae* L-AAO. The affinity of *A. oryzae* L-AAO towards various amino acids was assessed, as oxidative deaminating activity, under the standard assay conditions, using L-lysine as the authentic substrate. From Table 4, it can be seen that the enzyme displayed the maximum affinity to tyrosine (104%), followed by L-methionine (101%), and phenylalanine (86.3%), compared to lysine (100%). The enzyme had the lowest relative activity towards glutamine (15.5%), glycine (17.8%) and asparagine (19.8%). The enzyme also exhibited a proteolytic oxidative deaminating activity for bovine serum albumin and casein as substrates. Based on these results, tyrosine, phenylalanine, and methionine, together with L-lysine, were selected for further catalytic and kinetic studied.

The kinetic and catalytic parameters (K_m, V_{max}, K_{cat}) for L-lysine, tyrosine, L-metionine, and phenylalanine were summarized in Table 5. According to the Lineweaver–Burk plot for *A. oryzae* L-AAO (Fig. 4), the enzyme displayed the maximum catalytic affinity and reaction rate to L-lysine (K_m 3.39 mM, V_{max}

Table 3. Thermal kinetic parameters of *A. oryzae* L-AAO

Temperature, °C	Half-life time, h	Thermal denaturation rate, $\times 10^{-3}$ s^{-1}	Half-life temperature, °C
30	21.2	0.404	61.3
35	8.3	1.07	
40	3.6	2.6	
50	3.1	3.39	
60	2.6	3.94	
65	2.4	4.36	

0.27 U/mg min), followed by tyrosine (K_m 5.38 mM, V_{max} 0.24 U/mg/min), L-phenylalanine (K_m 6.63 mM, V_{max} 0.26 U/mg/min), and L-methionine (K_m 15.6 mM, V_{max} 0.26 U/mg/min). The catalytic efficiency of *A. oryzae* L-AAO was relatively similar for L-lysine (K_{cat} 0.040 s^{-1}), L-methionine, and phenylalanine (K_{cat} 0.039 s^{-1}), foldwed by L-tyrosine (K_{cat} 0.036 s^{-1}). Practically, the plausible specificity of *A. oryzae* L-AAO to basic amino acids, such as L-lysine, seems to be an important therapeutic criterion [10], compared to bacterial L-AAO. Thus, depending on the kinetic properties, the purified *A. oryzae* L-AAO displayed an obvious catalytic identity as L-lysine oxidase. Most of bacterial L-AAO display a broad specificity to various amino acids that hinders the therapeutic potency of these enzymes in vivo.

The effect of activators/inhibitors on activity of L-AAO was assayed by pre-incubation of the enzyme without substrates for 2 h at 4°C, then measuring its residual activity, as described above. From the obtained results (Table 6), the enzyme activity was significantly inhibited by hydroxylamine (36.8%), SDS (37.6%) and $HgCl_2$ (39.0%), with slight inhibition by EDTA, Cu^{2+} and Ca^{2+}. The drastic inhibition of the enzyme activity by hydroxylamine might be attributed to releases of flavin moieties as prosthetic groups from the holo-L-AAO giving a functionless apo-L-AAO, as was confirmed by the spectral analysis.

Cytotoxicity of *A. oryzae* L-AAO in vivo in mice. The cytotoxic effect of the purified enzyme was assayed in vivo using male Swiss Albino mice as described in Materials and Methods. The blood was collected 5 and 10 h after a single enzyme dose (70.0 U/mL) infusion, and the activities of AST and ALT were assessed. From the obtained results (Fig. 5), the activity of ALT was relatively unaffected by enzyme injection, while that of AST increased slightly (by about 10% after 6 h), followed by decreasing by about 18% after 10 h, compared to the negative controls.

The effect of the enzyme on platelet aggregation of the human plasma was assessed. No inducing effect on human plasma aggregation by the purified L-AAO was observed (data not shown). Moreover, the enzyme had no hemolytic activity, as was observed using human blood agar.

Table 4. Substrate specificity of *A. oryzae* L-AAO

Substrate, 10 mM	Relative activity, %
L-Lysine	100
L-Methionine	101
Phenylalanine	86.3
Arginine	27.6
Glycine	17.8
Asparagine	19.8
Alanine	21.2
Glutamine	15.5
Cysteine	29.5
Tyrosine	104
Glutathione	97
Bovine serum albumin	37
Casein	59

Table 5. Kinetic parameters of the purified *A. oryzae* L-AAO

Substrate	K_m	V_{max}	$K_{cat} \times s^{-1}$
L-Lysine	3.39	0.27	0.040
L-Methionine	15.6	0.26	0.039
L-Phenylalanine	6.63	0.26	0.039
L-Tyrosine	5.38	0.24	0.036

Molecular weight of native enzyme is 110 kDa.

Anticancer activity of *A. oryzae* L-AAO. The cytotoxic effect of L-AAO against human hepatocarcinoma (HepG2) and breast adenocarcinoma (MCF-7) cell lines was assessed using the MTT assay. According to the cytotoxicity profile (Fig. 6), the enzyme exhibits a higher anti-proliferative activity agaihst MCF-7 (IC_{50}, 370.6 μg/mL) and a somewhat lower one against HepG2 cells (IC_{50} 832.2 μg/mL).

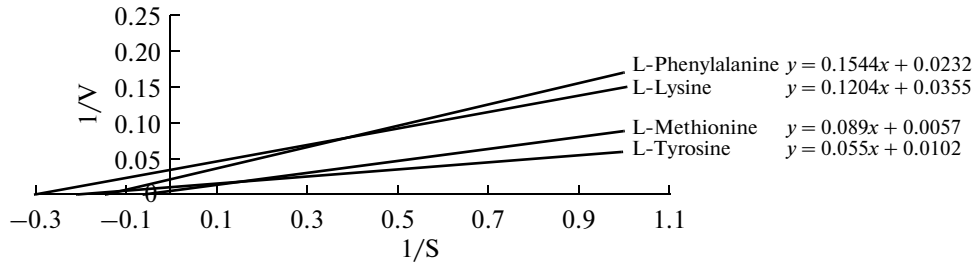

Fig. 4. Lineweaver–Burk plot for *A. oryzae* L-AAO for various amino acids as L-lysine, L-phenylalanine, L-methionine, and L-tyrosine.

Table 6. Effect of different activators/inhibitors on activity of *A. oryzae* L-AAO

Compound	Relative activity, %
Control	100
$CuSO_4 \cdot 7H_2O$	59.3
Hydroxylamine	36.8
EDTA	85.3
$LiSO_4$	46.2
$HgCl_2$	39.0
KCl	54.5
$CaCl_2$	74.1
SDS	37.6
NaSe	51
KI	46.5
DMSO	47.9

DISCUSSION

Recently, L-amino acid oxidases were shown to exhibit a potent activity in vivo against various tumors [4, 5, 35]. The enzymes from various bacterial genera and snake venoms were extensively purified and characterized [21]. However, L-AAO from various bacterial species and snakes usually have a broad range of amino acid specificity as substrates [10, 36, 37], which pivotally limits the therapeutic utility of this enzyme in vivo towards a unique amino acid. As it is well known, therapeutic enzymes from eukaryotic sources display a reasonable affinity to their substrates, which may usually be explained by the catalytic tertiary structure oriented during the enzyme posttranslational processing [18]. Thus, based on our previous work [21] we focused on characterization of *Aspergillus oryzae* as potent L-AAO producer, and the current study was extended to assess the biochemical and catalytic properties of the purified L-AAO. As a result of the purification procedure, the enzyme activity was increased by 3.4-fold, with a 8.8% yield, compared to the crude enzyme preparation, giving a single homogenous protein band under denaturing PAGE. A single homogenous band with molecular weight of about 55 kDa under SDS-PAGE and 105–110 kDa on native-PAGE were obtained, revealing the homo-dimeric identity of this enzyme, consisting of two identical subunits. However, other documented protocols resulted in higher purification yields of this enzyme from *Trichoderma viride* (66.1-fold) [4], *Chlamydomonas reinhardtii* (21-fold) [38] and *Bacillus carotarum* (112-fold) [10], compared to 3.4 fold for our enzyme. Thus, simplicity of our purification protocol might be able to decrease the probability of dissociation of the prosthetic group FAD from the apo-L-AAO enzyme, maintaining the tertiary structure and prosthetic groups as coincident with previous studies [39, 40].

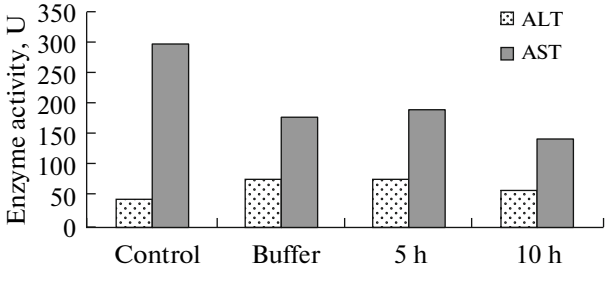

Fig. 5. Cytotoxic effect of the purified *A. oryzae* L-AAO on activities of AST and ALT of Swiss Albino mice, regarding to negative controls and potassium phosphate control, as described in Materials and Methods.

The homo-dimeric nature was previously documented for L-AAO from *T. viride* [4], *Streptomyces endus* [41], and *Cellulomonas cellulans* [42] with molecular weights from 49 to 55 kDa. However, L-AAO from *C. reinhardtii* [38] has a single band of 60 kDa on SDS-PAGE and 470 kDa on native PAGE revealing eight identical subunits in this enzyme.

The purified *A. oryzae* L-AAO displays neither surface glycosyl residues as detected by glucose oxidase/horseradish peroxidase assay, nor intrinsic metal ions according to atomic absorption analysis. Thus, *A. oryzae* L-AAO could be designated as non-glycoprotein and non-metallic enzyme. The lack of surface glycosyl residues is a privileged criterion from the therapeutic view for in vivo application, since glycosylation is one of the main causes for antigenicity and induction of neutralizing antibodies as was shown for various therapeutic glycoproteins [18].

According to the spectral analysis of the purified enzyme, the holo-L-AAO exhibits two maximum optima at 280 nm and at 341 nm, for the apo-L-AAO and FAD moieties, respectively. However, the explanation of the strong shift of the FAD spectra to 341 nm for this enzyme remains ambiguous. The flavoprotein nature of *A. oryzae* L-AAO is consistent with that of various L-AAOs [4, 10, 41].

According to the profile of optimum pH and pH stability, the enzyme has the highest activity at pH 7.8–8.0 and structural stability within the pH range from 7.2 to 7.6. In contrary to the highest stability at pH 7.2–7.6, the enzyme activity was strongly inhibited at acidic pH, revealing the neutral identity of constitutional intrinsic amino acids of *A. oryzae* L-AAO. A significant reduction of enzyme activity at acidic pH might be because of closeness to its intrinsic pI (4.0–5.0), dissociation of the prosthetic group (FAD), as reported for other amino acid hydrolyzing enzymes [21, 25]. Also, the drastic effect of enzyme activity at acidic pH might be attributed to the denaturation of enzyme subunits, and, thus, further studies are required to approve this hypothesis. In consistent to a slightly alkaline identity of *A. oryzae* L-AAO, the enzyme from *T. viride* [4], *B. carotarum* [10], *Pseudomonas* sp. [43] has an optimum pH 7.5–8.2.

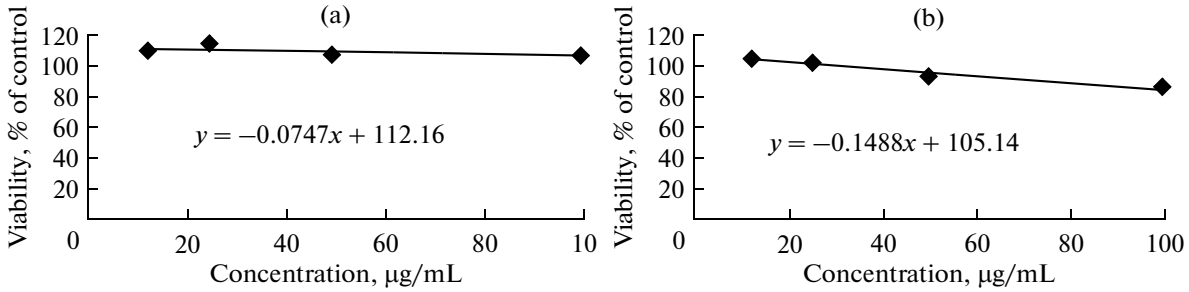

Fig. 6. Cytotoxic effect of *A. oryzae* L-AAO on tumor cells, human hepatocarcinoma HepG2 (a) and breast adenocarcinoma MCF-7 (b). The viability of tumor cells was assessed by MTT assay, as described in Materials and Methods.

The optimum of enzyme activity was recorded at 55°C, with a slight decrease (about 15%) at 65°C. A substantial increase on the enzyme activity with the reaction temperature reveals the acquisition of activation energy for binding of the substrate with the enzyme active sites. In agreement with that, L-AAO from *B. carotarum* [10] and *Ch. reinhardtii* [38] have their maximum activity at 50–55°C. From the profile of thermal stability, *A. oryzae* L-AAO has a plausible catalytic stability below 40°C, with subsequent inactivation with high temperature. The relative thermal stability seems to be an advantage from the therapeutic point of view. However, *B. carotarum* L-AAO has a half-life time of 4, 37, and 60 min at 37, 30, and 22°C, respectively [10]. At 40°C, the thermal structural stability of *A. oryzae* L-AAO is about 50 times higher than that of *B. carotarum* enzyme.

From the profile of the enzyme substrate affinity, the enzyme possesses a broad range of specificity towards various tested amino acids. Thus, the enzyme displays a high affinity to basic/aromatic amino acids, compared to S-amino acids, and, thus, demonstrating its unique catalytic identities to L-lysine, compared to other L-AAO [10, 40]. However, L-AAO from *T. viride* has a higher affinity to L-lysine (K_m 0.04 mM), L-ornithine (K_m 0.44 mM) and L-phenylalanine (K_m 14 mM), compared to our L-AAO [4]. Thus, it could be deduced that the enzyme from each organism has its specific catalytic and structural identity. Compared to L-AAO of *T. viride*, the lower substrate affinity of the *A. oryzae* enzyme towards basic amino acids, especially lysine, might be attributed to the accepted purification protocol. Unlike bacterial L-AAO, the plausible specificity of *A. oryzae* L-AAO to basic amino acids as L-lysine seems to be a favored criterion from the therapeutic point of view. Depending on its kinetic properties, *A. oryzae* L-AAO displays a plausible identity as L-lysine oxidase, in contrary to most of bacterial L-AAO that have a broad specificity to various amino acids forms.

The enzyme activity was significantly inhibited by hydroxylamine, carbonyl reagent, assuming the dissociation of FAD/FMN moieties (prosthetic groups) from the holo-L-AAO, similar to L-AAO from *Ch. reinhardtii* [38], *Gymnogongrus* sp. [44]. Also, a strong inhibition of the enzyme by SDS and $HgCl_2$ suggests its denaturation to free subunits, or interaction with the surface thiols. However, a slight inhibition of the enzyme activity by EDTA suggests the nonmetallic identity of this enzyme as consistent with those reported by Ito et al. [45] for *Amphiroa crassissima* L-AAO. Since bivalent cations as Cu^{2+} and Ca^{2+} have an inhibitory effect on *A. oryzae* L-AAO, this migh mean the interaction with the FAD/FMN moieties, similar to L-AAO from *A. nidulans* [46, 47].

The cytotoxic effect of the purified enzyme was assayed in vivo using male Swiss Albino mice. The activity of ALT was not significantly affected by the enzyme, while AST was slightly increased (1.1-fold after 6 h) if compared with the control. ALT and AST were the potent indicators of liver dysfunction [48]. There was no inducing effect on human plasma aggregation by the purified L-AAO. Since the enzyme was not observed to exhibit hemolytic activity on human blood agar, it is possible to assume the pharmacological compatibility of the enzyme in vivo [49].

The enzyme displayed higher anti-proliferative activity against MCF-7 (IC_{50} = 370.6 μg/mL) followed by HepG2 (IC_{50} = 832.2 μg/mL). L-Lysine α-oxidase from *T. harzianum* exhibited a wide potential activity against various tumors as ascitic hepatoma 22, melanoma B-16, mammary and colon adenocarcinoma [5].

In conclusion, L-AAO was purified from the solid cultures of *A. oryzae* using rice bran as substrates under SSF, by salting out, ion-exchange and gel-filtration chromatography. The biochemical properties of the purified enzyme were extensively studied. From the kinetic studies, the enzyme display a high affinity towards L-lysine, followed by the aromatic amino acids as phenylalanine, assuming the identity of enzyme as L-lysine oxidase than L-amino acid oxidase. The enzyme exhibits a higher potency to preserve their FAD as prosthetic group. The enzyme has no negative effect on the biochemical and hemolytic parameters as AST, ALT, platelet aggregation, and human blood hemolysis. Additionally, the enzyme displays a relative anticancer potency against hepatocar-

cinoma and breast adenocarcinoma. However, a further research work is ongoing in order to immobilize the enzyme on immunogenetic inert compounds and on nano-polymers, to fully explore their catalytic and therapeutic properties.

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