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EXPERIMENTAL ARTICLES

Isolation and Properties of L-Lysine-α-Oxidase from the Fungus *Trichoderma* cf. *aureoviride* RIFAI VKM F-4268D

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Abstract—L-lysine-α-oxidase (LO) synthesized by the fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D under salt stress conditions was isolated and characterized. The newly developed method for the isolation and purification of the enzyme was based on its precipitation from the culture liquid by copper sulfate. The subsequent LO purification by the methods of hydrophobic (Octyl Sepharose) and ion exchange (DEAE ToyoPearl) chromatography yielded a homogeneous enzyme preparation with a high degree of purification (310-fold) and high specific activity (90 U/mg protein). The molecular mass of the enzyme determined by gel filtration and native electrophoresis was 115–116 kDa. According to the data of SDS electrophoresis, LO was a dimer with identical subunits (57–58 kDa). The optical absorption spectrum of LO corresponded to the flavoprotein spectrum with maximums at 278, 390, and 465 (a shoulder at 490) nm. LO is a stereospecific enzyme oxidizing almost exclusively L-lysine (pH optimum 7.8–8.2). Insignificant activity was observed against L-ornithine and L-arginine. LO was shown to be stable at temperatures up to 50°C.

Keywords: fungus *Trichoderma aureoviride*, L-lysine-α-oxidase, biosynthesis, isolation, purification, substrate specificity

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L-lysine-α-oxidase (LO) is one of the enzymes that are promising for antitumor enzyme therapy based on the high sensitivity of tumor cells to the deficiency of growth factors, including amino acids [1]. LO induces the degradation of the essential amino acid L-lysine: oxidative deamination with the formation of ammonia, hydrogen peroxide, and pyrroline carboxylic acid [1, 2].

The L-amino acid oxidases have been found in mammals [3, 4], snakes [5], insects [6], marine organisms (mollusks and fish) [7, 8], and microorganisms including bacteria [9–12], yeasts [13] and fungi [14–20].

Among LO producers, the fungi of the genus *Trichoderma* draw particular attention [2, 17–20] as they are able to synthesize the extracellular enzyme. It is precisely the excretion of this enzyme into the growth medium that makes the process of its isolation and purification more technologically and economically effective.

The first homogeneous LO preparation (*Trichoderma viride* Y-244) was obtained by Japanese researchers with a yield of 8% and a specific activity of 66 U/mg from [17, 18]. The method of purification included eight steps and was long and labor-consuming.

German researchers developed an efficient method for purification of extracellular LO from the fungus *Trichoderma viride* i4, resulting in a 60% yield of the enzyme with a specific activity of 90 U/mg protein (degree of purification, 300) [19]. This method requires large amounts of acetone, up to 60% of the reaction mixture volume. Application of acetone under industrial conditions leads to environmental and accident prevention problems.

The goal of the present study was to develop an efficient method for LO production during submerged cultivation of the fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D under salt stress conditions.

MATERIALS AND METHODS

The fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D used in the work was obtained from the All-Russian Collection of Microorganisms (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences). The fungus under study is capable of extracellular LO biosynthesis when grown on wheat bran [20]. The producer was cultivated on Czapek medium containing the following

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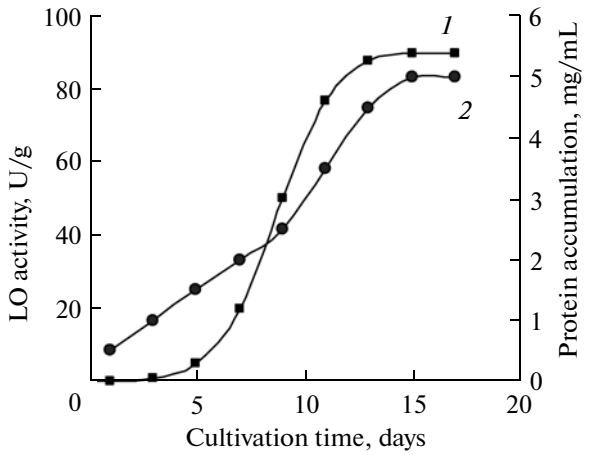

Fig. 1. Dynamics of L-lysine-α-oxidase accumulation during the cultivation of *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D on wheat bran. Activity (*1*) and protein accumulation (*2*).

(g/L): $NaNO_3$, 0.5; NaCl, 60.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; and Burkholder trace elements. Wheat bran (5–7%) was used as a growth substrate. Cultivation was performed in 0.75-L flasks containing 100 mL of the growth medium for 10–15 days on a shaker (220 rpm) at 28–29°C.

LO activity was assayed at 22°C by the rate of hydrogen peroxide production in 20 mM Tris-phosphate buffer (pH 8.0) in the presence of *o*-dianisidine (0.2 mM), peroxidase (5 μg/mL), and L-lysine (2 mM) on a Shimadzu spectrophotometer (E_{436} = 8.3 $mM^{-1} cm^{-1}$) [20]. The quantity of the enzyme catalyzing the oxidation of 1 μmol lysine per min was taken as a unit of activity.

Protein content was determined by the Bradford method.

The molecular mass of the enzyme was determined by gel filtration on a ToyoPearl HW-55 column (1.5 × 95 cm). Elution was performed with 0.025 M Tris-HCl buffer (pH 7.8). Alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), *a*-chymotrypsinogen (25 kDa), and cytochrome *c* (13 kDa) were used as standard proteins. The void volume (V_0) was measured with Blue Dextran 2000.

The homogeneity and molecular weight of the subunits were determined by 10% denaturing PAGE in a PROTEAN II xi system (Bio-Rad, United States). The analyzed sample (50 μL) containing 50 μg of the protein was mixed with 50 μL of the sample buffer (62.5 mM Tris-HCl buffer, pH 6.8) containing 0.1% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, and 1% sodium dodecyl sulfate. The treated samples were introduced into the gel in the amount of 10 μg of the protein per lane. The marker proteins used were β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), and RNase Bsp 981 (25 kDa).

The homogeneity of the resultant preparation was also confirmed by native gradient PAGE. The gel concentration gradient was 10 to 30%. Electrophoresis was carried out in a Helicon VE4 chamber. On completion of the electrophoresis, the gel was stained first by the *o*-dianisidine method and then with Coomassie Brilliant Blue R 250 to confirm the absence of foreign proteins.

The L-isomers of lysine, leucine, phenylalanine, citrulline, alanine, asparagine, histidine, ornithine, glutamine, threonine, tyrosine, isoleucine, and valine, as well as glycine and D-lysine, were used for determining the substrate specificity. The substrate and LO concentrations were 0.5 mM and 5 μg/mL, respectively.

The dependence of LO activity on pH was determined in the following buffer solutions: Tris-phosphate buffer, pH 6.0–8.5 (0.05 M), and glycine buffer, pH 8.0–9.5 (0.05 M). The LO concentration was 5 μg/mL.

Thermostability was determined by incubation of the enzyme (5 μg/mL) in Tris-HCl buffer, pH 7.8 (0.025 M) for 1 h at 20, 28, 37, 50, 60, 70 and 80°C. The activity was then measured as described above.

RESULTS AND DISCUSSION

Fig. 1 shows the data on accumulation of the extracellular LO in the culture liquid during the growth of the fungus in submerged culture on wheat bran under salt stress conditions (6% NaCl). Since wheat bran is an insoluble heterogeneous substrate, it was difficult to measure the biomass during cultivation; therefore, LO activity was calculated as U/g substrate (wheat bran). The figure shows that LO activity was first detected on day 3–5 of cultivation and reached the maximum value on days 12–13 (5 U/mL or 90 U/g) (curve *1*). Protein concentration in the culture liquid was

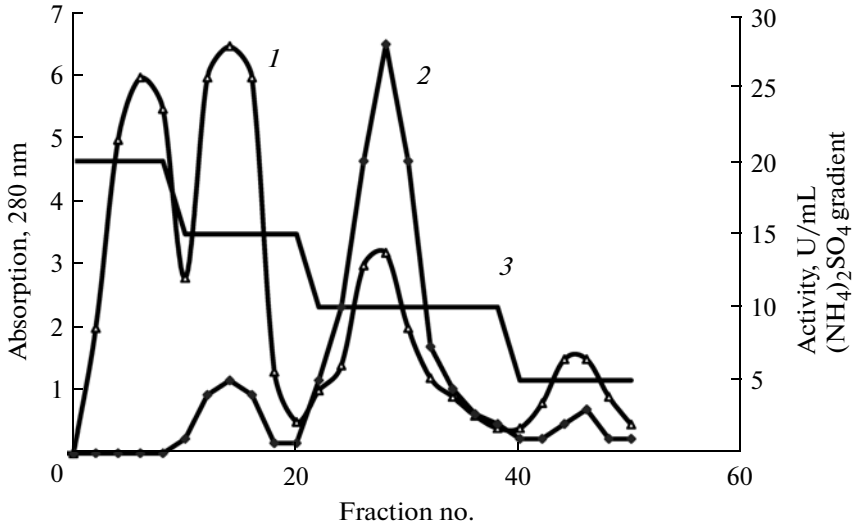

Fig. 2. Hydrophobic chromatography of L-lysine-α-oxidase on the Octyl Sepharose column. Absorption at 280 nm (*1*); activity, U/mL (*2*); and $(NH_4)_2SO_4$ stepwise gradient (*3*).

observed to increase simultaneously with the LO activity (curve *2*).

LO isolation and purification. The enzymatic protein was isolated and purified according to the following scheme.

Step 1. The culture liquid was separated from the mycelium and wheat bran by filtration through tissue and centrifugation at 6000 *g* for 30 min. The pellet was removed and copper sulfate was added to the supernatant under active stirring to a final concentration of 10 mM. The LO-containing precipitate formed after 2 h of incubation was harvested by centrifugation at 6000 *g* for 30 min. At this stage, more than 95% of the enzyme activity passed over to the precipitate. The collected precipitate was dissolved in 100 mL of 50 mM EDTA (pH 7.0) and its insoluble components were removed by centrifugation at 16000 *g* for 20 min. Ammonium sulfate and concentrated (1 M) Tris-HCl were added to the supernatant up to 25% of saturation and to 0.025 M (pH 7.5), respectively, and the mixture was incubated for 1 h at 4°C. The resultant precipitate was removed by centrifugation at 16000 *g* for 20 min. The enzyme from the supernatant was exposed to further purification.

Step 2. LO was purified by hydrophobic chromatography. Ammonium sulfate solution (25% of saturation) containing LO was injected with a peristaltic pump into an Octyl Sepharose column (2 × 30 cm) pre-equilibrated with Tris-HCl buffer (25 mM, pH 7.5) containing ammonium sulfate (25% of saturation). Then the column was washed with the same buffer. The enzyme was eluted under stepwise reduction of ammonium sulfate concentration in the buffer in the following order: 20, 15, 10, and 5% of saturation (Fig. 2). The major part of the enzymatic protein was eluted from the column when ammonium sulfate concentration decreased to 10% of saturation (Fig. 2, curve *2*), while the major part of admixture proteins was eluted from the column when the content of ammonium sulfate in the eluate was 20 and 15% of saturation (Fig. 2, curve *2*).

Step 3. LO was further purified by ion exchange chromatography on DEAE ToyoPearl. The combined active fractions of the enzyme after hydrophobic chromatography (elution by the buffer with ammonium sulfate, 10% of saturation) were dialyzed for 12 h against Tris-HCl buffer (5 mM, pH 7.5) and injected into the DEAE ToyoPearl column (2 × 40 cm) equilibrated with the Tris-HCl buffer (25 mM, pH 7.5). The column with the applied enzyme was washed with the same buffer. The enzyme was eluted at a stepwise increase of NaCl in the buffer in the following order: 0.1; 0.15; 0.2; and 0.3 M (Fig. 3). LO was found in the fraction eluted with 0.2 M NaCl (Fig. 3, curve *2*).

The results of 3-stage LO purification are summarized in the table.

Homogeneity of the resultant preparation was confirmed by denaturing and native gradient PAGE.

Determination of LO molecular mass. The molecular mass of LO (115 kDa) was determined by gel filtration on the ToyoPearl HW-55 column.

According to the data of denaturing PAGE (Fig. 4), the molecular mass of the enzyme subunit was 57–58 kDa.

Spectral characteristics of the enzyme. The spectra of LO purified to the homogeneous state are presented in Fig. 5. The spectrum was typical of a protein containing a flavin group, with maximums at 278, 390, and 465 (a shoulder at 490) nm.

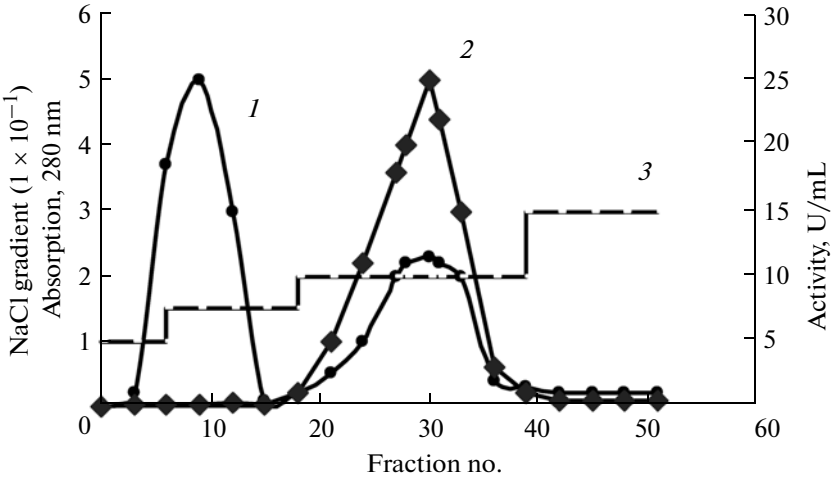

Fig. 3. Ion exchange chromatography of L-lysine-α-oxidase on DEAE ToyoPearl. Absorption at 280 nm (*1*); activity, U/mL (*2*); and NaCl stepwise gradient (*3*).

Substrate specificity of the enzyme. We found that the enzyme under consideration actually acted on L-lysine only. Insignificant activity was observed in the presence of arginine (5.8%) and L-ornithine (8.3%).

The L-isomers of leucine, phenylalanine, citrulline, alanine, asparagine, histidine, ornithine, glutamine, threonine, tyrosine, isoleucine, valine, as well as glycine and D-lysine, were also investigated as substrates. In the presence of these amino acids, the enzyme activity was zero.

Thus, the LO that we have isolated is a highly selective L-stereospecific enzyme.

Dependence of LO activity on pH. The maximum enzyme activity was observed at pH 7.8–8.2 (Fig. 6). The determined pH optimum of the isolated LO differed from the pH optimum of LO from *T. viride* Y-244 (pH 7.4) [17].

It should be noted that the study of kinetic characteristics of the isolated enzyme has revealed a cooperative effect. The peculiar features of LO kinetics (including the mechanism of cooperation) will be considered in the next report.

Thus, the 3-stage scheme for isolation and purification of L-lysine-α-oxidase synthesized by the fungus *T.* cf. *aureoviride* Rifai VKM F-4268D under salt stress conditions was developed. Two stages of fractional purification of the enzyme by salting out with ammonium sulfate and twofold centrifugation [17, 18] were replaced by a single stage of precipitation by copper sulfate. The following stages of hydrophobic (Octyl Sepharose) and ion exchange (DEAE ToyoPe-

The scheme for L-lysine-α-oxidase isolation from the culture liquid of *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D

Purification stage	Volume, mL	Total protein, mg	Total activity, U	Specific activity, U/(min mg protein)	Degree of purification, -fold
Culture liquid	1000	7000	2500	0.29	0
Precipitation by $CuSO_4$, 10 mM	100	500	2300	4.6	16
Hydrophobic chromatography on Octyl Sepharose	100	70	2000	28.5	95
Ion exchange chromatography on DEAE ToyoPearl	20	25	1800	90	310

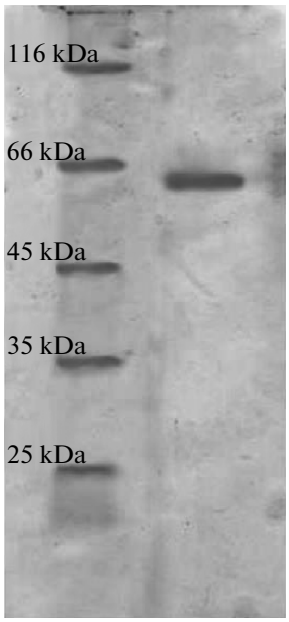

Fig. 4. SDS-PAGE of L-lysine-α-oxidase. Marker proteins: β-galactosidase, 116 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; lactate dehydrogenase, 35 kDa; Bsp 981 RNAse, 25 kDa.

arl) chromatography yielded a homogeneous enzyme preparation with a high degree of purification (310-fold) and high specific activity (90 U/mg of protein).

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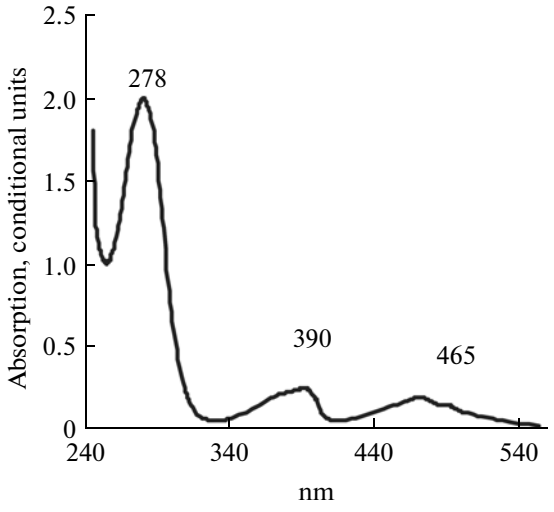

Fig. 5. Absorption spectrum of L-lysine-α-oxidase. 0.5 mg/mL LO in 0.025 M Tris-HCl, pH. 7.5.

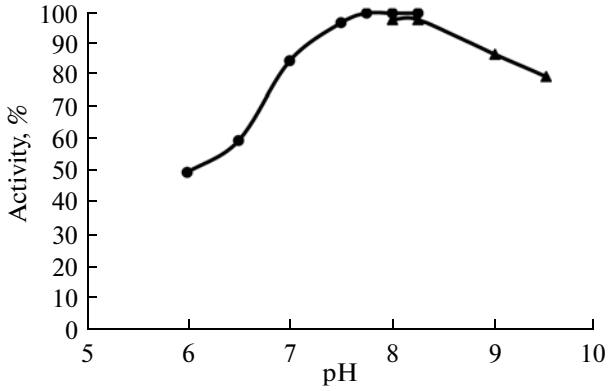

Fig. 6. Effect of pH on L-lysine-α-oxidase activity. pH 6.0–8.5, 0.05 M Tris-phosphate buffer (●–●); pH 8.0–9.5, 0.05 M glycine buffer (▲–▲).

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