# BIOPHYSICS AND BIOCHEMISTRY

# Isoforms of L-Lysine α-Oxidase from *Trichoderma sp.*

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Several purification methods were tested and the optimal procedure for obtaining L-lysine α-oxidase from fungi *Trichoderma sp.* and its isoform (minor L-lysine α-oxidase) was worked out. The enzyme and its isoform were obtained in a homogeneous state, the most important physicochemical properties were studied, and a number of differences between them were found. The most marked differences between L-lysine α-oxidase and its isoform were observed in the molecular weight (120 and 100 kD, respectively), in the isoelectric point (pI 4.4 and 5.6, respectively), and in the specific activity (90-95 and 17-20 U/mg) in experiments where L-lysine where used as substrate.

**Key Words:** *L-lysine α-oxidase; L-amino acid oxidase; isoform; purification of enzyme isoforms; pysicochemical properties*

Data on the purification of the antitumor enzyme L-lysine α-oxidase (LO) from fungi *Trichoderma sp.* [2,5,7] as well as on some catalytic and biological properties of this enzyme [3-6] were presented in previous papers. When the composition of culture fluid of *Trichoderma sp.* was examined, it was found that together with the already known LO it contains another protein which can also catalyze the oxidative deamination of L-lysine and some other L-amino acids and which was dubbed minor L-lysine α-oxidase (m-LO). Preliminary data on this new enzyme were published earlier [5].

Besides working out the optimal procedure for isolating and purifying m-LO, the aim of the present study was to obtain experimental evidence of m-LO existence and to study its main physicochemical properties.

## MATERIALS AND METHODS

For the separation of proteins the following chromatographic carriers were used; butyl-silochrom C-80 (Institute of Biotechnology Fermentas, Vilnius, Lithuanian Republic), diethylaminoethyl-granocel (DEAE-granocel) (Technical Institute, Kaunas, Lithuanian Republic), DE-52-cellulose (Whatman), DEAE-solose, DEAE-spheranite (Russian Institute of Ultrapure Substances, St. Petersburg), DEAE-spheron (Lachema), sulfopropyl-sephadex (Pharmacia), horseradish peroxidase (Reanal), o-dianisidine, L-lysine (Biolar, Latvian Republic), ovalbumin (Fluka), acrylamide, N,N'-methylenebisacrylamide, sodium dodecyl sulfate (SDS), glycine, bromophenol blue, TEMED, and Coomassie R-250 (Bio-Rad). The protein markers used were the following: phospholipase B (97.4 kD), bovine albumin (66.2 kD), ovalbumin (43 kD), trypsin inhibitor (21.5 kD), and egg lysozyme (14.4 kD) (Pharmacia). The *Trichoderma sp.* extract containing LO was obtained after cultivation of the fungi in a fermenter [1]. Different methods of enzyme purification were tested including the previously described chromotographic methods [2].

The molecular weight of subunits and enzyme homogeneity were determined by SDS polyacrylamide gel electrophoresis (PAGE). The protein prep-

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**TABLE 1.** Separation of Minor (m-LO) from Major (LO) L-Lysine α-Oxidase

| Stage of purification | Protein, mg | Total enzymatic activity, U | Specific activity, U/mg |
|---|---|---|---|
| Extract | 33516 | 4318 | 0.13 |
| Ammonium sulfate fractionation (60% saturation) | 929 | 4300 | 4.96 |
| Dialysis | 929 | 3181 | 3.40 |
| DEAE-solose chromatography | 280 | 1445 | 5.15 |
| Dialysis | 199 | 1290 | 6.50 |
| SP-sephadex chromatography m-LO | 9 | 93 | 10.3 |
| SP-sephadex chromatography LO | 18 | 1080 | 60 |

aration was boiled for 5 min in Tris-HCl buffer (pH 6.8) containing 2% SDS and 5% mercaptoethanol before being applied to the gel plate [9,11]. The electrophoresis procedure was performed in a Tris-glycine buffer system for 3-3.5 h [9]. After the electrophoresis the proteins were fixed for 2-12 h in an ethanol:water:acetic acid mixture (4.5:5.5:1.0) and stained with a 0.125% Coomassie R-250 solution in the above-described mixture for fixation. The molecular weight of the subunit was calculated according to a described method [10,11]. Isoelectric focusing of enzymes in PAGE was carried out after Westerberg [10,11] on ampholines in the pH range 3.5-10.0 at 4°C using the 8101 model assembly for electrophoresis (LKB).

The enzymatic activity of LO was assayed spectrophotometrically at 460 nm by measuring of $H_2O_2$ production using peroxidase and its chromogenic substrate o-dianisidine [1]. The standard reaction mixture contained o-dianisidine (0.19 mM) in 0.1 M sodium phosphate buffer, pH 7.4, 1.0 mM L-lysine, 0.25 μM peroxidase, and LO enzyme. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1.0 μM $H_2O_2$ per minute. Different amino acids (1.0 mM) were used as substrates for the investigation of LO substrate specificity. For the study of the influence of effectors on the enzymatic activity LO and m-LO (4 μg/ml) were incubated in 1.0 mM solutions of effectors for 2 h before the assay of enzymatic activity using L-lysine as substrate.

## RESULTS

Different carriers of the cellulose type were tested for the purification of LO: DEAE-granocel 2000, DE-52-cellulose, DEAE-solose, DEAE-spheranite, DEAE-spherone, SP-sephadex, and butyl-silochrom. Different schemes for the purification of oxidases employing the above-mentioned carriers were combined. The most rational scheme which enabled us to obtain the homogeneous enzyme preparations was chosen. It is necessary to mention that before the chromatography of LO on different carriers, it was established by the method of isoelectrofocusing of extract of *Trichoderma sp.* on ampholines that enzymatic activity was detected mainly in two protein bands — at pH 4.4 and 5.6. The protein with pI 4.4 possessing enzymatic activity was the already known and previously described enzyme LO [6]. The other protein with activity towards L-lysine was unknown prior to our experiments and we dubbed it m-LO. The differences in the isoelectric points of these two proteins were sufficient for their separation using ion-exchange chromatography. The scheme of m-LO purification is presented in Table 1. According to this scheme, it is possible to withdraw the bulk of waste proteins using ammonium sulfate fractionation, dialysis, and DEAE-solose chromatography and to concentrate LO and m-LO. The complete separation and purification of these two proteins was successfully achieved in the final stage of purification on SP-cellulose. It is best to carry out protein sorption at pH 4.75. Using this method the protein with pI 4.4 was the first to elute from the sorbent, and afterwards at pH 5.5-6.0 the other protein with enzymatic activity towards L-lysine was eluted. SDS-electrophoresis of separate fractions in PAGE attests to good separation of the two oxidases. The specific activities of LO were in the range 82-94 U/mg and of m-LO 10-17 U/mg.

**TABLE 2.** Substrate Specificity of Major (LO) and Minor (m-LO) L-Lysine α-Oxidase

| Substrate | Enzymatic activity, % | |
|---|---|---|
| | LO | m-LO |
| L-Lysine | 100 | 100 |
| L-Phenylalanine | 6.6 | 4.1 |
| L-Methionine | 0 | 3.4 |
| L-Ornithine | 5.0 | 7.8 |
| L-Histidine | 1.1 | 0 |
| L-Leucine | 2.0 | 3.8 |
| L-Arginine | 2.0 | 4.9 |
| $N_6$-Methyl-L-lysine | 200 | 86.8 |

**TABLE 3.** Influence of Effectors on Activity of L-Lysine α-Oxidase Isoforms

| Efector | Residual activity, % | |
|---|---|---|
| | LO | m-LO |
| $FeSO_4$ | 60.0 | 7.6 |
| $BaCl_2$ | | 94.0 |
| $NaOOCCH_3$ | 100.0 | 100.0 |
| $CoSO_4$ | | 85.2 |
| KCl | 100.0 | 100.0 |
| $CaCl_2$ | 100.0 | 100.0 |
| $MgSO_4$ | 88.0 | 80.6 |
| $CuCl_2$ | 65.0 | 51.3 |
| $ZnSO_4$ | | 46.2 |
| $MnCl_2$ | 100.0 | 53.2 |
| $LiSO_4$ | | 96.2 |
| FAD | 100.0 | 100.0 |
| PCMB | 0 | 2.8 |
| EDTA | 66.0 | 80.4 |
| o-Hydroxyphenanthroline | 42.0 | 100.0 |
| Hydroxyquinoline | 0 | 34.8 |
| Mercaptoethanol | 8.0 | 0 |

**Note.** Enzyme concentration 4 mg/ml; substrate L-lysine; effector concentration 1.0 μM; time of incubation 2 h. PCMB — p-chloromercuribenzoate, FAD — flavin adenine dinucleotide.

Using the scheme established (Table 1), a reasonable amount of m-LO was obtained and its properties were studied. m-LO differs significantly from LO in substrate specificity, especially in the velocities of $N_6$-methyl-L-lysine, L-arginine , L-histidine and L-methionine oxidative deamination (Table 2).

The most marked differences between LO and its isoform were found in molecular weight (120 and 100 kD, respectively) and in isoelectric point (pI 4.4 and 5.6, respectively). Significant differences were also observed in the levels of specific activity ($A_{sp}$) and Michaelis constant ($K_m$) in the reaction of L-lysine oxidative deamination: LO is characterized by $K_m$= 0.014 mM and $A_{sp}$=90-95 U/mg and m-LO is characterized by $K_m$=0.034 mM and $A_{sp}$=17-20 U/mg.

Table 3 presents data on the influence of effectors on LO isoforms. The most pronounced differences occur in the case of PCMB, o-hydroxyphenanthroline, and hydroxyquinoline.

In summary, a novel preparative method of separating and purifying LO isoforms from *Trichoderma sp.* was elaborated and their main physicochemical and catalytic properties were studied. On the basis of the data obtained it is possible to conclude that m-LO is an actually existing protein, as we speculated beforehand [5]. At present it is not possible to decide whether m-LO is a product of LO degradation or whether it is synthesized by the strain of *Trichoderma sp.* separately from the main LO. The latter question and the possibility of using m-LO as an anticancer agent call for special investigation.

## REFERENCES

1. T. T. Berezov, E. V. Lukasheva, and I. P. Smirnova, *Vopr. Med. Khim.*, **33**, No. 1, 127-132 (1987).
2. N. F. Laugalene, V. S. Vesa, and R. P. Yankevichene, *et al.*, *Ibid.*, **36**, No. 4, 88-90 (1990).
3. E. V. Lukasheva and T. T. Berezov, *Prikl. Biokhim.*, **24**, No. 4, 459-465 (1988).
4. E. V. Lukasheva, A. P. Zhukovskii, K. Soda, and T. T. Berezov, *Biokhimiya*, **56**, No. 6, 1069-1075 (1991).
5. O. L. Potapova, I. P. Smirnova, V. S. Vesa, *et al.*, *Vopr. Med. Khim.*, **38**, No. 1, 9-13 (1992).
6. S. Kh. Khaduev, E. V. Lukasheva, I. P. Smirnova, *et al.*, *Ibid.*, **34**, No. 4, 93-100 (1988).
7. N. B. Yankevich, N. F. Laugalene, V. S. Vesa, *et al.*, *Ibid.*, **35**, No. 2, 84-86 (1989).
8. O. H. Lowry, N. Z. Rosebrought, L. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265-273 (1951).
9. K. Weber, J. Pringle, and M. Osborn, *Methods Enzymol.*, **26**, 3-28 (1972).
10. O. Westerberg, and H. Swenson, *Acta Chem. Scand. [B]*, **200**, No. 3, 820-834 (1966).
11. O. Westerberg, T. Wadstrom, *et al.*, *Biochim. Biophys. Acta*, **133**, No. 13, 435-445 (1967).