



Kinetic characteristics of L-lysine α -oxidase from *Trichoderma cf. aureoviride* Rifai VKM F-4268D: Substrate specificity and allosteric effects



Vladimir I. Krupyanko^a, Alexander G. Medentsev^a, Elena V. Lukasheva^b,
Anna Yu. Arinbasarova^{a,*}

^a G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, RAS, Pushchino, Russia

^b RUDN University, RAMS, Moscow, Russia

ARTICLE INFO

Keywords:

L-lysine α -oxidase
Substrate specificity
Positive cooperativity
Hill coefficient
Michaelis–Menten constant
Kinetic scheme

ABSTRACT

The present work aims to investigate the kinetic characteristics of homodimer enzyme L-lysine α -oxidase from *Trichoderma cf. aureoviride* Rifai VKM F-4268D, taking into account allosteric effects. The enzyme was first shown to reveal positive cooperativeness, $h=2.05 \pm 0.15$. Using additional opportunities of Hill coefficient the value of the Michaelis–Menten constant has been estimated, $K_m=1.015 \cdot 10^{-5}M$, indicating high strength of substrate binding to the active site of each subunit. High selectivity and absolute *L*-stereospecificity of the enzyme were shown. The inhibition of L-lysine conversion by non-cleavable lysine analogs as well as the reaction product was found out to take place. These effects have been evaluated only as the inhibition coefficients (%). A more detailed study of these inhibition effects was complicated because of the cooperativeness of enzyme subunits mentioned above. The kinetic scheme of L-lysine α -oxidase was proposed involving parallel-subsequent action of each of two subunits in the catalytic act.

We think that the results obtained will be useful for studying the kinetic properties of other multi-subunit enzymes and improve understanding of the mechanisms of their action.

1. Introduction

The interest in L-amino acid oxidase studies is due to its potential application in biotechnology and medicine. L-Lysine α -oxidase (LysOx) (1.4.3.14) is one of the enzymes which are prospective for tumor enzyme therapy, based on a high sensitivity of tumor cells to the deficiency of growth factors including amino acids [1–4]. The enzyme catalyzes irreversible oxidative deamination of L-lysine with the yield of ammonia, hydrogen peroxide and α -keto- ξ -aminocaproic acid [3].

Earlier, we have reported [5] about LysOx isolated from the extracellular growth medium of *Trichoderma cf. aureoviride* Rifai BKM F-4268D. The homogenous enzyme with high specific activity (equal to 90 E/mg of protein) was obtained [5], and its significant anti-proliferative properties were demonstrated [4]. Considerable cytotoxicity and anti-tumor effects was shown *in vitro* against a panel of murine and human tumor cell lines and *in vivo* on murine tumors and on animals with human tumor xenografts (breast cancer SKBR3, melanoma Bro, colon cancer HCT116 and ovarian adenocarcinoma SCOV3) [4].

There are certain requirements to the enzymes for medical purposes: in addition to biological activity and low toxicity they should be characterized by suitable kinetic properties such as narrow substrate specificity and high activity. The combination of these properties determines a perspective of antitumor enzymes clinical application.

Literature data on the kinetic properties of L-amino acid oxidases including LysOx from various microorganisms are usually limited by the calculation of the Michaelis–Menten constants (K_m) and the maximum reaction rate [3,4,6–8]. Some attention was paid to the kinetic mechanism of the enzymes action [9,10], but mechanisms that would take into account the presence of two (or more) subunits and their possible cooperativity, have not been considered.

The purpose of this study is to examine a probable cooperativity of homodimeric enzyme L-lysine α -oxidase from *Trichoderma cf. aureoviride* Rifai VKM F-4268 D and evaluate Michaelis–Menten constant using Hill coefficient. The substrate specificity, the inhibition of L-lysine conversion by non-cleavable lysine analogs or the reaction product are also considered.

* Correspondence to: G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, RAS, Prospect Nauki, 5, Pushchino, Moscow region 142290, Russia.
E-mail address: ayu-arin@rambler.ru (A.Y. Arinbasarova).

2. Materials and methods

2.1. Isolation of L-lysine α -oxidase

LysOx was isolated from the fungus *Trichoderma cf. aureoviride* Rifai VKM F-4268D as described earlier [5]. Homogenous enzyme preparation of high purity (310 fold) with high specific activity (equal to 90 U/mg of protein) was used in the present work.

2.2. Determination of kinetic parameters

The rate of oxidative deamination of L-lysine or its analogs was determined spectrophotometrically at λ_{450} nm (a CF-4 DR two-beam spectrophotometer, Optica Milano, Italy). The reaction was carried out in a 0.05 M Tris-HCl buffer (pH 7.8) at 37 °C and ionic strength of 0.1 M (NaCl). The reaction medium contained o-dianisidine hydrochloride, horse radish peroxidase and the substrate at different concentrations. LysOx (5 μ g/ml) was added into the working cuvette to start the reaction.

The initial reaction velocity (v_0) was determined by the angle of the tangent slope to initial segments of the kinetic curves in not less than five parallel experiments.

To enhance the accuracy of determination of the initial reaction rates and to minimize the initial period of reaction recording (up to 2 s) a special device of fast stirring was used [11].

The value of maximum reaction rate (V) and Hill coefficient (h) were calculated using the plot of the dependence of v_0 on the L-lysine concentration (s) with software Sigma Plot 10 (USA), application of which has been illustrated in detail during analysis of enzyme inhibition and activation [12].

Relative activity of the enzyme (A , %) was determined as following:

$$A = v_{an} \cdot 100 / v_0 \quad (1)$$

The inhibition coefficients (n_i , %), was calculated by the formula:

$$n_i = (v_0 - v_i) \cdot 100 / v_0 \quad (2)$$

Root-mean-square deviations at five-fold determination were: n_i , v_0 and A does not exceed $\pm 2.5\%$, $S_{0.5}^h$, V and $h = \pm 10\%$, using the standard program "Statistic", Sigma Plot 10 (USA).

2.3. Substrate specificity

To characterize the substrate specificity of LysOx, L-lysine and the other L-amino acids were used, namely: arginine, leucine, phenylalanine, citrulline, alanine, asparagine, histidine, ornithine, threonine, tyrosine, isoleucine, valine, glutamine and glutamic acid. Glycine, D-lysine and the following analogs of L-lysine, such as N-2-trifluoroacetyl-L-lysine, 2,6-diaminopimelic acid, 6-aminocaproic acid, L-2,7-diamino heptanoic acid and 1,4-diaminobutane were also tested as possible substrates or inhibitors. α -Keto- ξ -aminocaproic acid was tested to reveal the effect of product inhibition. Concentrations of L-lysine analogs as the substrates were equal to 0.20 or 0.40 mM, and as inhibitors of L-lysine deamination –1 or 5 mM.

3. Results and discussion

3.1. Substrate specificity

We have undertaken comparative investigation of the interaction of LysOx with different L-lysine analogs. The parameters of this interaction including relative activity are given in the Table. It can be seen that the maximum activity (22%) was observed with 2,6-diaminopimelate. Some activity was registered in the presence of L-arginine (up to 12.8%) or L-ornithine (8.3%) compared with L-lysine. In the presence of the other tested substrates, including D-lysine (see "Materials and Methods") the enzyme activity was equal to 0. It should be noted that

the enzyme activity was observed when the substrates containing 3 or 4 -CH₂- groups at the radical were used. Any reduction (e.g. L-ornithine) or elongation (e.g. L-2,7-diamino heptanoic acid) of the chain by one C–C bond resulted in the loss of the capability of the enzyme to "recognize" (and convert) the substrate. Any modification of α -amino group (N-2-trifluoroacetyl-L-lysine), also leads to the loss of activity due to the difficulty of access to the active center of the enzyme.

In addition, given the fact that the length of a single C–C bond is equal to 1.53 Å [13], and that the angle between the bonds is 109°28', we can evaluate that the associative H₂NCH₂-group of the substrate is located at a distance of approximately 4.59 Å (4 CH₂-groups) from α -amino group oxidised. Assuming that the availability of the rotation angle has no significant effect on the length of the C–C bond, a distance between binding and catalytic centers of the enzyme can also be designated to be about 4.59 Å.

Thus, LysOx from *Trichoderma cf. aureoviride* Rifai VKM F-4268D was shown to be a highly selective and L-stereospecific enzyme. Among a number of substances tested, the enzyme activity was registered only with 4 substrates including L-lysine.

Both narrow substrate specificity and L-stereospecificity is characteristic of L-amino acid oxidases from various organisms [8,14]. The results obtained in our study are in agreement with literature data. The enzymes from *T. harzianum* Rifai [2], *T. viride* Y 244-2 [3], *Trichoderma* sp. 6 [6], *T. viride* i4 [7], were also shown to catalyze mainly oxidative deamination of L-lysine. Unique substrate specificity was also characteristic of recombinant LysOx from *Trichoderma viride* which was cloned in *Streptomyces lividans* TK 24 [15]. The enzymes were shown to reveal activity towards L-ornithine (18.3%), L-arginine (6.9%), L-phenylalanine (1.7%) and L-tyrosine (1.4%) along with L-lysine.

Some differences in the list of substrates converted (L-phenylalanine and L-tyrosine) may be explained by the features of measuring conditions along with possible differences in the structure of active sites of the enzymes. Moreover, the presence of impurities in the substrate samples (L-phenylalanine and L-tyrosine) may also be supposed, considering the small degrees of their conversion.

It should be noted, that high selectivity, absolute L-stereospecificity as well as high enzyme activity are underlying its use in oncological practice. On the other hand, amino acid oxidases with "narrow" substrate specificity represent as good candidates to obtain an enzyme more suitable for biotechnological applications by enlarging their substrate specificity by means of protein engineering [8].

3.2. Inhibition of L-lysine deamination by lysine analogs

We have also examined other aspects of the interaction of LysOx with L-lysine analogs. Some non-cleavable analogs of lysine, namely 1,4-diaminobutane, L-2,7-diamino heptanoic acid, 6-aminocaproic acid, L-leucine or L-alanine at high concentrations appeared to inhibit the conversion of L-lysine (Table 1). This inhibitory effect can be explained by the formation of nonproductive complexes of substrate analogs with the enzyme. The most effective inhibitor appeared to be 6-aminocaproic acid, which has the same structure and length of the carbohydrate side chain of the radical, as that of L-lysine.

The inhibition of lysine deamination by the reaction product, α -keto- ξ -aminocaproic acid, was also revealed, the inhibition coefficient (n_i) was equal to 14.4%.

It should be noted that inhibition effects above have been evaluated only in the form of n_i (Table 1).

3.3. Enzyme kinetics

As a first step of determining the enzyme kinetics the dependence of the reaction rate on the substrate concentration was investigated. As can be seen from the Figure the function of saturation of the enzyme by the substrate is represented as the sigmoid curve.

Table 1
Parameters of interaction of L-lysine-oxidase with L-lysine analogs.

Substrate analog	A, %		n_i , %	
Concentration, M	$2 \cdot 10^{-4}$	$4 \cdot 10^{-4}$	10^{-3}	$5 \cdot 10^{-3}$
L-lysine	100	–	–	–
$H_2NCH_2-(CH_2)_3-CHNH_2-COOH$				
D-lysine	0	0	0	0
2,6-diaminopimelic acid	12	22	–	–
$HOOC-CN_2H-(CH_2)_3-CHNH_2-COOH$				
L-glutamic acid	0	0	0	0
$HOOC-CH_2-CH_2-CHNH_2-COOH$				
L-glutamine	0	0	0.6	3.6
$OC-NH_2-CH_2-CH_2-CHNH_2-COOH$				
L-ornithine	8.3	–	0	0
$H_2NCH_2-CH_2-CH_2-CHNH_2-COOH$				
L-2,7-diamino heptanoic acid	0	–	4.7	29.0
$H_2NCH_2-(CH_2)_4-CHNH_2-COOH$				
L-leucine	0	0	0	13.7
$(CH_3)_2CH-CH_2-CHNH_2-COOH$				
L-alanine	0	0	0	19.4
CH_3-CHNH_2-COOH				
L-arginine	5.8	12.8	0	0
$(NH_2)(NH)C-HN-(CH_2)_3-CHNH_2-COOH$				
1,4-diaminobutane	–	–	1.7	–
$H_2NCH_2-CH_2-CH_2-CH_2-NH_2$				
N-2-trifluoroacetyl-L-lysine	–	0	–	0
$H_2NCH_2-(CH_2)_3-CH(C_2HO_2F_3)NH_2-COOH$				
6-aminocaproic acid	0	0	6.0	38.0
$NH_2CH_2-(CH_2)_4-COOH$				

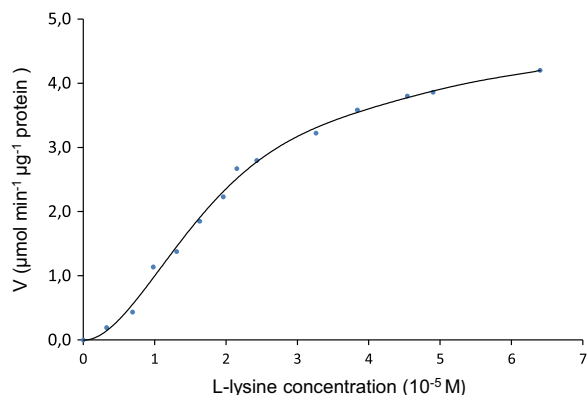


Fig. 1. Dependence of initial reaction velocity (V) on L-lysine concentration.

As it was shown earlier [5], LysOx is a homodimeric molecule with identical subunits (M.M. equal to 57–58 kDa), that assumes a cooperative substrate binding. With this in mind, we used three-parameter Hill equation to process the data presented in Fig. 1:

$$v_0 = V \cdot s^h / (s^h + s_{0.5}^h) \quad (3)$$

where v_0 is initial reaction rate, V is the maximum reaction velocity, $s_{0.5}$ is a substrate concentration at which the reaction rate is half of V , h – Hill coefficient. Dimensionless Hill coefficient, as is known [16], characterizes the amount of the enzyme's subunits which participate in the formation of an enzyme-substrate complex and the strength of their binding in complex simultaneously.

Processing these data with the program "Statistics" (Sigma Plot 10) yielded the following results: $h = 2.05 \pm 0.15$, $V = 4.36 \pm 0.14 \mu\text{mol} \cdot \text{min}^{-1} \mu\text{g}^{-1} \text{protein}$ and $s_{0.5} = (2.03 \pm 0.07) \cdot 10^{-5} \text{M}$. Hill coefficient (h) showed a positive cooperativity of two enzyme's subunits participating in the formation of an enzyme-substrate complex.

It was the effect of cooperativity that did not allow us to study the inhibition of lysine deamination both by non-cleavable lysine analogs and the reaction product in details. These effects have been estimated only as n_i .

It is clear from Eq. (4) that $s_{0.5}$ will take the meaning the Michaelis

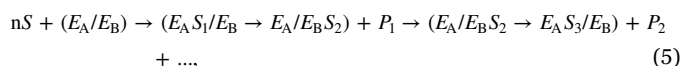
constant only when $h=1$.

$$v_0 = V s^h / (s^h + s_{0.5}^h) \rightarrow V s / (s + K_m) \rightarrow V / (1 + K_m/s) \quad (4)$$

Because h is not equal to 1 the parameter $s_{0.5}$ can't be used to characterize the binding strength of the substrate to the active site of enzyme.

3.4. Catalytic scheme of the reaction

We consider that the enzymatic mechanism should involve the participation of two identical subunits (two active sites) of LysOx in catalytic act. According to our vision at the first (associative) stage an active center of the first subunit "recognizes" terminal H_2NCH_2 -group of the substrate followed by landing on this site (associative site) and further relocation closer to the catalytic site. This strengthens the process of binding the next substrate molecule on the second active site of the enzyme (positive cooperativity $h > 1$). Removing the substrate molecule from the first active site after oxidation α -amino group is a signal to the oxidation of the second substrate on the second active center and landing the third substrate on the first active site, and the act of catalysis goes on.



where S is a substrate molecule, $E_A=E_B$ -enzyme subunits, P -product molecule.

Thus, the kinetic mechanism of LysOx was proposed to involving parallel-subsequent action of each of two subunits in the catalytic act.

3.5. Evaluation of the Michaelis-Menten constant

The kinetic investigations of two-subunit enzymes including LysOx or the other L-amino acid oxidases from various organisms are presented in the current literature [1–10], but no aspects of cooperativeness were taken into account for mathematical expression of the reaction kinetics. And Michaelis–Menten (K_m) constants have been estimated for enzymatic reactions controlled obviously by three-parameter Hill equation rather than two-parameter Michaelis–Menten equation [18].

To estimate the K_m we used the equation that has been developed earlier for cooperative (allosteric) enzymes using the Hill coefficient [17]:

$$K_{m(A)} = s_{0.5(A)}/h = s_{0.5(A)}/(1 + 1) \quad (6)$$

Substitution of the experimental data from Fig. 1 into the Eq. (6) allows us to calculate the constant:

$$K_{m(A)} = 2.03[1/(1_A + 1_B)] \cdot 10^{-5} \text{M} = 2.03(1/2) \cdot 10^{-5} \text{M} = 1.015 \cdot 10^{-5} \text{M} \quad (7)$$

The resulting value of K_m indicates high strength of substrate binding to the active site of each subunit ($\approx 10^{-5} \text{M}$). The affinity for the substrate is an important parameter of the therapeutic potential of the enzyme. The more enzyme capacity, the higher catalytic and therapeutic efficiencies.

On the other hand, the high affinity for the substrate and high specificity are essential for biocontrol and symbiotic interactions of the fungus-producer with plants, thereby predetermining the physiological role of the extracellular enzyme LysOx.

4. Conclusions

Thus, LysOx from *Trichoderma cf. aureoviride* Rifai VKM F-4268 D is high selective allosteric enzyme with positive cooperativeness, $h=2.05$. Using additional opportunities of Hill coefficient the value of the Michaelis–Menten constant has been estimated

($K_m=1.015 \cdot 10^{-5}$ M), indicating high strength of substrate binding to the active site of each subunit. The kinetic scheme of L-lysine- α -oxidase was proposed involving parallel-subsequent action of each of two subunits in the catalytic act. Allosteric effects make it difficult to study in detail the inhibition effects: the inhibition of L-lysine conversion both by non-cleavable analogs of lysine and the reaction product have been estimated only as n_i (%).

A high affinity for the substrate and strict selectivity revealed in this study, along with a high specific activity shown earlier, suggest both the possibility of therapeutic use in clinical oncology and a great biotechnological potential of the enzyme.

Moreover, these parameters may underlie physiological role of extracellular enzyme and its involvement in the relationships “*Trichoderma* – plant – pathogens”.

In addition, we think that the results obtained will be useful for studying the kinetic properties of other multi-subunit enzymes and improve understanding of the mechanisms of their action.

Acknowledgements

This work was partially supported by Scientific and technical program of Moscow Government “Development and practical realization in health care of new methods and drugs of prophylaxis, diagnosis and therapy of oncological, infection and other dangerous diseases”(Nos. 030521-3-000 and 30-7804/198 3K).

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.11.003>.

References

- [1] E.V. Lukasheva, T.T. Berezov, L-Lysine α -oxidase: physicochemical and biological properties, *Biochemistry* 67 (2002) 1152–1158 (Moscow).
- [2] H.M. Treshalina, E.V. Lukasheva, L.A. Sedakova, G.A. Firsova, G.K. Guerassimova, N.V. Gogichayeva, T.T. Berezov, Anticancer enzyme L-lysine alpha-oxidase: properties and application perspectives, *Applied Biochem, Biotechnol* 84 (2000) 267–273.
- [3] H. Kusakabe, K. Kodama, F. Kuninaka, Y. Yoshino, H. Misono, K. Soda, A new antitumor enzyme, L-lysine alpha-oxidase from *Trichoderma viride*. Purification and enzymological properties, *J. Biol. Chem.* 255 (1980) 976–981.
- [4] V.S. Pokrovsky, H.M. Treshalina, E.V. Lukasheva, L.A. Sedakova, A.G. Medentzev, A.Y. Arinbasarova, T.T. Berezov, Enzymatic properties and anticancer activity of L-lysine α -oxidase from *Trichoderma cf. aureoviride* Rifai BKMF-4268D, *Anti-Cancer Drugs* 24 (2013) 846–851.
- [5] A. Yu Arinbasarova, V.V. Ashin, K.V. Makrushin, E.V. Lukasheva, A.G. Medentzev, T.T. Berezov, Isolation and properties of L-Lysine α -oxidase from *Trichoderma cf. aureoviride* Rifai BKM F-4268D, *Microbiologia* 81 (2012) 549–554.
- [6] K.V. Makrushin, A.G. Medentzev, A. Yu, Arinbasarova, E.V. Lukasheva, T.T. Berezov, Isolation, purification and some properties of L-Lysine alpha-oxidase from *Trichoderma* sp. 6, in: S.D. Varfolomeev, G.E. Zaikov, L.P. Krylova (Eds.), *Biochemistry and Biotechnology: Research and Development*, Nova Science Publishers, 2012, pp. 7–16.
- [7] E. Weber, K. Tonder, C. Reinbothe, K. Unverhau, H. Weide, H. Aurich, L-Lysine α -oxidase from *Trichoderma viride* i4, *Purif. Charact., J. Basic Microbiol.* 34 (1994) 265–276.
- [8] L. Pollegioni, P. Motta, G. Molla, L-Amino acid oxidase as biocatalyst: a dream too far?, *Appl. Microbiol. Biotechnol.* 97 (2013) 9323–9341.
- [9] V. Massey, B. Curti, On the reaction mechanism of *Crotalus adamanteus* L-amino acid oxidase, *J. Biol. Chem.* 242 (1967) 1259–1264.
- [10] E. Sehanobish, S. Shin, A. Sanchez-Amat, V.L. Davidson, Steady-state kinetic mechanism of *LodA*, a novel cysteine tryptophylquinone-dependent oxidase, *FEBS Lett.* 588 (2014) 752–756.
- [11] V.I. Krupyanko, *A Vector Method of Representation of Enzymic Reactions*, Nauka, Moscow, 1990 (in Russian).
- [12] V.I. Krupyanko, Additional possibility of data analysis of enzyme inhibition and activation. 8. A choice of equations for calculation of the initial reaction rates in enzyme inhibition and activation, *J. Biol. Sci.* 7 (2007) 506–513.
- [13] S. Batsanov, *Structural Chemistry, Dialog*, MGU, Moscow, 2000.
- [14] G.S. Hossain, J. Li, H.D. Shin, G. Du, L. Liu, J. Chen, L-Amino acid oxidases from microbial sources: types, properties, functions, and applications, *Appl. Microbiol. Biotechnol.* 98 (2014) 1507–1515.
- [15] M. Amano, H. Mizuguchi, T. Sano, H. Kondo, K. Shinyashiki, J. Inagaki, T. Tamura, T. Kawaguchi, H. Kusakabe, K. Imada, K. Inagaki, Recombinant expression, molecular characterization and crystal structure of antitumor enzyme, l-lysine α -oxidase from *Trichoderma viride*, *J. Biochem.* 157 (2015) 549.
- [16] B.I. Kurganov, *Allosteric Enzymes: Kinetic Behavior*, John Wiley & Sons, New York, 1982.
- [17] V.I. Krupyanko, Additional possibility of using the Hill coefficients, *Eur. Chem. Bull.* 4 (2015) 340–342.
- [18] S. Schnell, Validity of the Michaelis–Menten equation – steady-state or reactant stationary assumption: that is the question, *FEBS J.* 281 (2014) 464–472.