METHODS

UDC 577.15.08 doi: https://doi.org/10.15407/ubj90.05.098

PURIFICATION PROCEDURE AND ASSAY FOR THE ACTIVITY OF LYSYL OXIDASE

O. O. GUDKOVA, N. V. LATYSHKO, O. V. ZAITSEVA, S. G. SHANDRENKO

Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv;
e-mail: gudkovahelga@gmail.com

The goal of the present study was to extract and purify lysyl oxidase from rodent's tissues by a fast, simple, effective and inexpensive method and to develop a sensitive, time-saving lysyl oxidase specific activity assay for routine in vitro experiments. Lysyl oxidase was purified by elaborated purification procedure which relies on negative adsorption principle, that is, an effective decrease in the concentration of ballast components by the polar hydrophilic adsorbent and increasing the concentration of the protein of interest. Peroxide-coupled lysyl oxidase activity quantification methods based on luminol chemiluminescence in the presence of horseradish peroxidase as a catalyst and fluorescent detection using folic acid and Cu(II) with 1,5-diaminopentane as the substrate, were designed. Lysyl oxidase was partially purified from urea extracts of rodent's tissues. Used purification procedure ensures the fast release of 93% of ballast proteins as shown by polyacrylamide gel electrophoresis. Lysyl oxidase specific activity after purification was 10-22-fold higher than that of the original extract. The molecular mass of murine lysyl oxidase from lung and heart was estimated to be ~32 kDa. We elaborated two sensitive methods for lysyl oxidase activity quantification and fast inexpensive procedure for partial enzyme purification useful in bulky in vitro experiments.

K e y w o r d s: lysyl oxidase, chemiluminescent method, fluorometric assay, 1,5-diaminopentane, negative adsorption, kaolinite, polyacrylamide gel, densitogram.

Lysyl oxidase (LOX; EC 1.4.3.13) and members of the LOX-like family, LOXL1 – LOXL4, are copper- and lysyl tyrosyl quinone-containing enzymes catalyzing lysine-derived cross-links in extracellular matrix proteins [1]. In addition to this generally assumed role required for normal collagen and elastin biosynthesis and maturation, different biological functions have been described for lysyl oxidases. LOXs activity is associated with multiple diseases including myocardial fibrosis, chronic liver disease, and hepatic fibrosis, metastatic cancer. LOX has complex roles in cancer in which the lysyl oxidase propeptide (LOX-PP) domain has tumor-suppressor activity, while the active enzyme promotes metastasis [2, 3]. It was found that LOXs are linked to the development and metastatic progression of breast cancers, whereas LOXL2 expression may serve as a clinical biomarker for breast cancer [4]. Very recently, novel functions, such as cellular senescence and chemotaxis, have been attributed to this family of amine oxidases [5, 6]. In connection with researchers growing interest in these enzymes, availability of simple, effective purification method and a sensitive lysyl oxidase specific activity assay will be significantly helpful in their functional studies. Lysyl oxidase has been isolated and purified from a number of sources. The methods currently used are time-consuming and expensive. Purification schemes mostly involved various combinations of ion exchange, affinity and gel filtration chromatography [7]. The activity of lysyl oxidase was usually measured by tritium-release assay by Pinnel and Martin with radiolabeled substrates involving laborious vacuum distillation of the released

tritiated water [8]. In view of this, a new approach to enzyme extraction and purification, as well as an assay for enzyme activity for routine *in vitro* and *in vivo* experiments, is urgent.

The goal of the present study was to extract and partially purify LOX from rodent's tissues by fast, simple, effective and inexpensive method and to develop a sensitive, simple, time-saving LOX specific activity assay.

Materials and Methods

Purified and lyophilized lysyl oxidase was obtained in the Enzyme chemistry and biochemistry department of Palladine Institute of Biochemistry from the production strain of *Brevibacterium* sp. grown at the Trypill Biochemical Plant (20.6 units/mg protein, 32 kDa), 1,5-diamino-pentane (cadaverine) and 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol) were purchased from Fluka, catalase from bovine liver (5.000units/mg protein) and 3-aminopropionitrile fumarate salt were purchased from Sigma. Kaolinite ($Al_2Si_2O_5(OH)_4$ and all other chemicals were of the highest quality and commercially available.

Animals. Male C57BL/6 4-week-old mice and male guinea pigs (~400 g) were maintained under standard conditions. All manipulations with animals before tissues isolation were performed in accordance with European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and national requirements for the care and use of laboratory animals.

Tissue samples. Samples of rodent's frozen tissue (lung and heart) – were prepared according to Zibadi et al. with modification [9, 10]. Briefly, 200 mg of frozen tissue were homogenized with liquid nitrogen and then mixed with 0.4 ml of 0.01 M Tris/HCl buffer pH 7.2 (tissue lysis/extraction reagent) and 1 ml of 50 mM sodium borate buffer, pH 8.2 with 1.2 M urea (carbamate), (buffer #1). After ultrasonication for 1 min, the mixture was centrifuged at 12,000 g for 10 min. The supernatant was used for purification steps and LOX activity determination.

Purification procedure. Negative sorption of ballast proteins on kaolinite was applied to easy purification of LOX from murine tissue urea extracts using two approaches: multistep procedure and purification in one step. In the first case, sorbent was added to the crude extract, supernatant after homogenization and extraction in several portions (gradual addition of 0.5, 5 and 15% of kaolinite (w/v)), whereas in the second case, one-time kaolinite addition 25% was performed. The mixture was thoroughly stirred at room temperature for 15 min followed by centrifugation at 12,000 g for 10 min without refrigeration. The pellet with ballast proteins was removed and the supernatant was collected at each step of the purification, with stirring for 15 min, and centrifugation as above. We monitored the procedure at each step by determining specific activity and by performing an SDS-PAGE analysis.

Protein estimation. The protein concentration was determined by the Bradford method [11] with bovine serum albumin as a standard.

Gel electrophoresis. Analytical gel electrophoresis was carried out according to Laemmli [12].

Determination of LOX activity. Lyophilized purified lysyl oxidase from Brevibacterium sp. and rodent's tissues extracts were used for the enzyme activity assay elaboration.

Chemiluminescence assay. LOX activity was measured according to Palamakumbura and Trackman [13] with modification (namely, released hydrogen peroxide was detected using enhanced chemiluminescence of the luminol-H_2O_2-horseradish peroxidase system) [14, 15]. The reaction mixture consisted of 500 μg of protein from any source, O_2-saturated buffer #1, 10 mM 1,5-diaminopentane (substrate), 0.1 mM luminol in 0.1 M sodium borate pH 10.5, 30-50 units of horseradish peroxidase (HRP) in the presence or absence of 50 units of catalase (control #2), whereas control #1 (blank control) contained all components except substrate. The assay was also performed with enzyme preincubated for 30 min with 0.2 mM β-aminopropionitrile, specific LOX inhibitor, in order to ensure that we estimated LOX specific activity. The reaction was started by the substrate addition. Chemiluminescence intensity (CLI) was monitored at wavelength 460 nm for 30 min at 3 min intervals with a microplate reader FLx800 (USA). Chemiluminescence intensity relative to blank control (Δ CLI) was considered as H_2O_2-coupled CLI. LOX activity was determined as the rate of increase in H_2O_2 specific CLI per mg of protein. The limit of detection for H_2O_2 is 0.05 μM according to D. Idrees et al. [16].

Fluorometric assay. The method is based on the formation of an intense fluorophore between folic acid and the hydrogen peroxide released during the oxidation of the substrate 1,5-diaminopentane (cadaverine) [17]. The reaction medium contained the enzyme sample (15-500 μg of protein), the substrate

(13 mM 1,5-diaminopentane), 1 mM NaN_3 and buffer #1, in a final volume of 260 µl. The assay was also performed with enzyme preincubated for 30 min with 0.2 mM β-aminopropionitrile. Following the incubation for 30 min under aeration at 37 °C the reaction was terminated by addition of 30% $ZnSO_4$. After centrifugation at 12,000 g for 10 min, the supernatants were used for detection of H_2O_2 released during the enzymatic reaction. Samples were aliquoted to a 96-well black plate. Folic acid ($1 \cdot 10^5$ M) and $CuCl_2$ ($2 \cdot 10^{-5}$ M) solutions were prepared and added to each of the wells. After incubation for 60 min at 37 °C in the dark, fluorescence was measured (λ_{excit} = 360 nm, λ_{emis} = 460 nm) and H_2O_2 production was calibrated with H_2O_2 (ε_{240} = 43.6 $M^{-1}cm^{-1}$), and corrected for the background noise. The limit of detection for H_2O_2 is 0.5 µM. The developed fluorescence intensity was measured in a microplate fluorometer (FLx800, 'Biotek', USA). The relative fluorescence unit obtained in test samples was expressed as specific activity of LOX – (µM H_2O_2/min/mg protein).

Data analysis. Data were analyzed by Student`s *t*-test using Excel. All results are expressed as mean ± SEM. A value of $P < 0.05$ was considered significant.

Results and Discussion

Two novel biochemical assays to detect LOX enzyme activity have been developed. We used the principle that amine oxidases, including LOX, catalyze amine substrates decomposition with hydrogen peroxide formation. H_2O_2, in turn, can be accurately quantified either by luminol chemiluminescence in the presence of horseradish peroxidase (HRP) as a catalyst or another system, in particular, fluorescent detection using folic acid plus Cu(II). Cadaverine serves as a substrate for all members of the LOX family. These chemiluminescence- and fluorescent-based assays are sensitive enough for the present research.

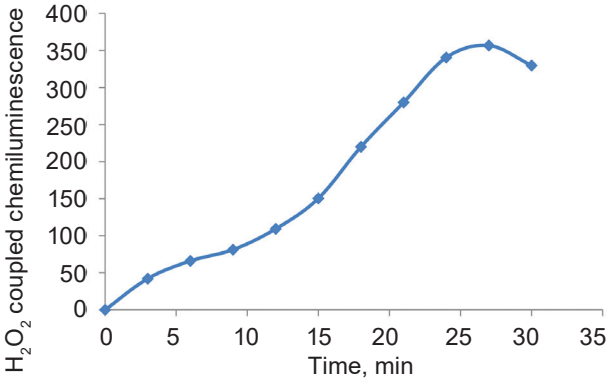

Fig. 1. H_2O_2 formation by 50 µg of lysyl oxidase from guinea pig lungs assayed by chemiluminescence-based method

Fig. 1 shows a typical time response curve of LOX from guinea pig lungs assayed by a chemiluminescence-based method. Fixed amounts of guinea pigs lung crude extract (8 mg protein/ml) were added to the reaction mixture and the reaction was run following the assay protocol as described in the Materials and Methods section. H_2O_2-dependent chemiluminescence was measured for 30 min at 3 min intervals. As seen, 25-30 min period is enough to assess LOX activity by this method. The method is sensitive and time-saving, however, some additional conditions must be observed, for example, required use of deionized water.

Data on time and dose dependence of lysyl oxidase from Brevibacterium sp. specific activity assayed by the fluorescent method are given in Fig. 2 and Fig. 3, respectively. Fixed amounts of LOX (1 mg/ml) were added to the reaction mixture and fluorescence was measured after 10, 20, 30 and 40 min. As seen from Fig. 2, 30 min is enough to

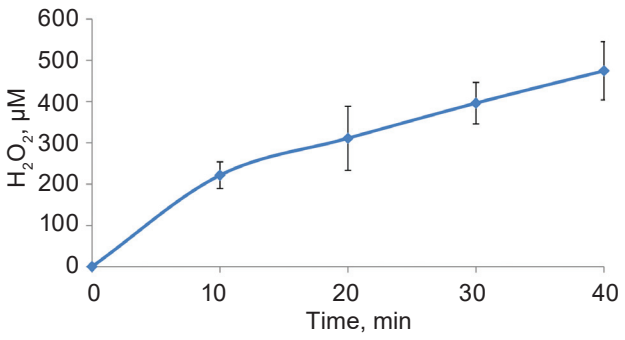

Fig. 2. H_2O_2 formation by 30 µg of lysyl oxidase from Brevibacterium sp. assayed by fluorescent method

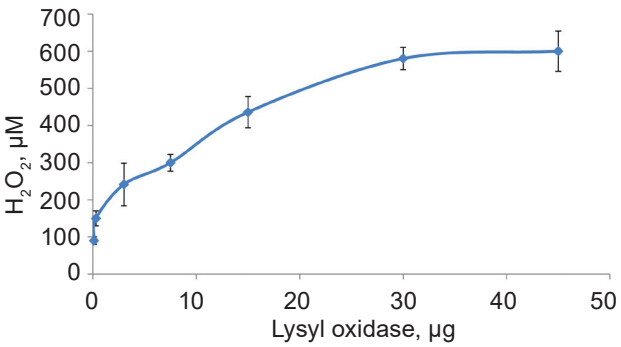

Fig. 3. Dependence of Brevibacterium sp lysyl oxidase activity on protein concentration

LOX activity assay by this method. The method is simple, sensitive, and time-saving.

Known amounts of LOX were added to the reaction mixture and reaction was run following assay protocol. The enzyme activity can be detected after 30 min of incubation at LOX protein concentration in incubation mixture as low as 1 μg/mL. High concentration of lysyl oxidase in incubation mixture (more than 2 mg/ml) may cause reduced fluorescence signal due to the non-fluorescent product formation (Fig. 3).

Results obtained by two mentioned methods of LOX activity quantification were comparable (data are not provided). At the same time, the chemiluminescent method was more laborious and complex than fluorometric one because of the need to comply with additional conditions. Therefore, the fluorometric method has been used in further studies.

Lysyl oxidase purification from rodents tissues extracts involved negative adsorption based on the principle where the enzyme molecules in incubation mixture cannot bind to the adsorbent and stay in solution (after the procedure) while separating from ballast proteins adsorbed on the polar sorbent. The factors affecting this separation process include a number of physical factors and chemical interactions that depend on adsorbent characteristics, nature of the adsorbate, its concentration, surface to volume ratio, incubation time as well as the characteristics of the background solution (pH, temperature). For this reason, the optimal conditions of the effective adsorption process were selected experimentally. Some of the major driving forces of protein adsorption include: surface energy, intermolecular forces (van der Waals dispersion forces, dipole-dipole interactions, and hydrogen bonding), hydrophobic interaction and ionic or electrostatic interaction. Electrostatic interactions play a major role in the adsorption of proteins at hydrophilic/charged surfaces [15]. Proteins can be purified by their adsorption to the polar adsorbent taking advantage of properties, such as total charge and the medium pH. It is well known that maximal protein adsorption occurs at medium pH equal to protein pI. Specifically, at a negatively charged surface such as kaolinite, only proteins with a net positive charge can be adsorbed, i.e., proteins with an isoelectric point pI higher than the pH of the solution (8.2 in our case). Thus, LOX with pI 6.2 remains in solution. The advantage of this purification method is that the adsorption does not require the sorbent activation and proceeds very quickly (15 min at room temperature).

Multistep kaolinite addition (0.5, 5, and 15% w/v) to crude extract and to active fractions obtained from previous purification steps have been used to determine the effective dose of the sorbent. The final kaolinite/protein ratio 1/4 used during the multistep procedure was applied to the one-stage purification protocol. The purification profiles of the lysyl oxidase from mouse heart and lung are shown in Table 1. The highest specific activity was obtained after the last step of the multistep procedure as well as one-stage purification protocol. LOX from mouse heart was purified 23.2-26.8 fold with an 80-78% yield, while the enzyme from mouse lung was purified 11.2-10.2 fold with a 43-46% yield. Crude extract and protein fractions containing LOX activity were subjected to electrophoresis according to Laemmli (Fig. 4). When the fractions enzyme activities were assessed by SDS-PAGE, bands at ~32 kDa were observed.

Fig. 4 shows a typical SDS-PAGE electrophoregram of mice lung and heart tissue preparations on purification steps. Lanes 1-5 correspond to preparations of mice lung LOX: lane 1 – crude extract, lanes 2-4 represent the samples after crude extract purification with 0.5, 5, and 15% of sorbent, respectively under the multistep procedure, lane 5 – one-step purification of the crude extract with 25% sorbent. Lanes 6-9, in turn, correspond to preparations of mice heart LOX: 6 – crude extract, lanes 7, 8 represent samples after purification with 5 and 15% of sorbent, respectively under the multistep procedure, lane 9 – one-step purification of the crude extract with 25% of sorbent. For these preparations, only the 32 kDa form of LOX is observed. Protein purity and relative quantitation values were calculated by Densital software from the gel densitograms of the initial extracts and purified samples. Fig. 5 and Table 2 show densitogram and an example of calculation of lysyl oxidase content in C57Bl/6 mice lung sample.

To save time and reduce workload, a one-step protocol of the enzyme purification was used in subsequent experiments since both protocols have the same efficiency with respect to yield and degree of purification.

Two sensitive methods for LOX activity quantification in biological samples from different sources were represented. Lysyl oxidase was extracted from murine tissues by a method similar to that of other

T a b l e 1. Purification of lysyl oxidase from mice tissues

Purification type	Purification step	Total protein, mg	Total activity, U	Spesific activity, U/mg	Yield, %	Purification (fold)
*Heart**						
Multistep	H0	5.32	34.5	6.50	100	1.0
	H1	1.39	30.2	21.7	87	3.3
	H2	0.18	27.7	151	80	23.2
One-step	H0	3.13	34.5	11.0	100	1.0
	H3	0.09	27.0	296	78	26.8
*Lung***						
Multistep	L0	5.66	55.3	9.77	100	1.0
	L1	4.10	42.6	10.4	77	1.1
	L2	1.76	22.5	12.8	41	1.3
	L3	0.22	23.6	109	43	11.2
One-step	L0	3.90	55.3	14.2	100	1.0
	L4	0.18	25.7	145	46	10.2

*H0 – crude extract of heart; H1 (1st step of multistep purification procedure) – supernatant H0 plus 5% caolinite (w/v); H2 (2nd step of multistep purification procedure) – supernatant H1 plus 15% caolinite (w/v); H3(one-step purification procedure) – supernatant H0 plus 25% caolinite (w/v). **L0 – crude extract of lung; L1 (1st step of multistep purification procedure) – supernatant L0 plus 0.5% caolinite (w/v); L2 (2nd step of multistep purification procedure) – supernatant L1 plus 5% caolinite (w/v); L3 (3rd step of multistep purification procedure) – supernatant L2 plus 15% caolinite (w/v); L4 (one-step purification procedure of lung) – crude extract L0 plus 25% caolinite (w/v).

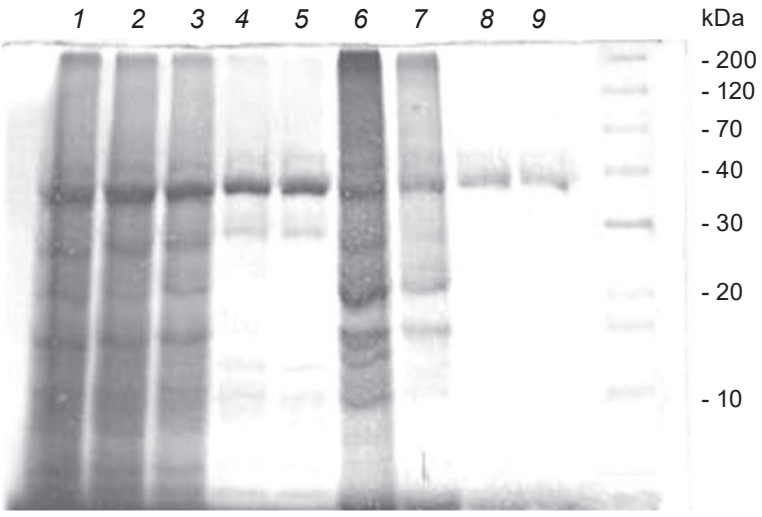

Fig. 4. SDS/PAGE analysis of partially purified lysyl oxidase from C57Bl/6 mice lung (lanes 1-5) and heart (lanes 6-9). 1 – crude extract of lung (L0), 2-4 – multistep purification procedure, 2 – supernatant L0 plus 0.5% caolinite (w/v) (L1), 3 – supernatant L1 plus 5% caolinite (w/v) (L2), 4 – supernatant L2 plus 15% caolinite (w/v) (L3), 5 – one-step purification procedure of lung crude extract L0 plus 25% caolinite (w/v) (L4), 6 – crude extract of heart (H0), 7,8 – multistep purification procedure of heart crude extract, 7 – supernatant H0 plus 5% caolinite (w/v) (L1), 7 – supernatant L1 plus 15% caolinite (w/v) (L2), 9 – one-step purification procedure of heart crude extract, supernatant H0 plus 25% caolinite (w/v) (H3), lane to the right contains molecular mass standard

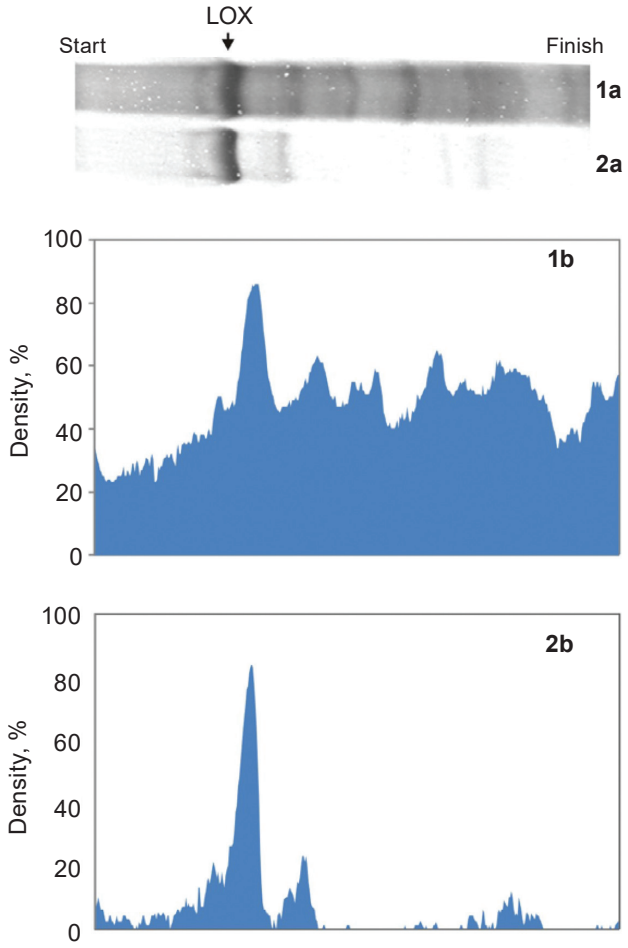

*Fig. 5. PAGE densitograms of lysyl oxidase content in C57Bl/6 mice lung crude extract (**1a**, and **1b**) and purified preparation (**2a**, and **2b**)*

Table 2. Purification of lysyl oxidase from C57Bl/6 mice lung by one-step procedure calculated by Densital software analysis of SDS/PAGE electrophoresis data (Fig. 5)

Kind of the peak	Density (Q)	
	Crude extract (line 1a)	Purified preparation (line 2a)
Total	1798	190
LOX protein	78	109
Yield, %	4.3	57.5

researchers, whereas the enzyme purification protocol was designed. LOX was purified from murine lung and heart tissues in multistep and one-step protocol (scheme) using hydrophilic sorbent kaolinite with 11.2-26.8 fold purification.

МЕТОДИ ОЧИСТКИ ТА ВИЗНАЧЕННЯ ЕНЗИМАТИЧНОЇ АКТИВНОСТІ ЛІЗИЛОКСИДАЗИ

О. О. Гудкова, Н. В. Латишко, О. В. Зайцева, С. Г. Шандренко

Інститут біохімії ім. О. В. Палладіна НАН України, Київ;
e-mail: gudkovahelga@gmail.com

Метою роботи було екстрагування та часткова очистка лізилоксидази, одержаної із тканин гризунів, простим та ефективним методом, а також розробка чутливого методу визначення його активності, який підходить для масштабних лабораторних експериментів *in vivo* та *in vitro*. Розроблений метод базується на принципі негативної сорбції полярного гідрофільного адсорбенту каоліну. Активність лізилоксидази оцінювали двома розробленими методами за кількістю пероксиду водню, який утворюється в ході реакції з 1,5-діамінопентаном як субстратом. H_2O_2 детектували або за допомогою хемілюмінесценції люмінолу в присутності пероксидази хрону, або флуорометрично з використанням фолієвої кислоти в присутності Cu (II). Застосований метод очистки лізилоксидази із тканин гризунів дозволив позбавитись від 93% баластних протеїнів, що показано за допомогою електрофорезу в ПААГ. Питома активність лізилоксидази після процедури часткового очищення була у 10–24 рази вища, ніж у вихідному екстракті. Молекулярна маса ензиму з тканин мишей становила приблизно 32 кДа. Запропоновані методи дозволяють економити час та матеріали у разі масштабних лабораторних досліджень.

Ключові слова: лізилоксидаза, 1,5-діамінопентан, хемілюмінесцентний метод, флуорометричний метод, негативна сорбція, каолін, поліакриламідний гель.

МЕТОДЫ ОЧИСТКИ И ОПРЕДЕЛЕНИЯ ЭНЗИМАТИЧЕСКОЙ АКТИВНОСТИ ЛИЗИЛОКСИДАЗЫ

О. А. Гудкова, Н. В. Латышко, О. В. Зайцева, С. Г. Шандренко

Институт биохимии им. А. В. Палладина НАН Украины, Киев;
e-mail: gudkovahelga@gmail.com

Целью работы было экстрагирование и частичная очистка лизилоксидазы, полученной из тканей грызунов простым и эффективным методом, а также разработка чувствительного метода определения его активности, который подходит для масштабных лабораторных экспериментов *in vivo* и *in vitro*. Лизилоксидазу очищали разработанным методом, основанном на принципе отрицательной сорбции полярного гидрофильного адсорбента каолина. Активность лизилоксидазы оценивали двумя разработанными методами: по количеству пероксида водорода, образованного в ходе реакции с 1,5-диаминопентаном в качестве субстрата. H_2O_2 детектировали либо с помощью хемилюминесценции люминола в присутствии пероксидазы хрена, либо флуорометрически с использованием фолиевой кислоты в присутствии Cu(II). Разработанный метод частичной очистки лизилоксидазы, полученной из тканей грызунов, позволил избавиться от 93% балластных протеинов, что показано с помощью электрофореза в ПААГ. Удельная активность лизилоксидазы после процедуры очистки была в 10–24 раза выше, чем в исходном экстракте. Молекулярная масса энзима из тканей мышей составляла около 32 кДа. Предложенные методы позволяют экономить время и материалы при масштабных лабораторных исследованиях.

Ключевые слова: лизилоксидаза, 1,5-диаминопентан, хемилюминесцентный метод, флуорометрический метод, отрицательная сорбция, каолин, полиакриламидный гель.

References

1. Rucker RB, Kosonen T, Clegg MS, Mitchell AE, Rucker BR, Uriu-Hare JY, Keen CL. Copper, lysyl oxidase, and extracellular matrix protein cross-linking. *Am J Clin Nutr.* 1998; 67(5 Suppl): 996S-1002S.
2. Bais MV, Ozdener GB, Sonenshein GE, Trackman PC. Effects of tumor-suppressor lysyl oxidase propeptide on prostate cancer xenograft growth and its direct interactions with DNA repair pathways. *Oncogene.* 2015; 34(15): 1928-1937.
3. Ikenaga N, Peng ZW, Vaid KA, Liu SB, Yoshida S, Sverdlov DY, Mikels-Vigdal A, Smith V, Schuppan D, Popov YV. Selective targeting of lysyl oxidase-like 2 (LOXL2) suppresses hepatic fibrosis progression and accelerates its reversal. *Gut.* 2017; 66(9): 1697-1708.
4. Janyasupab M, Lee YH, Zhang Y, Liu CW, Cai J, Popa A, Samia AC, Wang KW, Xu J, Hu CC, Wendt MK, Schiemann BJ, Thompson CL, Yen Y, Schiemann WP, Liu CC.Detection of Lysyl Oxidase-Like 2 (LOXL2), a Biomarker of Metastasis from Breast Cancers Using Human Blood Samples. *Recent Pat Biomark.* 2015; 5(2): 93-100.
5. Kim S, Park S, Kim Y. Alternative promoter activation leads to the expression of a novel human lysyl oxidase variant that functions as an amine oxidase. *Int J Mol Med.* 2014; 34(3): 894-899.
6. Trackman PC. Enzymatic and non-enzymatic functions of the lysyl oxidase family in bone. *Matrix Biol.* 2016; 52-54: 7-18.
7. Romero-Chapman N, Lee J, Tinker D, Uriu-Hare JY, Keen CL, Rucker RR. Purification, properties and influence of dietary copper on accumulation and functional activity of lysyl oxidase in rat skin. *Biochem J.* 1991; 275(Pt 3): 657-662.
8. Pinnell SR, Martin GR. The cross-linking of collagen and elastin: enzymatic conversion of lysine in peptide linkage to alpha-aminoadipic-delta-semialdehyde (allysine) by an extract from bone. *Proc Natl Acad Sci USA*. 1968; 61(2): 708-716.
9. Zibadi S, Vazquez R, Moore D, Larson DF, Watson RR. Myocardial lysyl oxidase regulation of cardiac remodeling in a murine model of diet-induced metabolic syndrome. *Am J Physiol Heart Circ Physiol.* 2009; 297(3): H976-H982.
10. Li S, Yang X, Li W, Li J, Su X, Chen L, Yan G. N-acetylcysteine downregulation of lysyl oxidase activity alleviating bleomycin-induced pulmonary fibrosis in rats. *Respiration.* 2012; 84(6): 509-517.

11. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72: 248-254.
12. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227(5259): 680-685.
13. Palamakumbura AH, Trackman PC. A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. *Anal Biochem.* 2002; 300(2): 245-251.
14. Diaz AN, Sanchez FG, Garcia JAG. Hydrogen peroxide assay by using enhanced chemiluminescence of luminal-H_2O_2-horse-radish peroxidase system: comparative studies. *Anal Chim Acta*. 1996; 327(2): 161-165.
15. Perez FJ, Rubio S. An improved chemiluminescence method for hydrogen peroxidase determination in plant tissues. *Plat Growth Regul.* 2006; 48(1): 89-95.
16. Khan P, Idrees D, Moxley MA, Corbett JA, Ahmad F, von Figura G, Sly WS, Waheed A, Hassan MI. Luminol-based chemiluminescent signals: clinical and non-clinical application and future uses. *Appl Biochem Biotechnol.* 2014; 173(2): 333-355.
17. Hirakawa K. Fluorometry of hydrogen peroxide using oxidative decomposition of folic acid. *Anal Bioanal Chem.* 2006; 386(2): 244-248.
18. Meissner J, Prause A, Bharti B, Findenegg GH. Characterization of protein adsorption onto silica nanoparticles: influence of pH and ionic strength. *Colloid Polym Sci.* 2015; 293(11): 3381-3391.

Received 26.12.2017