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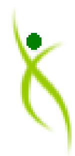

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Research Article

Characterization of L-Lysine-Α-Oxidase, A New Antitumor And Antiviral Drug Substance Synthesized By Trichoderma

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Abstract : The trigger to the studies of L-lysine-a -oxidase is of great interest for the scientists across the world. Regarding Russian Federation, the enzyme is under examination by several laboratories. Attracted by the polyfunctionality of the enzyme, the likelihood of utilizing this substance in the creation of multiple dosage forms of different therapeutic orientation. Thus, the main aim of the study is to analyze the new characteristics of L-lysine-a -oxidase as a new antitumor and antiviral drug substance synthesized by Trichoderma. To meet the study's aim, a biological experiments were carried out on guinea pigs weighing 200-250 g and CBA mice (CBA x C57Bl) weighing 18-20 g, C57 Bl, SHR.Native L-lysine-a -oxidase was injected into C57Bl mice five times intravenously at a dose of 35 U/kg. Blood was sampled every week for four weeks from the beginning of immunization (n=7), and the obtained sera of mice were analyzed by enzyme-linked immunosorbent assay according to a previously developed method. The sera were titrated in increments of 2. Tests of L-lysine-a -oxidase in mice and guinea pigs found low immunogenicity of L-lysine-a -oxidase when administered in mice at a dose of 35 U/kg, or 0.8 ml of protein/kg. Based on the results obtained, antibodies not only reduce its catalytic activity but also affect the affinity of the enzyme with the substrate. Experiments on guinea pigs have shown that native and modified L-lysine-a -oxidase at a dose of 35 U/kg *in vivo* and *in vitro* has a low allergenic activity. The allergenic activity of L-lysine-a -oxidase was investigated in comparison with other drugs. The results od this article, can be considered as one of a brand-new and effective methods for assessing the body sensitization caused by the use of enzymes and can contribute to the development of the respective field.

Keywords: Drug Substance, Antitumor, Antiviral Enzyme, L-Lysine-Α-Oxidase, Immunogenicity.

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1. INTRODUCTION

In the prevailing era, the trigger to the studies of L-lysine-α -oxidase is highly important subject for the scientists throughout the World. L-lysine α -oxidase (LO), which is deemed one of L-amino acid oxidases, defines L-lysine with the yield of H2O2, ammonia, and α -keto-ε -amino caproate[1,2]. Multiple in vitro and in vivo research have antimetastatic, cytotoxic, antitumor, and antitumor activity of LO. Contrary to asparaginase, LO holds a dual-action mechanism: depletion of L-lysine and build of H2O2, both targeting tumor expansion. Superior outcomes have been acquired on murine and human tumor models, such as human colon cancer xenografts HCT 116, LS174T, and T47D with maximum T/C 12, 37, and 36%, in turn[3,4]. The information acquired from human cancer xenografts in immunodeficient mice confirms the possibility of LO as an agent for colon cancer therapy[5-8]. The trigger to the studies of L-lysine-α -oxidase were the works by Japanese scientists, who showed its promise[1-4]. Now in the Russian Federation, the enzyme is being studied by many laboratories. Attracted by the polyfunctionality of the enzyme, the possibility of using this substance in the creation of several dosage forms of different therapeutic orientation [5-10]. However, as studies have shown, the enzyme can act as a strong inhibitor of especially dangerous plant diseases [11-13]. Plant objects may be considered as models for the study of this enzyme[14]. This paper is a continuation of studies related to the study of new properties of L-lysine-α -oxidase, an enzyme with antiviral and antitumor effect. The objective is to conduct in-depth studies of a new antiviral and antitumor drug substance, L-lysine-α -oxidase, synthetized by Trichoderma. The results of this study, can be deemed as one of a brand-new and efficient approaches to evaluate the body sensitization caused by the use of enzymes.

2. MATERIALS AND METHODS

Biological experiments[13,14] were carried out on guinea pigs weighing 200-250 g and CBA mice (CBA x C57Bl) weighing 18-20 g, C57 Bl, SHR.The study of the immunogenic properties of L-lysine-α -oxidase was carried out as follows. Native L-lysine-α -oxidase was injected into C57Bl mice five times intravenously at a dose of 35 U/kg. Blood was sampled every week for four weeks from the beginning of immunization (n=7), and the obtained sera of mice were analyzed by enzyme-linked immunosorbent assay according to a previously developed method. The sera were titrated in increments of 2.

2.1 *Study of the antigenic spectrum of L-lysine-α-oxidase*.

Hyperimmune rabbit sera[9,10] were used to assess the antigenic spectrum of the enzyme. The antigenic composition of the specimens was studied according to the data of immunoelectrophoresis (Grabar, Williams's method) at I=50 mA, t=1 h in Veronal buffer (pH=8.6), with an ionic strength of 0.05 in 1% agarose gel. The test batches of L-lysine-α -oxidase were insufficiently immunologically homogeneous specimens that contained two protein components. The values of electrophoretic mobility were as follows: for the first component - 5.6±0.24x 10^{-4} in $^{-1}$ cm^2 s^{-1}, for the second component - 7.0±0.22x10^{-4} in $^{-1}$ cm^2 s^{-1}.

2.2 Study of the allergenic activity of L-lysine-α-oxidase in vivo and in vitro

Anaphylactogenic properties of the enzyme were studied in guinea pigs in vivo, according to the data of active anaphylaxis.

2.3 Evaluation of the allergenic action of the enzymes

Active systemic anaphylaxis. The study of the allergenic activity of the native and modified L-lysine-α -oxidase of various batches[2,3] was carried out in guinea pigs. Animals were immunized intracardiacally once at a dose of 35 U/kg (estimated therapeutic dose). After 21 days, an anaphylaxis-provoking dose of 35 U/kg was administered. The experimental and control groups consisted of 8-10 animals each. The intensity of the anaphylactic reaction was assessed on a four-point scale. The experimental data were processed by calculating the anaphylactic index (AI) according to the following formula:

$$AI = \frac{(a \times 4) + (b \times 3) + (c \times 2) + (d \times 1) + (e \times 0)}{a+b+c+d+e},$$

where *a* is the number of animals who died (shock by 4+);
b is the number of animals with severe shock (3+);
c is the number of animals with moderate shock (2+);
d is the number of animals with mild shock (1+);
e is the number of shockless animals.

2.4 Study of Active Cutaneous Anaphylaxis

Active cutaneous anaphylaxis[12,13] was studied in guinea pigs, which were sensitized with a native and modified enzyme at a single dose of 35U/kg intracardiacally. Skin tests were performed by the method of allergometric testing on the 21st day, using the following enzyme concentrations: 100.0; 50.0; and 10.0 µ gb/ml. Active cutaneous anaphylaxis was performed in compliance with a laboratory-approved technique using a 0.5% Evans blue solution, which was administered intravenously before intradermal injections of the drugs. The test was considered positive if the reaction zone had dimensions of at least 5x5 mm, and the color intensity was not lower than 2+. The brightness of the color of the skin was determined on the basis of a three-plus scale. The results of skin tests were expressed in the form of two indicators: positive reaction index (PRI) and reaction intensity index (RII). PRI was calculated by the formula:

PRI = a/b-c/d/x 100,

where *a* is the number of animals with positive reactions in the experimental group;
b is the total number of animals in the experimental group;
c is the number of animals with positive reactions in the control group;
d is the total number of animals in the control group.
Each group consisted of at least five animals.
RII was calculated as follows:

$$RII = \frac{a/b - c/d}{5} \times 100, \quad (1)$$

where *a* is the total intensity (in points) of the reaction in the experimental group;
b is the total number of animals in the experimental group;
c is the total intensity of the reaction in the control group, point;
d is the total number of animals in the control group.

3. STATISTICAL ANALYSIS

In order to analyze the statistical results, Statistica for Windows Version 10.0 (Stat Soft inc., USA) is taken into consideration[7,11]. Parameters are presented in the form M±m, where M is the Mean, m is standard deviation. In the analysis of categorical group data, the criterion Pearson χ 2 with Yates correction was utilized[4-8]. Probability value (P) less than 0.05 was considered statistically significant.

4. RESULTS AND DISCUSSION

The experimental results of the study of the immunogenic properties of L-lysine-α -oxidase are presented in Table 1. It was found that the dynamics of the humoral immune response to the enzyme did not differ from the characteristics of antibody production in response to the introduction of protein antigens. The maximum content of antibodies was observed in animals on the 7-14th day of the experiment. In subsequent periods, mice showed a decrease in antibody titers.

Table 1. Immunogenic properties of L-lysine-α -oxidase

Mice line	Test day	Antiserum titer
C57Bl	7th	1/64
	14th	1/16-32
	21st	1/8

Table 2. Anaphylactogenic activity of various batches of native and modified L-lysine-α -oxidase

Drug batch	Number of guinea pigs per group	Anaphylactic index / AI M±*m*	P *	Sensitizing doze	
				U/kg	μ g protein/kg
1. L-lysine-α -oxidase / native /	8	0.8±0.3/4-1/	>0.05	35	0.77
2. L-lysine-α -oxidase / native /	10	1.2±0.2 /1-2/	>0.05	35	0.35
3. L-lysine-α -oxidase / native /	9	1.0±0.3/1-3/	>0.05	35	0.41
4. L-lysine-α -oxidase / native /	8	1.3±0.2 /1-4/	>0.05	35	0.35
5. L-lysine-α -oxidase / modified /	9	1.0±0.3/1-5/	>0.05	35	1.09

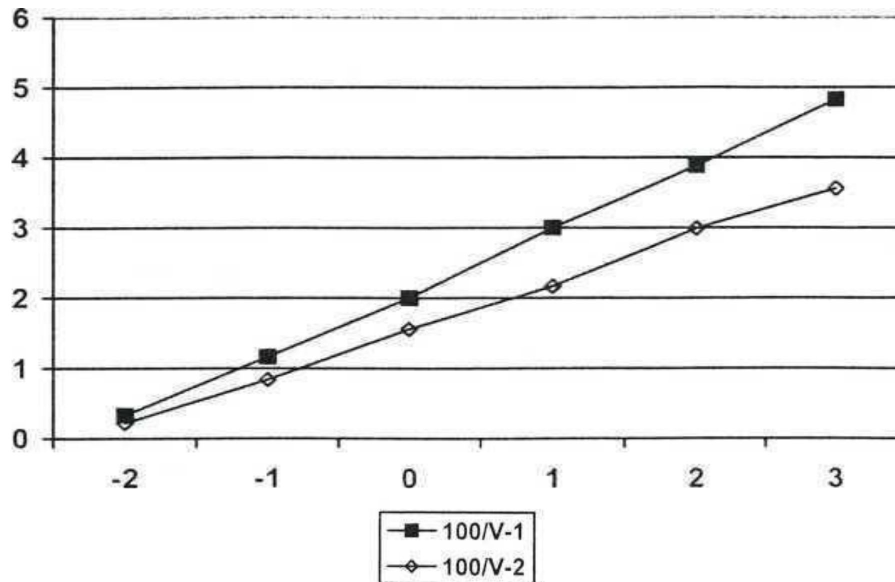
Notes:1. P* - the level of significance in comparison with the various groups indicated in brackets.

2. For sensitization, the drug was administered at a single therapeutic dose of 35 U/kg, intracardiacally. Permissive injection was performed 21 days later at the same dose, intravenously. The enzyme-linked immunosorbent assay (ELISA) showed very low titers, which indicates the low immunogenicity of the drug in the tested dose of 35 U/kg, or 0.8 mg protein/kg. These results can be compared with the studied immunogenicity of the *E. coli* L-asparagnase antitumor drug, approved by the Pharmaceutical Committee for clinical use. Five-fold administration of L-asparagnase at a dose of 300 U/kg, which corresponds to 2.0 mg/kg, leads to an increase in the antibody titer to 1/256, significantly exceeding the titers to L-lysine-α -oxidase (1/64)[10-14]. Thus, the intensity of the immune response to L-lysine-α -oxidase does not exceed or even is somewhat lower than other enzyme drugs approved for use.

4.1 Study of the antigenic spectrum of L-lysine-α-oxidase

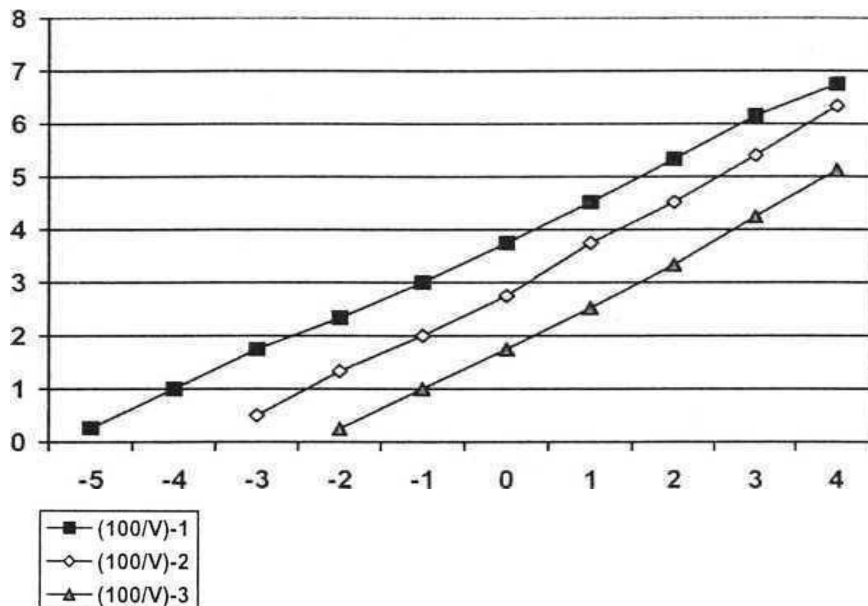
Results of the study of the effect of normal and immune blood sera on the activity of L-lysine-a-oxidase. At the first stage of the work, the relationship of the reaction rate on the substrate concentration was tudied according to the proposed earlier method [3]. Various concentrations of the L-lysine and enzyme substrate were studied in the range of 21.38 x 10^{-4} up to 10^{-3}M and 2.3 x 10^{-3} up to 13.85 x 10^{-3} U/ml, respectively. The reaction rate was calculated from the peak of the increase in optical density at 450 nm per unit time/OU/min/. The transformation in the Lineweaver-Burk coordinates made it possible to identify a regular increase in the maximum reaction rate with an increase in the amount of the introduced enzyme

in the selected range of enzyme concentrations. K_m, determined by the graphical method, is 4.76 x 10^{-4} (Fig. 1). When studying the effect of humoral factors on the enzyme, blood serum of normal and immune animals was used. The amount of blood serum added to the reaction mixture ranged from 0.675 to 6 μ l. Special experiments found that normal rabbit serum added to the enzyme solution in the indicated amounts practically does not affect the activity of L-lysine-a-oxidase[8-11] (Fig. 2).



1 – enzyme concentration 3.45×10^{-3}U/ml; 2 – enzyme concentration 2.3×10^{-3} U/ml; X axis – $\frac{1}{S}\times10^3$; Y axis – $\frac{1}{V}\times10^2$.

Fig. 1:Lineuyver-Burke graph



1 – 5 μl; 2 – 2.5 μl; 3 – no serum was added. X axis – $\frac{1}{S}\times10^3$; Y axis – $\frac{1}{V}\times10^2$.

Fig. 2: The effect of immune sera on the activity of L-lysine-α -oxidase

At the same time, immune sera had a suppressive effect on the enzyme activity which increased together with their content in the reaction medium. L-lysine-α -oxidase activity was noted in those cases when the immune sera were added immediately before the measurement of the activity. If the enzyme was preincubated with antibodies, the inhibitory effect increased[14,15]. This was observed with contact duration of less than one hour. In subsequent periods, up to two hours, the enzyme activity did not change. Therefore, most experiments were carried out with an incubation time of 60 min. Under these conditions, depending on the amount of added serum, a decrease in the maximum reaction rate by 50-60% was observed. As follows from the data presented below, antibodies not only reduce the catalytic activity of L-lysine-α -oxidase but also affect the affinity of the enzyme with the substrate. Since polyclonal antibodies were used in this work, these effects can be associated with different populations of antibodies[3,11].

4.2 Study of the allergenic activity of L-lysine-α-oxidase in vivo and in vitro in guinea pigs

As Table 2 shows, a single intracardiac injection of the enzyme at an assumed therapeutic dose of 35 U/kg caused a weak sensitization of the animals' organism. In no case were severe fatal reactions reported. A weak transient allergic reaction, noted in some experimental animals, indicates a low sensitizing activity of the native enzyme. Minor differences in the manifestation of allergic reactions in groups with different batches of the enzyme may be associated with the peculiarities of the component composition of the drugs. No significant differences were found between the groups. The study of the allergenic activity of the modified enzyme also did not reveal any noticeable allergic reactions, despite the fact that the protein sensitizing dose of the modified enzyme was slightly higher than that of the native drug (Table 2).Studies of the allergenic activity of the native and modified enzyme in cross-reactions, when sensitized with the modified enzyme (the anaphylaxis-provoking dose was administered with the native enzyme) did not reported sensitization of animals[2,3,16]. The anaphylactic index was 1.0±0.3. Based on the studies[4,6,17], we can conclude that L-lysine-α -oxidase, both native and modified, has a low allergenic activity when administered parenterally at a dose of not more than 35 U/kg, and in some cases even weaker than other enzyme drugs approved for use (Solizymum, Somilaza, Terridecase, Streptodecase, etc.).

4.3 Skin sensitizing activity

The results of the study of the allergenic activity of L-lysine-α -oxidase according to the data of skin tests are shown in Table 3.

Table 3. Evaluation of allergenic activity according to skin test data

Drug batch	Concentration of test allergen solution, μ gb/ml	Positive reaction index	Reaction intensity index	P *
L-lysine-α -oxidase / native /	100	20	8	>0.05
	50	20	4	>0.05
	10	10	0	>0.05
L-lysine-α -oxidase / modified /	100	20	4	>0.05
	50	20	12	>0.05
	10	20	12	>0.05

Note:P* - the level of significance in comparison with the corresponding control.

Table 4. Allergenic activity of L-lysine-α -oxidase and other enzymes

Drug	Route of administration of the sensitizing dose	Route of administration of the anaphylaxis-provoking dose	Anaphylactic index
L-lysine-α-oxidase	Intracardiac single dose	Intravenous	1.3±0.2
Solizymum	Orally multiple dose	Intravenous	1.2±0.3
A-amylase	Orally multiple dose	Intravenous	1.2±0.2
Terrilytin	Pulmonary multiple dose	Intravenous	1.2±0.3

As Table 3 shows, both native and modified enzymes cause reactions estimated by the positive reaction index (RI) from 0 to 20. There were practically no differences in the studied parameters between the control and experimental animals. Positive reactions to large doses of test allergens were observed in groups of intact animals that did not receive the enzyme. Such reactions are due to the nonspecific action of the enzyme at the injection site[2,17]. Equally slight reactions estimated by the positive reaction index / PRI / were noted in animals sensitized with both the native and modified enzyme[14,15]. However, the introduction of the modified enzyme with a small dose of the test allergen - 10.0 μ g protein/ml caused a slightly stronger reaction. The intensity of skin reactions (RII) in animals that received the native and modified enzyme in the tested concentration range of the test allergen was rather weak but the indicator for the modified drug was slightly higher than for the native one when using doses of 50 and 10.0 μ g protein/ml[9-11]. ××

5. CONCLUSION

Summarizing the results, we can conclude about the weak skin-sensitizing activity of the enzymes. There were no statistically significant differences between the native modified enzyme. The results obtained, apparently, can be considered as one of the methods for assessing the body sensitization caused by the use of enzymes. Thus, the results of the study of the anaphylactic activity of L-lysine-α -oxidase at a dose of 35 U/kg in vivo and in vitro indicate a low allergenic potential of the enzyme. The results of a comparison of the allergenic activity of L-lysine-α -oxidase with some other enzyme drugs approved by the Pharmaceutical Committee are presented in Table 4. L-lysine-α -oxidase at a dose of 35 U/kg, or 0.8 ml of protein/kg, tested in mice, has low immunogenicity. Antibodies not only reduce the catalytic activity of L-lysine-α -oxidase but also affect the affinity of the enzyme with the substrate. L-lysine-α -oxidase, both native and modified, at a dose of 35 U/kg has a low allergenic effect on guinea pigs both in vivo and in vitro. This present study, can be utilized as one of a new and extremely effective methods for assessing the body sensitization resulted from the utilization of enzymes and can be of great assistance in the development of the respective field.

6. AUTHORS CONTRIBUTION

A.N.S., and I.G.B. verified the analytical methods. I.P.S., and V.I.K, supervised the findings of this work. I.P.S., V.I.K, I.V.P., T.I.M., A.N.S., and I.G.B. carried out the experiment. All authors discussed the results and contributed to the final manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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