Definitions of antibodies to the antitumor enzyme L-lysine-α-oxidase, the study of its immunogenic properties and allergenic activity

Definiciones de anticuerpos contra la enzima antitumoral L-lisina-α-oxidasa, estudio de sus propiedades inmunogénicas y actividad alergénica

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Abstract

The determination of antibodies to the antitumor enzyme L-lysine-α-oxidase Trichoderma harzianum Rifai F-180 was studied using enzyme-linked immunosorbent assay. It was shown that when testing the enzyme in mice and guinea pigs at a dose of 35 U / kg, the antitumor enzyme L-lysine-α-oxidase has low immunogenicity. Antibodies not only reduce catalytic activity, but also affect the affinity of the enzyme with the substrate. Guinea pigs showed that native and modified L-lysine-α-oxidase at a dose of 35 U / kg in vivo and in vitro has a weak allergenic activity.

Keywords: the antitumor enzyme, L-lysine-α-oxidase, Trichoderma.

Resumen

Se estudió la determinación de anticuerpos contra la enzima antitumoral L-lisina-α-oxidasa Trichoderma harzianum Rifai F-180 mediante un ensayo inmunoabsorbente ligado a enzimas. Se demostró que al probar la enzima en ratones y cobayas a una dosis de 35 U / kg, la enzima antitumoral L-lisina-α-oxidasa tiene baja inmunogenicidad. Los anticuerpos no solo reducen la actividad catalítica, sino que también afectan la afinidad de la enzima con el sustrato. Los conejillos de Indias mostraron que la L-lisina-α-oxidasa nativa y modificada a una dosis de 35 U / kg in vivo e in vitro tiene una actividad alergénica débil.

Palabras clave: enzima antitumoral, L-lisina-α-oxidasa, Trichoderma.

Introduction

The search for treatments for malignant neoplasms continues to be an urgent problem of modern medicine. Enzymes take precedence in cancer chemotherapy. One of the reasons for their use is the numerous literature data on the absence or sharp decrease in the enzyme systems of the catabolism of nucleic acids, proteins, amino acids and enzyme systems of the anabolism of carbohydrates. The advantage of enzyme therapy is its selective catalytic effect, that is, high specificity with respect to the individual substrate.

The work of Japanese researchers has shown the ability of the fungus Tr. viride 244-2 to produce the enzyme L-lysine-α-oxidase (EC 1.4.3.2) with antitumor activity[1-4].

Search and selection of the producer of the anti-tumor enzyme L-lysine-α-oxidase from the collection of fungal cultures at the Department of Biochemistry named after T.T. Berezov, the domestic strain Trichoderma harzianum Rifai F-180 was selected, methods for determining its activity were developed and the biosynthesis of the enzyme by the producer strain was studied in depth. It was shown that the maximum formation of the enzyme occurs by four days of culture growth, and the L-lysine-α-oxidase inductor is wheat bran, created technological regulation of enzyme production on the basis of the Experimental technological installation of IBFM RAN named after G.K. Skryabin (Pushchino city)[5,7,13-18].

A set of scientific studies was carried out regarding the physicochemical, kinetic and biological properties of this enzyme[6-12]. The positive results of the influence of a homogeneous enzyme and culture fluid of the producer strain on tick-borne encephalitis and some mycoplasmas are obtained[22,23].

It was interesting and important to also look at the effect of the enzyme on microorganisms that cause plant diseases. For this purpose, microorganisms especially dangerous for plants were selected: viruses and bacteria that cause certain losses to agricultural farms in different countries and affect the environment. We have shown the positive effect of the culture fluid of the producer strain L-lysine-α-oxidase Trichoderma harzianum Rifai F-180 and a homogeneous enzyme on the inhibition of growth of Erwinia amylovora, INSV and TRSV viruses[19-21,24].

We continued research on the enzyme as a result of interest in it with a view to the possible practical use as an antitumor agent and as a means of different therapeutic orientation[25.26-28].

Purpose: In order to determine L-lysine-α-oxidase in biological fluids and to conduct pharmacodynamics and pharmacokinetics of the drug, the task was to develop an enzyme-linked immunosorbent assay to determine antibodies to L-lysine-α-oxidase, to study its immunogenic properties and allergic effect.

Homogeneous Enzyme

In the experiments, were used the enzyme L-lysine-α-oxidase allotted from the Trichoderma harzianum Rifai F-180 culture[5-7,10], obtained in a homogeneous state, diagnostic antibodies against mouse immunoglobulin's marked with peroxidase, produced by the Federal Research Center for Epidemiology and Microbiology N.F. Gamalei

Methods

Media and Methods

Different groups of the native and modified L-lysine-α-oxidase enzyme were used in the work (see table).

Biological experiments were performed on mice of CBA / CBA x C57B1 / lines, weighing 18-20 g, C57B1, SHR and guinea pigs, weighing 200-250 g.

The method of conducting enzyme-linked immunosorbent assay (ELISA) of mouse antisera.

In the wells of a 96-well polystyrene plate, L-lysine-α-oxidase was sorbed at a concentration of 0.5 μg / ml. This solution was obtained by diluting 0.1 mol of L-lysine-α-oxidase with a phosphoric acid buffer solution with a pH of 7.2, containing 0.145 mol / L sodium chloride. The solution was poured into 0.2 ml per well and sorbed for 12-18 hours at a temperature of 5-10°C. After washing 4 times with 0.1 mol / L sodium phosphate buffered saline containing 0.5 mol / L sodium chloride and 0.15 Tween-20 (buffer B) sorbed the mouse antiserum. 0.2 ml of antiserum diluted with buffer B from 0.5 to 1/1 thousand were poured into each well and incubated for 1.5 hours at 37°C. Then, 4 times were washed with buffer and 0.2 ml of a solution of diagnostic antibodies against mouse immunoglobulins marked with peroxidase were poured into each well.

After 1.5-hour incubation at 37°C, a 4-fold washing of the wells was carried out with buffer B. Then, 0.1 ml of a substrate mixture solution (10 mg of orthophenylenediamine- (O-PDA) was added to the wells — 24 ml of 0.1 mol / l phosphate-nitrate buffer pH 5.0 and 10 μl of H20 33%). After 10 minutes, the absorbance was measured at a wavelength of 490 nm. Dilution was taken as the serum titer, at which the optical density was 2 times higher than the background.

The study of immunogenic properties was performed on mice.

CBA (CBA x C57 Bl) weighing 18-20 g, C57 Bl, SHR

The immunogenic properties of L-lysine-α-oxidase were studied as follows. Native L-lysine-α-oxidase was administered to C57B1 mice five times intravenously at a dose of 35 U / kg. Weekly for four weeks from the start of im-

munization in animals (n=7), blood was drawn and the resulting mouse sera were analyzed by enzyme-linked immunosorbent assay according to our developed technique. Serum was triturated in 2 steps.

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

Hyperimmune rabbit serum was used to evaluate the antigenic spectrum of the enzyme. The antigenic composition of the drug was studied according to immunoelectrophoresis (Grabar, Williams method) at I = 50 mA, t = 1 h in veronal buffer (pH = 8.6), with an ionic strength of 0.05 in a 1% agarose gel. It was shown that the studied batches of L-lysine-α-oxidase turned out to be insufficiently immunologically homogeneous preparations and contained two protein components. The values of electrophoretic mobilities were as follows: for the first component - 5.6 ± 0.24x10-4 in -1cm2 s-1, for the second component - 7.0 ± 0.22x10-4 in-1cm2 s one.

For testing, used serums, obtained as a result of repeated immunization of rabbits with PAF enzyme (a complete set of Freund's adjuvants).

The study of the allergenic activity of L-lysine-α-oxidase in experiments in vivo and in vitro.

The anaphylactogenic properties of the enzyme were studied in guinea pigs in experiments in vivo, according to active anaphylaxis.

Evaluation of the allergenic effect of enzymes.

Active systemic anaphylaxis. The study of the allergenic activity of native and modified L-lysine-α-oxidase of various lots was carried out in guinea pigs. Animals were immunized intracardially once at a dose of 35 U / kg (the proposed therapeutic dose). After 21 days, a permissive dose of 35 U / kg was administered. In the experimental and control groups there were 8-10 animals. The intensity of the anaphylactic reaction was evaluated on a four-point scale.

Processing of experimental data was carried out by calculating the anaphylactic index (AI) according to the following formula:

$$AI = \_ \frac{(a \times 4) + (b \times 3) + (b \times 2) + (s \times 1) + (d \times O)}{a + b + c + c + d}$$

where a is the number of animals in which death has occurred (shock by 4+);

b - the number of animals with severe shock (3+);

in - the number of animals with moderate shock (2+);

c - the number of animals with mild shock (1+);

d is the number of animals without shock.

Results and Discussion

The use of quantitative immunochemical methods based on instrumental detection of the final result, allows you to increase the sensitivity of determining the concentration of specific antibodies by 2-3 orders of magnitude compared with the method of immunoprecipitation, based on visual control of the precipitation arc formed by the complex antigen-antibody. Thanks to the use of the highly sensitive ELISA method, it is possible to evaluate the manifestation of the immunogenicity of medications earlier than using visual methods, as well as to compare the immunogenicity and the effect on the immune response of a new drug with previously studied.

We have developed a method of heterogeneous ELISA for determining the concentration of antibodies in the sera of mice immunized with L-lysine-α-oxidase, shown schematically in Figure 1. The method is based on sorption of an enzyme or preparation containing the enzyme on a surface these wells of polystyrene plate, the interaction of the immobilized enzyme with serum containing antibodies to L-lysine-α-oxidase.

Table 1. Preparations of native and modified L-lysine-α-oxidase from trichoderma

Batchnumber / Drug	Protein, mg	Activity, ED	Specificactivity E / mg
1	14,7	660	**45**
2	5,5	555	**100**
3	9,0	880	**97**
4	25,0	2120	**84**
5	8,4	840	**100**
6 (modified)	11,7	539	**46**
7(modified)	**12,0**	**385**	**32**

At this stage, specific antibodies bind to the enzyme, whereas non-specific binding is suppressed by adding tween-20 to the sorption buffer, which reduces hydrophobic interactions. The higher the concentration of antibodies to L-lysine-α-oxidase, the greater their immobilization in the well of the tablet due to binding to antigenic determinants of the protein.

At the same time, immunoglobulins remain a free determinant, allowing them to bind anti-species antibodies. We used anti-mouse antibodies labeled with peroxidase, which allowed us to determine the concentration of antibodies at the level of immobilized peroxidase using a highly sensitive substrate - a mixture of hydrogen peroxide and orthophenylenediamine (O-FDA) 10-10 -10-11 M.

Fig. 1. Scheme of heterogeneous enzyme immunoassay.

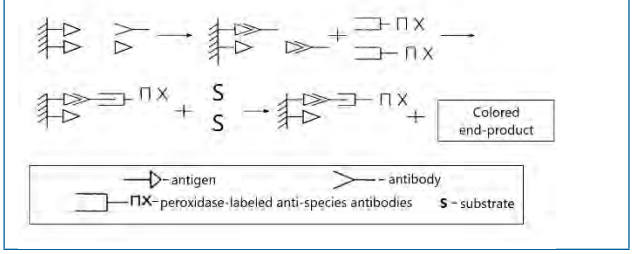
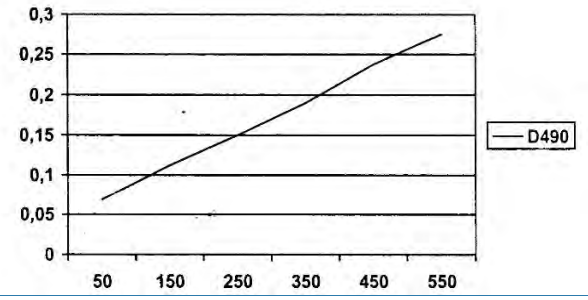


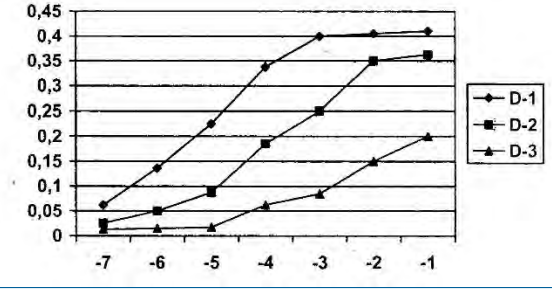
Fig. 2. The effect of the concentration of the sorbed solution of L-lysine-α-oxidase on the value of the analytical signal: on the abscissa axis - C ng / ml (concentration of the solution used for sorption on the tablet); the ordinate axis is D490 (optical density).



To work out the analysis scheme, it was necessary to study the conditions for enzyme immobilization on a tablet, the effect of the concentration of immobilized antigen on the number of bound antibodies, the effect of the incubation time of antibodies on the level of their binding in the well of a sensitized tablet.

Sorption of the sorbed enzyme L-lysine-α-oxidase (LO) in the wells of the tablet was carried out from phosphate-buffered saline with a pH of 7.2, since in preliminary experiments it was shown that during sorption of proteins in the pH range of 7.0 - 9.0 the amount of immobilized protein is almost the same. Sorption was carried out for 16-18 hours at a temperature of 4°C. Figure 2 shows the effect of the concentration of the solution used to adsorb LO on the tablet on the value of the analytical signal.

Fig. 3. Titration of mouse antiserum to L-lysine-α-oxidase (L-O) at various concentrations of adsorbed antigen: 1 - Sl-o = 500 ng / ml; 2 - With l-o = 100 ng / ml; 3 - With l-o = 50 ng / ml. On the x-axis - Log2X (X-serum dilution); along the ordinate axis - D490 (optical density).



As can be seen from Figure 3, with an increase in antigen concentration, the amount of immobilized LO also increases, which leads to a larger number of bound antibodies in the well. Therefore, to increase the sensitivity of the analysis, we chose the concentration of L-lysine-α-oxidase for sorption of 500 ng / ml. Title of a serum, i.e. the minimum tested concentration of immunoglobulins, while significantly higher than at concentrations of 100 and 50 ng / ml. The incubation time during the interaction of antibodies with an immobilized antigen and diagnostic antibodies with specific immunoglobulins is also essential for increasing the recorded signal.

Incubation was carried out at a temperature of 37°C, which significantly increased the rate of interaction.

Figures 4 and 5 show that an increase in incubation up to 1.5 hours led to an increase in signal, and a further increase in contact time did not affect the final result.

Thus, on the basis of our studies, we developed an analysis method for determining antibodies to LO, which makes it possible to identify the immunogenicity of the drug.

Fig. 4. Kinetic curve of sorption of diagnostic antibodies on a tablet sensitized with antibodies to L-lysine-α-oxidase (concentration of adsorbed antigen SL-o = 500 ng / ml): on the axis is the sorption time of mouse antisera; along the ordinate axis - D490 (optical density).

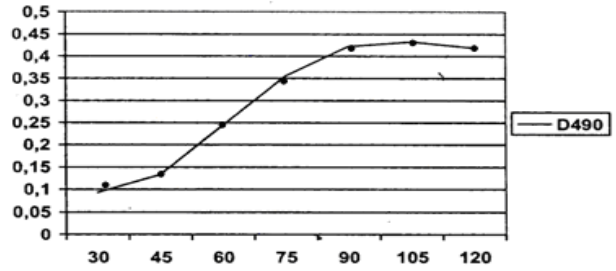


Fig. 5. Kinetic curve of sorption of mouse antiserum on a plate with immobilized L-lysine-α-oxidase: Sl-o = 500 ng / ml, the sorption time of diagnostic antibodies is 1.5 hours The abscissa shows the sorption time of diagnostic antibodies; along the ordinate axis - D490 (optical density).

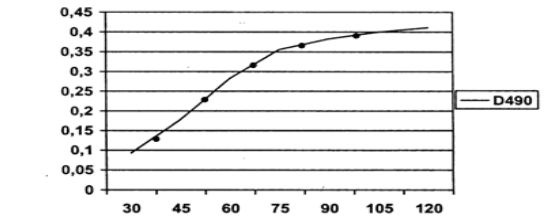


Fig. 6. Titration curves of the sera of mice immunized with L-lysine-α-oxidase and intact animals: 1 - immunized animals; 2 - intact animals. The abscissa axis is Log2X ((X is the dilution of antiserum); the ordinate axis is D490 (optical density).

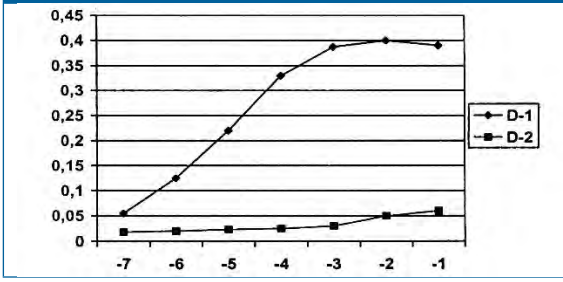


Figure 6 shows the titration curves of the sera of mice immunized with LO and intact animals. As can be seen from the figure, the titers of immune serums are small, which indicates a low immunogenicity of the drug, however, they are significantly higher than with titration of normal sera.

In further experiments, we studied immunogenic the properties of the enzyme and the allergenic effect of LO.

The results of experiments to study the immunogenic properties of L-lysine-α-oxidase are presented in table 2.

Table 2. Immunogenic properties of L-lysine-α-oxidase

Mouseline	Analysisday	Antiserumtiter
$C_{57}B1$	7-th	1/64
	14- th	1/16-32
	21- th	1/8

We found that the dynamics of the humoral immune response to the enzyme did not differ from the characteristics of antibody formation in response to the introduction of protein antigens. The maximum antibody content was observed in animals on the 7-14th day of the experiment. In subsequent periods, a decrease in antibody titers was observed in mice.

It should be noted that the titers in the enzyme immunoassay (ELISA) very low, which indicates a low immunogenicity of the drug in the tested dose of 35 U / kg or 0.8 mg protein / kg.

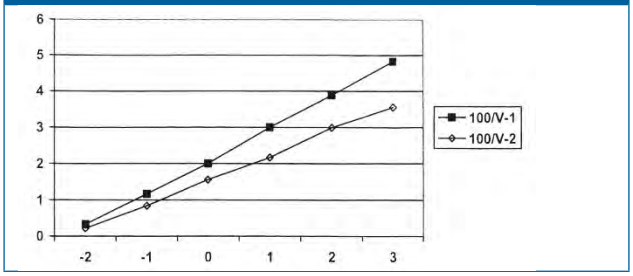
These results can be compared with the study of immunogenicity.

The well-known antitumor drug E. coli L-asparaginase approved by the Pharmacological Committee for clinical use. Five-time administration of L-asparaginase at a dose of 300 U / kg, which is 2.0 mg / kg, leads to an increase in antibody titer to 1/256, significantly exceeding the titers to L-lysine-α-oxidase (1/64).

Thus, the intensity of the immune response to L-lysine-α-oxidase does not exceed or even slightly lower than other enzyme preparations approved for use.

We studied the results of studying the effect of normal and immune serum on the activity of L-lysine-α-oxidase. At the first stage of the study, the dependence of the reaction rate on the substrate concentration was studied by the method developed above. Various concentrations of L-lysine substrate and enzyme were tested in the range of 21.38 x 10-4 to 10-3M and 2.3 x 10-3 to 13.85 x 10-3 U / ml, respectively. The reaction rate was calculated by the peak of the increase in optical density at a wavelength of 450 nm per unit time. The transformation in the Lineweaver-Burke coordinates made it possible to determine that in the selected range of enzyme concentrations, a regular increase in the maximum reaction rate with an increase in the amount of the introduced enzyme is observed. The CM determined by the graphical method is 4.76x10-4 (Fig. 7).

Fig. 7. Lineuiver-Burke Schedule:
1 - concentration of the enzyme 3.45 x 10 -3 U / ml;
2-concentration of the enzyme 2.3 x 10-3 U / ml;
The abscissa axis - 103; ordinates - 102.



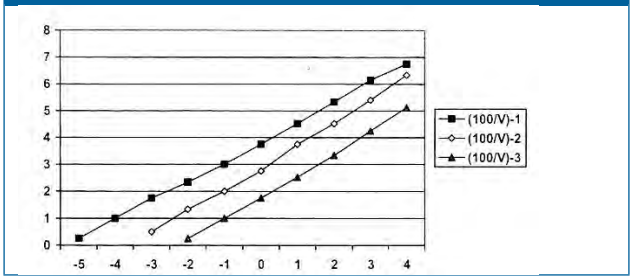
When studying the effect of humoral factors on the enzyme, we used blood serum from normal and immune animals. The amount of blood serum added to the reaction mixture ranged from 0.675 to 6 µl.

In special experiments, it was found that normal rabbit sera added to the enzyme solution in the indicated amounts practically did not affect LO activity (Fig. 8).

At the same time, immune serum suppressed the activity of the enzyme, and this effect increased with an increase in their content in the reaction medium. Appearance of L-lysine-α-oxidase activity was noted in those cases when immune serum was added immediately before the determination of activity. If the enzyme was preincubated with antibodies, the inhibitory effect increased. This was observed with a contact duration of one hour. In the subsequent periods, up to two hours, the enzyme activity did not change. Therefore, most experiments were carried out with an incubation time of 60 minutes. 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.

Fig. 8. Effect of immune sera on activity L-lysine-α oxidase.
1-5 µl; 2-2.5 µl; 3-serum was not added.
The abscissa axis - · 103; ordinates - 102.



The results of studying the allergenic activity of LO in in vivo and in vitro experiments on guinea pigs showed that a single, intracardiac administration of the enzyme at the estimated therapeutic dose of 35 U/kg caused a weak sensitization of the animal organism. In no case were severe

fatal reactions recorded. A weak transient allergic reaction observed in some experimental animals indicates a low sensitizing activity of the native enzyme. Slight differences in the manifestation of allergic reactions in groups with different lots of the enzyme may be associated with the peculiarities of the component composition of the drug. Significant differences between the groups could not be identified. When studying the allergenic activity of the modified enzyme, no noticeable allergic reactions were detected either, despite the fact that the sensitizing dose of the modified enzyme for the protein was slightly higher than that of the native drug (Table 3.).

Table 3. Anaphylactogenic activity of various parties native and modified L-lysine-α-oxidase.

Party thepreparations	Number of Guinea pigs in the Value groups	Anaphylactic index (AI) M ± m	P*	Sensitizing The dose	
				Units / kg	MGB / kg
1. L-lysine-α-oxidase (native).	8	0,8± /4-1/	> 0,05	35	0,77
2. L-lysine-α-oxidase (native).	10	1,2± /1-2/	> 0,05	35	0,35
3. L-lysine-α-oxidase (native).	9	1,0± /1-3/	> 0,05	35	0,41
4. L-lysine-α-oxidase (native).	8	1,3±/1-4/	> 0,05	35	0,35
5. L-lysine-α-oxidase (modified).	9	1,0±/1-5/	> 0,05	35	1,09

Notes: 1. The P * significance level is given in comparison with different groups indicated in parentheses.

2. For sensitization, the drug was administered in therapeutic dose 35ED / kg, intracardially, once.

Resolving injection was performed after 21 days in the same dose, intravenously.

Studies of the allergenic activity of the native and modified enzymes in cross-reactions, when they were sensitized with a modified enzyme and the resolving dose was administered with the native enzyme, did not reveal animal sensitization. The value of the anaphylactic index corresponded to 1.0 ± 0.3.

Based on the research, we can conclude that L-lysine-α-oxidase, native and modified, has a weak allergenic activity with parenteral administration at a dose of 35 U / kg no more, and in some cases even weaker than other enzyme preparations authorized for use (solism, somilase, streptodecase, etc.).

Thus, the results of the study of anaphylactic activity L-lysine α-oxidase at a dose of 35 U / kg in experiments in vivo and in vitro indicate a weak allergenic potential of the enzyme. The results of comparing the allergenic activity of L-lysine-α-oxidase with some other enzyme preparations authorized by the Pharmacological Committee are presented in table 4.

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

Drug	Method of administration of sensitizing doses.	Method for administering the allowable dose.	Anaphylactic index value
L-lysine-α-intracardiac oxidase	Intracardiac	onceintravenously	1,3± 0,2
Solizim	Orally	repeatedlyintravenously	1,2± 0,3
α-amilase	Orally	repeatedlyintravenously	1,2± 0,2
Terrilitin	Inhalation	repeatedlyintravenously	1,2± 0,3

Summary

The immunogenicity of the preparation was determined by the developed enzyme-linked immunosorbent assay for antibodies to L-lysine-α-oxidase from Trichoderma harzianum Rifai F-180.

It was shown that when testing the enzyme in mice and guinea pigs at a dose of 35 U / kg, the antitumor enzyme L-lysine-α-oxidase has low immunogenicity.

The proposed method can be used to determine enzyme in biological fluids and in the study pharmacokinetics and pharmacodynamics of the drug.

L-lysine-α-oxidase at a dose of 35 U / kg, tested in mice, has low immunogenicity.

Antibodies not only reduce catalytic activity L-lysine-α-oxidase, but also affect the affinity of the enzyme with the substrate.

L-lysine-α-oxidase, native and modified, has weak allergenic activity at a dose of 35 U / kg when in vivo and in vitro experiments on guinea pigs.

The intensity of the immune response to L-lysine-α-oxidase does not exceed or even slightly lower than some enzyme preparations, approved for using.

Conclusions

Thus, a method of heterogeneous ELISA was developed for determining the concentration of antibodies in the serum of mice immunized with L-lysine-α-oxidase, which makes it possible to detect the immunogenicity of the drug.

It was shown that the titers in the enzyme immunoassay (ELISA) are very low, which indicates a low immenogenicity of the drug at the tested dose of 35 U / kg or 0.8 mg of protein/kg. The results of studying the allergenic activity of LO in in vivo and in vitro experiments on guinea pigs showed that a single, intracardiac administration of the enzyme at the estimated therapeutic dose of 35 U / kg caused a weak sensitization of the animal organism.

Based on the research, we can conclude that L-lysine-α-oxidase, native and modified, has a weak allergenic activity with parenteral administration at a dose of 35 U / kg no more, and in some cases even weaker than other enzyme preparations authorized for use (solism, somilase, streptodecase, etc.).

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CONFLICT OF INTERESTS
The authors declare that the provided information has no conflicts of interest.

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