Volume 2, Issue 10, October 2024 ISSN (E): 2938-3765

METHODS OF DETERMINATION OF ENZYMATIC ACTIVITY IN ELYSA

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Abstract
The photometric method of recording enzyme activity is the most widely used in ELISA. In this case, enzyme substrates are substances whose transformation products are colored compounds or, conversely, the color of the substrates themselves changes during the reaction. Colored compounds absorb visible light, i.e. electromagnetic radiation with wavelengths of 400-700 nm. Light absorption obeys the Bouguer-Lambert-Bera law, according to which the optical density of a solution in a certain range is directly proportional to the concentration of the substance. A spectrophotometer is used to measure the optical density [3,5].

Introduction

Photometric Method

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Fluorimetric Method

Recently, substrates that form products registered by the fluorimetric method have become widespread in ELISA. When a molecule absorbs a photon, it changes from the ground electronic state to an excited state. The excited molecule can return to the ground state, with the excess energy transferred to heat, but there can be a reverse process of transition of the electron to the ground

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level, accompanied by the release of a quantum of light, which is called fluorescence. Due to the partial loss of energy during the transition of a molecule from excited to ground state, the wavelength of light emitted is always greater than the wavelength of light absorbed. The fraction of molecules that transitioned from the excited state to the ground state with the emission of light is determined by the quantum yield φ. The fluorescence intensity is proportional to the amount of light adsorbed by the sample [1,2,3].

Thus, it is directly proportional to the concentration of the dissolved substance and the absolute value of the initial light intensity, while in photometry the relative intensities adsorbed by the sample are compared. This fact makes it possible to increase the sensitivity of determination of a substance in solution by 1-2 orders of magnitude using the fluorimetric method compared to the photometric method [4,6].

Bioluminescence and Chemiluminescence

Enzymatic reactions whose energy is realized in the form of light radiation - bio- and chemiluminescence reactions - are used as detection systems in ELISA. The rate of such reactions is monitored by the luminescence intensity of the reaction system, which is recorded using a luminometer. Bioluminescence reactions are catalyzed by luciferases of fireflies and bacteria, and the reaction of oxidation of cyclic hydrazides by hydrogen peroxide (chemiluminescence reaction) is catalyzed by horseradish peroxidase [7,8].

Electrochemical Method

Electrochemical methods for determining the activity of enzymes used as tags in immunoassays are also known. Such sensors allow to determine the rate of enzymatic reactions in turbid media and are convenient for creating flow-through immunoassay cells. In immunoassay methods, both enzymes and their substrates can be used as tags for antigens and antibodies. If the label is an enzyme molecule, the chosen detection method must provide a signal that is proportional to the concentration of the enzyme, or, if a substrate is used as a label, to the concentration of the substrate. In the first case, the enzyme acts as a marker (it is co-valently bound to an antigen or antibody molecule), and in the second case, as a detector (free enzyme). After all immunochemical stages of any ELISA method, it is necessary to establish the concentration of the enzyme-labeled component of the immunochemical reaction, i.e. to determine the catalytic activity of the enzyme in the sample. The observed reaction rate is used to judge the concentration of the marker enzyme in the system. It should be noted that ELISA is always based on the comparative determination under identical conditions of the standard and measured sample, and therefore the requirement of speed and concentration proportionality is desirable rather than mandatory. The existence of a mutually unambiguous correspondence between the amount of the formed product of the enzymatic reaction and the amount of enzyme in the system is sufficient. However, fulfilling the condition of proportionality in a certain range of concentrations provides greater accuracy of the experiment and allows building a theoretical model with a description of the method for its optimization [4,9,10].

Main types of test systems depending on the antigens used depending on which antigens are used, immunoassay test systems are categorized into:

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- Lysate - which uses a mixture of native antigens (lysed or ultrasound-treated infectious agent obtained in culture);
- Recombinant - using genetically engineered protein analogs of specific protein antigens of the causative agent;
- Peptide - using chemically synthesized fragments of proteins.

The general direction of development of ELISA diagnostics is from lysate test systems, which are commonly called first generation test systems, to recombinant and peptide test systems. The technology of recombinant proteins production allows to obtain in a sufficiently pure form an analog of almost any single antigen. To create a high-quality recombinant test system, it is necessary to select antigens from the entire antigenic diversity of the pathogen that would be immunogenic (i.e., antibodies to these antigens should be produced in the body of an infected person) and highly specific (i.e., characteristic only for the given pathogen and, if possible, not cross-reacting with antibodies to other antigens). In addition, the quality of purification of recombinant proteins is of great importance. It is possible to obtain a recombinant test system with virtually with 100% specificity and high sensitivity [11].

Enzyme tags in ELISA There are a number of general requirements for the selection of enzyme tags in ELISA. The main ones are the following:

-high specificity and specific catalytic activity, allowing detection of the tag at low concentrations;
- availability of enzyme, possibility of obtaining sufficiently pure enzyme preparations that retain high activity after chemical modification to obtain conjugate with antigens or antibodies;
- stability under optimal conditions of antigen-antibody interaction;
- simplicity and sensitivity of the method of enzyme concentration determination.

The possibility of using enzymes as tags in ELISA is due to the high sensitivity of enzyme registration in solution. If conventional spectrophotometric or fluorimetric methods can register product formation at a concentration of 10-7 mol/L, the enzyme concentration will be 10-13 mol/L. Moreover, it is possible to significantly reduce the detection limits of enzymes both by increasing the time of enzymatic reaction and increasing the sensitivity of registration of the formed product. The most common in heterogeneous ELISA (where reagents immobilized on the surface of solid carriers are used) are:

- horseradish peroxidase,
- alkaline phosphatase
- β-D-galactosidase.

All three enzymes are determined at picomolar concentrations. Horseradish peroxidase is the most accessible. It contains carbohydrate residues easily oxidized by periodate, through which the enzyme can bind to antibodies or antigens. The substrate system for measuring peroxidase activity photometrically includes chromogens that give colored compounds when oxidized by peroxide. The catalytic activity of glucose oxidase is registered with the same chromogens as the activity of peroxidase, but the sensitivity of its determination is somewhat lower compared to peroxidase. The main advantage of the enzyme is its complete absence in blood plasma, which makes it possible to use this enzyme in homogeneous ELISA methods (reagents for all stages of ELISA are in aqueous solution) [5.6,7,8,9].

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Alkaline phosphatase and its conjugates have high stability and low detection limit, but have relatively high cost. Supersensitive enzymatic systems have been developed that allow detection of up to several thousand molecules of alkaline phosphatase in solution, based on the use of NADPH molecule as a substrate. The NAD product formed as a result of enzymatic hydrolysis is detected in the enzymatic system by cofactor regeneration [9,10].

β-D-Galactosidase is also a widely used enzyme in both homogeneous and heterogeneous ELISAs. It catalyzes the hydrolysis of lactose to form glucose and galactose. If 4 methylumbelliferyl-β -D-galactoside is taken instead of the natural substrate, the hydrolysis produces galactose and 4 methylumbelliferone, which is registered fluorimetrically. All commercial test systems use horseradish peroxidase, the choice of which is determined by its high specific catalytic activity, availability, stability, and ease of detection. As a substrate reagent, ortho-phenylenediamine (OPD) or tetramethylbenzidine (TMB) with hydrogen peroxide is most often used, the oxidation product of which is registered photometrically. To stop the enzymatic reaction, a “stop reagent” is used, which is added in equal amounts to all samples under test and control. Sulfuric acid is most often used as a “stop reagent”. The results are recorded spectrophotometrically at a wavelength of 450-490 nm.

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