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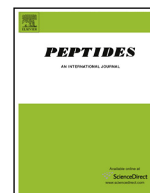

A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA

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ABSTRACT

Playing a critical role in the metabolic homeostasis of living systems, the circulating concentrations of peptides/proteins are influenced by a variety of patho-physiological events. These peptide/protein concentrations in biological fluids are measured using various methods, the most common of which is enzymatic immunoassay EIA/ELISA and which guide the clinicians in diagnosing and monitoring diseases that inflict biological systems. All the techniques where enzymes are employed to show antigen–antibody reactions are generally referred to as enzymatic immunoassay EIA/ELISA method. Since the basic principles of EIA and ELISA are the same. The main objective of this review is to present an overview of the historical journey that had led to the invention of EIA/ELISA, an indispensible method for medical and research laboratories, types of ELISA developed after its invention [direct (the first ELISA method invented), indirect, sandwich and competitive methods], problems encountered during peptide/protein analyses (pre-analytical, analytical and post-analytical), rules to be followed to prevent these problems, and our laboratory experience of more than 15 years.

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Introduction

Quantitative analytical methods that show antigen–antibody reactions through the color change obtained by using an enzyme-linked conjugate and enzyme substrate and that serve to identify the presence and concentration of molecules in biological fluids are generally called enzyme immunotests [enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA)] [16]. Very low-concentration molecules such as peptides/proteins, hormones, vitamins and drugs display a high level of specificity against antibodies or antigens developed for them [12,16,23]. This is because it is almost impossible for an antibody to be bound to a molecule other than its own antigen. Thus, this method can be used to measure even substances in very low concentrations with hardly any risk of interference. In other words, when we have the antigen which we know to be specific to a certain substance, we can identify the type and amount of its antibody and when we have the antibody, we can find out its specific antigen and the amount of antigen, using this method. All techniques and methods of analysis using enzymes to show antigen–antibody reactions are generally referred to as enzyme immunotests [12,16].

History of ELISA

Although the basic principle of ELISA and radioimmunoassay (RIA) techniques dates back to 1941 [11], RIA method was first used by Yalow and Berson in 1960s to measure the endogenous plasma insulin level [41]. In fact, ELISA method was invented simultaneously by two research teams at the same time [13,39]. However, ELISA method was pioneered largely by the Swiss scientists Engvall, and Perlmann who died in 2005 [13]. These two researchers developed the ELISA method in 1971 by modifying the RIA method [13]. In other words, they devised the immunological ELISA method by conjugating the tagged antigen and antibody radioisotopes in RIA with enzymes rather than radioactive iodine 125. They employed this new method to determine the levels of IgG in rabbit serum [13]. In the same year, a different research team succeeded in quantifying human chorionic gonadotropin amounts in the urine by using horseradish peroxidase (EC 1.11.17) enzyme with the EIA method [39]. The researchers applied for a patent both in the USA and Europe.

Abbreviations: AST, aspartate aminotransferase; CK, creatinine kinase; CZ, Czech Republic; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HCl, hydrochloric acid; H_2SO_4, sulfuric acid; LDH, lactate dehydrogenase; NaOH, sodium hydroxide; PRC, The People's Republic of China; QC1, quality control; RIA, radioimmunoassay; SA, South Africa; UK, The United Kingdom; USA, The United States of America.

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Following the invention of ELISA, a number of researchers used it: Carlson and colleagues in 1972 [10], Holmgren and Svennerholm in diagnostic microbiology in 1973 [15], Ljungstrom and colleagues to identify the presence of trichinosis in parasitology in 1974 [26], and Voller et al. to diagnose malaria in 1975 [40]. Bishai and Galli, Leinikki et al. and Ukkonen et al. made use of the ELISA method to identify infections caused by influenza, parainfluenza and mumps viruses in 1978, 1979, and 1981, respectively [6,22,38]. In 1980, Siegle et al. modified the ELISA test and incorporated microtitration plates to identify the concentrations of various hormones, peptides, and proteins [35]. The method which has found different fields of application and grown beyond infancy over time has become a routinely used method in research and diagnosis laboratories around the world.

How does the ELISA method work?

The antigen utilized in the ELISA method is bound to a solid phase. Tubes and microplates made of rigid polystyrene, polyvinyl and polypropylene are used as the solid phase. The microplates used must be able to appropriately adsorb the antigen and the antibody, but not adsorb the components in the other phases [13,41]. The enzymes that can be employed in ELISA include beta galactosidase, glucose oxidase, peroxidase, and alkaline phosphatase. Alkaline phosphatase can be stored at 4 °C with its conjugate sodium azide. Alkaline phosphatase and P-nitro-phenyl phosphate are used as substrates, are available in safe tablet forms, and produce a yellow color in positive reactions. For the peroxidase conjugate, 5 amino salicylic acid and orthophenylenediamine are used as the substrates and the production of a brown color is considered a positive reaction. If beta galactosidase is used, the sample must be read in a fluorometer. The catabolic effects of enzymes determine both the acceleration and the specificity of the immunological reaction during the enzyme-substrate reaction [12]. The enzyme-substrate reaction is usually completed within 30–60 min. The reaction can be stopped using sodium hydroxide (NaOH), hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) [16]. The results are read on a spectrophotometer and at 400–600 nm depending on the characteristics of the conjugate used.

Types of ELISA

Enzymatic immunoassay methods are considered under two general headings as homogeneous enzymatic immunoassay methods and heterogeneous enzymatic immunoassay methods [27] (Fig. 1). In the homogeneous enzymatic immunoassay methods, enzymes become inactivated when they are bound to the antibody, and thus, there is no stage (washing) where the antigen is separated from the medium. Homogeneous enzymatic immunoassay method is usually employed to measure substances in small quantities, like therapeutic drugs [27]. Homogeneous method is expensive and has low sensitivity. The only advantage it possesses is its ease of use.

As heterogeneous enzymatic immunoassay methods are more commonly used [27], the methods and types of this method are detailed in the following paragraphs. In this method, in order to the prevent interference of any molecule in the medium with it after the binding of the antigen and the antibody, the antigen–antibody complex is bound to the walls of the experiment tubes and anything other than the complex is removed from the medium through washing procedures. In other words, in heterogeneous enzymatic immunoassay methods, it is essential to have a washing stage to separate the bound antigen from the free antigen after the antigen–antibody interaction. Since the heterogeneous method is more sensitive than the homogeneous one, it is more commonly used. ELISA is a heterogeneous immunoassay technique used to detect specific antibodies and soluble antigens, and since the structure and the characteristics of the substances to be measured are not always the same, a variety of ELISA types have been developed to increase the specificity of measurement [27]. Schematic description of the homogeneous enzymatic immunoassay and heterogeneous enzymatic immunoassay methods is presented in Fig. 2.

Direct ELISA (antigen screening)

The technique was simultaneously developed in 1971 by Engvall and Perlmann [13] and by Van Weemen and Schuurs [39], the technique pioneered other ELISA types. Direct ELISA method is suitable for determining the amount of high molecule-weight antigens. The surface of the plate is coated directly with the antibody or antigen. An enzyme tagged antibody or antigen enables the measurement. Incubation is followed by washing which removes the unbound antigens or antibodies from the medium. Then the appropriate substrate is added to the medium to produce a signal through coloration. The signal is measured to determine the amount of the antigen or antibody [12,16].

Indirect ELISA

The technique was developed in 1978 Lindström and Wager [25], who were inspired by the direct ELISA method. The researchers reported measuring porcine IgG using this method. The reason why this method is called the indirect method is that what determines and separates the antigen to be measured is not the primary antibody, but another antibody that is placed in the medium. In this method, the diseased serum is added to the antigen-coated wells and the plates are incubated. During this incubation, the antibodies formed against the antigens in the diseased serum plaque produce an antigen–antibody complex. In order to render the antigen–antibody complex visible, a secondary antibody that recognizes the antibody in the serum and that is tagged with the enzyme is added. Then the substrate of the enzyme is added to the medium to produce color and the concentration is determined. This method utilized to identify antigens is used more commonly in endocrinology.

Sandwich ELISA (antibody screening)

The technique was developed in 1977 Kato and his co-workers [20]. In this ELISA method, the wells are coated with a capture antibody and blocked. The sample is added to the microplate wells coated with the antibody; then, the plate is incubated for some time and washed. Washing removes the unbound antigens. When the antigen specific to the bound antibody is found, these antigens cannot be removed. Following the washing step, antibodies that are tagged with the enzyme specific to the antigen are added and incubated. After incubation and washing, if there are antigens in the medium, these cannot be removed as the enzyme-tagged antibodies are bound to them. In order to reveal the enzyme activity, enzyme substrate is added to the medium and coloration is ensured. Coloration shows a positive result, while lack of coloration indicates lack of enzymes, or a negative result. As the relevant protein is stuck between two antibody molecules, this method is called Sandwich ELISA. Sandwich ELISAs have been reported to be 2–5 times more sensitive than all other ELISAs.

Competitive ELISA (antigen/antibody screening)

The technique was developed in 1976 Yorde and his coworkers [42]. In this method, the surface of the wells is coated with the antigen-specific antibody or antibody-specific antigen. The sample to be measured and the enzyme-tagged antigen or antibody

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