Enzyme-Linked Immunoassay of α-1-Fetoprotein in Serum by Differential Pulse Voltammetry

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Abstract
A method for the determination of α-1-fetoprotein (AFP) in human serum by using a horseradish peroxidase (HRP) label in an enzyme-linked immunosorbent assay (ELISA) is proposed. The method is based on the electrochemical determination of enzymatic reaction product with differential pulse voltammetry at a gold disk electrode. The assay consists of two successive steps. The first step is a conventional HRP-mediated ELISA for the formation of electroactive 2,2′-diaminoazobenzene by means of the *o*-phenylenediamine–H_2O_2–HRP system. At the second step, 2,2′-diaminoazobenzene exhibits a sensitive voltammetric response at −0.19 V in pH 2.0 PBS. The peak current is proportional to the concentration of AFP in the range of 0.5–400 ng/mL ($R = 0.9993$) under optimum conditions. The sensitivity of this method is higher than that of the traditional spectrophotometric ELISA procedure. The proposed method has been applied to the clinical determination of AFP in human serum with satisfactory precision and accuracy.

Keywords: α-1-Fetoprotein, Enzyme-linked immunosorbent assay, Electrochemical detection, Gold electrode, *o*-Phenylenediamine, Horseradish peroxidase, Differential pulse voltammetry

1. Introduction

α-1-Fetoprotein (AFP) is an important tumor marker for the diagnosis, and even early detection of original liver carcinoma. Its concentration in healthy human serum (in the age range of 18–40 years old) is as low as the average value of 3.4 ng/mL [1]. However, the AFP concentration rises to about 400 ng/mL in the serum of a severe liver cancer patient. Such concentrations have been determined with different methods such as one-step immunoradiometric assays (IRMA), one-step [2] or two-step enzyme-linked immunosorbent assays (ELISA) by chemiluminescene [3, 4], spectrometry [1, 5], or electrochemical detection [6–8]. Some sensors based on an oxygen probe [9, 10], pH electrode [11] or capacitance measurement [12] have also been developed to detect AFP content in serum. However, in most clinical practice the determination of AFP content is only performed with IRMA or spectrophotometric ELISA. Other developed methods have not been used in practical application yet. In IRMA technique, ^{125}I-labeled anti-AFP antibody is used as a tracer, which causes its own set of limitations such as the shelf life of ^{125}I-labeled antibody, radiation hazards and the special requirements for laboratories. In comparison with IRMA, spectrophotometric ELISA is a more welcome procedure and has been widely used to detect AFP [13]. This method is based on colorimetric reaction which is amplified by the catalysis of anti-AFP-horseradish peroxidase conjugated in the immunocomplex for decreasing the detection limit. As is well known, the sensitivity of the spectrophotometric ELISA is inadequate in many assays due to the influence of specimen color and some experimental factors. Thus, further development and refinement of ELISA is necessary for improving sensitivity, simplifying analytical procedures, shortening operation time [14], and even for in vivo clinical applications.

Among the different methods developed, electrochemical detection systems coupled with enzyme immunoassays have their own advantages, thus, they have been widely used for the development of ELISA [7–10, 15–17]. Kyoko [7] et al. proposed an amperometric detection method of AFP by using an alkaline phosphatase-labeled AFP-antibody in sandwich immunocomplex to catalyze the hydrolysis reaction of phosphate ester of ascorbic acid for the formation of electroactive ascorbic acid. Zheng et al. [8] reported an electrochemical detection system based on a sandwich enzyme-link immunosorbent assay with the HRP-labeled anti-AFP antibody. This method used a dropping mercury electrode (DME) as working electrode, which causes operational inconvenience and is difficult to further develop for its application to a micro-system. They determined AFP by utilizing linear sweep polarography at the peak potential of −800 mV at pH 9–11 with the linear range from 0.5 to 100 ng/mL, which is insufficient in clinical application.

This work uses a small-sized gold disk electrode as working electrode, thus avoiding all the disadvantages of DME. The effect of pH of detecting solution on peak current shows the optimum pH value should be controlled in the range of less then 4. Under optimum conditions, a wider linear range is obtained. In comparison with the spectrophotometric ELISA this method improves the sensitivity of AFP detection by about ten times. The results obtained in clinical specimens are in good agreement with those from IRMA.

2. Experimental

2.1. Reagents and Chemicals

ELISA kits were obtained from Zhengzhou Biocell Institute (China). Each ELISA kit contained 48 monoclonal anti-AFP coated wells, two AFP standard solutions of 20 and 400 ng/mL, a solution of horseradish peroxidase conjugated monoclonal anti-AFP and a buffered wash solution. *o*-Phenylenediamine and H_2O_2 with analytical grade were from the Shanghai Biochemical Reagent Company (China). AFP-IRMA kit, including 100 assay tubes coated with monoclonal anti-AFP, was purchased from

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Diagnostic Products Corporation (DPC, USA). This kit consisted of an iodinated monoclonal anti-AFP in liquid form, a series of standard solutions of AFP at different concentrations from 0 to 400 ng/mL and a buffered washing solution. All other reagents were of analytical grade. Twice distilled water was used for all experiments. 0.1 M phosphate buffer solutions (PBS) at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then adjusting the pH with 0.1 M NaOH and H_3PO_4. The substrate solution consisted of 2.5 mM *o*-phenylenediamine and 4.0 mM H_2O_2 at pH 4.0. All reagents were brought to room temperature (ca. 20 °C) before use.

2.2. Sera

Serum specimens were obtained from healthy human and clinically diagnosed patients with liver carcinoma. The sera were separated from the red blood cells, without hemolysis, and were stored at 4 °C for less than one week. Before assay, they were allowed to come to room temperature and to mix by gentle swirling.

2.3. Apparatus

Electrochemical measurements were performed with a BAS 100B electrochemical analyzer (BAS Inc. USA) using a three-electrode system with a SCE, a platinum wire and a gold disk electrode as reference, counter and working electrode, respectively.

Gold disk electrodes with the diameter of 0.5 mm were prepared by sealing polycrystalline gold wires (> 99.99 %) in soft glass tubes, abrading with successively finer grades of SiC paper. The electrodes were polished to a mirror-like finish before use with 0.3 μm and 0.05 μm alumina slurry on microcloth pads (Buehler), followed by rinsing with water and brief cleaning in an ultrasonic bath. The SCE with the diameter of 1 mm was homemade.

IRMA was carried out with a FMJ-182 Immunoradiometric Gamma Counter (Shanghai, China) according to the instruction and assay procedure in the operator's manual.

2.4. Procedures

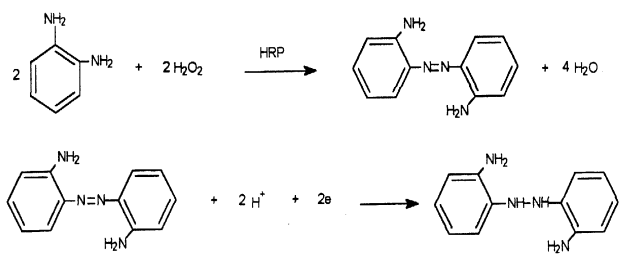
ELISA was based on a solid phase sandwich immunoassay. AFP present in sample was "sandwiched" between the antibodies. After dispensing 100 μL AFP standards or specimens into the coated wells and incubating them at 37 °C for 30 min, the incubation mixtures were removed by emptying the wells, followed by rinsing with washing solution and then with water five times. The residual water droplets were removed by striking the wells sharply onto absorbent paper. Then 100 μL HRP-conjugate reagent (used as received) was dispensed into each well and incubated at 37 °C for 15 min. At the end of the incubation, the contents in the wells were removed and the wells were washed as described above.

The enzymatic reaction was carried out by dispensing 200 μL substrate solution into the above mentioned wells and incubating at room temperature in the dark for 10 min. The reaction was stopped by adding 20 μL 2 M H_2SO_4 to each well [18]. After dispensing 9 μL 4 M NaOH solution into the microwell, the three electrodes were directly immersed into the microwell and the differential pulse voltammetric measurements were performed from +100 mV to −400 mV with the pulse amplitude of 50 mV and the pulse width of 60 ms.

3. Results and Discussion

3.1. Differential Pulse Voltammetric Response of Enzymatic Reaction Product

As shown in Figure 1, when the solution contained only pH 2.0 PBS, no peak was observed in the applied voltage range. When the pH 2.0 PBS contained 2.5 mM *o*-phenylenediamine and 4.0 mM H_2O_2, the differential pulse voltammetric curve changed slightly, but no peak occurred, indicating that the spontaneous oxidation rate of *o*-phenylenediamine by H_2O_2 was very slow. However, after 10 μL 1.0 mg/mL HRP was added into the system of 2.50 mL, the color of the solution changed quickly to tangerine, and the curve displayed a good peak at −0.19 V. The peak current increased with increasing time of enzymatic reaction and attained a constant value after a short time. Thus, the oxidation of *o*-phenylenediamine by H_2O_2 quickly went to completion under the catalysis of HRP. The product, 2,2′-diaminoazobenzene, exhibited a sensitive voltammetric response. The mechanism of enzymatic and electrode reactions can be expressed as shown in Scheme 1 [19].



Scheme 1: Enzymatic and electrode reactions.

3.2. Optimal Conditions of Enzymatic Reaction

3.2.1. Effect of pH

As is well known, the acidity of the solution greatly affects the enzyme activity. Most enzymes only display good activity in a

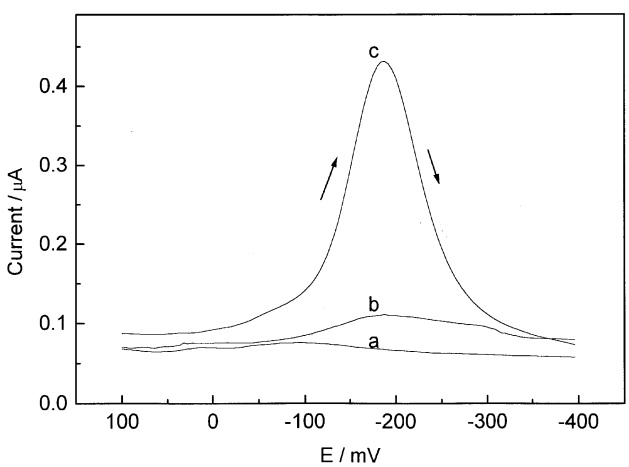


Fig. 1. Differential pulse voltammograms of a) 0.1 M PBS (pH 2.0), b) (a) + 4.0 mM H_2O_2 + 2.5 mM *o*-phenylenediamine and c) (b) + 4.0 μg/mL HRP at a gold disk electrode.

limited range of pH. Therefore, the voltammetric response of the product is related to the pH value of the enzymatic reaction solution. Figure 2 shows the effect of pH on the enzymatic reaction. When the pH value is very low or more than 8 the voltammetric response is very small. The maximum current appears at pH 4.0, exhibiting a maximum activity of enzyme catalysis. Thus, for this work the optimal pH value of the enzymatic reaction, pH 4.0, was chosen.

3.2.2. Optimal Concentration of o-Phenylenediamine

At low substrate concentrations, the peak current linearly increases with increasing *o*-phenylenediamine concentration. Here the concentrations of both HRP and H_2O_2 remain at constant values, thus the velocity of the enzymatic reaction is directly proportional to the *o*-phenylenediamine concentration (Fig. 3). At high substrate concentrations the velocity tends to a maximum value, while the rate becomes independent of the substrate concentration. The total concentration of 2,2′-diaminoazobenzene is only related to the amount of HRP and H_2O_2, showing a Michaelis-Menten's shape (Fig. 3). The apparent Michaelis-Menten constant (K_M^{app}) and the maximum current (I_{max}), obtained from the electrochemical transformation of the Lineweaver-Burk equation [20], were 0.21 ± 0.01 mM and 0.38 ± 0.02 μA, respectively, After the constant current occurs, both high sensitivity and low background value may be obtained, furthermore, the error from the slight change in substrate concentration was negligible. Thus, the value of 2.5 mM was chosen as the optimal concentration of *o*-phenylenediamine.

3.2.3. Concentration of H_2O_2

The effect of H_2O_2 concentration on voltammetric response also displays the curve of a Michaelis-Menten's shape as shown in Figure 4. The peak current increases with increasing H_2O_2 concentration, and then attains a maximum value. At low H_2O_2 concentration, both *o*-phenylenediamine and HRP are in excess, the amounts of 2,2′-diaminoazobenzene only depend on the H_2O_2 concentration. Thus, its reduction current was proportional to the H_2O_2 concentration. When the H_2O_2 concentration was higher than 2.5 mM, the product concentration became dependent on the amount of HRP. The current then trends to a constant value. The K_M^{app} and I_{max} values were 6.9 ± 0.2 mM and 0.86 ± 0.25 μA, respectively.

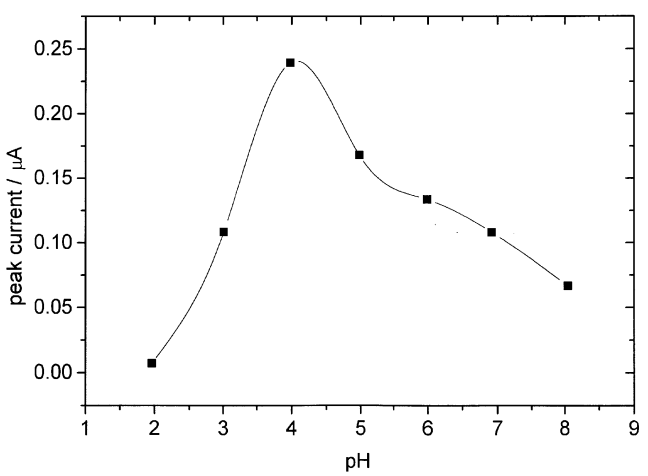


Fig. 2. Plot of peak current vs. pH of enzymatic reaction in 0.1 M PBS + 4.0 mM H_2O_2 + 2.5 mM *o*-phenylenediamine + 4.0 μg/mL HRP solution.

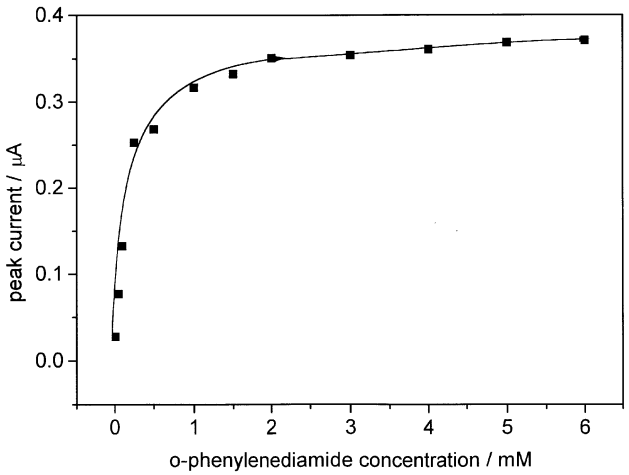


Fig. 3. Effect of *o*-phenylenediamine concentration on the peak current of the enzymatic product in 0.1 M pH 4.0 PBS + 4.0 mM H_2O_2 + 4.0 μg/mL HRP solution.

When the H_2O_2 concentration was higher than 8 mM, the peak current decreased with further increasing H_2O_2 concentration. This fact implies the denaturation of HRP to form irreversibly its higher oxidized and inactive form at higher H_2O_2 concentration [21]. 4.0 mM H_2O_2 concentration was chosen for the activity determination of HRP in a sandwich immunocomplex.

3.3. Effect of pH of Detection Solution on Reduction Current of 2,2′-Diaminoazobenzene

After the enzymatic reaction was stopped by adding H_2SO_4 solution into the system [18], the electrochemical measurements must be performed at an optimum condition for obtaining a higher sensitivity. Obviously, the electrochemical response was dependent on the solution pH value due to the participation of H^+ in the electrode process. The peak potential of the differential pulse voltammogram shifts to a more negative value with increasing pH. Figure 5 shows the relation between the peak current and the pH value of the detection solution. When the pH was controlled in the range of 1 to 4, the concentration of H^+ was high enough for the

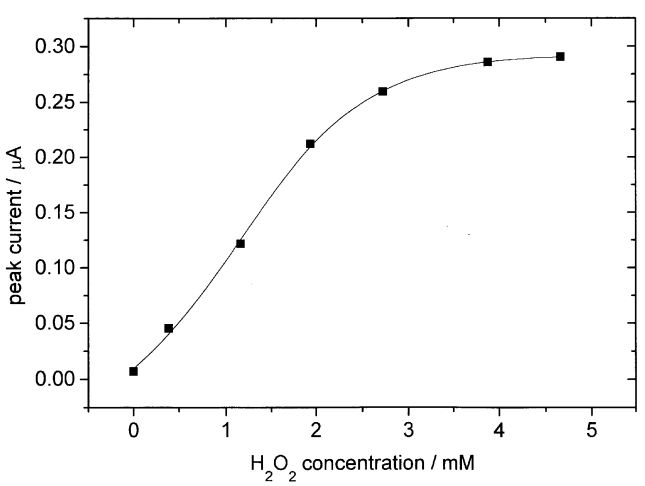


Fig. 4. Relation between the peak current of the enzymatic product and H_2O_2 concentration in 0.1 M PBS (pH 4.0) + 2.5 mM *o*-phenylenediamine + 4.0 μg/mL HRP solution.

reaction of 2,2′-diaminoazobenzene, the peak current maintains a steady value. With further increase of the solution pH, the peak current quickly decreases. After the pH value was greater than 8, not only the peak current did become very small, but also the peak-shape was very bad. Considering the changes in the peak current, the peak potential and the peak-shape, the optimum pH 2.0 was chosen at which the peak potential was −0.19 V.

3.4. Calibration Plot of AFP Determination

Following the standard immunometric sandwich and the enzymatic reaction procedures, the peak current of differential pulse voltammogram of enzyme reaction product was proportional to the concentration of AFP. Under optimal conditions, the plot of peak current vs. AFP concentration shows a linear relation in the range of 0.5–400 ng/mL (Fig. 6.). The correlation coefficient was 0.9993. It can be seen that the up-limit of the linear range could be extended if the standard solution of more than 400 ng/mL could be obtained. The linear range was wider than that of the spectrophotometric ELISA and that reported in [8]. Four determinations of the blank at −0.19 V resulted in a relative standard deviation of 7.5 %. The detection limit under the above conditions, calculated from three times the standard deviation of the blank, was about 0.06 ng/mL. The detection limit was ten times higher than that of traditional spectrophotometric ELISA and was the same as the one obtained in [8]. Thus, the presented method can be used in the determination of AFP for the clinical diagnosis of original liver carcinoma.

3.5. Precision, Accuracy and Clinic Application

Intra-assay precision was evaluated by assaying eight-fold replicates of one serum in the same run. Inter-assay precision was estimated by determining, in duplicate, AFP in one serum sample on eight different occasions. We used two sera to perform the precision tests. The variation coefficients (CVs) of intra-assay on this method were 5.6 % at the mean AFP concentration of 2.9 ng/mL and 1.6 % at 74 ng/mL AFP, while the CV values of inter-assay on this method were 9.6 % and 5.2 %, respectively. It has been noted that all the CV values were in the range

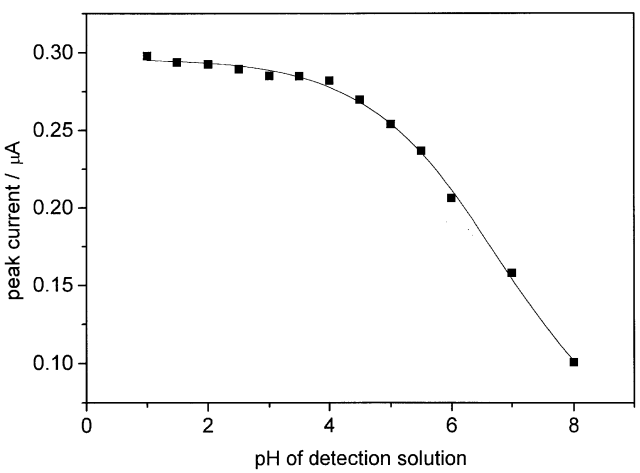


Fig. 5. Effect of the pH value of the detecting solution on the peak current of the enzymatic reaction product formed in 0.1 M PBS (pH 4.0) + 4.0 mM H_2O_2 + 2.5 mM *o*-phenylenediamine + 4.0 μg/mL HRP solution.

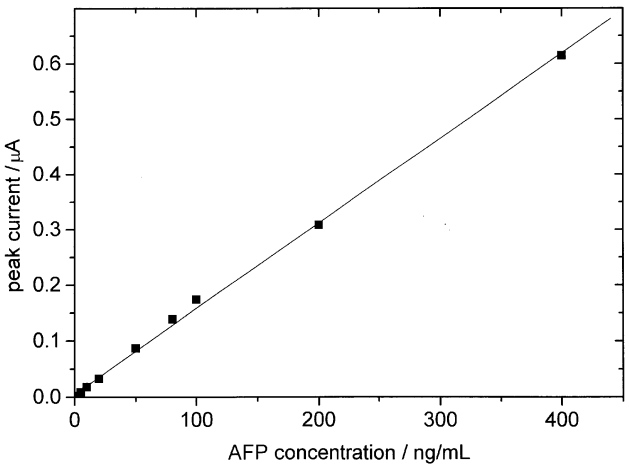


Fig. 6. Plot of peak current vs. AFP concentration in serum specimen under optimal conditions.

recommended in the manufacturer's manual of IRMA, thus this was sufficient in serum testing.

The accuracy of the AFP determination was examined by comparison of the results from this method and IRMA. In both techniques, the AFP contents in four sera were quantified by using a calibration plot with three replicates at each concentration. The mean AFP concentrations determined with this method were 2.9, 74, 332 and 412 ng/mL, while the values obtained from IRMA were 3.2, 69, 323 and 403 ng/mL, resulting in relative errors of −9.3%, 6.8%, 2.9% and 2.2%, respectively. These results by both methods were in good agreement. Thus, the proposed method can be satisfactorily applied to the clinical determination of AFP in human serum.

4. Acknowledgements

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