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Investigation of 3,3'-Diaminobenzidine-H_2O_2-Horseradish Peroxidase Voltammetric Enzyme-Linked Immunoassay System Used for the Detection of Prostate Specific Antigen

JUN YANG, CAIFENG BI*, YUHUA FAN and XIA ZHANG

Key Laboratory of Marine Chemistry Theory and Technology, Ministry of Education, College of Chemistry and Chemical Engineering, Ocean University of China, Qingdao, 266100, P.R. China

*Corresponding author: Fax: +86 532 66781932; Tel: +86 532 66781932; E-mail: bicaifeng301@163.com

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By introducing heterocyclic compound to immunoassay systems an electrochemical substrate, a new voltammetric enzyme-linked immunoassay system of 3,3'-diaminobenzidine (DAB)-H_2O_2-horseradish peroxidase (HRP) has been presented and used for the sensitive detection of prostate specific antigen (PSA) in human serum. In this proposed procedure, labelled horseradish peroxidase efficiently catalyzed the oxidation reaction of 3,3'-diaminobenzidine by H_2O_2 and generated the electroactive product, which produced a sensitive second-order derivative linear sweep voltammetric peak at potential of -0.62V (*versus* SCE) in Britton-Robinson (BR) buffer solution. The free horseradish peroxidase could be measured in a linear range from 2.5×10^{-6} - 2.5×10^{-2} unit mL^{-1} and a detection limit of about 1.5×10^{-6} unit mL^{-1}. Under the optimal experiment conditions, prostate specific antigen could be detected in the linear range from 0.20 to 32 ng mL^{-1} with a detection limit of 0.10 ng mL^{-1}. The proposed electrochemical enzyme-linked immunosorbent assay method is simple and inexpensive, which shows a promising alternative approach for detecting prostate specific antigen in the clinical diagnosis.

Key Words: 3,3'-Diaminobenzidine, Prostate specific antigen, Voltammetric enzyme-linked immunoassay.

INTRODUCTION

Prostate cancer is a deadly malignancy and no curative therapy is now available once the disease spreads the limits of the organ. To combat against the disease, therefore, accurate detection of prostate cancer at early stage while it is localized in the prostate gland offers the best hope. Prostate specific antigen (PSA), a glycoprotein in human serum, has been proved to be the most reliable and specific clinical tool for preoperative diagnosing and monitoring prostate cancer. Normally, prostate cancer is suspected if the total prostate specific antigen level is higher than 10 ng mL^{-1} [1-4]. Therefore, sensitive and specific detection of prostate specific antigen for early prostate cancer detection is of great significance.

Electrochemical immunoassay has been applied for the assay of a number of antigens and antibodies with satisfactory results[5-8]. The method not only has the advantages of high detection sensitivity, but it also like other substrate-based end point detection methods, couples well with the high selectivity of antibody-based techniques[9-14]. In addition, the electrochemical immunoassay methods, combined with modern separation techniques such as flow-injection[15-17] and capillary electrophoresis (CE)[18-20] are powerful analytical tools for determination of low levels of analytes and application in multianalyte immunoassays. ACE-based electrochemical immunoassay system, with *o*-aminophenol-H_2O_2-horseradish peroxidase (HRP) system, for simultaneous detection of three important tumor markers was developed by our group[21].

It is all known that *o*-phenylenediamine (OPD) is the most common substrate used in not only traditional spectrophotometric ELISA method but also traditional benzene cyclic substrate-based electrochemical immunoassay. For comparison, 3,3'-diaminobenzidine (DAB) as a substrate has been introduced to electrochemical immunoassay. The results of the study have showed that 3,3'-diaminobenzidine is a novel electrochemical oxidisable heterocyclic compound. The study has showed that 3,3'-diaminobenzidine is oxidized with H_2O_2 catalyzed by horseradish peroxidase and the resulting electroactive product produces a sensitive voltammetric peak at potential of -0.62V [*vs*. saturated calomel electrode (SCE)] in Britton-Robinson (BR) buffer solutions. By using this voltam-metric peak, free horseradish peroxidase and labeled horseradish peroxidase can be measured. Under the selected optimum conditions, the linear range for detection of free horseradish peroxidase was from 2.5×10^{-6}-2.5×10^{-2} unit mL^{-1} with a detection limit of about 1.5×10^{-6} unit mL^{-1}.

Sensitive and specific detection of prostate specific antigen (PSA) has been proved to be the most reliable clinical tool for preparative diagnosing and monitoring prostate cancer. Besides traditional spectrophotometric ELISA method, some techniques have been applied for the assay of prostate specific antigen with satisfactory results. Sarker *et al.* have developed amperometric biosensors for detection of prostate specific antigen with the detection limit of 0.25 ng mL^{-1} [22]. Xu and co-workers have reported a rapid enzyme immunoassay for serum prostate specific antigen at low concentrations by flow-injection electrochemical detection, which offers a much lower detection limit of 0.008 ng mL^{-1} [23].

In this paper, sensitive and specific detection of prostate specific antigen in human serum using 3,3'-diaminobenzidine-H_2O_2-horseradish peroxidase voltammetric enzyme-linked immunoassay system based on 3,3'-diaminobenzidine and horseradish peroxidase-labeled enzyme is presented. The detection limit of prostate specific antigen is 0.10 ng mL^{-1}, which is five times lower than that of traditional *o*-phenylenediamine spectrophotometric ELISA method. In addition, the linear range for prostate specific antigen detection is from 0.2 to 32 ng mL^{-1}, which is much wider than that of traditional *o*-phenylenediamine spectrophotometric ELISA method developed in our previous work. The proposed sensitive, specific, simple, inexpensive and rapid method for the detection of prostate specific antigen in human serum has been proved to be a reliable clinical tool for preparative diagnosing and monitoring prostate cancer.

EXPERIMENTAL

The electrochemical measurement was carried out with a MP-2 voltammetric analyzer (Shandong No.7 Electric Communication Corp., China). A three-electrode system was employed with a dropping mercury electrode or a hanging mercury drop electrode as working electrode, a platinum electrode as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. The instrumental conditions were as follows: initial potential, -0.20 V; mercury drop standing time, 7 s; potential scanning rate, 300 mV/s. OG3022A enzyme-linked immunity measure implement (Hua-dong Electronal Group Medical Treatment Instrument Ltd., China) was used for the spectrophotometric ELISA.

3,3'-Diaminobenzidine working solution was prepared by dissolving 0.0108 g 3,3'-diaminobenzidine (Acros Organics, 99 %) in doubly distilled water and diluted to 50.0 mL (1.0 mM). The solution of horseradish peroxidase (250 units per mg enzyme, Xueman Biochemical Technique Corp., China) was prepared by dissolving 10.00 mg horseradish peroxidase in 10.0 mL doubly distilled water (250 unit mL^{-1}) and then was stored at 4 ºC. The prostate specific antigen ELISA kit was purchased from Peking North-ward Biology Technique Graduate School and stored at 2-8 ºC. The kit included immunoplate precoated by anti-prostate specific antigen serum, horseradish peroxidase-linked anti-prostate specific antigen, standard prostate specific antigen sample, substrate solution (*o*-phenylendiamine solution), rinsing solution (PBS) and stop solution (H_2SO_4). Other chemicals were of analytical grade and were prepared by the doubly distilled water.

RESULTS AND DISCUSSION

The second-order derivative linear-sweep voltammograms differential pulse voltammetry, modern square wave voltammetry and linear sweep second-order derivative polarography all have excellent voltammetric peaks or polarographic waves in the detection of the product formed by H_2O_2 oxidizing 3,3'-diaminobenzidine, which is catalyzed by horseradish peroxidase in 0.2 M BR at pH 3.5. Among these methods, linear sweep second-order derivative polarography was the most optimal method with the advantages such as high sensitivity, low detection limit, short experimental time and simple manipulation. The product of the enzyme-catalyzed reaction has a well-defined voltammetric peak. Fig. 1 shows the results of the second-order derivative linear-sweep voltammograms. Curve 1 is the voltammogram of BR buffer solution, which has no voltammetric peak. Curve 2 is that of the BR-3,3'-diaminobenzidine-H_2O_2, which has a small voltammetric peak at -0.62 V. The small peak is due to the product of slow oxidation of 3,3'-diaminobenzidine by H_2O_2. Curve 3 is that of the enzyme-catalyzed reaction solution. Owing to the addition of horseradish peroxidase, which quickens greatly the oxidation of 3,3'-diaminobenzidine by H_2O_2, the reaction product produces a large and well-defined voltammetric peak at -0.62 V. Although the horseradish peroxidase content is as low as 1.5×10^{-6} unit mL^{-1}, a distinctive increase of this voltammetric peak still can be observed. The oxidation of 3,3'-diaminobenzidine by H_2O_2 yields a stable product, if the enzyme catalyzed reaction happens in 0.2 M BR at pH 5.0.

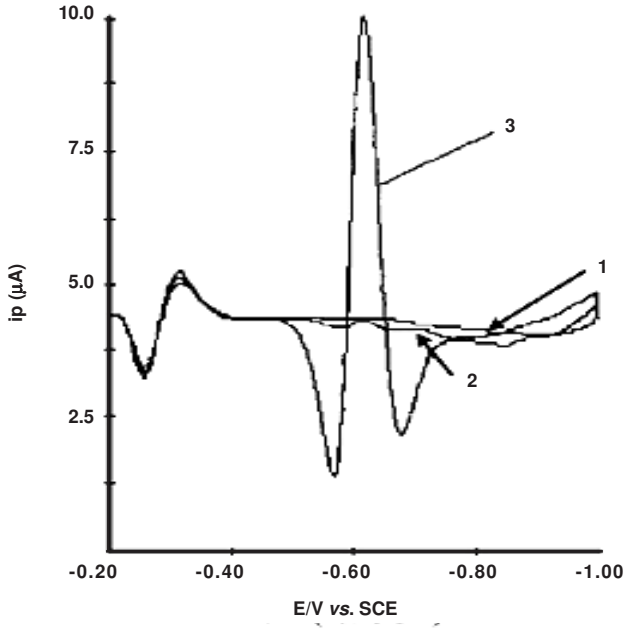
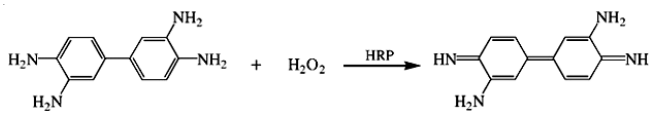


Fig. 1. Second-order derivative linear-sweep voltammogram of BR + H_2O_2 (**1**), BR + 3,3'-DAB + H_2O_2 (**2**), BR + DAB + H_2O_2 (**3**)

Optimal conditions for enzyme-catalyzed reaction: As seen from the voltammograms, horseradish peroxidase intensely catalyzed the oxidation reaction of 3,3'-diaminobenzidine by H_2O_2. Considering the structure of the product and catalysis cycle of horseradish peroxidase in reaction, the process of horseradish peroxidase-catalyzed oxidation reaction

of 3,3'-diaminobenzidine by H_2O_2 is concluded in **Scheme-I**. The enzymatic oxidation of 3,3'-diaminobenzidine yielded a stable product, 4,4'-diimino-bicyclohexylidene-2,5,2',5'-tetraene-3,3'-diamine in BR buffer solution at pH 3.5. Fig. 2 shows the results of the second-order derivative linear-sweep voltammograms.Through measuring the electrochemical response of the enzymatic product, the effect of pH on enzyme-catalyzed reaction was studied between 2.0 to 12.0. The electrochemical peak current reached the maximum value at pH 3.5 BR buffer solution. Additionally, the concentrations of each component of the substrate solution, including BR buffer solution, 3,3'-diaminobenzidine and H_2O_2, were also optimized. When the final 10 mL substrate solution consisted of 2.0 mL of BR buffer solution (0.2 M, pH 3.5), 2.0 mL 3,3'-diaminobenzidine solution (1.0 mM) and 1.0 mL H_2O_2 solution (1.0 mM), the electrochemical peak current reached the maximum value. Under the above optimal conditions, the electrochemical peak current of the product changed slightly after 40 min at 37 ºC, indicating the reaction reached the equilibrium. Thus, 40 min was selected as the optimal incubation time for the enzyme-catalyzed reaction.



Scheme-I: The process of the horseradish peroxidase-catalyzed oxidation reaction of 3,3'-diaminobenzidine by H_2O_2

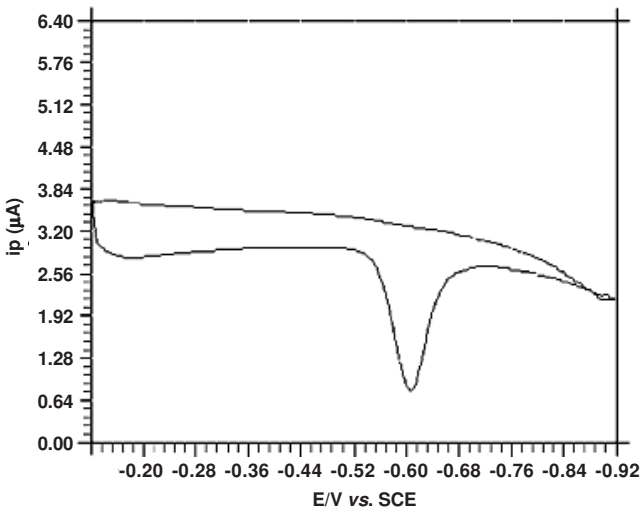


Fig. 2. Cyclic voltammograms of enzymatic product in BR buffer solution at pH 5.0. The enzymatic product was generated by oxidation reaction of 3,3'-diaminobenzidine in BR buffer solution at pH 3.5, T = 1s, v = 300mv/s

Optimal electrochemical conditions for the detection: The fine second-order derivative linear-sweep voltammetric peak for the enzyme-catalyzed product was obtained in BR buffer solution. After the enzyme-catalyzed reaction, the effect of pH value on the second-order derivative linear-sweep voltammetric peak of enzymatic product was investigated. Results showed that the peak potential shifted negatively with the increased pH. The peak current reached the maximum value at pH 5.0. Thus pH 5.0 was selected as the optimal pH value of BR for the electrochemical detection.

Electrochemical determination of free horseradish peroxidase: Different concentrations of free horseradish peroxidase were used to catalyze the oxidation reaction of 3,3'-diaminobenzidine by H_2O_2. 2 mL of 1 mM 3,3'-diaminobenzidine, 1 mL of 1 Mm H_2O_2 and 2 mL of 0.2 M BR at pH 3.5 was mixed with 1.0 mL of horseradish peroxidase solution with different concentrations. The mixture was diluted to 10 mL and kept at 37 ºC for 40 min in a water bath. Then, 3 mL of the above solution and 7 mL of 0.2 M BR at pH 5 were mixed in a 10.0 mL colourimetric tube and then was detected by the MP-2 voltammetric analyzer in a 10 mL electrolyte cell. The second-order derivative linear-sweep voltammogram was recorded. The peak current in BR exhibits a good linear relation with horseradish peroxidase concentration in the range of 2.5×10^{-6} - 2.5×10^{-2} unit mL^{-1} and a detection limit of about 1.5×10^{-6} unit mL^{-1}. The relative standard derivative (RSD, n=11) of horseradish peroxidase response to1.25×10^{-5} unit mL^{-1} horseradish peroxidase is 2.1 %.

Linear range, the detection limit and the precision of prostate specific antigen detection: Under the optimized conditions, the proposed new 3,3'-diaminobenzidine-H_2O_2-horseradish peroxidase system could detect prostate specific antigen in a linear range from 0.2-32.0 ng mL^{-1} with a detection limit of 0.1 ng mL^{-1}. The regression is y = 2.98 x + 6.95 (γ = 0.9948, n = 8), where y means Δip, ip is the peak current (μA), x is the concentration of prostate specific antigen. For TMB spectrophotometric ELISA method, the linear range of prostate specific antigen is 1.0-32.0 ng mL^{-1} with a detection limit of 0.5 ng mL^{-1}. The equation of linear regression is A = 0.0823 + 0.0467C (γ = 0.9974), where A is the absorbency, C is the concentration of prostate specific antigen. Therefore, the detection limit of our electrochemical enzyme-linked immunoassay method was 5 times lower than that of the TMB spectrophotometric ELISA method.

Determination of prostate specific antigen human serum samples: The human serum samples were detected using both electrochemical enzyme-linked immunoassay method and spectrophotometric ELISA method. The comparison results were listed in Table- 1. We could find that the two methods showed good agreement. The results of electrochemical method were linear proportional to that of spectrophotometric method. The regression was y = -0.22456 + 0.91952x (γ = 0.9994), where x was the results of electrochemical method, y was results of spectrophotometric method.

TABLE-1
THE COMPARISONS OF ELECTROCHEMICAL ELISA WITH SPECTROPHOTOMETRIC ELISA FOR THE DETECTION OF PSA IN HUMAN SERUM

Samples	Electrochemical ELISA (ng $mL^{?1}$)	Spectrophotometric ELISA (ng mL^{-1})
1	0.48	-
2	0.87	-
3	1.74	1.63
4	4.56	4.23
5	7.84	7.92
6	25.1	23.2
7	0.62	-
8	13.6	13.3
9	12.1	11.6
10	25.9	26.3

Specificity for detection of prostate specific antigen: The specificity for detecting prostate specific antigen (PSA) was investigated using dilutions of prostate specific antigen, α-fetoprotein (AFP), ferritin and full-mouthed disease (FMD). 0.2 ng/mL prostate specific antigen could be readily detected. However, there were no significant reactions with the AFP, ferritin and full-mouthed disease samples in the concentration of 10.0-1.0 μg mL^{-1}. In addition, uninfected human serum and human serum infected with AFP, ferritin and full-mouthed disease, respectively, gave no detectable reactions.

Conclusion

Based on the new system of 3,3'-diaminobenzidine-H_2O_2-horseradish peroxidase, the developed electrochemical enzyme-linked immunoassay showed potential performance for detection of prostate specific antigen (PSA) in human serum. The processes of the enzyme-catalyzed reaction and electro-reduction of the product on the electrodewas investigated. The detection limit for prostate specific antigen was five times lower than that of the traditional spectrophotometric ELISA method. The simple, inexpensive, reproducible and sensitive assay showed a promising alternative approach in clinical diagnosis.

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