An improved ELISA for the determination of southern bean mosaic virus with linear sweep voltammetry detection based on new system of PAP-H_2O_2-HRP

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An improved enzyme-linked immunosorbent assay (ELISA) for the determination of southern bean mosaic virus (SBMV) with linear sweep voltammetry based on a new system of *p*-aminophenol (PAP)-H_2O_2-horseradish peroxidase (HRP) has firstly been developed. The enzymatic product 3-[(4-hydroxyphenyl) amino]-4-(2-amino-5-hydroxyphenyl)-6-[(4-hydroxyphenyl)imino]-2,4-cyclohexadiene-1-one, produced from the oxidation of PAP with H_2O_2 catalyzed by HRP, yielded a sensitive linear sweep voltammetric response at −0.45 V (*vs.* SCE) in Britton-Robinson (BR) buffer solution. Based on the voltammetric peak, HRP can be measured with a detection limit of 0.4 mU/L and a linear range of $1.0—1.0\times10^2$ mU/L. The detection limit for the SBMV is 8.0 ng/mL and the highest dilution ratio for the detection of infected leaf sap is 1: 1.5×10^5.

Keywords Southern bean mosaic virus, horseradish peroxidase, *p*-aminophenol, voltammetry

Introduction

Southern bean mosaic virus (SBMV) is an important plant virus, which has a number of plant strains and wide spread hosts, and makes inroads on hundreds of plants such as soybean, mung bean, cucumis sativus, cucumber and so on. The sensitive determination of SBMV in various plants and seeds for the purpose of the provention and control of plant disease is very important. Hence, the sensitive assays for SBMV are needed. Enzyme-linked immunosorbent assay (ELISA) method is a conventional assay for clinic examination of SBMV,[1,2] but its detection limit is relatively poor by spectrophotometry. Therefore, a complete change of detection procedure may be key to improving sensitivity and detectability.

Electrochemical detection has been successfully coupled to enzyme immunoassay.[3-9] The high sensitivity of immunoassay with electrochemical detection makes the method competitive with that of radio immunoassay (RIA).[3] It can be used for colour or turbid media. A number of recent studies[4-7] on amperometric or potentiometric detection of electroactive species generated catalytically by alkaline phosphatase have appeared. Although amperometric or potentiometric detection coupled with liquid-chromatographic seperation or flow injection analysis has high sensitivity, the system is complicated.

On the other hand, voltammetric detection can also be coupled with enzyme immunoassay. In this paper, we report a new system of voltammetric enzyme-linked immunoassay for SBMV using HRP as a label enzyme and PAP-H_2O_2 as the substrate, which had not been investigated in spectrophotometric ELISA method. The enzymematic product is electroactive and can be measured by voltammetric techniques. The application of this method to quantitative assay of SBMV is described in

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this paper. The assay is simple, reproducible and more sensitive than the conventional method by spectrophotometry.

Experimental

Apparatus

MP-1 voltammetric analyser (Shandong Electric Communication Factory of No. 700) was used for electrochemical measurements with a three-electrode system composed of a dropping mercury or hanging mercury drop working electrode, a platinum wire auxiliary electrode and a saturated calomel reference electrode (SCE). JM-01 hanging mercury drop electrode was available from Jiangsu Electric Analysis Apparatus Factory. Incubation for the immue reaction was carried out with a Model HH.W21.Cr420 incubator (Guangdong Shantou Instrument Factory).

Reagents

HRP (250 units per mg enzyme) solution: 2.5×10^8 mU/L. 1% bovine serum albumin solution was prepared by dissolving 1 g bovine serum albumin in 100 mL of 0.2 mol/L phosphate buffer (pH 7.2). The purified SBMV and antiserum SBMV-As were obtained from Institute of Quarantine, Ministry of Agriculture. The infected leaf sap to be analyzed was obtained from following procedure: 2.0 mL of 0.2 mol/L phosphate buffer (PBS, pH 7.2) was added to 1 g of fresh infected leaves, ground and filtered. The filtrate was the infected leaf sap. Substrate solution: to a colorimetric tube of 10 mL were in sequence added 0.5 mL of PAP solution (5.0×10^{-3} mol/L), 1.0 mL of H_2O_2 solution (4.0×10^{-4} mol/L), 1.0 mL of Britton-Robinson buffer solution (0.02 mol/L) (BR, pH 4.7). Then the mixture was diluted to the desired scale and shaken to uniform. Water used was doubly deionized.

Measurement of HRP *activity*

A mixture of 0.5 mL of PAP (5.0×10^{-3} mol/L), 1.0 mL of H_2O_2 (4.0×10^{-4} mol/L), 1.0 mL of BR (0.02 mol/L, pH 4.7), 1.0 mL of HRP (2.5×10^2 mU/L) was diluted to 10 mL with water. Then it was kept at room temperature for 20 min. 10 mL of solution containing 5.0 mL of the mixture and 2.0 mL of BR (1.0 mol/L, pH 7.0) was prepared to record the second order derivative linear-sweep voltammogram with MP-1 voltammetric analyser. The working conditions are as follows: the initial potential, −0.10 V; the potential scanning rate, 535 mV/s and the mercury drop standing time, 12.8 s.

Structure identification of the product of the enzyme-catalyzed reaction

The product of the enzyme-catalyzed reaction was chromatographed on silica gel G (100—200 meshes) with ethyl acetate: cyclohexane of 95:5. The sample was separated into three layers. The solution of the first layer was collected. Then the solvent was evaporated to dryness in vacuum drying oven under reduced pressure of 8.0×10^3 Pa. The preliminary product was dissolved in a little tetrahydrofuran, then 2 g silica gel H (200—400 meshes) was added to prepare the sample. The sample was chromatographed on silica gel H (200—400 meshes) with ethyl acetate: cyclohexane: methanol: triethylamine of 28:60:10:2 for the second time. The sample was separated into three layers. The solution of the second layer was collected. The solvent was evaporated to dryness under reduced pressure and deaerated by N_2. The solid was dried in vacuum drying oven under reduced pressure of 8.0×10^3 Pa at room temperature for 12 h. Some pure product was dissolved in methanol to record UV-visible spectrum with a Philips 8720 spectrophotometer. ^{1}H NMR stectra were recorded with a Bruker 500-MHz NMR spectrometer in DMSO-d_6. FT-IR spectra were recorded with a 510 P FT-IR spectrometer. Mass spectra were recorded with a VG 7070-EQ instrument in the electron impact mode (70 eV). Elemental analysis was performed with a Perkin Elmer 2400 analyser.

(3-[(4-*Hydroxyphenyl*) *amino*]-4-(2-*amino*-5-*hydroxyphenyl*)-6-[(4-*hydroxyphenyl*) *imino*]-2, 4-*cyclohexadiene*-1-*one*) λ_{max}: 274.0, 408.9 nm. ν_{max}: 3322, 1703, 1609, 1571, 1513, 1453, 1255, 1170, 830, 864, 807 cm^{-1}. δ_c: 178.55, 155.05, 154.38, 154.00, 152.32, 151.02, 142.03, 141.90, 140.93, 129.92, 129.33, 125.62, 124.02, 122.70, 115.70, 115.41, 115.34, 95.27, 88.45. DEPT-135 spectrum: 125.62, 124.02, 122.70, 115.70, 115.41,

115.34,95.27,88.45. δ_H: 9.33;8.95;8.19;7.17, 7.15; 7.02, 7.00; 6.84, 6.83, 6.82, 6.81; 6.77, 6.75; 6.68, 6.67; 5.91, 5.90; 5.58. *m/z*(%): 413, 308, 206, 109. Anal. $C_{24}H_{19}N_3O_4$. Calcd: C, 69.72; H, 4.63; N, 10.16. Found: C, 69.84; H, 4.85; N, 9.78.

Method for the determination of SBMV

The purified SBMV and infected leaf sap were diluted with carbonate buffer (0.1 mol/L, pH 9.6), respectively. The wells of the polystyrene immunoplate were coated with the diluted antigens, 200 μL per well and incubated at 37℃ for 2 h, then placed in a refrigerator of 4℃ for 12 h. The wells were emptied, and then 300 μL of PBS buffer was added in each well. The wells were set for 3 min. This manipulation was repeated for three times. The wells were coated with 300 μL of 1% BSA and incubated at 37℃ for 30 min. Washed the wells as above. 200 μL of appropriate concentration of SBMV-As (1:6400) was added in each well and incubated at 37℃ for 2 h. Washed the wells as above. 200 μL of goat antirabbit IgG-HRP (1:640) was added in each well and incubated at 37℃ for 2 h. Washed the wells as above. 400 μL of substrate solution was added in each well and incubated at room temperature for 20 min. The reaction solution was transferred into a cell of 2 mL; 160 μL of BR (1.0 mol/L, pH 7.0) and 240 μL of water were added into the cell. The second order derivative linear-sweep voltammograms were recorded.

Results and discussion

Second order derivative linear-sweep voltammograms

The product of the enzyme-catalyzed reaction has 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 BR + PAP + H_2O_2, which has a small voltammetric peak at −0.45 V. The small peak is due to the product of slow oxidation of PAP by H_2O_2. Curve 3 is that of the enzyme-catalyzed reaction solution. Owing to the addition of HRP, which quickens greatly the oxidation of PAP with H_2O_2, the reaction product produces a big and well-defined voltammetric peak at −0.45 V. Although the HRP content is as low as 1.0 mU/L, a distinctive increase of this voltammetric peak can still be observed. The experiment has proved that, if the enzyme-catalyzed reaction happens in the BR buffer solution with pH 4.7, the oxidation of PAP by H_2O_2 yields the stable product 3-[(4-hydroxyphenyl)amino]-4-(2-amino-5-hydroxyphenyl)-6-[(4-hydroxyphenyl) imino]-2, 4-cyclohexadiene-1-one. This product is subject to electro-reduction, which can be detected by voltammetry. Curve 4 is the voltammogram of the pure product prepared with chemical method under the same experimental conditions. The peak potential and shape of curve 4 are the same as those of curve 3. It shows that the pure product prepared with chemical method and the product of the enzyme-catalyzed reaction in solution are the same.

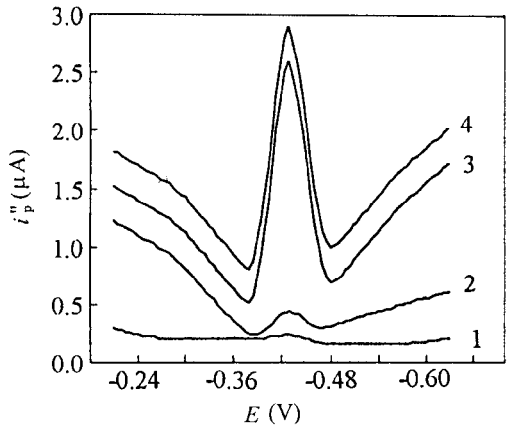
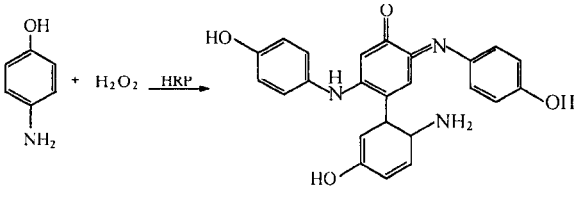


Fig. 1 Second order derivative linear-sweep polarograms. (1) BR buffer (0.002 mol/L, pH 4.7); (2) Reaction without HRP: BR buffer (0.002 mol/L, pH 4.7) + PAP (2.5×10^{-4} mol/L) + H_2O_2 (4.0×10^{-5} mol/L); (3) Reaction with HRP: 2 + HRP (25 mU/L); (4) Second order derivative linear-sweep voltammetric curve of the pure product prepared with chemical method under the same conditions.

Conditions of the enzyme-catalyzed reaction

HRP is widely used as labeled enzyme due to its good stability and low cost. It catalyzes intensely the oxidation reaction of PAP with H_2O_2, which has not been reported previously. From the structure of the product,

the enzyme-catalyzed reaction can be expressed as follows:



In a pH range of 2.0—10.0, the effect of the pH value of the BR buffer solution on the reaction was tested. In pH 4.5—4.9, a sensitive and stable voltammetric peak can be obtained. pH 4.7 was chosen in this reaction. The concentrations of BR buffer solution, PAP and H_2O_2 were also experimented. When 10 mL of the overall reaction solution includes 1.0 mL of BR buffer solution (0.02 mol/L, pH 4.7), 0.5 mL of PAP solution (5.0×10^{-3} mol/L) and 1.0 mL of H_2O_2 solution (4.0×10^{-4} mol/L), the peak is the highest and also stable.

Under the selected enzyme-catalyzed reaction conditions, the equilibrium will be achieved in 20 min at room temperature. And the peak height keeps stable within 2 h and the small blank peak has also no change. 20 min was selected as the time for the enzyme-catalyzed reaction at room temperature.

Detection conditions

A fine second order derivative linear sweep voltammetric peak of the product of the enzyme-catalyzed reaction can be obtained in BR buffer solution. BR buffer was selected as the supporting electrolyte for the voltammetric measurement. The effect of pH value of BR buffer on the voltammetric peak was investigated. The peak potential shifts negatively and the peak height advances with the increase of pH, and with a relative stability, before the peak height decreases with the further increase of pH. At pH 7.0, the peak is the highest and also stable. When 10 mL of the overall solution contains 1.0—5.0 mL of BR buffer solution (1.0 mol/L, pH 7.0), the highest peak appears.

The relation between the peak height of the product of the reaction and the static period τ was studied with the hanging mercury drop electrode. The peak height increases with increasing of the static time. When τ is more than 70 s, the peak height does not change any longer. For pH 4.7 BR buffer solution, the linear-sweep voltammetric peak increases with increasing the rate of scanning. But the plot of the peak current against the square root of the rate of scanning is not linear but an upward curve in the range of 120—1038 mV/s. The peak potential values shift to more negative values with increasing the rate of scanning. The electrocapillary curve of PAP-H_2O_2-HRP is compared with those of PAP-H_2O_2 and BR buffer solution, respectively. Between 0.00 and −2.00 V, the surface tension of the former solution is much less. Above results indicate that the product of the enzyme-catalyzed reaction is of adsorption on the mercury electrode.

The influence of pH on the peak potential is investigated for BR buffer solution of different pH. The peak potential value has a good linear relation with the pH value in the range of 3.0—12.0 with an equation of linear regression $E_p = 0.040—0.0601$ pH ($n = 13$, $\gamma = -0.9994$). According to the formula:[10] $-0.059\ x/n = -0.0601$, where n is the number of the electron transfer, x is the hydrogen ion number participating the reaction, $x \approx n = 2$.

The cyclic voltammetric experiment was run for the solution of the emzyme-catalyzed reaction. After reaction in pH 4.7 BR buffer solution, the cyclic voltammograms were recorded at different pH from 3.28 to 12.58. The results were shown in Fig. 2. Between pH 3.28 to pH 5.15, the cathodic peak and anodic peak both appear,

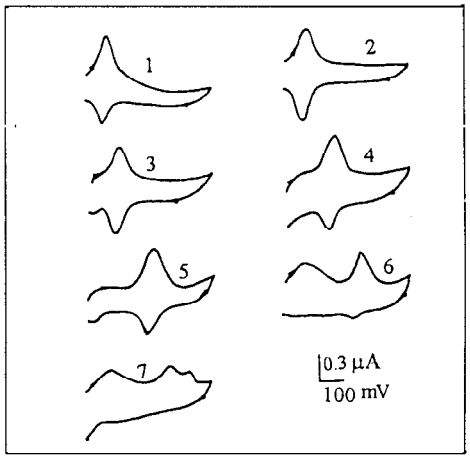
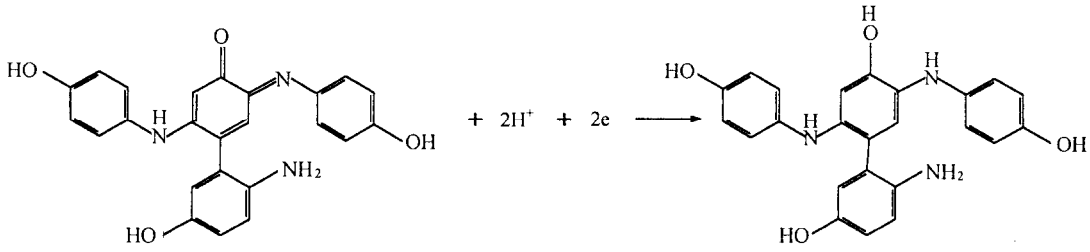


Fig. 2 Cyclic voltammograms. $\tau = 70$ s; $\nu = 96$ mV/s; scan potential range: −0.20—−0.80 V; (1) pH 3.28; (2) pH 5.15; (3) pH 7.30; (4) pH 8.78; (5) pH 10.50; (6) pH 12.10; (7) pH 12.58. All the solutions are deaerated with N_2.

but the height of the anodic peak is lower than that of the cathodic peak. The height of the anodic peak increases with the increase of pH value, and the ratio of the anodic to cathodic swells. Between pH 5.15 to pH 8.78, one pair of quite symmetric redox peaks appears. In the pH range of 5.15—8.78, the two peak heights are almost equal, which indicates that the voltammetric wave is a two-electron complete adsorption reversible wave. In the pH range of 8.78—12.10, the height of the cathodic peak decreases. Till pH 12.58, the cathodic wave is obviously divided into two waves, and the anodic peak disappears.

From above experimental results, there is a two-electron adsorptive reversible redox process of 3-[(4-hydroxyphenyl) amino]-4-(2-amino-5-hydroxyphenyl)-6-[(4-hydroxyphenyl) imino]-2, 4-cyclohexadiene-1-one under the selected conditions, which can be expressed as follows:



Determination of HRP

According to the experimental method, different quantities of HRP were used to catalyze the oxidation reaction of PAP with H_2O_2 and the second order derivative linear-sweep voltammograms were recorded. The HRP content from 1.0 to 1.0×10^2 mU/L has a good linear relation with the peak height in BR buffer solution. The relative standard deviation is 4.1% for eleven parallel determinations with 1.0 mU/L HRP, and the detection limit of HRP is 0.4 mU/L (3σ).

Determination of labelled HRP

The labelled HRP IgG-HRP was detected under the optimum experimental conditions. After the IgG-HRP was adsorbed on the solid-phase carrier, the effect of the solid-phase carrier on the determination was studied in order to use the system in the immunoassay of different viruses. The result was shown in Fig. 3. The detection result of the *o*-phenylenediamine spectrophotometric ELISA method was also shown in Fig. 3. As for the above mentioned labelled HRPs, the highest dilution ratios detected by this method are $1:9.0 \times 10^7$; $1:1.0 \times 10^6$. The highest dilution ratios detected by the *o*-phenylenediamine spectrophotometric ELISA method are $1:1.6 \times 10^7$; $1:2.1 \times 10^5$. The detection limits of this method are lowered by 5 times than those of the *o*-phenylenediamine spectrophotometric ELISA method.

Determination of SBMV

The determination of SBMV was carried out with indirect method. The advantages of which are that the common enzyme label suitable for various viruses, *e.g.* goat antirabbit IgG-HRP, was adopted, and the preparation of enzyme labelled-antibody for each detected virus is not necessary. So the manipulation is simpler, the detection cost lower and application easier. The more the detected virus (Ag) is, the more the IgG-HRP combined on the solid phase carrier, the higher the voltammetric peak is. Nonspecific adsorption of SBMV and labelled antibody can be suppressed by using Tween 20 and bovine serum albumin (BSA).

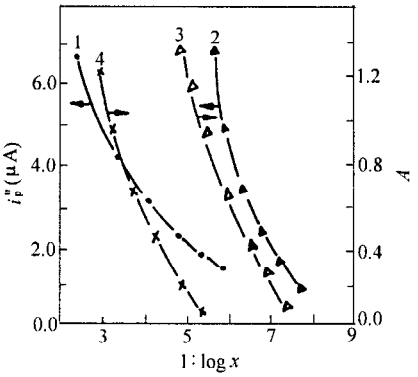


Fig. 3 Dilution curves of detecting the labelled HRP. (1), IgG-HRP with this method; (2), IgG-HRP adsorbed on the solid phase carrier with this method; (3), IgG-HRP with *o*-phenylenediamine spectrophotometric ELISA method; (4), IgG-HRP adsorbed on the solid phase carrier with *o*-phenylenediamine spectrophotometric ELISA method.

Optimum working concentrations of SBMV-As *and* IgG-HRP

Selection of working concentrations of SBMV-As *and* IgG-HRP The polystyrene immunoplates were coated with the purified SBMV of 10 μg/mL. The SBMV-As was multiply diluted with PBS-Tween 20. The experiment was conducted according to the procedure with 1:640 of IgG-HRP and SBMV-As of different dilution ratios. The peak height is the highest when the dilution ratio of SBMV-As is 1:6400. Similarly, the experiment was conducted with 1:6400 of SBMV-As and IgG-HRP of different dilution ratios. According to the principle that the labelled antibody concentration should be at the upper point of the one third of the linear portion of the concentration curve of the labelled antibody,[11] the selected working concentration of IgG-HRP is 1:640.

Linear range, detection limit and precision of purified SBMV *determination*

Under the optimum conditions, the linear range of the purified SBMV determination is 8.0—5000 ng/mL and the detection limit is 8.0 ng/mL. The equation of linear regression is $i_p'' = -24.15 + 11.54 \log_2 C$ ($n = 7$, $\gamma = 0.9864$). The relative standard deviation is 3.2% for eleven parallel determination with 60.0 ng/mL SBMV solution. Under the same conditons, the linear range of the purified SBMV determination is 40.0—5000 ng/mL and the detection limit is 40.0 ng/mL with *o*-phenylenediamine spectrophotometric ELISA method. The equation of linear regression is $A = -0.521 + 0.464 \log C$ ($n = 7$, $\gamma = 0.9827$). The detection limit of this method is 5 times lower than that of *o*-phenylenediamine spectrophotometric ELISA method.

Determination of SBMV-*infected leaf sap*

The infected leaf sap was diluted with carbonate buffer (0.1 mol/L, pH 9.6). Under the optimum conditions, the infected leaf sap was detected. The results of this method and *o*-phenylenediamine spectrophotometric ELISA method were listed in Table 1. The highest dilution ratio detected is $1:1.5 \times 10^5$ and the detection range is $1:1.5 \times 10^5$—1:50 with this method. The highest dilution ratio detected is $1:3.0 \times 10^4$ and the detection range is $1:3.0 \times 10^4$—1:50 with *o*-phenylenediamine spectrophotometric ELISA method. The detection limit of this method is 5 times lower than that of ELISA method. The result of this method is linearly proportional to that of *o*-phenylenediamine spectrophotometric ELISA method, and the equation of linear regression is $y = -47.96 + 1.06x$, (x is the results of this method; y is the results of ELISA method; $n = 5$, $\gamma = 0.9992$).

Table 1 Determination of SBMV-infected leaf sap

Dilution ratio	This method (ng/mL)	ELISA method (ng/mL)
$1:7.8 \times 10^5$	-	-
$1:1.5 \times 10^5$	14.4	-
$1:3.0 \times 10^4$	37.1	45.2
$1:6.0 \times 10^3$	65.7	66.6
$1:1.2 \times 10^3$	286.2	252.0
$1:2.5 \times 10^2$	896.1	1066.0
1:50	5770.0	5458.0

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