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Immunosensor for Rapid Detection of Gibberellin Acid in the Rice Grain

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A rapid, selective, sensitive, accurate, and inexpensive immunosensor for gibberellin acid detection was designed by coupling immunoassay with the square wave anodic stripping voltammetry (SWASV) technique involving copper ion labeled antigen in the competitive immunoreaction. The response signal expressed as the percentage of current reduction CR % (y) is linearly related to the concentration of GA (x) in the 1 μg/mL to ~150 μg/mL range with a regression equation of the form $y = 0.44x + 15.59$ and a correlation coefficient of 0.99. The results of the immunosensor assay were compared with those obtained by HPLC and ELISA, which show a satisfactory agreement. The immunosensor was used to determine the GA content in the hybrid rice grain samples taken in the growing period.

KEYWORDS: Immunosensor; gibberellin acid; competitive immunoreaction; copper ion labeled

INTRODUCTION

Gibberellin acid (GA) is a ubiquitous phytohormone essential for normal plant growth. In the whole life of the plant, it regulates many important physiological activities and determines most properties of the plant, such as stem shape, blossom, fruit, and gene expression (*1*, *2*). Monitoring and controlling GA in crops can ensure efficient development of the crops, which is very significant to agriculture and horticulture. Since the content of a phytohormone in a plant is very low, and easily decomposed by heat, light, and oxygen, the traditional methods such as GC (*3*), HPLC (*4*, *5*), CE (*6*), ELISA, and radioimmunoassay (*7*, *8*) are usable for the phytohormone assay, but some of them require sophisticated instrumentation or radioactive chemicals or are time consuming. So, exploring simple and convenient methods for phytohormone assay is of considerable interest to the phytohormone research.

Recently, biosensors continue to advance at an accelerated pace, and some of them are now being successfully used in clinical chemistry, food industry, and environmental fields. The principle of an immunosensor is the biosensor in the immunoassay based on the immunoreactions of antigen−antibody and measurement by the specific activity of the label such as radioactivity or enzyme activity, which made the immunosensor character of specificity and high sensitivity a fast, low-cost, reliable, and precise trace analysis tool (*9*, *10*). The advancement in the biosensors makes it is feasible and significant to design the immunosensor as a simple and convenient tool to assay phytohormone in the plant (*11*−*13*).

Owing to the various labels of enzyme, immunoassay with electrochemical detection has several advantages over the widely used spectrophotometric methods (*14*−*16*). Although some of them are labeled with enzymes such as alkaline phosphatase, horseradish peroxidase has the sensitivity comparable to radioimmunoassay, but to the plant extraction sample, it might be seriously affected by assay conditions such as pH, ionic strength, or coexisting substances in the sample. The novel metalloimmunoassays with electrochemical detection involving metal-based labels provide possibilities of overcoming those problems associated with enzyme labels and offer several advantages. Measurement in low volumes, in turbid media, had good sensitivity for a relatively inexpensive instrumentation (*17*−*19*). As an electrochemical assay technique, anodic stripping voltammetry is particularly well-adapted for sensitive measurements of heavy metals such as copper, which offer an attractive way of immunosensor development.

This paper reports the use of immunological technology, in conjunction with the square wave anodic stripping voltammetry (SWASV) detection, to design an immunosensor for the assay of GA in plants. The principle of this immunosensor as shown in **Figure 1** is based on the competitive immunoreaction. This reaction takes place between the Cu^{2+} labeled antigen (GA-BSA-Cu^{2+}) as the signal producer and GA as the analyte on the glassy carbon electrode (GC) surface assembled with the anti-GA IgG by the *Staphylococcal* protein A (SPA) that was absorbed on the nano-Au particles loaded by the amine groups on the surface of the silanized GC (*20*, *21*). This immunosensor can carry out a rapid, selective, sensitive, accurate, and

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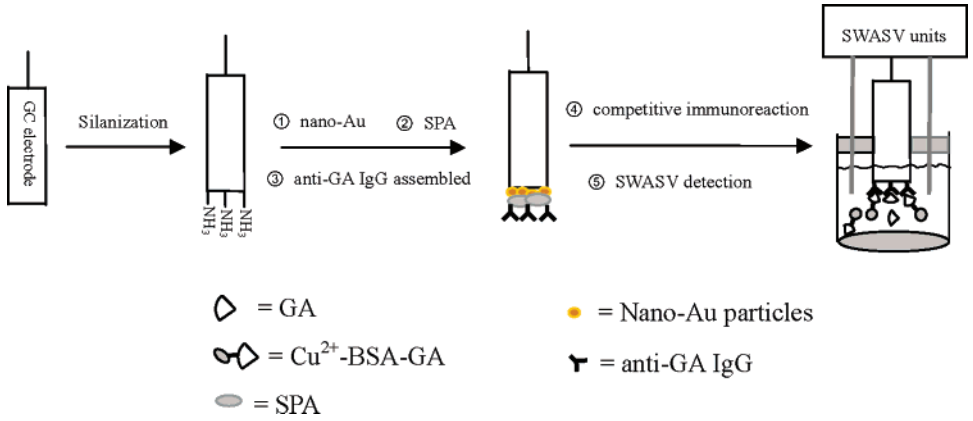

Figure 1. Schematic diagram of the working principle of the immunosensor.

inexpensive assay for GA detection in plants. GA was detected in the range of 1−150 μg/mL, and different types of hybrid rice grain were analyzed in this way with results in satisfactory agreement to those of HPLC and ELISA.

MATERIALS AND METHODS

Reagents. (Aminopropyl)triethoxysilane (APTES) was purchased from Wuda Chemicals Co. (Wuhan). Dimethylformamide (DMF), hydrofluoric acid (HF), sodium−potassium tartrate, and cupric sulfate were purchased from Beijing Chemicals Co. (Beijing). *Staphylococcal* protein A (SPA) was obtained from the Shanghai Institute for Bioreagents (Shanghai). Bovine serum albumin (BSA) was from Lizhu Biotechnology Company (Shanghai). Skim milk was from Inter Mongolia Milk Co. (Huhehaote). 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) and polyvinyl−pyrrolidone polymer (PVPP) were obtained from Sigma. Gibberellin acid was the product of ICN Biomedical Inc. Diethylaminoethyl-cellulose (DEAE) was purchased from Pharmacia. C_{18} Sep-Pak cartridges were purchased from Waters.

All other reagents were of analytical grade, and doubly distilled water was used throughout.

Apparatus. Quantitative determination of copper was carried out by square wave anodic stripping voltammetry (SWASV) in a three-electrode system consisting of an immunosensor, a Ag/AgCl reference electrode, and a platinum wire auxiliary electrode, using a microcomputer-based electrochemical analyzer LK98A of Tianjin Electrochemical Instruments (Tianjin, China). All potentials are referenced to Ag/AgCl. The assay cell with a stir bar was placed in a CJJ78-1 magnetic stirrer of Jintan Instruments (Jiangsu, China). A model CSS501 thermostat (Chongqing) was used to control the temperature.

A Benchmark Plus microplate spectrophotometer (Bio-RAD, Japan)-was used to read the absorbance on the ELISA plates at 405 nm. The HPLC system was an Agilent 1100 series.

Preparation of Coating Antigen. Coating antigen was prepared according refs *22* and *23*. GA (100 mmol) and EDC (140 mmol) were dissolved in 200 μL of 50% DMF (pH 5.3 borate buffer) and stirred for 15 min at room temperature. Then, the pH was adjusted to 6.5 with 0.1 M NaOH, and the mixture was added to 500 μL of 0.5% BSA (w/v, in 50% DMF solution). The reaction was carried on for 4 h under nitrogen atmosphere at 4 °C. The coating antigen of GA-BSA conjugate inside the dialyzer was obtained from the mixture solution purified by dialysis against 10% DMF at 4 °C overnight followed by lyophilization.

Immunization and Antiserum Collection. A male white rabbit was immunized. The GA-BSA conjugate (2 mg) dissolved in 0.9% sodium chloride solution (1 mL) and emulsified with complete Freund's adjuvant (1 mL) was injected. At 1, 2, and 5 weeks after the first injection, the same conjugate emulsified with incomplete Freund's adjuvant (1 mL) was repeatedly injected. One week after the last injection, about 60 mL of rabbit blood was collected and allowed to stand at room temperature for 1 h, then centrifuged at 3000 rpm at 4 °C for 15 min to separate out the antiserum, and stored at −20 °C until use.

Immunodiffusion. Agar (2 g) was dissolved in 200 mL of 0.9% NaCl in a 500 mL beaker while being heated. After the mixture was cooled to 50 °C, 4 mL aliquots were pipetted into sterile 30 × 90 mm glass boards and allowed to solidify. Agar plugs were removed by suction to form the desired pattern of wells. Solutions of antiserum (each of 100 μL) were pipetted into the peripheral wells, and BSA-GA (100 μL of 20 μg/mL) was introduced into the central well. The glass board was covered and placed inside a moisture chamber and stored at 37 °C for 24 h, and lines of precipitation were then examined.

Antigen Labeled by Cu^{2+}. Sodium hydroxide (4.0 g) and sodium−potassium tartrate (4.5 g) were dissolved in 400 mL of water, with 3.0 g of $CuSO_4$ added and diluted to 500 mL. 1 mL of the mixture solution was mixed with 1 mL of antigen (BSA-GA) with the concentration of 40 mg/mL (w/v, in 0.9% NaCl solution). The solution was kept at 4 °C for 2 h and dialyzed in 50 mM phosphate buffered saline (PBS, pH 7.0) for 24 h. Finally, the labeled antigen complex solution inside the dialysis tube was stored at 4 °C.

Preparation of the Immunosensor. The glassy carbon electrode (GC, 3 mm in diameter) of Jiangsu Electrochemical instruments (Jiangsu, China) was polished in a water slurry of 0.3 μmol of Al_2O_3 and cleaned in 70% nitric acid, followed by being washed in water and acetone. The cleaned GC was kept in a 5% HF solution for 30 min, in 10% H_2O_2 for 1 h, and then was silanized in 10% APTES in acetone (v/v) for 5 h. After the silanization, the GC was rinsed with acetone and dried for 30 min at 50 °C (*24*). The silanized GC was immersed in a gold−colloid solution for 2 h to modify the surface with the nano-Au particles (*20*, *25*), and after that it was washed with water, dried in air, and covered with 40 μL of 1 mg/mL SPA incubated at 35 °C for 1 h. The resulting electrode was washed with water and dried in air, and the modified GC surface was spread with 20 μL of a 1:40 antiserum solution (the stock antiserum solution diluted with 10 parts of 0.9% sodium chloride solution) and incubated for 1 h at 35 °C to assemble the anti-GA IgG. After being washed with 50 mM PBS pH 7.0, it was rinsed with water and dried in air. The anti-GA IgG assembled GC was treated with 10 μL of 5% milk (w/v) in 50 mM PBS (pH 7.0) at 35 °C for 15 min to prevent nonspecific adsorption, followed by being washed with 50 mM PBS ready for immunoassay.

Preparation of Plant Material Samples. Sample preparation followed published techniques (*26*). Fresh samples of hybrid rice (*Oryza sativa* L.) grain at the filling stage were frozen in liquid nitrogen and immediately placed in a lyophilizer for at least 48 h. All lyophilizer bottles were covered with aluminum foil to protect the samples from light exposure at all times. Each lyophilized sample was ground in a Willey mill and passed through a 40 mesh screen. An appropriate amount (1.0 g) of ground sample was placed in a vessel and mixed with 15 mL of 80% (v/v) methanol. The extraction process was continued overnight in a refrigerator. After rotary evaporation, the supernatant was collected after being centrifuged at 15 000 rpm for 20 min. The supernatant was separated by a PVPP-DEAE column system and collected by Waters C18 Sep-Pak cartridges. The phytohormones eluted out of the Sep-Pak with 5 mL of 50% (v/v) methanol, dried in a Jouan vacuum concentrating system, and methylated by 400 μL of diazomethane. The final sample extract was dissolved in 200 μL of 20% methanol for the following assay.

HPLC conditions. The HPLC protocol followed published techniques (*4*, *5*). The HPLC system was an Agilent 1100 series; an RP

C_{18} column (Hypersil ODS, 125 × 4 mm, 5 μm) protected by a guard column (Hypersil ODS, 4 × 4 mm, 5 μm) was used. The mobile phase consisted of methanol−water (40:60, v/v) with 1% acetic acid, which was at a flow rate of 1 mL/min. Diode-array and mutiple-wavelength detectors recorded the UV absorbance at 278 nm. HPLC-grade methanol and pure water were used throughout.

ELISA Assay. ELISA was carried out according to refs *5* and *27*. Antiserum was diluted with the carbonate/dicarbonate buffer (pH 9.6, 50 mM) at a fixed (1:300 v/v) concentration, and the diluted antiserum (100 μL) was added to a microplate and left overnight at 4 °C. After immobilization, the wells were washed three times with a solution of PBS-T (PBS + 0.05% Tween 20). A solution of 5% skimmed milk (200 μL) was used in blocking step (1 h at 37 °C). After being washed, the competition was performed as follows. Different concentrations of GA were added in triplicate to wells (100 μL of each) with 100 μL of GA-HRP (50 μg/mL, GA conjugated with horseradish peroxidase). The reaction was left to proceed for 30 min at 37 °C and then was rinsed with PBS-T. Finally, the chromogen/substrate solution was added to the wells, and the enzymatic reaction was stopped after 10 min at room temperature by the addition of H_2SO_4 (2 mol/L, 100 μL). Absorbance was read at 405 nm.

Immunosensor Assay Procedure. The assembled GC was immersed into 1 mL of 0.9 % NaCl (w/v), in which 100 μL of the 50 μg/mL Cu^{2+}-BSA-GA solution and 50 μL of sample solution were added. After being incubated for 30 min at 35 °C, the quantitative determination of copper ions was carried out by square wave anodic stripping voltammetry (SWASV) with the peak current recorded as I_x. When no GA existed in the competitive reaction, the peak current was recorded as I_0. The percent current reduction (CR %) of the competitive immunoreaction is given by

$$\mathrm{CR}\,\% = \frac{I_0 - I_x}{I_0}100 \qquad (1)$$

A series of standards of GA ranging from 1 to 150 μg/mL was used to construct the calibration curve, which could be used for the determination of GA in unknown samples.

After each immunoassay, the assembled GC was immersed in a cell with 5 mL of glycin-hydrochloric acid buffer (pH 2.4). After being stirred for 15 min, the GA and copper ion labeled antigen (Cu^{2+}-BSA-GA) absorbed on anti-GA IgG on the surface of the assembled GC electrode were rinsed out, and the electrode was regenerated for the next use.

RESULTS AND DISCUSSION

Conjugated Ratio in Antigen Preparation. Small molecules GA must be conjugated to a macromolecule BSA, a carrier protein, to become an antigen raising the antibodies specific against GA. Usually, a high ratio of the hapten conjugated to the carrier can provide good immunization results, and as for BSA, the molar ratio of hapten to the carrier should be in the range of 10:1 to 20:1 (*28*). However, a high conjugated ratio would change the structure of the protein greatly and sometimes block some of the critical sites of the carrier because of the hapten conjugating, which may reduce the immunogenic activity of the antigen. Having a strong absorbance at 280 nm, UV spectrophotometric determination of the conjugated compound indicates that the optimal ratio of GA conjugated to BSA in immunization is 12:1. The conjugated ratio (R) was given by

$$R = \frac{A_{\mathrm{BSA-GA}} - A_{\mathrm{BSA}}}{A_{\mathrm{GA}}} \qquad (2)$$

Figure 2 shows the change of the UV spectrum when the GA is conjugated to the BSA.

Characterization of Antiserum. The titer of the antiserum increases with the immunization schedule and reached 1:32 after 8 weeks from the first injection as **Figure 3(I)** shows. When the antibody is allowed to diffuse toward its antigen in a gel matrix, they will form a visible precipitin band at their optimal concentration, but there is no precipitin band when the antibody meets with the antigen without the affinity. In **Figure 3** (**II**), the antiserum from well c with a dilution of 1:100 (v/v, in 0.9% NaCl solution) formed a precipitin band with 20 μg/mL BSA-GA from well b, while no precipitin band appeared between well c and well a of 20 μg/mL BSA-IAA. In **Figure 3** (**III**), when 500 μg/mL GA blended in 20 μg/mL BSA-IAA in well b, the precipitin band became weak due to GA inhibiting BSA-GA combining to the antibody in the antiserum, indicating that the antibody in the antiserum has a very good specificity character.

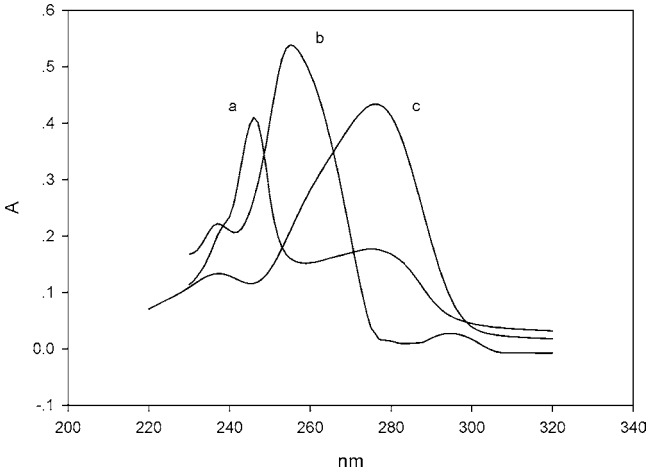

Figure 2. UV spectrum of a: GA; b: GA-BSA; and c: BSA.

Characteristic Performance of the Immunosenor. SWASV is one of the sensitive electroanalytical techniques that is employed to detect reversible redox systems. The copper ion, which was labeled on the antigen, was adsorbed onto the electrode surface by the immunoreactions and electrodeposited as copper. The electrodeposited copper was stripped from the electrode by anodic oxidation bringing a peak current that depended on various parameters such as the potential step increment, square wave amplitude, and frequency.

The effect of deposition potential on the stripping peak response was studied at different applied potentials. The maximal stripping peak current was obtained at the deposition potential of −1.0 V. The peak current is proportional to the frequency of the square wave (SW), and increasing the SW frequency would improve the analytical sensitivity; however, as the greater frequencies would increase the background also and distort the peak current, we find that 100 Hz is the optimal value. The sufficient preconcentration time made a sufficient amount of copper ion transfer to copper and deposit on the electrode surface, which brought a large peak current. It has been found that 300 s of preconcentration can guarantee both sensitivity and speed of the assay. The other SWASV conditions chosen in the experiment include a final potential of 0 V, a potential step increment of 5 mV, and a quiescent time before the potential scan started of 10 s (*29*, *30*).

Figure 4 shows the square wave anodic stripping voltammograms obtained with the biosensor immersed in 0.9% NaCl (w/v) solution. There is a current peak at −0.36 V (vs Ag/AgCl). Curve a was recorded after the competitive reaction between 50 μL of 20 μg/ml GA and 100 μL of 50 μg/mL Cu^{2+}-BSA-GA. When the amount of GA changed to 100 μg/mL, voltammetry curve b was recorded. The decrease of the current peak indicates that the GA inhibiting Cu^{2+}-BSA-GA combined with the anti-GA IgG on the electrode surface. Curve c shows the peak current

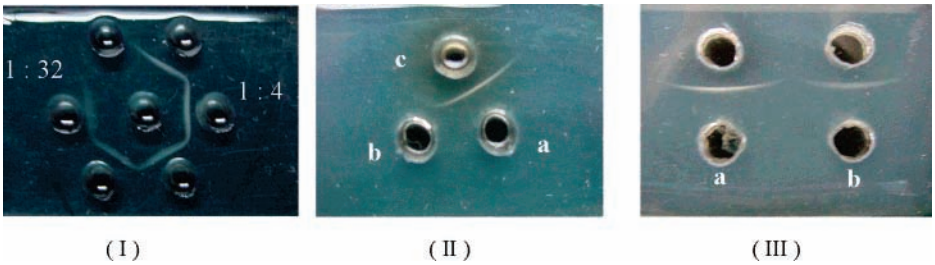

Figure 3. Specific characteristic of the antibody (**I**). 8 weeks after the first injection. (**II**) a: 100 μg/mL BSA-GA; b: 100 μg/mL BSA-IAA; and c: 1:100 antiserum (v/v). (**III**) a: the deposition line of 100 μg/mL BSA-GA combining with 1:100 antiserum (v/v); b: the same with 500 μg/mL GA in 100 μg/mL BSA-GA solution.

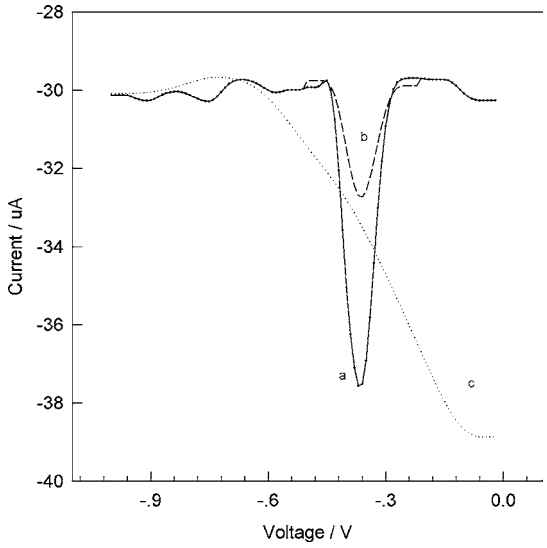

Figure 4. Square wave stripping voltammetry of the immunosensor in 0.9% NaCl solution after the competitive immunoreaction between 100 μL of 50 μg/mL Cu^{2+}-BSA-GA and 50 μL of a: 20 μg/mL GA; b: 100 μg/mL GA; and c: washed by a glycin-hydrochloric buffer of pH 2.4 after immunoassay.

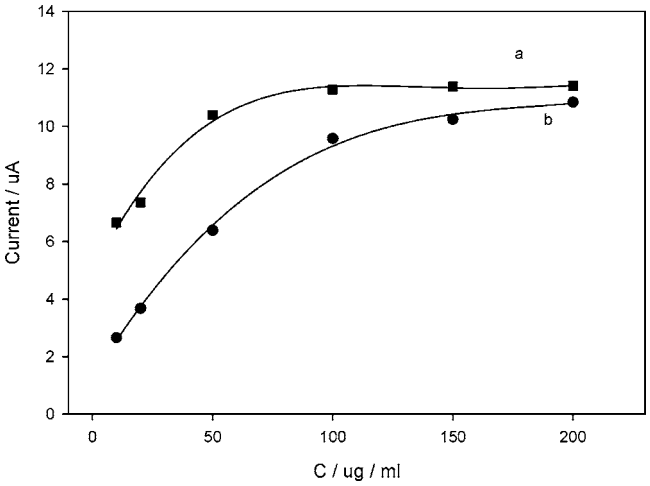

Figure 5. Peak current responses of the immunosensor incubating with different concentrations of Cu^{2+}-BSA-GA: a, without GA and b, with 50 μg/mL GA competing.

of the immunosensor washed by glycin-hydrochloric buffer of pH 2.4 after the immunoassay. Since Cu^{2+} was washed from the electrode, no current peak appeared.

Optimum Experimental Conditions for Immunosensor Assay. The current response of the immunosensor increased with the concentration of Cu^{2+}-BSA-GA in the assay solution as shown in **Figure 5**. The concentration of Cu^{2+} labeled antigen in the competitive immunoreaction in the assay solution is proportional to the amount of Cu^{2+} bound to the surface of immunosensor, which corresponds to the current response in SWSAV, so the current response increased with Cu^{2+}-BSA-GA in the assay solution. As curve a in **Figure 5** shows, the immunosensor gives a current response of about 7 μA to 25 μg/mL BSA-GA-Cu^{2+} in the assay solution, while it increases to 11 μA when the concentration of BSA-GA-Cu^{2+} is 150 μg/mL. Since the amount of the anti-GA IgG on the electrode surface corresponds to the number of Cu^{2+}-BSA-GA combining, the current response curve in **Figure 5** tended toward a constant value when the labeled antigen concentration was greater than 100 μg/mL. In the competitive immunoreaction, GA has inhibition to Cu^{2+}-BSA-GA, and as curve b in **Figure 5** shows, adding 50 μg/mL GA in the assay solution of 25 μg/mL Cu^{2+}-BSA-GA made the current response decrease to 3.6 μA from 7.1 μA reducing 49%. The same GA added in the assay solution of 150 μg/mL Cu^{2+}-BSA-GA caused the current response to decrease to 10 μA from 11 μA reducing only 9%, which indicates that GA has a larger inhibition to Cu^{2+}-BSA-GA with a low concentration than a high concentration during the competitive immunoreaction. Since the amount of GA in the sample is very small, to provide a nice competition environment with Cu^{2+}-BSA-GA, the concentration of the latter should not be too high in the reaction medium. We employed 50 μg/mL Cu^{2+}-BSA-GA in the experiment to guarantee both a sufficiently large current signal and a significant current change due to the competitive immunoreaction.

The dependency of the peak current on the antiserum dilution was investigated. After immobilization of protein A, a fixed volume (20 μL) of antiserum solutions of dilutions ranging from 1:5 to 1:160 was spread on the electrode surface followed by incubating, washing, and drying to accomplish immobilization of the sensor surface.

The results shown in **Figure 6** indicated that the peak current reached the highest value when the final antiserum dilution was 1:40 and then decreased as the antiserum concentration increased further. The amount of SPA on the electrode surface is fixed, which serves as Fc receptors immobilizing IgG oriented by binding its Fc portion, leaving the antigen-specific sites free. When the final antiserum dilution is greater than 1:40, the amount of the IgG in the antiserum solution is not enough to form sufficient antigen-specific sites at the sensor surface, and it would reduce the amount of Cu^{2+} labeled antigen combined on the sensor surface after the immunological reaction and decrease the peak current. When the antiserum dilution is smaller than 1:40, the number of anti-GA IgG bonds on the electrode surface increases. As the space on the sensor surface is limited, the antibodies may be immobilized very closely to each other, causing a steric hindrance and impairing the ability of the antibodies to bind to the antigen, and at the same time, a high

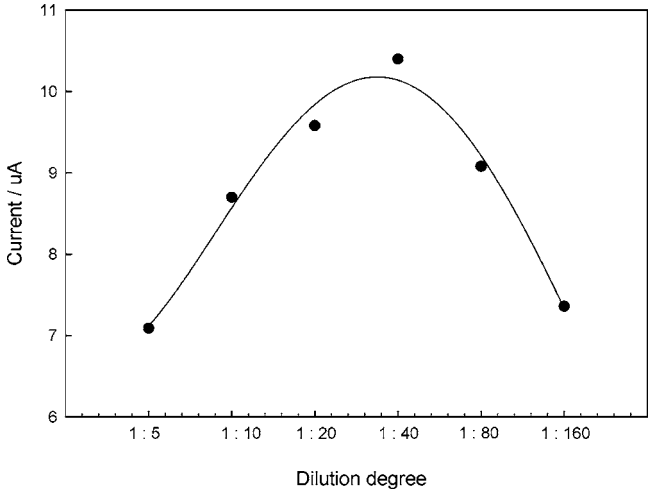

Figure 6. Current responses of the immunosensor plotted vs the dilution degrees of the antiserum.

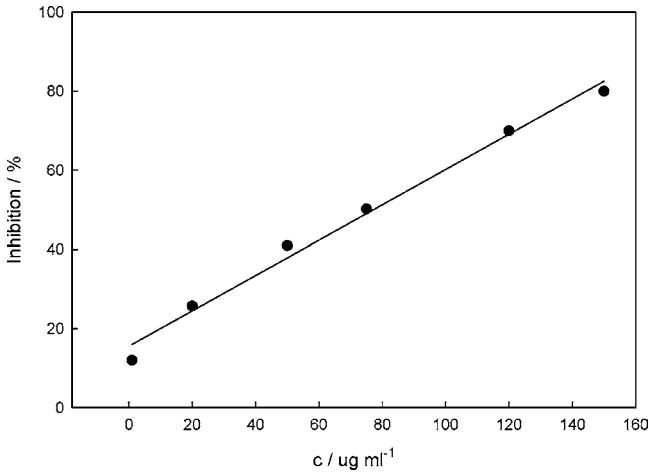

Figure 7. Calibration curve under the optimal experimental conditions.

concentration antiserum would increase the nonspecific adsorption on the electrode surface and reduce the amount of the immobilized anti-GA IgG. It seems that only with an optimum dilution ratio of the antiserum can one make the antibody so well self-assembled on the sensor surface as to induce the highest peak current. So an antiserum dilution ratio of 1:40 was used in the following experiments.

The influence of the temperature on the current response was investigated in the different temperature ranging from 20 to 45 °C under the same experimental conditions (data not shown). Generally, the rate of an immunological reaction increases with an increase of the reaction temperature, so the current response of the immunosensor increases with the temperature up to 35 °C in the experiment. While the optimal temperature of the immunoreaction is usually 37 °C, a temperature higher than this temperature might destroy the combined Ag-Ab and lessen the Cu^{2+} that was bond to the surface of the assembled GC by Ag-Ab combining, which made the current response decreased. So our experiments were conduced at the optimal temperature of 35 °C.

Calibration and Reproducibility of the Immunosensor. The calibration curve for GA was based on CR % versus [GA] as shown in **Figure 7**. The CR % (y) is linearly related to the concentration of GA (x) in the 1 μg/mL to ~150 μg/mL range with a regression equation of the form $y = 0.44x + 15.59$ and correlation coefficient of 0.99. To compare the results of the immunosensor with those of HPLC and ELISA, the results of

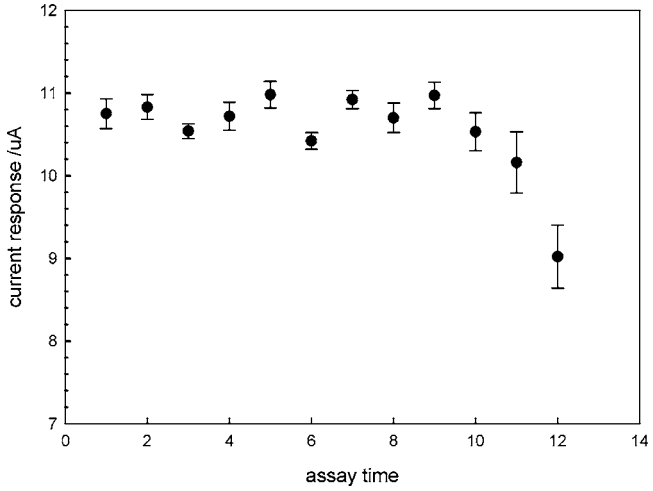

Figure 8. Repeated use of the immunosensor after regeneration. The immunosensor was incubated with 50 μg/mL Cu^{2+}-BSA-GA under the optimal assay condition for each time.

Table 1. Analysis of Hybrid Rice Grain Samples[a]

	concentration of GA, μg/g (mean ± s, $n = 3$)		
sample	immunosensor	ELISA	HPLC
A-6	129.3 ± 6.2	120.1 ± 12	122.8 ± 4.4
A-8	154.9 ± 7.0	156.9 ± 10	150.8 ± 4.8
B-4	137.9 ± 7.6	143.2 ± 12	140.1 ± 4.2
B-5	80.3 ± 7.8	90.5 ± 9.0	85.0 ± 4.6
C-6	131.8 ± 8.0	137.4 ± 11	132.5 ± 4.5

[a] The samples were obtained from Hunan Academy of Agriculture Science.

the analysis of rice sample determined by these three methods are presented in **Table 1**. The statistical elaboration of the data in **Table 1** was done using SAS (statistical analysis system) software, and an analysis of variance (ANOVA) procedure was applied, the results of which showed that there were significant differences ($F = 139.90$, $P < 0.0001$) among the concentrations of the different samples, but no significant differences ($F = 1.12$, $P = 0.2734$) among the results obtained from these three kinds of detection methods that indicated the immunosensor having a satisfactory agreement with ELISA and HPLC.

The immunosensor can be repeatedly used for 10 times with a RSD of 1.8% before the assembled layer becomes loose as shown in **Figure 8**. Five immunosensors made independently yielded a mean current change of 10.6 ± 1.2 μA.

Conclusion. The immunosensor described in this paper provides an alternative tool for determining GA in the plant samples. Its assay speed and efficiency allowing a rapid throughout of the extracted sample are particularly useful for the simultaneous analysis of large numbers of samples. The reliability of the method is shown by the satisfactory agreement of the analytical results with those obtained by HPLC and ELISA. The feasibility of the practical application of this proposed immunosensor for GA determination in the plant samples has been demonstrated by hybrid rice grain sample analysis.

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