A Novel Piezoelectric Biosensor for the Detection of Phytohormone β-Indole Acetic Acid

Jin LI,* Zhao-Yang WU,* Lang-Tao XIAO, Guang-Ming ZENG,* Guo-He HUANG,* Guo-Li SHEN,*† and Ru-Qin YU***

**State Key Laboratory of Chemo/Biosensing and Chemometrics, Chemistry and Chemical Engineering College, Department of Environmental Science and Engineering, Hunan University, ChangSha 410082, P. R. China*
***Key Laboratory of Phytohormones, Hunan Agricultural University, ChangSha, 410128, P. R. China*

A novel piezoelectric immunosensor has been developed for the determination of β-indole acetic acid (IAA) in dilute solutions. The detection is based on competitive immunoreaction between a hapten (IAA) and an antigen (IAA-BSA, hapten-protein conjugation) bound to an anti-IAA antibody, immobilized on a quartz crystal microbalance (QCM). The frequency change (y) of the sensor caused by antigen is linearly related to the logarithm of the concentration of IAA (x) in the range of 0.5 ng/ml – 5 μg/ml with a regression equation of the form $y = -23x + 151$ ($r = 0.9937$).

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Introduction

β-Indole acetic acid (IAA) is a ubiquitous phytohormone essential for normal plant growth. It is involved in almost every developmental process in the whole life cycle of a plant. Phytohormone is a natural substance which can regulate the life course and the physiological activities of plants, such as gene expression, response to outside stimulates, cell division, blossoming, fruit ripeness, and tissue decay. Much research indicates that phytohormone has the function of a signaling molecule in plants.[1,2] Like other phytohormones, the IAA content changes in different growth periods of a plant. It can promote plant growth at low concentration and can inhibit plant growth at a high concentration. Monitoring and controlling the IAA content in plants would help to keep plants growing efficiently. Phytohormone research is very significant to agriculture, horticulture and other related fields. It can help one to understand the basic laws of plant growth, which can also be applied to improve the output and quality of plants by adjusting the synthesis, transportation and distribution of phytohormone in plants. Phytohoemone research calls for a rapid, precise and convenient measurement procedure for phytohormone assay in plants. The content of phytohormone in a plant is very low, and the hormone is easy to be decomposed by heat, light and oxygen. The traditional detection methods GC,[3-5] HPLC,[6-8] CE,[9] enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA)[10-12] are usable, though some of these methods require sophisticated instrumentation or radioactive chemicals, or are time-consuming. Exploring new simple, low-cost methods for phytohormone assay is of considerable interest. This paper reports on the use of immunological technology in conjunction with piezoelectric detection to design an immunosensor for an IAA assay.

† To whom correspondence should be addressed.
E-mail: shenguo@public.cs.hn.cn

IAA as a hapten could be conjugated with bovine serum albumin (BSA) to become an antigen (IAA-BSA). Through immunization in a bio-body, like a white rabbit, one could obtain an antiserum which might be purified to an immunoglobulin (IgG). The protein A (SPA) is immobilized on the golden surface of a quartz crystal to form a film where the antibody is self-assembled by bonding the Fc portion with protein A.[13] A piezoelectric sensor, known as a quartz crystal microbalance (QCM), operates according to Sauerbery[14]:

$$\Delta F = -2.3 \times 10^6 F \Delta M / A. \quad (1)$$

ΔF is the change in the resonance frequency of the quartz crystal, F is the fundamental frequency of the quartz crystal, and A is the area of the quartz crystal. The detection limit of the sensor is about 10^{-12} g; it is still not sufficient for directly detecting IAA, because the mass difference between the immobilized films with and without bonding IAA is relatively small. By using a competitive immunoreaction[15] involving the antigen IAA-BSA with a relatively high molecular weight, an amplification effect can be realized, making it possible to prepare a QCM immunosensor capable of detecting IAA in dilute solutions in the range of 0.5 ng/ml – 5 μg/ml. The design and analytical characteristics of the immunosensor as an alternative tool for an IAA assay were studied in detail.

Experimental

Reagents

BSA (A. R; Shanghai Chemical Reagents), IAA (products of chromatographic purity from Fluka), staphylococcal protein A (SPA; Shanghai Institute for Bio-reagents), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Sigma), sodium alginate (Avocado Research Chemicals Ltd.), *N*-hydroxysuccinimid (Shanghai Chemical Reagents) were used as received. Freund's adjuvants were a Gibcobrl preparation. Other reagents were of analytical reagent grade. Double-

distilled water was used throughout.

Apparatus

Quartz-crystal resonators purchased from Chenxing Radio Equipments (Beijing) consisted of a 9 MHz, AT-cut quartz wafer with vacuum-deposited gold or silver electrodes with a diameter of 6 mm on both sides. A homemade transistor-transistor logic integrated circuit (TTL-IC) was used to drive the quartz crystal at its resonant frequency. The quartz crystal was one-side sealed with a rubber O-ring and a nylon sheet, maintaining an air environment on one side of the quartz crystal, while the crystal was immersed into an aqueous solution. The resonant frequency was monitored with a frequency counter (Model CN3165, Shijiazhuang Radio Instruments, Hebei). The incubating temperature was controlled with a Model CSS501 thermostat (Chongqing). An assay was carried out in a 3 ml water-jacked cell connected to a thermed bath (24.0±0.1˚C). The PPFs were prepared with a capacitive-coupled radio frequency plasma system (No. 48 Research Institute of Electric Ministry).

Preparation of the IAA antibody

Synthesis of an IAA-BSA conjugate. An IAA crystal was first dissolved in dimethylformamide (DMF). Then, isobutyl choroformate, BSA and sodium hydroxide were added and stirred at 0˚C for 5 h. Dialysis was successively conducted against DMF, after which water was deionized and then lyophilized.

Immunization and antiserum collection. A male New Zealand white rabbit was immunized according the following procedure. The conjugate in a complete Freund's adjuvant was injected after the 1st week, and then the conjugated in the incomplete Freund's adjuvant at 3rd and 6th week. Starting from the 7th week, antiserum was collected once every 2 weeks and the conjugate in an incomplete Freund's adjuvant was injected too.

Purification of antiserum

First, 3 ml of antiserum was taken and diluted with 3 ml of 0.9% sodium chloride solution. Then, 6 ml of a saturated $(NH_4)_2SO_4$ solution was slowly added and treated at 25˚C for 30 min. The dilution was centrifugated at 9000 rpm to obtain a deposit. Then 3 ml of a sodium chloride solution was added to dissolve the deposit, with 1.5 ml of a saturated $(NH_4)_2SO_4$ solution being slowly added. The solution was left standing again at 25˚C for 30 min. The obtained deposit by centrifugating at 9000 rpm was repeatedly treated in a similar way three times. The finally obtained deposit was dissolved with 3 ml of a sodium chloride solution and dialyzed in 25 mM of PBS (pH 7.0) untill a transparent solution was obtained. The concentration C of the stock solution of IgG was found to be 0.98 mg/ml, as calculated from the absorbance A at 280 nm ($C = A/1.4$). The purified immunoglobulin was stored at 4˚C with the addition of sodium azide (0.1% final concentration).

Immobilization procedure

Quartz crystals with gold electrodes were cleaned by exposure to a piranha solution (one part of 30% H_2O_2 to three parts of concentrated H_2SO_4; caution: piranha solution can react violently with organic compound). The crystals were cleaned three times, 3 min for each time. After that the crystals were washed successively with distilled water and ethanol, and then dried in air for immobilization. Three different immobilization methods were employed.

Immobilization via SPA-gold. To the gold surface on the crystal, 5 µl of an SPA solution (1 mg/ml in 50 mM phosphate buffer of pH 7.0) was added. After incubating at 35˚C for 30 min, the crystal was rinsed with distilled water and dried in air. Then, 20 µl of a 1:10 IgG solution (the stock IgG solution diluted with ten parts of 0.9% sodium chloride solution) was spread over the electrode surface of the crystal, and the crystal was incubated at 35˚C for 1 h. The crystal was washed alternatively with 50 mM PBS of pH 7.4 and distilled water three times each, and then dried in air. The crystal was stored at 4˚C.

Immobilization through a self-assembled PA-AA layer on PPFs. Crystals were coated with 1-butyl amine plasma-polymerized film (PPFs).[16] The crystals were placed in a stainless-steel reactor with a capacitive-coupled radio-frequency plasma system. 1-Butyl amine was used as a precursor, and pure hydrogen as a carrying gas. During the deposition process, the flowing rate of the carrying gas was kept at 50 ml/min, the pressure of the reactor chamber being at 2×133 Pa, and the apparent rf power being 80 W.

The copolymer of SPA and sodium alginate (PA-AA complex) was prepared as follows. EDC (1 mg) was added to a 0.5 ml of sodium alginate solution (2 mg/ml in water), the pH was controlled within 7.5 – 7.8. The mixture was incubated with stirring at 25˚C for 5 min, then mixed with 0.5 ml of 50 mM phosphate buffer of pH 7.8 containing 1 mg of *N*-hydroxysuccinimid and 1 mg of SPA. The mixture reacted with stirring at 25˚C for 2 h, and was then dialyzed in 1 L of 25 mM PBS of pH 7.0 at 4˚C for 24 h to obtain a PA-AA complex solution. The complex solution was dropped onto a crystal surface coated with the PPFs and incubated at 25˚C for 1 h; the PA-AA complex was self-assembled onto the PPFs covering the crystal surface. The surface was washing alternatively with pH 7.4 PBS and distilled water three times. Similar treatments were conducted after spreading 20 µl of IgG solution (1:10) on the surface, followed by incubation, washing and drying to yield the immobilized crystal, which was to be stored at 4˚C.

Immobilization via covalent binding. A self-assembled layer of cystamine was prepared on a cleaned gold surface by incubating the crystal in a cystamine solution (20 mg/ml in water) at 35˚C for 1 h, washing with distilled water three times and dried in air. Then, a glutaraldehyde solution (2.5% in water) was dropped onto the crystal to react at 35˚C for 1 h. The crystal was washed with distilled water three times and dried in air. Afterward, 5 µl of a 1 mg/ml protein A solution in 50 mM phosphate buffer of pH 7.0 was spread on the crystal surface and incubated at 35˚C for 1 h. The crystal was washed alternatively with 50 mM phosphate-buffered saline (PBS) of pH 7.4 and distilled water three times each, and then dried in air. A similar procedure was used after spreading 20 µl of an IgG solution (1:10) on the crystal surface, followed by incubating, washing, and drying to accomplish immobilization of the crystal surface, which was then stored at 4˚C.

Immunoassay procedure

Before using as an immunosensor, the assembled crystal was treated with 1% BSA (w/v) in 50 mM phosphate buffer of pH 7.4 at 35˚C for half an hour in order to prevent non-specific adsorption, followed by washing with 50 mM PBS of pH 7.4 and distilled water to remove any excess BSA. The immunosensor was immersed in 3 ml of a 0.9% (w/v) saline water solution (physiological saline) and placed in a thermostated cell at 24.0±0.1˚C. The resonance frequency was monitored for an initial period of 10 – 20 min until the signal stabilized to ±1 Hz. After the response frequency tended to stabilize, 30 µl of 20 µg/ml IAA-BSA and 30 µl of an IAA

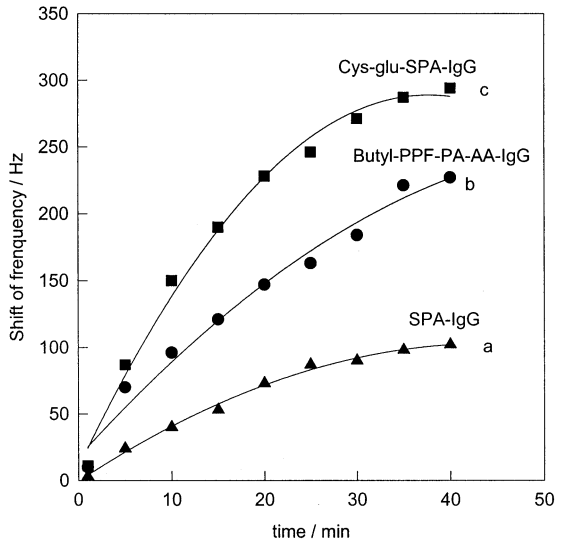

Fig. 1 Comparison of three different immobilization methods.

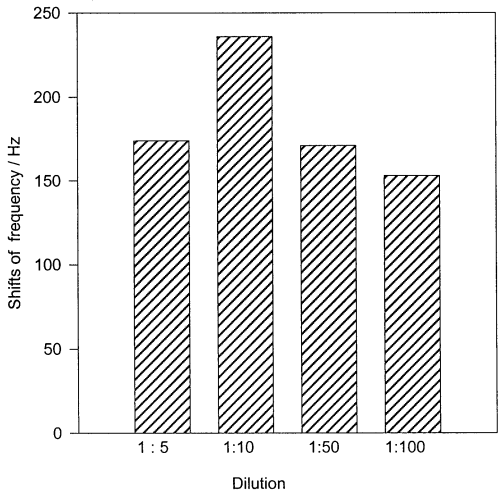

Fig. 2 Immunosensor response under the condition of different IgG dilution. The ratio 1:*x* (v/v) refers to the dilution of the stock IgG solution with *x* parts of 0.9% sodium chloride solution (see the text for details).

sample were added to the solution. At the same time, the solution was gently stirred with a magnetic bar; a stabilization period of 15-s was allowed, after which the frequency was continuously monitored. The frequency change (ΔF_t) was calculated as the difference between the frequency value (F_t) recorded after 30 – 40 min immunoreation and the initial value (F_0) recorded after 15-s waiting period. It is given by

$$\Delta F_t = F_t - F_0. \quad (2)$$

A series of standards of IAA were used to construct the calibration curve, which could be used for the determination of IAA in unknown samples.

Results and Discussion

Selection of the immobilization method

The frequency responses of the QCM immunosensors prepared by three immobilization methods after a 40-min reaction are shown in Fig. 1. The frequency response of the QCM immunosensor with immobilization of SPA is 102 Hz (Fig. 1, a). The frequency response of QCM with the immobilization of the PA-AA layer on PPFs is 227 Hz (Fig. 1, b). The immunosensor with covalent immobilization by cys-glu-SPA cross-linking exhibits a response of about 294 Hz (Fig. 1, c). The crystal immobilized by cys-glu-SPA cross-linking has the largest response, though the immobilization procedure is relatively time-consuming. The immobilization with SPA adsorption is the simplest one, while the response signal of the immunosensor prepared is also the smallest one. A method involving immobilization by the PA-AA layer on PPFs with an intermediate response value is also relatively simple, because the prepared PPF layer is sufficiently stable to allow the bio-layer immobilized on it to be regenerated and used repeatedly. Since a large frequency response is desirable in methodological studies, we used cys-glu-SPA cross-linking immobilization to construct the QCM immunosensor for the IAA assay.

Optimization of the assay condition

After immobilization of protein A, a fixed volume (20 µl) of IgG solutions of dilutions ranging from 1:5 to 1:100 was spread on the crystal surface followed by incubating, washing and drying to accomplish immobilization of the sensor surface, as described in the section concerning immobilization *via* covalent binding. The dependency of the frequency response on the IgG dilution was investigated; the results are shown in Fig. 2. It can be seen that the frequency change reached the highest value when the final IgG dilution was 1:10; it decreased with the antibody concentration either decreasing or increasing. The SPA immobilized on the crystal surface serves as Fc receptors; they can immobilize IgG oriented by binding with its Fc portion, leaving the antigen-specific sites free. The amount of SPA on the crystal surface is fixed when the final antibody dilution is greater than 1:10; the amount of the IgG in the solution is not enough to form sufficient antigen-specific sites at the sensor surface. This would make the amount of antigen that can be combined on the sensor surface by an immunological reaction reduce, so that the frequency change would decrease. When the antibody dilution is smaller than 1:10, the number of IgG bonds on the crystal increases. While the space on the sensor surface is limited, the antibodies may be immobilized very closely to each other. The neighboring antibodies may cause a steric hindrance, and thus impairing the ability of the antibodies to bind to the antigen. This might be the reason why a dilution smaller than 1:10 also causes the frequency change to decrease. It seems that only with an optimum dilution ratio the antibody can be well self-assembled on the sensor surface, inducing the highest frequency. An antibody dilution ratio of 1:10 was used in the following experiments.

Figure 3 shows the dependency of the response of the sensor on the antigen (IAA-BSA) concentration. The increase in the IAA-BSA concentration seems to be beneficial for an obtaining increased frequency response. When the concentration of the antigen is higher than 20 µg/ml, the frequency response becomes sluggish, which might be attributed to the interaction between the macromolecules, leading to undesirable absorption and desorption and an abnormal frequency change. In the following experiment an antigen concentration of 20 µg/ml was used.

The dependency of the frequency shift on the reaction temperature is shown in Fig. 4. The highest frequency change of the sensor was obtained at 35˚C. Generally, the rate of an

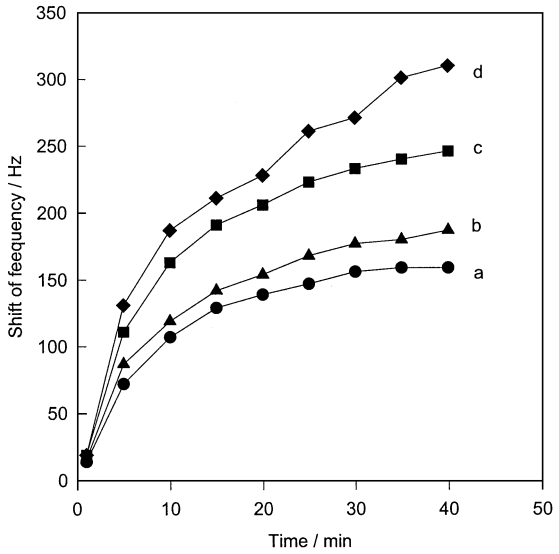

Fig. 3 Frequency response recorded under different concentration of IAA-BSA: (a) 5 μg/ml; (b) 10 μg/ml; (c) 20 μg/ml; (c) 40 μg/ml.

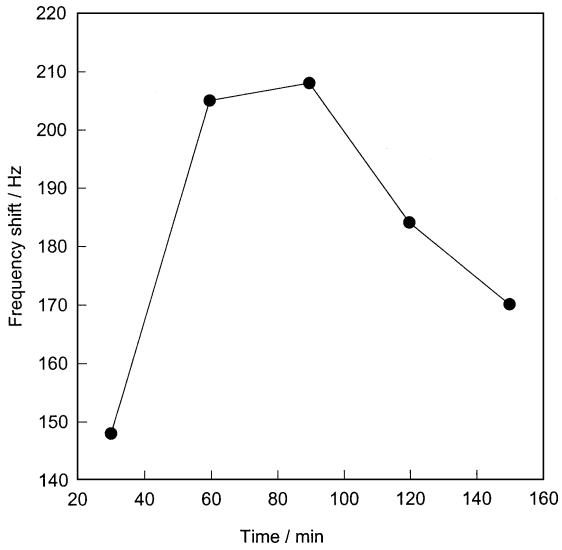

Fig. 5 Frequency response of an immunosensor prepared with different incubated times.

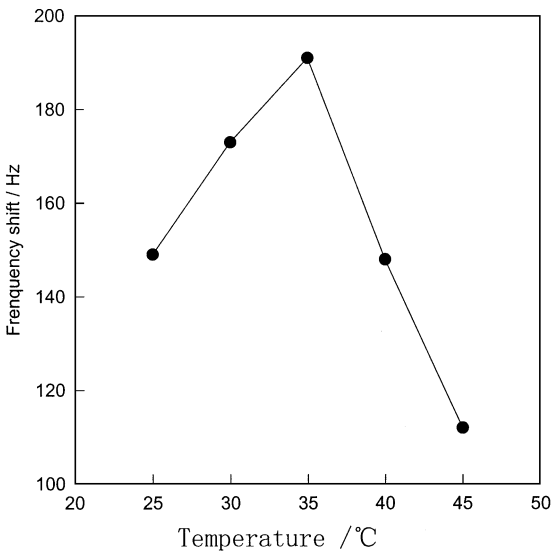

Fig. 4 Frequency response at different temperatures.

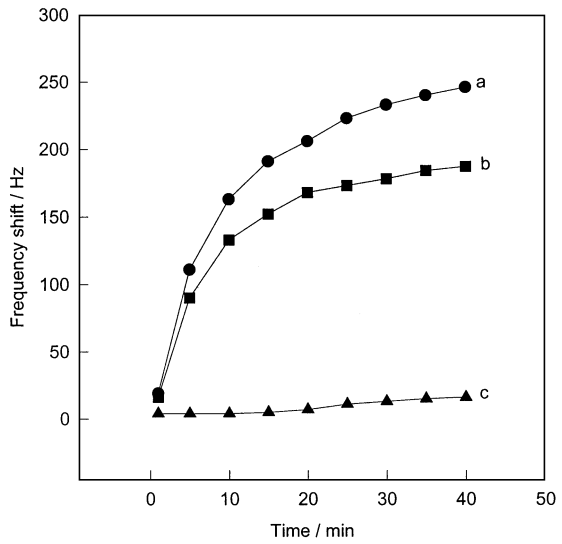

Fig. 6 Typical frequency response of the immunosensor in: (a) 20 μg/ml antigen (IAA-BSA); (b) 20 μg/ml antigen (IAA-BSA) + 1 ng/ml IAA; (c) 20 μg/ml BSA.

immunological reaction increases with an increase in the reaction temperature. A temperature higher than 35˚C may, however, impair the antigen and antibody and cause the rate of the immunological reaction to decrease. Therefore, the following experiments were conduced at 35˚C.

The dependency of the frequency shift on the incubation time during the immobilization of IgG on the surface of the crystal can be seen from Fig. 5. An incubation time shorter than 60 min seems to be insufficient for IgG immobilization with sufficient coverage of the sensor surface. A relatively long incubation time, say, more than 90 min, results in a decrease of the frequency response. We thus used 60 min as the incubation time in the immobilization step.

Figure 6 shows the frequency response of the immunosensor in solutions containing IAA-BSA or BSA. A comparison of curves a and b, obtained upon the addition of 20 μg/ml antigen of IAA-BSA without and with 1 ng/ml IAA, respectively, clearly shows the amplification effect by introducing a competitive immunoreaction involving the antigen IAA-BSA with relatively high molecular weight compared to IAA, itself. The mass change due to the added amount of IAA is clearly too small to cause a measurable frequency shift. During a competitive immunoreaction, this small amount of IAA could replace a relatively large mass of IAA-BSA, making the frequency response significantly amplified (curve b). By adding the basic fact that free IAA is more attractive to IgG immobilized on the sensor surface compared to BSA-IAA, one could imagine that the smaller IAA molecule could diffuse faster than the BSA-IAA macromolecule in solution during a competitive process. Curve c of Fig. 6 shows the frequency changes resulting from 20 μg/ml of BSA added into the solution. BSA, itself, seems not to cause any adsorption problem, and does not have a specific immunoreaction with the antibody. The change rate of the frequency decreases gradually

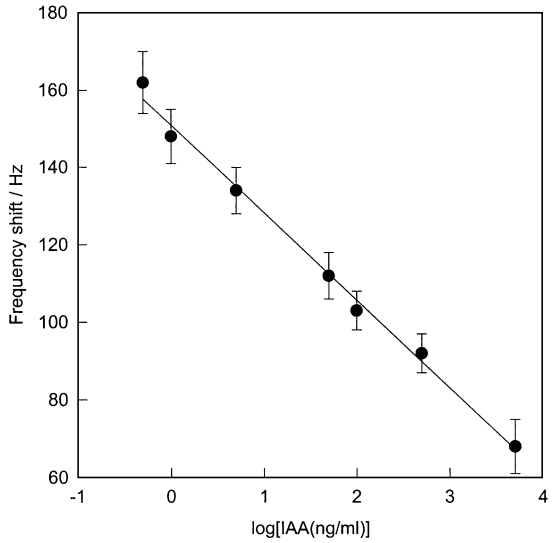

Fig. 7 Calibration graph for IAA. The vertical bars designate the standard deviation for the mean of 4 repeat tests.

with the reaction time, and tends to be stable after 30 min of the immunoreaction.

Measurement with the immunosensor

The calibration curve for IAA is based on the frequency shift (ΔF_t) *vs*. log[IAA], which is presented in Fig. 7. The frequency shift (y) is linearly related to the logarithm of the concentration of IAA (x) in the 0.5 ng/ml – 5 μg/ml range with a regression equation of the form $y = -23x + 151$ and a correlation coefficient of 0.9937. This indicates that the immunosensor can quantitatively detect IAA were a relatively wide range. The results of the analysis of four samples are presented in Table 1.

Conclusion

By using a piezoelectric immunosensor in conjuction with a competitive immunoreaction it is possible to detect IAA in dilute solutions. By using the ampliphication effect of a competitive immunoreaction involving IAA-BAS with a relatively large molecular weight, the sensitivity of the assay is substantially improved. The regeneration of the sensor is an unsolved problem and research along this line is in progress.

Acknowledgements

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Table 1 Applications of the sensor for determination of IAA (ng/ml) in solution

Sample No.[a]	Add	Found[b]	Recovery, %
1	1.00	1.08 ± 0.16	108
2	5.00	4.98 ± 0.50	99.6
3	100.00	95.57 ± 24.46	96
4	1000.00	1158.91 ± 229.32	116

a. Samples were 96% IAA (chromatogram degree, produce of Fluka) in pure water pH 3.4.
b. Mean ± *s* of three measurements.

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