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High-Sensitivity Assay for Pesticide Using a Peroxidase as Chemiluminescent Label

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The application of a newly isolated transgenic tobacco peroxidase (TOP) as a chemiluminescent label for immunoassay purposes is described for the first time. The enzyme has been oxidized with *m*-periodate and subsequently coupled to the model compound 2,4-dichlorophenoxyacetic acid (2,4-D) using a carbodiimide method. As compared to the native horseradish peroxidase used in control experiments, the TOP enzyme showed significantly higher efficiency of coupling to the antigen and no loss of the specific activity was observed. The obtained 2,4-D−TOP conjugate demonstrated unique properties in chemiluminescent detection. The latter allowed the minimization of the conjugate concentration due to the superior chemiluminescent activity of the enzyme. A highly sensitive capillary chemiluminescent immunoassay using the 2,4-D−TOP conjugate as labeled competitor is reported. Direct competitive ELISA has been performed using a specific monoclonal antibody immobilized onto the sol−gel treated glass capillary surface. A modified photomultiplier tube with a special holder for a capillary was used for the resulting chemiluminescent signal detection. The typical standard calibration curve for the 2,4-D pesticide detection is linear between 30 pg and 500 ng/mL.

Horseradish peroxidase (HRP; EC 1.11.1.7) is one of the most widely used enzymes for analytical purposes. The enzyme catalyzes oxidation of numerous natural and artificial electron donor substrates with hydrogen peroxide:

$$\mathrm{HRP} + \mathrm{H_2O_2} \rightarrow \mathrm{Cpd\ I} + \mathrm{H_2O} \qquad (k_1) \qquad (1)$$

$$\mathrm{Cpd\ I} + \mathrm{AH_2} \rightarrow \mathrm{Cpd\ II} + \mathrm{AH^\bullet} \qquad (k_2) \qquad (2)$$

$$\mathrm{Cpd\ II} + \mathrm{AH_2} \rightarrow \mathrm{HRP} + \mathrm{AH^\bullet} + \mathrm{H_2O} \qquad (k_3) \qquad (3)$$

where HRP is the ferric enzyme, Cpd I and Cpd II are the oxidized intermediates, compounds I and II, respectively, and AH_2 and $AH^\bullet$ are the electron donor substrate and the radical product of its one-electron oxidation, respectively.

The high specific activitity of the enzyme allows high-sensitivity methods to be developed on the basis of its desired application. Enzyme immunoassay (EIA) is one of the techniques where HRP is commonly used. Depending on the substrate used, the products of its oxidation can be detected spectrophotometrically (visibly), by chemiluminescence or fluorescence. The chemiluminescent system developed for the HRP detection, the so-called enhanced chemiluminescence, e.g., the simultaneous use of luminol with a substrate enhancer of phenolic origin, provides superior sensitivity levels down to 10^{-13} M enzyme.[1] However, the sensitivity of a particular immunoassay depends on a number of factors: (a) the efficiency of peroxidase coupling to antigen/antibody/receptor/etc., which includes both the yield of the active conjugate and retention of the specific activity of the enzyme marker; (b) the optimization of reaction conditions to minimize the enzyme inactivation during the reaction course; and (c) the affinity constant for the antibody−antigen interaction. If the last parameter could only worsen during the course of coupling, the first two could, in principle, be improved by using novel enzymes with the desired properties. We have recently shown that the newly isolated tobacco peroxidase from transgenic tobacco plants superproducing the enzyme[2] exhibits unique properties as a catalyst of the chemiluminescent luminol reaction.[3] The goal of the present work was to test this novel enzyme for the first time in an EIA using 2,4-dichlorophenoxyacetic acid (2,4-D) as a model pesticide compound. The results obtained clearly demonstrate the advantage of the new enzyme marker over the commonly used HRP.

EXPERIMENTAL SECTION

Materials. Chemicals and Immunoreagents. 2,4-Dichlorophenoxyacetic acid (2,4-D), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), hydrogen peroxide, sodium periodate, sodium borohydride, 2,4,6-trinitrobenzenesulfonic acid, and dimethylformamide (DMF) were purchased from Sigma Chemical (St. Louis, MO). 1,3-Diaminopropane was from Merck-Schuhardt. HRP and 2,2′-azinobis[3-ethylbenzthiazolinsulfonate (6)] (ABTS) were from Boehringer Mannheim. Tetraethoxysilane was from Aldrich (Al-

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drich Chemical Co., Milwaukee, WI). Monoclonal antibodies (clone E2/G2) against 2,4-D were raised[4] at the Veterinary Research Institute (Brno, Czech Republic) and kindly provided by Dr. Sergei Eremin, Moscow State University, Russia. Rabbit antimouse IgG was purchased from DAKO (Dakopatts Co.). Tobacco anionic peroxidase (MW 36 000, p*I* 3.5) was purified as described earlier.[2] All reagents were of analytical grade and used without further purification.

Borosilicate glass capillaries, 75 × 1 mm (i.d.) for the chemiluminescent immunoassay were purchased from World Precision Instruments Inc. (Sarasota, FL), and microtiter plates coated with antimouse IgG for ELISA were from Boehringer Mannheim.

Buffer solutions and standards were prepared using highly purified distilled and deionized water. Phosphate-buffered saline (PBS), pH 7.4 contained 0.136 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. Washing buffer solution (PBST1) contained PBS with 0.1% Tween-20, and reaction buffer solution was PBS with 0.05% Tween-20 (PBS-T2). The blocking solution containing protein mixture from proteolytic degradation of gelatin in 50 mM Tris-HCl was from Boeringer Mannheim. Sodium carbonate buffer was used for coating and contained 13 mM Na_2CO_3 and 85 mM $NaHCO_3$, pH 9.6. Substrate solution for chemiluminescent reaction contained 1 mM luminol and 3 mM H_2O_2 in 0.1 M Tris buffer, pH 8.5. Substrate solution for ELISA contained 1.8 mM ABTS and 6 mM H_2O_2 in 1 mM sodium acetate buffer, pH 4.5.

The stock solution of 2,4-D (10 mg/mL) was prepared in methanol. For calibration purposes, a serial dilution of the stock solution was prepared in 10 mM PBS from 0.001 to 1000 ng/mL.

Methods. *Apparatus.* The photomultiplier tube (PMT) Sensor Module HC 135-01 with embedded microcontrol system for chemiluminescence intensity detection was purchased from Hamamatsu (Hamamatsu Corp., Bridgewater, NJ) and mounted in a light-tight holder for capillary made in black Delrin. The standard LKB Wallac 1250 luminometer (EG & G Wallac, Turku, Finland) was used as a reference detector. A multiscan MCC/340 (Labsystems, Helsinki, Finland) was used as the microtiter plate reader.

The synthesis of 2,4-D−tobacco peroxidase (TOP) and 2,4D−HRP conjugates was based on the periodate oxidation method[5] with minor modifications as described elsewhere.[6] Briefly, (a) to 1 mL of 10 mg/mL TOP solution in 1 mM Na-acetate buffer, pH 4.5, was added 0.5 mL of 35 mg/mL $NaIO_4$ solution in the same buffer. The reaction mixture was stirred for 30 min at room temperature and thereafter purified with gel filtration through a G-25 column using 1 mM Na-acetate buffer as eluent. Next, 1 mL of 36 mM diaminopropane in 0.1 M sodium carbonate buffer, pH 9.2, was added to the enzyme solution, and the reaction mixture was incubated overnight at room temperature. Modified enzyme solution was purified with gel-filtration. (b) To activate the antigen molecule, to 9 mmol of 2,4-D in 1.2 mL of DMF, 17 mmol of NHS and 48 mmol of DCC were added, and the reaction mixture was stirred for 1.5 h at room temperature. During this period, approximately 40 mmol of DCC was added to the mixture. (c)

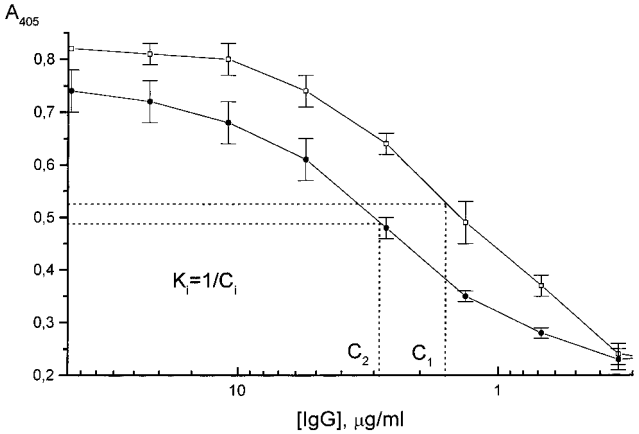

Figure 1. Equilibrium binding constant determination for TOP−2,4-D (open squares), and HRP−2,4-D (filled circles) conjugates. The measurement was performed in duplicate.

The activated 2,4-D was added to the activated TOP solution, and the mixture was incubated for 2 h at room temperature with constant stirring and left overnight at 4 °C. The conjugate obtained was purified twice by gel filtration through Sephadex G-25 packed in a 40 × 1(i.d.) cm column.

The 2,4-D−HRP conjugate was synthesized in the same way, except that 40 mg of enzyme was used for the coupling reaction, and diaminohexane was used for the enzyme modification. The 2,4-D−enzyme molar ratios of the conjugates obtained were determined by comparing the number of surface amino groups in the native and conjugated enzymes using the 2,4,6-trinitrobenzenesulfonic acid method.[7,8]

The sol−gel coating procedure described elsewhere[9] was used to increase the adsorption properties of the surface of the glass capillaries. Initially, capillaries were washed with boiling 10% HNO_3 for 4 h and thereafter rinsed excessively with water. Then, 2.25 mL of tetraethoxysilane, 0.7 mL of water, and 50 μL of 0.1 M HCl were mixed in a 10-mL tightly closed glass bottle. The mixture was sonicated for 2−3 min until the solution became turbid. Next, the mixture was stirred vigorously for at least 4 h at RT, until the solution became clear. The obtained sol was further diluted with deionized water in 1:4 ratio of sol to water. The diluted sol was used for further experiments. Cleaned glass capillaries were filled with the sol solution and incubated for 1 h on a shaker to facilitate uniform coating. The excess sol was removed, and the coated capillaries were left overnight at RT to obtain the dry gel. Later, the capillaries were washed, first with buffer and then with abundant amounts of water, and, finally, dried at RT.

Apparent Binding Constants. Anti-2,4-D IgG were serially diluted in PBS-T2, applied to a microtiter plate coated with anti-mouse IgG, and incubated for 1 h at 37 °C. The plate was washed with PBS-T1, and unoccupied sites were blocked with the blocking solution for 30 min. The plate was washed, and the conjugates diluted to appropriate concentrations in PBS-T2 were added. The plate was incubated for 2 h at RT and washed thereafter. ABTS substrate solution was added, and the absorption at 405 nm was measured after a 45-min incubation at RT. The absorbance vs IgG concentration profile was plotted, and the apparent binding constants were calculated using the values representing 50% of the bound IgG fraction.

Assay Procedure. *(a) Chemiluminescent ELISA Using a Standard Luminometer.* Aliquots of 500 μL/tube of 10 μg/mL rabbit anti-mouse IgG in 50 mM sodium carbonate buffer, pH 9.6, were incubated overnight at 4 °C. The tubes were washed with PBS-T1 three times, and unoccupied sites were blocked with the

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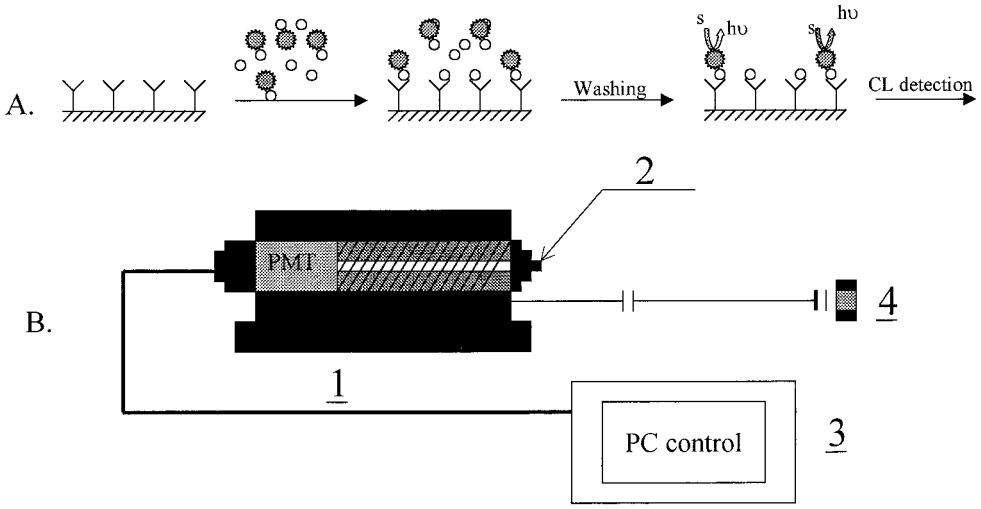

Figure 2. Experimental setup used: (A) direct competitive ELISA used in the work (see details in the Experimental Section); (B) PMT built into a black Delrin holder (1), with a locking channel for capillary insertion (2), on-line PC control (3), 5-V power supply (4).

blocking solution. A 500-μL aliquot of 10 μg/mL anti-2,4D IgG in PBS-T2 was added to each tube and the mixture incubated for 1 h at 37 °C. The tubes were washed, and 100 μL/tube of the 2,4D standards or analyte in 10 mM PBS was added and preincubated for 15 min. Thereafter, 400 μL of 30 pM 2,4D−TOP conjugate was added to each tube, and after a 2-h incubation at RT, the unbound fraction was washed out with PBS-T1. A 500-μL aliquot of luminol substrate solution was added to each tube, and the maximum chemiluminescent intensity was measured with a standard LKB luminometer.

(b) Chemiluminescent ELISA Using Glass Capillaries and a PMT-Based Detection System. Initially, 50 μL/capillary of 10 μg/mL rabbit anti-mouse IgG in 50 mM sodium carbonate buffer solution, pH 9.6, was incubated overnight at 4 °C. The capillaries were washed three times with PBS-T1 and then twice with water. Thereafter, 50 μL of 10 μg/mL anti-2,4D IgG in PBS-T2 was added to each capillary and the mixture incubated for 1 h at 37 °C. The capillaries were washed with washing buffer and water. Thereafter, a 50-μL mixture of 2,4D−TOP and standards or analyte was added to each capillary, and the competitive reaction was allowed to proceed by incubation for 2 h at RT. To determine nonspecific binding, four capillaries received no specific IgG and no pesticide, to determine the maximum chemiluminescent intensity of the bound fraction of the labeled competitor. Finally, the capillaries were washed and 50 μL/capillary of luminol substrate solution was added. The maximum chemiluminescent intensity was measured with the modified Hamamatsu PMT.

RESULTS AND DISCUSSION

TOP Conjugation and Characterization of the Obtained Conjugate. Beside the purity of water and reagents used, the sensitivity of the ELISA employed in this work depends on the preserved specific and immunological activities of the obtained conjugates. The quality of the conjugate synthesized by the commonly used periodate method was controlled by measuring the peroxidase specific activity, immunological activity of the conjugate and its hapten/protein ratio.

(1) Specific Activity of the Enzyme. The peroxidase activity was referred to the absorbance of the Soret band at 403 nm corresponding to peroxidase heme protein. No changes in this activity were observed after the coupling procedure, and the conjugate activity corresponded to that of unmodified TOP (2500 units/mg of heme protein with ABTS as a substrate). Thus, the novel enzyme was stable and showed no loss in catalytic activity upon overnight modification at pH 9.2 in the presence of 10% DMF. For comparison, as will be shown later, the specific activity of the HRP was significantly diminished during the coupling procedure.

(2) Immunological Activity of the Obtained Conjugates. The immunochemical properties of the prepared conjugates have been assessed in both noncompetitive and competitive conditions. Initially, apparent binding constants of the antibody−antigen noncompetitive interaction were determined. In this experiment, a constant amount of the 2,4-D−peroxidase conjugate was allowed to bind specifically to the serially diluted anti-2,4-D IgG's immobilized on a microtiter plate surface. The amount of IgG binding 50% of the labeled antigen molecules was determined on the constructed dilution curve, and the apparent equilibrium binding constants of the antibody−labeled antigen interaction were calculated (Figure 1). The values obtained were equal to 0.9×10^8 and 1.25×10^7 M^{-1} for the 2,4-D−TOP and 2,4-D−HRP conjugates, respectively.

The competitive capabilities of the obtained conjugates were estimated with direct chemiluminescent ELISA format (Figure 2 A) in which labeled and unlabeled 2,4-D molecules compete for the binding sites of the specific monoclonal anti-2,4-D IgG immobilized on precoated solid support suface. The resulting chemiluminescent signal from the specifically bound 2,4-D−enzyme conjugate reaction was detected by a modified PMT detector (Figure 2B). We should emphasize that the TOP enzyme does not need any enhancer. This makes the assay cost-effective since the currently used HRP chemiluminescent substrates present the mixture of luminol and a proprietary enhancer, which makes their use rather expensive. Figure 3 shows the standard calibration curves obtained with the competitive ELISA performed in luminometer cuvettes with highly diluted but equal concentrations of both conjugates. With the used conjugate concentration, 30 pM, the HRP conjugate gives no signal of immunological or enzyme activity, whereas the TOP conjugate shows a linear plot over the range 10 pg/mL−5 ng/mL 2,4-D with reliable enzymatic activity values. Considering the fact that both the conjugates have approximately the same affinity to the antibody used, the sharp contrast in the results obtained seems to be mainly due to the loss of the HRP specific activity. The loss of the specific activity

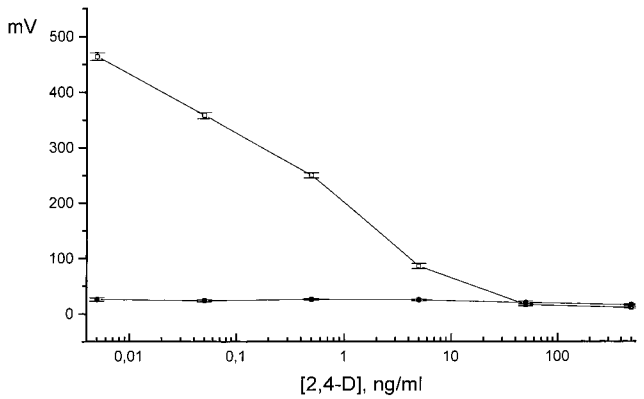

Figure 3. Comparison of the two obtained conjugates in competitive ELISA with chemiluminescent detection using standard luminometer: TOP−2,4-D (open squares) and HRP−2,4-D (filled circles). The assay was performed in duplicate.

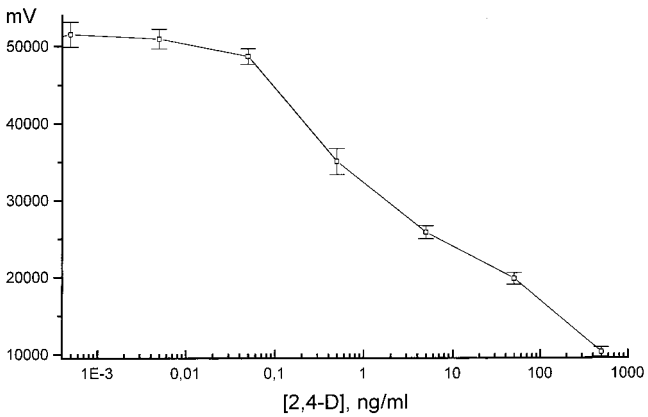

Figure 4. Standard calibration curve for 2,4-D determination using TOP−2,4-D conjugate and capillary chemiluminescent ELISA. All samples were analyzed in quadruplets except that for the "0" standard which was analyzed in 10 replicates.

could be partly due to the high dilution factor and partly due to the coupling procedure employing the *m*-periodate oxidation method. The use of the latter may cause up to 40% of the native enzyme activity to be lost.[10] However, the higher specific activity of the 2,4-D−TOP conjugate can be explained by the high reactivity of the enzyme's compound II toward luminol (see the catalytic scheme in the introduction section) and increased stability of the holoenzyme with respect to heme dissociation.[3] The better catalytic activity of the TOP conjugate was also confirmed with colorimetric ELISA where the conjugate demonstrated at least 3 times higher activity than that obtained with the HRP conjugate (the data are not shown here).

(3) Hapten/Enzyme Ratio. Often in immunoassays, a labeled competitor can have significantly higher affinity to the binding sites of antibody compared to unlabeled antigen. This is due to the formation of a bivalent immune complex[11] by only the labeled antigen and such a complex binds to the antibody with much higher affinity,[12] thus shifting the reaction equilibrium and, consequently, decreasing the assay sensitivity. The probability of such bivalent interactions and conditions for the competing unlabeled antigen strongly correlates with the density of antigenic determinants on the conjugate surface.[13] Herein, we have estimated the compositions of the obtained conjugates by the trinitrobenzenesulfonic acid method, and the hapten/enzyme ratios were found to be 8:1 and 12:1 for 2,4-D−TOP and 2,4-D−HRP conjugate, respectively.

Capillary Chemiluminescent Immunoassay Using 2,4-D−TOP Conjugate. As we have demonstrated earlier, capillary chemiluminescent assays may provide a better sensitivity due to the waveguiding abilities.[14,15] Combined with the modified PMT detector and sol−gel coating technique, capillaries could facilitate improved and sensitive detection of chemiluminescent intensity. Although, in this particular case we observed no advantages of this type of assay compared to the luminometer-based one, a high-sensitivity assay has been developed and the obtained calibration curve was linear over a wider range of 2,4-D concentrations, e.g., from 30 pg/mL to 500 ng/mL (Figure 4). The better linear range could be attributed to the sol−gel treatment of the capillary surface.

CONCLUSION

A novel and highly sensitive chemiluminescent enzyme marker is proposed for use in EIA and, in particular, EIA of pesticides. The enzyme has a number of advantages over the commonly used HRP: (a) it shows no loss of specific activity during the coupling procedure, (b) it demonstrates unique properties in chemiluminescent detection, allowing the minimization of the conjugate concentration due to its high chemiluminescent activity, and (c) it avoids the use of expensive enhancer substrates allowing the assay to be simplified and significantly less costly. Taking into account the overproduction of the tobacco peroxidase in transgenic plants (tobacco,[2] tomato[16]), one can consider it as a promising substitute for HRP in chemiluminescent detection systems.

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