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Laccase from *Coriolus hirsutus* as Alternate Label for Enzyme Immunoassay

Determination of Pesticide 2,4-Dichlorophenoxyacetic Acid

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Abstract

A new label—laccase from the fungus *Coriolus hirsutus*—was applied for solid-phase enzyme-linked immunosorbent assays of the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D). Two proposed assays are based on (1) competitive binding of antibody-laccase conjugate with immobilized 2,4-D-protein conjugate and 2,4-D in tested sample, and (2) competition of 2,4-D and 2,4-D-laccase conjugate for binding with immobilized antibodies. Kinetic and concentration dependencies for these reactions were studied, and the ELISAs were optimized in accordance with the data obtained. The elaborated systems permit the detection of 2,4-D in concentrations down to 10–20 ng/mL; time of the assays is 1.5–2 h. The main advantage of the laccase label, in comparison with the widely used peroxidase one, lies in the lack of hydrogen peroxide from substrate mixture, because dissolved oxygen plays the role of oxidizer.

Index Entries: Enzyme immunoassay; laccase; pesticide; 2,4-dichlorophenoxyacetic acid.

Introduction

The choice of the best labels for different immunoanalytic systems is a problem of much interest. Currently, horseradish peroxidase (HRP) label is the most commonly used for enzyme immunoassays of various compounds *(1–3)*. However, in the HRP case the assay results depend significantly on the quality of the hydrogen peroxide (H_2O) and on the enzyme

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inhibition by the reaction products. Therefore, the search for alternate labels for enzyme immunoassay continues *(4)*.

Earlier the possibility of applying extracellular laccase (E.C. 1.14.18.1) from the fungus *Coriolus hirsutus* as such label was shown *(5)*. Solid-phase enzyme-linked immunosorbent assays (ELISAs) of insulin and human IgG with its use were developed and characterized *(6)*.

The purpose of the present investigation is to compare laccase and HRP in respect to their accomplishment for ELISA of pesticide. The pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) has been chosen as the model object.

On the one hand, the development of ELISA for this compound of low molecular weight permits the careful study of the properties and suitability of both labeled antibody and labeled antigen. On the other hand, immunodetection of pesticides is a problem of significant practical maintenance. Pesticides have become an indispensable part of modern agricultural technologies, being highly toxic compounds at the same time. Various techniques of pesticide detection in soil, water, and agricultural products are now intensively elaborated *(2,3,7–10)*. Hence, it is worthwhile to characterize a new label in this wide field of applications.

Materials and Methods

Chemicals

The following chemicals were used in this study: 2,4-D; sodium periodate; sodium borohydride; sodium dodecyl sulfate (SDS); polyethylene glycol (PEG) (M_w 6 kDa); dimethylformamide; Tween-20 (Serve, Heidelberg, Germany); phenol; phenoxyacetic acid; 2,4,5-trichlorophenol; 2,4,5-trichlorophenoxyacetic acid; pentachlorophenol; 5-aminosalicylic acid; bovine serum albumin (BSA); albumin from chicken eggs; *N*-hydroxysuccinimide (Sigma, Diesenhofen, Germany); 2,2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid) (ABTS) diammonium salt (Boehringer Mannheim, Mannheim, Germany); 3,3',5,5'-tetramethylbenzidine dihydrochloride; *o*-phenylenediamine dihydrochloride (Fluka, Buchs, Switzerland); 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide (CalBiochem, San Diego, CA); soybean trypsin inhibitor (STI) (Reanal, Budapest, Hungary); HRP (RZ = A_{403}/A_{280} = 3.0; Biolar, Latvia); protein A of *Staphylococcus aureus* (Vostok, Novosibirsk, Russia); 2,4,6-trinitrobenzensulfonic acid (Chemapol, Praha, Czech Republic); catechol (Reachim, Moscow, Russia). Constituents of buffer solutions and other chemicals were of analytical grade.

Obtaining Laccase

Enzyme was obtained from cultural liquid of the basidial fungus *C. hirsutus (Fr.) Quel* according to the following technique *(11)*. Filtered and concentrated cultural liquid was centrifuged at 2500*g* for 15 min. Precipitate was dissolved and then repeatedly centrifuged. Liquid fractions were united, worked up by $(NH_4)_2SO_4$ solution (90% saturating concentration),

and centrifuged at 2500*g* for 1 h. The obtained precipitate was dissolved in distilled water and dialyzed against 5 m*M* K-phosphate buffer, pH 6.5. Then the preparation was added to DEAE-cellulose cotton wool (Whatman, Maidstone, UK) equilibrated with the same buffer. Bound molecules were threefold eluted by 0.2*M* K-phosphate buffer, pH 6.5. The eluate was then precipitated by the same $(NH_4)_2SO_4$ solution, centrifuged at 2500*g* for 15 min, redissolved, and dialyzed against distilled water.

The preparation was applied onto a DEAE-cellulose (Whatman) column equilibrated with 5 m*M* K-phosphate buffer, pH 6.5. Bound molecules were eluted by linear gradient of the buffer, increasing its molarity up to 0.2*M*. Catalytically active fractions were united and rechromatographed in the same way. The preparation was chromatographed on a Toyopearl HW-55 (ToyoSoda, Tokyo, Japan) column equilibrated with 5 m*M* K-phosphate buffer, pH 6.5, and finally on a DEAE-Trisacryl (ToyoSoda) column using the previously described gradient elusion.

The chromatographic fractions were tested on catalytic activity with the use of catechol as a substrate (*see* Measurement of Peroxidase and Laccase Activity).

Homogeneity of the obtained enzyme was proved by gradient electrophoresis in polyacrylamide gel and high-pressure gel filtration on a TSK-3000 column (7.5 × 300 mm; LKB, Durham, NC). The molecular weight of the laccase was 55 kDa.

Synthesis of 2,4-D Conjugates with Protein Carriers

For 2,4-D activation was conjugated with BSA, STI, and ovalbumin (OvA) according to the following technique *(12)*. For 2,4-D activation, 15 mg of 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide and 4 mg of *N*-hydroxysuccinimide were added to 4 mg of 2,4-D dissolved in 0.5 mL of dimethylformamide. The mixture was incubated by stirring for 2 h at room and temperature and then added to the protein solution in water (10 mg/mL). Hapten:protein initial molar ratio in the reaction mixture was 50:1 for BSA and 20:1 for STI or OvA. The resultant mixture was incubated at room temperature by stirring for 2 h and a further at 4°C for 16 h. The conjugates were separated from low molecular weight compounds by dialysis and/or gel filtration on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column.

Synthesis of 2,4-D Conjugates with Enzyme Labels

2,4-D was conjugated also with enzymes for applying these reactants in ELISA. The technique of the 2,4-D-HRP conjugate synthesis was the same as the one just described. The hapten:protein initial molar ratio in the reaction mixture was 20:1.

For synthesis of 2,4-D-laccase, the following modification of the carbodiimide technique was used. Two milligrams of 1-cyclohexyl-3 (2-morpholinoethyl) carbodiimide and 0.5 mg of *N*-hydroxysuccinimide were added to 0.5 mg of 2,4-D dissolved in 0.25 mL of dimethylformamide. The mixture was incubated with stirring for 3 h at room temperature. Then the activated

2,4-D was cooled to 4°C and added to 2 mg of laccase (dissolved in 20 µL of 50 m*M* K-phosphate buffer, pH 6.5). Thus, the hapten:protein initial molar ratio was 125:1. The resultant mixture was incubated at 4°C for 16 h, and the conjugate was separated by dialysis.

Determination of the Conjugates' Composition

The composition of the conjugates obtained was calculated by comparing the quantity of amino groups on the surface of the original protein and the conjugate. The following technique of detection of amino groups was used *(13,14)*. Solutions of the protein and its conjugate (1 mg/mL) in water were prepared. Fifty-microliter aliquots of protein or conjugate, 50 µL of saturated $NaHCO_3$ solution, and 50 µL of 2,4,6-trinitrobenzenesulfonic acid solution (2 mg/mL) were added into the wells of an ELISA plate. The mixture was incubated at 37°C for 2 h. Then 25 µL of a 10% SDS solution and 25 µL of 0.5*M* HCl were added. The concentration of the amino groups is proportional to an increase of optical density at 405 nm, which was measured by vertical photometer for ELISA (MR-600; Dynatech, Alexandria, VA).

Immunization

For raising antibodies against 2,4-D, *Chinchilla* rabbits weighing 3 to 4 kg were immunized according to the following procedure *(12)*. 2,4-D-BSA was dissolved in 0.05*M* K-phosphate buffer, pH 7.4, with 0.1*M* NaCl (hereafter named PBS) at a 1 mg/mL concentration. The solution was emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI). At d 1, 15, and 29 of immunization, 1 mL of the mixture was injected intracutaneously into several sites on the back from scapula to sacrum. After 2 mo (at d 89), the first cycle of reimmunization was carried out: rabbits were boosted intravenously with 0.3 mL of the immunogen dissolved in PBS at the same concentration and bled 7 d later. Then boosting and bleeding was repeated two to four times on a monthly basis (boosting—at d 119, 149, 179, and 209; bleeding—at d 126, 156, 186, 216).

Antibody Separation

Antisera were extracted by settling blood samples at +4°C for 12 h. Upper layers were carefully collected, divided into aliquots, and stored at –20°C.

IgG was precipitated by two cycles consisting of the addition of 20% PEG to antisera (v/v = 1: 1), centrifugation, and redissolving *(15)*.

Synthesis of Antibody-HRP Conjugate

The following technique was used for the synthesis of antibody-HRP conjugate *(15,16)*. To 0.5 mL of HRP water solution (4 mg/mL) was added 0.1 mL of freshly prepared 0.1*M* $NaIO_4$. The mixture was incubated for 20 min at room temperature under stirring, with pH being conducted at 4.4–4.5. The obtained HRP aldehyde was dialyzed against 1 m*M* Na-acetate buffer,

pH 4.4. Then the pH was changed to 9.0–9.5 by 0.2*M* Na-carbonate buffer, pH 9.5, and 1 mL of IgG solution (8 mg/mL) in 10 m*M* Na-carbonate buffer, pH 9.5, was added immediately. The reaction mixture was incubated under continuous stirring at room temperature for 2 h and dialyzed against PBS at 4°C for 16 h. Then 0.1 mL of freshly prepared 0.1*M* $NaBH_4$ was added. The obtained mixture was incubated at room temperature for 2 h and dialyzed against PBS at 4°C for 16 h.

Synthesis of Antibody-Laccase Conjugate

Twice 0.3 mL of laccase solution (3.3 mg/mL) in 50 m*M* K-phosphate buffer, pH 6.3, was dialyzed against distilled water. Then 0.1 mL of freshly prepared 0.12*M* $NaIO_4$ was added. The mixture was incubated at room temperature under stirring for 40 min. The obtained laccase aldehyde was dialyzed for 16 h against 5 m*M* Na-acetate buffer, pH 4.6, and then for 2 h against 0.01*M* Na-carbonate buffer, pH 9.0. Then it was united with 0.2 mL of IgG solution (10 mg/mL) in the carbonate buffer. The reaction mixture was incubated at room temperature under stirring for 2 h. Finally, it was dialyzed against 50 m*M* K-phosphate buffer, pH 6.3, at 4°C for 16 h.

2,4-D ELISA with Immobilized Antibodies

Basic Technique

Aliquots of 0.1 mL of IgG in PBS were incubated into the wells of an ELISA plate. Then the wells were fourfold washed by PBS with 0.05% Tween-20 (PBST). Next, 50 µL of pesticide-containing sample and 50 µL of 2,4-D-enzyme solution were added. The microplate was incubated and fourfold washed, and the enzyme activity was measured.

Technique with Immobilization via Protein A

Aliquots of 0.1 mL of protein A in PBS were incubated into the wells of an ELISA plate. Next, after fourfold PBST washing, 0.1-mL aliquots of IgG were added into the wells and incubated. All following stages were the same as described previously.

For both techniques, the initial plate coatings were carried out either at 4°C for 16 h, or at 37°C for 1.5 h. All further stages were carried out at 37°C. Table 1 gives the optimal durations of the ELISA stages and concentrations of immunoreactants.

2,4-D ELISA with Immobilized 2,4-D-Protein Conjugate

Aliquots of 0.1 mL of 2,4-D-STI or 2,4-D-OvA conjugates in PBS were incubated into the wells of an ELISA plate at 37°C for 1.5 h (or at 4°C for 16 h). After washing, 50 µL of IgG-enzyme conjugate solution in PBST and 50 µL of the analyzed sample were added simultaneously and incubated in the wells. Then the plate was repeatedly washed and enzyme activity was detected. The temperature regime was the same as in the previous case. Table 2 gives the optimal durations of the ELISA stages and concentrations of immunoreactants.

Table 1
Optimal Regimes of 2,4-D ELISA
with Immobilized Antibodies for Laccase and HRP Labels

	Laccase	HRP
Concentration of protein A during adsorption	3.0 μg/mL	3.0 μg/mL
Concentration of IgG (antiserum dilution)	6.2 μg/mL (1:2000)	1.6 μg/mL (1:7500)
Duration of reaction between protein A and antibodies	45 min	45 min
Concentration of pesticide-enzyme conjugate	0.25 μg/mL	0.1 μg/mL
Duration of immunochemical reaction	60 min	60 min
Duration of color development	≥30 min	30 min

Table 2
Optimal Regimes of 2,4-D ELISA
with Immobilized Pesticide-Protein Conjugate for Laccase and HRP Labels

	Laccase	HRP
Concentration of pesticide-OvA conjugate during adsorption	0.2 μg/mL	0.5 μg/mL
Concentration of IgG-enzyme conjugate	2.5 μg/mL	6.2 μg/mL
Duration of immunochemical reaction	60 min	60 min
Duration of color development	≥30 min	30 min

Measurement of Peroxidase and Laccase Activity

Substrate solutions for the laccase activity measurements were prepared by the following techniques *(15,17)*:

1. *o*-Phenylenediamine (0.4 mg/mL) was dissolved in 30 m*M* Na-acetate buffer, pH 4.5. The plate was incubated with the substrate in darkness, and then the reaction was stopped by 2*M* H_2SO_4 solution.
2. 5-Aminosalicylic acid (0.8 mg/mL) was dissolved in water on heating (50–60°C), and then pH was corrected to 6.0 by KOH.
3. 3,3',5,5'-Tetramethylbenzidine (0.1 mg/mL) was dissolved in 0.1*M* Na-citrate buffer, pH 6.0.
4. ABTS diammonium salt (0.44 mg/mL) was dissolved in the same buffer. In some experiments, the reaction was stopped by 3*M* NaF solution.
5. Catechol (1.1 mg/mL) was dissolved in 0.1*M* K-acetate buffer, pH 4.5.

For HRP testing, H_2O_2 was added to substrates 1–4. Its concentration was equal to 1.8 m*M* for substrates 1–3 and 0.9 m*M* for substrate 4. Substrate 5 was used only for the measurements of laccase activity in this work.

Optical densities were measured by vertical photometer (MR-600, Dynatech) at 490, 450, 450, and 405 nm for substrates 1–4 respectively, or by double-beam spectrophotometer 557 (Hitachi) at 410 nm for substrate 5.

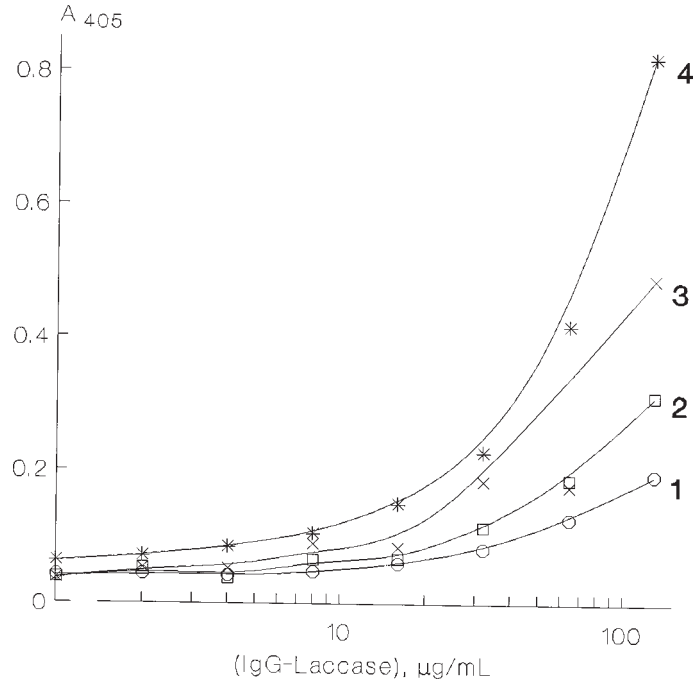

Fig. 1. Optimization of regime for ELISA with antibody labeling: choice of 2,4-D-OvA concentration for adsorption. Curves 1–4 represent 2,4-D-OvA concentrations (by protein) 0.3, 1, 3, and 10 µg/mL. *x*-axis, concentration (by total protein) of IgG-laccase conjugate; *y*-axis, detected optical density of the ABTS oxidation product.

Results and Discussion

The indispensable approach in ELISA of monovalent antigens (haptens) is the competition between hapten to be determined and hapten-protein conjugate for binding with antibodies. The main two formats of competitive ELISA are the ones with immobilization of either antibodies or hapten-protein conjugate.

The necessary immunoreactants were prepared for both of these formats. The conjugates of laccase and HRP were synthesized and characterized according to their composition. It was shown that by applying carbodiimide-activated 2,4-D, appropriate laccase conjugates could be obtained. They contain nearly five to six 2,4-D groups at the enzyme molecule. About 60–80% from the initial catalytic activity is preserved during syntheses. This parameter is no worse than that for the HRP conjugates.

By using the reactants obtained, competitive ELISAs of 2,4-D were developed. The conducted studies have sought to optimize the immobilization method, the immunoreactants' concentrations, the durations of the assay stages, and the enzyme substrate.

Optimal conditions of immobilization were determined both for 2,4-D-protein (Fig. 1) and for IgG (Fig. 2) adsorption. The outcome is that

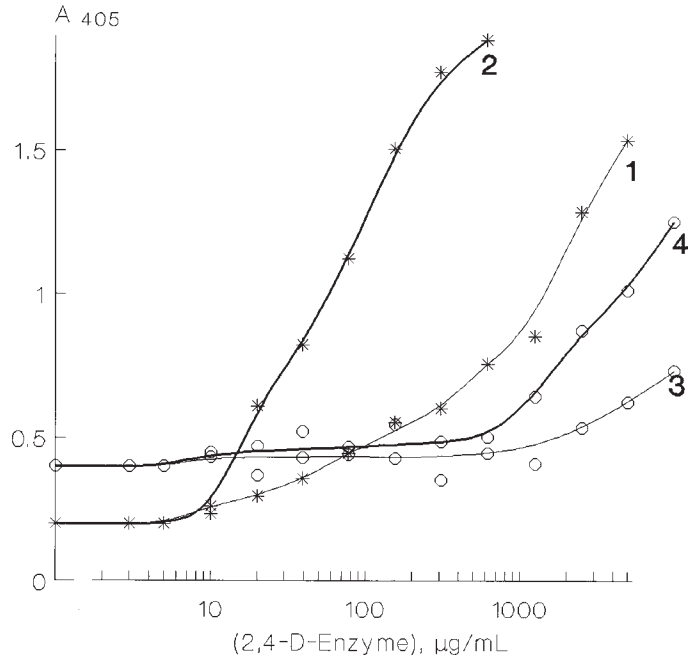

Fig. 2. Optimization of regime for ELISA with antigen labeling: choice of IgG immobilization way. Curves 1 and 3 represent direct IgG adsorption (from 10 μg/mL), curves 2 and 4 immobilization of IgG (10 μg/mL) through protein A adsorbed from 3 μg/mL. Antibodies were detected by 2,4-D-laccase conjugate (curves 1 and 2) or by 2,4-D-HRP conjugate (curves 3 and 4). *x*-axis, concentration (by protein) of 2,4-D-enzyme conjugates; *y*-axis, detected optical density of the ABTS oxidation product.

attachment through preliminary adsorbed staphylococcal protein A is the most effective way of antibody immobilization. Previously the same phenomenon was described for testosterone ELISA *(18)*. It may be explained by either favorable orientation of antigen-binding sites owing to binding of the antibodies through their Fc portions or selection of IgG subclasses having higher affinity to antigen.

Concentration dependencies for antibody-antigen interactions were obtained (*see* Fig. 3). The reactant concentrations for ELISA were optimized in accordance with these data. The durations of the antibody-antigen reactions were chosen by references to the studied kinetics of the conjugates' binding. Figure 4 shows that short immunochemical stages as well as prolonged ones cause a decrease in ELISA sensitivity. In the first case (curves 1 and 2), the decrease is a consequence of low amplitude of the detected signals. In the second case (curves 4 and 5), it is probably connected with the realignment of antibody-conjugate complexes. The formation of bivalent bounds may be proposed as a possible mechanism of the realignment by virtue of the fact that it is attended by growth of the affinity of the complexes *(19–21)*.

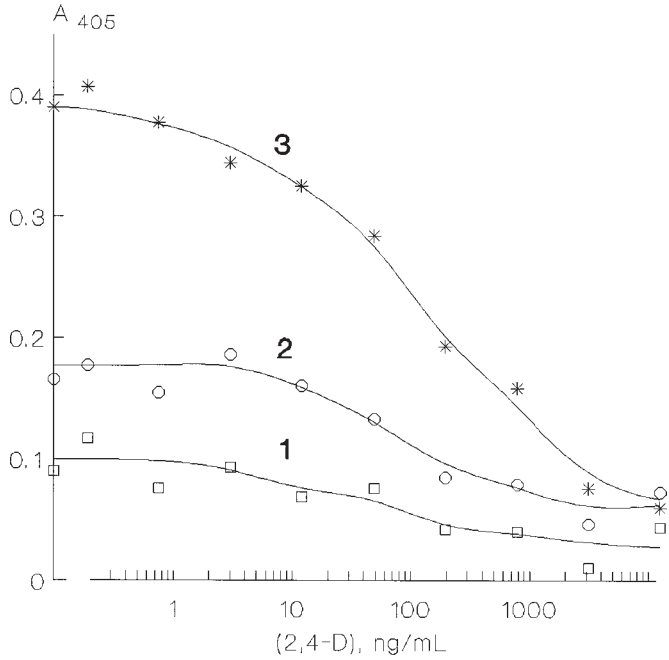

Fig. 3. ELISA with antibody labeling: choice of IgG-laccase concentration. Curves 1–3 represent IgG-laccase concentrations (by enzyme) 1, 2, and 4 µg/mL. 2,4-D-OvA conjugate was adsorbed from 3 µg/mL. *x*-axis, concentration of competing 2,4-D in the sample tested; *y*-axis, detected optical density of the ABTS oxidation product.

We have compared ELISA formats with and without preincubation of tested samples with antibodies (before addition of the competitor). For both assays—with immobilization of antibodies and hapten-protein conjugate—preincubation did not influence sensitivity. This property of the obtained antibodies was established earlier *(22)* for peroxidase-based competitive ELISA of 2,4-D. Although the majority of immunoassay systems increase their sensitivity after preincubation, the described phenomenon is not a unique one. A possible explanation is that a high rate of dissociation for the immunocomplexes induces rapid chemical equilibrium independently of the prior stages of the assay.

Different substrates (*o*-phenylenediamine, 5-aminosalicylic acid, 3,3',5,5'-tetramethylbenzidine, and ABTS) have been tested regarding their effectiveness for the detection of laccase at ELISA. ABTS appears to be the best substrate because of its high growth of optical density and low level of nonspecific oxidation.

Tables 1 and 2 give the chosen parameters of the ELISAs. The developed systems permit the detection of 2,4-D in concentrations down to 10–20 ng/mL. Total assay time is 1.5–2 h (including 60 min of the immunochemical interactions and 30 min of color development). The following interassay coefficients of variation (CV) were determined (n = 4): 8% for 20 ng/mL of 2,4-D, and 5% for 40 ng/mL of 2,4-D. A row of 2,4-D analogs

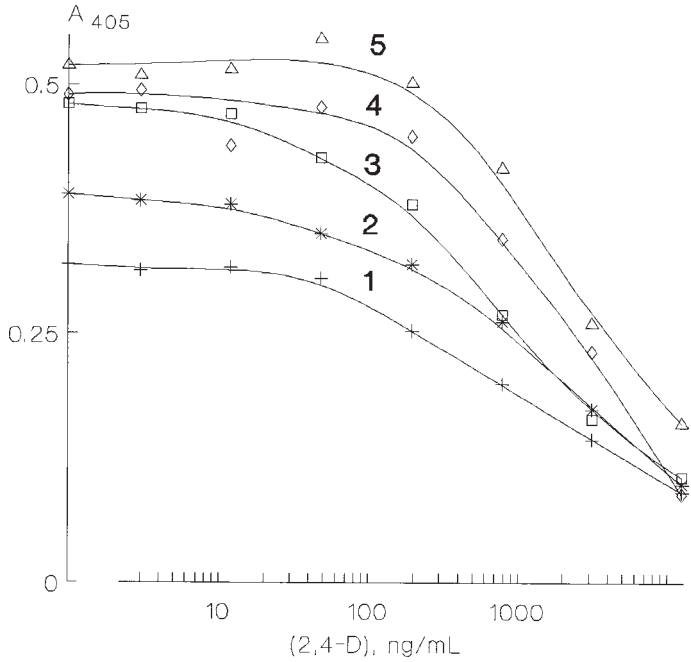

Fig. 4. ELISA with antigen labeling: choice of the immunochemical stage duration. Curves 1–5 represent 20-, 40-, 60-, 100-, and 120-min durations. IgG (10 μg/mL) was immobilized through protein A adsorbed from 3 μg/mL; concentration of 2,4-D-laccase (by enzyme) was equal to 2 μg/mL. *x*-axis, concentration of competing 2,4-D in the sample tested; *y*-axis, detected optical density of the ABTS oxidation product.

(phenol, phenoxyacetic acid, 2,4,5-trichlorophenol, pentachlorophenol, 2,4,5-trichlorophenoxyacetic acid) does not give significant crossreactions at the assays.

Comparison of ELISAs with laccase label and with HRP label has shown that both assays have equal optimal durations (*see* Tables 1 and 2) and identical sensitivities. HRP-based ELISA was somewhat less accurate: CV (n = 4) was equal to 12% for 20 ng/mL of 2,4-D, and 9% for 40 ng/mL of 2,4-D.

Sensitivities of previously proposed HRP-based immunoenzyme methods of 2,4-D detection *(12,23–28)* vary in intervals of 1–10^2 ng/mL. The attained level depends significantly on the quality (binding constants) of the antibodies used. Comparison of the laccase-based ELISA of 2,4-D with the HRP-based one using the same antiserum *(19)* demonstrates close levels of sensitivity.

The primary advantage of laccase label lies in the absence of H_2O in its substrate solutions, because the O_2 dissolving from air plays the role of oxidizer. Consequently, the preparation and standardization of analytical systems become easier.

Another advantage is the ability of laccase to convert substrate molecules over a number of hours without product-induced inhibition, result-

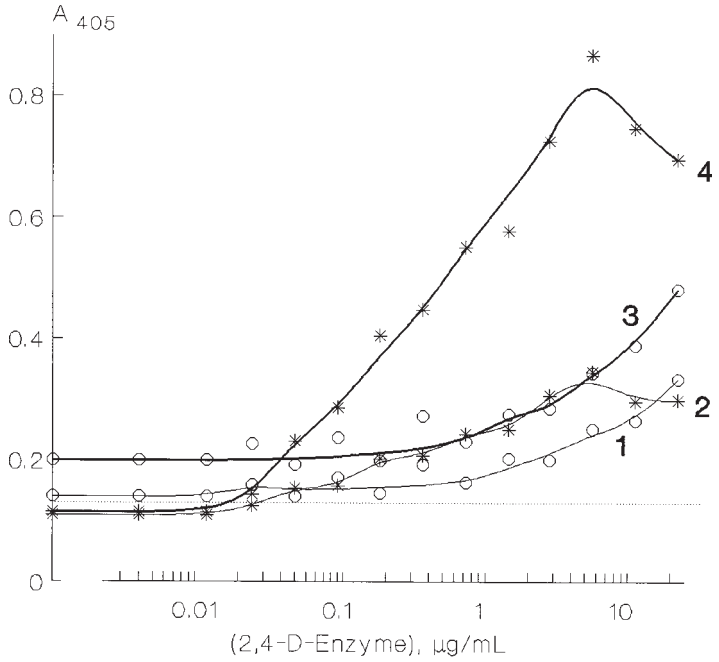

Fig. 5. Influence of duration of catalytic reaction on detection of bound ELISA label. The duration was equal to 30 min for curves 1 and 2, and 4 h for curves 3 and 4. Curves 1 and 3 represent the 2,4-D-HRP conjugate use, and curves 2 and 4 the 2,4-D-laccase. The conjugates had been immobilized by the formation of their complexes with protein A and anti-2,4-D antibodies. *x*-axis, concentration (by protein) of 2,4-D-enzyme conjugates; *y*-axis, detected optical density of the ABTS oxidation product. Dotted line represents initial optical density of the substrate.

ing in considerable growth of the measured signal at a wide interval of the conjugate concentrations (Fig. 5). Hence, ELISA may be realized for extremely low label concentrations. This tendency permits better conditions for the pesticide molecules in the tested sample at their competition with the pesticide groups that have been conjugated with the enzyme. Moreover, the level of nonspecific substrate oxidation (catalyzed by heavy metal ions) for O_2 is lower as compared with H_2O_2 *(5)*.

We have not applied this property of laccase in the proposed ELISA techniques, since the total duration of 2,4-D assay should not be increased significantly. Nonetheless, the usage of laccase may be a subject of particular interest for tasks of continual ecological monitoring (primarily for two-site formats of immunoassay).

Conclusion

The developed ELISAs of 2,4-D with laccase label are characterized by high performance and stability and may be used for solving agricultural, ecological, and medical tasks.

The data obtained show that laccase from the fungus *C. hirsutus* is an appropriate alternate label for ELISA of pesticides. Its main advantages are as follows:

1. Simple and easy system for the detection of catalytic activity (because of the absence of H_2O_2 in the substrate solution)
2. Higher accuracy of ELISA compared with peroxidase-based assays
3. Possible to detect increased substrate conversion over a number of hours with a low level of nonspecific oxidation (because of the absence of product-induced inhibition)

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