DEVELOPMENT OF SOLID-PHASE ENZYME-LINKED IMMUNOASSAY FOR DETECTION OF PROPANIL IN RICE

A. I. Krasnova*, V. S. Krikounova*, S. A. Eremin*, and A. Scekacs**

An Enzyme-linked Immunosorbent Assay (ELISA) for quantitative determination of herbicide propanil was developed. The influence of coating conjugate structure on ELISA analytical parameters was studied. The method detection limit is 0.1 ng/ml of propanil. Conditions providing propanil monitoring in rice grain were optimized. The range of the detectable concentrations in rice grain is 1 to 100 μg/g. The developed ELISA could efficiently measure propanil in rice without clean-up at the regulatory maximum residue limit (10 μg/g) postulated by code of federal regulations.

Propanil (I) is a very effective and, therefore, widely used in an agriculture herbicide for inhibition of photosynthesis of weeds in rice crops [1] and other cultures (wheat, barley, oats, rye). The monitoring of propanil residues is very important to assess the safety of environment and food.

The conventional methods for analysis of propanil are gas chromatography (GC) with a mass-spectrometry detector or diode-array detector [2, 3]. However, the GC methods are time -consuming and expensive due to the long clean-up procedure and the sophisticated analytical instrumentation. The immunoassays are very sensitive, rapid and simple to perform. The principle of competitive immunoassay is based on a competition of free and immobilized on a solid phase an antigen for the binding centers of specific antibodies. Recently in our group ELISAs for determination of pesticides simazine and atrazine [4], 2,4-dichlorphenoxyacetic (2,4-D) and 2,4,5-trichorphenoxyacetic acids (2,4,5-T) [5] were performed.

The preparation of samples for ELISA is simple compared with GC methods. Nevertheless matrix impurities can considerably influence the sensitivity of ELISA. A sensitive ELISAs for detection pesticides 2,4,5-T [6] and flutolanil [7] in rice were described. In these papers authors proposed the simple extraction procedures with methanol followed by dilution in assay buffer.

In this work we describe the development of competitive indirect ELISA for quantitative analysis of propanil in rice.

Materials and Methods

Chemical reagents were supplied from Sigma Chemicals Co (St.Louis, MO, USA). Organic solvents and inorganic salts were purchased from Reakhim (Moscow, Russia).

Following buffers were used: phosphate buffer saline (PBS) 0.01 M, containing 0.01 M NaCl (pH 7.4); PBST—PBS containing 0.05 or 0.2% Tween-20 v/v; carbonate buffer—0.01 M sodium carbonate buffer (pH 9.3); citrate buffer (CB)—0.1 M sodium citrate buffer (pH 5.0).

Production of polyclonal anti-propanil antiserum against immunogen 3,4-DCA coupled to succinilated BSA was described in [10].

ELISA was carried out in high capacity 96-well microplates (Nunc, DK). Measurements of optical density were performed on a iEMS microplate reader (LabSystems, Helsinki, Finland). 2,4-DCA-ovalbumin and goat anti-rabbit IgG-HPR conjugates was kindly provided by A. Szekacs from Plant Protection Institute (Budapest, Hungary).

Synthesis of 3,4-DCA-gelatine was carried out by conjugation of 3,4-DCA to succinylated protein using carbodiimid method. Thus, 2 mg of succinic anhydride in 200 μl DMF was added dropwise to a solution 7 mg of gelatin in 2 ml of water. The pH of reaction was kept at 9. The reaction mixture was stirred within 2 h at 20°C was then dialyzed against water within 1 day and against 0.15 M phosphate buffer (pH 5.6) within two following days. 5 mg of 1-ethyl-3-(dimethylaminopropyl)carbodiimid and 1.6 mg of 3,4-DCA in 200 μl DMF was added to the solution of activated protein. Reaction mixture was stirred overnight at room temperature and then was dialyzed within the two days against 0.2% solution of sodium chloride. The conjugate obtained was diluted in 50% glycerol up to concentration 1 mg/ml and stored at -20 °C until use.

Preparation of extracts of a rice flour. Rice was ground in a ball mill. Then 100 ml of methanol were added to various amounts of a rice flour and then were extracted during 12 h at vigorous shaking. The extracts were filtered and used for the analysis.

The recovery test. Rice samples were dried overnight at 60°C. Then the samples (5 g) were spiked with different aliquots of analytical grade purity propanil diluted in methanol and were dried on the air. Further, prepared extracts were treated as described above. Percentage recoveries were calculated as a ratio of the found concentration to added concentration.

Competitive ELISA. The wells of the plates were coated with 100 μl of coating conjugate in carbonate buffer in concentration range 0.01–10 μg/ml for 16 h at 4°C. The wells were washed with 0.2% PBST (4×300 μl) and were then blocked with a 150 μl of the 0.5% gelatin solution in PBS for 1h at 37°C. Aliquots of 50 μl propanil standard

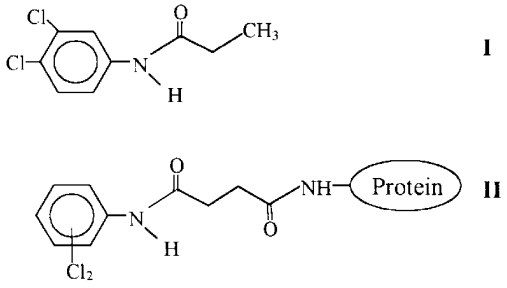
* Department of Enzymology, Faculty of Chemistry, M.V. Lomonosov Moscow State University, Moscow, Russia.
** Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary.

solutions or sample solutions were added to wells. Then 50 μl diluted antiserum in PBST were added to the wells and the plates were incubated for 1 h at 20°C. The wells were washed with 0.2% PBST (4×300 μl). 100 μl of goat anti-rabbit IgG-HPR conjugate solution diluted 1/12 000 in 0.5% PBST were added to the wells followed by another 1-h incubation at 37°C and washing with PBST (4×300 μl). 200 μl fresh prepared substrate solution was added to each well. The substrate solution contained 5 mg *o*-phenylenediamine and 10 μl hydrogen peroxide per 25 ml CB. The color reaction was stopped after 15 min using 50 μl 4 M H_2SO_4 solution. The optical densities were read at 492 nm.

Detection limit of propanil was determined according to the recommendations IUPAC [9].

Results and Discussion

The first step of indirect competitive ELISA is the adsorption of coating conjugate on a microplates. A structure of this coating conjugate can considerable affects the sensitivity of the analysis. In previous study it was shown that the variation of hapten structure in coating conjugates results in differences in binding with specific antibodies. The titers dramatically decrease from 2,4-DCA-OVA to 3,4-DCA-OVA and 3,5-DCA-OVA [11].



Scheme

In the present work we prepared conjugate 3,4-DCA-gelatin and compared it with 2,4-DCA-OVA. The structure of these conjugates is represented on Scheme (II). We studied the binding of these conjugates with specific antibodies and their behaviour in the competitive analysis. For each conjugate series of titration curves were obtained and titers of antibodies were calculated. The results of titration test are presented in Table 1. The optimal concentration for conjugate 2,4-DCA-OVA was 5 μg/ml whereas for conjugate 3,4-DCA-gelatin 0.1 μg/ml. Using optimal conjugate concentration series of calibration curve at several dilutions of antiserum were established. Calibration curves under optimal conditions are presented in Fig. 1. The best IC_{50} value (analyte concentration giving 50% inhibition) for conjugate 2,4-DCA-OVA was 14 ng/ml and 17 ng/ml for 3,4-DCA-gelatin. The quantification range was between 0.1 and 1000 ng/ml for both conjugates.

The organic solvents are commonly used in sample extraction. Therefore we studied the effect of methanol on assay performance. It was shown that the methanol content in a reaction mixture in the range 0–2.5% does not influence the analysis, i. e., the IC_{50} value is a constant and equal

Table 1

Titers of antiserum for different concentrations of coating conjugates

Coating conjugate	Concentration of coating conjugate, μg/ml	Titer of antiserum
2,4-DCA-OVA	0.02	1/810
	0.1	1/2400
	0.5	1/4700
	1	1/4600
	5	1/5500
	10	1/35700
3,4-DCA-gelatin	0.02	1/53000
	0.1	1/91000
	0.5	1/88000
	1	1/84000
	5	1/71000
	10	1/61000

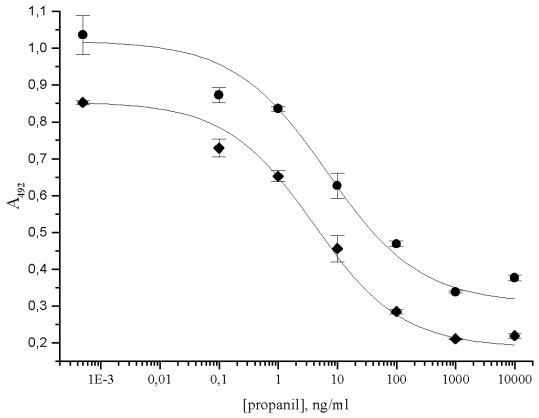


Fig. 1. ELISA calibration curves of propanil for different coating conjugates: 3,4-DCA-gelatin (●); 2,4-DCA-OVA (♦).

18.3±0.5 ng/ml. 5% of methanol results in loss of sensitivity on 23%, and at 15%—more than in 4 times. Thus the extraction samples contained up to 5% of methanol could be applied in ELISA.

The main difficulty during ELISA performance for food samples is connected with matrix interferences and low detection limit [4, 5]. The tolerances for herbicide propanil established by Codex Federal Regulation in rice grain, rice hulls, rice pollishing and rice mill fraction are 10 μg per g rice [8]. Therefore following stage of work was to investigate the influence of components of a rice extracts on the sensitivity of ELISA for propanil. The various techniques of preparation of extracts were examined, thus extracts prepared with different amount of rice flour were spiked with a propanil and analyzed by ELISA. The resulting calibration curves are presented in Fig. 2. The optimum ratio rice flour/methanol was found to be 10 g of rice flour per 100 ml of methanol. Under these conditions the detection of propanil was unaffected by rice extract interferences.

The recovery tests were made to assess the applicability of our ELISA in the quantitative analysis of propanil in rice. Rice grain samples spiked with propanil were applied to indirect ELISA. The recoveries obtained in the range 1–20 μg/g rice averaged between 78 and 150% (Table 2), what is considered to be acceptable for propanil monitoring

Table 2

Recovery of propanil from spiked rice grain samples by the indirect competitive ELISA (N = 3, P = 0.95)

Propanil added, μg/g rice grain	Mean $\pm$ SD*, μg/g	Recovery*, %	Mean $\pm$ SD**, μg/g	Recovery**, %
1	1.4 ± 0.5	140 ± 35	0.8 ± 0.3	80 ± 30
2	3 ± 0.9	150 ± 30	1.4 ± 0.6	70 ± 30
5	3.9 ± 0.7	78 ± 18	3.4 ± 0.8	68 ± 16
10	9.5 ± 1.2	95 ± 12	8.3 ± 2.1	83 ± 21
20	18.7 ± 4.9	93 ± 26	17.0 ± 3.0	85 ± 15
50	94 ± 25	188 ± 27	78.2 ± 17.8	156 ± 36
100	228 ± 68	228 ± 30	130 ± 23	130 ± 23

* Coating conjugate 2,4-DCA-OVA.
** Coating conjugate 3,4-DCA-gelatin.

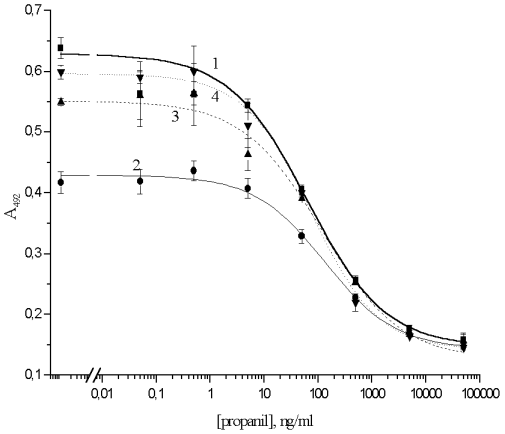


Fig. 2. Influence of rice matrix on calibration curves for propanil: (1) PBST containing 5% methanol; methanol (100 ml) extracts of milled rice: 50 g (2); 25 g (3); and 10 g (4).

in rice. It was demonstrated that the ELISA could detect 10 μg per g rice, which is the regulatory maximum residue limit for propanil in rice [8].

Thus, a sensitive competitive indirect ELISA for propanil was developed. The range of detectable concentrations is 0.1–1000 ng/ml. The ELISA was optimized for quantitative analysis of propanil residues in rice. It was demonstrated, that the content of methanol in reaction mixture up to 5% does not affect the assay. The optimal extraction conditions that allow minimize matrix effect was found to be 10 g of rice per 100 ml of methanol. The range of detectable concentration is 1–100 μg of propanil per g of rice. The ELISA could efficiently measure propanil in rice at regulatory maximum residue limit.

Acknowledgements

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