SYNTHESIS OF ENZYMATIC MARKER 3,6-DICHOLORO-2-METHOXY-BENZOIC-ALKALINE PHOSPHATASE AND EVALUATION OF THE AFFINITY AGAINST HOMOLOGUE ANTIPESTICIDE ANTIBODY

İ. DOROBANŢU, LİVİA HARANGUŞ, M. RADU

"Horia Hulubei" National Institute for Physics and Nuclear Engineering (IFIN-HH), 407, Atomiştilor st., Măgurele, 077125, Romania, mradu@nipne.ro

Abstract. A procedure of synthesis of the enzymatic marker 3,6-dichloro-2-methoxy-benzoic-alkaline phosphatase is described. This bioconjugate is used as reagent in ELISA technique to monitor this pesticide from environmental samples. According to the procedure, 3,6-dichloro-2-methoxybenzoic acid is activated with N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in anhydrous medium of dimethylformamide and then coupled to alkaline phosphatase. The enzymatic activity and the evaluation of the affinity against homologue antipesticide antibody are also described.

Key words: immunoassay, dicamba, affinity, antipesticide, antibody.

INTRODUCTION

Dicamba (3,6-dichloro-2-methoxybenzoic acid) (presented in Fig. 1) is a herbicide used against annual and perennial broadleaf weeds in grain crops and grasslands. In combination with a phenoxyalkanoic acid or other herbicide, dicamba in formulations of this herbicide as Banvel, Oracle and Vanquish are used for agricultural purposes to protect cereals and fruit crops (citrus, apple, pear and peach) and for aquatic uses to control weeds in ditches, canals, rivers, etc. [5, 6]. In soil dicamba is converted to 3,6-dichlorosalicylic acid (3,6-DCSA) by soil bacterium *Pseudomonas maltophylia* and lacks herbicidal activity [3, 4]. The reported half-life in soil is 1 to 6 weeks [5]. Residues of this pesticide occur as a consequence of release from spraying on fields and can contaminate groundwater from agricultural areas and surface water so it can be a real threaten for drinking water supplies. Taken by plants, this pesticide can be sometimes found in cereals or fruit crops. Livestock may graze dicamba-treated areas and the pesticide can be found in their tissues or into milk. Dicamba can present toxic effects by ingestion, inhalation or dermal exposure as loss of appetite, vomiting, muscle weakness,

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effects on heart, kidney and central nervous system [5, 6]. Dicamba is very irritating and corrosive and can cause severe damage to the eyes [6]. This pesticide is also suspected of being a human teratogen [6]. The use of dicamba in agriculture and its toxicity led to the necessity of monitoring of environmental samples for the presence of this pesticide. Analytical methods as high performance liquid chromatography (HPLC), gas chromatography or UV-spectrometry were used in monitoring of pesticides in environmental samples [4]. Most of these techniques being expensive, cheaper and easy to use the immunochemical methods such as ELISA (enzyme linked immunoassay) were proposed. ELISA is used mainly to detect the presence of an antibody or an antigen in a sample. This technique uses specific reagents likes antibodies and enzymatic markers that can be obtained by covalent linking of the antibody or antigen to an enzyme.

Enzymatic markers for detection of pesticides in environmental samples based on linking of antidicamba antibody to peroxidase are presented in the literature [1], but covalent linking of carboxy pesticide as antigen to peroxidase is difficult to obtain. Horse radish peroxidase contains only six lysine residues in its structure and only one or two of those are accessible to be used to link to carboxy compounds (such as antigens) and, consequently, the enzymatic markers are difficult to obtain. Bovine alkaline phosphatase contains fortytwo lysine residues in the structure and presents an enzymatic specific activity higher than peroxidase. From these reasons we chose alkaline phosphatase to be used in obtaining of enzymatic markers.

The paper presents a chemical procedure for the synthesis of the enzymatic marker 3,6-dichloro-2-methoxybenzoic-alkaline phosphatase and an evaluation of the affinity of this label against homologue antipesticide antibody.

MATERIALS AND METHODS

3,6-dichloro-2-methoxybenzoic acid (dicamba), 1ethyl-3-(3-dimethyl-aminopropyl), carbodiimide, N-hydroxysuccinimide (NHS), dimethyl-formamide (DMF), alkaline phosphatase and other chemicals were purchased from Sigma-Aldrich CO, USA.

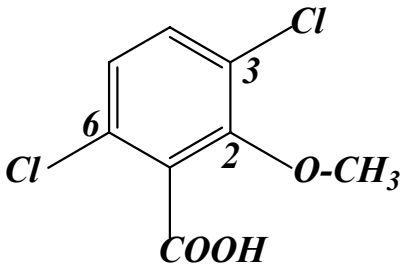

Fig. 1. 3,6-dichloro-2-methoxybenzoic acid (dicamba) molecular structure.

SYNTHESIS OF 3,6-DICHLORO-2-METHOXYBENZOIC-ALKALINE PHOSPHATASE CONJUGATE

The antidicamba antiserum was obtained in our laboratory by immunizing of the New Zealand rabbits with dicamba-hemocyanin as immunogenic conjugate. A mixture of 10 mg 3,6-dichloro-2-methoxy benzoic (dicamba), 5 mg N-hydroxysuccinimide (NHS) and 20 mg 1 ethyl-3-(3-dimethylaminopropyl) carbodiimide (CDI) in 0.5 mL of anhydrous dimethyl-formamide (DMF). The reaction mixture was stirred at room temperature for 4 h. 200 µL activated pesticide mixture was then added to 0.8 mL (≈1 mg alkaline phosphatase) solution. Reaction mixture was stirred and incubated for 24 h at 4 ºC to form pesticide-enzyme conjugate (covalent coupling between pesticide and enzyme). The enzymatic marker was purified by chromatography on a Sephadex G25 column. The fractions containing enzyme activity were collected and an equal value of ethylene glycol was added and kept at –20 ºC for future uses.

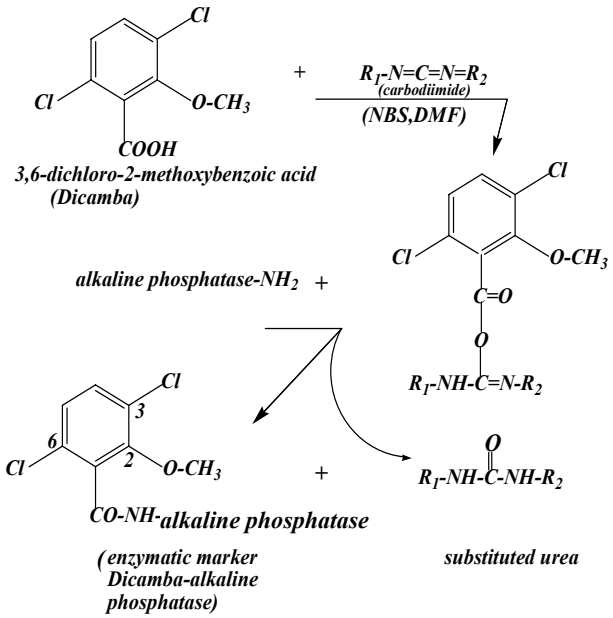

Fig. 2. Chemical reactions for synthesis of enzymatic marker dicamba-alkaline phosphatase.

IMMUNOASSAY PROCEDURE

Microtiter plates were coated with anti-dicamba antibodies in 50 mmol/L sodium carbonate buffer pH 9.6 (100 µL/well). The plates were sealed and incubated overnight at 4ºC. The plates were washed four times with 10mM

phosphate buffer pH 7.2 containing 0.05% Tween 20. Different quantities of pesticide were introduced into wells (100 µL) and then dicamba-alkaline phosphatase solution (100 µL) was added in all wells and incubated for 4 h at the room temperature. After incubation the contents of the wells was removed and the plates were washed three times with phosphate buffer as above described. The activity of enzyme was evaluated measuring the color intensity produced by the transformation of the substrate sodium p-nitrophenylphosphate. To run the reaction, 100 µL sodium p-nitrophenylphosphate 1mg/mL in 50 mM sodium carbonate pH 9.6 was added in each well and the enzyme reaction was stopped by adding 100 µL of 0.5 M NaOH and the color intensity was measured at the wavelength of 400 nm with a Varian Cary 100 spectrophotometer.

RESULTS AND DISCUSSIONS

Procedure of obtainment of the enzymatic marker was realized by coupling of 3,6-dichloro-2-methoxy benzoic acid activated with N-hydroxysuccinimide and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide in anhydrous medium of dimethylformamide followed by reaction with alkaline phosphatase. The enzymatic marker was purified on Sephadex G25 and the results of the measurements are shown in Figure 3. A specific peak for enzymatic conjugate was obtained. The enzymatic specific activity of the marker resulted to be 560 u/mg.

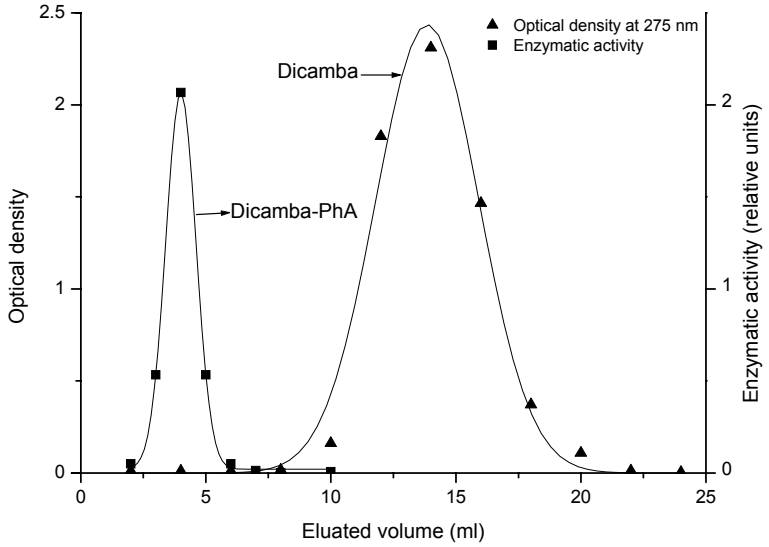

Fig. 3. Chromatography of dicamba-alkaline phosphatase on sephadex G25, eluent 10 mM phosphate buffer pH 7.2.

EVALUATION OF THE AFFINITY OF ENZYMATIC MARKER LABELED AS ANTIGEN AGAINST ANTIPESTICIDE ANTIBODY

Interaction antibody-antigen is characterized by relatively weak binding forces. These forces consist of Van der Waals, electrostatic hydrogen linking or hydrophobic interactions that imply a perfect match between an epitope (antigenic valence) and the binding sites of antibodies. The intrinsic association constant or equilibrium constant that characterizes the antibody binding site with an epitope or a hapten is called affinity.

To calculate the affinity constant between 3,6-dichloro-2-methoxybenzoic-alkaline phosphatase conjugate (dicamba-alkaline phosphatase conjugate) as antigen and the antidicamba antibody, the following algorithm describing the equilibrium kinetics of the antigen (*Ag*)-antibody (*Ab*) system was developed. The system formed by antidicamba antibody coupled on a solid phase (immunosorbent) and enzymatic labelled or unlabelled dicamba as antigen are typical examples of bimolecular reversible reactions.

$$Ab_{\mathrm{b}} + Ag \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} Ab_{\mathrm{b}} \circ Ag \tag{1}$$

$$Ab_{\mathrm{b}} + Ag^{*} \underset{K^{*}_{-1}}{\overset{K^{*}_{+1}}{\rightleftharpoons}} Ab_{\mathrm{b}} \circ Ag^{*} \tag{2}$$

where Ab_{b} is the antidicamba antibody bound on solid phase (immunosorbent);

Ag is the dicamba antigen in solution (unlabelled antigen);

Ag^{*} is the dicamba-alkaline phosphatase conjugate (labelled antigen);

$Ab_{\mathrm{b}} \circ Ag$ and $Ab_{\mathrm{b}} \circ Ag^{*}$ are the immune complexes formed on the surface of immunosorbent;

K_{+1}, K_{-1}, K^{*}_{+1} and K^{*}_{-1} are rate constants of the forward and, respectively, backward chemical reactions.

If we note:

a – the initial concentration of antidicamba antibody bound on the solid phase;

b – the initial concentration of dicamba from solution;

c – the initial concentration of dicamba-alkaline phosphatase conjugate from solution;

x – the concentration of the unlabelled immune complex bound on the solid phase;

y – the concentration of the enzymatic labelled immune complex bound on the solid phase,

then the formation rate of unlabelled immune complex bound on the solid phase is:

$$v_{\mathrm{f}} = \frac{dx}{dt} = K_{+1}\left[\mathrm{a} - \left(x + y\right)\right]\left(b - x\right) \tag{3}$$

and the formation rate of enzymatic labelled immune complex bound on the solid phase is:

$$v_{\mathrm{f}}^{*} = \frac{dy}{dt} = K_{+1}^{*}\left[\mathrm{a} - \left(x + y\right)\right]\left(\mathrm{c} - y\right) \tag{4}$$

The dissociation rate of the unlabelled immune complex, $Ab_{\mathrm{b}} \circ Ag$ in Ab_{b} and Ag is $K_{-1}x$, and the dissociation rate of the enzymatic labelled immune complex, $Ab_{\mathrm{b}} \circ Ag^{*}$ in Ab_{b} and Ag^{*} is $K_{-1}^{*}y$.

After reaching the chemical equilibrium, the rates become zero, and these result:

$$K_{\mathrm{e}} = \frac{K_{+1}}{K_{-1}} = \frac{x_{\mathrm{e}}}{\left[\mathrm{a} - \left(x_{\mathrm{e}} + y_{\mathrm{e}}\right)\right]\left[b - x_{\mathrm{e}}\right]} \tag{5}$$

$$K_{\mathrm{e}}^{*} = \frac{K_{+1}^{*}}{K_{-1}^{*}} = \frac{y_{e}}{\left[\mathrm{a} - \left(x_{\mathrm{e}} + y_{\mathrm{e}}\right)\right]\left[\mathrm{c} - y_{\mathrm{e}}\right]} \tag{6}$$

where K_{e} and K_{e}^{*} are the equilibrium constants of unlabelled respectively labelled immune complexes;

x_{e} and y_{e} represent the concentrations of the unlabelled respectively labelled immune complex at chemical equilibrium.

Assuming that affinities of the labelled and unlabelled antigen against antibody are the same:

$$K = K_{\mathrm{e}} = K_{\mathrm{e}}^{*} \tag{7}$$

there results:

$$K\left[\mathrm{a} - \left(x_{\mathrm{e}} + y_{\mathrm{e}}\right)\right] = \frac{x_{\mathrm{e}}}{\left(b - x_{\mathrm{e}}\right)} = \frac{y_{\mathrm{e}}}{\left[\mathrm{c} - y_{\mathrm{e}}\right]} \tag{8}$$

Or, $x_{\mathrm{e}} = \frac{b}{\mathrm{c}} y_{\mathrm{e}}$, and eq. (8) becomes:

$$K\left[\mathrm{a} - y_{\mathrm{e}}\left(\frac{b + \mathrm{c}}{\mathrm{c}}\right)\right] = \frac{y_{\mathrm{e}}}{\left[\mathrm{c} - y_{\mathrm{e}}\right]} \tag{9}$$

where a and c are constant and b is variable, and for the sample "*i*", it results:

$$K\left[\mathrm{a}-y_{ei}\left(\frac{b_i+\mathrm{c}}{\mathrm{c}}\right)\right]=\frac{y_{ei}}{\left[\mathrm{c}-y_{ei}\right]} \tag{10}$$

For $b_i=0$, it results $y_{ei\max}$ (maximal enzymatic activity):

$$K=\frac{y_{ei\ \max}}{[\mathrm{c}-y_{ei\ \max}][\mathrm{a}-y_{\mathrm{e}\max}]} \tag{11}$$

The concentration of the enzymatic labelled immune complex bound on the solid phase can be calculated by measuring the optical density of p-nitrophenol obtained in the reaction between alkaline phosphatase-dicamba conjugate with sodium p-nitrophenylphosphate as substrate.

The concentration of the enzyme bound to immunosorbent for variable (b_i) is correlated with the concentration of the enzyme from solution by means of the relation:

$$\frac{y_{ei}}{\mathrm{c}-y_{ei}}=\frac{A_\mathrm{b}}{A_\mathrm{f}}=\frac{OD_\mathrm{b}}{OD_0}\approx\alpha_i \tag{12}$$

where: A_b is the enzymatic activity of immunosorbent;

A_f is the enzymatic activity from solution;

OD_b is the optical density of p-nitrophenol measured for immunosorbents;

OD_0 is the optical density of p-nitrophenol measured for free enzyme marker, OD_0 and OD_b, being measured for each value of b_i.

Replacing eq. (12) in (11), it results the equilibrium constant (the affinity constant):

$$K_i=\frac{\alpha_i}{\left[\mathrm{a}-\alpha_i\left(b_i+\mathrm{c}\right)\right]} \tag{13}$$

For b_i in the range of $0 - 10^{-7}$ M, the values of OD_b and OD_0 were measured and the factor α_i and the affinity constant K_i were calculated according to equations (12) and (13), respectively. The results are presented in Table 1. The value of the initial concentration of dicamba antibody bound on the solid phase was $\mathrm{a} = 1.84\cdot10^{-10}$ M and the value of the initial concentration of dicamba-alkaline phosphatase conjugate from solution was $\mathrm{c} = 1.1\cdot10^{-8}$ M. The mean value of the calculated affinity constant values is $\overline{K}=\left(1.43\pm0.54\right)\cdot10^{7}\left(\mathrm{L/mol}\right)$.

Table 1

The values of the equilibrium constants (affinity constants) for the system antidicamba antibody respectively antigen dicamba, dicamba-alkaline phosphatase conjugate

b_i(M)	α_i	K_i(L/mol)	$\overline{K}$(L/mol)
0	$3.20 \cdot 10^{-3}$	$2.10 \cdot 10^{7}$	$(1.43 \pm 0.54) \cdot 10^{7}$
$2 \cdot 10^{-8}$	$2.43 \cdot 10^{-3}$	$1.10 \cdot 10^{7}$	
$5 \cdot 10^{-8}$	$1.37 \cdot 10^{-3}$	$1.65 \cdot 10^{7}$	
$10 \cdot 10^{-8}$	$0.74 \cdot 10^{-3}$	$0.90 \cdot 10^{7}$	

CONCLUSIONS

Solid-phase immunoassay is based on the reactivity of antibodies or antigens when adsorbed on solid surfaces and thus there is a difference between reaction kinetics in solution and those occurring on the solid phase. Although chemical coupling of protein to solid phase is possible in our study we used noncovalent adsorption which is essentially hydrophobic in nature. Most proteins tend to adopt a conformation in which hydrophilic groups tend to be on the outside of the protein and hydrophobic groups within it. Hydrophobic binding to polymer surfaces causes conformational changes in the adsorbed protein, in our case antipesticide antibody, and the number of active sites and its affinities for the antigen dicamba or dicamba-alkaline phosphatase conjugate are modified [2, 7]. The mean affinity constant in our experiments was $(1.43 \pm 0.54) \cdot 10^{7}$ L/mol and represents only a characteristic of the system that uses solid phase and dicamba-alkaline phosphatase as labeled antigen respectively. The determined affinity value is different comparing to the affinity of the system in which antigen and antibody react in a homogeneous solution. The importance of the estimation of the affinity constant is that the sensitivity of the ELISA technique for monitoring of the pesticide from environmental samples is finally limited only by the residual experimental errors and the equilibrium constant.

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REFERENCES

1. CLEGG, B.S., G.R. STEPHENSON, J.C. HALL, Development of an enzyme-linked immunosorbent assay for the detection of dicamba, *J. Agric. Food Chem*., 2001 May, **49(**5**)**, 2168–2174.

2. HOWELL, E.E., J. NASSER, K.J. SCHRAY, Coated tube enzyme immunoassay: factors affecting sensitivity and effects of reversible protein binding to polystyrene, *Journal of Immunoassay*, 1981, **2**(3&4), 205–225.
3. MORAIS, S., P. CASINO, M.L. MARTIN, Assessment of enzyme linked immunosorbent assay for the determination of 2,4,5-TP in water and soil, *Anal. Bioanal. Chem.,* 2002, **374**, 262–268.
4. ROY, J.W., J.C. HALL, G.W. PARKIN, C.W. RIDDLE, B.S. CLEGG, Seasonal leaching and biodegradation of dicamba in turfgrass, *Journal Environmental Qual*., 2001, **30**, 1360–1370.
5. US Department of Agriculture. Forest Service Pacific Northwest Region, Dicamba-Herbicide Information Profile, http://www.fs.fed.us/r6/nr/fid/pubsweb/dicamba_99.pdf, 1999.
6. US Environmental Protection Agency, Reregistration Eligibility Decision for Dicamba and Associated salts, http://www.epa.gov/oppsrrd1/REDs/dicamba_red.pdf, June 8, 2006.
7. WILD, D., *The immunoassay handbook*, Third edition, Elsevier Ltd. ed., New York, 2005.