Budapest Technical and Economical University
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DEVELOPMENT AND APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS

PhD thesis

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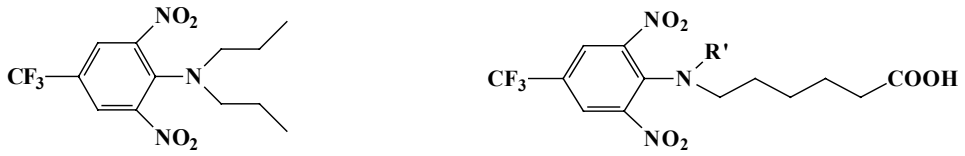
1. Development of an ELISA system for the detection of the herbicid trifluralin

The target compound: Trifluralin is a selective, preemergence dinitroaniline herbicide used to control annual grasses and broadleaf weeds in a large variety of fruit trees, nuts, vegetables, and grain crops. Although pure trifluralin is not acutely toxic, certain formulated products may be more toxic than the technical material itself, and single dose treatments or long time exposure to trifluralin over lifetime at health advisory level have been shown to cause liver and kidney damage. In addition, because of its slow decomposition, this pesticide has a potential environmental persistence. In order to allow rapid monitoring of trifluralin, we developed an ELISA system for this analyte.

Hapten synthesis: In the first step, in order the structure of trifluralin should remain unchanged as much as possible, the parent structure was modified with a carboxyl group, and three ω-carboxy derivatives were prepared. In these compounds, instead of the *N*-propyl group of trifluralin, a 5-carboxypentyl group was placed. In addition, in in the position of the other *N*-propyl group of the base molecule, the presence of a H, methyl or propyl group was also varied. In the **immunogen** I used the *N*-propyl-*N*-(5-carboxy-pentyl) derivative of trifluralin as hapten, in **surface antigens** I varied the carboxyalkyl group and the length of the other *N*-alkyl group (*Figure 1*).



(a) **(b)**

Figure 1 The chemical structure of the herbicide trifluralin (**a**) and haptenic compounds (**b**). R`: H, Me, Pr

Conjugation, and the efficiency of conjugation: After hapten synthesis the prepared haptens were bound to different proteins (BSA, CONA, KLH), and the efficiency of conjugation was determined by **UV-spectroscopy** and **MALDI-ToF MS**. In UV-spectroscopy experiments I measured the absorbance intensities of the haptens and their conjugates at their maximal absorbance wavelengths (380-420 nm). Using the calculated molar extinction coefficients (ε = 1900 5300 1/mol/cm) I calculated the molar concentrations of the haptens in the conjugates, and compared these data with the concentration of the carrier proteins. In MALDI-ToF MS measurements I received immediate information on the average molecular weight of the conjugates.

The results of these two methods were in good correlation, although I got systematically higher hapten/protein ratios calculated by UV-spectroscopy. The reason of this difference may be the presence of non-bound haptens after dialysis, causing increased absorbancies. The calculated hapten/protein molecular ratios represent proper proportions of hapten/protein reagent ratios in the conjugation reaction (the theoretical hapten/protein ratios were 1.0, 0.2, 0.039 μmol hapten / mg protein), although the measured and theoretical ratios were in closer agreement with each other at lower reagent rates**.**

Immunization, optimization: Using the KLH-conjugate of hapten *N*-(2,6-dinitro-4-trifluoromethyl)phenyl-*N*-propyl-6-amino-hexanoic acid, rabbits were immunized, and antisera were obtained with multiple with bleedings. Using antisera against the hapten conjugates, I developed immobilized antigen-based, competitive inhibition ELISA systems, and optimized them for their main analytical parameters (serum titer, concentration of the coating antigen applied, mid-point of inhibition (IC_{50}) and limit of detection (LOD)). With optimal conditions (after 3rd immunization, 1 μg/ml coating antigen concentration, 1:3000 dilution of antisera) the developed system can determine trifluralin concentrations from 0,1 to 100 ng/ml, the IC_{50} values were 2,56 – 3,55 ng/ml. I compared the optimized ELISA system with an instrumental analytical (GC-MS) method (using solid phase microextraction (SPME) for sample preparation) in buffer, at 0 – 25 ng/ml concentration range. The ELISA and the GC-MS methods gave nearly identical concentrations in the concentration range of 1 – 10 ng/ml ($r^2 = 0,988$).

Method stability, cross-reactivity: In the optimized trifluralin ELISA system I studied the effects of the main analytical parameters (the effect of the pH, organic solvents, cross-reactivity). Several water-miscible organic **solvents** (ethanol, acetonitril, aceton, dimethyl sulfoxide (DMSO), dimethyl formamide (DMF)) under the concentration of 2 V/V% do not modify the sensitivity of the method, on the other hand the IC_{50} value increased to over double in the presence 0.5 V/V% methanol, did not further deteriorate at 1 – 5 V/V% methanol concentrations, and continued the increasing tendency at concentrations of methanol over 10 V/V%. I tested the effect of **pH** (pH = 4,65 - 9,38) on assay performance, and the assay worked the best at pH = 6.4. Within optimized ELISA system I studied the **cross-reactivity** of compounds with structure similar to trifluraline (dinitroaniline herbicides, haptens, synthetic intermediates). I found the ELISA system specific for trifluralin, as only its close structural analog haptens showed significant (10 – 28%) cross-reactivity, while the cross-reactivity of closely analogous herbicides were under 5.2 %.

Practical applications: I applied the developed method for the determination of trifluralin content in surface water and vegetable drinks (carrot, pumpkin and tomato juice). In surface water (after the pH was set to 7.4) the method is applicable for detection of trifluralin at a LOD of 0.85 ng/ml. Although this value is higher than the Europian drinking water standard, it is suitable for environmental monitoring. In sewage water the method is not applicable because of a severe matrix effect. To determine the trifluralin content in pumpkin and tomato juice, it is necessary to dilute the sample 1:10, while with carrot juice a dilution of 1:5 is necessary to eliminate the matrix effect.

New scientific results:

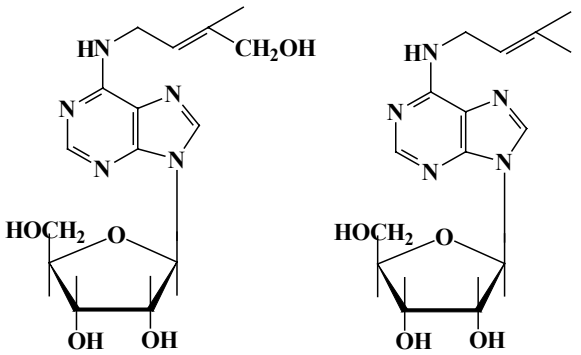
2.2. I coupled haptenic derivatives of the target analyte trifluralin to BSA and KLH proteins using different hapten/protein ratios. With UV and mass spectroscophy (MALDI-ToF) I verified, that the hapten density in the conjugates is near the calculated values (1.02, 0.16 and 0.10 μmol hapten/mg protein, respectively).

2.3. Using the antisera against the KLH-conjugate, a sensitive ELISA system (LOD: 0,85 ng/ml) was developed to determine trifluralin concentration in the low ppb range. I optimized the system for its main assay parameters (the effect of pH and organic solvents), and I checked the specificity for the target analyte. The advantage of the optimised system is its high specificity to trifluralin among dinitroaniline herbicides. The system can tolerate the pH in a wide range (4.6 9.4), the methanol content up to 5 V/V%, and the ethanol, acetonitrile, acetone, DMSO and DMF content up to 2 V/V%.

2.4. I validated the system in the trifluralin concentration range of 1 – 10 ng/ml using SPME/GC-MS. I studied applicability of the method to the determination of trifluralin concentration in different surface water and vegetable juice samples.

2. Development of an ELISA system for the detection of cytokinin hormones

The target compound: The level of cytokinin type hormones, such as N^6-(2-isopentenyl)-adenosine (IPA) and *trans*-zeatin riboside (ZR) (*Figure* 2), present in plant tissues, is a good indicator of the resistance of plants to abiotic environmental stresses and necrotrophic pathogens, as well as plant juvenility or senescence. Using traditional analytical procedures to measure the cytokinin hormone levels in plant is a quite difficult, time-consuming and costly task due to the complex matrix, because of heat-lability of cytokinins, and because the detection methods often rely on chemical derivatization. Moreover, these hormones occur in trace amounts, together with numerous compounds that are similar in structure. Therefore, a high thoughput, simple, rapid, economic and quantitative ELISA detection method was developed for IPA and ZR type cytokinins.



(**a**) (**b**)

Figure 2 The chemical structure of zeatin riboside (**a**) and isopentenyl-adenosine (**b**)

Hapten synthesis and conjugation: The conjugation reaction was carried out based on methods described in the literature utilizing the riboside moiety in *trans*-zeatin riboside and in isopentenyl-adenosine, or presumably through the hydroxypentenyl side chain of *trans*-zeatin. The riboside vicinal diols of IPA and ZR were converted, through cleavage of the C-C bond of the riboside ring, to the corresponding dialdehyde using sodium periodate; and these dialdehydes were conjugated to the amino groups of carrier proteins (BSA, OVA). A similar approach was also carried out to directly conjugate zeatin presumably through its hydroxypentenyl side chain. Hapten incorporation rates on the carrier proteins were followed in both types of conjugation reaction by isoelectric focusing of the BSA conjugates along with the carrier protein. Higher hapten incorporation rates were detected when hormones were coupled through their riboside moiety.

Immunization, optimization: Using the OVA-conjugates (in a similar manner that was seen for the trifluralin ELISA), rabbits were immunized and antisera were prepared from the blood. Hapten-homologous and hapten-heterologous competitive indirect enzyme-linked immunosorbent assays (ELISAs) were developed with BSA-conjugates as coating antigens. I optimized these systems for their main analytical parameters (concentration of coating antigen, serum titer, the inhibition mid point (IC_{50}), limit of detection (LOD), as well as incubation and preincubation time). The optimized hapten-homologous system for ZR (concentration of coating antigen 1 μg/ml, serum dilution 1:800, LOD 0.4 ng/ml) and for IPA (coating antigen concentration 5 μg/ml, serum dilution 1:1100, LOD 0.7 ng/ml) allowed IC_{50} values of 19 ng/ml and 18 ng/ml, respectively, while the hapten-heterologous system for ZR (using ZR-BSA as coating antigen and Z-OVA as immunogen) allowed a moderate titer, and no interpretable inhibition curve was obtained in this system with ZR.

Method stability, cross-reactivity: The optimized hapten-homologous ELISA systems for both cytokinins can tolerate the **pH** from 4.6 to 9.2. I studied the effect of various commonly used water miscible organic **solvents** for extraction Both assays appeared to produce practically unchanged standard curves when methanol and ethanol were present at up to 8%, and the uninhibited assay tolerated these solvents at up to 32%. In contrast, DMF and DMSO had significant detrimental effects even on the uninhibited assay signal at concentrations above 2%. **Cross-reactivities** were determined in each assay under optimized conditions. In these determinations, six cytokinins, namely Z, ZR, 2-iP and IPA as major natural cytokinins, as well as benzyladenine and kinetin were used. The anti-ZR antibody practically did not cross-react with the other types of cytokinins examined, and similarly, superior sensitivities were seen for 2-iP and IPA in the hapten-homologous system for IPA. This indicates that the presence of a single OH group in the side chain of the hormone in the immunogen causes a great change in antisera specificity, and because of the specific detection of the IPA type and ZR type cytokinins we can measure the concentration of these hormones when both of them are present in the sample.

Practical application: In the process of the development of the ELISA systems, the first applied tracer enzyme was peroxidase, but in some cases the high level of peroxidases in certain plant tissues caused severe artifacts even though these plant peroxidases are introduced into the ELISA system at an earlier step before detection, and in principle should have been removed by the subsequent washing steps. The matrix problem was resolved by using another label enzyme, alkaline phosphatase (AP). The optimized ELISA systems were readily applied on various control and genetically modified plant samples with elevated cytokinin levels. Samples, extracted with methanol had to be diluted with buffer for he ELISA tests, therefore, in contrast

to ZR, I could not measure the IPA concentration of the samples in all cases as its physiological level is lower than that of ZR. Results obtained with the optimized immunoassays have clearly demonstrated that the above-mentioned manifestations of improved stress resistance are truly due to elevated cytokinin (expecially ZR) levels in these plants.

New scientic results:

2.1. I coupled the two target hormones (ZR, IPA) to carrier proteins (BSA, OVA) and verified the efficiency of conjugation with SDS-PAGE.

2.2. Using antisera against KLH-conjugates I developed a quick ELISA system (as a result of experiments with the incubation time) to analyse two types of cytokinin hormones. The IC_{50} values under optimized system parameters in the ZR and IPA ELISA systems are 19 ng/ml and 18 ng/ml, respectively. I optimized the systems for their main assay parameters (the effect of pH and solvent, incubation and preincubation time). I examined the pH (systems can tolerate from 4.6 to 9.2) and water miscible organic solvent (systems can tolerate methanol and ethanol content up to 8 V/V%, and acetonitril, aceton, DMF and DMSO content up to 2 V/V %) and the specificity for target hormones. I changed the peroxidase tracer enzyme to phosphatase to eliminate the matrix effect in plants, and verify that systems are suitable to measure the cytokinin concentration of plant samples.

ABBREVIATIONS

ELISA – enzyme-linked immunosorbent assay
IC_{50} – inhibition mid-point (inhibitor concentration causing a 50% decrease of maximal signal)
LOD – limit of detection
BSA – bovine serum albumin
CONA – conalbumin
KLH – hemocyanin (from keyhole limpet)
OVA – ovalbumin
GC-MS – gas chromatography – mass spectrometry
MALDI ToF MS – matrix-assisted laser desoprtion/ionization time of flight mass spectrometry
SPME – solid phase microextraction
DMSO – dimethyl sulfoxid
DMF – dimethyl formamide
IPA – isopentenyl-adenosine
ZR – zeatine riboside

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PUBLICATIONS, AS THE BASIS OF THE DOCTORAL THESIS

1. **Hegedűs, Gy.**; Bélai, I. and Székács, A. (2000) Development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide trifluralin. *Anal. Chim. Acta*, **421**, 121-133.
2. Székács, A.; **Hegedűs, Gy.**; Tóbiás, I.; Pogány, M. and Barna, B. (2000) Immunoassays for plant cytokinins as tools for the assessment of environmental stress and disease resistance. *Anal. Chim. Acta*, **421**, 135-146.

COMPLETE LIST OF PUBLICATIONS

Papers in scientific periodicals:

1. H.M. Le; **Gy. Hegedűs** and A. Székács (1998) Differential detection of *N*-heterocyclic compounds and their *N*-methylated derivatives by immunoanalysis. *Acta Biologica Hungarica*, **49**, 455-462. [IF 0,219]
2. **Gy. Hegedűs**; I. Bélai and A. Székács (2000) Development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide trifluralin. *Anal. Chim. Acta*, **421**, 121-133. [IF 1,894]
3. A. Székács; **Gy. Hegedűs**; I. Tóbiás; M. Pogány and B. Barna (2000) Rapid immunoassays for plant cytokinins as tools for environmental stress and disease resistance. *Anal. Chim. Acta*, **421**, 135-146. [IF 1,894]
4. V. Krikunova; **Gy. Hegedűs**; H.M. Le; L. Jouravleva; S. Eremin; M. Natangelo; E. Benfenati and A. Székács (2001) Development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide propanil. *Int. J. Envir. Anal. Chem.*, **82**, 865-878. [IF 0,797]
5. **Gy. Hegedűs**; V. Krikunova; I. Bélai; S. Eremin and A. Székács (2002) An enzyme-linked immunosorbent assay (ELISA) for the detection of acetochlor. *Int. J. Envir. Anal. Chem.*, **82**, 879-891. [IF 0,797]

[Σ IF 5,585]

Conference presentations and posters:

1. H.M. Le, **Gy. Hegedűs** and A. Székács (1998) Immunodetection of *N*-heterocyclic compounds. Poster presented at the *4th International Conference on the Role of Formaldehyde in Biological Systems - Methylation and Demethylation Processes* (Budapest, Hungary, July 1-4, 1998)
2. A. Székács, **Gy. Hegedűs**, D. Knopp and R. Niessner (1998) A modified enzyme-linked immunosorbent assay (ELISA) for polyaromatic hydrocarbons. Poster presented at *The Immunochemistry Summit VII and the 3rd Workshop on Biosensors and Biological Techniques in Environmental Analysis* (Las Vegas, Dec. 1-3, 1998)
3. **Gy. Hegedűs**, H.M. Le, A. Székács, A. Krasnova, S. Eremin, M.-C. Hennion, M. Natangelo and E. Benfenati (1999) Inter-laboratory trials of GC-MS versus immunoanalytical detection of environmental pollutants. Poster presented at *The 9th Symposium on Handling of Environmental and Biological Samples in Chromatography* (Porto, Portugal, Oct. 10-13, 1999)
4. **Gy. Hegedűs**, I. Bélai and A. Székács (1999) Development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide trifluralin. Lecture presented at *The 4th Workshop on Biosensors and Biological Techniques in Environmental Analysis* (Mao, Spain, Dec. 1-3, 1999)
5. **Gy. Hegedűs**, A. Székács, I. Tóbiás and B. Barna (1999) Immunoassays for plant cytokinins: rapid monitoring methods of environmental stress. Poster presented at *The 4th Workshop on Biosensors and Biological Techniques in Environmental Analysis* (Mao, Spain, Dec. 1-3, 1999)
6. **Gy. Hegedűs**, A. Székács, I. Tóbiás and B. Barna (2000) Immunanalitikai renmdszerek kifejlesztése növényi hormonok meghatározására. Előadás a *46. Növényvédelmi Napok*on (Budapest, Feb. 22-23, 2000), Összefoglalók, 99. old.
7. **Gy. Hegedűs**, I. Bélai és A. Székács (2000) Enzim-jelzéses immunoassay (ELISA) fejlesztése trifluralin kimutatására. Előadás a *Vegyészkonferencia 2000* rendezvényen (Debrecen, Jul. 5-7, 2000)
8. **Gy. Hegedűs**, Székács, A., Le, H.M., Krikunova, V., Eremin, S., Natangelo, M. and Benfenati, E. (2000) Development of an propanil. Poster presented at *The 4th Euroconference on Environmental Analytical Chemistry* (Visegrád, Sep. 14-19, 2000)
9. **Gy. Hegedűs**, I. Bélai and A. Székács (2000) Enzyme-linked immunosorbent assay (ELISA) for monitoring the herbicide trifluralin. Poster presented at *The 4th Euroconference on Environmental Analytical Chemistry* (Visegrád, Sep. 14-19, 2000)
10. A. Székács és **Gy. Hegedűs** (2001) Növényvédő szer-maradékok meghatározása műszeres és immunanalitikai módszerekkel. Előadás a *47. Növényvédelmi Tudományos Napok* rendezvényen (Budapest, Feb. 27-28, 2001).
11. A. Székács, G. Novák és **Gy. Hegedűs** (2001) Növényvédő szer hatóanyagok meghatározása műszeres és immunanalitikai módszerekkel. Előadás a *XV. Országos Környezetvédelmi Konferencia* rendezvényen (Siófok, Sep. 11-13, 2001)