Development of ELISA Kit for Detection of Glyphosate-Resistant Genetically Modified Soybean

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Recieved: February 01, 2019; Accepted: March 14, 2019
DOI: 10.17721/moca.2019.xx-xx

The goal of research was development of competitive ELISA kit for detection of genetically modified plants resistant to glyphosate. As the source for gene isolation we have used leaves of GM soybean RoundUp Ready, line 40-3-2 (Monsanto). Using polymerase chain reaction the gene encoding the enzyme 5-enolpiruvilshykimat-3-phosphate synthase (CP4 EPSPS) was isolated, which provides resistance to the herbicide glyphosate. Isolated gene has been cloned into plasmid vector pET24(a), creating genetic construct pET-CP4EPSPS. E.coli (BL 21(DE3)) has been transformed by genetic construct and over expressed after induction by 0.5 mM IPTG. Recombinant enzyme CP4 EPSPS with molecular weight 45 kD was purified as inclusion bodies and used as antigen for 96-well polystyrene plates immobilization. Monoclonal antibodies CP4 EPSPS mAb3 (Artron BioResearch Inc., Canada) was used for synthesis of horseradish peroxidase conjugate and receiving diagnostic reagent. We have shown that in the competitive ELISA we can detect at least 0.1% of GM plants resistant to glyphosate, on the example of soybean (line RR 40-3-2), moreover we can also detect rapeseed (line GT73) and sugar beet (line H7-1).

Keywords: genetically modified plants, enzyme immunoassay, 5-enolpyruvylshikimate-3-phosphate synthase, monoclonal antibody

Розробка імуноензимного діагностикуму для виявлення гліфосат-резистентної генетично модифікованої сої

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Надійшла: 01 лютого 2019 р; Прийнята: 14 березня 2019 р
DOI: 10.17721/moca.2019.xx-xx

В роботі представлено етапи розроблення імуноензимного діагностикуму для виявлення генетично-модифікованих рослин, стійких до гліфосату. В якості моделі використовували ГМ сою RoundUp Ready, лінія 40-3-2 (Монсанто). За допомогою полімеразної ланцюгової реакції з ГМ-сої було виділено ген, що кодує ензим 5-енолпірувілшикімат-3-фосфат синтазу (CP4 EPSPS), яка забезпечує резистентність до гербіциду гліфосату. У процесі роботи виділений ген CP4 EPSPS було клоновано в плазмідний вектор для бактерійної експресії та отримано рекомбінантний ензим CP4 EPSPS. Моноклональні антитіла (CP4 EPSPS mAb3, Artron BioResearch Inc., Канада) використовували для синтезу імуноензимного кон'югату з пероксидазою хрону та подальшого конструювання діагностикуму. Було продемонстровано, що ІЕА у конкурентному варіанті дозволяє виявляти не менше 0.1% ГМ-рослин, стійких до гліфосату, на прикладі сої (лінія RR 40-3-2). Крім того, показана можливість ідентифікації ГМ-ріпаку (лінія GT73) та ГМ-цукрового буряку (лінія H7-1).

Ключові слова: генетично модифіковані рослини, імуноензимний аналіз, 5-енолпірувілшикімат-3-фосфат синтаза, моноклональні антитіла

The use of transgenic plants has become one of the most significant innovations in the agrarian sector. Over the past 19 years of commercialization of transgenic crops, the area size has grown to 182 million hectares in the world, indicating that biotechnological approaches in crop production are considered to be number one in modern agriculture [1].

Around 60% of all genetically modified agricultural crops are transgenic crops resistant to herbicides, and the glyphosate-resistant soy has the first place among it with more than 260 million tons of annual production. So far, the United States of America, Brazil and Argentina, with more than 80% of world soybean production, are the leaders. The growth of soybeans production is determined by high profitability of the crop, state support and high demand as a high protein feed for the livestock industry. With due regard to the steady growth of livestock product demand in the

world, soybeans are considered as a strategic culture for food security in future [1].

Glyphosate is the active ingredient of wide-spectrum herbicide, more commonly known under the “Roundup” trademark (Monsanto); it is an enzyme inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), (EC 2.5.1.19). This enzyme catalyzes the penultimate stage of the shikimate pathway of bacteria, yeasts, algae, fungi and higher plants biosynthesis of aromatic amino acids [2].

At the beginning of 1990’s, a glyphosate-tolerant enzyme of EPSPS with a gene isolated from *Agrobacterium tumefaciens* sp. (CP4 strain) from wastewater of the glyphosate plant was patented by the Monsanto scientists. Biotechnically, the CP4 EPSPS gene was introduced into the soybean genome, which led to the buildup of a 40-3-2 glyphosate-resistant event. It marked the beginning of a new era of transgenic lines of herbicides-resistant crops such as soybeans, corn, rape, cotton, sugar beet, etc. commercialization [3].

In Ukraine, the production interest of soybeans grows annually. Thus, according to the State Statistics Committee of Ukraine, the gross soybean output was 4.27 million tons for 2018, and it was 27.6% more than in 2017 [4]. Herewith, more and more cases of glyphosate-resistant genetically modified soybeans cultivation (lines 40-3-2) are registered in Ukraine.

Taking into account that the latest data prove the oncogenicity of glyphosate, which leads to the development of non-Hodgkin’s lymphoma in humans, the use of glyphosate is already prohibited in European countries, and its trace amount is strictly regulated in the final agricultural product. Thus, the necessity of continuous monitoring of the distribution of glyphosate-resistant GM plants is indisputable [5].

The generally accepted method for studying GM plants is the polymerase chain reaction (PCR), based on the detection of transgenic elements in the genome of GM plants. Despite the apparent benefits of PCR, such as hypersensitiveness and high specificity, the main drawback of the method is that it is based on databases available data on the nucleotide sequence of transgenic elements and their location in the plant genome. It is impossible to develop methods for the detection of nucleic acids if there is lack of such data [6]. Therefore, the detection of transgenic proteins [7] that affect the phenotypic peculiarities of organisms, such as resistance to herbicides or to insect pests is an alternative approach for the study of GM plants. This is especially true for research of plant material before its processing.

The aim of work was to develop an immunoenzymatic assay (IEA) for determination of CP4 EPSPS foreign protein that provide genetically modified plants with characters of toleration to glyphosate, on the GM soya model (RoundUp Ready 40-3-2). To achieve the aim, the following tasks were solved: 1 – to clone the gene encoding the CP4 EPSPS transgenic enzyme; 2 – to receive the recombinant enzyme CP4 EPSPS in the bacterial expression system; 3 – to synthesize the immunoenzymatic conjugate of specific monoclonal antibodies CP4 EPSPS mAb3 with horseradish peroxidase; 4 – to construct competitive immunoenzymatic test system for detection of glyphosate-resistant GM soybeans.

Materials and methods

Reagents and equipment

Reagents: Oligonucleotide primers GTS-F (5’-at gcgtcgacgcgaggacgtcatcaatac-3’) and GTS-R (5’at gcctcgagagccttcgtatcggagagttcg-3’) synthesized by BioBasic Inc. (Canada); PCR reagents, endonuclease restriction reagents and GeneJET PCR Purification Kit by Fermentas Thermo Scientific Group (Lithuania); BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA); competent E. *coli* cells of BL21(DE3) strain and *F-ompT hsdSB(rB-mB-) gal dcm (DE3)* genotype, by NEB (USA); chemical reagents of “Molecular biology grade” qualification by Sigma (USA), Carl Roth (Germany), Amresco (USA); Difco nutrient mediums (BD Group); Sarstedt polystyrene 96-well plates (Germany); CP4 EPSPS mAb3 monoclonal antibodies by Artron BioResearch Inc. (Canada).

Equipment: Thermocycler 2720 Thermal Cycler (Applied Biosystems, USA), BioLogic LP low pressure chromatographic system (Bio-Rad, USA), BioSpec-nano spectrophotometer (Shimadzu Biotech, Japan), SmartSpec Plus spectrophotometer (Bio-Rad, USA), genetic analyzer 3130 Genetic Analyzer (Applied Biosystems, USA), electroporation unit 2510 (Eppendorf, Germany), Multifuge 3 S-R centrifuge (Heraeus, Germany), Mini Protean Tetra Cell Systems vertical electrophoresis chamber (Bio-Rad, USA), Microtron MB 550 experimental mill (Kinematica AG, Switzerland), Humatemp microplate hatcher unit (Human, Germany), PW40 automatic microplate washer (Bio-Rad, USA), Humareader Plus automatic microplate spectrophotometer (Human, Germany) etc. The samples used in the work are presented in Table 1.

Cloning of a gene encoding a CP4 EPSPS *transgenic enzyme.* DNA was extracted from GM soya leaves (RoundUp Ready 40-3-2 line) routinely [8]. The amplification of the gene encoding the CP4 EPSPS transgenic enzyme was performed with the GTS-F and GTS-R specific oligonucleotide primers in a 50 mcl of reaction mixture containing 20 mM Tris-HCl (pH 8.8, 25°C), 10 mM $(NH4)_2SO_4$, 10 mM KCl, 0.1 mg/ml of BSA, 0.1% of Triton X-100, 2.0 mM $MgSO_4$, 0.25 mM of each of dNTPs, 1.0 mcM of each of the primers, 100 ng of extracted genomic DNA and 1.5 units of Pfu DNA polymerase. The thermal amplification profile had the following stages: initial DNA denaturation (5 min, 95 ^{0}C), 30 consecutive DNA denaturation cycles (10 seconds, 95 ^{0}C each), annealing of primers (15 seconds, 55 ^{0}C), DNA synthesis (1 min, 72 ^{0}C), and the final stage of DNA

synthesis (7 min, 72 ^{0}C).

After amplification, a specific PCR product (1200 base pair) was separated by electrophoresis in 1.0% agarose gel, stained with bromic ethidium (0.5 ug/ml), and cuted in UV light rays (245 nm). A specific DNA fragment was extracted from agarose gel using a GeneJET PCR Purification Kit. The thus-purified specific PCR product was treated with SalI and XhoI restriction enzyme (3 hours, 37 ^{0}C), the pET-24a plasmid vector (Novagen, USA) was treated with the appropriate restriction endonuclease simultaneously. Subsequent cloning of a specific DNA fragment, representing the CP4 EPSPS enzyme gene, in the pET-24a plasmid vector was performed according to standard genetic engineering methods [9]. At the final stage, the competent cells of *E. coli*, BL21 (DE3) strain were transformed with the recombinant plasmid pET-CP4 EPSPS by electroporation. The expression analysis of CP4 EPSPS recombinant enzyme was performed by electrophoresis in 12% of SDS-PAGE [10] before and after induction of 100 ml of bacterial culture (OG_{600} - 0.5) using 0.5 mM intact PTH. The recombinant CP4 EPSPS protein was extracted from bacterial lysate in as inclusion bodies, by consequent washing with 2M urea. After 3-4 rounds of washing the precipitation of inclusion bodies was dissolved in 8 M urea and used as an antigen for the immunization of the chicken.

Conjugation of monoclonal CP4 EPSPS mAb3 antibodies with horseradish peroxidase and constructing a competitive IEA. The classical periodate method was used for the synthesis of the immunoensymatic conjugate of monoclonal antibodies with horseradish peroxidase [11]. The specificity of the conjugate (mAb3-HRP) was checked by reciprocal titration. While doing so, the optimal concentration of sensitized antigen on the plate and the conjugate titer in a competitive variant of the immunoenzymatic analysis were determined using the aqueous extract of 1.0% reference standard of the positive GM soya (RoundUp Ready 40-3-2) and the negative soybean standard (blank).

Table 1. The main characteristics of samples.

Plants	Template	Event	Transgene	GMO certified value, %
Soybeans	Leaves	Roundup Ready	CP4EPSPS	100.0
	ERM*-BF410a	Blanc	—	0
	ERM-BF410bk	Roundup Ready	CP4EPSPS	0.1
	ERM-BF410c	Roundup Ready	CP4EPSPS	0.5
	ERM-BF410dk	Roundup Ready	CP4EPSPS	1.0
	ERM-BF410e	Roundup Ready	CP4EPSPS	2.0
	ERM-BF410f	Roundup Ready	CP4EPSPS	5.0
	ERM-BF410gk	Roundup Ready	CP4EPSPS	10.0
	AOCS** 0906-B	GenuityRoundup Ready 2 Yield	CP4EPSPS	100.0
	Seeds	Liberty Link	PAT	100.0
	Seeds	«Cheremosh»	—	0
Corn	ERM-BF414f	GA21	CP4EPSPS	5.0
	ERM-BF415f	NK603	CP4EPSPS	5.0
	Seeds	«Khmelnytsky»	—	0
Sugar beet	ERM-BF419b	H7-1	CP4EPSPS	100.0
	Seeds	«Oleksandriya»	—	0
Rape	AOCS 0304-B	GT73	CP4EPSPS	100.0
	Seeds	«Bahira»	—	0

*Reference Materials: * Institute for Reference Materials and Measurements (IRMM), Belgium; ** American Oil Chemistry Society (AOCS), USA.*

Conducting a competitive IEA using reference samples. Samples for the study were weighed out by 100 mg and 1000 mcl of distilled water was added. The certified reference material with different content of GMO (0.1; 0.5; 1.0; 2.0; 5.0; 10 and 100%) was analyzed to determine the sensitivity parameters of the method. The reference standards of GM-soya, A2704-12 line (Liberty Link) were used for the research of cross-reactivity. All the samples were analyzed triply to determine the within-assay reproducibility of the developed method. The samples were mixed thoroughly and incubated at room temperature for 5-10 minutes. After the incubation, the aqueous extracts were centrifuged at 3000 rpm for 1 min. The supernatant was used for research in the competitive version of the IEA.

The CP4 EPSPS recombinant anti-gene was sorbed in 96-well polystyrene plates.

50 mcl of aqueous extracts of the study samples were added to the wells of the sensitized plate, than 50 mcl of the synthesized conjugate (mAb3-HRP) with a predefined titer (1: 400) were added. The plate was incubated in a thermostat for 60 minutes at 37^0C. After the incubation, the wells were four times washed with a solution with an automatic plate washer. Conjugate control (CC) was used to determine the background signal of the IEA, only the solution of the conjugate was introduced into the wells, without adding the study samples.

The chromogene solution was injected in 100 mcl per well and incubated for 30 minutes at room temperature in a dark place.

The colour enzymatic reaction was stopped by adding 50 mcl of stop reagent (2 M sulphuric acid) to the wells of the plate. The reaction was recorded using an automatic spectrophotometer in two-wave mode at 495 and 620 nm.

The indicator “the percentage of inhibition” (PI) calculated by the formula was used for the results interpretation of a competitive IEA:

$$PI = \frac{OD(CC) - OD(Sample)}{OD(CC)} \cdot 100\%,$$

where the OD(CC) – optical density of conjugate control.

Statistical data was processed with the Microsoft Excel program. The difference between the indicators and the control (blank) was estimated by the standard method of variation statistics and considered reliable at $P < 0.05$.

Results and discussion

IEA is a highly sensitive research method that make possible to detect the analyte-antigens at the subnanomolar level, while the key component in any variant of the IEA is the usage of specific antibodies and antigens. Therefore, the first step of the IE diagnostic kit for the detection of glyphosate-resistance GM plants development was to obtain a specific antigen-analogue of the CP4 EPSPS enzyme. To do this, the gene was extracted and cloned and a recombinant analogue of the CP4 EPSPS enzyme was obtained. Fig. 1 shows a general scheme for plasmid encoding a recombinant CP4 EPSPS construction. The gene of CP4 EPSPS enzyme (1200 base pairs in size) was amplified by a polymerase chain reaction and cloned into a vector for pET-24-a bacterial expression.

The obtained genetic construct of pET-SR4EPSPS (6510 base pairs in size) was used to transform E. *coli* of BL-21 (DE3) strain. Electrophoretic analysis of of recombinant E. *coli* clones lysate after the intact PTH induction has revealed that the target protein locates in the inclusion bodies. The latter was washed with 2 M urea solution and then dissolved in 8 M urea. Thus, a fairly pure preparation of recombinant CP4 EPSPS was obtained. The actual size of the recombinant CP4 EPSPS, according to electrophoresis data, corresponded to 45 kD in 12% of SDS-PAGE, which is resilient to the theoretically derived data (Fig. 1, D).

Determination of the nucleotide sequence of a specific fragment of the pET-CP4EPSPS genetic construct and alignment of the amino acid sequences of the pET-CP4EPSPS recombinant enzyme gene and the Roundup Ready GM soybeans, line 40-3-2 (GenBank No. AB209952) transgene (Fig. 2), make it possible to suggest that a full-fledged SR4EPSPS enzyme, outside of the first N-terminal 40 amino acid residues, which are the *Petunia hybrida* chloroplast-transit peptide, was obtained. In addition, the CP4EPSPS recombinant enzyme contains a C-terminus tag of six amino acid residues of histidine, to purify the protein from bacterial lysate by Immobilized Metal Affinity Chromatography.

CP4 EPSPS mAb3 monoclonal antibodies were used to conjugate synthesize with horseradish peroxidase and construct a competitive variant of the IEA. In Fig. 3 is an IEA scheme in a competitive variant: the wells of the polystyrene plate are sensitized with the CP4 EPSPS recombinant enzyme (gray triangles). When inserting the test samples into the plate wells, the CP4 EPSPS transgenic enzyme expressed by GM plants (green triangles) competes with the recombinant analogue for the binding sites with the specific conjugate. Without the CP4 EPSPS transgenic enzyme in plant extract (Fig. 3, A), the conjugate interacts with the recombinant analogue on the plate and the enzymatic activity of the horseradish peroxidase can be detected by the color reaction at the final stage of the assay. Otherwise, if there is CP4 EPSPS transgenic enzyme in the plant extract (so the plant contains the GMO attributes), the conjugate interacts with the transgenic enzyme and is partially washed out of the plate wells during washing, in this case there is no color reaction (Fig. 3, B).

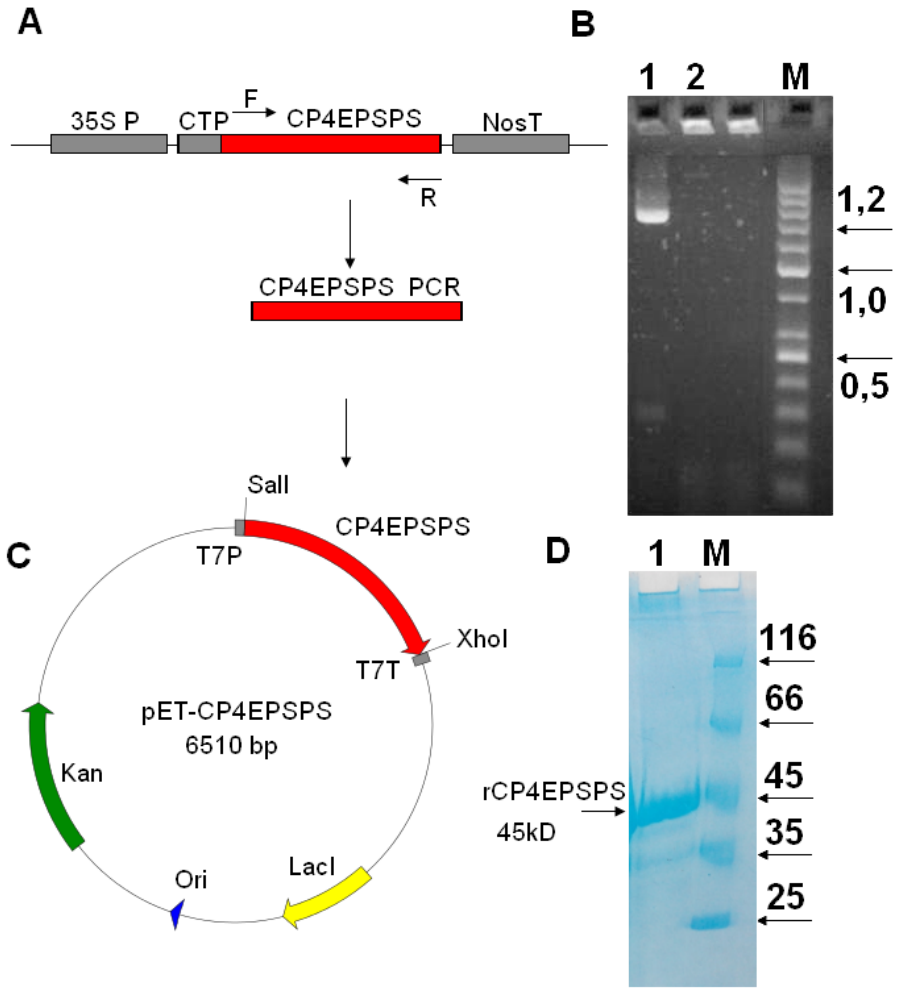

Fig.1. Strategy for obtaining the CP4 EPSPS recombinant transgenic enzyme. A - scheme of transgenic cassette in the GM soya genome (RR 40-3-2). 35S P and NosT, are the promoter and transcription terminator of CP4EPSPS gene, respectively, CTP - chloroplast transit peptide, F and R - annealing positions for the PCR primers; B - electrophoregram of PCR product separation: 1 - GM-soybeans (RR 40-3-2), 2 – negative soybeans, M - marker of DNA fragments' length , the product separated in 1% agarose gel; C - genetic map of the pET-CP4EPSPS plasmid, D - electrophoregram of separation of recombinant CP4EPSPS in 12% of SDS-PAGE.

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                1        10        20        30        40        50        60        70        80        90       100       110       120
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
rCP4                                                    MASMTGGQQMGRGSEFGEDVINTGKAMQAMGARIRKEGDTWIIDGVGNGGLLAPEAPLDFGNAATGCRLTMGLVGVYDFDSTFI
nCP4            MSHGASSRPATARKSSGLSGTVRIPGDKSISHRSFMFGGLASGETRITGLLEGEDVINTGKAMQAMGARIRKEGDTWIIDGVGNGGLLAPEAPLDFGNAATGCRLTMGLVGVYDFDSTFI

              121       130       140       150       160       170       180       190       200       210       220       230       240
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
rCP4            GDASLTKRPMGRVLNPLREMGVQVKSEDGDRLPVTLRGPKTPTPITYRVPMASAQVKSAVLLAGLNTPGITTVIEPIMTRDHTEKMLQGFGANLTVETDADGVRTIRLEGRGKLTGQVID
nCP4            GDASLTKRPMGRVLNPLREMGVQVKSEDGDRLPVTLRGPKTPTPITYRVPMASAQVKSAVLLAGLNTPGITTVIEPIMTRDHTEKMLQGFGANLTVETDADGVRTIRLEGRGKLTGQVID

              241       250       260       270       280       290       300       310       320       330       340       350       360
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
rCP4            VPGDPSSTAFPLVAALLVPGSDVTILNVLMNPTRTGLILTLQEMGADIEVINPRLAGGEDVADLRVRSSTLKGVTVPEDRAPSMIDEYPILAVAAAFAEGATVMNGLEELRVKESDRLSA
nCP4            VPGDPSSTAFPLVAALLVPGSDVTILNVLMNPTRTGLILTLQEMGADIEVINPRLAGGEDVADLRVRSSTLKGVTVPEDRAPSMIDEYPILAVAAAFAEGATVMNGLEELRVKESDRLSA

              361       370       380       390       400       410       420       430       440       450       460462
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+-|
rCP4            VANGLKLNGVDCDEGETSLVVRGRPDGKGLGNASGAAVATHLDHRIAMSFLVMGLVSENPVTVDDATMIATSFPEFMDLMAGLGAKIELSDTKALEHHHHHH
nCP4            VANGLKLNGVDCDEGETSLVVRGRPDGKGLGNASGAAVATHLDHRIAMSFLVMGLVSENPVTVDDATMIATSFPEFMDLMAGLGAKIELSDTKAA
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Fig. 2. Alignment of the amino acid sequences of the recombinant (rCP4) and native (nCP4) CP4 EPSPS transgenic enzyme (http://multalin.toulouse.inra.fr/multalin/).

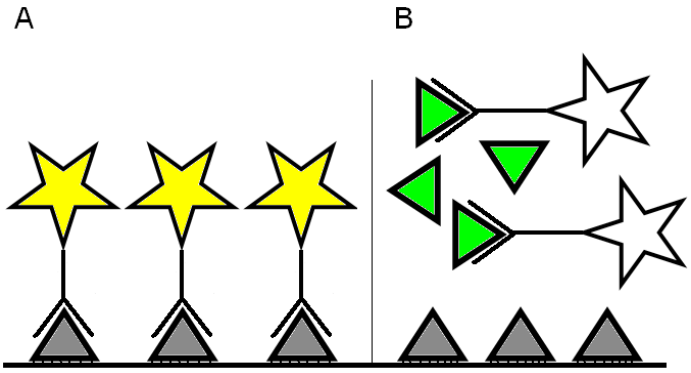

Fig. 3. IEA principle in a competitive variant (the scheme). A - in the absence of the analyte under study; B - in the presence of the analyte under study (see explanation in the text).

A certain titer of conjugate is the critical parameter in the development of a competitive IEA; it is determined experimentally using positive and negative samples. By titration, a dilution of the conjugate with the maximum optical density (OD) in the study of the negative sample, and with the minimum OD in the study of the positive sample, is used in the IEA. It is also required to collate a competitive concentration of CP4 EPSPS recombinant enzyme while the sensitization of polystyrene plates.

Fig. 4 shows the curves of reciprocal titration of monoclonal antibodies mAb3 conjugate with horseradish peroxidase in the research of certified reference samples with 0% content of GM soybeans (BF410a blank) and 1.0% of GM Roundup Ready 40-3-2 (ERM-BF410 dk) soybeans. Experimentally, the optimal concentration of antigen on the plate (3 ug/ml) and the conjugate titer (1/400) were found.

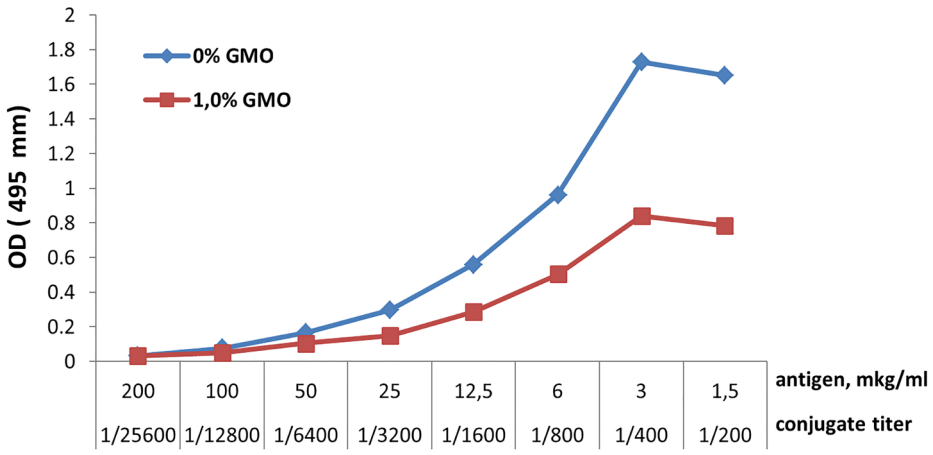

Fig. 4. Curves of reciprocal conjugate titration and antigen concentration on a plate in an IEA competitive variant with 1.0% positive standard of GM soybeans (RoundUp Ready, 40-3-2) and negative standard soybeans (0% GMO blank).

The main IEA parameters to confidently distinguish 1.0% positive GM-soybeans from the negative one were determined. OD of the negative standard (1.728) at least twice as many the value of OD in the study of a 1.0% positive standard (0.840).

Titration of the parameters of the IEA competitive variant with 1.0% positive GMO standard was chosen not coincidentally; according to the 1829/2003 EU Directive (Article 12, paragraph 2) the GMO content should not exceed 0.9% in negative samples, which is a certain limit below which the GMO is pointless, as it is considered accidental or technically inevitable [12].

Fig. 5 shows a diagram of quantitative assay of GM soya as percentage of inhibition (PI) using the developed test system which applies certified standards to a wide concentration range (from 0.1-100%). It was shown that for the quantitative assay of GMO content more than 1.0% the limit is 77.27 ± 1.29 PI.

Besides, in the study of 0.1% reference standard the minimum GMO detection limit was 68.27 ± 2.64 PI, so it is possible to discriminate this standard sample from the negative sample (blank) with confidence - 57.26 ± 4.29 PI ($P < 0.05$). The linear range (from 0.5% to 10%, $R^2 = 0.9$) was also determined for the quantitative assay of GM soybeans content using the developed method (Fig. 6).

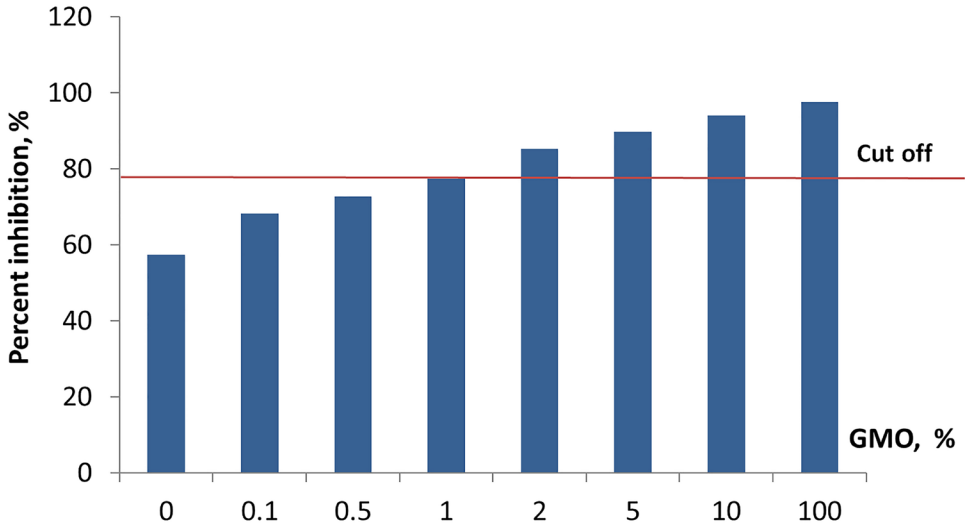

Fig. 5. Results of the study of various concentrations of GM soybeans in the competitive variant of the IEA ($P < 0.05$).

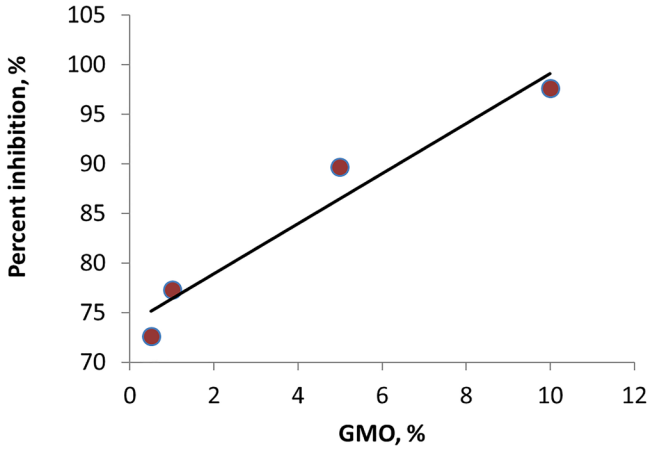

Fig. 6. Calibration plot of the dependence of GM soybeans content from the PI in the competitive variant of the IEA ($y = 2.4431x + 72.6$; $R^2 = 0.8843$).

Upon that, the difference of the obtained data does not exceed 10% in 3 iterations, which indicates the reproducibility of the developed method (Table 2).

The studies found that there is no cross-reaction with GM phosphinothricin-resistant soya samples of A2704-12 line (Liberty Link) (60.89±5.56 PI). In addition, the detection of transgenic soybeans (GenuityRoundup Ready 2 Yield (Monsanto)), which represents the second generation of glyphosate-resistant soybeans, is also possible due to the developed method. The capacity to identify the other types of glyphosate-resistant GM plants such as sugar beet (H7-1 line) and rape (GT73 line), with the expression of the CP4 EPSPS gene, which also was developed by Monsanto, was demonstrated (Table 2). Although, in the study of rape samples, the difference between 100% GM rape (GT73) and rape of Ukrainian selection (Bahira breed) was just 14 DI.

According to our reckoning, this owes to the low efficiency of protein hydroextraction for rich in oil rape seeds. However, the inability to identify the GM corn of NK603 line (Monsanto) which expresses the CP4 EPSPS enzyme is ambiguous. In our opinion, this can be explained by the need to collate the parameters of water-soluble proteins extraction in preparation of corn and rape samples for the assay.

Thus, the CP4 EPSPS recombinant enzyme analogue was obtained and the quantitative IEA protocol with CP4 EPSPS mAb3 monoclonal antibodies to detect the glyphosate-resistant GM soybeans (Roundup Ready, 40-3-2) with a minimum limit of 0.1% , as well as other types of GM glyphosate-resistant plants such as rape (GT73 line) and sugar beet (H7-1 line), was developed.

According the developed method, a sample of soya is considered genetically modified (with more than 1.0% of GMO content) if the DI value is higher than 77.27±1.29%.

It should be noted that the rapidity of the research is the bulge of the developed method of competitive IEA on the real-time PCR. For the study of 96 samples by the IEA method the timing is 1.5-2 hours minimum, versus to at least 8-12 hours for the study of 96 samples by the PCR. The reason is the fewer

operating procedures, since the competitive IEA consists of only two procedures: sample preparation and assay, while the real-time PCR has three stages: sample preparation, genomic DNA purification and assay.

More affordable and common laboratory equipment available in the most of Ukrainian laboratories is needed for the IEA, which is a substantial margin of the developed method over the PCR.

Table 2. Results of quantitative assay of GM plants according to the competitive IEA method.

Plants	Template	Strain/breed	ABI±SD*	Interpretation, % GMO
Soybeans	ERM-BF410a	Roundup Ready (Blank)	57.26±4.29	Negative
	ERM-BF410bk	Roundup Ready	68.27±2.64	Positive 0.1
	ERM-BF410c	Roundup Ready	70.49±1.27	Positive 0.5
	ERM-BF410dk	Roundup Ready	77.27±1.29	Positive 1.0
	ERM-BF410e	Roundup Ready	85.40±1.99	Positive 2.0
	ERM-BF410f	Roundup Ready	90.07±0.73	Positive 5.0
	ERM-BF410gk	Roundup Ready	94.18±0.04	Positive 10.0
	AOCS 0906-B	GenuityRoundup Ready 2 Yield	96.76±0.25	Positive 100
	Seeds	Liberty Link	60.89±5.56	Negative
	Seeds	«Cheremosh»	57.27±0.71	Negative
Corn	ERM-BF414f	GA21	18.5±6.45	Negative
	ERM-BF415f	NK603	23.16±9.37	Negative
	Seeds	«Khmelnytsky»	29.25 ±8.03	Negative
Sugar beet	ERM-BF419b	H7-1	96.45±0.50	Positive 100
	Seeds	«Oleksandriya»	14.92±5.80	Negative
Rape	AOCS 0304-B	GT73	84.14±1.24	Positive 100
	Seed	«Bahira»	70.64±2.68	Negative

*Notes: * - average PI ± standard deviation*

Considering this, the developed method is provided as a primary tool for the screening of glyphosate-resistant GM soya (Roundup Ready 40-3-2) in Ukraine to help domestic producers of GMO free organic products establish a control system and take the lead in this cluster.

It is planned to continue the research on parameters determination for water-soluble proteins extraction during the corn and rape samples preparation, as well as transferring the competitive variant of the IEA into rapid tests with the immunochromatographic assay technology and the possibility of field assay.

The authors express their gratitude to S.V. Khyzhnyak, Dr. Sci. (Biology) and S.V. Mikhalap, Cand. Sc. (Biology) for critical editing of the manuscript.

The work was carried out within the framework of the 110/31l-pr research “Development of immunoenzymatic diagnosticums for the detection and identification of herbicide-resistant genetically modified agricultural plants”, state registration #00493706 #0113U00382.

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