Enzyme-Linked Immunosorbent Assay for the Determination of Isoflavones in Alimentary Important Plants

M. VÍTKOVÁ[1]*, Z. MACKOVÁ[2], R. KOBLOVSKÁ[2] and O. LAPČÍK[2]

[1]Department of Biochemistry and Microbiology and [2]Department of Chemistry of Natural Substances, *E-mail: vitkovam@vscht.cz, Institute of Chemical Technology, Prague, Czech Republic

Abstract: The development of polyclonal antibody-based enzyme-linked immunosorbent assays (ELISA) for the determination of individual isoflavones, i.e. daidzein, genistein and biochanin and their homologues, is presented in this work. Isoflavone conjugates with bovine serum albumin were used as immunogens, coupled at the position C 7 and C 4′ via a carboxy methyl spacer. The developed ELISAs are highly specific, I_{50} values of the standard curves range between 0.3–1.2 ng/ml. The cross reactivities to other isoflavones are in acceptable range and the interference of non-isoflavonoid molecules is negligible. The immunoassays have been used for monitoring of changes in isoflavone levels in alimentary important plants, such as *Medicago sativa*, during germination; and during different vegetation stages of the *Rutaceae* family plants.

Keywords: ELISA; isoflavone; daidzein; genistein; biochanin A; *Ruta*; *Citrus*

INTRODUCTION

Isoflavonoids are a varied group of polycyclic compounds and plant secondary metabolites that occur widely in legumes, however they were found also in certain non-leguminous taxa. Their biosynthesis was described in over 20 families e.g. *Rosaceae, Poaceae, Asteraceae, Iridaceae*, etc. The important sources of isoflavones for human are soybeans, soy products, and other leguminous plants. Recently, the isoflavone concentrates of soybean and clover origin have been introduced to the market as food additives.

Isoflavones are known to interact with estrogen receptors and to influence steroid metabolizing enzymes and several other signaling pathways playing a role in the cell growth and differentiation [1]. Epidemiological studies have connected the consumption of isoflavone rich diet with lower incidence of cardiovascular diseases and of several types of cancer [2].

Here we present the development of five indirect competitive ELISA methods for the determination of individual isoflavones. The ELISA methodology has substantial advantages when compared with commonly used chromatographic techniques [3], particularly: it is rapid, sensitive, technically simple, it does not require special cleaning steps and enables to analyze a great number of samples in a short time.

EXPERIMENTAL

Immunoreagents. Daidzein, genistein and biochanin A conjugates with bovine serum albumin were used as immunogens, coupled at the position C 7 and C 4′ *via* a carboxy methyl spacer. Synthesis and characterization of conjugates were performed as described in our previous paper [4]. Rabbit polyclonal antibodies against daidzein-4′-BSA, daidzein-7-BSA, genistein-4′-BSA, genistein-7-BSA and biochanin A-7-BSA were obtained.

Indirect competitive ELISA procedure. The polystyrene microplates were filled with BSA-isoflavone conjugates diluted in carbonate-bicarbonate buffer, pH 9.6 (0.1 ml/well) and incubated overnight at

laboratory temperature for absorption. The coated plates were washed four times with 0.01M PBS containing 0.05% Tween 20 (PBS-Tw) to remove unbound antigens. Aliquots (50 µl) of the antigen solution in PBS-Tw (isoflavone standard solution in the range of 80 ng/ml – 20 pg/ml, or diluted samples) and 50 µl of antibody solution in the PBS/Tw with 0.1% BSA were added to the wells. After incubation for 1.5 h at laboratory temperature and washing four times with PBS-Tw, the conjugate of "second antibody" (swine anti-rabbit IgG) with horseradish peroxidase diluted 1:2000 in PBS-Tw was added (100 µl). Plates were incubated for 1.5 h at laboratory temperature and washed four times with PBS-Tw. 100 µl of solution of peroxidase substrate and chromogenic compound (50 mg of *o*-phenylenediamine in 100 ml of 0.1M citrate/phosphate buffer pH 5.0 containing 0.03% hydrogen peroxide) were added into each well. After incubation at laboratory temperature for 15 min the enzyme reaction was stopped by addition of 50 µl of 2M H_2SO_4, and absorbance was measured at 492 nm by a Labsystem Multiscan MCC/340. The absorbance was inversely proportional to the amount of antigen in solution.

Alfalfa seedlings. Alfalfa (*Medicago sativa*) seeds were purchased from the "Country Life" food store, which specializes in healthy nutrition products. Seeds were germinated 8 days according to the recommendations of the producer, using tap water for their wetting twice daily. Samples were taken every day and kept deep frozen until lyophilized. The dry material was disintegrated in a grinder and extracted 4 h with 80% ethanol. The extracts were filtered and adjusted on final volume 25 ml of extract per 1.0 g dry matter. Before the analysis, the extracts were diluted 100 times with the assay buffer.

Samples of the Rutaceae family plants. Tested plant material (leaves and flowering tops) was collected from plants growing in a subtropical greenhouse of the Czech University of Agriculture in Prague. Samples were frozen immediately after collecting and kept at –20°C until lyophilization. Dry samples were milled in the electric mill Fex IKA A11. Powdered material was extracted by maceration in 80% ethanol (20 ml/g, dry weight) and these crude extracts were analyzed by immunoassay or fractionated using HPLC and than analyzed.

RESULTS AND DISCUSSION

The indirect competitive format of ELISA for biochanin, daidzein and genistein was optimized using polyconal antibodies formed against the isoflavone conjugate with bovine serum albumin.

The parameters of calibration curves obtained for individual isoflavones are listed in Table 1. The detection limits ranged from 22 pg/ml (the daidzein assay) to 105 pg /ml (the biochanin assay) and I_{50} were between 0.3–1.2 ng/ml. The methods are very sensitive and enable the determination of the isoflavones in the pg level. These values are comparable with previously reported immunoassays and in some cases are even better [5].

A panel of isoflavonoids, flavonoids and lignans was used for testing specificity of individual ELISA methods, and the signal was compared to that of the particular analyte. The cross-reactivity was expressed as the ratio of fifty percent intercept of the analyte to fifty percent intercept of a cross-reactant for all substances where this parameter did not exceed 10 ng per tube. Due to the fact, that the position used for construction of the immunogen is immunochemically indiferent, each of the presented methods is specific for a narrow group of isoflavones that are homologous to the particular hapten. The cross reactivities to other isoflavones are in acceptable range and the interference of non-isoflavonoid molecules is negligible.

The biochanin A ELISA was used for the analysis of real samples of alfalfa seedlings during germina-

Table 1. Parametres of calibration curves

Antibody against	Biochanin-7-BSA	Daidzein-4′-BSA	Daidzein-7-BSA	Genistein-4′-BSA	Genistein-7-BSA
Analyte	biochanin	daidzein	daidzein	genistein	genistein
I(50) (pg/ml)	1212.3 ± 135.8	302.4 ± 57.2	916.3 ± 51.0	315.9 ± 55.8	667.4 ± 114.8
Detection limit (pg/ml)	105.1 ± 23.7	22.0 ± 8.5	102.9 ± 17.3	58.1 ± 14.4	30.3 ± 5.5
Working range of calibration curve (ng/ml)	80 – 0.1	5 – 0.02	20 – 0. 08	5 – 0.02	80 – 0. 02
Dilution of Ab	1:32 000	1:40 000	1:20 000	1: 60 000	1:20 000

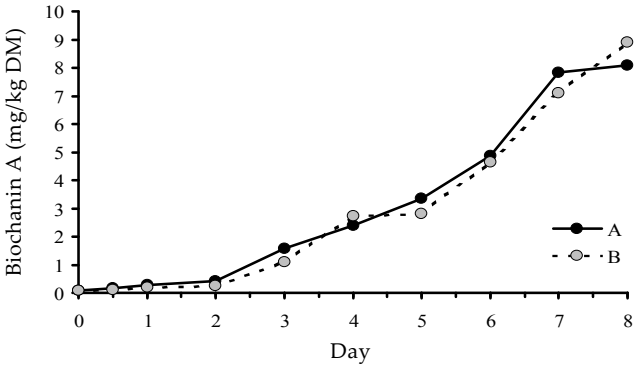

Figure 1. Biochanin A immunoreactivity in alfalfa seedlings incubated under a glass lid (A) or a non-transparent lid (B)

tion under light and dark. Biochanin A content in alfalfa sprouts increased during the germination from 0.081 mg/kg at the day 0 up to 8.91 mg/kg (dry weight) at the day 8 (Figure 1). No significant differences were found between the light and dark germination.

In the further work, all five ELISAs were employed for screening of isoflavones in the *Rutaceae* family. Combining the ELISA methods with HPLC pre-separation of the samples we were able to identify many known isoflavones in the *Rutaceae*, namely: glycosides-daidzin, genistin, sissotrin and aglycones-daidzein, genistein, formononetin, biochanin A (Figure 2). All *Ruta* and *Citrus* species contained the above mentioned isoflavonoids in ng/g – μg/g levels (dry weight), aglycones as well as glycosides. Both *Ruta* species were relatively rich in sissotrin while *Citrus* species contained lower levels of this compound. 4′-Methoxyisoflavones (formononetin, biochanin A) prevailed to 4′-hydroxyisoflavones (daidzein, genistein).

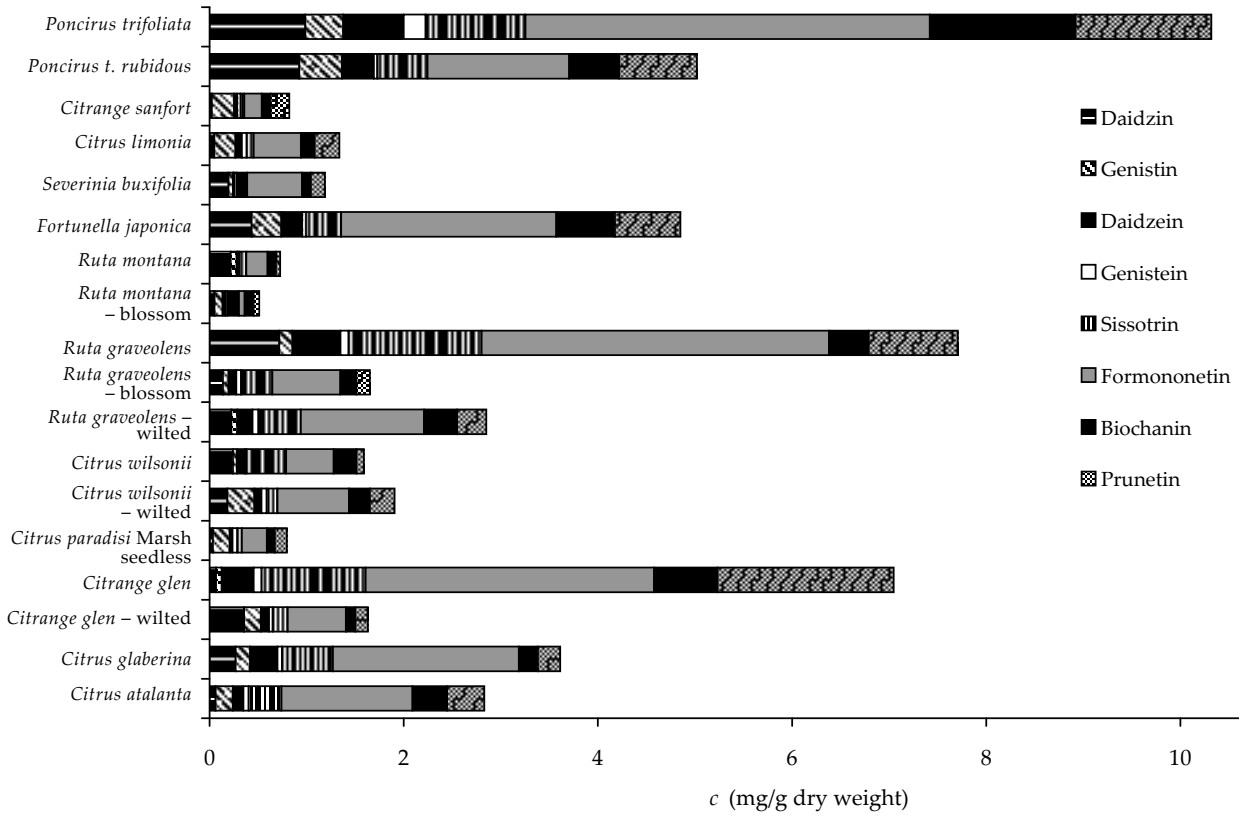

Figure 2. Isoflavone levels in selected plants of *Ruta* and *Citrus* genera

Table 2. Crossreactivity (%) of individual antibodies

Analyte	Antibody against				
	biochanin-7-BSA	daidzein-4′-BSA	daidzein-7-BSA	genistein-4′-BSA	genistein-7-BSA
Biochanin	100	0.48	<0.01	178.20	0.41
Sissotrin	120.00	0.02	<0.01	0.13	4.80
Daidzein	< 0.01	100	100	14.70	9.30
3′-OH-Daidzein	< 0.01	3.52	3.55	0.06	2.70
Daidzin	< 0.01	0.03	42.10	0.05	1.23
Genistein	2.80	0.57	8.27	100	100
5-Methoxygenistein	< 0.01	0.03	5.20	0.15	1.35
7,4′-Dimethoxygenistein	46.50	< 0.01	< 0.01	0.01	0.22
Genistin	< 0.01	0.21	53.20	0.03	32.00
Formononetin	0.3	192.19	5.60	6.54	< 0.01
Isoformononetin	< 0.01	0.16	342.50	0.01	42.16
Prunetin	5.00	0.02	33.85	2.42	215.00

CONCLUSION

We developed the sensitive and rapid indirect ELISA methods for the most important isoflavones. The developed ELISAs are highly sensitive and allows us to analyze isoflavones in wide range of concentrations and different matrices.

The immunoassays have been used for screening of isoflavones levels in the *Rutaceae* family and in *Medicago sativa* sprouts. We propose further use of these ELISA methods for studying isoflavonoid distribution and metabolism in different materials and biological models, e.g. food samples, body fluids, cell culture media and other biological material.

Acknowledgement: This work was supported by the Grant GA CR 525/03/0352.

References

[1] Adlercreutz H. (1997): Reproductive and Developmental Toxicology. Marcel Dekker Inc., New York.

[2] Yamamoto S., Sobue T., Kobayashi M., Sasaki S., Tsugane J. (2003): J. Natl. Cancer I, **95**: 906.

[3] Wu Q.L., Wang M.F., Simon J.E. (2003): J. Chromatogr. A, **1016**: 195.

[4] Lapčík O., Hampl R., Hill M., Wähälä K., Nawaf A.M., Adlercreutz H.J. (1998): Steroid Molec. Biol., **64** : 261.

[5] Bennetau-Pelissero C., Le Houerou C., Lamothe V., Le Menn F., Babin P., Bennetau B. (2000): J. Agric. Food Chem., **48**: 305.