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Research article

The determination of potentially allergenicity of selected herbs

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Abstract: *The aim of the research was to compare the content of allergens in herbs from the Lamiaceae (basil, oregano) and Apiaceae (cumin, fennel, parsley, anise, coriander) family. Herbal plants from conventional and organic crops were subjected to research. In the extracts of herbs, the content of protein as well as the content of Bet v I analogs and profilin were determined using the immunoenzymatic indirect method.*

Protein content in conventional crops determined by the Bradford method ranges between 160-204 mg/g, and Pierce determined between 105-394 mg/g. In samples of organic herbs the results are as follows: Bradford method 149-196 mg/g, and Pierce method 109-333 mg/g.

In the case of plants grown using conventional methods, the content of Bet v I analogues, based on a commercial test, ranged from 0.5 to 1.15 μg/g in method I, whereas in the method developed by us from 0.22 to 0.68 μg/g. In herbs from organic farming, the range of results according to the commercial test is 0.86-1.54 mg/g, and use by the test we developed 0.5-0.63 mg/g. The results of profilin content were as follows: in samples grown with conventional methods, they ranged from 1.00 to 18.13 ng/g, while organics - from 3.27 to 12.62 ng/g. The calculated p-value is less than the assumed $\alpha = 0.05$, – this result is statistically significant.

The correlation between the results of the method I and II in both crops is strongly statistically significant.

Keywords: *profilins, Bet v I, Bet v II, Elisa tests, Total Extractable Protein, Bradford, Pierce.*

Introduction

The reason for pollen-food syndromes underlying cross-reactions between inhalation and food allergens is the similarity of the epitopes of sensitizing molecules [1]. This term means the occurrence of hypersensitivity symptoms after consumption of specific types of fruit and vegetables in people previously sensitized to plant pollen. These symptoms are mainly caused by two groups of allergens: proteins of the PR-10

group (pathogenesis-related proteins), whose most important representative is birch pollen Bet v1 and profilins [2].

The profilins are 12-16 kDa, actin-binding proteins (monomeric form), found in all eukaryotic cells and some viruses, with the exception of some protists. Profiles promote the polymerization of actinium fibrils and monomers, and are therefore involved in the production of the cytoskeleton and movement [3].

The variety of 50 identified profilins suggests a significant role in many more complex molecular processes as well as in signal transduction [4, 5].

Profiles are widely distributed, present in all eukaryotic cells. The occurrence of their homologous varieties in many foods and pollen is the reason for the existence of such clinical syndromes as: birch pollen – celery – spices, pollen flea – celery – spices, pollen tree – hazelnut syndrome or grass pollen – celery – carrot syndrome.

In the case of PR-10 proteins, epitopes with a homologous structure to Bet v I of the main allergenic birch pollen epitope are characteristic. Cross-reactions are possible not only between pollen from various taxonomic beech trees, but also with numerous vegetables and fruits, such as apple or cherry. This is related to the occurrence of local symptoms such as an allergy syndrome of the mouth after ingesting some of the above-mentioned fruit or vegetables (carrots, celery or soybeans) and hazelnuts [2, 6].

Experimental

Materials

In this work herbal plants from conventional as well as organic cultivations were subjected to research. The analysis of herbs from the *Lamiaceae* family (basil, oregano) and *Apiaceae* (cumin, fennel, parsley, anise, coriander) was analyzed. In the plant samples, the content of Bet v I and profilin analogs was determined indirectly using the immunoenzymatic method.

Methods

Chemicals and reagents

1. Total protein extraction.

Reagents:

- lysis buffer SN-009, Total Protein Extraction Kit For Plant Tissues, Invent Biotechnologies, INC.

Protein determination:

2. Bradford's method.

Reagents:

- 1 mg/ml solution of BSA bovine serum albumin,
- Bradford reagent, Chempur Company,

3. Pierce's method.

Reagents:

- Pierce™ BCA Protein Assay Kit (Thermo Fisher, 23225),
- Tris-glycinebuffer, pH 8.3 (0.05 M Tris; 0.33 M glycine),
- Bovine ScientificThermoScientific™ 23209,
- protein Assay Reagent„A”,
- protein Assay Reagent„B”,
- WR reagent (ang. *WR Working Reagent*) consisting of reagent A and reagent B mixed in 1:50 proportions,
- protease inhibitor (pROTEASE Coctail inhibitor, Sigma P9599).

Determination of allergens:

4. The commercial Bet v I Elisa 2.0 test

All extracts used for the determinations were diluted three times in carbonate buffer. Before the test, all the reagents from which the solutions below were taken were brought to room temperature.

Reagents:

Set an InBioo™ Product, INDOOR biotechnologies kit, Bet v I ELISA 2.0,

- wash buffer: 15 ml of the concentrate contained in the Elisa test was added to 135 ml of deionized H_2O,
- test buffer: adding 3 ml of concentrate to 9 ml of deionized H_2O,
- antibody/conjugate detection mixture: consisted of 10 µl biotinylated 2E10 antibodies and 10 µl of peroxidase labeled avidin, this mixture was added to 10 ml of assay buffer,

5. Intermediate Elisa test for the determination of Bet v I analogues.

Reagents:

- 3% solution of skimmed milk powder,
- PBS Tween 20 Wash Buffer,
- 3 molar NaOH solution,
- mouse antibodies against Bet v I from Dendritic, catalog number MKI67: 10 µl of antibodies were added to 10 ml of deionized H_2O,
- anti-mouse antibodies (phosphatase enzyme conjugate), polyclonal, produced in goat, Sigma-Aldrich®, catalog number A0168: 10 µl of antibodies were added to 10 ml of deionized H_2O,
- PNPP solution – p-nitrophenol phosphate – ready-made solution, Sigma Life Science.

6. Intermediate test Elisa for the determination of Bet v II analogues

The test was performed in the same way as in section 5, only other antibodies that detect profilins were used:

Dendritic Company mouse antibodies were changed to I rabbit antibodies against H CUSABIO Company profilins, while II anti-mouse antibodies from Sigma-Aldrich® to II Sigma-Aldrich® anti-rabbit antibodies.

Reagents:

- main Pollen allergen profilins-1 Bet v II from H CUSABIO Company, catalog number 0354B – standard in the determinations carried out using the developed procedure from which the standard curve was applied to the plate,
- rabbit antibodies against H CUSABIO profilins, catalog number P7624: 10 µl of antibodies were added to 10 ml of deionized H_2O,
- anti-rabbit antibodies (phosphatase conjugate), polyclonal, produced in goat, Sigma-Aldrich®, catalog number A3687: 10 µl of antibodies were added to 10 ml of deionized H_2O,
- PNPP solution – phosphate paranitrophenol – ready solution.

Results and Discussion

The formula used to calculate the protein content is as follows:

$$c = \frac{x \times f \times v}{m}$$

where:
x – the value determined from the standard curve,
f – dilution of the extract,
v – volume of the sample [ml],
m – sample weight [g].

A comparison of two methods for the determination of protein extracts used in the Bradford and Pierce methods.

Table 1. Comparison of protein determination methods

Methods	Curveequation	Coefficient of correlation R^2	Range of applicability [µg/ml]
Bradford'smeth od	Y = 0.0185x + 0.1678	0.9821	from 0.5 to 7.5
Pierce'smethod	Y = 0.0015x + 0.2144	0.9923	from 0 to1000

The protein content was determined using two methods receiving different results. Differences in the results obtained by the two methods, Bradford and Pierce (Table 2), result from the fact that other amino acids react with the reagents used for the protein determination reaction.

Table 2. Protein content [mg/g] in herbal samples

Sample	Conventional cultivation		Ecological cultivation	
Apiaceae	Bradford's method	Pierce's method	Bradford's method	Pierce's method
Cumin	204.73 ± 2.6	162.6 ± 7.3	150.57 ± 38.1	206.66 ± 13.7
Fennel	154.48 ± 0.5	204.1 ± 29.8	149.78 ± 16.1	333.67 ± 0.1
Parsley	162.57 ± 2.4	133.51 ± 15.6	149.44 ± 0.2	109.65 ± 1.9
Anise	191.45 ± 1.9	210.33 ± 9.6	196.67 ± 6.3	197.98 ± 5.4
Coriander	160.53 ± 16.0	105.64 ± 4.2	175.22 ± 3.0	121.36 ± 13.3
Lamiaceae				
Basil	157.36 ± 0.6	371.41 ± 16.0	–	–
Oregano	158.25 ± 0.6	394.09 ± 16.0	–	–

The Bradford method uses the Coomassie Brilliant Blue G-250 staining (CBB-G250) with amino groups of proteins by means of ionic and hydrophobic bonds. The Arg residues mainly react with the dye; in a minimal degree His, Lys, Tyr, Trp and Phe residues. CBB in acidic environment has a brown color, which after reaction with the protein turns into blue. This involves changing the maximum absorption from 465 to 595 nm. The intensity of the color is proportional to the protein content in the solution. In the Bradford method, peptides below 30 KDa do not undergo any reaction.

In the Pierce method, however, protein determination takes place in two stages. In the first stage, copper ions of Cu^{2+} to Cu^{+} are reduced through the protein resulting in a light blue protein-copper complex, whereas in the second the resulting Cu^{+} ion reacts the Bicinchoninic Acid (BCA) as a result of chelating 2 molecules of BCA with 1 Cu^{+} ion forms a purple complex whose color is proportional to the protein concentration.

In the Bradford method (Table 2) the results of conventional cultivation protein determination range between 160-204 mg/g, and in Pierce 105-394 mg/g. In samples of organic herbs, the results are as follows: in the Bradford method 149-196, and in the Pierce method 109-333, however, experience has shown that the determination of protein content depends on the method used.

At the same time, no statistically significant differences were found in protein content in organic and conventional samples.

Commercial test Bet v I Elisa 2.0 - Method I and the test modified to the analogues Bet v I - Method II

Table 3. Comparison of Bet v I and Bet v II determination method

Method	Curve equation	Coefficient of correlation R^2	Range of applicability [ng/ml]
Method for the determination of Bet v I analogues	y = 0.0242x - 0.0697	0.8758	from 0.5 to 50
Method of determination profilins (our method)	y=0.0017x + 0.7415	0.9942	from 5 to 100

Table 4 summarizes the results of the Bet v I analogues in herbal samples.

In the case of plants grown using conventional methods, the content of Bet v I analogues on the basis of a commercial test ranged from 0.5 to 1.15 μg/g in method I, while in method II from 0.22 to 0.68 μg/g. In herbs from organic farming, the scope of method I is 0.86-1.54 μg/g, and methods II 0.5-0.63 μg/g. Higher values were obtained based on a commercial test.

It turns out that the content of analogues is slightly higher for herbs from organic farming.

Table number 5 indicates the correlations between methods I and II in both types of crops. All results are given in [μg/g].

Table 4. Content of Bet v 1 analogues in herbal samples [μg/g]

Sample		Conventional cultivation	Ecological cultivation	
Apiaceae	Metod I	Metod II	Metod I	Metod II
Cumin	0.80 ± 0.1	0.46 ± 0.06	1.54 ± 0.2	0.52 ± 0.08
Fennel	1.07 ± 0.2	0.68 ± 0.3	1.4 ± 0.3	0.5 ± 0.09
Parsley	1.13 ± 0.03	0.6 ± 0.3	0.98 ± 0.3	0.63 ± 0.04
Anise	1.15 ± 0.3	0.45 ± 0.07	1.15 ± 0.1	0.55 ± 0.03
Coriander	0.95 ± 0.08	0.42 ± 0.04	0.86 ± 0.04	0.6 ± 0,1
Lamiaceae				
Basil	0.63 ±0.3	0.42 ± 0.1	-	-
Oregano	0.5 ±0.05	0.22 ± 1.0	-	-

Based on the Table 5, it can be seen that the R^2 coefficient in the first and fourth columns shows a positive correlation of 0.3-1, which means that it is statistically significant and there is a strong correlation between them.

In method II the obtained results are lower, but they are proportional, and one can notice correlations in the range of 0-0.3 with the results obtained by method I, as indicated in the third column. This means that the correlation is moderate, but statistically significant.

Table 5. Comparison of correlation coefficients between methods and crops

Method	Comparison of method I in conventional and ecological cultivation	Comparison of method II in conventional and ecological cultivation	Comparison of methods I and II in conventional cultivation	Comparison of methods I and II in ecological cultivation
R^2	0.8085	0.3509	0.2224	0.3509

Intermediate test Elisa for the determination of profilin analogs

Table 6 shows the results of the profilin content determination.

The results of fruit profilin content were as follows: in samples grown using conventional methods, they ranged from 1.00 to 18.13 ng/g, while organics from 3.27 to 12.62 ng/g.

The content of these allergens is comparable in samples from organic and conventional crops.

Table 6. Profilins content in herb samples [ng/g]

Sample	Conventional cultivation	Ecological cultivation
Apiaceae		
Cumin	10.12 ± 4.0	9.9 ± 4.2
Fennel	4.7 ± 5.4	3.75 ± 3.9
Parsley	9.4 ± 1.5	3.27 ± 3.2
Anise	1.0 ± 0.8	3.42 ± 4.2
Coriander	18.13 ± 1.3	12.36 ± 3.1
Lamiaceae		
Basil	12.62 ± 10.3	–
Oregano	3.76 ± 2.7	–

Statistical analysis and significance test for differences

Statistical calculations were also made for each result. Statistical analysis confirmed that the content of allergens does not depend on the method of cultivation of the tested plants.

The p-value values were also calculated, assuming that $p < 0.05$, where $\alpha = 0.05$.

P-value results:

p-value is the parameter of specific observations (statistically) in the above tests. The P-value allows you to directly assess the credibility of the hypothesis. The higher the p-value, the more hypothesis H0 is true. The small p-value testifies against the null hypothesis.

The protein content was compared in herbal and organic crop samples tested using the Bradford, Pierce method, Methods I and II, and the Elisa Intermediate Test for the determination of Bet v II analogs

Table 7. p-value results between different crops tested by two different methods

Conventional Cultivation Bradford and Pierce	Ecological Cultivation Bradford and Pierce
p-value = 0.00033526*	p-value = 0.00003099*
Conventional and Ecological Cultivation Bradford	Conventional and Ecological Cultivation Pierce
p-value = 0.01782801*	p-value = 0.01867236*
Conventional and Ecological Cultivation Method I	Conventional and Ecological Cultivation Method II
p-value = 0.0409605*	p-value = 0.0002791*
Conventional Cultivation Methods I and II	Ecological Cultivation Methods I and II
p-value = 0.00067921*	p-value = 0.00927747*
Conventional and Ecological Cultivation profilins	
p-value = 0.32321653**	

* *strongly statistically significant,*

** *moderately statistically significant.*

In all results it can be noted that the p-value value between conventional and organic crops is less than the assumed αwhich was assumed to be equal = 0.05. The interpretation of the above results is that they are statistically significant.

The only exception is the calculated value in Method II. The result is greater than the assumed p-value, which means too low statistical power.

Interpreting Table 7, it can also be seen that the results obtained by the method can be compared, not the results obtained between the methods, for example, results from Conventional and Organic Crops made using Method I, not the results obtained from the Ecological Crops markings between Method I and II

Learning about the research related to bet v II allergens, the only similar article to our work was the cantilever work Villalta [7] and his team team because they studied sera from 43 patients allergic to profilin using two tests. Each of them gave results very similar to ours in the range of 0 to 18 ISU/l (expressed as standardized ISAC units (ISU/L), they are indirectly related to the international reference formulation of

the World Health Organization for human IgE 75/502 serum) in our case, the results range from 0 to 18 ng/g. Unfortunately, publications on profilin, and research related to them, are few, which only confirms us in the good direction of laboratory tests and deepen knowledge about profilin.

Summary

In the extracts of various samples of herbal plants, the contents of the analogues Bet v I and profilin were determined.

The results obtained from the two methods, Bradford and Pierce are different. Differences arise from the essence of the method - other amino acids are involved in the reaction, Pierce's method is faster and simpler, which also increases reagent savings.

The results of the Bet v I analogue determination obtained by the commercial method are higher than the results obtained by the indirect Elisa test on Bet v I analogues, but proportionality is noticeable between these results, which is also confirmed by the correlation coefficients. A method for determining profilin in samples of herbal plants was developed.

The content of allergens in a sample of conventional and organic herbs is comparable, it does not differ statistically significantly.

The developed tests proved to be suitable for this type of determination. The obtained results fall within the range obtained by other authors [7].

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