Determination of pea proteins allergenicity with the use Balb/c mouse

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Abstract

One of important criteria in evaluating the allergenicity of proteins in human diet is the resistance of their epitopes to thermal denaturation and hydrolytic processes as affected by digestive enzymes. The effect of the cooking process on the immunogenic properties of pea proteins was determined experimentally on an animal model. Experimental Balb/c mice were fed diets consist protein originated from flour obtained from either raw or cooked pea that was added to diets at different doses. The control group was administered with casein. On termination of the experiment, the animals were weighed, anaesthetized and exsanguinated. The serum obtained was determined with the ELISA method for the level of IgA and IgG antibodies specific to pea proteins. Minimal differences (statistically insignificant) were demonstrated in the level of IgA between particular groups of mice. In contrast, significant differences occurred in the amount of specific IgG. The highest IgG titre was recorded in serum of mouse from the group fed a diet with the addition of flour from raw pea seeds. The immunoblotting assays confirmed those results. A specific reaction was observed to occur between antibodies and protein fractions with molecular mass of ca. 50 kD (vicilin subunits), 70 kD (convicilin) and ca. 60 kD (legumin proteins). Cooking the pea seeds did not influence on the loss of protein immunogenicity. In mice serum there were found specific antibodies primarily to high-molecular subunits of vicilin. This points to strong potential allergenic properties of pea vicilin.

Key words: *pea proteins, allergenicity, mice, ELISA*

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Introduction

The application of grain legume seeds in human nutrition and animal feeding has currently been observed to increase [1-4]. These results from the fact that those seeds contain valuable protein, dietary fibre and other nutrients [5, 6]. The chemical composition of grain legume seeds includes also compounds that may exert a negative impact on the utilization of nutrients or even on health, i.e. first of all protease inhibitors, tannins, alkaloids and lectins [7, 8]. In recent years, however, some of those compounds have been demonstrated to positively affect human body, e.g. protease inhibitors [9]. The latest reports point to the presence of proteins allergenic in character in legume seeds [10].

Soybeans and peanuts are the cause of most of food-borne allergic reactions in The United States, The United Kingdom or Japan, where their intake is high [11]. In contrast, the intake of chick-pea, lentil or pea is the highest in the Mediterranean countries as well as some Asian countries (India) [12, 13]. It is most likely the reason why in Spain 10% of patients suffering from food allergies manifest clinical symptoms after the consumption of lentil seeds [14]. Although the clinical cross-reactivity between proteins of grain legumes is believed to be relatively rare [15] (those investigations refer mainly to seeds of soybean and peanuts), over 70% of patients with allergy to pea, lentil or chick-pea react positively in open challenge tests to proteins of all those seeds [16]. In Poland, cases of allergies to proteins of grain legume seeds other than soybean or peanuts have rather been sporadic so far. Perhaps it results from their weak allergenic properties or the fact that they are not covered by diagnostic allergic tests.

Currently, food allergy has been enumerated as one of the most wide-spread civilization diseases. That problems

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affects nearly 1-2% of the adult population. In the case of children, especially infants, it exceeds 8% [17]. This is linked most of all with the hypersensitivity of small children to food products newly-incorporated into their diet (milk protein, egg white, meat, fruit, vegetables, etc.) as well as with anomalies connected with improperly-developed mucous barrier enabling increased penetration of food antigens from the intestinal lumen to the circulatory system.

One of important criteria in evaluating the allergenicity of proteins of dietary raw materials is the resistance of their epitopes to thermal denaturation and digestive processes. Reference data provide reports on the behaviour of some proteins of grain legume seeds estimated *in vivo* in animal studies [18-20]. Atkinson et al. [21] have conducted a research addressing food allergy with the use of the Brown Norway rat model. Dearman and Kimber [22] have suggested that the Balb/c mouse may provide an appropriate model for the identification and characterization of protein allergens.

The study was aimed at determining, in experiments carried out on an animal model, the allergenicity of pea proteins and stability of their epitopes during thermal treatment.

Materials and methods

Animals and diets

Ten-week-old female Balb/c mice, obtained from the Animal House-Warsaw (Poland), were used in the experiment. All mice were acclimatized before the experiment and fed a diet without legume proteins. The experiment was carried out in plastic cages at a temperature of 20˚C. The mice were fed for 28 day (free access to food ad libitum) a balanced diet containing ca. 10% of total protein, 8% of fat (olive oil), 1% of cholesterol and standard doses of a mineral mix [23] and vitamin mix [24]. The composition of diets for particular groups was provided in the table 1.

Table 1. Composition of experimental diets (g/kg)

	Diets				
	C	**SG+C**	**SG**	**GG+C**	**GG**
casein	138.5	69.8	–	69.8	–
pea seeds (raw)	–	280	557	–	–
pea seeds (cooked)	–	–	–	248	493
methionine	1.5	2.3	3	2.3	3
olive oil	**50**	**30**	**30**	**30**	**30**
potato starch	50	–	–	–	–
corn starch	710	567.9	360	599.9	424
mineral mix	**40**	**40**	**40**	**40**	**40**
vitamin mix	10	10	10	10	10

The experiment was carried out on 40 laboratory Balb/c mice that were divided into five experimental groups (eight animals each): 1 – control – with casein as a source of proteins (diet C); 2 – with flour from raw pea seeds and casein as sources of proteins (1:1) (diet SG+C); 3 – with flour from raw pea seeds as a source of proteins (diet SG); 4 – with flour from cooked pea seeds and casein as sources of proteins (1:1) (diet GG+C); and 5 – with flour from cooked pea seeds as a source of proteins (diet GG).

The animals were weighed before commencing the experiment and after 1, 2, 3 and 4 weeks. After 28 days of the experiment, the mice were anaesthetized and exsanguinated. Their spleens and livers were dissected, blood was coagulated, centrifuged for 5 min (2000 g) and stored at a temperature of -20˚C until analyzed.

The research was conducted in agreement with Ethical Commision's regulations.

Enzyme immunometric assay (ELISA) for IgA and IgG levels in serum

The procedure was followed: the microtitre plate was coated with 100 µL/well of antigen (pea protein) diluted in 50 mM – carbonate buffer, pH 9.8, and incubated for 12-18 h at 4˚C. The plate was then washed 2 times with 10 mM – phosphate buffered saline, pH 7.4, containing 0.1% Tween-20 (PBS-T), and 2 times with PBS without Tween. This washing system was used after each analytical step. Residual free binding sites were blocked with 200 µL/well of 1.5% gelatine in coating buffer for 30 min at 25˚C. The plate was washed, filled with 100 µL/well of diluted mice serum and incubated for 1 h at 37˚C. After washing, the plate was incubated for 1 h at 37˚C with 100 µL/well of goat anti-mouse IgA biotin conjugate (Sigma) or goat anti-mouse IgG biotin conjugate (Sigma). After washing, 100 µL/well of ExtrAvidin peroxidase conjugate was added. Incubation was continued for 1 h/37˚C. Then 3,3",5,5"-tetramethylbenzidin (TMB) in a 9 mM citrate buffer, pH 5.0, was used as a substrate. After incubation the plates for 30 min, 50 µL of 2 M sulphuric acid was added to stop the enzymatic reaction. Absorbance was read at 450 nm on an automated plate reader (Sunrise, Tecan).

Electrophoresis

Electrophoretic separations of protein were performed with 12% polyacrylamide gel (SDS-PAGE) according to Laemmli [25]. Before electrophoresis, all protein samples were boiled for 3 min in the presence of SDS (3 % w/v) and 2-mercaptoethanol (0.1% v/v). Low molecular markers (Sigma) ranging from 6.5 to 66 kD were used as a standard. The gels were run in a Tris-glycine buffer, pH 8.3 and proteins in gels were stained with Coomassie Brilliant Blue R-250.

Immunoblotting

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane in the apparatus for the so-called

„wet" electrotransfer using a Tris-glycine buffer with methanol, pH 8.3 (192 mmol/L glycine, 25 mmol/L Tris and 20% v/v methanol) [26].

In order to detect antigenic fractions, the membrane was incubated overnight at 4°C in serum solution containing serum of mouse. Antigen – antibody complexes were stained on the membrane by placing them in the solution of species--specific antibodies, horseradish peroxidase conjugated goat anti-mouse IgG. The reaction of the enzyme with the substrate (H_2O_2/4-chloro-1-naphtol) produced navy blue bands at the site of conjugated antibodies.

Statistical analysis

The results obtained were elaborated statistically by means of Statistica 6.0 software by Statsoft. Use was made of one-way analysis of variance ANOVA and Duncan's multiple range test. The statistical significance of differences was determined at a level of $P \leq 0.05$.

Results

Data describing feed utilization over the experimental period, changes in body weight, spleen mass and liver mass were elaborated statistically and collected in the table 2. Over the entire experimental period, feed intake by mice was alike in particular groups and accounted for ca. 97.3 – 104 g. Only in the group fed a diet with flour from cooked pea seeds was the feed intake statistically significantly reduced – ca. 82 g. The greatest body weight gain was observed in the control group fed a casein-containing diet. In groups of animals receiving diets with the addition of pea flour, a slight decrease in the body weight was recorded over 4 weeks, as compared to the control group. The greatest mean body weight loss occurred in mice fed a diet containing flour from cooked pea seeds (by ca. 2.7 g as compared to the initial body weight in that experimental group).

Minimal differences (statistically insignificant) between particular groups were demonstrated for the level of pea proteins specific IgA (Tab. 2). In contrast, significant differences occurred in the level of IgG in serum (Tab. 2). The highest titre of anti-pea IgG (mean absorbance of 1.8582) was recorded in the serum of mice from the group fed raw pea seeds (group 3). The application of the 50% dose of pea proteins in a diet has already caused some increase in the serum level of specific IgG antibodies (absorbance of 1.877), as compared to the control group (mean absorbance of 1.7303).

Figure 1 presents results of the electrophoretic separation of pea proteins obtained from raw and cooked seeds. In the extract containing proteins of pea not subjected to thermal treatment there were observed typical fractions with molecular mass ranging from 10 to 70 kD. They were predominated by vicilin proteins with molecular mass of ca. 50 and 33 kD, acidic (40 kD) and base (20 kD) subunits of legumin and proteins constituting albumins with molecular mass of 28 kD and under 14 kD. The process of thermal treatment affected the reduction in the number of high-molecular fractions in protein extracts and partial denaturation of some legumin subunits. Next five figures (Fig. 2-6) illustrate results of the immunoblotting assay that was carried out by incubating electrophoretically-separated proteins with serum of each mouse from particular experimental groups. A specific reaction was observed primarily for fractions with molecular masses of ca. 50 kD (vicilin subunits) and 70 kD (convicilin).

Discussion

The immune GALT system is an integral part of the gastrointestinal tract. As a result of challenge with food antigens, a complex mechanism of reactions is activated. One of the first lines of systemic defense is so-called secretory immunoglobulin A (sIgA), and the next one – an increase in the level of immunoglobulin G (IgG) circulating

Table 2. Effect of diet on body and organ weights of mouse and titre of specific IgA and IgG in serum

	Groups					SEM
	1	**2**	**3**	**4**	**5**	
initial body weight (g)	29.00	29.01	28.79	29.17	28.99	0.19
final body weight (g)	31.15^{a}	28.54^{ab}	27.74^{b}	28.13^{ab}	26.28^{b}	0.50
feed diet (g)	103.15^{a}	104.07^{a}	102.97^{a}	97.27^{a}	82.35^{b}	1.11
liver weight (g/100 g body weight)	3.76	3.72	3.59	3.67	3.64	0.05
spleen weight (g/100 g body weight)	0.35	0.31	0.34	0.34	0.33	0.03
specific IgA	1.25	1.27	1.29	1.27	1.27	0.01
specific IgG	1.73^{c}	1.79^{b}	1.86^{a}	1.73^{c}	1.76^{bc}	0.01

Legend: **1** – group fed with casein (diet – C); **2** – group fed with flour from raw seeds of pea and casein (diet – SG+C); **3** – group fed with flour from raw seeds of pea (diet – SG); **4** – group fed with flour from cooked seeds of pea and casein (diet – GG+C); **5** – group fed with flour from cooked seeds of pea (diet – GG). **SEM** – standard error of means; **a, b, c** – values within rows followed by the same letter are not significantly different at P=0.05

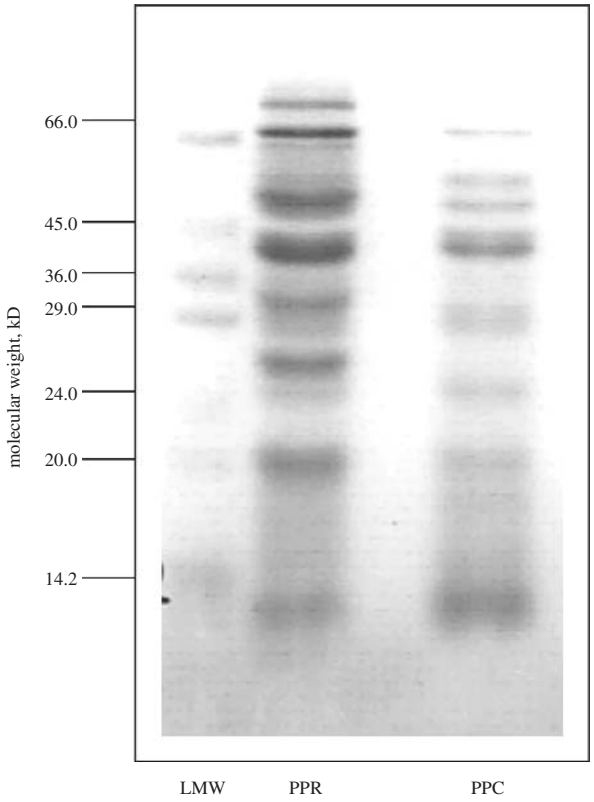

Fig. 1. SDS-PAGE separation of pea proteins: LMW – low molecular weights; PPR – pea proteins from raw seeds; PPC – pea proteins from cooked seeds

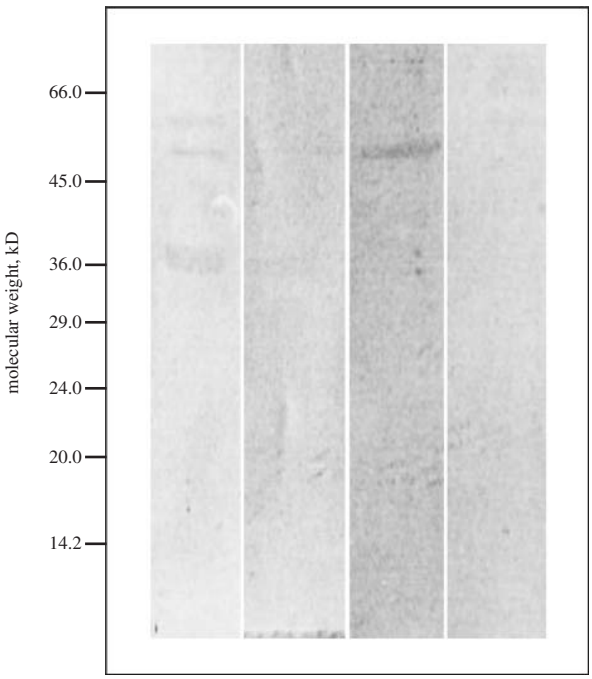

Fig. 2. Immunoblots with serum from mouse fed control diet containing casein (group 1)

in blood. Among other parameters, the levels of IgA and IgG in mice serum are a sort of a measure of the activity of food antigens. The reported experiment carried out on an animal model was aimed at determining the effect of proteins obtained from raw or cooked pea seeds on the immune system. The use of Balb/c mice in that study has been justified by previous investigations [27, 28].

Lalles et al. [29] have reported that the immunogenicity of selected anti-nutrients, e.g. lectins or proteins of grain legume seeds, might indicate that the immune response is linked with nutrition disorders, reduced availability of nutrients and growth inhibition. The reported experiment demonstrated that, generally, the addition of pea flour caused a reduction in body weight of mice, as compared with the control group fed a casein-containing diet. This is likely to be due to the presence of anti-nutrients in pea seeds that may inhibit nutrient absorption and negatively affect the growth of the animals. A lower body gain of the mice fed pea flour as compared to the group administered with casein has also been described elsewhere [30, 31]. Whereas no statistically significant differences were observed in the mass of liver and spleen when expressed per 100 g body weight of experimental animals. Nevertheless, the animals fed a diet in which pea constituted the source of protein were characterized by negligibly lower relative mass of liver and spleen when compared with the group fed casein (Tab. 2). Similar findings have been reported by Esparza et al. [31].

Growth disorders evoked by the intake of pea proteins did not affect the level of specific IgA antibodies in mouse serum, determined with the ELISA method. Other researchers have also used the ELISA method to determine specific IgA and did not demonstrate any significant differences in the titre of those antibodies in the serum between experimental piglets fed grain legume seeds and the control ones receiving casein. In contrast, when applying the immunoblotting method, those authors have demonstrated a number of specific bands recognized by IgA present in the serum of animals with the highest titre of that immunoglobulin. These were mainly fractions of vicilin proteins and, to a lower extent, legumin ones. In our experiment, we have failed to conduct similar immunoblotting analyses. Difficulties might have resulted from a low titre of IgA antibodies in the serum of mice. Yet, the immunoblotting method has been successively applied for the determination of the most immunogenic fractions of pea proteins based on their reactions with specific IgG.

In the serum of control mice, there was observed a small number of IgG antibodies that reacted with the proteins analyzed. On immunoblotts, a very weakly-colored band appeared at the height corresponding to the molecular mass of ca. 70 kD (Fig. 2). Prior to the experiment, the animals were fed a diet devoid of grain legume proteins and the

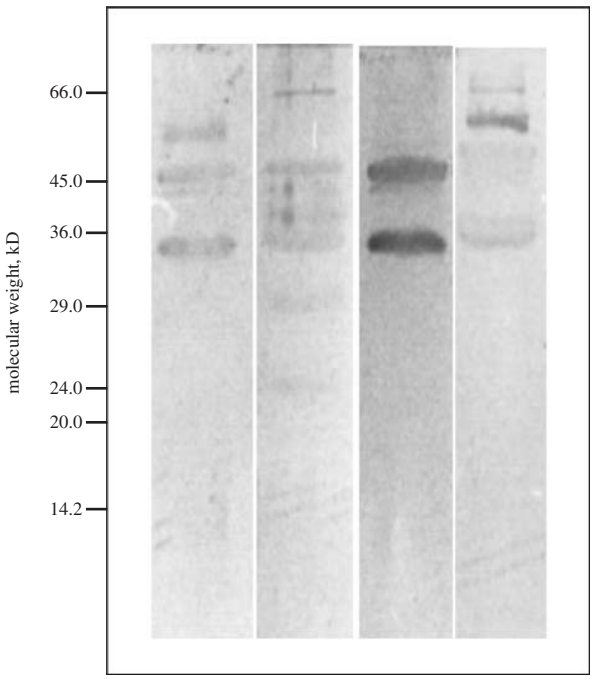

Fig. 3. Immunoblots with serum from mouse fed diet containing pea proteins (from raw seeds) and casein (group 2)

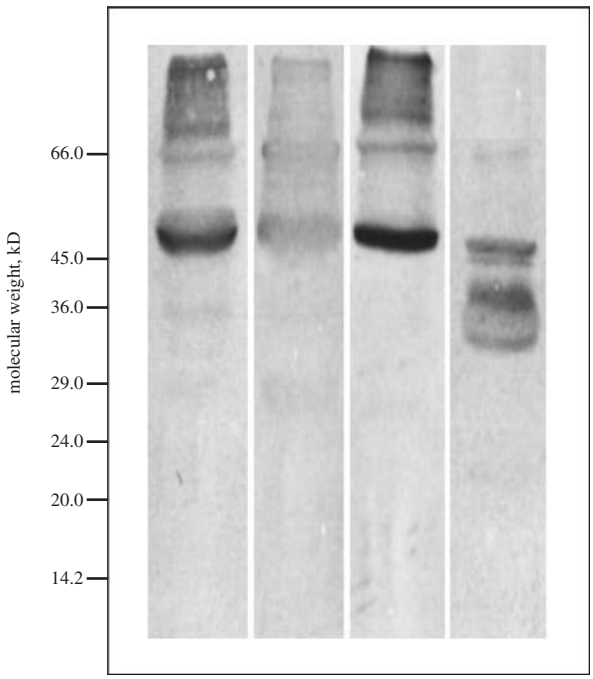

Fig. 4. Immunoblots with serum from mouse fed diet containing pea protein (from raw seeds) (group 3)

presence of specific antibodies in their serum may indicate passive transfer of the antibodies to the circulatory system from mothers still in the intrauterine life. It is also likely that the mothers were fed a diet containing proteins of soybean or other legume seeds. Due to cross-reactions, the antibodies produced against those proteins might have also recognized epitopes occurring in pea extracts.

The highest number and the most clear bands were demonstrated on immunoblotts made with sera of mice fed a diet containing raw pea seeds. The specific reaction was observed mainly for fractions with molecular masses of ca. 50 kD (vicilin subunits), 70 kD (convicilin) and ca. 60 kD (legumin proteins) (Fig. 4). Similar fractions were recognized by antibodies present in the serum of mice from the group fed casein with the addition of raw pea seed flour. Still, they were considerably less intensively colored (Fig. 3).

Martínez et al. [32] have also observed an increased level of IgG in the serum of mice receiving grain legume seeds with their diet.

Piglets fed five different raw seeds of grain legumes (pea, bean, lupine, chick-pea and black chick-pea) demonstrated an elevated level of IgG antibodies to vicilin fractions [33]. Exactly the same observations were made by Seabra et al. [34] who fed the animals with three other legumes. This seems surprising since 7S and 11S globulins are claimed to be structurally-related. Nevertheless, those families of proteins have also structural differences considered responsible for specific biochemical, rheological and, probably, immunological properties. Little is known about relations between the structure and immunogenicity of globulin proteins. Various immunogenic properties of vicilin and legumin proteins may be elucidated, to some extent, by different susceptibility of those proteins to hydrolysis with digestive enzymes. In the case of soybean, Sissons and Thurston [35] have demonstrated that fraction 7S (β-conglycinin) was more resistant than the fraction 11S (glycinin) to *in vitro* digestion with pepsin assay. Our previous studies have also indicated a relatively high resistance of pea proteins, especially vicilin, to pepsin hydrolysis under *in vitro* conditions [36]. Lalles [37] have reported on a high resistance of vicilin proteins to digestion in experiments *in vivo*.

In our study, it seemed a significant finding that the mean level of specific IgG in groups fed cooked seeds was higher, as compared to that of the control group whose diet was based on casein proteins only, and only negligibly lower than the results obtained for mice fed flour from untreated pea seeds. This may indicate a little effect of thermal treatment of pea seeds on diminishing the immunogenic capacity of proteins. Literature data indicate that also allergens of other legumes demonstrate a high resistance to thermal processing [38, 39].

The serum of mice fed a diet based primarily on cooked pea seeds was found to contain specific antibodies mainly towards high-molecular subunits of vicilin (Fig. 5, 6). This results from the fact that epitopes present in those proteins are characterized by a high resistance to thermal denaturation and degradation with digestive enzymes. It may be concluded then

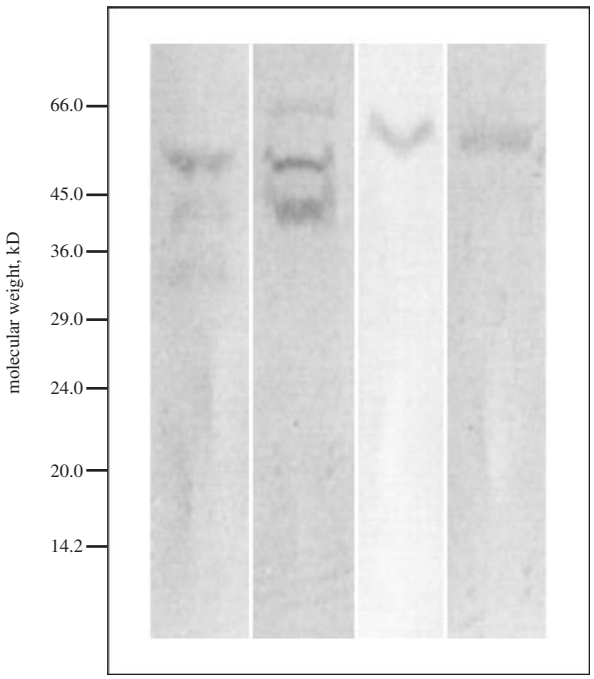

Fig. 5. Immunoblots with serum from mouse fed diet containing pea proteins (from cooked seeds) and casein (group 4)

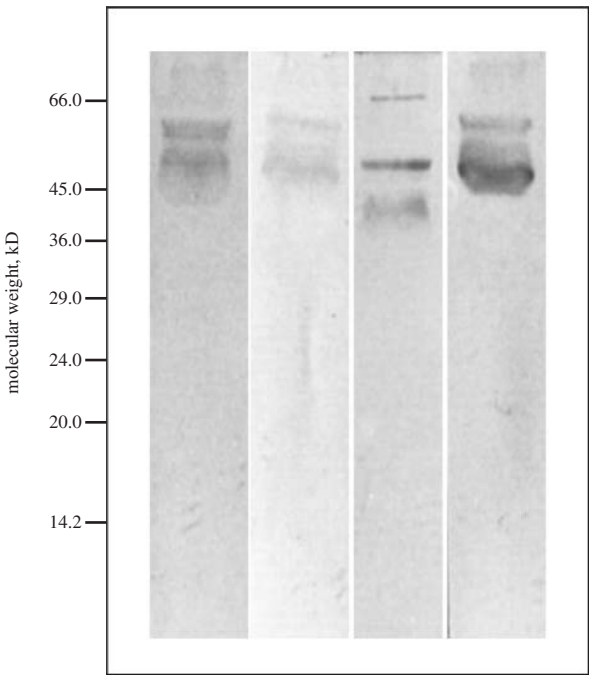

Fig. 6. Immunoblots with serum from mouse fed diet containing pea proteins (from cooked seeds) (group 5)

that proteins occurring in the vicilin fraction can be responsible for most of allergic reactions induced by pea.

The vicilin proteins – 7S are relatively widely distributed and occur not only in the representatives of grain legume seeds. A number of strong allergens are exactly 7S type proteins [38-42]. The best recognized allergenic vicilin is the major allergen of peanuts – Ara h 1 – which has been reported to be responsible for most of cases of anaphylaxis induced by food [43]. In the last decade, a number of research have addressed the analysis of that strong allergens. They have demonstrated that three monomers of Ara h 1 form highly stable structure of trimer. This, in turn, protects the molecule against digestion by proteases, makes it resistant to denaturation and enables its transfer through the small intestine [44]. The stable structure is undoubtedly the major reason of high allergenic of that protein. Vicilins of grain legume seeds display a high affinity to an amino acid sequence, e.g. at a level of 60-65% in the case of vicilins of pea and peanuts (Ara h1). Wensing et al. [45] have claimed that the affinity between pea vicilin and Ara h 1 is the molecular basis of the IgE-dependent cross-reactivity determined *in vitro* between peanuts and pea.

They have also proved the existence of the clinical cross-reactivity between those proteins. Apart from legumins, vicilins belong to the main storage proteins of various seeds consumed world-wide by people and the problem of their allergenicity seems highly significant and undoubtedly requiring further extensive research.

Conclusions

Mouse-animal model experiments were useful in evaluating the allergenicity of pea proteins. Subjecting pea seeds to the cooking process resulted in only negligible diminution of the immunogenic properties of pea proteins. In further studies, it seems advisable to use extracts from the small intestine of mice for determination of sIgA levels.

Vicilin appeared to be the protein that stimulated mouse organism for the production of specific IgG antibodies to the greatest extent. These points to strong potential allergenic properties of pea vicilin.

References

1. Deshpande SS (1992): Food legumes in human nutrition: a personal perspective, critical review. Food Sci Nutr 32: 333-363.
2. Friedman M (1996): Nutritional value of protein from different food sources, a review. J Agric Food Chem 44: 6-29.
3. Jongen WM, Meerdink G (2001): Pea proteins based food products as meat replacers: the Profetas concept. Nahrung 45: 402-404.
4. Waggle DH, Steinke FH, Shen JL: Isolates soy proteins. In: Legumes, Chemistry, technology and human nutrition. Ed. RH Matthews, Dekker, New York 1989, 99-138.
5. Costa GE, da Silva Queiroz-Monici K, Reis SM, et al. (2006): Chemical composition, dietary fibre and resistant starch contents of raw and cooked pea, common bean, chick pea and lentil legumes. Food Chem 94: 327-330.
6. Duranti M, Gius C (1997): Legume seeds: protein content and nutritional value. Field Crops Res 53: 31-45.

7. Kozłowska H (1994): Przeciwżywieniowe substancje w nasionach roślin strączkowych. Przem Spoż 48: 183-191.
8. Rehman Z, Shah WH (2005): Thermal heat processing effects on antinutrients, protein and starch digestibility of food legumes. Food Chem 91: 327-331.
9. Duranti M (2006): Grain legume proteins and nutraceutical properties – review. Fitoterapia 77: 67-82.
10. Breiteneder H, Radauer C (2004): A classification of plant food allergens. J Allergy Clin Immunol 113: 821-830.
11. Bruijnzell-Koomen C (1995): Adverse reactions to food. Position paper of the European Academy of Allergy and Clinical Immunology. Madrid, 1995.
12. Pascual CY, Fernandez-Crespo J, Sanchez-Pastor S, et al. (1999): Allergy to lentils in Mediterranean pediatric patients. J Allergy Clin Immunol 103: 154-158.
13. Patil SP, Niphadkar PV, Bapat MM (2001): Chickpea: a major food allergen in the Indian subcontinent and its clinical and immunochemical correlation. Ann Allergy Asthma Immunol 87: 140-145.
14. Crespo JF, Rodriguez J, Vives R, et al. (2001): Occupational IgE-mediated allergy after exposure to lupine seed flour. J Allergy Clin Immunol 108: 295-297.
15. Sicherer SH, Sampson HA, Burks AW (2000): Peanut and soy allergy: a clinical and therapeutic dilemma – review. Allergy 55: 515-521.
16. Ibaňez D, Martinez M, Sanchez JJ, et al. (2003): Legume: cross-reactivity. Allergol Immunopathol 31: 151-161.
17. Helm RM, Burks AW (2000): Mechanisms of food allergy. Curr Opin Immunol 12: 647-653.
18. Dréau D, Lallés JP, Salmon H, et al. (1995): IgM, IgA, IgG1 and IgG2 specific responses in blood and gut secretion of calves fed soyabean products. Vet Immunol Immunopathol 47: 57-67.
19. Lallés JP, Duvaux-Ponter C, Sissons JW, Toullec R (1998): Small intestinal motility disorders in preruminant calves chronically fed a diet based on antigenic soya: characterization and possible mediators. Vet Res 29: 59-72.
20. Salgado P, Freire JPB, Ferreira R, et al. (2003): Immunodetection of legume proteins resistant to small intestinal digestion in weaned piglets. J Sci Food Agric 83: 1571-1580.
21. Atkinson HAC, Johnson IT, Gee JM, et al. (1996): Brown Norway rat mode of food allergy: effect of plant components on the development of oral sensitization. Food Chem Toxicology 34: 27-32.
22. Dearman RJ, Kimber I (2001): Determination of protein allergenicity: studies in mice. Toxicol Lett 120: 181-186.
23. NRC: National Research Council. Nutrient requirements of laboratory animals. National Academy Press. Washington, DC 1976.
24. Reeves PG (1997): Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr 127: 838-841.
25. Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
26. Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350-4354.
27. Adel-Patient K, Creminon C, Bernard H, et al. (2000): Evaluation of a high IgE-responder mouse model of allergy to bovine β-lactoglobulin BLG: development of sandwich immunoassays for total and allergen-specific IgE, IgG1 and IgG2a in BLG-sensitized mice. J Immunol Methods 235: 21-32.
28. Bernard H, Nergroni L, Chatel JM, et al. (2000): Molecular basis of IgE cross-reactivity between human β-casein and bovine β-casein, a major allergen of milk. Molecular Immunol 37: 161-167.
29. Lallés JP, Salmon H, Bakker NPM, et al.: Effect of dietary antigens on health, performance and immuno system of calves and piglets. In: Recent Advances in Antinutritional Factors in Legume Seeds. Ed: AFB van der Poel, J Huisman and HS Sini. EAAP Publication No 70. Wageningen 1993, 253-270.
30. Martínez JA, Esparza ML, Larralde J (1995): Immunological changes in growing mice fed on diets containing casein or peas (*Pisum sativum* var. Belinda) as the source of protein. Br J Nutr 73: 87-97.
31. Esparza ML, Larralde J, Martínez JA (1996): Development--related nutritional utilization and immune function in mice fed on two different protein sources. Nutr Res 16: 829-839.
32. Martínez JA, Macarulla MT, Marcos R, et al. (1992): Nutritional outcome and immunocompetence in mice fed on diet containing raw field beans (*Vicia faba* var. Minor) as the source of protein. Br J Nutr 68: 493-503.
33. Salgado P, Freire JPB, Ferreira R, et al. (2002): Legume proteins of the vicilin protein are more immunogenic than those of legumin family in weaned piglets. Food Agric Immunol 14: 51-63.
34. Seabra M, Carvalho S, Freire JPB, et al. (2001): *Lupinus luteus*, *Vicia sativa* and *Lathyrus cicera* as protein sources for piglets: ileal and total tract apparent digestibility of amino acids and antigenic effects. Animal Feed Sci Technol 89: 1-16.
35. Sissons JW, Thurston SM (1984): Survival of dietary antigens in the digestive tract of calves intolerant to soybean products. Res Vet Sci 37: 242-246.
36. Szymkiewicz A, Jędrychowski L (2005): Reduction of immunoreactive properties of pea globulins as the result of enzymatic modification. Acta Alimentaria 34: 295-306.
37. Lallés JP: Soy products as protein sources for preruminants and yung pigs. In: Soy in animal nutrition. Ed. JK Drakley. Federation of Animal Science Society. Savoy. IL, USA 1999, 106-126.
38. Sánchez-Monge R, Pascual CY, Diaz-Perales A, et al. (2000): Isolation and characterization of relevant allergens from boiled lentils. J Allergy Clin Immunol 106: 955-961.
39. López-Torrejón G, Salcedo G, Martin-Esteban M, et al. (2003): Len c 1, a major allergen and vicilin from lentil seeds: protein isolation and cDNA cloning. J Allergy Clin Immunol 112: 1208-1215.
40. Beyer K, Bardina L, Grishina G, et al. (2002): Identification of sesame seed allergens by 2-dimensional proteomics and Edman sequencing: Seed storage proteins as common food allergens. J Allergy Clin Immunol 110: 154-159.
41. Pastorello A E, Farioli L, Pravettoni V, et al. (2004): Lipid transfer protein and vicilin are important walnut allergens in patients not allergic to pollen. J Allergy Clin Immunol 114: 908-914.
42. Wang F, Robotham JM, Teuber SS, et al. (2002): Ana o 1, a cashew (*Anacardium occidental*) allergen of the vicilin seed storage protein family. J Allergy Clin Immunol 110: 160-166.
43. Burks W, Bannon GA, Sicherer S, Sampson HA (1999): Peanut-induced anaphylactic reactions. Int Arch Allergy Immunol 119: 165-172.
44. Shin DS, Compadre CM, Malekis J (1998): Biochemical and structural analysis of the IgE binding sites on Ara h 1, an abundant and highly allergenic peanut protein. J Biol Chem 273: 13753-13759.
45. Wensing M, Knulst AC, Piersma S, et al. (2003): Patients with anaphylaxis to pea can have peanut allergy caused by cross-reactive IgE to vicilin (Ara h 1). J Allergy Clin Immunol 111: 420-424.