POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

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Pol. J. Food Nutr. Sci.
2008, Vol. 58, No. 3, pp. 345-350

EFFECT OF ACYLATION AND ENZYMATIC MODIFICATION ON PEA PROTEINS ALLERGENICITY

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Key words: pea proteins, enzymatic hydrolysis, acylation of proteins, allergenicity of pea proteins

In the study chemical modifications (acetylation or succinylation) and enzymatic hydrolysis (with Alcalase) were applied in order to reduce the allergenicity of particular pea proteins. Application of Alcalase after acylation lowered the immunoreactive properties of pea vicilin from 14-17% down to 2-2.5% as compared to that of the native fraction. Under these conditions, the immunoreactivity of legumin and albumins was reduced by nearly 100%. Proteolysis of pea proteins under conditions optimal for Alcalase decreased the immunoreactivity of vicilin to about 24%. Application of acetic anhydrides for further modification led to lowering their level to 6% and 9%, during acetylation and succinylation, respectively. The immunoreactivity of pea legumin and albumins was reduced down to 1-3%.

The lower immunoreactive properties of particular pea proteins do not correspond with lower allergenicity of total proteins. Of all the applied methods acetylation of previously hydrolysed proteins proved to be the most effective; it resulted in about 70% allergenicity decrease (on the average) of thus modified samples as compared to native pea proteins. Yet, the reaction showed an individual character and may be different in individual patients.

INTRODUCTION

Plant tissues consumed by humans contain thousands of different proteins, many of which may cause allergic reactions in atopic individuals [Breitender & Radauer, 2004]. Many allergens belong to the cupin superfamily (7S and 11S seed storage proteins) [Chapman *et al*., 2007]. Globulins 7S and 11S, both revealing allergenic properties, mostly occur in the seeds of leguminous plants (soy, peanuts, lentils, pea) and nuts [Astwood *et al*., 2002; Mills *et al*., 2002]. These proteins have a high structural similarity [Breiteneder & Mills, 2005]. The studies have proved that proteins of 7S type show stronger immunogenic properties than globulins 11S [Salgado *et al*., 2002]. Technological processes applied to food may reduce or increase its allergenicity [Sathe *et al*., 2005]. The molecular basis for changes in allergenic activity is destruction of epitope structures, formation of new epitopes, or better access of cryptic epitopes caused by denaturation of the native allergen [Besler *et al*., 2001]. Epitopes of the majority of natural allergens in native state have conformational structure which guarantees better matching for the structure of antibody paratope [Wal, 1998]. Deformation or destruction of the conformational structure of a protein allergen particle caused by heating, denaturation or joining by hapten leads to destruction of conformational epitopes. Linear epitopes are more resistant to the action of denaturation than the conformational ones but they may undergo changes during enzymatic modification as a result of polypeptide chain fragmentation or blocking certain functional groups by chemical modifications [Bredehorst & David, 2001].

The most popular methods used to reduce protein allergenicity are enzymatic processes [Clemente *et al*., 1999a; Gauthier *et al*., 2006, Peñas *et al*., 2006]. The main reason for lowering antigenicity during protein proteolysis are conformational changes and decomposition of polypeptide bonds within epitopes. Another significant factor is the reduction of particles size [Van Beresteijn *et al*., 1994; Van Hoeyveld *et al*., 1998]. Too strong protein hydrolysis may, however, result in lowering the nutritional value and disadvantageous changes in taste and flavour due to the presence of bitter and astringency peptides, which considerably limits the usability of such hydrolysates in oral nutrition. Too high amount of low-molecular polypeptides in hydrolysates may also cause an increase in product osmosity to a level excluding its applicability as a formula component [Leman, 2001]. An alternative solution might be application of chemical modifications.

The primary aim of chemical modifications of proteins used for nutritional purposes is the application of non-toxic reagents so as to provide proteins with suitable functional properties in accordance with the requirements for ready food products with simultaneous preservation or even improvement of their nutritional value. The most frequently used methods are acylation with acid anhydrides, phosphorylation and oxidation [El-Adawy, 2000; Vidal *et al*., 2002]. Combining chemical and enzymatic methods may lead to obtaining a product with lowered allergenic properties and beneficial functional properties. The application of plant proteins on the industrial scale is often limited by their insufficient solubility at average acidity (pH 3-6) [Ross & Bhat-

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nagar, 1989], which occurs during, for example, production of coffee cream, yoghurt or instant sauces. In order to improve the functional properties of plant proteins usually limited hydrolysis is applied, which, however, does not guarantee improvement of their solubility at low pH values. Although succinylation leads to an increase in protein solubility, succinylated proteins with high molecular mass may undergo precipitation at low pH. Achouri *et al*. [1998] and Achouri & Zhang [2001] made an attempt at combining enzymatic and chemical modifications in order to obtain a product with beneficial functional properties.

Food allergy affected as many as 6% of young children and 3–4% of adults [Sicherer & Sampson, 2006]. Legumes (peanut, soybean) are common allergens often implicated in severe anaphylactic reactions, which eliminates the use of these legume proteins as a supplement in the production of anti-allergenic food [Chapman *et al*., 2007]. Pea proteins have a high structural similarity to peanut or soy proteins, but they are not showing so strong allergic properties [Breiteneder & Mills, 2005]. Pea proteins can probably be used as an additive in the production of anti-allergenic food. The modification of pea proteins may increase the safety this food and improve its functional properties.

The aim of the study was to apply both chemical and enzymatic modifications, one after another, to reduce allergenicity of pea proteins.

MATERIAL AND METHODS

Plant material

Pea seeds of the Kwestor variety (obtained from Poznań Plant Breeding Station Ltd., Tulice n/Poznań, Poland) were used in the study.

Pea protein extraction

The pea seeds were ground, sieved (Ø 0.25 mm) and kept in a refrigerator at about 4°C until use. Proteins were extracted from pea flour (1:10 v/w) for 2 h with 0.1 mol/L Tris-HCl buffer containing 0.1 mol/L NaCl (pH 8.5). The extract was centrifuged for 30 min (20,000×g), dialysed against 0.05 mol/L NH_4HCO_3, frozen, lyophilized and stored at –20°C until use.

Isolation and purification of pea antigens

Globulins used as antigens were isolated from pea flour according to the method reported by Freitas *et al*. [2000]. Particular globulin fractions (legumin and vicilin) were further purified on an ion-exchange chromatography column (DEAE-Sepharose).

Albumins were isolated according to Lu *et al*. [2000] and purified by gel chromatography (Sephadex G-75).

The purified fractions were lyophilised and stored at –70°C until use.

SDS-PAGE analysis of pea antigens

Electrophoretic separation of pea antigens was performed with 12.5% polyacrylamide gel (SDS-PAGE) according to the method of Laemmli [1970]. Protein samples (concentration 2 mg/mL) were boiled for 3 min in the presence of SDS and 2-mercaptoethanol. Low molecular weight 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. The proteins were stained with Coomassie Brilliant Blue R-250.

Production of polyclonal antibodies

The polyclonal antibodies against purified pea proteins were obtained through subcutaneous and intramuscular quadruple immunization of two rabbits at 2-week intervals. Each time, 1 mL of a solution containing 10 mg of protein was used. The first immunization was performed in the presence of complete Freund adjuvant, and the subsequent – in the presence of incomplete Freund adjuvant. The indirect ELISA method was used to determine the titre of the obtained antibodies. The research was conducted in agreement with the Ethical Commission's regulations (No. 39/N).

Acylation of pea proteins

Acylation of 2% pea proteins solution (in 0.05 mol/L phosphate buffer, pH 8) was carried out according to the method described by El-Adawy [2000] with some modification. The proteins were acylated by reaction with succinic or acetic anhydrides, by adding these reagents in concentrations of 0.2 g of anhydride/g of proteins. The pH of the solution was maintained at about 8 by 0.5 mol/L NaOH and with constant stirring for 2 h at room temperature. It was then dialysed for 48 h against distilled water and lyophilized.

The degree of acylation was measured by reactions of an epsilon amino group of lysine and N-terminal amino groups of proteins with 2,4,6-trinitrobenzensulfonic acid (TNBS) [Concon, 1975]. The ratio of free lysine in the acylated protein to lysine of native protein is the degree of acylation (%). The total protein (N x 6.25) was measured with the standard Kjeldahl's method [AOAC, 1990].

Hydrolysis

Hydrolysis was carried out with Alcalase 2.4 L FG (*Subtilisina Carlsberg*, Novo Nordisk). The proteolytic activity of the enzyme was determined according to Anson [Mejbaum-Katzenellenbogen & Mochnacka, 1969] and calculated for Alcalase 1.83 AU/mL.

The enzymatic hydrolysis was carried out for 120 min at pH 8.0 in pH-stat, with constant stirring. During the hydrolysis process, pH was maintained at a constant level by adding 0.5 mol/L NaOH from a burette. The enzyme was added as a water solution (1 mL) in the amount of 30 mAU/g of protein. The proteolytic enzyme was inactivated by heating at the end of hydrolysis at 90°C for 5 min. After cooling out, the hydrolysates were frozen, lyophilised and subjected to further analysis.

The degree of hydrolysis was calculated on the basis of the quantity of free amino groups in reaction with *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol [Panasiuk *et al*., 1998]. The total number of α-amino groups was determined according to Hajós *et al*. [1988].

Competitive enzyme-linked immunosorbent assay (ELISA)

Microplates were coated with an antigen (pea proteins fractions: albumin, legumin or vicilin; 10 μg/mL) in a 9 mmol/L carbonate buffer solution, pH 9.6, and incubated for 18 h at 4°C. After rinsing with PBS-T (0.1 mol/L phosphate buffered saline, containing 0.15 mol/L NaCl, pH 7.4 with 0.5% Tween

20) and blocking with a 1.5% gelatin solution. Then wells were filled with the sample (containing the antigen in a concentration of 0.001 to 1 mg/mL as standard or modified proteins) and polyclonal rabbit antibodies obtained against the given antigen (50 μL of each solution of adequate concentration per well). Goat anti-rabbit IgG peroxidase solution conjugate (A-6154, Sigma) (1:5000) was used as a conjugate. The substrate TMB (3,3',5,5'-tetramethylbenzidine; T-5525, Sigma) was added and after 30 min the reaction was stopped by 2 mol/L H_2SO_4. Absorbance was measured at 450 nm using a Sunrise-Tecan automatic reader. The obtained results were processed with the Immunofit™ EIA/RIA software (Beckman). The experiments were performed in triplicate and means of three values were reported.

The result obtained were elaborated statistically with the ANOVA test (α=0.05).

Determining reduction rate of allergenicity of modified pea proteins

Blood serum for *in vitro* study was obtained from 24 patients at NZO Allergology Centre in Łódź.

The reduction rate of the allergenicity of modified pea proteins was determined by assessing specific antibodies level in patients' serum.

The percentage of the loss of antigenic activity of pea proteins was calculated based on the formula [Clemente *et al*., 1999a]:

$$A\ [\%] = (1 - A_M / A_N)\ x\ 100$$

where: A_M – absorbance of modified sample, and A_N – absorbance of native sample

IgE level in blood serum of patients with food allergy was determined with the ELISA method. Microplates were coated with the antigen (10 μg/mL) in a 9 m mol/L carbonate buffer solution, pH 9.6, incubated for 18 h at 4°C and blocked with a 1.5% gelatin solution. Next, wells were filled with human serum in a solution of 1:5. After 2-h incubation at 37°C biotinylated goat anti-human IgE (5069, Nordic) was added in a solution of 1:1000 and then ExtraAvidin Peroxidase Conjugate (E 2886, Sigma) in a solution of 1:1000. The substrate (TMB) was added and after 30 min the reaction was stopped by 2 mol/L H_2SO_4. Absorbance was measured at 450 nm using a Sunrise-Tecan automatic reader.

RESULTS AND DISCUSSION

The major proteins of pea seeds are storage proteins, which are classified as 7S and 11S globulins according to their sedimentation coefficients (S) and albumins. SDS-PAGE separations of the purified pea protein fractions are shown in Figure 1. Vicilin (7S globulin) purified on DEAE-Sepharose column yielded bands corresponding to the molecular masses of 50 kD, about 33 kD and three 19-16 kD bands. As a result of chromatographic separations the purified legumin fraction (11S globulin) was obtained which on SDS-PAGE gel yielded bands corresponding to the molecular masses of 40 kD (acidic subunits) and 20 kD (basic subunits). The pea albumins are proteins with molecular weight of 26 kDa and below 6.5 kDa (Figure 1). The same pea protein fractions were reported in literature [Bacon *et al*., 1987; Lu *et al.,* 2000; O'Kane *et al.,* 2004]. Polyclonal antibodies, produced against pea vicilin, legumin and albumins are characterised by a high specificity and no cross reactivity has been detected between them (results not shown).

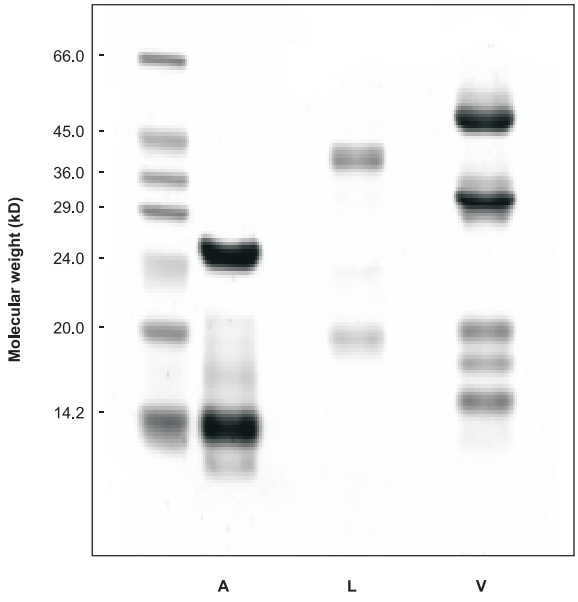

FIGURE 1. SDS-PAGE separation of purified pea antigens: A – albumins; L – legumin; V – vicilin.

Chemical modifications like acetylation of proteins of leguminous plant seeds lead first of all to a change in the charge and hydrophobicity of proteins, which in turn increases their solubility and improves emulsification and foaming properties. There are also observed changes within the structure of chemically-modified proteins [Bora, 2002; Krause *et al.*, 1999, 2001; Lawal, 2005]. These changes may result in lowering protein immunoreactivity. In an earlier study [Szymkiewicz & Jędrychowski, 2008] pea proteins were modified with acetic or succinic anhydride in different concentrations (0.01-1.0 g per g protein). The research indicated that the extent of changes in the immunoreactivity of individual pea proteins depended on the dose and also on anhydrides used. The greatest reduction of the immunoreactivity of albumins and legumin were observed after acylation with 0.2 g anhydrides (by 91-99% and 78-97% after succinylation and acetylation, respectively). Under these conditions, the immunoreactivity of vicilin fraction was reduced down to 12-17% [Szymkiewicz & Jędrychowski, 2008]. The residual immunoreactivity of albumins and vicilin (12-22%) eliminates the chemically-modified pea proteins as a supplement in the production of non-allergenic food. In the presented study the chemically-modified pea proteins (with 0.2 g acylating agent/g protein) were further enzymatically hydrolysed with Alcalase. Alcalase effectively lowered the allergenicity of non-modified pea proteins [Szymkiewicz & Jędrychowski, 2005]. The highest degree of hydrolysis rate in all samples was observed during the first 30 min of the process (Figure 2). Next, it decreased considerably and after *ca*. 90 min it stabilised. The maximum DH for samples was 10.5% for previously acetylated samples and 11% for succinylated ones (Figure 2).

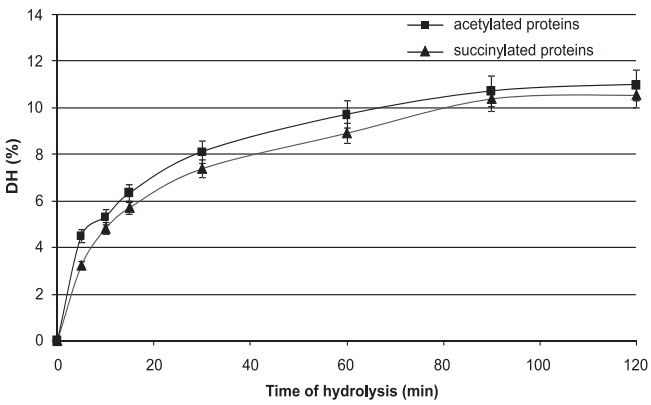

FIGURE 2. Kinetics of hydrolysis of chemically modified pea proteins.

The hydrolysis of chemically-modified pea proteins caused significant reduction in the immunoreactivity of particular pea proteins fractions, especially vicilin, whose epitopes were the most resistant to the modifications, both chemical [Szymkiewicz & Jędrychowski, 2008] and enzymatic one [Szymkiewicz & Jędrychowski, 2005], applied separately. After acetylation the remaining immunoreactivity of vicilin was about 17% (Figure 3) and after succinylation about 12% (Figure 4). The application of Alcalase lowered the immunoreactive properties of this fraction to 2-2.5% in respect of native vicilin. Under such conditions the immunoreactivity of legumin and albumin decreased by nearly 100% (Figures 3 and 4).

The proteolysis of pea proteins under conditions optimal for Alcalase resulted in lowering the immunoreactivity of pea legumin and albumins to 7.5% and 2%, respectively (Figure 5) whereas immunoreactive properties of vicilin remained at the level of about 24%. Application of acetic anhydrides for further modification led to lowering their level to 6% and 9%, during acetylation and succinylation, respectively (Figure 5). Immunoreactivity of protein fraction in hydrolysed sample was statistically different ($p < 0.05$) as compared to that in hydrolysed and chemically-modified samples as it was show by ANOVA test.

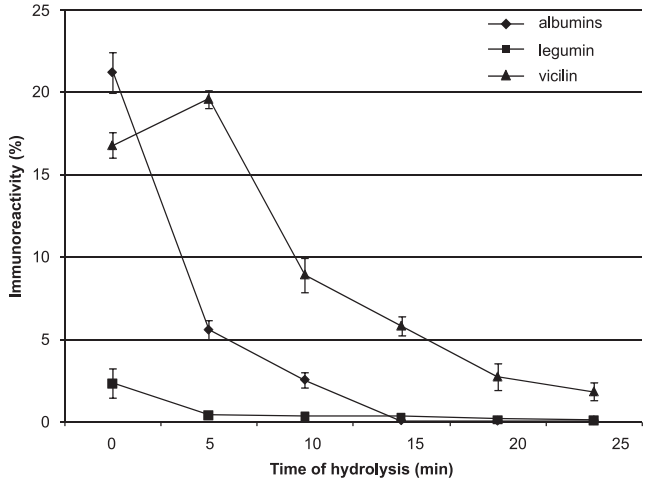

FIGURE 3. Residual immunoreactivity of pea protein modified by acetylation and Alcalase hydrolysis.

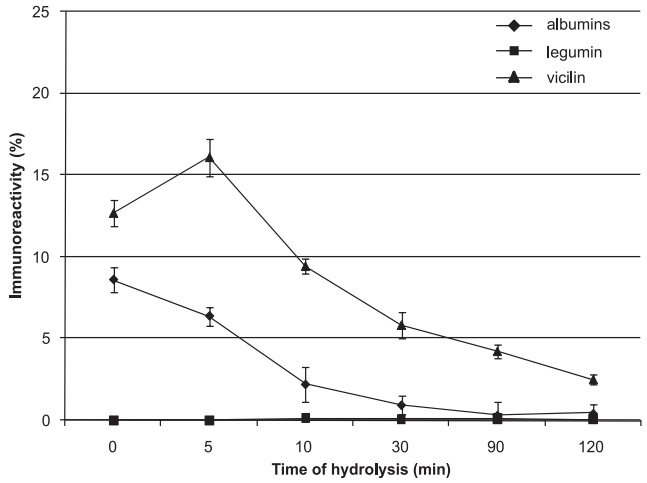

FIGURE 4. Residual immunoreactivity of pea proteins modified by succinylation and Alcalase hydrolysis.

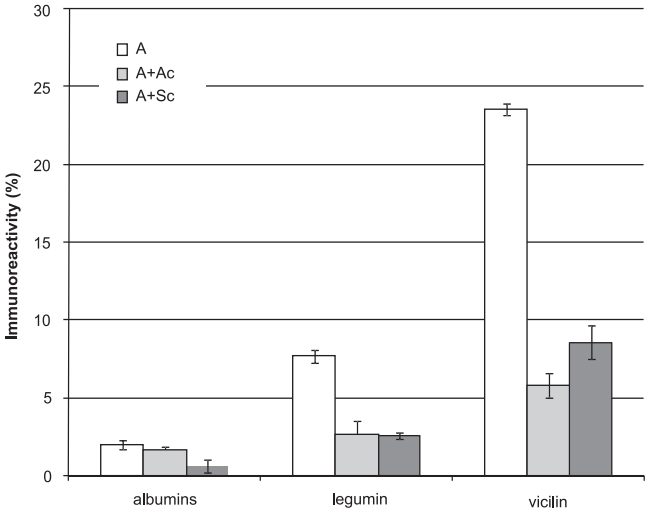

FIGURE 5. Residual immunoreactivity of pea proteins modified by Alcalase hydrolysis (A), Alcalase hydrolysis and acetylation (A+Ac) or Alcalase hydrolysis and succinylation (A+Sc).

A considerable decrease in the immunoreactive properties of proteins not always guarantees their lower allergenicity. The serum of patients with a higher level of immunoglobulin E specific against proteins of leguminous seeds was used to determine allergenicity of modified pea protein. It was used to study modified samples of pea proteins characterised by the highest reduction of immunoreactivity of particular protein fractions determined earlier by the ELISA method *in vitro* with the use of rabbit polyclonal antibodies.

Depending on the modification type and analysed serum sample, antigenic properties of pea proteins were reduced by 40-75% (Table 1). One-step modification type, enzymatic hydrolysis or acetylation, resulted in 60% or 58% reduction on average, respectively. The production of hypoallergenic formulas is based mostly on the use of the enzymatic hydrolysis process in pH 6-8 and temperature of 40-60°C [Clemente, 2000]. It is assumed that reducing product's antigenicity by 90% may cause that it no longer induces allergy symptoms [Clemente, 2000]. The application of such products in nutri-

TABLE 1. Percentage reduction of allergenicity of pea proteins after modifications.

Kind of modification of pea proteins	Reduction of allergenicity (%)											
	Number of serum											
	2	4	5	12	15	17	18	19	20	22	23	24
Alcalase hydrolysis	61.66	68.41	65.55	63.31	56.21	59.87	56.69	64.03	59.23	46.97	63.04	53.51
Alcalase hydrolysis and acetylation	65.62	74.54	69.95	69.87	72.54	74.74	70.51	68.42	70.24	57.85	71.40	59.97
Acetylation	56.44	62.92	64.16	63.74	57.29	55.70	62.38	68.48	62.03	41.93	57.71	49.02
Acetylation and Alcalase hydrolysis	59.98	72.07	39.75	62.23	46.53	55.62	61.22	72.46	64.83	49.78	61.97	33.28

tion, especially for children or hypersensitive patients, seems highly risky if not hazardous; and should be preceded by individual provocation tests. In the presented study, although the applied techniques of enzymatic modification resulted in significantly reduced immunoreactivity of pea proteins, their antigenicity still remained at a high level (Table1). It might be due to the fact that patients' IgE recognised mainly the epitopes occurring in vicilin fraction. The presented results concern the use of antibodies produced against particular pea proteins fractions to indicate that they are the most resistant to enzymatic hydrolysis and applied methods of chemical modification. Astwood *et al*. [2002] reported high resistance of vicilins to digestion and technological processes applied to food. Using acetic anhydride for acylation of pea protein hydrolysate reduced antigenic activity by another 4-11% compared to the samples hydrolysed only with Alcalase; their allergenicity was lowered approximately by 69% (Table 1). Combining various modification methods may turn out a valuable guideline in the search for a non-allergenic product. It was found that the succession of modification types is of key significance in the elimination of allergenicity. The enzymatic hydrolysis of proteins previously modified with acetic anhydride did not have a positive effect on their allergenicity. It was found that allergenicity of thus modified pea proteins even increased from 62% to over 64% on average. In some individual cases (serum samples No 5, 15 or 24), the increased turned out to be considerable (10-24%). The breakdown of peptide bonds caused by enzymatic hydrolysis may lead to the release of epitopes hidden inside the macromolecules [Clemente *et al*., 1999b; Mahmoud *et al*., 1992].

While preparing a product with reduced allergenicity it is most often tested with the so-called "pool serum". The results presented in Table 1 show that individual reactions of particular patients may vary significantly, which results from the fact that each patient may be allergic to a different pea protein. The process applied to modify proteins may change them to a different extent; therefore, it is vital that methods enabling a reduction of allergenicity of all proteins should be used or that different types of modification should be applied simultaneously.

CONCLUSIONS

In the study it was shown that chemical modification (acetylation or succinylation) and enzymatic hydrolysis used successively can be applied to reduce the allergenicity of pea proteins. Chemical modifications are not so often applied in the food industry regarding complex procedures of removing chemical agents and biological inactivation of amino acids, particularly lysine. The application of chemical modification is still controversial regardless the fact that the modified proteins are characterised by better functional and immunoreactive properties as it has been shown. Combining various methods of protein modification may be recommended for preparing products with reduced allergenicity which could be used as food additives.

ACKNOWLEDGEMENTS

This study was financed under the grant No. PO6T07826.

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Received February 2008. Revision received April and accepted May 2008.