EFFECT OF ACYLATION AND ENZYMATIC MODIFICATION ON PEA PROTEINS
ALLERGENICITY

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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 [Bredelhorst & David, 2001].

The most popular methods used to reduce protein allergenicity are enzymatic processes [Clemente et al., 1999a; Gauthier et al., 2006, Péñas 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-
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-allergic 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-allergic 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 x g), dialysed against 0.05 mol/L NH₄HCO₃, 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 lyophilized 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 [Meijaumb-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 mA/U/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-phthalaldehyd (OPA) and 2-mercaptoethanol [Panasuik 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
Pea proteins allergenicity

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 of the reaction was stopped by 2 mol/L H₂SO₄. 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]:

\[ A \% = \left(1 - \frac{A_{\text{M}}}{A_{\text{N}}} \right) \times 100 \]

where: \( A_{\text{M}} \) – absorbance of modified sample, and \( A_{\text{N}} \) – 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 H₂SO₄. 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-Sephacore 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).

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 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 legumin (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).
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.

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.

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<tr>
<th>Kind of modification of pea proteins</th>
<th>Reduction of allergenicity (%)</th>
<th>Number of serum</th>
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<td></td>
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<td>2</td>
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<tr>
<td>Alcalase hydrolysis</td>
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<td>Acetylation</td>
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<td>Acetylation and Alcalase hydrolysis</td>
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REFERENCES