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# EXAMINATION OF IMMUNOGENIC PROPERTIES OF HYDROLYSED MILK AND PEA PROTEINS – APPLICATION OF IMMUNOBLOTTING TECHNIQUE

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The paper describes an application of immunoblot analysis of two different protein materials (pea and milk) hydrolysed with selected enzymes (Alcalase, pronase and papain).

"One-step" hydrolysis of whey protein concentrate (WPC) resulted in hydrolysates characterized by insignificantly decreased immunoreactivity of both α-lactalbumin (α-la) and β-lactoglobulin (β-lg). "Two-step" hydrolysis of WPC in pronase-Alcalase system contributed to greater lowering of immunoreactive properties of WPC. When sodium caseinate was hydrolysed in both "one-step" and "two-step" system, the immunoreactivity of casein proteins (α-casein, β-casein and κ-casein) was lowered so that there were observed no reactions with polyclonal antibodies directed against α-, β-, and κ-casein on immunoblotting membranes.

Hydrolysis of pea proteins with Alcalase resulted in significant lowering of vicillin immunoreactivity and much higher lowering of legumin immunoreactivity. It was found by immunoblotting method that there were no reactions between pea hydrolysed proteins and anti-legumin antibodies.

## INTRODUCTION

Recent advance in analytical techniques has greatly contributed to enriching the research facilities and general knowledge. Electrophoretic and immunochemical (ELISA, immunoblotting) techniques have played a crucial role in recognition of food composition.

The method of transferring electrophoretically--separated proteins onto porous membranes under the influence of electric current has been called *western blotting*. This technique was used for the first time at the end of 1970s [Towbin *et al.,* 1979]. Proteins transferred onto membranes (nitrocellulose NC, made of nylon or polyvinylidene fluoride PVDF) can be subjected to various processes. Protein replicates have been most often applied for the reaction with antibodies - *immunoblotting*, elution of proteins from membrane, amino acid analysis, protein sequencing, and reactions with probes other than antibodies. The immunoblotting has a wide range of applications from research fields allowing detection of a particular protein to diagnostic fields methods aiming at detection of causes of viral and bacterial diseases, some necroses and allergies.

The immunoblotting is a method commonly used in diagnostics for identification of properties of important protein allergens, characteristics of antibodies from different samples of polyclonal serum, and purification of specific antibodies from the polyclonal serum.

Allergens are components commonly occurring in food. Most often allergies are caused by the following food products: cow milk, eggs, peanuts, soybean, fish, crustacea, and wheat [Bruijnzell-Koomen, 1995; Matsuda *et al.*, 1993]. Nowadays, allergenic diseases constitute a serious medical and societal problem since they involve *ca.* 25% of the human population.

In the light of the latest studies, it is claimed that immunogenic properties of food products are determined mainly by proteins of the molecular weight ranging from 15 to 40 kD [Leszczyńska, 1999], but there have also been noted incidences of allergic reactions to peptides with molecular weight of *ca.* 3 kD as well as to proteins with the molecular weight over 70 kD [Barej, 1996]. A tendency for the application of protein isolates with a high content of short-chain peptides and amino acids in the production of hypallergenic food as well as for elimination of allergenic fractions from protein raw materials seems therefore highly reasonable [Schmidl *et al.*, 1994]. A great importance is ascribed also to the possibility of modifying protein properties upon the application of physical, chemical and biotechnological processes, including enzymatic modifications [Kananen *et al.*, 2000; Adler-Nissen, 1986].

One of the main methods applied to modify proteins is enzymatic hydrolysis with the use of proteolytic enzymes [Gonzales-Tello *et al.*, 1994]. Hydrolytic activity of the enzymes towards proteins changes their properties leading to increased availability and solubility, decreased allergenicity and viscosity, *etc.* [Adler-Nissen, 1976]. These properties are highly determined by the degree of hydrolysis which depends on hydrolysis conditions, namely type and specificity of the enzymes, substrate and enzyme concentrations, pH, temperature, and time of reaction. While the degree of hydrolysis of proteins depends on their

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further application. Protein hydrolysates are used as components of *e.g.* milk-replacing-, dietetic- and food--enriching formulas. In order to obtain the most beneficial (from the nutritional point of view) ratio of amino acids to di- and tripeptides, the proteins should be hydrolysed with the use of exo- and endoproteases. Such an activity is shown by pancreatin. Moreover, hydrolysis of proteins can be performed with non-selective plant enzymes: papain, bromelanin and ficin; or more selective enzymes of microbiological origin, *e.g.* Alcalase (serine protease isolated from *Bacillus subtilis*).

The presented work is a part of research focused on the possibilities of lowering immunoreactive properties of food proteins. The aim of this work was to determine the effect of enzymatic hydrolysis on the immunoreactive properties of milk whey and pea proteins with the use of immunoblotting technique.

## MATERIALS AND METHODS

**Materials**. The study involved enzymatic hydrolysis of proteins from cow milk and pea seeds. The experimental material consisted of whey protein concentrate (WPC) and sodium caseinate, originating from the Laktopol company, Suwałki, Poland; and seeds of Kwestor pea variety originating from Plant Breeding Station of Poznań Sp. z o.o. in Tulce near Poznań, Poland.

Hydrolysis was carried out with Alcalase 2.4 L FG (*Subtilisina Carlsberg*, Novo Nordisk), pronase isolated from *Streptomyces griseus* (Sigma), and papain - EC 3.4.22.2 (Sigma). Proteolytic activity of the enzymes, determined according to the method of Anson [Mejbaum--Katzenellenbogen & Mochnacka, 1969], reached 1.83 AU/mL for Alcalase, 10.12 AU/g protein for pronase, and 3.15 AU/g protein for papain.

**Hydrolysis**. Enzymatic hydrolysis was performed in a pH-stat, with constant stirring, at a temperature of 50°C and pH optimal for respective proteolytic enzymes. During the hydrolysis process, pH was maintained at a constant level by dosing 0.5 M solution of NaOH from a burette. The enzymes were added as water solutions (1 mL) in the amount of 15 mAU/g protein.

The proteins were extracted from pea flour with 0.1 M NaCl solution (pH 8). After extraction, the crude extract was diluted, so the protein content in the solution was 4%, then pea protein solution was hydrolysed with Alcalase for 120 min. Next, the hydrolytic enzyme was inactivated by heating at a temperature of 90°C for 5 min. After cooling out, the hydrolysates were frozen, freeze-dried and subjected to further analysis.

Hydrolysis of 10% milk protein solution was conducted in a "one-step" and "two-step" system. The "two-step" hydrolysis was carried out with the enzyme systems: pronase-Alcalase and Alcalase-papain. "Two-step" hydrolysis was carried out with the first enzyme for 100 min. Next, the process was stopped by inactivation of enzymes by heating at a temperature of 90°C for 5 min. The mixture was cooled down to the temperature optimal to the next hydrolytic enzyme to be used and then hydrolytic process was continued till the DH was constant. Next, the hydrolytic enzyme was inactivated as described above. After cooling out the hydrolysates were frozen, freeze-dried and subjected to further analysis.

**Electrophoresis.** Electrophoretic separations of protein extracts were performed with 15% polyacrylamide gel (SDS--PAGE) according to Laemmli [Laemmli, 1970]. 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 Coomassi 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) [Towbin *et al*., 1979].

In order to detect antigenic fractions, the membrane was incubated overnight at 4°C in serum solution containing rabbit polyclonal antibodies against analysed proteins. Antigen-antibody complexes were stained on the membrane by placing them in the solution of species-specific antibodies, horseradish peroxidase conjugated goat anti-rabbit 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.

For the estimation of electrophoretic protein separation and electrotransfer efficiency, gels were stained with a Coomassi Brilliant Blue R-250.

The blots were probed with anti-pea proteins (anti--legumin and anti-vicilin) antibodies or with anti-milk proteins (anti-α-lactalbumin, anti-β-lactoglobulin, anti-α--casein, anti-β-casein, anti-κ-casein) antibodies.

The α-la, β-lg, α-casein, β-casein and κ-casein antigens were purchased from Sigma Co., and legumin and vicilin antigens were isolated and purified according to the method of Freitas *et al.* [2000].

**Production of polyclonal antibodies.** Polyclonal antibodies were obtained through subcutaneous and intramuscular quadruple rabbit immunisation at 2-week intervals. Each time, 1 mL solution containing 100 mg protein was used. The first immunisation was performed in the presence of complete Freund adjuvant, and the subsequent - in the presence of incomplete Freund adjuvant. Indirect ELISA method was used to determine the titre of the antibodies obtained.

## RESULTS AND DISCUSSION

Electrophoregrams of pea protein hydrolysates obtained with the use of Alcalase were presented in Figure 1A. Crude extract of pea proteins (position 2) was characterised by the presence of fractions with the molecular weight ranging from 14.2 to 66 kD, which was typical of pea proteins. As a result of enzymatic hydrolysis with Alcalase, fractions with a high molecular weight were found to disappear, while those with molecular weight of 14-20 kD as well as peptides were found to increase (Figure 1A). Not later than after 10 min of hydrolysis, disappearing of fractions with the highest molecular weight (over 50 kD)

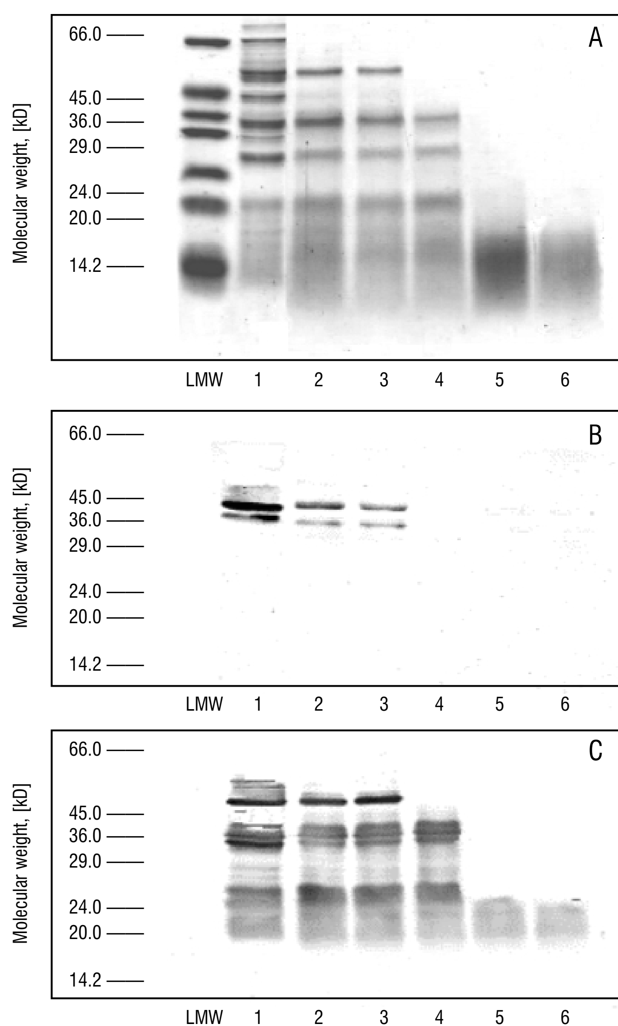


FIGURE 1. A. SDS-PAGE separation of pea proteins and hydrolysates with Alcalase; B. Immunoblotting with anti-vicilin rabbit antibody; C. Immunoblotting with anti-legumin rabbit antibody.
1. pea proteins; 2. after 10 min-hydrolysis; 3. after 20 min-hydrolysis; 4. after 40 min-hydrolysis; 5. after 90 min-hydrolysis; 6. after 120 min-hydrolysis.

was observed (Figure 1B). At the same time, a high resistance was observed of vicilin fraction with the molecular weight of *ca*. 50 kD to the activity of Alcalase. This vicilin fraction disappeared no sooner than after 40 min of hydrolysis (Figure 1C). The immunometric method applied ensured identification of the presence of vicilin fraction with the molecular weight of *ca.* 30 kD in the hydrolysate. This protein disappeared in the hydrolysate after 90 min of hydrolysis process. Vicilin fraction with the molecular weight of *ca.* 20 kD appeared the most resistant to hydrolysis with Alcalase. The results obtained allow assuming that the structure of epitopes occurring in a particular fraction and responsible for reactions with specific antibody remains unchanged under hydrolysis conditions.

Legumin fraction present in the hydrolysed pea extract was found more susceptible to the activity of Alcalase. After 40 min of hydrolysis, the extract was devoid of proteins the epitopes of which would be able to react with the antibody produced against legumin fraction (Figure 1B).

Alcalase is an endopeptidase with relatively low specificity of activity which attacks peptide bonds at random, thus ensures a high degree of hydrolysis with this enzyme and a rapid decrease in the immunoreactivity of the investigated pea proteins. Mahmoud *et al.* [1992] claimed that a rapid antigenicity decrease during hydrolysis with Alcalase might have been caused by immediate destruction of surface epitopes characterised by a high activity. Proceeding hydrolysis, however, may destroy more stable epitopes which can be either more stable surface epotipes or partially masked and potentially protected epitopes occurring in the inner part of protein molecules. The remaining antigenicity may result from the presence of monovalent fragments of epitopes [Mahmoud *et al.*, 1992].

Clemente [1999] performed studies on the effect of enzymatic hydrolysis with Alcalase (at temp. 50°C, pH 8.0) on the protein quality and antigenicity of protein isolates of chickpea. At a high degree of hydrolysis (DH 27%), the hydrolysate demonstrated the presence of high amounts of small peptides and free amino acids in the SDS-PAGE image, compared to proteins with a low molecular weight. Contrary to pea proteins, hydrolysates of chickpea proteins demonstrated the presence of legumin fraction proteins after hydrolysis with Alcalase, although base peptide chains of this fraction bound strongly with IgE antibodies than acidic units [Clemente, 1999].

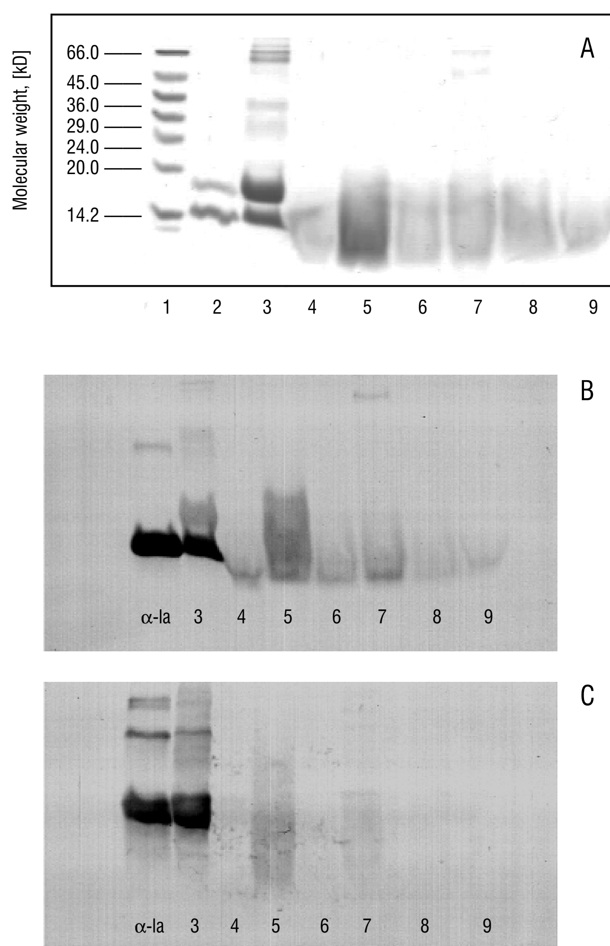


FIGURE 2. A. SDS-PAGE separation of whey milk proteins (WPC) and hydrolysates. B. Immunoblotting with anti-α-lactalbumin rabbit antibody C. Immunoblotting with anti-β-lactoglobulin rabbit antibody.
1. LWM standard; 2. α-la and β-lg; 3. WPC; 4-9 hydrolysates WPC with;4. Pronase; 5. Papain; 6. Alcalase and papain; 7. Alcalase; 8. Pronase; 9. Pronase and Alcalase.

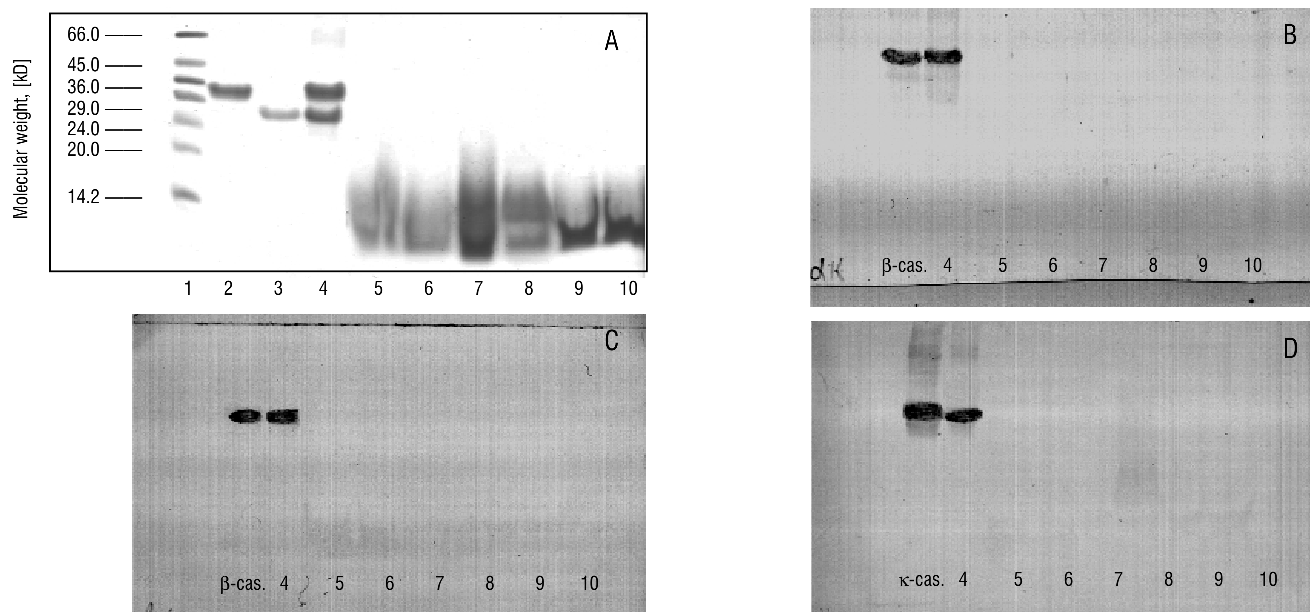


FIGURE 3. A. SDS-PAGE separation of isolate of sodium caseinate proteins and hydrolysates. B. Immunoblotting with anti-α-caseine rabbit antibody; C. Immunoblotting with anti-β-caseine rabbit antibody. D. Immunoblotting with anti-κ-caseine rabbit antibody. 1. LWM standard; 2. α-caseine; 3. β-caseine; 4. sodium caseinate; 5-10. hydrolysates of sodium caseinate with; 5. pronase; 6. papain; 7. Alcalase and papain; 8. Alcalase; 9. pronase; 10. pronase and Alcalase.

Upon electrophoretic separation, fractions of α-la and β-lg as well as traces of other fractions of milk proteins with the molecular weight of *ca.* 33 kD (α-casein), 24 kD (β-casein), and *ca.* 66 kD were observed in the WPC. The major components of WPC are α-la and β-lg, but other proteins (casein, BSA) could be observed, which results from the industrial technology of WPC production. In all hydrolysates, fractions α-la and β-lg were hydrolysed (Figure 2A). It can be seen in the electrophoregrams as a wide band reflecting the presence of peptides with molecular weight lower than 14.2 kD.

The immunoblotting of the WPC concentrate and its hydrolysates indicated a weak binding reaction of protein fractions (<14.2 kD) present in all hydrolysates with polyclonal antibodies against α-la (Figure 2B). The use of antibodies against β-lg enabled observation of their reactions with protein fractions of WPC hydrolysate obtained with the use of papain (Figure 2C).

In the electrophoretically-analysed (SDS-PAGE) sodium caseinate, two fractions of casein were present: α-casein (*ca.* 33 kD) and β-casein (*ca.* 24 kD) (Figure 3A). Hydrolysates of sodium caseinate obtained with the use of papain and Alcalase contained proteins with the molecular weight of *ca.* 14 kD (technological contamination of sodium caseinate with whey proteins), while fractions of α-casein and β-casein were hydrolysed completely. Electrophoresis of hydrolysates obtained as a result of consecutive administration of two enzymes (Alcalase-papain and pronase-Alcalase) confirmed the presence of polypeptides and peptides, which was demonstrated by a wide band composed of units with the molecular weight lower than 14.2 kD. Sensitivity of immunoblotting of the sodium caseinate hydrolysates enabled finding out that none of the hydrolysates contained epitopes binding antibodies against α-, β- and κ-casein, which seems to confirm the reduction of substrate immunoreactivity (Figures 3B, C, D).

Rosendal & Barkholt [2000] analysed *in vitro* 12 infant formulas based on different proteins with various hydrolysis degree: non-hydrolysed Nan 1 (cow milk proteins), partially hydrolysed Nan HA, Beba HA, Nutrilon Pepti, Nutrilon Pepti Plus (whey proteins), Aptamil Hypoantigen (whey proteins and casein), Aptamil HA (bovine collagen and soybean proteins), extensively hydrolysed Alfare, Pepti Junior, Profylac (whey proteins), Pregomin (bovine collagen and soybean proteins), Nutramigen and Pregestimil (casein). The SDS-PAGE electrophoresis revealed the presence of molecules with the molecular weight above 20 kD [Rosendal & Barkholt, 2000]. The obtained hydrolysates contained two major allergens of cow milk whey, *i.e.* α-la and β-lg with the molecular weight of 14.2 and 18.6 kD, respectively. Technological processes used for the production of hypoallergenic formulae are not able to reduce totally the immunoreactivity of proteins which are the main material for their production. Therefore searching for new technological solutions is a necessity in science and their control should proceed with the application of immunometric techniques.

## CONCLUSIONS

The presented work shows that the effectiveness of enzymatic hydrolysis (used as the method of lowering the immunoreactive properties of food proteins) depends on the protein to be hydrolysed and the hydrolytic enzyme employed.

The most favourable lowering of the WPC proteins immunoreactivity was obtained in a "two-step" (pronase--Alcalase) system. A weak binding reaction of hydrolysed WPC protein with polyclonal antibodies against α-la and β-lg was shown by the immunoblotting technique, and therefore the application of "two-step" hydrolysis in lowering immunoreactive properties of milk whey proteins

is suggested to be more favourable as compared to "one-step" hydrolysis.

Hydrolysis of sodium caseinate in both "one-" and "two-step" system allowed lowering the immunoreactivity of casein proteins (α-casein, β-casein and κ-casein) to such extent that there were observed no reactions between proteins and polyclonal antibodies directed against α-, β-, and κ-casein on immunoblotting membranes.

Regarding pea proteins immunoreactivity, enzymatic hydrolysis can be considered as very effective tool which could be employed in order to lower the immunoreactive properties of legumin fraction. Lowering the immunoreactivity of vicilin fraction however appears not to be satisfactory in the systems examined, and hydrolysis effectiveness was shown to be dependent on the enzyme used and reaction conditions, therefore it is justified to carry out the searching for such hydrolytic system which could result in the total reduction of the immunoreactivity of pea proteins.

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