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# Immunoreactive properties of pea protein extract and its trypsin hydrolysates*

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ABSTRACT

The study was undertaken to examine the immunogenic potential of pea protein of Polish variety Rodan and its trypsin hydrolysates differing in degree of hydrolysis. The physicochemical characteristic of pea protein extract and its hydrolysates, DH 2.0 and 5.0, were determined by SDS-PAGE electrophoresis, chromatofocusing, affinity chromatography, and sequential analysis. The immunogenic properties of pea protein and its trypsin hydrolysates, DH 2.0 and 5.0, were investigated by direct and competitive ELISA methods. The results confirmed that protein extract is a stronger immunogen than hydrolysates, while hydrolysate DH 2.0 was a stronger immunogen than DH 5.0. The dominant antigen isolated from pea protein extract and both trypsin hydrolysates had a molecular weight of about 20 kDa and was in the glycoprotein fraction. The N-terminal sequence of this antigen was determined to be: Thr-Glu-Thr-Thr-Ser-Phe-Leu-Ile-Thr-Lys-.

KEY WORDS: pea protein, immunonogenic properties, glycoproteins

## INTRODUCTION

Pea seeds contain about 20-25% protein, depending on the variety. The majority of pea proteins consists of salt-soluble globulins, or storage proteins,

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that are synthesized during seed development, stored in protein bodies, and hydrolysed during germination to provide nitrogen and carbon skeletons for the developing seedling (Wang et al., 2003). The remainder are albumins that include many housekeeping proteins, lectins, and lipooxygenases. From the nutritional point of view, globulins are very important; they consist of two classes, 7S and 11S, based on their sedimentation coefficients. These proteins determine not only the nutritional value of peas for humans and animals, but also the immunogenic and allergenic potential of pea proteins (Hayakawa et al., 1999; Wensing et al., 2003). Before consumption by humans, pea seeds are thermally processed. This provides many benefits, but also brings about changes in allergenicity. It is necessary to recognize the full immunogenic potential of pea proteins in order to devise effective methods of reducing pea allergenicity. In particular, there is little information about the allergenic properties of glycoproteins. The authors hypothesise that many glycoproteins have not only allergenic properties, but can also undergo non-enzymatic glycosylation and be a source of neoallergens. Investigations of the immunogenic potential of protein enzymatic hydrolysates are also important from the practical point of view, because the hydrolysates usually have lower allergenic properties.

A study was undertaken to examine the immunogenic potential of pea protein isolated from the Polish pea variety, cv. Rodan.

## MATERIAL AND METHODS

Pea seeds of the Polish variety, cv. Rodan, were purchased from the Breeding and Seed Gardening Production Plant (Nochow, Poland) and the Plant Breeding Company (Łagiewniki, Poland).

*Extraction of proteins.* Pea proteins were extracted by McLeester's method (1973). 9.1 g of pea flour were suspended in 25 ml of buffer (0.5 M NaCl and 0.25 M ascorbic acid, pH 2.5) and homogenized for 5 min at room temperature. Proteins were extracted for 1 h at 5°C. The mixture was centrifuged at 30 000 *g* for 30 min at 4ºC. The supernatant was dialysed for 48 h at 5°C against distilled water and then lyophilized. The protein content in the extract was determined by the Bradford and Kjeldahl methods (Bradford, 1976; AOAC, 1984).

*Enzymatic hydrolysis of protein.* A solution of protein extract (3.57 g/32 ml 0.2 M phosphate buffer, pH 8.0) was prepared and heated to 50°C. Then 0.022 g of trypsin (15 mAU/g of extract) dissolved in 3.7 ml of phosphate buffer was added and the solution mixed. Enzymatic hydrolysis was performed at 50°C and kept at pH 8.0 by constant addition of a 1 M NaOH solution. Samples were taken after 0, 10, 20, 30, 60 and120 min of incubation. To a 5 ml aliquot of the hydrolysate, 5 ml of a 1% sodium dodecyl sulphate (SDS) solution were added and the mixture was

heated at 90°C for 15 min to inactivate the enzyme. The final volume was adjusted to 25 ml. This solution will be referred to as "the examined sample". The degree of hydrolysis (DH) of the extract was determined by the TNBS method.

TNBS assay was performed according to Adler-Nissen (1979) with the reagent volume being reduced to 50% of the original volume. In brief, to a 0.125 ml aliquot of the sample diluted 2.5-fold, 1 ml of a phosphate buffer (pH 8.2, 0.212 M) was added along with 1 ml of a freshly prepared aqueous solution of TNBS (0.1%). Tubes were shaken in the dark at 50°C for 1 h. The reaction was stopped by the addition of 2 ml of a 100 mM HCl solution. After 20 min 4 ml of water was added and after another 10 min the absorbance was read at 340 nm. The blank was prepared identically and L-leucine was used as the standard (0-5 x 10-7 mol/0.125 ml of sample).

The total content of α-amine groups (h/total) was determined according to Hajos et al. (1988). A solution of 0.5 g of protein extract in 10 ml of 6 M HCl was placed in a glass ampoule, saturated with nitrogen and closed. Hydrolysis was conducted at 105°C for 12 h. The solution was filtrated, neutralized with 6 M NaOH, and adjusted with phosphate buffer (pH 8.2) to a final volume of 100 ml. The content of α-amine groups was determined by the TNBS method (Adler-Nissen, 1979). The degree of hydrolysis (DH) was computed from the following equation (Adler-Nissen, 1979):

$$DH\ (\%) = h/htotal \times 100$$

where: h - number of the hydrolysed peptide bonds (mEq Leu - $NH_2$/g of protein); htotal - total number of peptide bonds in the protein (mEq Leu - NH2/g of protein). Two tryptic hydrolysates, DH 2.0 and DH 5.0 were prepared.

*Antibodies.* Antibodies were produced using six rabbits. Immunogene prepared for the first immunization contained 0.5 ml of antigen (1.5 mg of pea protein extract or its tryptic hydrolysates, DH 2.0 and 5.0) solution in 0.9% sodium chloride (2.0 mg/ml) emulsified with the equivalent of Freud's complete adjuvant (F 5881, Sigma, Poznań, Poland). Next, four immunizations were made at weekly intervals in the presence of Freud's incomplete adjuvant (F 5506, Sigma, Poznań, Poland) with the same volume and antigen concentration as described previously. All immunization injections were given subcutaneously. The production of antibodies and an increase in their titer were controlled using the indirect ELISA method by taking blood samples from the marginal vein of a rabbit 2-3 days prior to the subsequent scheduled immunizations. Ten days after the last immunization the rabbits were exsanguinated. Blood was incubated for 1 h at 30°C. Following centrifugation at 1500 *g* for 20 min, serum IgG antibodies were obtained at 20% saturation with sodium sulphate. After centrifugation at 1500 *g* for 30 min, the pellet was dissolved in a phosphate buffer of pH 8.8,

dialysed for 15 h at 4°C with a phosphate buffer being changed four times and the IgG fraction was lyophilized.

*Direct ELISA method (Engval and Perlman, 1971).* A microtitre plate (Nunc®) was coated with 1 µg/ml of antigen (pea protein extract or its tryptic hydrolysates DH 2.0 and 5.0) in 50 mM carbonate buffer of pH 9.8 and incubated overnight at 4°C. After washing with TPBS (10 mM of PBS, pH 7.4 with 0.5% of Tween 20), residual free binding sites were blocked with 150 µl/well of 1.5% gelatine (G 9382, Sigma, Poznań, Poland) for 30 min at 25°C. Next, the dilutions of rabbit blood serum (1:50 to 1:102400) samples were added to the antigen-coated and gelatine-blocked well for 1 h at 37°C. After washing, the plate was incubated for 1 h at 37°C with 100 µl/well of peroxidase-conjugated goat anti-rabbit immunoglobulin G (A 6154, Sigma, Poznań, Poland), followed by washing and addition of *o*-phenylene-diamine dihydrochloride (P 8287, Sigma, Poznań, Poland). After 30 min of incubation, 100 µl/well of 4 M sulphuric acid were added to stop the reaction. Absorbance was read at 492 nm on an automatic plate reader (Reader 510, Organon Teknika, Brussels, Belgium).

*Competitive ELISA method (Engval and Perlman, 1971).* The microtitre plate (Nunc®) was coated with 100 µl/well of antigen (pea protein or its tryptic hydrolysates DH 2.0 and 5.0) in 50 mM carbonate buffer at a pH of 9.8 and incubated overnight at 4°C. After washing, residual free binding sites were blocked with 150 µl/well of 15% gelatine (G 9382, Sigma, Poznań, Poland) for 30 min at 25°C. Next, the solution of rabbit antibodies [(50 µl/well diluted with PBS (1:12000) and the sample examined (50 µl/well)] were added simultaneously to the antigen-coated and gelatine-blocked well for 1 h at 37°C. After washing, the plate was incubated for 1 h at 37°C with 100 µl/well of peroxidase-conjugated goat anti-rabbit immunoglobulin G (A 6154, Sigma, Poznań, Poland), followed by washing and addition of *o*-phenylene-diamine dihydrochloride (P 8287, Sigma, Poznań, Poland). After 30 min of incubation, 100 µl/well of 4 M sulphuric acid were added to stop the reaction. Absorbance was read at 492 nm on an automatic plate reader (teader 510, Organon Teknika, Brussels, Belgium).

*Affinity chromatography.* Sepharose 4B (column: 10 × 2.5 cm) was activated with bromine cyanide. 100 mg of the rabbit immunoglobulin against the antigen (pea protein extract) was bound to the activated Sepharose 4B. Next, 60 mg of antigens (tryptic pea protein hydrolysate DH 2.0 and DH 5.0) dissolved in 1 ml of PBS (pH 7.4) were loaded on the top of the column and left for 12 h at 4°C. The unbound antigens were washed out from the column with PBS until absorbance at 220 nm reached 0.02. The bound antigens were released from the column with 0.1 M glycine-HCl buffer (pH 2.8) at 0-2°C. The fractions were immediately alkalized with carbonate buffer (pH 8.2), dialysed against distilled water at 4°C, and lyophilized.

*SDS-PAGE electrophoresis.* Polyacrylamide gel at a concentration of 15% and an acrylamide-to-methylenebisacrylamide ratio of 37:1 was prepared according to Laemmli (1970). The separation of proteins was carried out with TRIS-glycine buffer (pH 8.3) and a voltage drop of 40 V/cm using a Flat Bed Apparatus FBE 300 (Pharmacia, Sweden). Proteins were stained with Coomassie brilliant blue R-250 and additionally glycoproteins were stained with Kit Pro-Q™ - Emerland 300 Glycoprotein Gel Kit (P-21855; Molecular Probes Inc. Eugene, OR, USA). The stained gels were scanned at 510 nm on a spectrophotometer (Specord M-40, Germany) with a scanner transport system.

The molecular weight of the protein bands was calculated from a standard curve prepared with Sigma standards (Mw range 6 500-205 000), using the KODAK 1D (3.5.4) programme.

*Sequential analysis.* The sequence analysis of the N-terminal fragments of the antigens was performed by a gas-phase sequencer (Model 491, USA) (Gendel, 1998). Phenylhydantoin derivatives of amino acids were analysed by an integrated HPLC system (Microgradient Delivery System, Model 140 C, USA).

## RESULTS

The protein content of pea seeds was 21.6%. The SDS-PAGE electrophoresis of pea protein extract showed sixteen bands with molecular weights ranging from 10 to 50 kDa (Figure 1). The maximum degree of

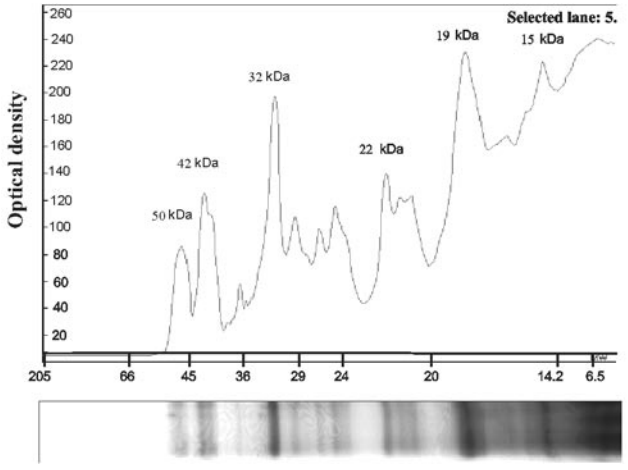


Figure 1. SDS-PAGE electrophoresis of pea protein extract

hydrolysis (DH 5.0) of pea protein extract by trypsin was reached after about two h (Figure 2). The electrophoretic separations of trypsin hydrolysates showed the presence of three main fractions with molecular weights of 14, 17 and 20 kDa. The 20 kDa fraction dominated (Figure 3). The changes in polyclonal antibody titre during immunization of the rabbits are presented in Figure 4.

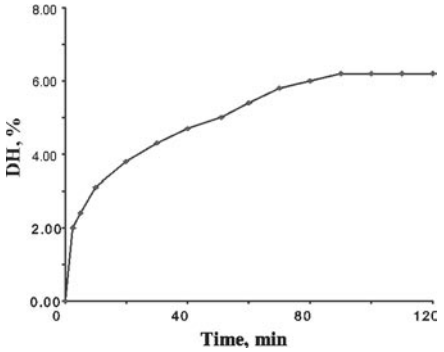


Figure 2. Kinetics of the hydrolysis of pea protein extract by trypsin

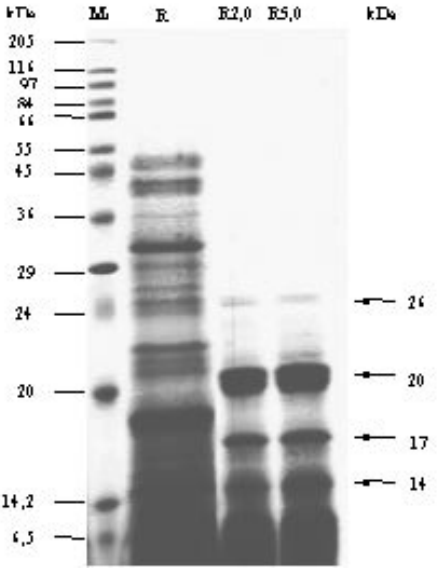


Figure 3. SDS-PAGE electrophoresis of pea protein extract and its trypsin hydrolysates (DH 2.0 and 5.0). Mw - markers, R –pea protein extract, R 2.0 and R 5.0 - trypsin hydrolysates with DH 2.0 and 5.0

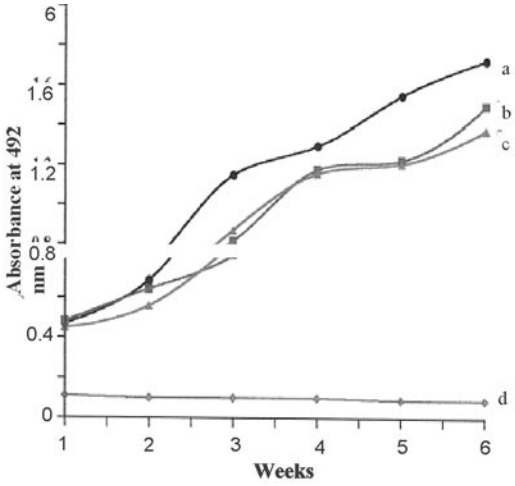


Figure 4. Changes of titre of polyclonal rabbit antibodies during immunization
a -●-●-●-● - pea protein extract
b -■-■-■-■- trypsin hydrolysate (DH 2.0) of pea protein extract
c -▲-▲-▲-▲- trypsin hydrolysate (DH 5.0) of pea protein extract

Statistically significant differences in the increase of rabbit antibody titres were observed between the pea extract and its trypsin hydrolysates with DH 2.0 and 5.0. These results showed (Table 1) that the pea protein extract displays a stronger

Table 1. Antibody titres against the pea protein extracts and its trypsin hydrolysates with DH 2.0 and 5.0

| Antigen | Antibody titre |
|---|---|
| Pea protein extract | 1 : 25600 |
| Trypsin hydrolysate (DH 2.0) | 1 : 6400 |
| Trypsin hydrolysate (DH 5.0) | 1 : 3200 |

immunogenic potential (1:25 600) than its trypsin hydrolysates with DH 2.0 (1:6 400) and DH 5.0 (1:3 200). The antigens were additionally characterized with the competitive ELISA test. This test enables determining the competition of antigens against the antibodies produced against pea protein extract (Figure 5). The results indicate that the pea protein extract was most strongly bound to the antibodies while the trypsin hydrolysates differed in competition in relation to antibodies (Figure 5). A higher affinity to the antibodies was shown by trypsin

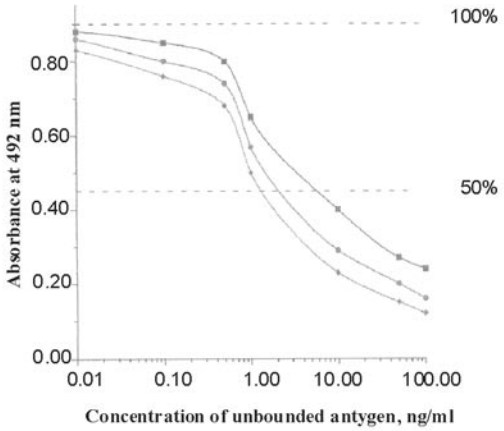


Figure 5. Compeptitive ELISA of pea protein and its hydrolysates
a -●-●-●-● - pea protein extract cv. Rodan
b -■-■-■-■- trypsin hydrolysate (DH 2.0) of pea protein extract cv. Rodan
c -▲-▲-▲-▲- trypsin hydrolysate (DH 5.0) of pea protein extract cv. Rodan

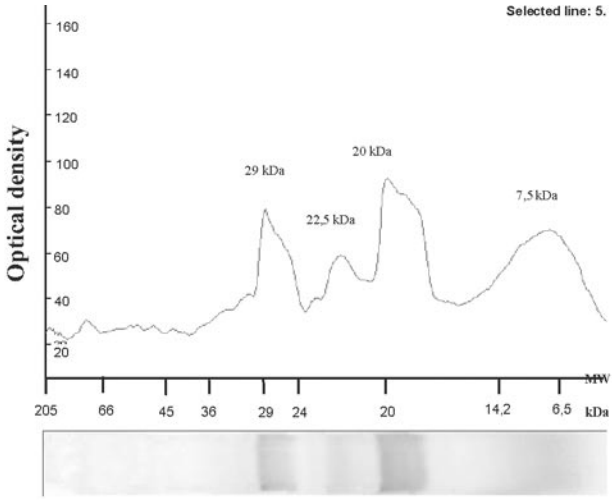


Figure 6. SDS-PAGE electrophoresis of antigen fraction of pea protein extract

hydrolysates with DH 2.0 than DH 5.0. The electrophoretic characterization of the antigens is presented in Figures 6 to 8. Three main bands with molecular weights of 7.5, 20.0 and 22.5 kDa were observed on all electrophoregrams. An

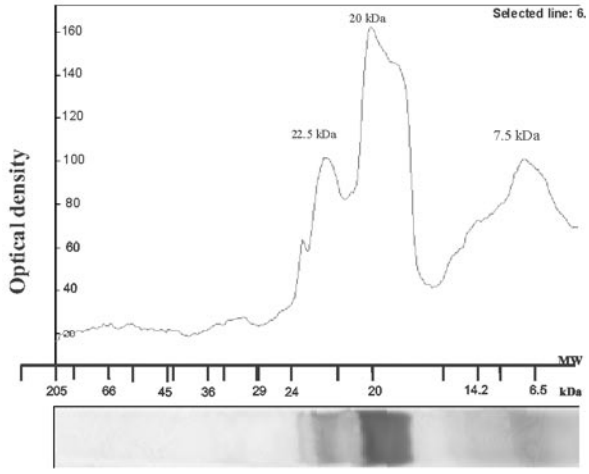


Figure 7. SDS-PAGE electrophoresis of antigen fraction of trypsin hydrolysate DH 2.0

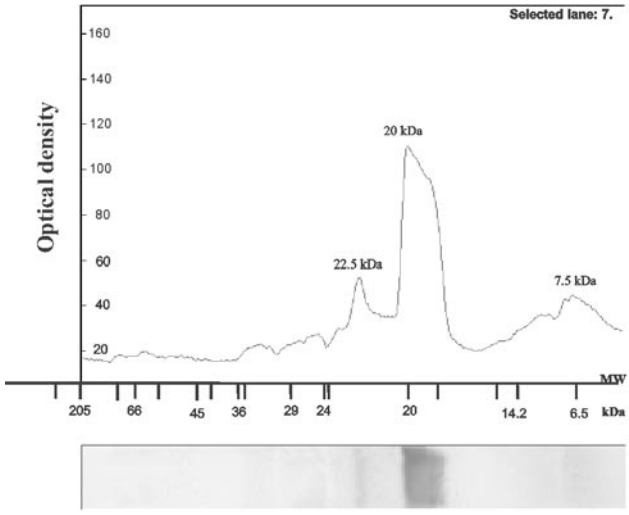


Figure 8. SDS-PAGE electrophoresis of antigen fraction of trypsin hydrolysate DH 5.0

additional band with a molecular weight of 29.0 kDa was found in the pea protein extract. The dominant antigen in all cases was in the band with a molecular weight of 20.0 kDa. The N-terminal of this antigen was found to have the sequence Thr-Glu-Thr-Thr-Ser-Phe-Leu-Ile-Thr-Lys. In addition, it was shown that this antigen is a glycoprotein with $pH_1$ 4.44 (Figures 9 and 10) (Hefle, 1996).


Figure 9. SDS-PAGE electrophoresis of antigen glycoproteins with molecular weights: 1-7.5 kDa, 2-20.0 kDa, 3-22.5 kDa, Mw - marker (44 kDa)

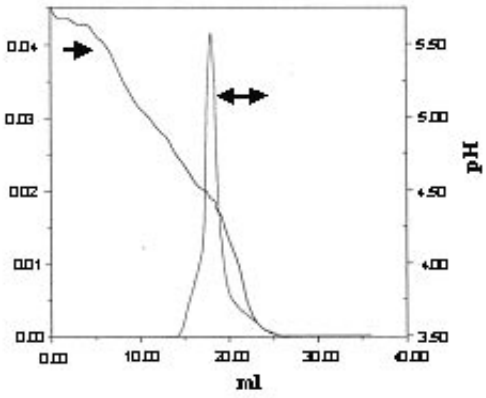


Figure 10. Diagram of isoelectric $pH_I$ of antigen glycoprotein 20.0 kDa
→ - gradient of pH
↔ - fraction of antigen glycoprotein 20 kDa

## DISCUSSION

The protein content in pea seeds depends on both the botanical variety of the pea and the abiotic conditions of the cropping. Gueguen and Barbol (1988) reported that the differences in the ratio of pea albumins to pea globulins range from 12 to 38% of total proteins. These facts may suggest that the immunogenic potential of pea proteins and their enzymatic hydrolysates can be different and dependent on the variety of pea. The protein content in pea seeds can influence enzymatic hydrolysis. Crevieu-Gabriel (1999) proved that the constituent proteins

of feed peas (i.e. globulins, albumins, insolubles) and their subfractions are highly variable in terms of their digestibilities. Protein structure, including hydrophobicity, glycosylation, beta-sheets, compact tertiary structure and disulphide bonds, can have negative a impact on protein hydrolysis. From the nutritional point of view, the immunogenic and allergenic potential of pea proteins is very important. Unfortunately, pea proteins are allergenic. In particular, the major potential allergens from pea are vicilin and convicilin (Sanchez-Monge et al., 2004). In the present study it was found that the pea protein extract was a stronger immunogen than its trypsin hydrolysates. That means that enzymatic hydrolysis reduces rather than eliminates the immunogenicity of pea proteins. These results seem to confirm the view of Ena et al. (1985) that the immunogenic potential of proteins and their hydrolysates depends not only on the amino acid sequences of the epitopes but also on their molecular weight and conformation. Our results also suggest that the cause of this phenomenon was the degradation of the antigen fraction with a molecular weight of 20.0 kDa. However, the difference in the immunogenic potential of the trypsin hydrolysates with DH 2.0 and 5.0 was probably caused by different amounts of antigens in the trypsin hydrolysates. Sanchez-Monge et al. (2004) proved that pea vicilin and one of its proteolytic fragments (32 kDa), reacted with more than half of the individual sera tested. Additional proteolytic subnits of vicilin (36, 16 and 13 kDa) bound IgE from approximately 20% of the sera. Our results proved that the fraction with a molecular weight of 20 kDa had the greatest antigenic potential, suggesting that this antigen could have allergenic properties. An additional argument for this suggestion can be the fact that this antigen is a glycoprotein with an isoelectric $pH_I$ of 4.44 (Hefle, 1996). According to Ueda and Ogawa (1999), many glycoproteins have the allergenic properties.

## CONCLUSIONS

Data obtained in this study demonstrated the presence of four antigens in the protein extract of pea cv. Rodan, and three antigens in its trypsin hydrolysates. Pea protein extract had a higher immunogenic potential than its trypsin hydrolysates. Moreover, the trypsin hydrolysates of pea protein differed in their immunogenic potentials. Thus, our results seem to confirm the thesis that the immunogenic potential of proteins and peptides is determined not only by specific amino acid sequences but also by their molecular weights. The glycoprotein fraction with a molecular weight of 20 kDa was the most immunogenic, which confirms the high immunogenic potential of glycoproteins. Finally, it can be assumed that pea proteins and their enzymatic hydrolysates are the potential allergens for humans and animals.

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