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IMMUNOGENIC POTENTIAL OF ANTIGENS ISOLATED FROM TRYPSIN PEA PROTEIN HYDROLYSATES

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Key words: pea protein, protein hydrolysates, immunogenic properties, glycoproteins

The study was undertaken to examine the immunogenic potential of pea protein of Polish cultivar Maraton and its trypsin hydrolysates differing in a degree of hydrolysis. The physicochemical characteristics of a pea protein extract and its hydrolysates, DH 2.0 and 5.0, was conducted by means of 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 confirm that the protein extract is a stronger immunogene than the hydrolysates, while hydrolysate with DH 2.0 was a stronger immunogene than that with DH 5.0. The dominant antigen isolated from the pea protein extract and both trypsin hydrolysates had a molecular weight of about 20 kDa and was in glycoprotein fraction. The N-terminal sequence of this antigen was determined to be: Thr-Glu-Thr-Thr-Ser-Phe-Leu-Ile-Thr-Lys. Its precursor is probably pea lectin.

INTRODUCTION

Peas (*Pisum sativum L.*) have a high nutritional value and are used as a constituent of animal feedstuffs or human diet. Pea seeds contain about 13.7–30.7% of protein, depending on cultivar [Tzitzikas *et al*., 2006; Frączek *et al*., 2007]. The majority of pea proteins consist of salt-soluble globulins or storage proteins that are synthesized during seed development, stored in protein bodies, and hydrolysed during germination to provide nitrogen and carbon skeletons for a developing seedling [Wang *et al*., 2003]. The others are albumins that include many housekeeping proteins, lectins, and lipooxygenases. Tzitzikas *et al*. [2006] proved that the total globulin content ranged from 49.2 to 81.8% of the total pea protein extract (TPPE). Legumin content varied between 5.9 and 24.5% of the TPPE. Vicilin was the most abundant protein of pea, and its content varied between 26.3 and 52.0% of the TPPE. Convicilin was the least abundant globulin, and its content ranged from 3.9 to 8.3%. Finally, these authors concluded that the globulin-related proteins were present in amounts ranging from 2.8 to 17.3%. Generally, globulins consist of two classes, 7S and 11S, based on their sedimentation coefficients: the 7S fraction (vicilin, convicilin) and 11S fraction (legumin). These two groups differ considerably in molecular weight and structure. Vicilin is a protein of 47-50 kDa, which can form trimers of a molecular mass of ~150 kDa [Tzitzikas *et al*., 2006]. Some of vicilins are glycosylated, and glycosylation occurs close to the C terminus of the γ fragment [Sheldon *et al*., 1996]. Convicilin is a protein of ~70 kDa, which can form trimers with a molecular mass of ~210 kDa [Casey, 2003; O'Kane *et al*., 2004]. Convicilin is not known to undergo any post-/co-translational modifications other than the removal of a signal peptide, and is not glycosylated [Newbigin *et al*., 1990]. Legumin is expressed as protein of 60 -80 kDa, which is usually present in a haxameric form [Casey & Domoney, 1999].

Pea is an important protein-rich European crop. In spite of this fact, peas and pea proteins are not widely used in food application due to the competitiveness of soybean. The increase of the competitiveness of peas in relation to soybean requires further research of the biological values of all pea proteins. One of those biological values are the immunogenic/ allergenic properties of the pea proteins and the products of their enzymatic hydrolysis [Sanchez-Monge *et al*., 2004].

The aim of this study was to examine the immunogenic potential of antigen fractions of a pea protein extract and its trypsin hydrolysates from Polish pea cv. Maraton.

MATERIALS AND METHODS

Materials

Pea seeds of the Polish cv. Maraton were purchased from the Plant Breeding Company (Łagiewniki, Poland).

Extraction of proteins

Pea proteins were extracted from pea seeds with McLeester's method [1973]. Pea flour (9.1 g) was suspended in 25 mL of buffer (0.5 mol/L NaCL and 0.25 mol/L 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 x g for 30 min at 4°C. The supernatant

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was dialysed for 48 h at 5°C against distilled water and then lyophilized.

Determination of proteins

Protein content of the extract was determined according to the Kjeldahl's method [AOAC, 1990].

Determination of the degree of hydrolysis (DH)

Pea protein extract was used for tryptic hydrolysis. A solution of the protein extract (3.57 g/32 mL, 0.2 mol/L phosphate buffer, pH 8.0) was prepared and heated to 50°C. Then, 0.022 g of trypsin (15 mAU/g of extract) resolved in 3.7 mL of phosphate buffer was added and the solution was mixed. Enzymatic hydrolysis was performed at 50°C and kept at pH 8.0 with constant addition of 1 mol/L NaOH solution. Samples were taken after 0, 10, 20, 30, 60 and 120 min of incubation. To 5 mL of hydrolysate aliquot, 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 solution was then transferred to a volumetric flask and adjusted to a final volume of 25 mL. This solution will be referred to as "the examined sample". The degree of hydrolysis (DH) of the extract was determined with the TNBS method.

TNBS assay was performed according to the method reported by Adler-Nissen [1979] with the reagent volume being reduced to 50% of the original volume. In brief, to 0.125 mL aliquot of the examined sample diluted 2.5-fold, 1 mL of a phosphate buffer (pH 8.2, 0.212 mmol/L) was added along with 1 mL of a freshly prepared aqueous solution of TNBS (0.1%). Tightly secured 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 mmol/L HCL solution. After 20 min, 4 mL of water were added and after another 10 min the absorbance was read at 340 nm. The blank was prepared identically and L-leucine was used as a 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 the method reported by Hajos *et al.* [1988]. The solution of 10 mL of 6 mol/L HCL and 0.5 g of protein extract was put in a glass ampoule and saturated with nitrogen. Next, the ampoule was closed by heating. Hydrolysis was conducted at 105°C for 12 h. The solution was filtrated and neutralized with 6 mol/L NaOH, and transferred to a volumetric flask and adjusted with phosphate buffer (pH 8.2) to a final volume of 100 mL. The content of α-amine groups was determined with TNBS method [Adler-Nissen, 1979]. The degree of hydrolysis (DH) was computed from the following equation [Adler-Nissen, 1979]: DH (%) $= h/h_{total}$ x 100, where: h – number of hydrolyzed peptide bonds (mEq Leu – NH_2/g of protein), and h_{total} – total number of peptide bonds in protein (mEq Leu – NH_2/g of protein).

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 hydrolysate, DH 2.0 and 5.0) solution in 0.9% sodium chloride (2.0 mg/mL) emulsified with 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 antigene concentration as described previously. All immunization injections were given subcutaneously. The production of antibodies and an increase in their titre 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 thus obtained was lyophilized.

Direct ELISA method [Engval & Perlman, 1971]

The microtitre plate (Nunc®) was coated with 1 μg/mL of antigen (pea protein extract or its tryptic hydrolysate with DH 2.0 and 5.0) in 50 mmol/L carbonate buffer of pH 9.8 and incubated overnight at 4°C. After washing with TPBS (10 mmol/L 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 1:102,400) samples were added to the antigen-coated and gelatine was well blocked 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 incubation, 100 μL/well of 4 mol/L 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 & Perlman, 1971]

The microtitre plate (Nunc®) was coated with 100 μL/well of antigen (pea protein or its tryptic hydrolysate with DH 2.0 and 5.0) in 50 mmol/L carbonate buffer of pH 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: 12,000)) 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 incubation, 100 μL/well of 4 mol/L sulphuric acid was 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 x 2.5 cm) was activated with bromine cyanide. Rabbit immunoglobulin (100 mg) against

the antigen (pea protein extract) was bound to the activated Sepharose 4B. Next, 60 mg of antigens (extract or tryptic pea protein hydrolysates with 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 the absorbance at 220 nm has reached 0.02. The bound antigens were released from the column with 0.1 mol/L glycine-HCL buffer (pH 2.8) at 0-2°C. The fractions were immediately alkalized with the carbonate buffer (pH 8.2), dialysed against distilled water at 4°C and lyophylized.

SDS-PAGE electrophoresis

Polyacrylamide gel with the concentration of 15% and an acrylamide to methylenebisacrylamide ratio of 37:1 were prepared according to the method described by Laemmli *et al*. [1970]. The separation of proteins was carried out with TRIS-glycine buffer (pH 8.3) and the 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. Eugenie 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 (Mr range 6500 – 205,000), by KODAK 1D (3.5.4) programme.

Sequential analysis

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

RESULTS

The content of protein in pea seeds cv. Maraton, expressed as the per cent of meal, was 20.3%.

The maximum degree of hydrolysis (DH 5.0) of the pea protein extract by trypsin was reached after about two hours (Figure 1).

The SDS-PAGE electrophoresis of the pea protein extract showed sixteen bands with molecular weight ranging from 14 to 55 kDa (Figure 2). The electrophoretic separations of trypsin hydrolysates showed the presence of three main fractions with molecular weights of 14, 17 and 20, and a fraction with molecular weight of ~ 26 kDa (Figure 2).

Changes in polyclonal antibody titre during immunization of rabbits are presented in Figure 3. 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 displayed a stronger immunogenic potential than its trypsin hydrolysates with DH 2.0 and 0.5.

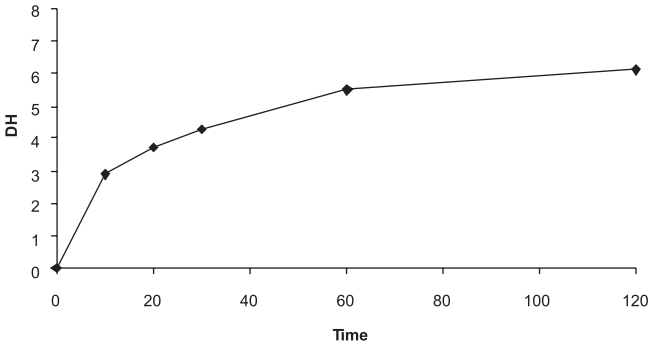

FIGURE 1. Kinetics of hydrolysis of the pea protein extract by trypsin.

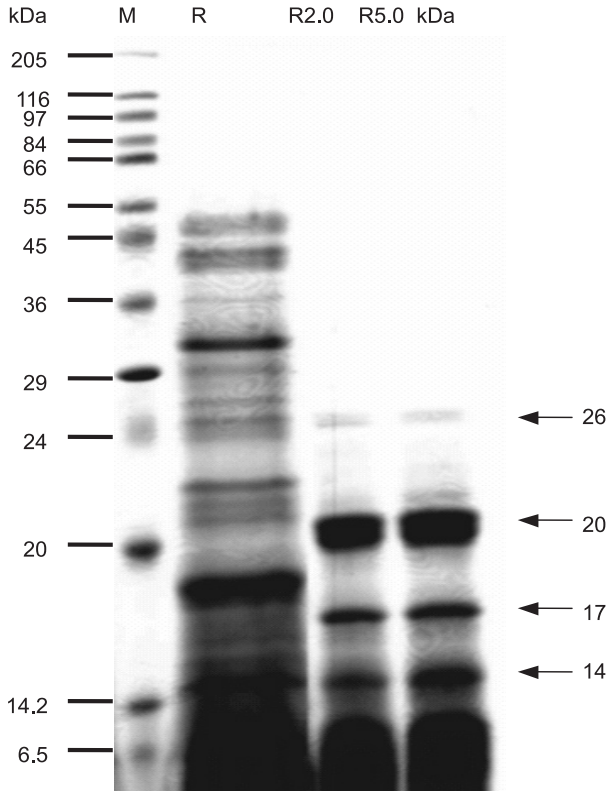

FIGURE 2. SDS-PAGE electrophoresis of the pea protein extract and its trypsin hydrolysates (DH 2.0 and 5.0). M – markers, R – pea protein extract, R 2.0 and R 5.0 – trypsin hydrolysates with DH 2.0 and 5.0.

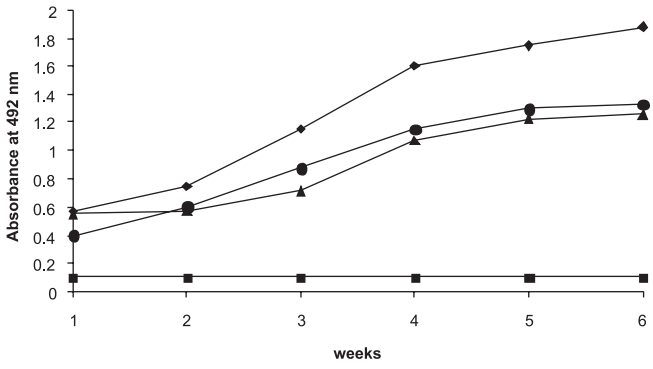

FIGURE 3. Changes of titre of polyclonal rabbit antibodies during immunization (-●-●-●-●- pea protein extract, -■-■-■-■- trypsin hydrolysate (DH 2.0) of pea protein extract, -▲-▲-▲-▲- trypsin hydrolysate (DH 5.0) of pea protein extract).

TABLE 1. Antibody titres against the pea protein extract and its trypsin hydrolysates DH 2.0 and 5.0.

Antigen	Antibody titre
Pea protein extract	1: 12800
Trypsin hydrolysate (DH 2.0)	1: 6400
Trypsin hydrolysate (DH 5.0)	1: 3200

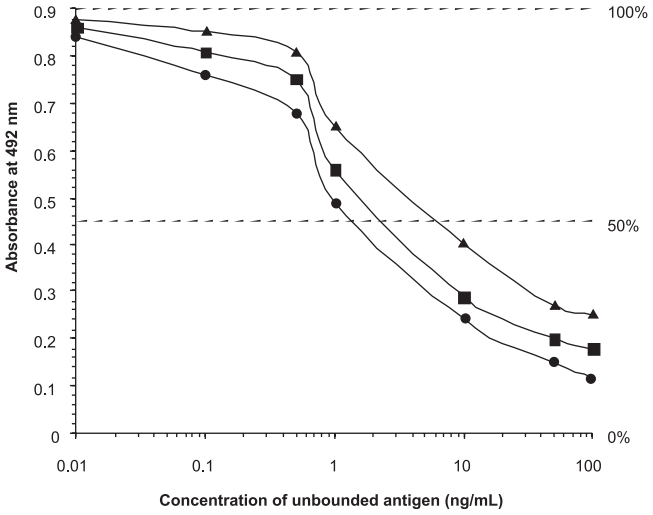

FIGURE 4. Competitive ELISA of pea protein and its hydrolysates (-●-●-●-●- pea protein extract, -■-■-■-■- trypsin hydrolysate (DH 2.0) of pea protein extract, -▲-▲-▲-▲- trypsin hydrolysate (DH 5.0) of pea protein extract).

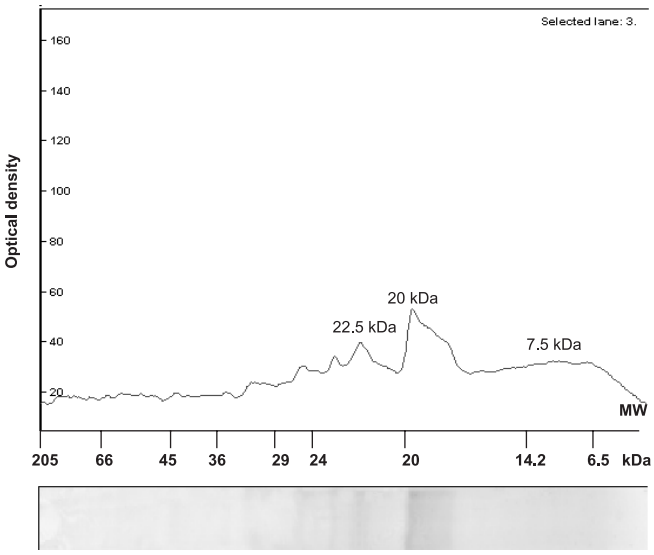

FIGURE 5. SDS-PAGE electrophoresis of an antigen fraction of the pea protein extract.

The antigens were additionally characterised with a competitive ELISA test (Figure 4). 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 4). Higher affinity to the antibodies was shown by trypsin hydrolysates with DH 2.0 than by those with DH 5.0.

The electrophoretic characterization of the antigens is presented in Figures 5 to 7. Three main bands with molecular weights of 7.5, 20 and 22.5 kDa were observed on all electrophoregrams. In all cases, the dominant antigen was in the band with a molecular weight of 20 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 was a glycoprotein (Figure 8).

DISCUSSION

A research by Gruppen *et al*. [1993] and our previous study [Frączek *et al*., 2007] indicated that low molecular pea proteins can also provoke the antigenic response. This fact suggests that the total immunogenic/allergic potential of pea proteins is not else fully characterised. An additional argument for this statement are the results concern-

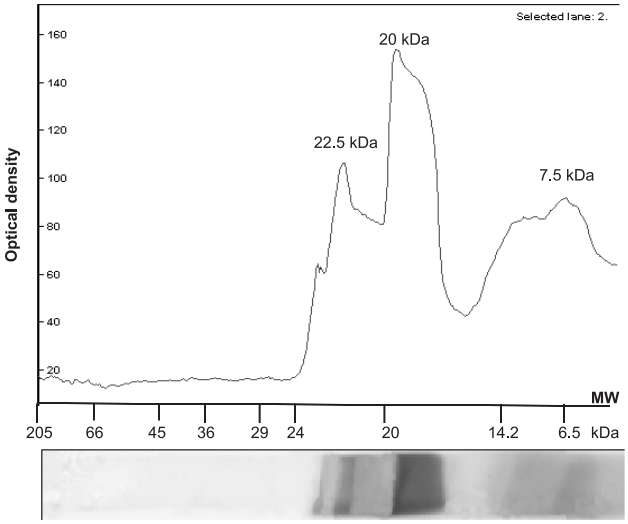

FIGURE 6. SDS-PAGE electrophoresis of na antigen fraction of trypsin hydrolysate DH 2.0.

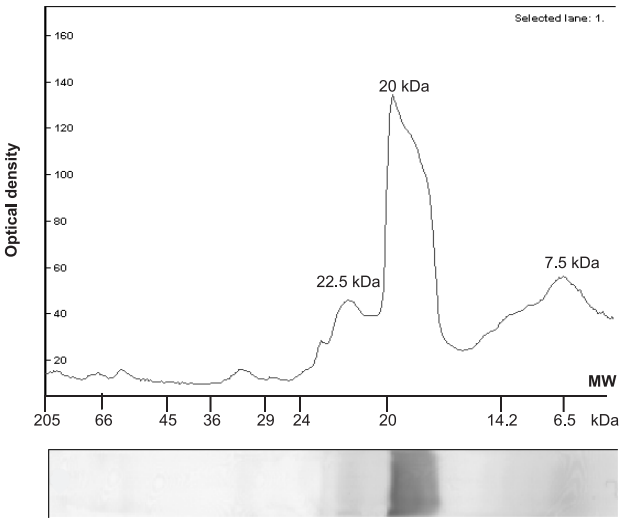

FIGURE 7. SDS-PAGE electrophoresis of an antigen fraction of trypsin hydrolysate DH 5.0.

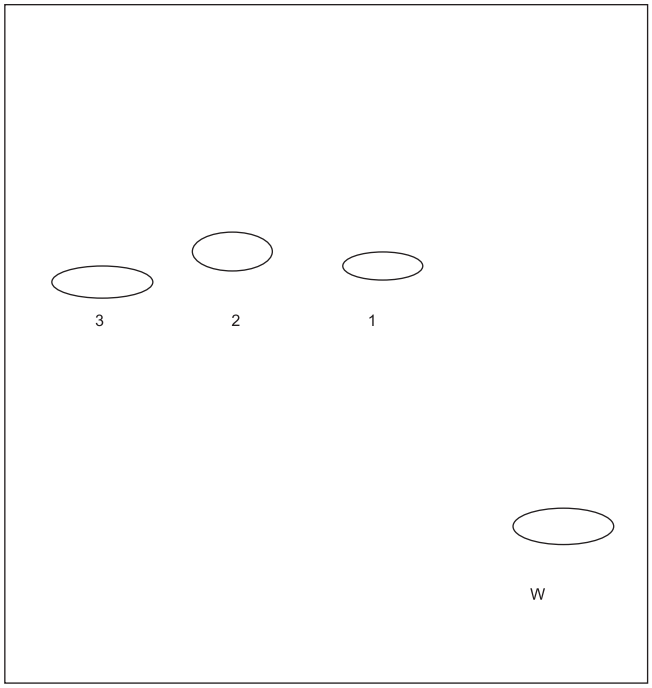

FIGURE 8. Schematic electrophoregram of pea protein fractions which reacted positively with reagent identifying glycoproteins: 1 – 7.5 kDa, 2 – 20.0 kDa, 3 – 22.5 kDa, W – marker (44.0 kDa).

ing the genetic variations in pea seed globulin composition [Tzitzikas *et al*., 2006]. Our early researches on pea protein extract from the pea seeds of the Polish cultivar Rodan [Frączek *et al*., 2007] proved that the strongest antigenic properties were displayed by glycoprotein with molecular weight of ~20 kDa and N-terminal sequence: Thr-Glu-Thr--Thr-Ser-Phe-Leu-Ile-Thr-Lys, which is in agreement with the results obtained in this work. The identified N-terminal sequence suggests that the precursor of this polypeptide could be pea lectin [Guzmán-Partida *et al*., 2004]. This result is different from findings of Gruppen *et al*. [1993], who demonstrated that a 29 kDa pea protein of cv. Solara showed the most distinct antigenic response. The obtained results seem to confirm the view of Ena *et al.* [1995] and Hyun Soo Shin *et al.* [2007] 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. This dependence confirms our earlier and present results. The same three main bands with molecular weights of 7.5, 20.0 and 22.5 kDa were observed on all electrophoregrams of the antigen fractions of pea protein extract cv. Rodan [Frączek *et al*., 2007] and cv. Maraton. An additional band with a molecular weight of 29.0 kDa was found in the pea protein extract of cv. Rodan. The presence of this fraction in the pea protein extract was probably the cause of twice higher an immunogenic potential of this extract in comparison with pea protein extract of cv. Maraton. Some confirmation of this statement could be the fact that this fraction was hydrolysed by trypsin and an immunogenic potential of these hydrolysates with DH 2.0 and 5.0 was the same both for protein hydrolysates of cv. Rodan and cv. Maraton.

CONCLUSION

Pea protein is characterised by a higher immunogenic potential than its trypsin hydrolysates. The trypsin hydrolysates of pea proteins differ in their immunogenic potential. The hydrolysates with a higher degree of hydrolysis show lesser immunogenicity. The obtained results seem to confirm the view that the immunogenic potential of proteins and peptides is determined not only by amino acid sequence but also by molecular weight and, in consequence, by their conformation.

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REFERENCES

1. Adler-Nissen J., Determination of the degree of hydrolysis of food protein hydrolyzates by trinitrobenzenesulfonic acid. J. Agr. Food Chem. 1979, 27, 1256–1260.
2. AOAC. 1990. Official Methods of Analysis, 15th ed; Association of Official Analytical Chemists: Washington DC.
3. Casey R., Pea legumins and vicilins. 2003, *in*: Idustrial Proteins in Perspective. Progress in Biotechnology (eds. W.Y Aalbersberg *et al*.). Elsevier Science: Amsterdam, The Netherlands, pp. 23, 55–62.
4. Casey R., Domoney C., Pea globulins. 1999, *in*: Seed Proteins (eds. R.P Shewry, R. Casey). Kluwer Academic Publisher: Amsterdam, The Netherlands, pp. 171–208.
5. Ena J.M., Van Beresteijn E.C.H., Robben J.P.M., Schmidt D.G., Protein antigenicity reduction by fungal proteinases and a pepsin/pancreatin combination. J. Food Sci., 1995, 60, 104–110.
6. Engval E., Perlman P., Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry, 1971, 3, 871–876.
7. Frączek R.J., Kostyra E., Kostyra H., Krawczuk S., Immunoreactive properties of pea protein extract and its trypsin hydrolysates. J. Anim. Feed Sci., 2007, 16, 472–484.
8. Gendel S.M., Sequence database for assessing the potential allergenicity of proteins used in transgenic foods. Adv. Food Nutr. Res., 1998, 42, 63–91.
9. Gruppen H., de Groot J., van Oort M.G., Identification and partial isolation of an antigenic protein *Pisum sativum cv. Solara.* 1993, *in*: Recent Advances of Research on Antinutritional Factors in Legumes Seeds. (eds. A.F.B. van der Poel *et al*.). Wageningen Press, The Netherlands, pp. 293–297.
10. Guzmán-Partida A.M., Robles-Burgueño M.R., Ortega-Nieblas I., Vázquez-Moreno I., Purification and characterization of complex carbohydrate specific isolectins from wild legume seeds: *Acacia constricta* is (vinorama) highly homologous to *Phaseolus vulgaris lectins*.. Biochimie, 2004, 4–5, 335–342.
11. Hajos G., Elias I., Halask A., Methionine enrichment of milk protein by enzymatic peptide modification. J. Food Sci., 1988, 53, 739–742.
12. Hyun Soo Shin, Sang Bum Kim, Soo Cheol Kang, Muhammad Ajmal Khan, Hyeon Shup Kim, Hyun Jung Shin, Chi Hoon

Chang, Production of low antigenic cheese whey protein hydrolysates using mixed proteolytic enzymes. J. Sci. Food Agric., 2007, 87, 2055–2060.
13. Laemmli U.K., Cleavage of structural proteins during the assembly of the head bacteriophage T 4. Nature, 1970, 227, 680–685.
14. McLeester R.C., Hall T.C., Sun S.M., Bliss F.A., Comparison of globulin proteins from *Phaseolus vulgaris* with those from *Vicia faba*. Phytochemistry, 1973, 2, 85–93.
15. Newbigin E.J., Do Lumen B.O., Chendler P.M., Gould A., Pea convicilin: structure and primary sequence of the protein and expression of a gene in the seeds of transgenic tobacco. Planta, 1990, 180, 461–470.
16. O'Kane F.E., Happe R.P., Vereijken J.M., Gruppen H., Van Boekel MAJS., Characterisation of pea vicilin. 2. Consequences of compositional heterogeneity on heat-induced gelation behavior. J. Agr. Food Chem., 2004, 52, 143–154.
17. Sanchez-Monge R., Lopez-Torrejon G., Pascual CY., Varela J., Martin-Esteban M., Salcedo G., Vicilin and convicilin are potential major allergens from pea. Clin. Exp. Allergy., 2004, 34, 1747–1753.
18. Sheldon P.S., Keen J.N., Bowles D.J., Post-translational peptide bond formation during concavalin A processing *in vitro*. Biochem. J., 1996, 320, 865–870.
19. Tzitzikas E.N., Vincken J.P., De Groot J., Gruppen H., Visser R G.F., Genetic variation in pea globulin composition. J. Agr. Food Chem., 2006, 54, 425–433.
20. Wang T.L., Domoney C., Hedley L., Casey R, Grusak M.A., Can we improve the nutritional quality of legume seeds? Plant Physiol., 2003, 131, 886–891.

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