

PROTEOLYTIC MODIFICATION OF SELECTED LEGUME FLOURS*

Barbara Baraniak, Michał Świeca

Agricultural University of Lublin

Abstract. The influence of pepsin (EC 3.4.1.1) and trypsin (EC 3.4.4.4) action on the chemical composition of legume flours was the aim of this study. The level of proteins and lipids in hydrolysed flours was changed significantly. In comparison to the raw flours also fatty acid composition in treated flours was altered. In the lentil flours both trypsin and pepsin digestion conditions have decreased the level of unsaturated fatty acid. It is noteworthy that in all investigated, hydrolysed flours ratio linoleic: oleic fatty acid was significantly decreased in comparison to unhydrolysed flours (about 40%-pea; 60%-lentil). Our investigations were also focused on the potential implementations of IMAC method in the separation and purification of peptides. Generally, peptides separation profiles, performed on immobilized Zn (II), were dependent on the kind of flour and enzyme used in the hydrolysis process. In the lights of our results is clearly visible that investigated peptides had a weak affinity to the chelated metal ions. It is noteworthy, that in some cases the influences of chelating factor on separation profiles were noticeable.

Key words: IMAC, legumes, lipid profile, peptides, proteolysis, zinc

INTRODUCTION

Legume seeds are a good source of proteins (about 30% of total composition). The level of protein is closely bound with the kind of plant and cultivation conditions. Flours obtained from milled grains have also high levels of starch, non-starch polysaccharides and other phytotherapeutic compounds [Duranti and Gius 1997, Duranti 2006]. Their usefulness in the food industry often is limited by presence toxic and antnutritive components. The quality of this product can be improved by enzymatic proteolysis. Over the last years, using limited protein digestion in food processing industry has increased rapidly. In legume flours case enzymatic proteolysis can remove their "beany" flavour,

* Project PBZ/KBN/P06/99 from the State Committee for Scientific Research 2001-2004 supported this research.

Corresponding author – Adres do korespondencji: Prof. dr hab. Barbara Baraniak, Department of Food Sciences and Biotechnology of Agricultural University of Lublin, Skromna 8, 20-704 Lublin, Poland, e-mail: barbara.baraniak@ar.lublin.pl

reduce level of polyphenols, phytic acid or proteases inhibitors [Periago et al. 1998]. Protein modification by enzymatic proteolysis can improve functional properties of protein isolates and also is used for the production of special food, such those destined for children, the old or sportsmen [Clemente 2000, Whitehurst and Low 2002].

The possibility to get peptides with desirable properties is one of benefits of limited proteolysis [Gill et al. 1996, Korhonen and Pihlanto 2006]. By use-selected condition in digestion process (kind of enzyme, time, pH, temperature, enzyme: substrate ratio) we are able to get peptide with biological function. Bioactive peptides have been defined as specific protein fragments that have an impact on the body function or condition and may ultimately influence health [Kitts and Weiler 2003]. Since today, a lot of studies have shown a numerous known peptides exhibiting, e.g., antioxidative, immunomodulatory, antifungal, antihypertensive activities [Gill et al. 1996, Korhonen and Pihlanto 2006].

It is necessary for complete characteristic of bioactive peptides to purify these peptides from his source. Isolation of peptides from food is difficult because they are present in a complex mixture containing various substances such as free amino acids, sugars, salts and acids. Generally, for separation of peptides are used classical, preparative, chromatographic methods based on their hydrophobicity, molecular size and net charge [Careri and Mangia 2003]. In the recent years in peptidomic has been notified an increase of employment immobilized metal ion chromatography (IMAC). Binding of peptides to immobilised metal ions is based on interaction between an electron-donating group of peptides and metal ions presenting accessible coordination sites. Although separation of peptides on IMAC mainly based on acting amino acids residues and the metal ions, many other factors govern their adsorption and desorption. Complex formation depends on chelate structure, type of metal ions, peptide structure, pH, type of buffer, ionic strength and a presence of detergents [Ueda et al. 2003].

Changes in the proteins, peptides content and lipids profile in enzymatically modified pea and lentil flours and characteristic of isolated peptides by IMAC method were the aim of ours studies.

MATERIAL AND METHODS

The raw materials used in this work were pea (*Pisum sativum*, cv. 'Grapis') and lentil (*Lens culinaris*, cv. 'Anita') seeds. Enzymatic treatment of the legume flours proteins involved two proteases, trypsin (EC 3.4.4.4; 1.13 BaEE U/mg) and pepsin (EC 3.4.1.1; 515 U/mg), commercially available by Sigma chemicals, St. Louis, Mo, USA. Hydrolysis was carried out for 30 minutes, in temperature 37°C, pH optimal for enzymes (trypsin 7.5, pepsin 2.5). Briefly, into 10 g of flour dissolved in 100 ml of buffer was added 10 ml of enzyme solution (50 mg/50 ml TRIS-HCl buffer, pH 7.5: enzyme: substrate ratio 1:10). Inactivation was made by heating in 90°C for 15 min. After that, hydrolysates were centrifuged 20 minutes (4000 × g). Supernatants and pellets obtained after centrifugation were regarded in the further analysis as hydrolyzates and flours after hydrolysis, respectively.

Crude protein contents in raw and hydrolysed flours were determined according to the micro-Kjedhal method (AOAC... 1990). Soluble proteins in hydrolysates were determined follow Lowry method with bovine albumin as a standard [Lowry et al. 1951].

Degree of hydrolysis (DH) was calculated in foothold about protein levels. Peptides from hydrolysed flours were isolated with 1% trichloroacetic acid (TCA) and their levels were determined in reaction with trinitrobenzenesulfonic acid (TNBS) [Hebeeb 1966].

Lipids were extracted with n-hexan in the Soxtec HT-6 and after those fatty acids analysis was performed by gas chromatography method on the Unicam 610. The degree of saturation (DU) was determined as described by Porzucek and Raznikiewicz [1990] using following equation:

$$DU = [1x (\%wt \text{ MUFA}) + 2x (\%wt \text{ DUFA}) + 3x (\%wt \text{ PUFA})]/100,$$

where: MUFA represents monounsaturated fatty acids,
DUFA – diunsaturated fatty acids,
PUFA – polyunsaturated fatty acids.

Peptides extracted from the treated flours and present in the hydrolysates were separated by IMAC method. Four grams of Sephadex G25 was mixed with 375 mg NaBH₄, 10 ml 2M NaOH and 1 ml of epichlorohydrine. After 2 hours incubation 10 ml 2M NaOH and 5 ml of epichlorohydrine were added. Gel activation was finished after 12 hours by washing in water. After drying gel was mixed with 25 ml of solution (5.3 g Na₂CO₃, 2.5 g iminodiacetic acid (IDA)) and incubated 12 hours in 60°C. In the next step, zinc ions (ZnSO₄, 1 mg/1 ml) were immobilized onto gel. Chromatographic column was equilibrated with 0.05 M Tris-HCl buffer pH 7.5. Peptides elution was performed with gradient of 0.05 M Tris-HCl buffer (7.5, 5.5, 4.5, 7.5) and their level was determined in reaction with TNBS.

RESULT AND DISCUSSION

Proteins in investigated flours were vulnerable on enzymatic treatment. It is noteworthy that pepsin was much more effective than trypsin. Level of proteins in hydrolysed flours was significantly lower in comparison with raw flour. In hydrolysed flours it was decreased about 58% in lentil flour treated with pepsin and the pea flours hydrolysed with pepsin and trypsin. Decrease about 35% was observed in the case of the lentil flours treated with trypsin (Table 1). Total lipids content in pea, raw flour was higher about 65% in comparison with lentil flour. Process of enzymatic digestion caused an increase of total lipids contents in hydrolysed flours (Table 1). We suggest the following explanations. Enzymes impact released large amount of proteins from flour. This action could influence on the percentage composition of flours (it was counted on 100 g of dry mass). It is also possible that lipids in hydrolysed flours were more vulnerable on extraction. According to chemical analysis (Table 1, 2) the protein and lipid contents in the investigated legume flours are correspond to the results obtained by others authors [Grela and Gunter 1995, Subagio 2006].

Fatty acid composition of the raw and hydrolysed flour is shown in Table 2. The results show the presence of saturated and unsaturated fatty acids. The dominant fatty acids in both pea and lentil flour were palmitic, oleic and linoleic. It is noteworthy that fatty acids concentration was changed in flours digested with trypsin and pepsin. Level of palmitic acid was increased in both trypsin and pepsin hydrolysates obtained from pea flour (22.7% and 21.03% respectively). Similar results were received in the case of lentil

Table 1. Proteins and lipids contents in the raw and treated pea and lentil flours
 Tabela 1. Zawartość białka oraz tłuszczu w surowych i trawionych mąkach grochu i soczewicy

Flour Mąka	Proteins content Zawartość białka %	DH*	Lipid contents mg/g of flour Zawartość tłuszczu mg/g mąki	DU
Pea – Groch				
raw – surowa	21.15	–	1.68	1.142
trypsin hydrolysis hydrolizowana trypsyną	11.66	9.8	1.91	1.467
pepsin hydrolysis hydrolizowana pepsyną	11.68	25.6	1.58	1.035
Lentil – Soczewica				
raw – surowa	30.46	–	1.02	1.155
trypsin hydrolysis hydrolizowana trypsyną	19.33	6.3	1.3	1.016
pepsin hydrolysis hydrolizowana pepsyną	16.83	17.9	1.35	1.056

*The data were already published in the Acta Sci. Pol., Biotechnologia 5 (1-2) 2006, 49-59.

*Wyniki zostały opublikowane w Acta Sci. Pol., Biotechnologia 5 (1-2) 2006, 49-59.

flour (48% and 39.3% respectively). The ratio linoleic: oleic acids, in obtained hydrolysates, have been changed after hydrolysis of raw pea and lentil flours. In pea flour this ratio was significantly decreased from 1.162 in raw flour to 0.762 (trypsin) and 0.883 (pepsin). This factor has changed also in lentil flours, from 1.538 to 0.772 and 0.635 respectively.

Baraniak et al. [2004] have also investigated changes in fatty acids composition of pea flours. The presence of high levels unsaturated fatty acids in the investigated flours is important from the nutritional point of view. Lower concentration of linoleic acid in hydrolysed lentil flour may suggest that it has better stability properties than hydrolysed pea flours. As suggested by Porzucek and Raznikiewicz [1990] relative high levels of unsaturated fatty acids in the investigated flour are potentially desirable because of health benefits.

Chromatograms obtained by separation of the pea and lentil hydrolysates show the highest concentration of peptides in the first eight fractions (Fig. 1). It is clearly visible that in these fractions are present at least two peaks. Probably there are peptides with the highest molecular weight and weak affinity to the investigated support. It is also noteworthy that trypsin treatment of pea flour generates much more peptides with affinity to immobilized zinc ions, than pepsin digestion. The effect of chelating ion factor during separation of pea hydrolysates was not significant. Pepsin treatment of lentil flour released three main fractions of peptides (fractions no. 1-6, fractions no. 20-23, fractions no. 54-59). Separation of trypsin hydrolysates, generated from lentil flour, was depended on the kind of zinc chelator. Contrary to OPS-Zn column, chromatograms performed on the column with Zn ions bound by IDA show three additionally peaks (fractions no. 9-12, fractions no. 18-22, fractions no. 29). High concentration of peptides was also detected in the fraction 51 during separation on the OPS – Zn support (Fig. 1 A). Chromatograms

Table 2. Fatty acids composition of raw and enzymatically modified pea and lentil flours, percentage of total fatty acids

Tabela 2. Zawartość kwasów tłuszczowych w surowych i enzymatycznie modyfikowanych mąkach grochu i soczewicy, procent całkowitej puli kwasów tłuszczowych

Fatty acid Kwas tłuszczowy %	Molecular name Wzór	Pea – Groch			Lentil – Soczewica		
		raw surowa	trypsin hydrolysis hydroliza trypsyną	pepsin hydrolysis hydroliza pepsyną	raw surowa	trypsin hydrolysis hydroliza trypsyną	pepsin hydrolysis hydroliza pepsyną
Myristic Mirystynowy	C _{14:00}	0.38	0.54	0.54	0.68	0.95	0.65
Myristoleic Oleomirystynowy	C _{14:01}	0.21	0.13	nd	nd	0.23	0.22
Palmitic Palmitynowy	C _{16:00}	15.01	16.2	19.33	14.71	17.05	15.48
Palmitoleic Oleopalmitynowy	C _{16:01}	nd	0.08	nd	nd	0.99	nd
Stearic Stearynowy	C _{18:00}	3.75	3.93	4.68	2.17	3.29	3.45
Oleic Oleinowy	C _{18:01}	34.16	41.38	37.34	28.39	39.1	45.95
Linoleic Linolowy	C _{18:02}	39.69	31.55	32.97	43.32	30.2	29.17
Linolenic Linolenowy	C _{18:03}	5.22	4.43	1.9	7.81	5.5	2.42
Arachidic Arachidowy	C _{20:00}	0.42	0.45	0.2	0.45	0.87	1.09
Eicosaenoic Gadoleinowy	C _{20:01}	1.16	1.31	3.05	2.47	1.83	4.58

nd – not detected.
nd – nie oznaczono.

received with material obtained from lentil, hydrolysed flours show high concentration of peptides (trypsin – 9.81 mg/ml, pepsin – 5.08 mg/ml). In spite of this fact any dominant fractions have not been observed, chromatograms shown a presence a lot of peaks with relatively low concentration of peptides (Fig. 1 B). Chromatograms of peptides isolated from the pea flours, treated with pepsin, exhibit a presence of peptides mainly in fractions no. 2-7 (separation on the OPS). Separation of the trypsinized, pea flour isolates on OPS support shows only one peak in fractions no. 37-40 (0.85 mg/ml). As it can be seen on Figure 1 peptides released from pea flours have had much higher affinity to zinc ions immobilized by OPS than IDA. Generally, separation profiles were conditioned by both, the kind of flour and enzyme used in hydrolysis and zinc-chelating factor (OPS or IDA). Differences between separation profiles generated on zinc ions immobilized by OPS and IDA can suggest that tested chelators bind metal ions distinctively.

By the principles of hard and soft acids and bases theory (HSAB) zinc belongs to the borderline acids and coordinates favorably with aromatic nitrogen atoms (borderline bases) and sulfur atoms (soft bases). It also known that zinc affinity to peptides is mainly con-

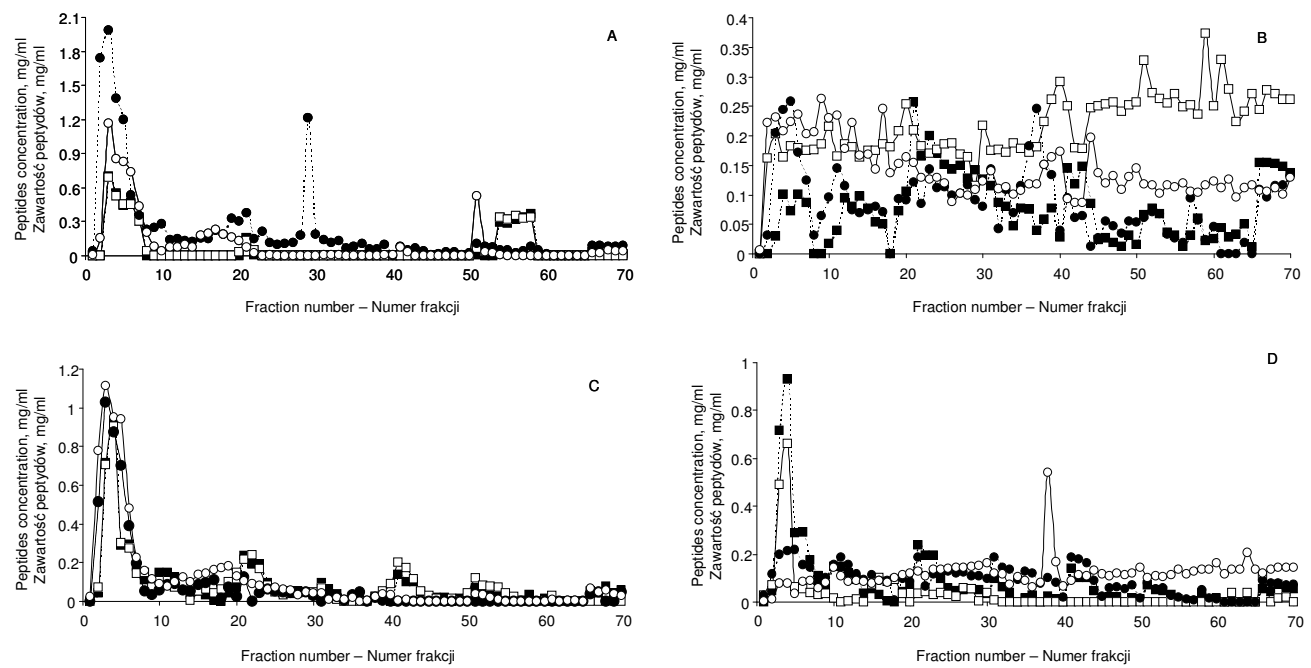


Fig. 1. Separation of peptides obtained by hydrolysis of legume flours: A – lentil flour hydrolysates, B – lentil hydrolysed flours, C – pea flour hydrolysates, D – pea hydrolysed flours. Legend: ---■--- pepsin Zn-IDA, —□— pepsin Zn-OPS, ---●--- trypsin Zn-IDA, —○— trypsin Zn-OPS

Rys. 1. Rozdziály peptydów otrzymanych w wyniku hydrolizy mąk strączkowych: A – hydrolizaty mąki soczewicy, B – hydrolizowana mąka soczewicy, C – hydrolizat mąki grochu, D – hydrolizowana mąka grochu. Legenda: ---■--- pepsyna Zn-IDA, —□— pepsyna Zn-OPS, ---●--- trypsyna Zn-IDA, —○— trypsyna Zn-OPS

ditioned by presence of histidine cluster. As shown in our study the retention strength depends on the kind of chelating factor. Different selectivity and adsorption activity towards separated peptides can be explained by differences in structure of the zinc ions complex with the IDA and OPS [Ueda et al. 2003]. Interactions between immobilized Zn ions and amino acids residues of peptides and protein were already studied. Binachi et al. [1994] investigated the interaction of mammalian and human protamines on the columns with Zn immobilized by IDA. In the case of human protamines P1 the strongest interactions were found to be related with presence of tyrosine, serine and threonine closely spaced to the histidyl side chain. Comparison studies of adsorption properties different metal ions (Cu, Ni, Zn) were performed by Yip et al. [1989]. They show that IDA-Cu (II) and IDA-Zn columns show very similar selectivity, however Zn ions display a weaker affinity for investigated bioactive peptides. Also Pasquinelli et al. [2000] identified several Zn fusion tags that can be used in purification of protein and peptides on Zn bound ions. The retention of peptides is primarily due to the metal affinities to their amino acid sequences. It is very difficult though to postulate their structure based only on their resolution on the immobilized metal.

CONCLUSIONS

Our studies clearly show that the investigated legume flours protein were good substrates for used enzymes. The hydrolysis process caused also positive changes in the lipids composition. Both trypsin and pepsin released a large amount of peptides. Generally, the influence of ions chelating factor on the separation of the hydrolysates and peptides isolated from the hydrolysed flours was not significant. It is also noteworthy that the investigated peptides show weak affinity to the immobilized Zn ions.

REFERENCES

- AOAC. Official Methods of Analysis 1990. Association of Official Analytical Chemists., Washington.
- Baraniak B., Niezabitowska M., Porzucek H., 2004. The content of total protein, trypsin inhibitor, and strachyoase in protein preparation coagulated from pea flour by various methods. *Food* 3(40), 87-97.
- Bianchi F., Rousseaux-Prevost R., Hublau P., Rousseaux J., 1994. Interaction of mammalian sperm nuclear protamines and peptides derived thereof with immobilized zinc. *Int. J. Pept. Protein Res.* 43(3), 410-416.
- Careri M., Mangia A., 2003. Analysis of food protein and peptides by chromatography and mass spectrometry. *J. Chromat. A.* 1000, 609-635.
- Clemente A., 2000. Enzymatic protein hydrolysates in human nutrition. *Trends Food Sci. Technol.* 11, 254-262.
- Duranti M., 2006. Grain legume protein and nutraceutical properties. *Fitoterapia* 77, 67-82.
- Duranti M., Gius C., 1997. Legume seeds: protein content and nutritional value. *Field Crops Res.* 53, 31-45.
- Gill I., Lopez-Fandino R., Jorba X., Vulfson E., 1996. Biologically active peptides and enzymatic approaches to their production. *Enzyme Microb. Technol.* 18, 162-183.
- Grela E.R., Gunter K.D., 1995. Fatty acid composition and tocopherol content of some legume seeds. *Anim. Feed Sci. Technol.* 52, 325-331.

- Habeeb A.F.S.A., 1966. Determination of free amino group in protein by trinitrobenzenesulfonic acid. *Anal. Biochem.* 14, 328-336.
- Kitts D.D., Weiler K., 2003. Bioactive protein and peptides from food source. Applications of bioprocesses used in isolation and recovery. *Curr. Pharmaceut. Des.* 9, 1309-1323.
- Korhonen H., Pihlanto A., 2006. Bioactive peptides: production and functionality. *Int. Dairy J.* 16, 945-960.
- Lowry O., Rosebriught N., Farr A., Randal R.J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 256-268.
- Pasquinelli R.S., Shepherd R.E., Koepsel R.R., Zhao A., Atai M.M., 2000. Design of affinity tags for one-step protein purification from immobilized zinc columns. *Biotechnol. Prog.* 16, 86-91.
- Periago M.J., Vidal M.L., Ros G., Rincon F., Martinez C., Lopez G., Rodrigo J., Martinez I., 1998. Influence of enzymatic treatment on the nutritional and functional properties of pea flour. *Food Chem.* 63, 71-78.
- Porzucek H., Raznikiewicz L., 1990. Fatty acid composition and lipooxygenase activity of flours and protein isolates from leguminous plant. *Swed. Agric. Res.* 20, 31-34.
- Subagio A., 2006. Characterization of hyacinth bean (*Lablab purpureus* (L.) sweet) seeds from Indonesia and their protein isolate. *Food Chem.* 95, 65-70.
- Ueda E.K.M., Gout P.W., Morganti L., 2003. Current and prospective application of metal ion protein binding. *J. Chromat.* 988, 1-23.
- Whitehurst R.J., Low B.A., 2002. Enzymes in food technology. Chapter 6. Blackwell Publ. Sheffield.
- Yip T.T., Nakagawa Y., Porath J., 1989. Evaluation of the interaction of peptides with Cu (II), Ni (II) and Zn (II) by high-performance immobilized ion affinity chromatography. *Anal. Biochem.* 15, 159-171.

MODYFIKACJA PROTEOLITYCZNA MĄK WYBRANYCH ROŚLIN STRĄCZKOWYCH

Streszczenie. W pracy badano wpływ działania pepsyny (EC 3.4.1.1) i trypsyny (EC 3.4.4.4) na skład chemiczny mąk otrzymanych z nasion grochu odmiany 'Piast' i soczewicy odmiany 'Anita'. W modyfikowanych mąkach znacząco zmienił się poziom białka i lipidów oraz zawartość poszczególnych kwasów tłuszczowych. W porównaniu z mąkami niepoddawany modyfikacji, zarówno w mąkach hydrolizowanych z użyciem trypsyny, jak i pepsyny, uległ obniżeniu poziom nienasyconych kwasów tłuszczowych oraz stosunek kwasu linolowego do oleinowego (ok. 40% dla grochu i 60% dla soczewicy). Badano także potencjalne możliwości zastosowania chromatografii powinowactwa na unieruchomionych jonach metali do rozdzielania i oczyszczania peptydów. Profile rozdzielania peptydów, wykonane na unieruchomionych jonach cynku (II), zależały od rodzaju rośliny i enzymu użytego w procesie hydrolizy, jak również od rodzaju zastosowanego czynnika immobilizującego jony metalu (kwasu iminodioctowego – IDA i ortofosfoseryny – OPS). Badane peptydy wykazywały na ogół słabe powinowactwo do unieruchomionych jonów cynku.

Słowa kluczowe: cynk, IMAC, peptydy, profil lipidowy, proteoliza, rośliny strączkowe

Accepted for print – Zaakceptowano do druku: 4.12.2007

For citation – Do cytowania: Baraniak B., Świeca M., 2008. Proteolytic modification of selected legume flours. Acta Sci. Pol., Technol. Aliment. 7(1), 41-48.