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Antioxidant Activity of Hydrolysates Prepared from Flaxseed Cake Proteins Using Pancreatin

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Key words: flaxseed protein hydrolysates, pancreatin, degree of hydrolysis, antioxidant activity

Proteins were isolated from defatted flaxseed cake and hydrolysed with pancreatin. The hydrolysis process was conducted at a stable temperature of 50°C and pH 7.5, and monitored with the pH–stat method. The obtained hydrolysates with a degree of hydrolysis (DH) of 5, 10, 15, 20, 25% were investigated in terms of antioxidant properties. The radical scavenging activity was assayed against DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, the reducing ability – with FRAP assay, and the capability to bind Fe(II) – by reaction with ferrozine. SE–HPLC analysis was used to determine molecular weight distribution of hydrolysis products.

The antiradical activity of pancreatin hydrolysates of flaxseed proteins was increasing along with an increasing DH and for the hydrolysate with DH 25% the EC_{50} value determined with the DPPH assay accounted for 0.083 mg/assay, and the ABTS$^{\bullet+}$ scavenging activity – for 0.218 mmol Trolox/g. This hydrolysate was constituted mainly by peptides with low molecular weights (MW) of 0.238–0.556 kDa. In turn, the Fe(II) binding capability increased from 44.5% to 64.9% in the case of hydrolysates with DH 5–20% and decreased in the case of the hydrolysate with DH 25%. A similar dependency was observed in the ability of pancreatin hydrolysates of flaxseed proteins to reduce Fe(III). The maximum value of reducing ability reached 0.25 mmol Fe(II)/g for the hydrolysate with DH 20% that was predominated by polypeptides and peptides with MW of 0.238–1.046 Da.

INTRODUCTION

Recently, a growing interest has been observed in the enzymatic hydrolysis of proteins as a process which ensures the final product with enhanced biological activity. Ample studies have demonstrated that enzymatic hydrolysis of proteins results in the release of antihypertensive (ACE inhibitory), antioxidative, immunomodulatory, antimicrobial, osteoprotective, antilipemic and opioid peptides [Hartmann & Meisel, 2007; Möller *et al.*, 2008; Zambrowicz *et al.*, 2013]. The bioactive peptides may be released upon immediate effects of proteases on protein substrates as well as during gastrointestinal digestion of proteins and protein hydrolysis by microorganisms applied in fermentative processing of foods [Korhonen & Pihlanto, 2006].

The enhanced biological potential of a hydrolysate compared to the native proteins is affected by the quantity and chemical structure of released biopeptides. The amino acid composition, amino acids sequence and the length of the peptide chain determine the beneficial health effects [Hartmann & Meisel, 2007; Samaranayaka & Li–Chan, 2011]. The size of active sequences may vary from two to twenty amino acid residues [Korhonen & Pihlanto, 2006]. Certainly, this sequence of amino acids should occur in a substrate protein, and proteases selected for the hydrolysis process should be able to disrupt the appropriate peptide bonds and release the appropriate fragment of the peptide chain. Highly significant is also the adjustment of hydrolysis conditions, *i.e.* temperature (T), pH, initial concentration of substrate (S_0), enzyme to substrate ratio (E/S), and time (t) [Adler–Nissen, 1986; Karamać *et al.*, 2002]. Adler–Nissen [1986] demonstrated that four of these parameters, *i.e.* T, S_0, E/S and t, might be controlled with simultaneous determination of a hydrolysis degree (DH). For a given protein–enzyme system, DH monitoring is therefore sufficient for complete control of the process conducted at stable pH of the reaction medium.

Substrates applied in the production of enzymatic hydrolysates with a biological activity include proteins of both animal and plant origin. It is a frequently applied solution to manage high–protein by–products of the food industry [Karamać *et al.*, 2005; Popovic *et al.*, 2013]. These by–products include, among others, flaxseed cake being a residue after oil solvent extraction from seeds or seeds pressing. Flaxseed cake contains from 31.3 to 40.9% of protein [Oomah & Mazza, 1993; Mueller *et al.*, 2010]. Its proteins are characterised by a beneficial amino acid composition with relatively high contents of aspartic acid, glutamic acid, leucine and arginine [Oomah & Mazza, 1993]. Some reports show that their nutritive value and amino acid profile are comparable to these of soybean proteins [Rabetafika *et al.*, 2011]. Despite that, flaxseed cake is applied only as feedstuff and the protein component of flaxseed in rarely investigated in the aspect of nutritional applications.

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So far, the greatest attention of researchers has been focused on enzymatic hydrolysates of flaxseed proteins with the antihypertensive activity [Udenigwe *et al.,* 2009a; Udenigwe & Aluko, 2010; Marambe *et al.*, 2008, 2011]. By using Alcalase or Flavourzyme it is also possible to produce flaxseed protein hydrolysates with the antioxidant activity [Marambe *et al.*, 2008; Silva *et al.*, 2013]. In turn, Udenigwe *et al.* [2009b] compared the antiradical and anti–inflammatory activity of low molecular weight and cationic peptide fractions isolated from hydrolysates produced using Alcalase, pepsin, ficin, trypsin, papain, thermolysin and pancreatin.

In view of the above, the goal of this study was to isolate proteins from flaxseed cake, to produce pancreatin hydrolysates with various degrees of hydrolysis (DH) and to determine their antioxidant activity depending on their DH.

MATERIAL AND METHODS

Material

The initial material in the study was cake of flaxseeds of Recital cultivar obtained from the Oil Production Plant in Grodzisk Wielkopolski (Poland). Flaxseed cakes contained 38.2±0.46% proteins (N × 6.25), 10.3±0.78% lipids, 6.4±0.06% ash, 8.4±0.06% moisture (n=3) [AOAC, 1990] and 36.7% carbohydrates (calculated by difference).

Isolation of proteins from flaxseed cake

Flaxseed cake (*ca.* 200 g) was disintegrated in a laboratory mill and oil residues were removed by 3×2 h extraction with n–hexane (1:4, w/v) at a room temperature. Protein was isolated from defatted and dried cake according to the procedure described by Dev & Quensel [1988] with modification proposed by Udenigwe *et al*. [2009b] and small modifications made for the purpose of this study. An aqueous suspension of cake (1:20, w/v) was adjusted to pH 5.0 with 2 mol/L HCl and placed in a water bath with shaking at 37°C. Cellulase from *Aspergillus niger* (Sigma–Aldrich, activity ~0.8 units/mg) was added to the suspension in a dose of 10 mg/g cake in order to hydrolyse mucilage of the cake. The reaction was arrested after 4 h by changing pH into 9.5 using 2 mol/L NaOH. Proteins were extracted for 2 h in alkaline conditions, at room temperature, under continuous stirring. After centrifugation (10°C, 30 min, 4000×g, MPW–350 centrifuge, MPW Med. Instruments, Poland), the supernatant was decanted and the precipitate was suspended in the NaOH solution with pH 9.5, and extraction was repeated. Afterwards, 0.2 mol/L HCl was added to the combined supernatants till pH 4.0 has been reached. The precipitated proteins were centrifuged at 10°C for 30 min at 8700×g. The precipitate was then suspended in a small volume of water, neutralised (0.2 mol/L NaOH), and dialysed for 48 h at 4°C against deionised water that was exchanged 8 times. Dialysis was conducted using molecular porous membrane tubing of MW cut–off at 6–8 kDa (Spectra/Por, Spectrum Laboratories, USA). The resultant isolate was dried by lyophilisation for ~48 h at –50°C and 0.021 mbar (FreeZone 6 Liter Freeze Dry System, Labconco, USA), and stored at 4°C.

Hydrolysis of flaxseed proteins

The flaxseed protein isolate was hydrolysed with pancreatin (Sigma–Aldrich). A protein suspension in water (1:20 w/v) was adjusted to pH 7.5, and then the enzyme was added in a dose of 15 mAU/g. The reaction was run under stable pH and temperature (50°C) using the ETS 822 end point titration system working in the pH–stat mode (Radiometer Analytical, Denmark). Based on the volume of 0.5 mol/L NaOH used in the reaction, the degree of hydrolysis (DH) was determined acc. to the equation provided by Adler–Nissen [1986]:

$$DH = \frac{B \times M_b}{\alpha \times P \times h_{tot}} \times 100\%$$

where: B – base consumption (mL), M_b – molarity of the base (mol/L), α – average degree of dissociation of the α–NH_2 groups, P – mass of protein (g), and h_{tot} – total number of peptide bonds in the protein (meqv Leu–NH_2/g protein). A series of hydrolytic reactions were carried out that were arrested when DH reached 5, 10, 15, 20 and 25%, by heating at 100°C for 5 min.

The content of protein in the isolate (82.2±0.39%, n=6) was determined with the Kjeldahl's method (N×6.25) [AOAC, 1990], whereas the total number of peptide bonds in proteins (h_{tot}) was assayed based on the number of the –NH_2 groups released during acidic hydrolysis of protein in 6 mol/L HCl at 105°C and determined using the spectrophotometric method with *o*–phthalaldehyde (OPA) [Panasiuk *et al*., 1998].

SE–HPLC separation

The SE–HPLC separation of hydrolysates was conducted on a TSKgel G2000SW_{XL} column (5 μm, 7.8 × 300 mm, Tosoh Bioscience, Tokyo, Japan) coupled with a Shimadzu HPLC system: LC–10AD_{Vp} pump, SPD–M10A_{Vp} photo–diode array detector, and SCL–10A_{Vp} system controller (Kyoto, Japan). Acetonitrile : water : trifluoroacetic acid (45:55:0.1 v/v/v) was applied as a mobile phase with a flow rate of 0.5 mL/min. Then, 20–μL portions of hydrolysate solutions with the concentration of 5 mg/mL or the solution of a mixture of molecular weight (MW) standards (Sigma–Aldrich): albumin from chicken egg white (45 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa), angiotensin II acetate (1.046 kDa), leucine enkephalin (0.556 kDa), VAL–TYR–VAL (0.379 kDa) and GLY–TYR (0.238 kDa) were injected onto the column. Detection was monitored at 216 nm.

ABTS assay

The ability of hydrolysates to inactivate $ABTS^{\bullet+}$ was determined with the method of Re *et al.* [1999] with modification enabling carrying out the reaction on multi–well plates. ABTS (96 mg) was activated in a 2.45 mmol/L solution of potassium persulfate for 16 h. The stock solution was diluted in water at the rate of 1:50 (v/v). Hydrolysates were dissolved in a 0.1 mol/L phosphate buffer (pH 7.0) in the concentration of 2 mg/mL. Then, 10–μL portions of the sample were applied onto a 96–well plate and 200 μL of ABTS solution heated to 30°C were added. The plate was fixed in an Infinite M1000 microplate reader (Tecan, Switzerland) and shaken. The ab-

sorbance was read out after 6 min at λ=734 nm. The results were calculated using the standard curve for Trolox (r=0.999) or reduced L–glutathione (r=0.991) and expressed in mmol of standard equivalents per g of hydrolysate.

DPPH assay

The DPPH$^{\bullet}$ scavenging activity of the hydrolysates was assayed with the method of Brand–Williams *et al.* [1995] adjusted to absorbance detection in multi–well plates. The hydrolysates were dissolved in a 0.1 mol/L phosphate buffer (pH 7.0) in concentrations ranging from 0.4 mg/mL to 2 mg/mL. To 100 μL of hydrolysate solutions, there were added 100 μL of 0.3 mol/L solution of DPPH in methanol. The reaction was continued for 30 min at room temperature. Absorbance was read out at λ=517 nm using an Infinite M1000 microplate reader. Solutions of Trolox and reduced L–glutathione were applied onto the plate in parallel. Curves of the dependency of % scavenged DPPH$^{\bullet}$ on sample concentration were plotted for each hydrolysate and standard. The resultant curves enabled determining the EC_{50} value that indicates the quantity of hydrolysate (mg/assay) necessary to inactivate 50% of DPPH radicals.

FRAP assay

The reducing ability of hydrolysates was estimated *via* the ferric reducing antioxidant power (FRAP) assay [Benzie & Strain, 1996]. To this end, 40–μL portions of hydrolysates dissolved in deionized water (1 mg/mL) or solutions of standard antioxidants – Trolox (0.0625 mg/mL) and reduced L–glutathione (0.125 mg/mL), were applied onto a 96–well plate. Afterwards, 200–μL portions of heated to 37°C reagent consisting of: 0.3 mol/L acetate buffer with pH 3.6, 10 mmol/L 2,4,6–Tris(2–pyridyl)–*s*–triazine in 40 mmol/L HCl and 20 mmol/L $FeCl_3 \times 6H_2O$ in the ratio of 5:1:1 (v/v/v), were added to the samples. After 30–min incubation at 37°C, absorbance was detected at λ=593 nm (Infinite M1000 plate reader). Results were expressed in mmol of Fe(II) per g of hydrolysate/standard based on standard curve plotted for $FeSO_4 \times 7H_2O$ (r=1).

Fe(II) binding activity

The ability of hydrolysates to bind Fe(II) ions was determined using the spectrophotometric method with ferrozine [Karamać & Pegg, 2009]. The reaction was carried out on a 96–well plate. After mixing 200 μL of aqueous solutions of hydrolysates (2 mg/mL) with 20 μL of 0.4 mmol/L $FeCl_2 \times 4H_2O$, 40 μL of 5 mmol/L ferrozine were added to wells. Solutions absorbance was read out after 10 min at λ=562 nm using an Infinite M1000 plate leader. The percentage of bound Fe(II) was calculated for each sample.

Statistical analysis

All analyses were carried out in at least three replications. Results in figures are presented as mean values with standard deviation. The results of antioxidant tests were analysed by a one–way ANOVA statistical model with Tukey's multiple comparison test (GraphPad Prism version 6.04 for Windows, GraphPad Software, USA). The differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

The applied conditions of hydrolysis (initial concentration of substrate – 20%, pancreatin dose – 15 mAU/g, temperature 50°C, pH 7.5) enabled producing hydrolysates with DH 5, 10, 15, 20 and 25%. The maximum DH value – 25% is comparable to the values obtained during pancreatin hydrolysis of proteins from seeds of other plants [Valdez–Ortiz *et al.*, 2012; Betancur–Ancona *et al.*, 2014].

Molecular weight distribution of products of flaxseed proteins hydrolysis with pancreatin was presented in Figure 1. Pancreatin is a mixture of enzymes that contains proteases characterised by major activities of tryptic, chymotryptic and elastase types, and displaying carboxypeptidase activity [Andriamihaja *et al.*, 2013]. Owing to that, it is capable of releasing simultaneously polypeptides with various lengths of the peptide chain and single amino acids from proteins. Hence, the hydrolysate with the lowest DH (5%) contained products of hydrolysis with a wide range of molecular weights (MW) from ≤0.238 to 12.4 kDa (Figure 1A). The chromatogram shows also a peak with retention time of 12.9 min which corresponds to proteins with MW ≥45 kDa. An increase of DH to 10% caused an increase in the amount of hydrolysis products with MW 0.555–6.5 kDa. Further increase in DH value was accompanied by tangible successive decrease of peaks of the polypeptides with the highest molecular weights (12.4––1.046 kDa) and an enlargement of peaks corresponding to lower MW. The hydrolysate with DH 20% was predominated by compounds with MW 0.238–1.046 kDa. In turn, based on the chromatogram from the separation of the hydrolysate with DH 25% it may be concluded that it contained mainly peptides with MW 0.238–0.556 kDa (Figure 1E). Successive degradation of polypeptides to oligopeptides and amino acids in the course of pancreatin hydrolysis of peanut meal was demonstrated by Zheng *et al.* [2013]. These authors fractionated hydrolysates based on MW and observed that the large peptide fraction with MW >5 kDa decreased drastically, while the smaller peptide fractions of 3–5 kDa and <3 kDa increased.

The antiradical activity of pancreatin hydrolysates of flaxseed proteins with various DH values was analysed against two synthetic radicals: ABTS$^{\bullet+}$ (Figure 2) and DPPH$^{\bullet}$ (Figure 3). In the case of both radicals, a gradual increase was noted in radical scavenging activity along with an increasing DH of hydrolysates. A similar correlation regarding ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ was demonstrated for hydrolysates of hot– and cold–pressed peanut meals treated with pepsin followed by pancreatin [Zheng *et al.,* 2013]. Also the ABTS$^{\bullet+}$ scavenging activity of buckwheat proteins digested with the same system of enzymes was increasing in the course of hydrolysis [Ma *et al*., 2010]. A few studies demonstrated that fractions of peptides with the lowest MW separated from pancreatin hydrolysates were characterised by a high antiradical activity against ABTS$^{\bullet+}$ or DPPH$^{\bullet}$ [Ma *et al*., 2010; Alashi *et al*., 2014]. Results of our study are consistent with this observation. The hydrolysate with DH 25%, which was the most effective in radicals scavenging, contained mainly small peptides with MW 0.238–0.556 kDa.

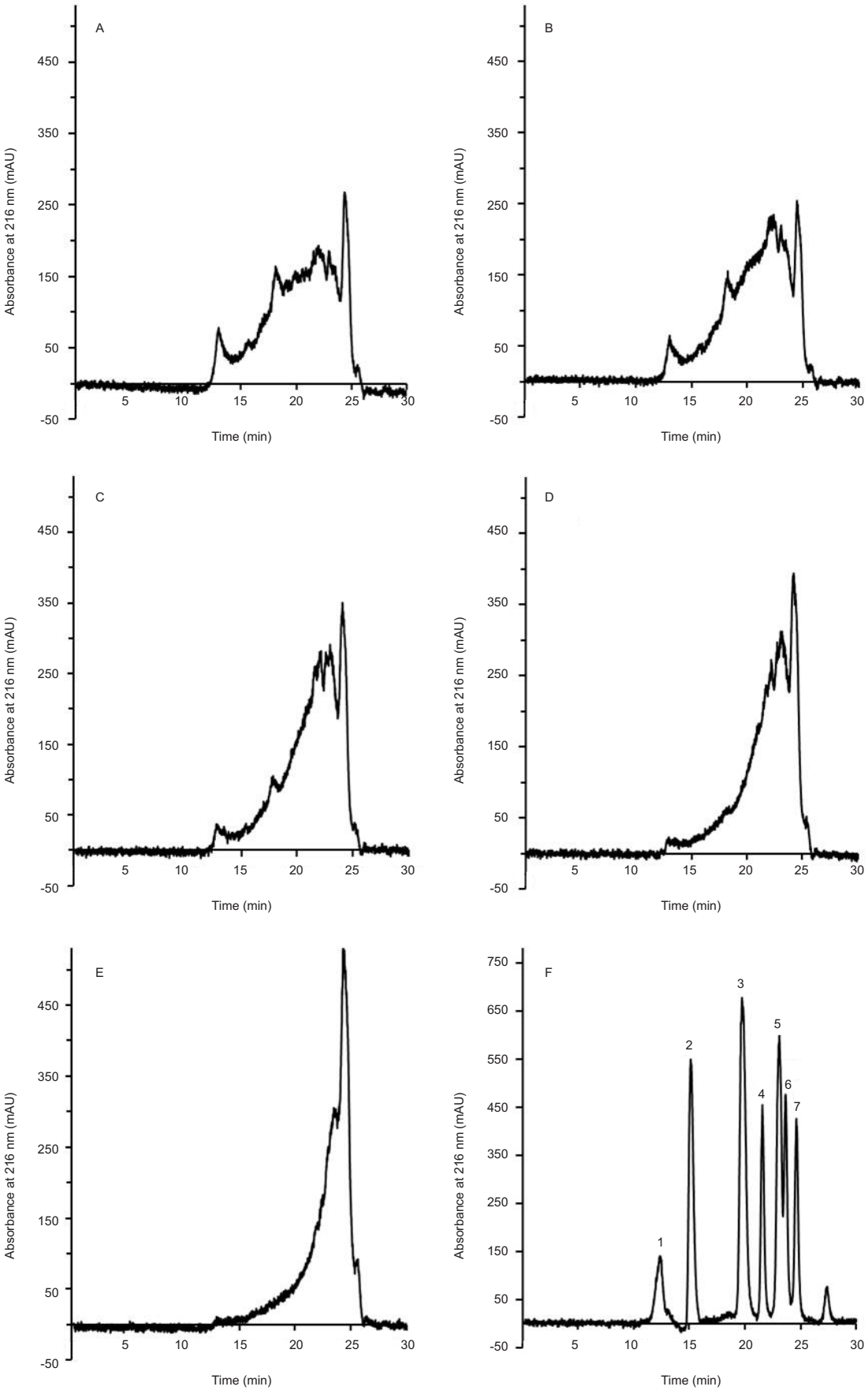

FIGURE 1. SE–HPLC separation of flaxseed cake protein hydrolysates with a different degree of hydrolysis (DH): A – 5%, B – 10%, C – 15%, D – 20%, E – 25% and molecular weight standards – F: 1 – albumin from chicken egg white (45 kDa), 2 – cytochrome C (12.4 kDa), 3 – aprotinin (6.5 kDa), 4 – angiotensin II acetate (1.046 kDa), 5 – leucine enkephalin (0.556 kDa), 6 – VAL–TYR–VAL (0.379 kDa), 7 – GLY–TYR (0.238 kDa).

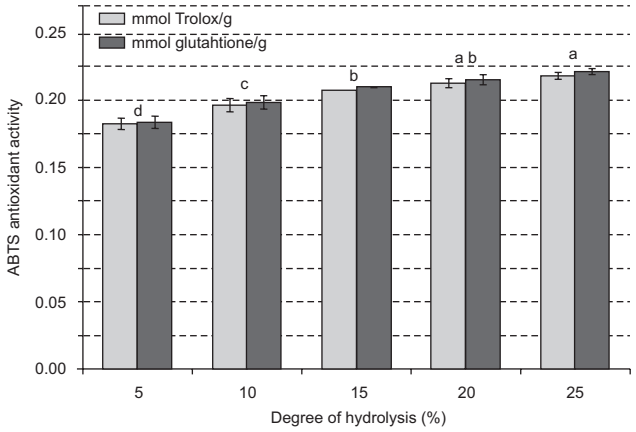

FIGURE 2. ABTS$^{\bullet+}$ antioxidant activity of flaxseed cake protein hydrolysates with a different degree of hydrolysis. The bars with different letters are significantly different at $p<0.05$.

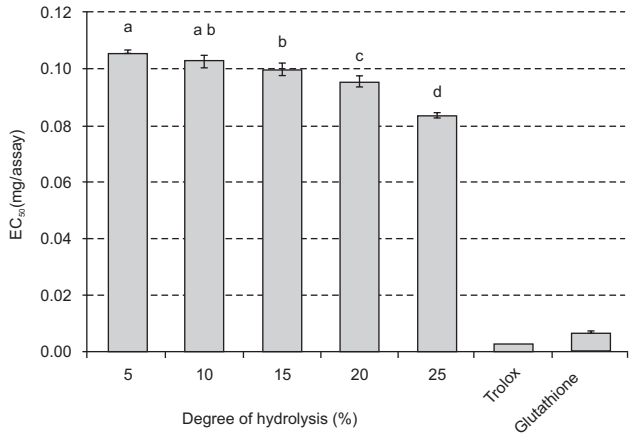

FIGURE 3. DPPH$^{\bullet}$ scavenging activity of flaxseed cake protein hydrolysates with a different degree of hydrolysis. The bars for hydrolysates with different letters are significantly different at $p<0.05$.

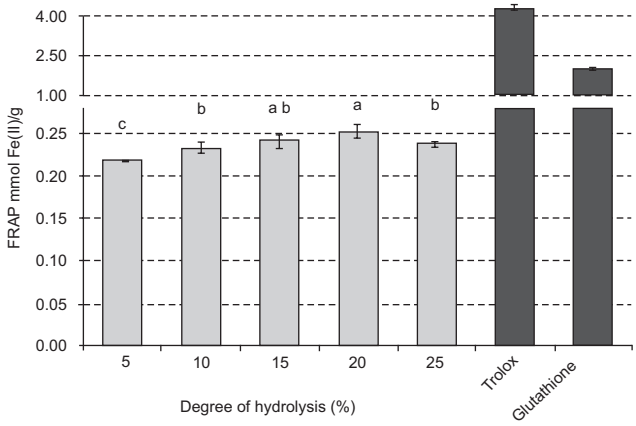

FIGURE 4. Ferric reducing antioxidant power (FRAP) of flaxseed cake protein hydrolysates with a different degree of hydrolysis. The bars for hydrolysates with different letters are significantly different at $p<0.05$.

According to the procedure by Re e*t al*. [1999] and common practice, the ABTS$^{\bullet+}$ antioxidant activity of hydrolysates was expressed in Trolox equivalents. In the case of protein hydrolysates, peptides are the potential antioxidant compounds, hence results of the ABTS test in our study were additionally expressed in equivalents of reduced glutathione as an example of antioxidant with a chemical structure of peptide (Figure 2). The obtained results expressed per both standards were similar and ranged from 0.182 to 0.218 mmol Trolox/g and from 0.184 to 0.221 mmol glutathione/g. Similar values reaching 70–327 μmol Trolox/g were achieved by Ng *et al.* [2013] for trypsin hydrolysates from palm kernel cake proteins with DH 30–50%. Slightly higher values were recorded by Zheng *et al.* [2013] for cold– and hot–pressed peanut meals digested with a pepsin–pancreatin system, *i.e.* 509 and 527 μmol Trolox/g, respectively. In turn, a commercial isolate of pea proteins hydrolysed with papain demonstrated significantly lower ABTS$^{\bullet+}$ scavenging activity – 28 mmol Trolox/kg d.m. [Zilic *et al.*, 2012].

The lowest EC_{50} value – 0.835 mg/assay – determining the maximum DPPH$^{\bullet}$ scavenging activity was noted for the flaxseed protein hydrolysate with DH 25%. The EC_{50} values for Trolox and reduced glutathione were, respectively, 28.6 and 13.2 times lower than the value assayed for the most active hydrolysate. Only negligibly lower antiradical activity against DPPH$^{\bullet}$ compared to reduced glutathione was observed by Alashi *et al*. [2014] for canola meal protein hydrolysate obtained using pancreatin. Glutathione was characterised by *ca.* 7–fold lower EC_{50} than the hydrolysate. In contrast, significantly lesser differences in DPPH$^{\bullet}$ scavenging activity of hydrolysates and standard were reported by other authors [Pownall *et al*., 2010; Ajibola *et al*., 2011]. However, in these investigations results of DPPH assay were expressed as % of scavenged radical.

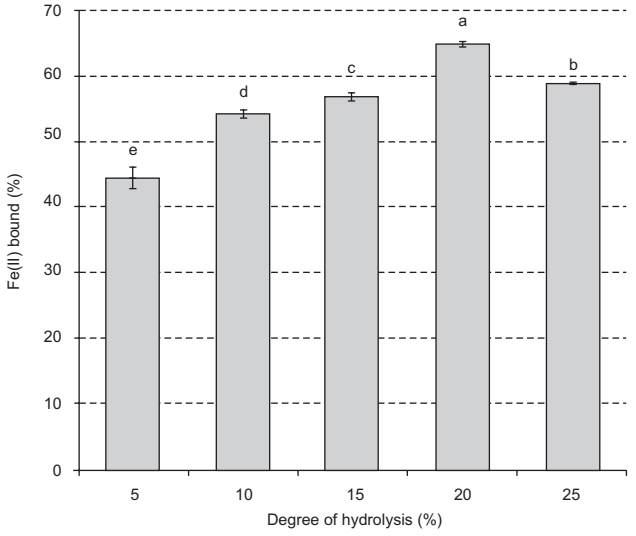

FIGURE 5. Fe(II) binding ability of flaxseed cake protein hydrolysates with a different degree of hydrolysis. The bars with different letters are significantly different at $p<0.05$.

The antioxidant activity of compounds is determined not only by their radical scavenging activity but also by their capability to participate in redox reactions, and to be more specific – by their reducing ability. Therefore, another test was conducted to determine the capability of pancreatin hydrolysates of flaxseed proteins to reduce Fe(III) ions to Fe(II) (Figure 4). The lowest reducing power was demonstrated for the hydrolysate with DH 5%. The ability of hydrolysates with DH 5–20% to reduce Fe(III) was increasing successively. The reducing power of the hydrolysate with DH 25% was lower than that of the hydrolysate with DH 20%. The lower reducing activity of the hydrolysate containing mainly peptides with low MW is quite unexpected. In many works, fractionation of hydrolysates demonstrated that smaller size peptides exhibited better reducing ability than high MW fractions [Li *et al.*, 2008; Ajibola *et al*., 2011]. It should, however, be noticed that differences observed in reducing ability between particular hydrolysates of flaxseed proteins were small.

The FRAP values determined for pancreatin hydrolysates of flaxseed proteins ranged from 0.21 to 0.25 mmol Fe(II)/g. The standard antioxidants – reduced glutathione and Trolox – acted significantly stronger and were characterised by, respectively, *ca.* 7.5–fold and 17–fold higher reducing ability than the analysed hydrolysates. Even weaker reducing activity compared to glutathione was demonstrated by African yam bean seed proteins hydrolysed with Alcalase and by pea protein hydrolysate produced using Thermolysin [Ajibola *et al*., 2011; Pownall *et al*., 2010].

It is known that reduced forms of some transition metal ions accelerate oxidative processes by participating in the Fenton/Haber–Weiss reactions that result in the formation of very reactive hydroxyl radicals [Borg, 1993]. The action of antioxidants consists in chelation of ions of pro–oxidative metals. In the case of pancreatin hydrolysates of flaxseed proteins with various DH, their ability to bind with Fe(II) was analysed in the study (Figure 5). Likewise in the case of reducing ability, Fe(II) complexation by hydrolysates was increasing along with their DH increasing from 5% to 20%, and for the hydrolysate with the highest DH – it was observed to decrease. The maximum value of bound Fe(II) reached 64.9%, and the minimal – 44.5%. Zhang *et al.* [2014] investigated the effect of DH on iron binding capacity of soy protein hydrolysates and demonstrated that, likewise in our study, the ability of Flavourzyme hydrolysates to bind Fe(II) reached the maximum value at some DH and then decreased. In turn, Ajibola *et al*. [2011] noted that the metal ion chelating effect of peptide fractions with MW <1 kDa and fractions with higher MW did not differ statistically.

CONCLUSIONS

Flaxseed cake proteins treated with pancreatin exhibited antioxidant activity. Pancreatin hydrolysates were capable of scavenging DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, reducing Fe(III) ions and binding Fe(II). Their antioxidant activity depended on their degree of hydrolysis (DH). The study showed that the most active scavengers of free radicals were flaxseed protein hydrolysates with the highest degree of hydrolysis. However, the reducing ability and ability to form complexes with metal ions reached the maximum values in the case of the hydrolysate with a slightly lower DH. It may, therefore, be concluded that – depending on the mechanism of action – the most effective antioxidants are pancreatin hydrolysates of flaxseed proteins with DH 20–25%. Their antioxidant activity is ascribed to peptides with MW 0.238–1.046 kDa released from proteins.

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