**Identification of peptides released from flaxseed (*Linum ussitatissimum*) protein by
Alcalase[®] hydrolysis: antioxidant activity**

Fernanda Guimarães Drummond e Silva^a, Blanca Hernández-Ledesma^b, Lourdes Amigo^b,
Flavia Maria Netto^a, Beatriz Miralles^{b*}

^a Faculty of Food Engineering, University of Campinas, UNICAMP, Monteiro Lobato 80,
13083-862 Campinas, SP, Brazil

^b Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI
UAM+CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain

*Corresponding author: email: beatriz.miralles@csic.es Telephone +34-910017932; Fax:
+34-910017905

Abstract

In this study, the hydrolysis of a flaxseed protein isolate with Alcalase[®] was performed as a strategy to generate antioxidant peptides. A chromatographic separation of the hydrolysate was conducted by RP-HPLC. Both hydrolysate and six collected fractions were subjected to ORAC and FRAP assays to evaluate their antioxidant capacity. The higher antioxidant values were shown by fractions containing predominantly low molecular weight peptides, as it was demonstrated by MALDI analysis. Four peptides were identified by LC-MS/MS and one by Edman degradation. The peptide with sequence GFPGRLDHWCASE was synthesised showing a notable ORAC activity, 3.20 μmol Trolox equivalents/ μmol of peptide. This value was higher than that reported for butylated hydroxyanisole. Therefore, the contribution of this peptide to the activity of the fraction where it had been found was 61%. The identified sequences represent an advance in the molecular characterization of the flaxseed protein fraction.

Keywords: Flaxseed, peptides, antioxidant capacity, Alcalase[®] hydrolysis, tandem mass spectrometry

1. Introduction

Flax is one of the most ancient crops that has been used until the nineteenth century for cloths and paper manufacturing. The major industrial product is flaxseed oil because it is the richest known plant source of α -linolenic acid, while the residual meal remaining after oil extraction is used in animal feed. At present time, flaxseed has new prospects in food because of the growing consumers' interest for functional food with health benefits (Shim, Gui, Arnison, Wang & Reaney, 2014). The presence of n-3 fatty acids, soluble fibers, vitamin E, lignans, and other phenolic and peptide compounds in flaxseed has been considered responsible for the health benefits attributed to this plant (Cardoso Carraro, Dantas, Espeschit, Martino & Ribeiro, 2012). In the body tissues, free radicals are continually generated as byproducts of oxidative metabolism. Although they are essential on cellular processes, their accumulation and the absence of antioxidant endogenous defenses that neutralize their oxidative action can lead to oxidative stress status. Many studies associate this status with the occurrence and the progression of various chronic diseases such as diabetes, cancer, neurodegenerative and cardiovascular disorders, and aging (Fiaschi & Chiarugi, 2012). Therefore, the interest of these compounds with the ability to prevent these disorders is evident.

In whole and ground/milled flaxseeds stored at room temperature limited oxidative deterioration was observed, which is indicative of an efficient antioxidant system. In accordance to the composition and existing research, this could be attributed to lignans, concretely secoisolariciresinol diglucoside and phenolic acids, i.e. p-coumaric, vanillic, sinapic and ferulic present in the seed (Hosseinian, Muir, Westcott & Krol, 2006; Johnsson et al., 2002). On the other hand, it has been reported that polar compounds in flaxseed oil increase its oxidative stability (Sharav, Shim, Okinyo-Owiti, Sammynaiken, & Reaney, 2014). Barthet, Klensporf-Pawlik, & Przybylski (2014) studied the types of components

involved in the antioxidant properties of the flaxseed meal. Solvents of different polarity such as water, acetone and methanol were used to extract diverse potential antioxidant components. Lignans and phenolic acids were found partially responsible for the flaxseed antioxidative system, and the participation of some proteins was suggested (Barthet et al., 2014). In accordance to these findings, we have observed that flaxseed protein products exert antioxidant capacity with different contribution to each mechanism, i.e. ferric reduction or oxygen radical scavenging, and this bioactivity was not impaired by submitting the samples to sequential hydrolysis with pepsin at pH 2.0 and pancreatin at pH 6.5 to simulate digestion (unpublished data).

In flaxseed, proteins represent 35–45% on dry oil-free matter basis, and 56–70% is concentrated in aleuronic grains in cotyledons (Rabetafika, Van Remoortel, Danthine, Paquot, & Blecker, 2011). Their solubility in various solvents reveals two major fractions namely globulin (linin, 11-12S; 64-73% of total seed protein) and albumin (conlinin, 1.6-2S; 27-42% of total seed protein) that have molecular masses of 252-298 and 16-17 kDa, respectively (Chung, Lei & Li-Chan., 2005; Marambe, Shand & Wanasundara, 2013). Additional proteins with molecular weights between 9 and 17 kDa have been detected by electrophoretic techniques (Sammour, 1999).

The proteinaceous materials, when hydrolysed, are known to yield hydrolysates with different bioactive and nutritional values. The strategies to recover and characterize bioactive components from flaxseed proteins include hydrolysis or simulated gastrointestinal digestion of protein isolates followed by size exclusion or cation exchange chromatographic separation, (Marambe, Shand & Wanasundara, 2011; Udenigwe, Lu, Han, Hou & Aluko, 2009), electrophoresis (Liu, Shim, Poth & Reaney, 2016) or electrodialysis with ultrafiltration membranes (Doyen, Udenigwe, Mitchell, Marette, Aluko, & Bazinet, 2014). The obtained peptide fractions have shown angiotensin converting enzyme (ACE) inhibitory and hydroxyl

radical scavenging activity (Marambe et al., 2008), calmodulin inactivation with inhibition of CaM-dependent phosphodiesterase and renin inhibition (Udenigwe & Aluko, 2012), increased glucose uptake in L6 cells, and even antihypertensive activity on spontaneously hypertensive rats (Doyen et al., 2014).

Due to the incomplete sequence description of flaxseed proteins, only in very few cases peptide identification has been achieved. However, the assessment of peptides linked to their accountability for the elicited effects is necessary because the observed activity cannot be only attributed to the degree of hydrolysis, as the decisive factor is the structure of the peptides and the sequence of amino acids that build them (Dryáková, Pihlanto, Marnila, Curda, & Korhonen, 2010). The aim of the present study was to evaluate the antioxidant behavior of fractions from a flaxseed protein isolate prepared by an optimized hydrolysis process with Alcalase[®], and to identify peptides responsible for this activity.

2. Materials and Methods

2.1. Preparation of the flaxseed protein hydrolysate (FPH)

Partially defatted brown flaxseed meal (Cisbra Ltd., Panambi, RS, Brazil) was defatted with hexane in a ratio of 1:3 (w/v). Then, three consecutive extractions with 63% ethanol (v/v) were performed to remove phenolic compounds. The phenolic reduced defatted flaxseed meal was dispersed in deionized water at a flour:water ratio of 1:10 (w/w). After adjusting the pH of the solution to 9.0, it was stirred at room temperature for 120 min, and centrifuged (2500 x g/30 min; 25°C). The pH of the supernatant was adjusted to 4.2, and the precipitated protein was separated by centrifugation (2500 x g/30 min), suspended in deionized water, and the pH adjusted to 6.0. It was then freeze-dried and stored at -20 °C until use. The hydrolysis of flaxseed protein isolate was performed with Alcalase[®] 2.4 L (enzyme activity ≥ 2.4 activity units/g) purchased from Sigma (St. Louis, MO, USA) at 60°C, during 180 min. The

hydrolysis conditions were optimized through a two factor central composite rotatable design (Silva, O'Callaghan, O'Brien, & Netto, 2013). The protein concentration was 5% (w/v), the pH 8.5, and the enzyme:substrate ratio was 1:90 (w/w). The hydrolysis reaction was monitored by a pH-stat method using an automatic titrator DL model Metler 21 (Schwerzenbach, Switzerland) with a stirring system coupled to a thermostatic bath. The pH of the hydrolysate was adjusted to 6.0, freeze-dried and stored at -20°C. The degree of hydrolysis (% DH) of FPH was 17.20%, calculated using the equation described by Adler Nissen considering the total number of peptide bonds in the protein substrate determined in vegetal proteins, 7.8 meq/g protein (Adler-Nissen, 1986).

For the subsequent analyses, an aqueous extract was obtained by suspension of FPH in deionized water (1% w/v), stirring for 30 min, centrifugation at 36,000 x g for 30 min at 10°C, filtration through N°1 Whatman qualitative filter paper, and freeze-drying.

2.2. Fractionation of the flaxseed protein hydrolysate (FPH) by RP-HPLC

The method reported by Hernández-Ledesma, Dávalos, Bartolomé & Amigo (2005) with slight modifications was used. FPH, at a concentration of 5.0 mg/mL, was separated using a Waters Nova-Pak HR C18 (300 mm × 7.8 mm internal diameter) column, in a Waters 600 HPLC (Waters Corp., Milford, MA, USA) equipped with two pumps (module delta 600), a pump controller (module 600), an autosampler (module 717), and a diode array detector (module 996) in combination with an automatic fractions collector (module II). The digests were eluted by using 0.37% (v/v) trifluoroacetic acid (TFA) in water as solvent A and 0.27% (v/v) TFA in acetonitrile as solvent B, at a flow rate of 3.5 mL/min. The injection volume was 350 µL. Peptides were eluted with a linear gradient of solvent B in A going from 0 to 40% over 45 min. Detection was carried out at 214 nm. Data were processed by using Empower 2

Software (Waters Corp.). Six fractions were collected from separate RP-HPLC runs, pooled, lyophilized, and stored at -20°C until further analyses.

2.3. Characterization of the flaxseed protein hydrolysate (FPH) and its fractions

The protein content of the FPH and the collected fractions was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA), using bovine serum albumin as standard protein.

Amino acids were analyzed in duplicate by RP-HPLC using a liquid chromatograph, consisting of a Waters 600 Controller programmable solvent module (Waters Corp.), a WISP 710B autosampler (Waters Corp.), and a HP 104-A fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA). Samples were submitted to automatic precolumn derivatization with ortho-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol following the method described by Moreno-Arribas, Pueyo, Polo & Martín-Álvarez (1998). Separation was carried out on a Waters Nova Pack C18 column (150 × 3.9 mm i.d., 60 Å, 4 µm) column. Detection was performed by fluorescence ($\lambda_{\text{excitation}} = 340 \text{ nm}$, $\lambda_{\text{emission}} = 425 \text{ nm}$), and chromatographic data were collected and analyzed with a Millenium 32 system (Waters Corp.). The FPH was previously hydrolyzed with 6 N HCl for 21 h at 110°C. Trp content was determined after alkaline hydrolysis of the sample with 4.2 N NaOH for 21 h at 110°C.

For analysis of peptide mass distribution both in the hydrolysate and its fractions, MALDI-TOF mass spectrometry (MS) was performed on a Bruker Autoflex Speed® (Bruker Daltonik GmbH, Bremen, Germany) as previously reported (Lozano-Ojalvo, Molina, & Lopez-Fandiño, 2016). Samples were spotted on a Bruker Anchorchip target with α -CHCA matrix in acetonitrile/water (30:70) containing 0.1% TFA. Mass spectra were acquired in positive reflectron mode by accumulating 1000 laser pulses on average. Calibration was

performed with the Peptide Calibration Standard I and II (Bruker Daltonik). To refine fraction characterization, m/z signals appearing in more than two fractions were discarded.

2.4. Antioxidant activity of the flaxseed protein hydrolysate (FPH) and its fractions

2.4.1. Oxygen radical absorbance capacity (ORAC)

An oxygen radical absorbance capacity (ORAC)-fluorescein (FL) assay (ORAC-FL) was used based on that optimized for protein hydrolysates and peptides by Hernández-Ledesma et al. (2005). Briefly, the reaction was carried out at 37°C in 75 mM phosphate buffer (pH 7.4). The final assay mixture (200 μ L) contained FL (70 nM), 2,2'-azobis (2-methylpropionamide)-dihydrochloride (AAPH, 14 mM) and antioxidant [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 0.2-1.6 nmol) or sample (at different concentrations)]. Fluorescence was recorded during 137 min (104 cycles) in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FLUOstar Control ver. 1.32 R2 software for fluorescence measurement. Three independent runs were performed for each sample. Final ORAC-FL value was expressed as μ mol Trolox equivalent per mg protein or μ mol peptide.

2.4.2. Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was carried out according to Benzie & Strain (1996) with modifications. In the dark, 30 μ L sample extract, standard or blank was mixed with 90 μ L of water and 900 μ L of the FRAP reagent (450 μ L of 0.3 M acetate buffer, pH 3.6; 225 μ L of 10 mmol tripyridyltriazine (TPTZ) in 40 mmol HCl and 225 μ L of 20 mmol FeCl_3). The mixture was incubated at 30°C for 30 min. The absorbance was measured at 595 nm in a Synergy™ HT Multi-Mode Microplate Reader (Biotek®, Vermont,

USA). Trolox was used as standard and the results were expressed as μmol of Trolox equivalent per mg of protein.

2.5. Peptide identification and synthesis

2.5.1 Peptide identification by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

FPH and its fractions were injected on an Acquity Ultrahigh Performance LC (UPLC) from Waters Corp., coupled to a Microtof-QII (Bruker Daltoniks). The LC-MS system was controlled by the HyStar 3.2 software (Bruker Daltonics). The column employed for the analyses was an Acquity UPLC BEH 130 C₁₈ of 2.1 mm $\times$ 50 mm (Waters Corp.), with a particle size of 1.7 μm . The injection volume was 5 μL , and the flow was set at 0.2 mL/min. Freeze-dried samples were dissolved (2 mg/mL) in 0.1% (v/v) formic acid (FA). Solvent A was a mixture of Milli-Q water/FA (100:0.1, v/v), and solvent B contained acetonitrile (HPLC grade)/FA (100:0.1, v/v). The gradient was 45% of solvent B in 27 min, after which the percentage of solvent B increased to 70% in 2 min, and remained 3 min. Data processing was done by using Data AnalysisTM (version 4.0; Bruker Daltoniks). For peptide sequencing, the matched MS/MS spectra were interpreted by using BioTools from Bruker, MASCOT from Matrixscience and X! Tandem from the Global Proteome Machine Organization (GPM), using a homemade database that includes the main proteins of flaxseed.

2.5.2. Peptide identification by Edman degradation

The N-terminal sequence of the main compound of the most active fraction was identified by sequence analysis with a Perkin-Elmer/Applied Biosystems Procise 494 microsequencer (Uberlingen, Germany) running in pulsed liquid mode.

2.5.3. Peptide Synthesis

The sequence of the peptide determined by Edman degradation was prepared in our laboratory by the conventional Fmoc solid-phase synthesis method with a 433A peptide synthesizer (Applied Biosystems, Warrington, UK), and its purity was verified by LC-MS/MS (Sánchez-Rivera et al., 2014).

3. Results and discussion

3.1 Characterization of the hydrolysed product

Different flaxseed products are being studied for their potential to present antioxidant activity. Most research explored the role of polyunsaturated fatty acids and lignan in these properties while flaxseed protein has been studied less often. However, hydrolysis of protein concentrates from this source with several proteases and digestive enzymes has been attempted as an effective method to promote several biological activities, such as ACE inhibitory (Marambe, & Shand, 2008) or anti-inflammatory (Udenigwe et al., 2009). In the case of antioxidant properties, flaxseed hydrolysates with proteases (Alcalase[®], Pronase, Flavourzyme[®]) have shown scavenging capacity on different radicals. Concretely, Alcalase[®] hydrolysates have been evaluated for scavenging 2,2-diphenyl-1-picrylhydrazyl radical, superoxide anion radical, electron-spin resonance-detected hydroxyl radical and nitric oxide with variable results for the different radicals (Udenigwe et al., 2009). The Alcalase[®] hydrolysed product analysed in the present study was prepared under the conditions selected based on a mathematical model for obtaining the highest antioxidant capacity measured by ORAC and FRAP (Silva et al., 2013). These two assays are widely used to evaluate the antioxidant activity of food protein/peptides (Power, Jakeman, & FitzGerald, 2013). The ORAC and FRAP values obtained for the hydrolysate were 0.93 ± 0.04 μmol Trolox equivalent/mg protein and 0.02 ± 0.00 μmol Trolox equivalent/mg protein, respectively. The

differing values obtained with both assays can be attributed to the implicated mechanism, i.e. hydrogen atom transfer where the hydrogen of peptides neutralizes the radicals formed for ORAC, and ferric ion reducing by electron transfer for FRAP. A number of works have reported the ability of Alcalase[®] to release antioxidant peptides from other plant proteins, although the activity showed by the hydrolysates was dependent on the protein source. As an example, canola meal protein Alcalase[®] hydrolysate showed an ORAC value of 2.20 μmol Trolox equivs/mg of protein, higher than that found in our flaxseed hydrolysate (Alashi et al., 2014) while Alcalase[®] hydrolysate obtained from common bean (*Phaseolus vulgaris*) protein showed an ORAC value of 0.33 μmol Trolox equivs/mg of protein (Oseguera-Toledo, González de Mejia, and Amaya-Llano, 2015).

The RP-HPLC analysis confirmed the extensive hydrolysis of the product by this highly nonspecific endopeptidase, and allowed the separation of a remarkable number of peptides that were collected in six fractions of increasing hydrophobicity (Figure 1A). The antioxidant capacity of the fractions was determined by the methods above mentioned and the values were expressed as μmol Trolox equivalent per mg of protein. All fractions showed activity in the ORAC assay (Figure 1B). Thus, taken as a whole, the separated peptides have a high potential to disrupt reactions that involve peroxy radicals but the highest value corresponded to fraction F6 (3.58 ± 0.12 μmol Trolox equivs/mg protein). This is consistent with studies where high radical scavenging activities for the protein hydrolysates or peptides are usually associated with hydrophobicity or high hydrophobic amino acid content (Rajapakse, Mendis, Byun, & Kim, 2005). In the FRAP assay, only fractions F3, F4 and F5 exhibited antioxidant capacity. The reducing power of the peptides might be attributed to the exposure of electron-dense amino acid side chain groups, such as polar or charged moieties (Bamdad, Wu, & Chen, 2011).

The peptide mass distribution profile of the FPH determined by MALDI-MS confirmed that Alcalase[®] had digested the proteins in small peptide fragments with overall sizes under 4000 Da with 70% of peptides in the range 500 to 1500 Da (Figure 2A). Despite the higher abundance of low m/z signals in all samples, the individual analysis of the fractions showed different compositions (Figure 2B). The analysis revealed that some fractions comprised peptides with the whole range of molecular weights, i.e. fractions F2 and F5. In contrast, fractions F3 and F6 showed predominantly small size peptides. Fractions F1 and F4 showed an intermediate mass profile. The most antioxidant fraction comprised only small size peptides, which suggested that the activity might be ascribed to these minor size ($M_w < 1500$) peptides. This would be in line with the statement that the majority of the antioxidative peptides derived from food sources have molecular weights between 500 and 1800 Da (Samaranayaka & Li-Chan, 2011). Onuh, Girgih, Aluko, & Aliani (2014) reported that the higher ORAC values of the small peptides may be due to increased ability to interact and donate electrons to the free radical when compared to bigger peptides that may have reduced ability to interact with the free radical.

The amino acid composition in protein hydrolysates is known to play an important role in the antioxidant capacity when it has to be attributed to peptides. Table 1 shows the amino acid distribution in the FPH. The hydrophobic amino acids represented 34.7% of the total amino acids. This might increase the solubility of the peptides in lipids, facilitating a better interaction with free radicals. Moreover, the aromatic amino acids might donate protons to electron deficient radicals, maintaining their stability. Their content in the hydrolysate was also important, 18.5%. Therefore, the amino acid composition of the Alcalase[®] hydrolysate was compatible with the presence of peptides with antioxidant capacity.

3.2 Identification of peptides

The analysis by LC-MS/MS allowed to detect many peptide compounds but only four fragments matched sequenced proteins from flaxseed (Table 2). As an example, Figure 3 shows the tandem MS spectrum of ion m/z 1012.45. Following sequence interpretation this ion found in fraction F2 corresponded to fragment 35-47 of conlinin, one of the high abundant seed storage proteins, consisting of 168 amino acids. The fragment in fraction F5 belonged to cellulose synthase, a protein involved in the pathway of plant cellulose biosynthesis which consists of 1097 amino acids. The fragments found in fractions F5 and F6 corresponded each to the sequence of UDP-glycosyltransferase-1, based on protein homology. The identified peptides might be candidates for the antioxidant activity due to the presence of Tyr and Met in their sequence. Previous studies on the amino acid composition-activity relationship have demonstrated the relevance of those amino acids. Besides, antioxidant behavior using the thiobarbituric acid method has been observed in sequences with Gly in the C-terminal end (Kim *et al.* 2001). However, the correspondence of these sequences with an enzyme protein limits their abundance in the source.

The predominant compound in the LC-MS/MS analysis of fraction F6 (Figure 4) could not be assigned to any known protein although it had also been detected in the MALDI-MS analysis. In order to identify the compound, fraction F6 was submitted to Edman degradation. A 13 amino acids sequence, GFPGRLDHWCASE, was identified although it could not be allocated in any of the sequenced flaxseed proteins. It must be kept in mind that not all flaxseed proteins sequences are known to date. The amino acid residues present in the sequence anticipated antioxidant activity. Hence, His, Pro, Ala have been reported to contribute to the scavenging of free radicals in soybean conglycinin antioxidant peptides (Chen, Muramoto & Yamauchi, 1995). Furthermore, Trp is an amino acid that has shown potent ORAC activity (Hernández-Ledesma, Amigo, Recio & Bartolomé, 2007).

The identified sequence was synthesised and its ORAC activity was assayed. A value of 3.20 ± 0.24 μmol Trolox equivalents/ μmol peptide was determined. This value indicates a potent antioxidant capacity in relation with the disruption of reactions involving peroxy radicals. The antioxidant capacity of this peptide is higher than that of butylated hydroxyanisole (2.43 μmol Trolox equivalents/ μmol), a synthetic antioxidant currently used in the food industry (Marteau et al., 2016). Taking into account the Trolox equivalents per mg of protein of fraction F6, the activity shown by the peptide would account for 61% of the total activity of the fraction.

To date, few peptides released from flaxseed proteins have been identified. Marambe et al. (2011) analysed selected ACE inhibitory fractions collected from a flaxseed hydrolysate. Two probable amino acid sequences assigned by the authors to the non-sequenced protein linin were considered as responsible for the effects. In a next study, the chromatographic fractions of the Alcalase[®] hydrolysate were subjected to tandem MS peptide sequencing, and short flaxseed protein-derived peptides were provided although in most cases they were potential sequences comprising multiple options (Udenigwe & Aluko, 2012). Recently, several peptides corresponding to conlinin were identified by 2D electrophoresis and MALDI-MS, and this protein was described to contribute to the emulsification properties of flaxseed gum (Liu et al., 2016). However, the identified peptides were not assayed for this property nor they can be assigned to a particular biological activity.

The specific contribution of the protein fraction to the antioxidant capacity of the whole flaxseed products is not fully elucidated. The present study showed that the protein fraction of flaxseed can be the source of peptides with a notable antioxidant capacity as determined by ORAC and FRAP assays, and both the size and the sequence of some of the identified peptides are compatible with the observed effects. This has allowed assigning the radical disruption activity of a flaxseed hydrolysate to the peptides generated with Alcalase[®].

The sequences provided in this work, and especially the peptide GFPGRLDHWCASE which shows antioxidant activity itself, represent an advance in the molecular characterization of the flaxseed protein fraction.

Acknowledgments

This work has received financial support from projects AGL2015-66886-R and FAPESP (2010/52680-7). F.G.D. Silva acknowledges the CNPq for the scholarship granted, and B. Hernández-Ledesma thanks the Ministry of Economy and Competitiveness (MINECO) for her “Ramón y Cajal” post-doctoral contract. The authors thank Dr. A. Guerrero (UC Davis, USA) for his help on peptide identification.

Conflict of interest

The authors declare that they have no competing interests.

References

Adler-Nissen, J. (1986). *Enzymic hydrolysis of food proteins* (1st ed.). New York, pp 110-131: Elsevier Applied Science Publishers.

Alashi, A.M., Blanchard, C.L., Mailer, R.J., Agboola, S.O., Mawson, A.J., He, R., Girgih, A., Aluko, R.E. (2014). Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chemistry*, 146, 500-506.

Bamdad, F., Wu, J., & Chen, L. (2011). Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. *Journal of Cereal Science*, 54, 20-28.

Barthet, V.R.J., Klensporf-Pawlik, D., & Przybylski, R. (2014) Antioxidant activity of flaxseed meal components. *Canadian Journal of Plant Science*, 94, 593-602.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “Antioxidant Power”: The FRAP assay. *Analytical Biochemistry*, 239, 70-76.

Cardoso Carraro, J. C., Dantas, M. I. d. S., Espeschit, A. C. R., Martino, H. S. D., & Ribeiro, S. M. R. (2012) Flaxseed and human health: Reviewing benefits and adverse effects. *Food Reviews International*, 28, 203-230.

Chen, H.-M., Muramoto, K., & Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean beta-conglycinin. *Journal of Agricultural and Food Chemistry*, 43, 574-578.

366 Chung, M. W. Y., Lei, B., & Li-Chan, E. C. Y. (2005). Isolation and structural
 367 characterization of the major protein fraction from NorMan flaxseed (*Linum usitatissimum*
 368 L.). *Food Chemistry*, 90, 271-279.

369

370 Doyen, A., Udenigwe, C. C., Mitchell, P. L., Marette, A., Aluko, R. E., & Bazinet, L. (2014).
 371 Anti-diabetic and antihypertensive activities of two flaxseed protein hydrolysate fractions
 372 revealed following their simultaneous separation by electro dialysis with ultrafiltration
 373 membranes. *Food Chemistry*, 145, 66-76.

374

375 Dryáková, A., Pihlanto, A., Marnila, P., Curda, L., & Korhonen, H. T. (2010). Antioxidant
 376 properties of whey protein hydrolysates as measured by three methods. *European Food*
 377 *Research and Technology*, 230, 865-874.

378

379 Fiaschi, T., & Chiarugi, P. (2012). Oxidative stress, tumor microenvironment, and metabolic
 380 reprogramming: a diabolic liaison. *International Journal of Cell Biology*, Art. ID. 762825.

381

382 Hernández-Ledesma, B., Amigo, L., Recio, I., & Bartolomé, B. (2007). ACE-Inhibitory and
 383 radical-scavenging activity of peptides derived from β -lactoglobulin f(19-25). Interactions
 384 with ascorbic acid. *Journal of Agricultural and Food Chemistry*, 55, 3392-3397.

385

386 Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005). Preparation of
 387 antioxidant enzymatic hydrolysates from α -lactalbumin and β -lactoglobulin. Identification of
 388 active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, 53, 588-593.

389

Hosseinian, F., Muir, A., Westcott, N., & Krol, E. (2006). Antioxidant capacity of flaxseed lignans in two model systems. *Journal of the American Oil Chemists' Society*, 83, 835-840.

Johnsson P, Peerlkamp A, Kaml-Eldin R E, Andersson L N, Lundgren L N, & Aman P. (2002). Polymeric fractions containing phenol glucosides in flax. *Food Chemistry*, 76, 207-212.

Kim, S.-K., Kim, Y.-T., Byun, H.-G., Nam, K.-S., Joo, D.-S., & Shahidi, F. (2001). Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska Pollack skin. *Journal of Agricultural and Food Chemistry*, 49, 1984-1989.

Liu, J., Shim, Y. Y., Poth, A. G., & Reaney, M. J. T. (2016). Conlinin in flaxseed (*Linum usitatissimum* L.) gum and its contribution to emulsification properties. *Food Hydrocolloids*, 52, 963-971.

Lozano-Ojalvo, D., Molina, E., & Lopez-Fandino, R. (2016). Hydrolysates of egg white proteins modulate T- and B-cell responses in mitogen-stimulated murine cells. *Food & Function*, 7, 1048-1056.

Marambe, H. K., & Shand, P. J. (2008). An in-vitro investigation of selected biological activities of hydrolysed flaxseed (*Linum usitatissimum* L.) Proteins. *Journal of the American Oil Chemists' Society*, 85, 1155-1164.

Marambe, H. K., Shand, P. J., & Wanasundara, J. P. D. (2011). Release of angiotensin I-converting enzyme inhibitory peptides from flaxseed (*Linum usitatissimum* L.) Protein under

415 simulated gastrointestinal digestion. *Journal of Agricultural and Food Chemistry*, 59, 9596-
416 9604.

417
418 Marambe, H. K., Shand, P. J., & Wanasundara, J. P. D. (2013). *In vitro* digestibility of
419 flaxseed (*Linum usitatissimum* L.) protein: effect of seed mucilage, oil and thermal
420 processing. *International Journal of Food Science and Technology*, 48, 628-635.

421
422 Marteau, C., Guitard, R., Penverne, C., Favier, D., Nardello-Rataj, V., & Aubry, J.-M. (2016).
423 Boosting effect of ortho-propenyl substituent on the antioxidant activity of natural phenols.
424 *Food Chemistry*, 196, 418-427.

425
426 Moreno-Arribas, M. V., Pueyo, E., Polo, M.C., & Martín-Álvarez, P. J. (1998). Changes in
427 the amino acid composition of the different nitrogenous fractions during the aging of wine
428 with yeast. *Journal of Agricultural and Food Chemistry*, 46, 4042-4051.

429
430 Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2014). *In vitro* antioxidant properties
431 of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chemistry*,
432 150, 366-373.

433
434 Oseguera-Toledo M. E., González de Mejia, E., & Amaya-Llano, S.L. (2015). Hard-to-cook
435 bean (*Phaseolus vulgaris* L.) proteins hydrolyzed by alcalase and bromelain produced
436 bioactive peptide fractions that inhibit targets of type-2 diabetes and oxidative stress. *Food*
437 *Research International*, 76, 839-851.

439 Power, O., Jakeman, P., & FitzGerald, R. J. (2013). Antioxidative peptides: enzymatic
 440 production, in vitro and in vivo antioxidant activity and potential applications of milk-derived
 441 antioxidative peptides. *Amino Acids*, 44, 797-820.

442

443 Rabetafika, H. N., Van Remoortel, V., Danthine, S., Paquot, M., & Blecker, C. (2011).
 444 Flaxseed proteins: food uses and health benefits. *International Journal of Food Science &*
 445 *Technology*, 46, 221-228.

446

447 Rajapakse, N., Mendis, E., Byun, H.-G., & Kim, S.-K. (2005). Purification and in vitro
 448 antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative
 449 systems. *The Journal of Nutritional Biochemistry*, 16, 562-569.

450

451 Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A
 452 review of their production, assessment, and potential applications. *Journal of Functional*
 453 *Foods*, 3, 229-254.

454

455 Sammour, R. H. (1999). Proteins of linseed (*Linum usitatissimum* L.), extraction and
 456 characterization by electrophoresis. *Botanical Bulletin of Academia Sinica*, 40, 121-126.

457

458 Sánchez-Rivera, L., Ares, I., Miralles, B., Gómez-Ruiz, J. Á., Recio, I., Martínez-Larrañaga,
 459 M. R., Anadón, A., & Martínez, M. A. (2014). Bioavailability and kinetics of the
 460 antihypertensive casein-derived peptide HLPLP in rats. *Journal of Agricultural and Food*
 461 *Chemistry*, 62, 11869-11875.

462

Sharav, O., Shim, Y. Y., Okinyo-Owiti, D. P., Sammynaiken, R., & Reaney, M. J. T. (2014).
Effect of Cyclolinopeptides on the Oxidative Stability of Flaxseed Oil. *Journal of
Agricultural and Food Chemistry*, 62, 88-96.

Shim, Y. Y., Gui, B., Arnison, P. G., Wang, Y., & Reaney, M. J. T. (2014). Flaxseed (*Linum
usitatissimum* L.) bioactive compounds and peptide nomenclature: A review. *Trends in Food
Science & Technology*, 38, 5-20.

Silva, F. G. D., O'Callaghan, Y., O'Brien, N. M., & Netto, F. M. (2013). Antioxidant
capacity of flaxseed products: The effect of *in vitro* digestion. *Plant Foods for Human
Nutrition*, 68, 24-30.

Udenigwe, C. C., Lu, Y.-L., Han, C.-H., Hou, W.-C., & Aluko, R. E. (2009). Flaxseed
protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-
induced nitric oxide production in murine macrophages. *Food Chemistry*, 116, 277-284.

Udenigwe, C., & Aluko, R. (2012). Multifunctional cationic peptide fractions from flaxseed
protein hydrolysates. *Plant Foods for Human Nutrition*, 67, 1-9.

Figure captions

Figure 1. RP-HPLC chromatographic separation of the flaxseed hydrolysate (A) and determined antioxidant activity in the collected fractions by ORAC (gray) and FRAP (black) assays.

Figure 2. Peptide mass frequency distribution of the flaxseed hydrolysate (A) and the RP-HPLC collected fractions (B).

Figure 3. Tandem MS spectrum of ion m/z 1012.45. Following sequence interpretation it matched to flaxseed conlinin.

Figure 4. (A) UV-chromatogram and total ion current chromatogram (TIC) of fraction F6. (B) Compound m/z 1476.9 and +2 (738.9) and +3 (492.9) species.