LC-ESI-QTOF-MS identification of novel antioxidant peptides obtained by enzymatic and microbial hydrolysis of vegetable proteins

Abbreviated running title:

LC-ESI-QTOF-MS identification of novel vegetable antioxidant peptides

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ABSTRACT

Bioactive antioxidant peptides are more and more attracting the attention of food manufacturers for their potential to transform food in functional food, able to prevent a variety of chronic diseases associated with oxidative stress. In the present study proteins extracted from different vegetable sources (KAMUT® khorasan wheat, emmer, lupine and pea) were hydrolyzed with commercial enzymes and *Lactobacillus* spp. strains. Hydrolysates were separated by size exclusion chromatography and purified fractions were analyzed for their antioxidant activity. Peptides from the fractions with the highest activity were identified by nanoLC-ESI-QTOF-MS and thirteen peptides were selected for synthesys on the basis of their sequence. Four peptides (VLPPQQQY, TVTSLDLPVLRW, VTSLDLPVLRW, FVPY) were found able to scavenge superoxide anion and hydroxyl radicals, organic nitro-radicals (ABTS, DPPH) and to inhibit lipid peroxidation. The impact of this work is targeted to add hydrolysed vegetable proteins to reformulated functional food or to produce health-promoting ingredients and nutraceuticals.

Keywords: antioxidant peptides, microbial hydrolysis, enzymatic hydrolysis, Lactobacillus, nano LC-ESI-QTOF-MS, vegetable proteins

Abbreviations: AA: ascorbic acid; AAeq: ascorbic acid equivalents; ABTS: *2,2,-azino-bis(3-ethylbenz-thiazoline-6-sulfonic) acid*; KKW: KAMUT® Khorasan Wheat; DPPH: 1,1-DiPhenyl-2-PicrylHydrazyl; GSH: L-glutathione reduced; SDS-PAGE: Sodium Dodecyl Sulfate-PoliAcrylamide Gel Electrophoresis; SEC: Size Exclusion Chromatography; SD: Standard Deviation; MW: Molecular Weight; LC-ESI-QTOF-MS: Liquid Chromatography-ElectroSpray Ionization-Quadrupole Time Of Flight-Mass Spectrometry.

1. Introduction

Biologically active peptides are defined as specific protein fragments (from 2 to 20 amino acid residues and molecular masses less than 6.0 kDa) that have positive effects on body functions or conditions and may influence human health (Kitts & Weiler, 2003). A large spectrum of biological activities has been assigned to these compounds, including antihypertensive, opioid, immunomodulatory, mineral sequestering, antioxidant, antimicrobial (Kits et al., 2003), antithrombotic (Shimizu et al., 2009) and hypocholesterolemic (Zhong, Liu, Ma & Shoemaker 2007).

Bioactive peptides correspond to cryptic sequences from parent food proteins, which are latent until they are released and activated by hydrolytic reactions that take place during food fermentation and processing or during gastrointestinal digestion (Korhonen & Pilhanto, 2007). The release of these peptides can be obtained also by treatment of proteinaceous food sources with proteases or combination of proteases like alcalase, chymotrypsin, pancreatin, pepsin, thermolysin and enzymes from bacterial and fungal sources (Kits et al., 2003; Sarmadi & Ismail, 2010; Zhang, Zhang, Wang, Guo, Wang & Yao, 2010). Whole cell systems, based on the hydrolytic activity of cultured bacterial cells, have been used with different food matrices, as well (Kits et al., 2003; Sarmadi et al., 2010; Coda, Rizello, Pinto & Gobbetti, 2012).

Information on peptide sequences are extremly relevant to understand the molecular mechanisms involved in peptide bioactivities and is required to develop medical applications (Lee, Bae, Lee & Yang, 2006). In fact, the specific bioactivity of food peptides against various molecular disease targets depends on their structural properties such as chain length and physicochemical characteristics of the amino acid residues, like hydrophobicity, molecular charge and side-chain bulkiness (Pripp, Isaksson, Stepaniak, Sorhaug & Ardo, 2005).

In the recent years, interest in antioxidant peptides has particularly grown, as there is evidence that they can prevent oxidative stress associated with numerous degenerative diseases like cancer and

77 artherosclerosis (Coda et al., 2012; Chakrabarti, Jahandideh & Wu, 2014). These peptides have
78 been mainly studied in meat, poultry, fish and the traditional animal origin fermented products
79 (Sarmadi et al., 2010). Plants are known for antioxidant properties mostly because of their
80 polyphenolic compounds (Chakrabarti et al., 2014), but recently the antioxidant properties of
81 vegetable proteins and peptides have been increasingly explored both in *in vitro* and *in vivo* studies.
82 For a consistent report of vegetable proteins and peptides with antioxidant activity see García,
83 Puchalska, Esteve and Marina (2013) and for vegetal substrate for their biosynthesis Rizzello,
84 Tagliazucchi, Babini, Sefora Rutella, Taneyo Saa and Gianotti (2016).
85 This research field (i.e. the identification of antioxidant and generally bioactive peptides in
86 vegetable food proteins) follows the growing interest of Food and Nutrition Science towards
87 vegetable foods, due to their higher sustainability with respect to animal foods and the increased
88 cunsumer requirements of healthy and balanced vegetable diets. Cereals (supplying half the world's
89 protein needs) and legumes are the main target of this research, being both rich sources of proteins
90 with a complementary spectrum of amino acids. Hence, they have the potential to be good
91 substrates for the formulation of functional foods, nutraceuticals and natural drugs.
92 In this light, the present study was aimed to use LC-ESI-QTOF-MS to investigate the antioxidant
93 potential of vegetable peptides obtained by enzymatic and whole cell hydrolysis of cereal and
94 legume protein extracts. In particular, KAMUT® khorasan wheat (KKW - a registered trademark of
95 Kamut Internatuinal, Ltd. And Kamut Enterprises of Europe, bvba certifying that grain is 100%
96 ancient khorasan wheat organically grown) and emmer were selected as substrates for their relevant
97 protein content, which is higher than that of modern wheat (Bonafaccia, Galli, Francisci, Mair,
98 Skrabanja & Kreft, 2000; Dvoracek & Curn, 2003; Marconi et al., 1999; Piergiovanni, Laghetti &
99 Perrino, 1996; Ranhorta, Gelroth, Glaser & Lorenz, 1996) although it seemed to be most suitable
100 for non-celiac wheat-sensitive people (Carnevali, Gianotti, Benedetti, Tagliamonte, Primiterra,
101 Laghi, Danesi, Valli, Ndaghijimana, Capozzi, Canestrari & Bordoni, 2014). The choice of pea and
102 lupine flours, among legumes, was instead dictated by their suitability to be incorporated into high

carbohydrate foods, resulting in significant increase in proteins and fibers, reduction in refined carbohydrates, and little change in product acceptability (Aguilera & Trier, 1978).

2. Materials and Methods

2.1. Materials

Two KKW sourdoughs were used, one from a southern italian bakery (Altamura, Italy) and the other from a french bakery. KKW whole flour was purchased from a local mill (Cesena, Italy), emmer bran from *Azienda Agricola Prometeo* (Urbino, Italy) while pea and lupine flours from *Bongiovanni & C Snc* (Cuneo, Italy).

Reagents were analytical grade from Sigma (USA), Merck (Germany) and Oxoid (England). Reagents and standard for sodium dodecyl sulfate-poliacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Italy). Enzymes Alcalase® 2.4L , Neutrase® 0.8L , Flavourzyme® 500U/g were from Sigma (USA). Synthetic peptides (purity = 95%) were purchased from ChinaPeptides Co., Ltd (China).

2.2. Microrganisms

L. casei lbcd, *L. fermentum* MR13, *L. paracasei* 1122, *L. plantarum* 98a, *L. rhamnosus* C1272, *L. rhamnosus* C249, *L. sanfranciscensis* bb12 and *L. brevis* 3BHI belong to the collection of the Department of Agri-Food Sciences and Technologies of the University of Bologna (Italy). Bacteria were grown for 24 h at 37 °C in MRS broth modified by the addition of yeast extract (5%, v/v) and 28 mM maltose, at pH 5.6. The enumeration of bacteria was carried out by plating serial dilutions of dough on modified MRS agar medium at 37 °C for 48 h.

2.3. Sourdough fermentation

KKW flour was used to prepare 8 different doughs containing 100 g of flour and 400 g of tap water. Fermentation with the pool of selected bacteria at their late exponential phase (initial cell load of $5*10^7$ CFU/g of dough) was carried out at 37 °C for 72 h under mild agitation on a rotary shaker.

Control dough without bacterial inoculum was prepared and incubated under the same conditions. The pH was measured, in triplicate, at the beginning and at the end of fermentation.

2.4. Protein extraction

Proteins were extracted from substrates according to the method originally described by Osborne (1907) and further modified by Weiss, Vogelmeier & Gorg (1993). For each dough, an aliquot containing 7.5 g of flour was diluted with 30 mL of 50 mM Tris-HCl (pH 8.8), held at 4 °C for 1 h, vortexing each 15 min, and centrifuged at 25432 rcf for 20 min. The supernatant, containing the water/salt-soluble nitrogen fraction, was stored at -80 °C. The same procedure was applied to 7.5 g samples of KKW flour and emmer bran. The protein fractions of pea and lupine flours were obtained after defatting the flours using the cold extraction method (Dei Più et al. 2014). Flour was mixed with n-hexane, stirred for 16 h and then filtered. This step was repeated twice. The filtrate was open air dried at room temperature, then ground to pass through a 70 mesh screen and finally stored at -80 °C.

2.5. Hydrolysis reactions

Hydrolysis with bacteria was made using cultures grown for 12 hours in modified MRS broth. Cells were harvested by centrifugation at 11381 rcf for 10 min at 4 °C, washed twice with 20 mM phosphate buffer (pH 7.0) and resuspended in the same buffer at a concentration of ca. 10^9 CFU/mL. The assay mixture, containing 6 mL of cellular suspension and 30 mL of protein extract, was incubated at 37 °C for 72 h and then centrifuged at 7741 rcf for 5 min to remove cellular debris. Hydrolysis with commercial enzymes was carried out according to Dei Più, Tassoni, Serrazanetti, Ferri, Babini, Tagliazucchi and Gianotti (2014) for 2 h using enzymes at a ratio of 0.3 UA/g. Reactions conditions were: pH 8 and 55 °C for Alcalase; pH 6.5 and 40 °C for Neutrase; pH 6 and 60 °C for Flavourzyme. Reactions were stopped by heating at 80 °C for 20 min and samples were centrifuged to remove the insoluble material.

Ten g of KKW dough and 10 mL of hydrolysed samples were freeze-dried under vacuum at -56 °C for 48 h, in a Heto Power Dry LL 3000 lyophilizer, Thermo Electron Corporation (USA), and stored at -80 °C. Before use, freeze dried samples were solubilized in 0.1 M phosphate buffer (pH 7.0) and centrifuged at 27216 rcf for 10 min.

2.6. Protein pattern analysis by SDS-PAGE

SDS-PAGE was performed using hand-cast 12% polyacrylamide gels and Mini-PROTEAN® equipment from Bio-Rad (Italy). Sample volumes were optimized to get the best separation of proteins and Precision Plus Protein Standard (Bio-Rad, Italy) was used as marker.

2.7. Purification of peptides by size exclusion chromatography (SEC)

Peptides from 50 □L samples were fractionated by SEC using phosphate buffer 0.1 M (pH 7.0) as eluent, on a TSKgel G2500PW$_{XL}$ column from Tosoh Bioscience SRL (Italy) with AKTA FPLC equipment from GE Healthcare (Sweden). Fractions of 500 µL volume were collected and analysed for DPPH activity and peptide sequence.

2.8. Antioxidant activity assays

2.8.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant activity of soluble extracts of substrates and doughs, of their hydrolysates, of purified fractions from SEC and of synthetic peptides (the latter dissolved in DMSO to a concentration of 0.5 mM) was measured by the DPPH free radical scavenging assay, according to the method of Govindarajan et al. (2003). The sample was added to 1:10 volumes of 100 □M DPPH in methanol. The mixture was shaken, left for 30 min at room temperature in the dark and the absorbance was read at 517 nm. The value was corrected for the blank (DMSO) and the DPPH activity was expressed as µg ascorbic acid (AA) eq/mL or mmol glutathione (GSH) eq/mol peptide,

by means of calibration curves obtained with AA (0 to 10 □M) or GSH (1 to 5 □M), in the same assay conditions.

The antioxidant activity of synthetic peptides (in 0.5 mM DMSO solution) was determined also with other common assays, hereafter described.

2.8.2 *2,2,-azino-bis(3-ethylbenz-thiazoline-6-sulfonic) acid (ABTS) assay*

The ABTS assay was performed according to the method of Ozgen, Reese, Tulio, Scheerens & Miller (2006). Briefly, equal amounts of ABTS 7 mM in 20 mM sodium acetate (pH 4.5) and 2.45 mM potassium persulfate were mixed and allowed to stand for 12-16 h at room temperature in the dark, until reaching a stable oxidative stress. The solution was then diluted with 20 mM sodium acetate (pH 4.5) to an absorbance of 0.700 ± 0.01 at 734 nm. For each peptide, the sample was added to 1:10 volume of $ABTS^{\cdot+}$ solution, shaked, and after 30 minutes at room temperature in the dark, the absorbance was read at 734 nm. The value was corrected for the blank (DMSO) and the ABTS scavenging capacity was expressed as mmol GSH/mol peptide, by means of a calibration curve obtained with GSH (1 to 5 □M), in the same assay conditions.

2.8.3 *Ferric reducing antioxidant power (FRAP) assay*

The ferric reducing potential of the synthetic peptides was tested following the method reported by Benzie & Strain (1999) adapted to a microplate reader. Working FRAP reagent was prepared by mixing 10 volumes of acetate buffer (300 mM, pH 3.6), 1 volume of 10 mM TPTZ (2,4,6-tripyridyl-S-triazine) dissolved in HCl 10 mM and 1 volume of 20 mM $FeCl_3$. Then, 150 µL of working FRAP reagent were mixed with 5 µL of peptide, into a clear bottom 96-well plate. The absorbance at 595 nm was read after 6 min of reaction at room temperature, using a microplate reader. Data are reported as mmol GSH/mol peptide, by means of a calibration curve obtained with GSH (0.5 to 5 mM), in the same assay conditions.

2.8.4 *Hydroxyl radical scavenging assay*

The capacity to scavenge hydroxyl radicals was evaluated according to a method reported by Ajibola, Fashakin, Fagbemi & Aluko (2011), with some modifications. The assay consisted of mixing 50 µL of 3 mM TPTZ dissolved in HCl 50 mM, 50 µL of 3 mM $FeSO_4$, 50 µL of synthetic peptide or GSH (at concentration ranging from 1 to 10 mM), and 50 µL of 0.01% (v/v) hydrogen peroxide, in a clear bottom 96-well plate. The mixture was incubated for 1 h at 37°C and the absorbance was measured at 595 nm using a microplate reader. The hydroxyl radical scavenging capacity was expressed as mmol glutathione/mol peptide.

2.8.5 *Superoxide anion radical scavenging activity assay*

The superoxide anion radical scavenging activity of the synthetic peptides was determined by the method of Bamdad & Chen (2013). An aliquot (80 µL) of peptide or GSH at different concentrations (0.125–2 mM) was mixed with 80 µL of 50 mM Tris-HCl buffer (pH 8.3) containing 1 mM EDTA in a 96-well microplate and 40 µL of 1.5 mM pyrogallol in 10 mM HCl. After 10 min of incubation at room temperature, the superoxide anion radical-induced polymerization of pyrogallol was measured as increase in absorbance at 405 nm using a microplate reader. The superoxide anion scavenging capacity was expressed as mmol glutathione/mol peptide.

2.8.6 *Assay of metal ion chelation*

The metal chelating activity of the synthetic peptides was assessed following the method developed by Karama & Pegg (2009), adapted to a microplate reader. An aliquot (200 µL) of 0.5 mM peptide sample solution was combined with 20 µL of $FeSO_4$ (2 mM) and 40 µL of TPTZ (3 mM in HCl 10 mM) in a clear bottom 96-well plate. After 10 min of incubation at room temperature, absorbance of sample was measured using a microplate reader at 595 nm. GSH was used as standard compound.

232 **2.8.7** ***Lipid peroxidation inhibition assay***

233 This assay was carried out using a linoleic acid emulsion system according to the methods of Osawa
234 & Namiki (1985) with some modifications. For that purpose 200 µL of sample (synthetic peptides
235 or GSH at concentration of 0.5 mM dissolved in DMSO), 200 µL of 99.5% ethanol and 2.6 µL of
236 linoleic acid were mixed and the total volume was adjusted to 500 µL with sodium phosphate
237 buffer, 50 mM, pH 7.0. The mixture was incubated at 40°C in the dark for seven days. The amount
238 of generated lipid hydroperoxide was measured by the FOX assay as reported by Tagliazucchi et al.
239 (2010). The lipid peroxidation inhibitory activity of the peptides and GSH was expressed as
240 percentage of inhibition respect to a control reaction in which the sample was replaced with DMSO.
241

242 ***2.9. Identification and sequencing of antioxidant peptides by nano-LC-ESI-QTOF-MS***

243 SEC fractions showing the highest antioxidant activity were subjected to nano LC-ESI-QTOF-MS
244 analysis for peptide identification.

245 Experiments were performed according to Dei Più et al. (2014). For identification, MS^2 spectra
246 were converted to .mgf files and then searched against the Swiss-Prot database using MASCOT
247 Matrix Science (USA) and Protein Prospector (USA) protein identification softwares. The
248 following parameters were considered: enzyme, none; peptide mass tolerance, ± 40 ppm; fragment
249 mass tolerance, ± 0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal
250 number of PTMs permitted in a single peptide 2. Only peptides with a best expected value lower
251 than 0.05 that corresponded to $P < 0.01$ were considered. De novo peptide sequencing was
252 performed using Peaks 6 software from Bioinformatics Solutions Inc. (Canada) and the same
253 parameters as described above.
254

255 ***2.10. Statistical analysis***

Antioxidant activity assays mean values were reported along with standard deviation (SD). ANOVA analysis was done to evaluate the significance of antioxidant activity. Statistical analysis for peptide identification was described into the specific section.

3. Results and Discussion

3.1. Dough fermentation

Cell load of KKW doughs fermented by lactic acid bacteria reached an average value of 7.39 log CFU/g after 72 h at 37 °C. The lowest value was found in the dough fermented by *L. casei* lbcd (5.6 log CFU/g), the highest in Altamura dough (9.42 log CFU/g). Before fermentation, pH values were 6.69 ± 0.06 and at the end 3.61 ± 0.25.

3.2. Antioxidant activity of doughs

For some traditional fermented products deriving from milk, soy and rice the impact on health of microbial fermentation of proteins has been deeply evaluated. In the recent years fermentation has been considered a tool to increase the nutritional value of other vegetable substrates, testing the biological activity of protein hydrolysates with different bacterial strains. Here, the effect of fermentation by *Lactobacillus* strains on antioxidant activity was tested on KKW, Altamura and french bakery doughs, using the DPPH assay as preferred method, accordingly to Coda et al. (2012) (Table 1). The resulting activities were significantly different in comparison to each control flour (Table 1S). They were very similar with only minor differences with respect to control (62.39 ± 0.01 µg AAeq). The highest value was found in *L. plantarum* 98a dough (68.75 ± 0.05 µg AAeq), the lowest in *L. paracasei* 1122 (58.11 ± 0.13 µg AAeq) and *L. brevis* 3BH1 (57.79 ± 0.02 µg AAeq), doughs. Doughs with the highest activity (*L. plantarum* 98a, *L. rhamnosus* C249 and Altamura) were subjected to SEC separation for identification of peptides potentially responsible of the detected activity.

3.3. Protein pattern and antioxidant activity of hydrolysates

Protein pattern of hydrolysates was analysed on SDS-PAGE for KKW flour and emmer bran (for lupine and pea flours, no significant results were obtained as the extraction method made the

285 samples not suitable for this technique). As expected, different hydrolytic activities were detected
286 for both substrates, depending on strains and enzymes. Figure 1 shows, as an example, the SDS-
287 PAGE of emmer bran. The protein pattern of hydrolysates looks different with only a partial
288 fragmentation of proteins, as it can be seen by the presence, in all samples, of medium and high
289 molecular weight (MW) proteins.
290 Antioxidant activity was measured for bacterial and commercial enzyme hydrolysates of all the
291 substrates (Table 1).
292 For KKW flour hydrolysis by Alcalase and Neutrase increased the activity of about 6.6 (410.15 ±
293 0.24 µg AAeq) and 9 times (568.35 ± 0.03 µg AAeq) with respect to control sample (62.39 ± 0.01
294 µg AAeq). For emmer bran almost all the strains, except *L. casei* lbcd, increased the activity of the
295 not hydrolysed sample (227.67 ± 0.04 µg AAeq) and in particular *L. plantarum* 98a (588.30 ± 0.42
296 µg AAeq), *L. paracasei* 1122 (445.12 ± 0.09 µg AAeq) and *L. sanfranciscensis* bb12 (422.33 ±
297 0.05 µg AAeq). Alcalase and Neutrase were also highly active, giving hydrolysates with antioxidant
298 values of 318.44 ± 0.4 µg AAeq and 318.35 ± 0.02 µg AAeq, respectively.
299 Despite hydrolysis usually results in the release of peptides with higher antioxidant activity respect
300 to the parent proteins,, for lupine and pea flours hydrolysis with both lactic acid bacteria and
301 commercial enzymes did not increase the activity with respect to control samples (234.43 ± 0.05 µg
302 AAeq and 174.96 ± 0.05 µg AAeq, respectively). Lupine and pea flours, differently from Kamut
303 and emmer bran, contain protein (such as SOD-like proteins) and protein-phenolic complexes
304 which conferred high radical scavenging activity (Nice, Robinson and Holden (1995); Martínez-
305 Villaluenga, Zieliński, Frias, Piskuła, Kozłowska and Vidal-Valverde (2009)). Hydrolysis could
306 result in the degradation of antioxidant protein culminating in a decrease in the antioxidant activity.
307 On the other hand, (Frias, Miranda, Doblado and Vidal-Valverde (2005) reported that *Lupinus albus*
308 fermentation produced a reduction of 23% of the antioxidant activity measured with the DPPH
309 assay. In pea flour, the antioxidant activity of Flavourzyme hydrolysate was significantly higher
310 (Table 1) than hydrolysates produced by other enzymes. This results are in agreement with that

obtained by Huminski and Aluko (2007), which showed that the hydrolysis of pea proteins with Flavourzyme gave higher antioxidant activity (DPPH assay) than that obtained by hydrolysis with Alcalase and other enzymes. Results reported here suggested that the antioxidant activity of the hydrolysates depends on the substrate, the type of fermentation and/or enzyme utilized.

Neverthless, considering that the aim of the study was to identify new short antioxidant peptides that can be used in functional food and pharmaceutical applications, hydrolysates obtained by *L. fermentum* MR13, lupine flour hydrolysed by Neutrase and pea flour hydrolysed by Flavourzyme were selected for further characterization.

3.4. Antioxidant activity of SEC fractions of doughs and hydrolysates

Doughs and hydrolysates with the highest antioxidant activity, together with control samples, were subjected to SEC in order to separate the low MW peptides accounting for this activity. The DPPH values of SEC fractions, for each substrate, are reported in Table 2. For clarity, only samples whose SEC fractions had activity, have been included. All DPPH activities were significantly different in comparison to their control (total hydrolysed flour), as reported in statistical analysis (Table 2S).

For KKW dough, DPPH values reported in Table 2A, show that some fractions had an activity higher than their total hydrolysates and control, and in particular: fraction 1 and 3 from *L. plantarum* 98a dough (126.69 ± 0.01 µg AAeq and 135.00 ± 0.01 µg AAeq), fraction 8 from *L. rhamnosus* C249 dough (110.01 ± 0.02 µg AAeq) and fraction 4 from Altamura dough (118.33 ± 0.02 µg AAeq).

Interestingly, for KKW flour, none of the fractions obtained with commercial enzymes showed antioxidant activity, despite the high values observed for their total hydrolysates. This might be due to the presence of antioxidant compounds other than small peptides (both larger peptides and/or different molecules like polyphenols, flavonoids, oligosaccharides) which become free after the hydrolytic reaction and are lost during the separation by SEC, the latter aimed to purify only small petides.

For emmer bran (Table 2B), many fractions from whole cell or commercial enzyme hydrolysates had high antioxidant activity and were selected for peptide identification and particularly: fractions 6 and 7 from *L. rhamnosus* C249 hydrolysate (234.97 ± 0.05 and 218.34 ± 0.04 µg AAeq); fractions 2, 3 and 7 from *L. plantarum* 98a hydrolysate (310 ± 0.05, 218.30 ± 0.04 and 284.97 ± 0.05); fractions 3, 5 and 6 from Neutrase hydrolysate (218.37 ± 0.03, 351.65 ± 0.04, and 118.32 ± 0.02 µg AAeq). As already observed for KKW flour, the antioxidant activity of some emmer bran hydrolysates resulted to be higher than that of purifed SEC fractions. For lupine and pea flours (Table 2C), despite the low antioxidant activity of total hydrolysates, it was possible to find SEC fractions with activity equivalent or higher than that of the not hydrolysed (control). In particular, for lupine flour fraction 4 of Neutrase hydrolysate had activity (234.87 ± 0.2 µg AAeq) equivalent to control. For pea flour many fractions in the three hydrolysates had increased activity with respect to control and in particular: fractions 1 and 4 of the control (335.01 ± 0.01 and 185.00 ± 0.03 µg AAeq); fractions 1, 2 and 3 of Flavourzyme hydrolysate (318.35 ± 0.01, 318.30 ± 0.02 and 193.34 ± 0.01 µg AAeq).

As previously reported (Coda et al., 2012), purified fractions can show higher antioxidant activity than the control as the consequence of the higher concentration of the active compound compared to that of the other constituents of the matrix. Indeed, complex food matrices may lead to antagonistic effect in the antioxidant activity assays. For example, it has been shown that the combination of polyphenols and gluthatione may result in antagonistic interactions (Pereira, Sousa, Costa, Andrade, Valentão, 2013)).

The results so far obtained show that by commercial enzymes and whole microbial cells it is possible to increase the antioxidant activity of the selected vegetable substrates.

3.5. Peptide identification by LC-ESI-QTOF-MS

Fractions obtained from SEC with the highest antioxidant activity were subjected to nano LC-ESI-QTOF-MS analysis, in order to identify the active peptides.

363 In Table 3 only the peptides with alleged antioxidant activity are reported. The inclusion criteria
364 were: (i) the presence in the peptide of at least one antioxidant amino acid (triptophan, tyrosine,
365 methionine and cysteine); (ii) the presence in the peptide of a sequence with previously reported
366 antioxidant activity.
367 For each peptide, the reported MW was estimated using mass spectrometry. Sixty two potentially
368 bioactive peptides were selected on the basis of their predicted antioxidant activity. Specifically for
369 KKW (Table 3), fractions 98a_3 and C249_8 showed the highest antioxidant activity. Here, most of
370 the peptides contained tyrosine in their sequence which is a good scavenger of free radicals because
371 of the presence of a phenolic moiety (Nimalaratne, Lopes-Lutz, Schieber & Wu, 2011). Some
372 peptides in these two fractions also contained sequences with previously demonstrated antioxidant
373 activity, like PYPQ isolated from human milk (Hernandez-Ledesma, Quiros, Amigo & Recio,
374 2007). The Altamura fraction 4 contained a single peptide having a potential antioxidant activity
375 due to the presence of a cysteine residue whose activity depends on the reducing SH group (Elias,
376 McClements & Decker, 2005).
377 In emmer (Table 3), the fractions 2, 3 and 7 resulting from the proteolysis with *L. plantarum* 98a,
378 contained respectively five, five and two peptides with putative antioxidant activity. The *L.*
379 *rhamnosus* C249 fraction 1 showed two peptides accounting for a certain antioxidant activity. In
380 particular one with MW of about 6.0 kDa which contained in its sequence two tyrosines, two
381 methionines and five histidines and fraction C249_6 with a single peptide with two cysteines.
382 Fraction C249_7 did not contain peptides but free methionine which could be responsible of the
383 detected high antioxidant activity (Elias et al., 2005).
384 The hydrolysis of emmer bran by commercial enzymes generated short peptides rich in cysteyne
385 and methionine (Table 3).
386 For pea hydrolysates (Table 3), the most interesting peptides resulted from the action of
387 flavourzyme and were found, in particular, in fractions 2 and 3 which are rich in tyrosine and
388 tryptophan-containing peptides. Some peptides possess a C-terminal –RW sequence which has been

389 previously identified as antioxidant peptide released from egg white protein (Saito et al., 2003). The
390 presence of the indole group makes this amino acid the one with the highest radical scavenging
391 activity (Nimalaratne et al., 2011).
392 Finally among lupinee hydrolysates (Table 3), the fractions resulting from the proteolytic activity of
393 Neutrase, and in particular, fractions 1, 3, 4 and 5 contained, respectively, one, two, two and two
394 peptides with antioxidant amino acids. Fraction 4 also contained a peptide with the antioxidant
395 sequence VPY at the C-terminus (Saito et al., 2003).
396
397 ***3.6 In vitro evaluation of antioxidant properties of synthetic peptides***
398 On the basis of their amino acid sequences, thirteen peptides identified in the SEC fractions with the
399 highest antioxidant activity (KKW flour 98a_3, emmer bran 98a_2, pea flour flavourzyme_2 and
400 lupine flour neutrase_4) were chemically synthesized.
401 These peptides were selected in order to fulfil certain structural requirements, such as the presence
402 of W and Y at the C-terminus (Saito et al., 2003) and/or the presence of sequences with previously
403 demonstrated antioxidant activity in the peptide chain (PYPQ, RW and VPY). The fraction 98a_2 in
404 hydrolyzed emmer bran did not contain peptides that meet the above structural requirements.
405 Therefore in this fraction three peptides, which contain Y and/or W in their sequence (but not at the
406 C-terminus) were selected for synthesis.
407 The antioxidant properties of a molecule depend on many factors such as the ability to directly
408 scavenge reactive oxygen species, the capacity to donate a hydrogen atom or to transfer an electron
409 to a radical compound as well as the capability to chelate transition metals such as copper and iron
410 (Lu, Lin, Yao, Chen, 2010). Given the different chemistries involved in each mechanism and the
411 diverse rates of reactions with the different free radicals, seven different antioxidant assays that
412 measured the capacity to scavenge reactive oxygen species (superoxide anion and hydroxyl
413 radicals) or organic nitro-radicals (ABTS and DPPH), the capacity to reduce Fe^{3+} (FRAP assay), the
414 capacity to inhibit lipid peroxidation and the ability to chelate Fe^{2+} were used in this work.

All of the synthesized peptides were able to scavenge the organic nitro-radicals ABTS and DPPH (Table 4). The presence of W or Y at the C-terminus seems to be primary for the ABTS radical scavenging activity of the peptides since the peptides that lack of this strucutral characteristic exhibited the lowest ABTS scavenging activity. This can be also inferred by comparing the ABTS scavenging activity of the peptides FLGQQQPFPPQQPYPQPQPFPSQQPY and FLGQQQPFPPQQPYPQPQPFPSQQP. They have the same sequence (with a Y residue inside the chain and the antioxidant sequence PYPQ) but the second peptide (which showed significant lower antioxidant activity) lack of the Y residue at the C-terminus. Indeed, the presence of a W residue at the C-terminus conferred higher ABTS scavenging properties since the peptides TVTSLDLPVLRW, VTSLDLPVLRW and TSLDLPVLRW were the most active. The presence of Y or W at the C-terminus appeared to be less important in determing the DPPH scavenging activity of the synthesized peptides (Table 4).

None of the tested peptides showed reactivity in the FRAP assay (data not shown) suggesting that single electron transfer (SET) is not the predominant antioxidant mechanism. On the other hand, Gu et al. (2012) suggested that for antioxidant peptides containing Y the predominant antioxidant mechanism is hydrogen atom transfer (HAT) whereas SET mechanism is the predominat for cysteine containing peptides.

Synthetic peptides were also tested for their ability to scavenge two reactive oxygen species, superoxide anion and hydroxyl radicals. Nine synthesized peptides exhibited a certain degree of hydroxyl radical scavenging activity (Table 4). Generally, the presence of an antioxidant amino acid (Y or W) at the C-terminus is mandatory for the scavenging capacity since the four peptides devoided of activity lack of this structural requirements. The peptides isolated from KKW flour 98a_3 SEC fraction were the most active against hydroxyl radicals without showing no significant differences each other. This four peptides had in common the sequence PPQQ which can be important for the activity. No clear relationship was found between the superoxide anion scavenging capacity and the structure of the tested peptides (Table 4).

441 Regarding the ability of the peptides to inhibit linoleic acid oxidation, we found nine peptides with
442 higher inhibitory activity respect to glutathione (Figure 2). Two peptides, the KKW flour-derived
443 peptide FLGQQQPFPPQQPYPQPQPFPSQQP and the emmer bran-derived peptide
444 NSSYFVEWIPNNVK, totally inhibited the formation of lipid hydroperoxide during one week of
445 incubation. Phenylalanine seems to be important for the lipid peroxidation inhibitory activity since
446 the peptides with the lowest (or absent) activity did not contains this amino acid in their sequence.
447 Traansition metal chelating capacity was found to be not important for the ability to inhibit lipid
448 peroxidation since none of the thirteen peptides were able to chelate iron (data not shown).

4. Conclusions

The present study explored the potential of vegetable proteins from cereals and legumes to become a source of antioxidant peptides in the form of proteinaceous hydrolysates obtained by commercial enzymes and whole microbial cells. In particular KAMUT® khorasan wheat, emmer bran, lupine and pea, already known for the high content of proteins with high nutritional value, turned out to be good substrates for the preparation of hydrolysates with antioxidant activity to be used as ingredients for the preparation of functional fermented food or nutraceuticals for different food formulations. The lactic acid bacteria ability to increase the antioxidant properties of cereal and legumes was accounted to phenolic compounds, catechins and other flavonoids delivered by depolymerisation of plant matrix (Hur, Lee, Kim, Choi, & Kim, 2014, Gianotti et al., 2011). However, according to our results, they also increased antioxidant peptides by their proteolytic activity. Specifically, the analysis of antixodant activity of synthetic peptides showed that four of them (the KAMUT® khorasan wheat-derived peptide VLPPQQQY, the pea-derived peptides TVTSLDLPVLRW and VTSLDLPVLRW, and the lupine-derived peptide FVPY) were able to scavenge reactive oxygen species (superoxide anion and hydroxyl radicals) and organic nitro-radicals (ABTS and DPPH) and to inhibit lipid peroxidation. Despite further studies using cell-based and *in vivo* systems will be required to validate the results so far obtained, these peptides can be considered as good candidates for use as natural antioxidants in controlling oxidative reactions (especially lipid oxidation) in food and in improving the antioxidant properties of functional foods.

Acknowledgments

The authors are grateful to Kamut Enterprises of Europe, Belgium for financial support for part of this study and to Dr. Robert M. Quinn for his kind english review.

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Figure captions

Figure 1. SDS-PAGE of emmer bran (e.b.) hydrolyzed by lactic acid bacteria and commercial enzymes. St: Precision Plus Protein Standard from Bio-Rad; C: e.b. control; 1: e.b. hydrolyzed by *L. paracasei* 1122; 2: e.b. hydrolyzed by *L. plantarum* 98a; 3: e.b. hydrolyzed by *L. sanfranciscensis*; 4: e.b. hydrolyzed by *L. rhamnosus* C1272; 5: e.b. hydrolyzed by *L. brevis* 3BHI; 6: e.b. hydrolyzed by *L. fermentum* MR13; 7: e.b. hydrolyzed by *L. rhamnosus* C249; 8: e.b. hydrolyzed by Neutrase; 9: e.b. hydrolyzed by Flavourzyme; 10: e.b. hydrolyzed by Alcalase.

Figure 2 Lipid peroxidation inhibitory activity of the thirteen selected peptides and glutathione (GSH) expressed as percentage of inhibition respect to a control reaction carried out in the same assay condition but without peptides or GSH. Syntethic peptides and GSH were dissolved in DMSO and tested at concentration of 0.5 mM. Each sample was run in triplicate and results are reported as mean values ± SD. Columns with the same letter are not significantly different ($P > 0.05$). The numbers on the *x* axis correspond to the peptide sequences as shown in Table 4. N.A. means no activity.