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

3 Abbreviated running title:

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

5

6 Elena Babini¹, Davide Tagliazucchi², Serena Martini², Lucilla Dei Più¹, Andrea Gianotti^{1*}

7

8 ¹ Department of Agri-Food Sciences and Technologies, *Alma Mater Studiorum* - University of
9 Bologna, Piazza Goidanich, 60 – 47521 Cesena, Italy, ² Department of Life Sciences, University of
10 Modena and Reggio Emilia, Via Amendola, 2 - 42122 Reggio Emilia, Italy

11

12 *: *corresponding author* **Andrea Gianotti, Ph.D**

13 Department of Agri-Food Sciences and Technologies,

14 *Alma Mater Studiorum*, University of Bologna,

15 Via Fanin, 50 – 40127 Bologna, Italy

16 Tel +39 051 2096577

17 Piazza Goidanich, 60 - 47521 Cesena, Italy

18 Tel +39 0547 338134

19 Fax +39 0547382348

20 e-mail: andrea.gianotti@unibo.it

21

22 e-mail of co-authors:

23 Elena Babini: elena.babini2@unibo.it

24 Davide Tagliazucchi: davide.tagliazucchi@unimore.it

25 Serena Martini: serena.martini@unimore.it

26 Lucilla Dei Più: lucilla2.deipiu@gmail.com

27 **ABSTRACT**

28

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

41

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

44

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

51 **1. Introduction**

52

53 Biologically active peptides are defined as specific protein fragments (from 2 to 20 amino acid
54 residues and molecular masses less than 6.0 kDa) that have positive effects on body functions or
55 conditions and may influence human health (Kitts & Weiler, 2003). A large spectrum of biological
56 activities has been assigned to these compounds, including antihypertensive, opioid,
57 immunomodulatory, mineral sequestering, antioxidant, antimicrobial (Kitts et al., 2003),
58 antithrombotic (Shimizu et al., 2009) and hypocholesterolemic (Zhong, Liu, Ma & Shoemaker
59 2007).

60 Bioactive peptides correspond to cryptic sequences from parent food proteins, which are latent until
61 they are released and activated by hydrolytic reactions that take place during food fermentation and
62 processing or during gastrointestinal digestion (Korhonen & Pilhanto, 2007). The release of these
63 peptides can be obtained also by treatment of proteinaceous food sources with proteases or
64 combination of proteases like alcalase, chymotrypsin, pancreatin, pepsin, thermolysin and enzymes
65 from bacterial and fungal sources (Kitts et al., 2003; Sarmadi & Ismail, 2010; Zhang, Zhang,
66 Wang, Guo, Wang & Yao, 2010). Whole cell systems, based on the hydrolytic activity of cultured
67 bacterial cells, have been used with different food matrices, as well (Kitts et al., 2003; Sarmadi et al.,
68 2010; Coda, Rizello, Pinto & Gobbetti, 2012).

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

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

77 atherosclerosis (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 consumer 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

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

105 **2. Materials and Methods**

106

107 **2.1. Materials**

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

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

117

118 **2.2. Microorganisms**

119 *L. casei* lbcd, *L. fermentum* MR13, *L. paracasei* 1122, *L. plantarum* 98a, *L. rhamnosus* C1272, *L.*
120 *rhamnosus* C249, *L. sanfranciscensis* bb12 and *L. brevis* 3BHI belong to the collection of the
121 Department of Agri-Food Sciences and Technologies of the University of Bologna (Italy). Bacteria
122 were grown for 24 h at 37 °C in MRS broth modified by the addition of yeast extract (5%, v/v) and
123 28 mM maltose, at pH 5.6. The enumeration of bacteria was carried out by plating serial dilutions of
124 dough on modified MRS agar medium at 37 °C for 48 h.

125

126 **2.3. Sourdough fermentation**

127 KKW flour was used to prepare 8 different doughs containing 100 g of flour and 400 g of tap water.
128 Fermentation with the pool of selected bacteria at their late exponential phase (initial cell load of
129 $5 \cdot 10^7$ CFU/g of dough) was carried out at 37 °C for 72 h under mild agitation on a rotary shaker.

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

132

133 **2.4. Protein extraction**

134 Proteins were extracted from substrates according to the method originally described by Osborne
135 (1907) and further modified by Weiss, Vogelmeier & Gorg (1993). For each dough, an aliquot
136 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,
137 vortexing each 15 min, and centrifuged at 25432 rcf for 20 min. The supernatant, containing the
138 water/salt-soluble nitrogen fraction, was stored at -80 °C. The same procedure was applied to 7.5 g
139 samples of KKW flour and emmer bran. The protein fractions of pea and lupine flours were
140 obtained after defatting the flours using the cold extraction method (Dei Più et al. 2014). Flour was
141 mixed with n-hexane, stirred for 16 h and then filtered. This step was repeated twice. The filtrate
142 was open air dried at room temperature, then ground to pass through a 70 mesh screen and finally
143 stored at -80 °C.

144

145 **2.5. Hydrolysis reactions**

146 Hydrolysis with bacteria was made using cultures grown for 12 hours in modified MRS broth. Cells
147 were harvested by centrifugation at 11381 rcf for 10 min at 4 °C, washed twice with 20 mM
148 phosphate buffer (pH 7.0) and resuspended in the same buffer at a concentration of ca. 10⁹
149 CFU/mL. The assay mixture, containing 6 mL of cellular suspension and 30 mL of protein extract,
150 was incubated at 37 °C for 72 h and then centrifuged at 7741 rcf for 5 min to remove cellular debris.
151 Hydrolysis with commercial enzymes was carried out according to Dei Più, Tassoni, Serrazanetti,
152 Ferri, Babini, Tagliazucchi and Gianotti (2014) for 2 h using enzymes at a ratio of 0.3 UA/g.
153 Reactions conditions were: pH 8 and 55 °C for Alcalase; pH 6.5 and 40 °C for Neutrase; pH 6 and
154 60 °C for Flavourzyme. Reactions were stopped by heating at 80 °C for 20 min and samples were
155 centrifuged to remove the insoluble material.

156 Ten g of KKW dough and 10 mL of hydrolysed samples were freeze-dried under vacuum at -56 °C
157 for 48 h, in a Heto Power Dry LL 3000 lyophilizer, Thermo Electron Corporation (USA), and
158 stored at -80 °C. Before use, freeze dried samples were solubilized in 0.1 M phosphate buffer (pH
159 7.0) and centrifuged at 27216 rcf for 10 min.

160

161 **2.6. Protein pattern analysis by SDS-PAGE**

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

165

166 **2.7. Purification of peptides by size exclusion chromatography (SEC)**

167 Peptides from 50 µL samples were fractionated by SEC using phosphate buffer 0.1 M (pH 7.0) as
168 eluent, on a TSKgel G2500PW_{XL} column from Tosoh Bioscience SRL (Italy) with AKTA FPLC
169 equipment from GE Healthcare (Sweden). Fractions of 500 µL volume were collected and analysed
170 for DPPH activity and peptide sequence.

171

172 **2.8. Antioxidant activity assays**

173 **2.8.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay**

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

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

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

185

186 **2.8.2 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic) acid (ABTS) assay**

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

196

197 **2.8.3 Ferric reducing antioxidant power (FRAP) assay**

198 The ferric reducing potential of the synthetic peptides was tested following the method reported by
199 Benzie & Strain (1999) adapted to a microplate reader. Working FRAP reagent was prepared by
200 mixing 10 volumes of acetate buffer (300 mM, pH 3.6), 1 volume of 10 mM TPTZ (2,4,6-
201 tripyridyl-S-triazine) dissolved in HCl 10 mM and 1 volume of 20 mM FeCl₃. Then, 150 μ L of
202 working FRAP reagent were mixed with 5 μ L of peptide, into a clear bottom 96-well plate. The
203 absorbance at 595 nm was read after 6 min of reaction at room temperature, using a microplate
204 reader. Data are reported as mmol GSH/mol peptide, by means of a calibration curve obtained with
205 GSH (0.5 to 5 mM), in the same assay conditions.

206

207 **2.8.4 Hydroxyl radical scavenging assay**

208 The capacity to scavenge hydroxyl radicals was evaluated according to a method reported by
209 Ajibola, Fashakin, Fagbemi & Aluko (2011), with some modifications. The assay consisted of
210 mixing 50 μ L of 3 mM TPTZ dissolved in HCl 50 mM, 50 μ L of 3 mM FeSO₄, 50 μ L of synthetic
211 peptide or GSH (at concentration ranging from 1 to 10 mM), and 50 μ L of 0.01% (v/v) hydrogen
212 peroxide, in a clear bottom 96-well plate. The mixture was incubated for 1 h at 37°C and the
213 absorbance was measured at 595 nm using a microplate reader. The hydroxyl radical scavenging
214 capacity was expressed as mmol glutathione/mol peptide.

215

216 **2.8.5 Superoxide anion radical scavenging activity assay**

217 The superoxide anion radical scavenging activity of the synthetic peptides was determined by the
218 method of Bamdad & Chen (2013). An aliquot (80 μ L) of peptide or GSH at different
219 concentrations (0.125–2 mM) was mixed with 80 μ L of 50 mM Tris-HCl buffer (pH 8.3) containing
220 1 mM EDTA in a 96-well microplate and 40 μ L of 1.5 mM pyrogallol in 10 mM HCl. After 10 min
221 of incubation at room temperature, the superoxide anion radical-induced polymerization of
222 pyrogallol was measured as increase in absorbance at 405 nm using a microplate reader. The
223 superoxide anion scavenging capacity was expressed as mmol glutathione/mol peptide.

224

225 **2.8.6 Assay of metal ion chelation**

226 The metal chelating activity of the synthetic peptides was assessed following the method developed
227 by Karama & Pegg (2009), adapted to a microplate reader. An aliquot (200 μ L) of 0.5 mM peptide
228 sample solution was combined with 20 μ L of FeSO₄ (2 mM) and 40 μ L of TPTZ (3 mM in HCl 10
229 mM) in a clear bottom 96-well plate. After 10 min of incubation at room temperature, absorbance of
230 sample was measured using a microplate reader at 595 nm. GSH was used as standard compound.

231

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, MS² 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**

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

259 **3. Results and Discussion**

260

261 **3.1. Dough fermentation**

262 Cell load of KKW doughs fermented by lactic acid bacteria reached an average value of 7.39 log
263 CFU/g after 72 h at 37 °C. The lowest value was found in the dough fermented by *L. casei* lbcd (5.6
264 log CFU/g), the highest in Altamura dough (9.42 log CFU/g). Before fermentation, pH values were
265 6.69 ± 0.06 and at the end 3.61 ± 0.25 .

266

267 **3.2. Antioxidant activity of doughs**

268 For some traditional fermented products deriving from milk, soy and rice the impact on health of
269 microbial fermentation of proteins has been deeply evaluated. In the recent years fermentation has
270 been considered a tool to increase the nutritional value of other vegetable substrates, testing the
271 biological activity of protein hydrolysates with different bacterial strains. Here, the effect of
272 fermentation by *Lactobacillus* strains on antioxidant activity was tested on KKW, Altamura and
273 french bakery doughs, using the DPPH assay as preferred method, accordingly to Coda et al. (2012)
274 (Table 1). The resulting activities were significantly different in comparison to each control flour
275 (Table 1S). They were very similar with only minor differences with respect to control ($62.39 \pm$
276 $0.01 \mu\text{g AAeq}$). The highest value was found in *L. plantarum* 98a dough ($68.75 \pm 0.05 \mu\text{g AAeq}$),
277 the lowest in *L. paracasei* 1122 ($58.11 \pm 0.13 \mu\text{g AAeq}$) and *L. brevis* 3BH1 ($57.79 \pm 0.02 \mu\text{g}$
278 AAeq), doughs. Doughs with the highest activity (*L. plantarum* 98a, *L. rhamnosus* C249 and
279 Altamura) were subjected to SEC separation for identification of peptides potentially responsible of
280 the detected activity.

281

282 **3.3. Protein pattern and antioxidant activity of hydrolysates**

283 Protein pattern of hydrolysates was analysed on SDS-PAGE for KKW flour and emmer bran (for
284 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 Neutralse increased the activity of about 6.6 ($410.15 \pm$
293 $0.24 \mu\text{g AAeq}$) and 9 times ($568.35 \pm 0.03 \mu\text{g AAeq}$) with respect to control sample (62.39 ± 0.01
294 $\mu\text{g AAeq}$). For emmer bran almost all the strains, except *L. casei* lbcd, increased the activity of the
295 not hydrolysed sample ($227.67 \pm 0.04 \mu\text{g AAeq}$) and in particular *L. plantarum* 98a (588.30 ± 0.42
296 $\mu\text{g AAeq}$), *L. paracasei* 1122 ($445.12 \pm 0.09 \mu\text{g AAeq}$) and *L. sanfranciscensis* bb12 ($422.33 \pm$
297 $0.05 \mu\text{g AAeq}$). Alcalase and Neutralse were also highly active, giving hydrolysates with antioxidant
298 values of $318.44 \pm 0.4 \mu\text{g AAeq}$ and $318.35 \pm 0.02 \mu\text{g AAeq}$, 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 ($234.43 \pm 0.05 \mu\text{g}$
302 AAeq and $174.96 \pm 0.05 \mu\text{g AAeq}$, 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, Piskula, 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

311 obtained by Huminski and Aluko (2007), which showed that the hydrolysis of pea proteins with
312 Flavourzyme gave higher antioxidant activity (DPPH assay) than that obtained by hydrolysis with
313 Alcalase and other enzymes. Results reported here suggested that the antioxidant activity of the
314 hydrolysates depends on the substrate, the type of fermentation and/or enzyme utilized.
315 Nevertheless, considering that the aim of the study was to identify new short antioxidant peptides
316 that can be used in functional food and pharmaceutical applications, hydrolysates obtained by *L.*
317 *fermentum* MR13, lupine flour hydrolysed by Neutrase and pea flour hydrolysed by Flavourzyme
318 were selected for further characterization.

319 320 **3.4. Antioxidant activity of SEC fractions of doughs and hydrolysates**

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

326 For KKW dough, DPPH values reported in Table 2A, show that some fractions had an activity
327 higher than their total hydrolysates and control, and in particular: fraction 1 and 3 from *L.*
328 *plantarum* 98a dough ($126.69 \pm 0.01 \mu\text{g AAeq}$ and $135.00 \pm 0.01 \mu\text{g AAeq}$), fraction 8 from *L.*
329 *rhamnosus* C249 dough ($110.01 \pm 0.02 \mu\text{g AAeq}$) and fraction 4 from Altamura dough ($118.33 \pm$
330 $0.02 \mu\text{g AAeq}$).

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

337 For emmer bran (Table 2B), many fractions from whole cell or commercial enzyme hydrolysates
338 had high antioxidant activity and were selected for peptide identification and particularly: fractions
339 6 and 7 from *L. rhamnosus* C249 hydrolysate (234.97 ± 0.05 and 218.34 ± 0.04 $\mu\text{g AAeq}$); fractions
340 2, 3 and 7 from *L. plantarum* 98a hydrolysate (310 ± 0.05 , 218.30 ± 0.04 and 284.97 ± 0.05);
341 fractions 3, 5 and 6 from Neutrased hydrolysate (218.37 ± 0.03 , 351.65 ± 0.04 , and 118.32 ± 0.02 μg
342 AAeq). As already observed for KKW flour, the antioxidant activity of some emmer bran
343 hydrolysates resulted to be higher than that of purified SEC fractions. For lupine and pea flours
344 (Table 2C), despite the low antioxidant activity of total hydrolysates, it was possible to find SEC
345 fractions with activity equivalent or higher than that of the not hydrolysed (control). In particular,
346 for lupine flour fraction 4 of Neutrased hydrolysate had activity (234.87 ± 0.2 $\mu\text{g AAeq}$) equivalent
347 to control. For pea flour many fractions in the three hydrolysates had increased activity with respect
348 to control and in particular: fractions 1 and 4 of the control (335.01 ± 0.01 and 185.00 ± 0.03 μg
349 AAeq); fractions 1, 2 and 3 of Flavourzyme hydrolysate (318.35 ± 0.01 , 318.30 ± 0.02 and $193.34 \pm$
350 0.01 $\mu\text{g AAeq}$).

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

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

359

360 **3.5. Peptide identification by LC-ESI-QTOF-MS**

361 Fractions obtained from SEC with the highest antioxidant activity were subjected to nano LC-ESI-
362 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 (tryptophan, 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 cysteine
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.

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

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

432 Synthetic peptides were also tested for their ability to scavenge two reactive oxygen species,
433 superoxide anion and hydroxyl radicals. Nine synthesized peptides exhibited a certain degree of
434 hydroxyl radical scavenging activity (Table 4). Generally, the presence of an antioxidant amino acid
435 (Y or W) at the C-terminus is mandatory for the scavenging capacity since the four peptides
436 devoided of activity lack of this structural requirements. The peptides isolated from KKW flour
437 98a_3 SEC fraction were the most active against hydroxyl radicals without showing no significant
438 differences each other. This four peptides had in common the sequence PPQQ which can be
439 important for the activity. No clear relationship was found between the superoxide anion
440 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 FLGQQQPFPPQQPYQPQPFPSQQP 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).

449 **4. Conclusions**

450

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

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607 **Figure captions**

608

609 **Figure 1.** SDS-PAGE of emmer bran (e.b.) hydrolyzed by lactic acid bacteria and commercial
610 enzymes. St: Precision Plus Protein Standard from Bio-Rad; C: e.b. control; 1: e.b. hydrolyzed by *L.*
611 *paracasei* 1122; 2: e.b. hydrolyzed by *L. plantarum* 98a; 3: e.b. hydrolyzed by *L. sanfranciscensis*;
612 4: e.b. hydrolyzed by *L. rhamnosus* C1272; 5: e.b. hydrolyzed by *L. brevis* 3BHI; 6: e.b. hydrolyzed
613 by *L. fermentum* MR13; 7: e.b. hydrolyzed by *L. rhamnosus* C249; 8: e.b. hydrolyzed by Neutrase;
614 9: e.b. hydrolyzed by Flavourzyme; 10: e.b. hydrolyzed by Alcalase.

615

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