Response Surface Methodology to Optimize Hydrolysis Parameters in Production of Antioxidant Peptides from Wheat Germ Protein by Alcalase Digestion and Identification of Antioxidant Peptides by LC-MS/MS

Z. Karami\textsuperscript{1}, S. H. Peighambardoust\textsuperscript{1*}, J. Hesari\textsuperscript{1}, and B. Akbari-Adergani\textsuperscript{2}

ABSTRACT

The antioxidant capacity of wheat germ protein hydrolyzed by Alcalase was optimized using Response Surface Methodology (RSM). The optimum hydrolyzing parameters were found at temperature of 52.28°C, time 233 minutes, and E/S 1.46 %. The amino acids profiles of intact and hydrolyzed proteins showed that Wheat Germ Protein Hydrolysate (WGPH) had higher percentage of hydrophobic amino acids than that of intact protein. WGPH prepared in optimum condition was fractionated by RP-HPLC. The obtained fractions were subjected to ABTS assay for antioxidant capacity evaluation. The fraction with higher antioxidant value was then exposed to further analysis by LC-ESI/MS/MS. The sequences of the peptides were found to be TVGGAPAGRIVME (1257.66 Da) and GNPIPREPGQVPAY (1494.77 Da).

Keywords: ABTS assay, Amino acids profiles, Bioactive peptides, RSM.

INTRODUCTION

Flour industry generates large amounts of wheat germ as waste materials that have a poor utility for human consumption unless they are processed and stabilized. There is a growing interest among researchers to discover effective methods for the recovery of waste material and to produce added value substances (Deng et al., 2012). Defatted wheat germ involves ~30% protein (rich in essential amino acids) which is a suitable source of protein to provide beneficial products (Ge et al., 2000). Bioactive peptides, known as functional food ingredients, are specific protein fractions with amino acids that are inactive when they are part of proteins, but might show broad applications in cosmetics, food additives, nutraceuticals, and pharmaceuticals (antimicrobial, antioxidant, antithrombotic, and ACE-inhibitory activities) (Chen et al., 2012; Farzamirad and Aluko, 2008; Li et al., 2014; Sadeghi et al., 2018; Sedaghati et al., 2014; Wang et al., 2016). Bioactive peptides could be produced under controlled conditions by enzymatic hydrolysis (Dadzie et al., 2013; You et al., 2010) and it has been shown that size, composition, molecular weight, structure, amino acids content of peptide, and charge distribution on the molecule determine its biological features (Xiong et al., 2013). For instance, Zhang et al. (2013) illustrated that the type of enzyme used to digest affects antioxidant properties. Also, it has been demonstrated that the presence of certain amino acids can cause higher antioxidant activities (Nagasawa et al., 2001). Therefore, to earn more potent...
antioxidant peptides, it is significant to apply appropriate condition to hydrolyze wheat germ protein. Thus, the aim of this study was to optimize hydrolysis parameters in production of wheat germ protein hydrolysates with the highest antioxidant capacity using response surface methodology and, then, fractionate the obtained hydrolysates under optimized conditions by RP-HPLC and recognize the fraction that displays the highest ABTS radical scavenging by nano LC-ESI/MS/MS proteomics.

MATERIALS AND METHODS

Defatted Wheat Germ Powder Preparation

Wheat germs obtained from an industrial mill were cleaned to remove impurities and then its fat was removed with hexane for 8 hours. The process was repeated several times with clean hexane until the fat content was less than 1% (Soxhlet method). Then, it was air-dried at room temperature and sieved by a 70-mesh screen (Zhu et al., 2006).

Preparation of Wheat Germ Protein Isolates (WGPI)

Wheat germ powder was interspersed in sodium chloride solution (1M, 1:8, w/v). It was agitated for 30 minutes and pH was set to 9.5 by using sodium hydroxide (1M). Then, centrifugation was done at 8,000xg at 4°C for 20 minutes. The supernatant pH was set to 4.0 by using hydrochloric acid (1M) for protein precipitation. Re-centrifugation was executed at 8,000xg for 20 minutes at 4-8°C. After that, precipitates were interspersed in distilled water, and set to pH= 7.0 by using sodium hydroxide (1M). Then, the final product was freeze-dried (FDB-5503, Operon) (Zhu et al., 2006).

Preparation of Wheat Germ Protein Hydrolysate (WGPH)

A protein isolate solution (10% (w/v)) was hydrolyzed by using Alcalase 2.4 L under the following conditions: 0.5 to 2% w/w enzyme–substrate ratio; 45-55°C temperature and during 2-5 hours. The process was managed in a 200 mL reaction vessel that was equipped with a thermometer, pH electrode, and stirrer. To halt hydrolysis, heat treatment was carried out at 90°C for 10 minutes. Centrifugation was done at 8,000xg for 20 minutes to separate irresoluble ingredients and remaining enzyme. Then, the hydrolysates were lyophilized and kept at -20°C until next analyses (Zhu et al., 2006).

Calculation of the Hydrolysis Degree (%DH)

The hydrolysis degree of WGPH solutions in optimized point was defined as explained by González-García et al. (2014), with some modification. First, the OPA (OrthoPhthalaldehyde) reagent was provided with sodium tetrahydroborate (7.6 g) and SDS (20 mg) dissolved in 15 mL deionized water, to which 16 mg OPA in 400 µL 96% ethanol and 40 µL β-ME in 5 mL water were added. A calibration curve was then prepared from serial dilutions of a GSH solution (1-5 mg mL^{-1}). Then, 36 µL of the GSH dilution was added to 270 µL of OPA reagent, kept at room temperature for 2 minutes, and absorbance was read at 340 nm (PowerWave XS2 microplate reader, Biotek Instruments Inc., USA). For WGPH samples, the above OPA protocol was also used, and the resulting readout interpolated into the standard GSH curve to determine the peptide content. Protein content was determined by the biuret test. A calibration curve was first provided from a dilution series of a 1-5 mg mL^{-1} BSA stock solution, then, WGPH protein content was determined by interpolation as above. Finally, DH was calculated as:
Optimization of Hydrolysis Conditions of Wheat Germ

\[
DH = \frac{\text{peptide content}}{\text{protein content}} \times 100
\]

**DPPH Radical-Scavenging Assay**

This method was done according to Bougatef et al. (2009). A 500 µL of WGPH sample was blended with 125 µL of 0.02% DPPH in ethanol (99%) and then diluted with 500 µL of ethanol 99%. The mixture was kept in dark at room temperature for 60 minutes, and reduction of the DPPH radical was evaluated at 517 nm (Cecil 2041 UV/VIS spectrophotometer, UK) (Distilled water was applied as control). This value was determined as follows:

\[
\text{DPPH radical-scavenging activity (\%) = } \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100
\]

**ABTS Radical Scavenging Assay**

This method was evaluated as explained by Zhuang et al. (2013), with minor modifications. At first, the ABTS stock solution (7 mM) was added to potassium persulfate (2.45 mM) to obtain the ABTS radical cation. Then, it was incubated in dark at room temperature for 24 hours. The ABTS radical solution was then added to phosphate buffered saline (5 mM), pH 7.4, to reach the absorbance of 0.70±0.02 at 734 nm. For the assay, 980 µL of the diluted ABTS radical solution was mixed with 20 µL of the sample and the absorbance was read at 734 nm. The same volume of phosphate buffered saline was applied as control. The ABTS scavenging activity was determined (in %) as the difference in absorbance between the control and the sample relative to the absorbance of control.

**Total Antioxidant Capacity Assay**

Samples of 0.1 mL with several concentrations were added to 1 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate) and then stored at 90°C for 90 minutes. After a while, their absorbance was read at 695 nm. In this method, the control solution consisted of distilled water instead of sample. The antioxidant capacity of the hydrolysate was Reported as \(\alpha\)-tocopherol equivalent (µmol mL\(^{-1}\)) (Bougatef et al., 2009).

**Ferrous (Fe\(^{2+}\))-Chelating Activity Assay**

This method was determined as described by Zhuang et al. (2013). A sample of 0.1 mL of 2 mM FeCl\(_2\) solution was added to 3 mL of samples, and was left for 3 minutes, afterwards, 0.2 mL of ferrozine (5 mM) was surcharged and the mixture was retained at mild temperature for 10 minutes. Finally, the absorbance value was recorded at the wavelength of 562 nm. The control was similarly provided with distilled water instead of the sample. The calculation of ferrous chelating activity was accomplished as follows:

\[
\text{Ferrous chelating activity (\%) = } \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100
\]

**Optimization of Hydrolysis Using RSM**

The Statistics 8.0 software (Stat soft Inc., Tulsa, Oklahoma, USA) was used for experimental design. A Central Composite Rotatable Design (CCRD) at the central point with four axial points in three replicates (with 20 runs) was applied to evaluate the pattern of response and create a model. In the experimental design, temperature \((x_1)\), time \((x_2)\), and Enzyme to Substrate ratio \((E/S) (x_3)\) were chosen as independent factors, whereas ferrous chelating activity, DPPH, ABTS radical-scavenging, and total antioxidant capacity were used as dependent factors. The model was fitted based on a second-order equation (de Castro and Sato, 2015) as follows:
\[ Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j \]

Where, \( Y \) is estimated response, \( i \) and \( j \): values from 1 to the number of factors (n), \( \beta_0 \): Intercept factor, \( \beta_i \): Linear coefficient, \( \beta_{ij} \): Quadratic coefficient, \( x_i \) and \( x_j \): Coded independent factors.

**Amino Acid Analysis**

Sample of WGPH in optimized point (150 mg) was subjected to ion chromatography analysis (Metrohm 844 UV/Vis Compact IC equipped with a post-column reactor, Metrohm Herisau, Switzerland). Prior to chromatographic analyses, 120 mg of WGPH freeze-dried samples were subjected to hydrochloric acid (6 mol L\(^{-1}\)) hydrolysis under nitrogen, for 24 hours at 110°C. Amino acid analysis was based on 18 standards commonly found after wheat germ hydrolysis. All standards were prepared in 0.1 mM HCL as 5.0 mM solutions as well as 0.5-5.0 mM dilution range, which were used for calculation of amino acid contents in the samples. Chromatographic separation was attained on a Metrosep 5 µm, 100×4 mm analytical column in a 50°C oven. For the mobile phase, a degassed mixture of lithium citrate (42.6 mM) and phenol (10.6 mM), pH 2.8, was prepared and delivered in isocratic mode at a flow rate of 0.4 mL min\(^{-1}\). Derivatization of amino acids was performed by flowing a mixture of ninhydrin (0.11M) and hydridantin (2.5 mM) in DMSO and lithium acetate (2.0M, pH 5.2) at a flow rate of 0.2 mL min\(^{-1}\), with the post column reactor adjusted to 120°C. All analyses were carried out at 570 nm and the chromatographic data was obtained and processed using the Metrohm manager software (Version 2.3 IC net).

**Fractionation of Wheat Germ Protein Hydrolysate (WGPH) by RP-HPLC**

WGPH was suspended in deionized water (200 mg 10 mL\(^{-1}\)), stirred for 30 minutes, centrifuged at 36,000xg for 30 minutes at 10°C and filtered through Whatman No 1 paper. Ten mL of the filtrate was injected onto a Luna C\(_{18}\) preparative column (250×21.2 mm, 10 µm; Phenomenex, Jupiter, CA) fitted to an LC–8A system (Shimadzu, Japan). The eluting of column was accomplished with a 0-60% of solvent B by linear gradient (0.1% TriFluoroAcetic acid (TFA) in ACN) into solvent A (0.1% TFA in H\(_2\)O) over 30 minutes, at a flow rate of 25 mL min\(^{-1}\), with UV detection at 220 nm. Significant peaks observed in consecutive, reproducible runs were manually collected and pooled into eight fractions, freeze-dried and kept at -20°C until next analyses.

**Peptide Identification in WGPH Fractions**

In order to identify active peptides, WGPH fraction with higher antioxidant activity (ABTS radical scavenging) was examined in an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a Proxeon Easy LC (Thermo Fisher Scientific, San Jose, CA, USA). Samples were loaded onto a C\(_{18}\) NanoTrap column (2 cm×100 μm, 5 μm particle size; Thermo Fisher Scientific) and isolated by RP-LC on a C\(_{18}\) column (12 cm×75 μm, 1.9 μm particle size; NikkyoTechnos Co., Ltd. Japan). Elution at a flow rate of 300 nL min\(^{-1}\) was started at 97% solvent A (0.1% formic acid in H\(_2\)O) for 3 minutes. Then, a linear gradient was applied into 15% of solvent B (0.1% formic acid in ACN) for 1 minute, and increased to 35% of solvent B over 8 minutes. After each run, the column was washed with 10% solvent A for 10 minutes, for restarting conditions. The mass spectrometer was managed in positive ionization mode with the nano spray voltage set at 2.5 kV and the source temperature at 200°C. Ultrapak 1621 (Thermo Fischer Scientific) was used for external calibration. Therewith, internal calibration was also completed using the background polysiloxane ion signal (m/z 348.0767).
The apparatus was worked in Data-Dependent Acquisition (DDA) mode and full MS scans over a m/z 350-1500 mass range were acquired in the Orbitrap at 60,000 resolutions, with auto gain control and dynamic exclusion set at 1E6 and 5 s, respectively, and charge state filtering applied to discard singly charged peptides. In each DDA cycle, the top five most intense, multiply charged ions above a threshold ion count of 5,000 following each survey scan were designated for fragmentation at normalized collision energy of 35%. Fragment ion spectra produced via collision-induced dissociation were generated in the ion trap setting of AGC to 5E4, isolation window of 2.0 m/z and maximum injection time of 50 ms, respectively. Data acquisition was performed with Xcalibur software v2.2. Proteome Discoverer software suite (v1.4, Thermo Fisher Scientific) and the Mascot search engine (v2.5, Matrix Science, London) were implemented for peptide identification. Samples were searched against a NCBInr database (No. of sequences: 35,149,712) and all the corresponding decoy entries. No enzyme was chosen and oxidation (M) and acetylation (N-terminal) were used as variable modifications. Searches were accomplished utilizing a peptide tolerance of 7 ppm, with a product ion tolerance of 0.5 Da. The output data files filtration was regulated on FDR < 5%. This attitude was repeated in statistical analysis, too.

Statistical Analysis
ANOVA was done with Tukey comparison tests by TGraphPad Instat 3 software. The confidence level was chosen at 95%. Proteome Discoverer software suite (v1.4, Thermo Fisher Scientific) and the Mascot search engine (v2.5, Matrix Science (Perkins et al., 1999)) were applied for peptide identification. Samples were examined against a NCBInr database (Number of sequences: 35149712) and all the corresponding decoy entries. No enzyme was chosen and oxidation (M) and acetylation (N-terminal) were used as variable modifications.

RESULTS AND DISCUSSION
To optimize the complex processes such as hydrolysis, where numerous factors and interactions could affect the response, response surface methodology could be a useful method (Karami et al., 2015). The antioxidant capacity of functional sample is influenced by several factors that cannot be adequately evaluated in a single experiment. It is therefore advisable to use several methods to obtain a more comprehensive picture of how the sample is capable of scavenging radicals (González-García et al., 2014; Karami et al., 2013).

The CCRD matrix with the independent factors (temperature, time, and E/S ratio) and the mean results for the total antioxidant capacity, DPPH and ABTS radical scavenging activity and ferrous chelating of WGPH by Alcalase are presented in Table 1. These experimental data were beneficent enough to determine the second-order polynomial equations coefficients (Table 2).

Effects of Process Conditions on Antioxidant Activity of WGPH
ANOVA illustrated that the P-value for the 4 models of DPPH and ABTS radical scavenging, total antioxidant capacity, and metal chelating activity were all < 0.01 (Table 2), which indicated that the models were significant at a 99.99% confidence level. The lack of fit values of the 4 models were 0.099, 0.171, 0.107 and 0.142, respectively, (P< 0.05), showing that the fitness of all models were significant. The R² values for the 4 models were 0.97, 0.97, 0.99 and 0.99, respectively. High R² values demonstrated that models were well adapted to the responses. The linear terms of time and E/S had significant effects on DPPH and
ABTS radical scavenging (P < 0.05 for x₁ and x₃) and the quadratic terms of hydrolysis temperature exert a significant effect on response (P < 0.05) but its linear effect was not significant (P= 0.09, P= 0.07> 0.05 for X₁). The interaction terms of the three factors showed a significant effect (P < 0.01). The linear and quadratic terms of temperature and interaction terms of the three factors had a significant effect on total antioxidant capacity (P < 0.05) and the quadratic terms of temperature and interaction terms of the three factors had a significant effect on metal chelating activity. However, the effects of time and E/S were not significant on metal chelating activity. Based on the ANOVA results, the relationship between DPPH, ABTS radical scavenging, total antioxidant capacity, metal chelating activity and independent variables can be described by the following equations:

DPPH radical scavenging assay = +68.64 + 1.15x₁ + 2.86x₂ + 9.39x₃ + 0.36x₁x₂ - 1.80x₁x₃ - 7.15x₂x₃ + 7.76x₁² - 4.16x₂² - 0.47x₃²

ABTS radical scavenging assay = +73.13 + 1.31x₁ + 2.75x₂ + 9.12x₃ + 0.43x₁x₂ - 1.84x₁x₃ - 7.04x₂x₃ + 7.39x₁² - 3.54x₂² - 0.83x₃²

Total antioxidant capacity (μmol α-tocopherol mL⁻¹) = +235.81 + 3.06x₁ + 23.81x₂ + 24.48x₃ + 1.36x₁x₂ + 4.30x₁x₃ - 6.64x₂x₃ - 21.88x₁² - 3.04x₂² + 0.028x₃²

Metal chelating activity = +52.93 + 8.37x₁ + 3.75x₂ + 5.64x₃ + 2.65x₁x₂ - 0.79x₁x₃ - 0.29x₂x₃ + 2.59x₁² - 1.89x₂² - 5.43x₃²

Where, x₁, x₂ and x₃ are variable values of temperature, time, and Enzyme to Substrate (E/S).

The effects of E/S, time and temperature on antioxidant properties were demonstrated in surfaces response (Figure 1). Previous works showed that, under the appropriate conditions, the hydrolysis can release the antioxidant peptides and increase antioxidant activity of the hydrolysates (You et al., 2010). It is possible that some
Table 2. Analysis of variance (ANOVA) for the quadratic model.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>Mean scavenging activity (%)</th>
<th>Mean -shelating activity (%)</th>
<th>Mean -tioxidation capacity (α-tocopherol equivalent µmol/g)</th>
<th>Mean -tioxidation capacity (α-tocopherol equivalent µmol/g)</th>
<th>Mean -tioxidation capacity (α-tocopherol equivalent µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>2</td>
<td>1597.34</td>
<td>798.67</td>
<td>2.39</td>
<td>0.125</td>
<td>81.83</td>
<td>45.08</td>
<td>38.65</td>
<td>38.65</td>
<td>38.65</td>
</tr>
<tr>
<td>X1</td>
<td>1</td>
<td>88.53</td>
<td>88.53</td>
<td>0.27</td>
<td>0.610</td>
<td>81.83</td>
<td>45.08</td>
<td>38.65</td>
<td>38.65</td>
<td>38.65</td>
</tr>
<tr>
<td>X2</td>
<td>1</td>
<td>13.13</td>
<td>13.13</td>
<td>0.04</td>
<td>0.841</td>
<td>81.83</td>
<td>45.08</td>
<td>38.65</td>
<td>38.65</td>
<td>38.65</td>
</tr>
<tr>
<td>X1X2</td>
<td>1</td>
<td>31.76</td>
<td>31.76</td>
<td>0.09</td>
<td>0.731</td>
<td>81.83</td>
<td>45.08</td>
<td>38.65</td>
<td>38.65</td>
<td>38.65</td>
</tr>
<tr>
<td>X1X1</td>
<td>1</td>
<td>14.55</td>
<td>14.55</td>
<td>0.04</td>
<td>0.841</td>
<td>81.83</td>
<td>45.08</td>
<td>38.65</td>
<td>38.65</td>
<td>38.65</td>
</tr>
<tr>
<td>X1X2X2</td>
<td>1</td>
<td>13.76</td>
<td>13.76</td>
<td>0.04</td>
<td>0.841</td>
<td>81.83</td>
<td>45.08</td>
<td>38.65</td>
<td>38.65</td>
<td>38.65</td>
</tr>
</tbody>
</table>

**Note:**
- DPPH: 2,2-Diphenyl-1-picrylhydrazyl.
- X1: pH.
- X2: Reaction temperature (°C).
- X1X2: Interaction between pH and reaction temperature.
- X1X1: Interaction between pH and pH.
- X1X2X2: Interaction between pH, reaction temperature, and another factor.

**References:**

**Data:**
- Mean scavenging activity: 81.83 ± 4.508.
- Mean -shelating activity: 38.65 ± 2.386.
- Mean -tioxidation capacity: 38.65 ± 2.386.

**Analysis:**
- The model is significant at the 0.05 level.
- The pH and reaction temperature have a significant interaction effect on the antioxidant activity.

**Conclusion:**
- The optimal conditions for maximizing antioxidant activity are pH 8 and reaction temperature of 50°C.

---

835
Figure 1. Response surface for the effects of time (h), temperature (°C), and E/S (%w/w) on DPPH radical-scavenging assay (A), ABTS radical scavenging activity (B), Total Antioxidant Capacity (TAC) (C) and, Metal Chelating Activity (MCA) (D).
hydrolysis treatments either generate peptides with or without antioxidant capacity or convert them to amino acids, leading to a decrease in the antioxidant capacity of hydrolysate (Zhuang and Sun, 2011). As depicted in Figures 1-A and -B, it is clear that the DPPH and ABTS scavenging activities of the hydrolysates followed a similar pattern, although the amounts of ABTS scavenging activity were higher than the DPPH scavenging activity. Overall, it can be seen that both were increased with an increase in the E/S ratio and time. Also, apart from a brief fall from 45 to 50°C, there was a rise in the radical scavenging activity with increase in the temperature. Our results showed that time and E/S ratio had positive linear effects (P<0.001), while temperature had a quadratic effect (P<0.001). There was a striking similarity in these results and the findings of Seo et al. (2015) who illustrated that radical scavenging activity rose gently with a surge in hydrolysis time in bovine plasma protein hydrolysates. Figure 1-C displays an increase in the total antioxidant capacity with the increase in temperature at the beginning of hydrolysis following a slow decrease, while total antioxidant capacity increased by increasing the time and E/S ratio. Table 2 shows that all variables have linear and quadratic effect on the total antioxidant capacity as well as interaction between variables that had a significant effect on the total antioxidant capacity (P<0.05). In Figure 1-D, metal chelating activity reduced accessibility of transition metals and inhibited oxidative chain reactions were increased with the increase in E/S ratio, time, and temperature. All variables had linear and quadratic effect, in addition to interaction between variables that had a significant effect on chelating activity (P<0.05). It is evident that protein hydrolysates obtained in the enzymatic approach contain peptides and free amino acids in different concentrations and compositions compared to the isolated substrates, which can be ascribed to the synergistic or antagonistic effects. It is also expressed that some amino acids like Trp (W), Lys (K), His (H), Met (M), Tyr (Y), and Gly (G) exhibit antioxidant or pro-oxidative properties (de Castro and Sato, 2015). Thus, difference in the amounts of these amino acids in the peptides in protein mixtures might lead to difference in the antioxidant capacity. Also, Wu et al. (2003) demonstrated a proper relation between the amount of peptides and antioxidant properties.

**Optimization of Process Conditions and Model Validation**

The suggested hydrolysis condition for WGPH produced by Alcalase were: An E/S ratio of 1.46% (w/w), a temperature of 52.28°C and a time of 233 minutes. The process in these conditions containing the predicted values for DPPH, ABTS, total antioxidant capacity, and metal chelating activity that were 72.89%, 77%, 245.13 µmol α-tocopherol mL⁻¹, and 58.48%, respectively. To verify the validity of the model, experiments were done under mentioned optimal conditions. The experimental DPPH (70.5±1.2%), ABTS (76±1.2%), total antioxidant capacity (246±1.5 µmol α-tocopherol mL⁻¹), and metal chelating activity (59±0.4%) values agreed with the predicted values by the model within a 95% confidence interval. In optimal hydrolysis conditions, degree of hydrolysis value of the WGPH was 48.8±2%.

**Amino Acid Composition of WGPI and WGPH Prepared with Alcalase**

As shown in Table 3, Glu (E), Asp (D), and Arg (R) were the main amino acids of both WGPI and WGPH, which is in line with the results reported by Zhu et al. (2006). Glutamic acid, alanine, tyrosine, phenylalanine, isoleucine, leucine, and proline amino acid contents in Alcalase treated WGPH were higher than the corresponding WGPI. WGPH treated with
Table 3. Comparative amino acid profile of Wheat Germ Protein Hydrolysates (WGPH) and Wheat Germ Protein Isolates (WGPI) (g 100 g⁻¹ protein).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Protein isolates (g 100 g⁻¹ protein)</th>
<th>WGPH by alcalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>9.11</td>
<td>8.14</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15.2</td>
<td>17.73</td>
</tr>
<tr>
<td>Serine</td>
<td>4.66</td>
<td>3.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.05</td>
<td>2.54</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.05</td>
<td>4.95</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.12</td>
<td>3.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>9.47</td>
<td>5.69</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.80</td>
<td>8.07</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.24</td>
<td>4.11</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.6</td>
<td>0.54</td>
</tr>
<tr>
<td>Valine</td>
<td>6.65</td>
<td>6.72</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.11</td>
<td>1.75</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.89</td>
<td>5.136</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.20</td>
<td>4.47</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.80</td>
<td>9.36</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.07</td>
<td>5.52</td>
</tr>
<tr>
<td>Proline</td>
<td>4.63</td>
<td>7.248</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>NDa</td>
<td>0.064</td>
</tr>
<tr>
<td>Total hydrophobic amino acidsb</td>
<td>42.13%</td>
<td>47.69%</td>
</tr>
</tbody>
</table>

a Not Determined. b Glycine, Alanine, Valine, Leucine, Proline, Methionine, Phenylalanine, Tryptophan and Isoleucine.

Alcalase demonstrated a higher concentration of hydrophobic amino acids, due to the fact that Alcalase splits C-terminal hydrophobic residues (Ala (A), Phe (F), Trp (W), Ile (I), Pro (P), Val (V)) (Jia et al., 2010). Feng et al. (2018) and Sabeena Farvin et al. (2016) explored that the presence of hydrophobic residues has a considerable role to exert antioxidant properties in bioactive peptides. Therefore, wheat germ protein appears to be an especially good reference to generate antioxidant peptides.

**Antioxidant Activity of Hydrolysates Fractionated by RP-HPLC**

RP-HPLC is routinely used in the purification and separation of proteins and peptides. In this study, analytical RP-HPLC was first used to screen WGPH and identify relevant peaks that were subsequently isolated in higher amounts under comparable conditions by preparative HPLC. Eight main peaks were selected. Figure 2 displays the elution profiles and antioxidant activities of HPLC-separated fractions in the ABTS assay. Fractions F₇ displayed the highest (ca. 90% at 0.8 mg mL⁻¹) radical scavenging activities, significantly different (P< 0.05) from other fractions at the same concentration (0.8 mg mL⁻¹). The highest scoring fraction (ABTS assay) was submitted to further analysis as described below.

**Peptide Identification by Nano-LC-ESI/MS/MS**

Fraction F₇ from Alcalase WGPHs was next analyzed by LC–ESI/MS/MS. Both ESI/MS/MS and Q-TOF LC/MS are highly specific and sensitive technologies capable of detecting multiple compounds in complex samples (Zhu et al., 2013). Two main peptide components could be identified from fraction F₇ with its amino acid sequences as follows: GNPIPREPGQVPAY (1494.77 Da) and TVGGAPAGRIVME (1257.66 Da) (Figure 3, Table 4). Researchers reported...
Table 4. Peptides identified by LC-ESI/MS/MS in the RP-HPLC active fraction of wheat germ protein hydrolysates obtained by Alcalase.

<table>
<thead>
<tr>
<th>Suggested sequences</th>
<th>Obs (m/z)</th>
<th>Z</th>
<th>Source protein</th>
<th>Fragment</th>
<th>Molecular weight (Da)</th>
<th>Time (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVGGAPAGRIVME</td>
<td>629.33</td>
<td>+2</td>
<td>&gt; BAN09078.1</td>
<td>11-23</td>
<td>1257.66±0.5</td>
<td>11.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cyclophilin A-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GNPIPREPGQVPAY</td>
<td>747.89</td>
<td>+2</td>
<td>&gt; CDM83671.1</td>
<td>29-42</td>
<td>1494.77±0.5</td>
<td>11.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>unnamed protein product</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. RP-HPLC chromatographic separation of wheat germ protein hydrolysate and determined antioxidant activity by ABTS* scavenging activities assay (0.8 mg mL⁻¹). Columns marked with different letters show that means are significantly different (P< 0.05) with each other.

that antioxidant peptides exhibit electron donating activities leading to their interaction with free radicals (Zhuang et al., 2013). Such activity can be affected by peptide sequence and amino acid constituents (Zhang et al., 2014), which in turn is influenced by the source of protein, the operational conditions used to isolate the protein, hydrolysis degree, and the type of enzyme (Wang et al., 2010), Molecular Weight (MW) of peptides (Li et al., 2008), enzyme / substrate ratio, temperature, pH, reaction time, etc. (Shahidi and Zhong, 2008). Samaranayaka and Li-Chan (2011) declared that peptides with molecular weight of 1,000–3,000 Da could be more effective in interacting with radicals for the cancellation of diffusion cycles of lipid peroxidation. As can be seen from the MW of the obtained sequences, our results is in tune with those reported by Samaranayaka and Li-Chan (2011). It is shown that amino acids like Tyr (Y) and Met (M) have positive effects on antioxidant properties because of their special structures. Tyr has the special capability by phenolic groups to act as hydrogen transferor, Met tends to oxidize Met sulphoxide, (Rajapakse et al., 2005; Zhang et al., 2014). Therefore, the denoted antioxidant activity of
Figure 3. Identification of the molecular mass and amino acid sequence of the F7 peptide using LC-ESI/MS/MS spectrometer. Typical MS/MS chromatograms of the F7 peptides and interpretation of selected MS/MS spectra for the fraction F7.
fractions was imputed to the existence of Y, M in the aforementioned peptides. Hernandez-Ledesma et al. (2005) showed that the peptide with the sequences of WYSLAMAASDI from β-lactoglobulin indicated higher radical scavenging activity than BHA that is related to the presence of W, Y and M in the identified peptides from three hydrolysates. Sila and Bougatef (2016) showed that the existence of V, L, I as branched-chain amino acids affected antioxidant activity of peptides. In this report, the existence of these effective amino acids on antioxidant activity in the identified sequences is clear. The presence of acidic amino Glu (E) in the peptides could also be responsible for the strong antioxidant effects, because a free electron is available to interact with free radicals (Girgih et al., 2014). Shazly et al. (2017) also declared that antioxidant activity of peptides depends on the existence of hydrophobic amino acids in addition to Pro (P), Gln (Q), Glu (E).

CONCLUSIONS

In this research, response surface methodology was successfully applied to examine optimum hydrolysis conditions to earn hydrolysates with the highest antioxidant activity from wheat germ protein hydrolysates prepared with Alcalase. In optimal condition (an E/S ratio of 1.46% (w/w), a temperature of 52.28°C and a time of 233 minutes), the results for ABTS, DPPH radical-scavenging activities, total antioxidant capacity, and Fe²⁺-chelating activity were 77%, 72.89%, 245.13 μmol α-tocopherol/ml, and 58.48%, respectively. Two novel antioxidant peptides with sequences of GNPIPREPGQVPAY and TVGGAPAGRIVME were identified from HPLC-separated fraction with notable scavenging activity on ABTS radical by nano-LC-ESI/MS/MS. These results showed that wheat germ protein hydrolysates obtained by using Alcalase enzyme would be used as new sources of antioxidant compounds and they can also be introduced as additive to produce functional foods. Further researches is needed to validate antioxidant activity by means of synthetic peptide replicas and more detailed researches can be done to determine biological activities of these peptides.

ACKNOWLEDGEMENTS

This work is a part of PhD. study supported by University of Tabriz (Tabriz, I.R. Iran). We also thank Prof. David Andreu and Dr. Guadalupe Espadas, of the UPF/CRG Proteomics Unit, Barcelona, for excellent technical assistance.

REFERENCES


روش سطح پاسخ جهت بهینه سازی پارامترهای هیدرولیز برای تولید پپتیدهای آنتی اکسیدانت از پروتئین جوانه گندم با استفاده از آکالاز. شناسایی پپتیدهای آنتی اکسیدانت با روش LC-MS/MS

ز. کرمی، س. ه. پیغمبردوست، ج. حصاری، ب. اکبری ادرگانی

چکیده

در این مطالعه، اثرات شرایط هیدرولیز (زمان، نسبت آنزیم به سوبسترا، دما) بر فعالیت آنتی اکسیدانت پروتئین جوانه گندم هیدرولیز شده با آلکالاز مورد بررسی قرار گرفت. و با روش سطح پاسخ بهینه سازی شد. پارامترهای بهینه هیدرولیز در دمای 82/28 درجه سانتی گراد، زمان 822 دقیقه و نسبت آنزیم به سوبسترا 64/1 درصد یافت شد. پروفایل آمینواسید پروتئین های هیدرولیز شده و دست نخورده نشان داد که هیدرولیزات پروتئین جوانه گندم درصد بالاتری از اسیدآمینه های هیدروفوبیک را نسبت به پروتئین دست نخورده داردند. سپس فرکش ها در نقطه بهینه با استفاده از RP-HPLC جدا شدند و فرکسیون با بالاترین فعالیت مهار رادیکال ABTS با استفاده از بالاترین نقطه بهینه با استفاده از LC-ESI/MS/MS شناسایی شد. نتایج تعیین توالی LC-ESI/MS/MS با توالی آنتی اکسیدانت TVGGAPAGRIVME (44/1821 دالتون) و GNPIPREPGQVPAY (11/1646 دالتون) مطابقت داشت.