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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

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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

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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 8,000×g 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 8,000×g 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 8,000×g 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 (Ortho-PhthalAldehyde) 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:

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$$DH=\frac{peptide\ content}{protein\ content}\times 100$$

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 α-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:

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$Y= \beta_0+\Sigma \beta_i X_i+\Sigma\beta_{ii}X_i^2+\Sigma\beta_{ij}X_iX_j$

Where, Y is estimated response, i and j: values from 1 to the number of factors (n), β_0: Intercept factor, βi: Linear coefficient, βij: 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 110ºC. 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 hydrindantin (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 36,000×g for 30 minutes at 10ºC 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_2O) 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 -20ºC 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_2O) 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. Ultramark 1621 (Thermo Fischer Scientific) was used for external calibration. Therewith, internal calibration was also completed using the background polysiloxane ion signal (m/z

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445.12). 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 selected and Met oxidation and *N*-terminal acetylation were applied 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 R^2 values for the 4 models were 0.97, 0.97, 0.99 and 0.99, respectively. High R^2 values demonstrated that models were well adapted to the responses. The linear terms of time and E/S had significant effects on DPPH and

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Table 1. Experimental design and results of CCD of response surface methodology.

Run	Temperature (℃) (x_1)	Time (h) (x_2)	E/S(%w/w) (x_3)	DPPH radical-scavenging assay (%)	ABTS radical-scavenging assay (%)	Total antioxidant capacity (µmol ∝-tocopherol mL^{-1})	Metal-chelating activity (%)
1	50	3.5	1.25	68.15	73.15	234.5	53
2	55	3.5	1.25	77.75	81.75	219.67	64.06
3	50	2	1.25	59.1	64.1	211.15	47.64
4	45	2	0.5	50.2	55.2	158	32.26
5	50	3.5	1.25	67.5	72.5	233.67	53.1
6	50	3.5	2	76.6	80.6	261	53.11
7	50	3.5	1.25	69.3	74.3	236	52.2
8	55	2	0.5	55.07	60.07	152.72	44.5
9	45	5	2	76.79	80.8	244	47.51
10	45	3.5	1.25	76.010	80	210.25	47.28
11	50	5	1.25	70.83	75.8	256.45	54.747
12	55	2	2	85.71	90.1	223	55.625
13	50	3.5	1.25	67.042	71	234.8	52.832
14	45	2	2	87.17	91	212.54	45.02
15	45	5	0.5	67.57	71.57	217.5	34.39
16	50	3.5	1.25	67.44	71.44	235	53.07
17	50	3.5	1.25	70.49	75	236.8	52.76
18	50	3.5	0.5	60.71	64.71	212.74	42.2
19	55	5	0.5	74.75	79.75	216.17	58.75
20	55	5	2	75.92	80	261.35	67.19

ABTS radical scavenging ($P< 0.05$ for x_1 and x_3) 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 X_1). 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:

$$\text{DPPH radical} - \text{scavenging assay} = +68.64 + 1.15x_1 + 2.86x_2 + 9.39x_3 + 0.36x_1x_2 - 1.80x_1x_3 - 7.15x_2x_3 + 7.76x_1^2 - 4.16x_2^2 - 0.47x_3^2$$

$$\text{ABTS radical} - \text{scavenging assay} = +73.13 + 1.31x_1 + 2.75x_2 + 9.12x_3 + 0.43x_1x_2 - 1.84x_1x_3 - 7.04x_2x_3 + 7.39x_1^2 - 3.54x_2^2 - 0.83x_3^2$$

$$\text{Total antioxidant capacity} = +235.81 + 3.06x_1 + 23.81x_2 + 24.48x_3 + 1.36x_1x_2 + 4.30x_1x_3 - 6.64x_2x_3 - 21.88x_1^2 - 3.04x_2^2 + 0.028x_3^2$$

$$\text{Metal chelating activity} = +52.93 + 8.37x_1 + 3.75x_2 + 5.64x_3 + 2.65x_1x_2 - 0.79x_1x_3 - 0.29x_2x_3 + 2.59x_1^2 - 1.89x_2^2 - 5.43x_3^2$$

Where, x_1, x_2 and x_3 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

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Table 2. Analysis of variance (ANOVA) for the quadratic model.

	DPPH radical scavenging activity (%)				ABTS radical scavenging activity (%)				Total antioxidant capacity (∝-tocopherol µmol mL^{-1})				Metal-chelating activity (%)			
Source	Sum of squares	*df*	Mean Square	*P*-value	Sum of square	*df*	Mean Square	*P*-value	Sum of squares	*df*	Mean squares	*P*-value	Sum of squares	*df*	Mean square	P-value
Model	1597.34	9	177.48	0.0001	1512.65	9	168.07	0.0001	15103.2	9	1678.14	0.0001	1367.1	9	151.90	0.0001
X_1	13.13	1	13.13	0.0973	17.16	1	17.16	0.0731	93.76	1	93.76	0.0002	699.98	1	699.98	0.0001
X_2	81.85	1	81.85	0.0010	75.35	1	75.35	0.0018	5667.2	1	5667.26	0.0001	140.94	1	140.94	0.0001
X_3	881.53	1	881.53	0.0001	831.74	1	831.74	0.0001	5990.7	1	5990.75	0.0001	317.59	1	317.59	0.0001
X_1X_2	1.05	1	1.05	0.6160	1.45	1	1.45	0.5730	14.69	1	14.69	0.0431	56.15	1	56.15	0.0001
X_1X_3	25.85	1	25.85	0.0281	27.20	1	27.20	0.0303	148.09	1	148.09	0.0001	4.98	1	4.98	0.0007
X_2X_3	409.27	1	409.27	0.0001	396.92	1	396.92	0.0001	352.98	1	352.98	0.0001	0.68	1	0.68	0.1047
X_1^2	165.40	1	165.40	0.0001	150.05	1	150.05	0.0001	1316.79	1	1316.79	0.0001	18.38	1	18.38	0.0001
X_2^2	47.58	1	47.58	0.0059	34.43	1	34.43	0.0177	25.45	1	25.45	0.0123	9.84	1	9.84	0.0001
X_3^2	0.61	1	0.61	0.7025	1.91	1	1.91	0.5193	2.114	1	2.114	0.9784	81.08	1	81.08	0.0001
Residual	39.25	10	3.92		42.80	10	4.28		27.40	10	2.74		2.12	10	0.21	
Lack of fit	30.47	5	6.09	0.0991	30.46	5	6.09	0.1718	21.20	5	4.24	0.1079	1.56	5	0.31	0.1428
Pure Error	8.78	5	1.76		12.33	5	2.47		6.20	5	1.24		0.56	5	0.11	
Cor Total	1636.59	19			1555.45	19			15130.6	19			1369.2	19		
CV%				0.8497				2.77				0.74				0.91
R^2				0.97				0.97				0.99				0.99
Adj-R^2				0.95				0.94				0.99				0.99

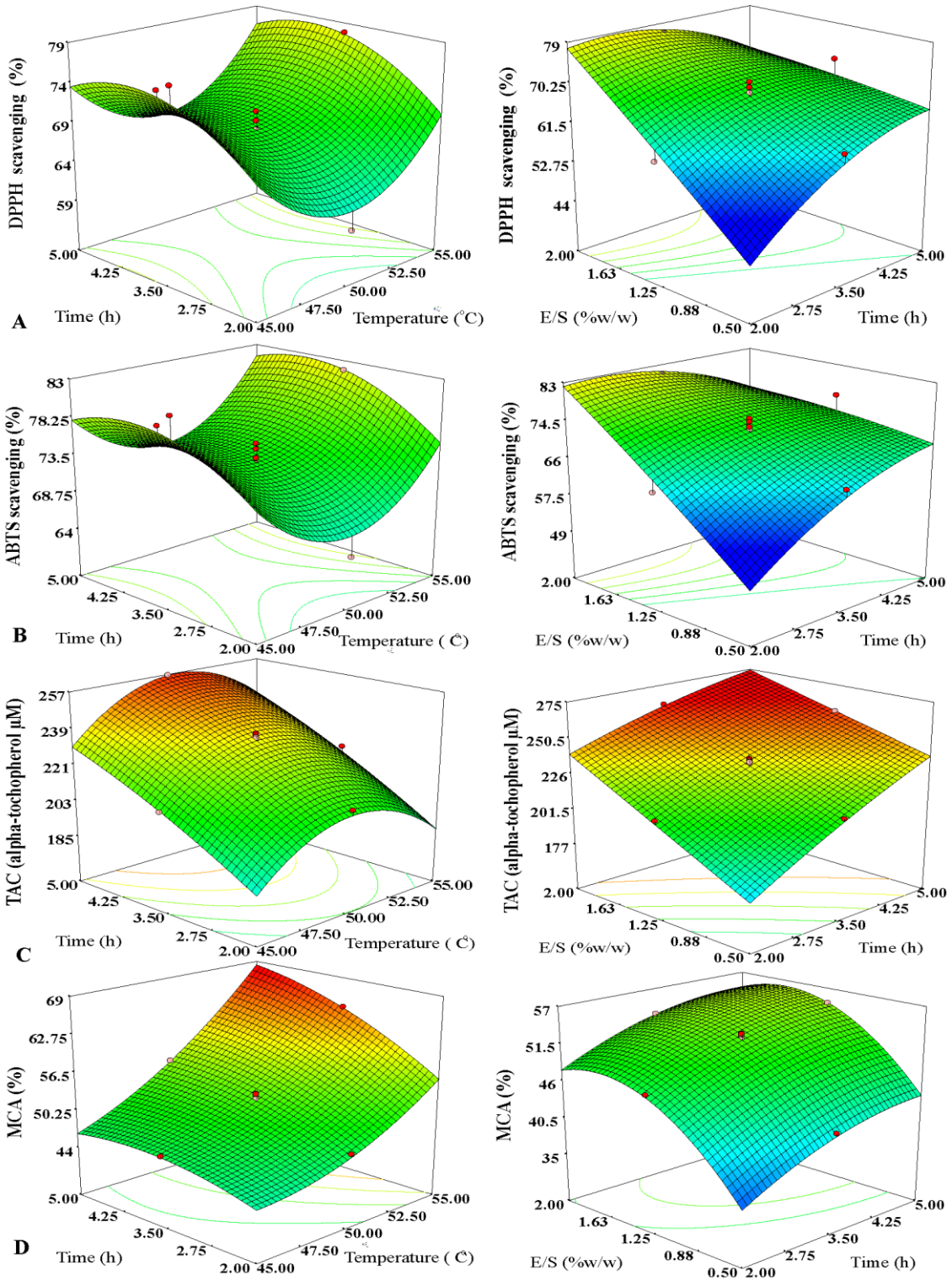

Figure 1. Response surface for the effects of time (h), temperature (℃), and E/S (%w/w) on DPPH radical-scavenging assay (A), ABTS radical scavenging activity (B), Total Antioxidant Capacity (TAC) (C) and, Metal Chelating Activity (MCA) (D).

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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℃, 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 produced by Alcalase, 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

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Table 3. Comparative amino acid profile of Wheat Germ Protein Hydrolysates (WGPH) and Wheat Germ Protein Isolates (WGPI) (g 100 g^{-1} protein).

Amino acid	Protein isolates (g 100 g^{-1} protein)	WGPH by alcalase
Aspartic acid	9.11	8.14
Glutamic acid	15.2	17.73
Serine	4.66	3.3
Histidine	3.05	2.54
Glycine	6.05	4.95
Threonine	4.12	3.1
Arginine	9.47	5.69
Alanine	5.80	8.07
Tyrosine	3.24	4.11
Cysteine	0.6	0.54
Valine	6.65	6.712
Methionine	2.11	1.75
Phenylalanine	4.89	5.136
Isoleucine	4.20	4.47
Leucine	7.80	9.36
Lysine	7.07	5.52
Proline	4.63	7.248
Tryptophan	ND[a]	0.064
Total hydrophobic amino acids[b]	42.13%	47.69%

[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 F_7 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 F_7 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 F_7 with its amino acid sequences as follows: GNPIPREPGQVPAY (1494.77 Da) and TVGGAPAGRIVME (1257.66 Da) (Figure 3, Table 4). Researchers reported

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Table 4. Peptides identified by LC-ESI/MS/MS in the RP-HPLC active fraction of wheat germ protein hydrolysates obtain by Alcalase.

Suggested sequences	Obs (m/z)	*Z*	Source protein	Fragment	Molecular weight (Da)	Time (Min)
TVGGAPAGRIVME	629.33	+2	> BAN09078.1 cyclophilin A-1	11-23	1257.66±0.5	11.58
GNPIPREPGQVPAY	747.89	+2	> CDM83671.1 unnamed protein product	29-42	1494.77±0.5	11.90

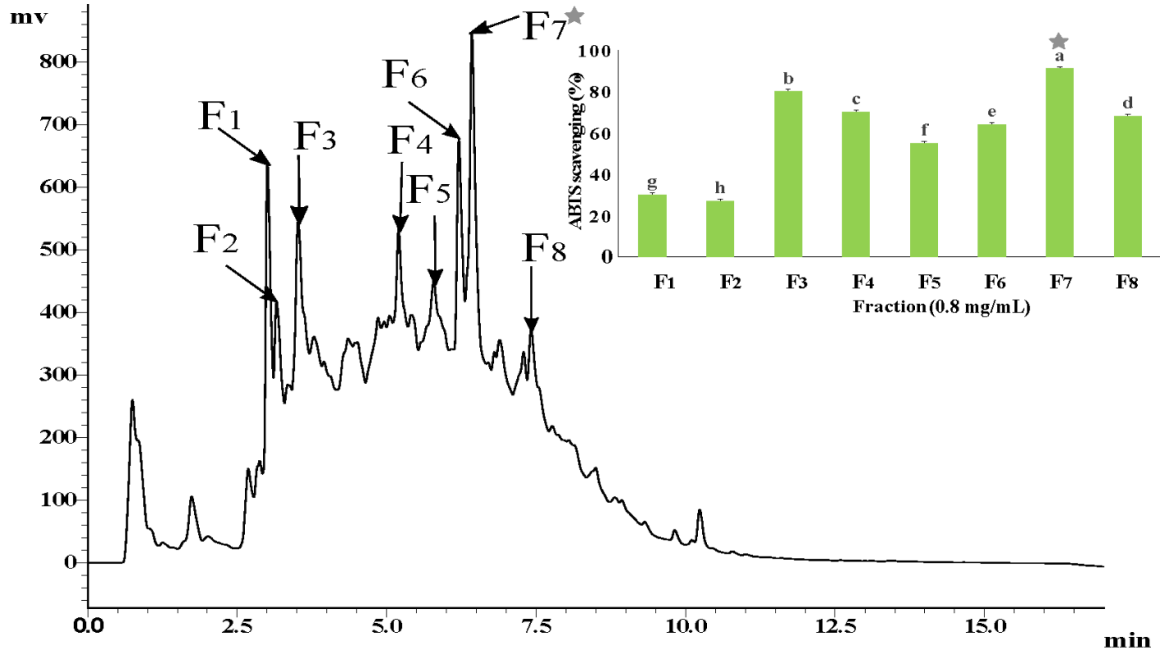

Figure 2. RP-HPLC chromatographic separation of wheat germ protein hydrolysate and determined antioxidant activity by $ABTS^{+}$ scavenging activities assay (0.8 mg mL^{-1}). Columns marked with different letters show that means are significantly different ($P< 0.05$) with each other.

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

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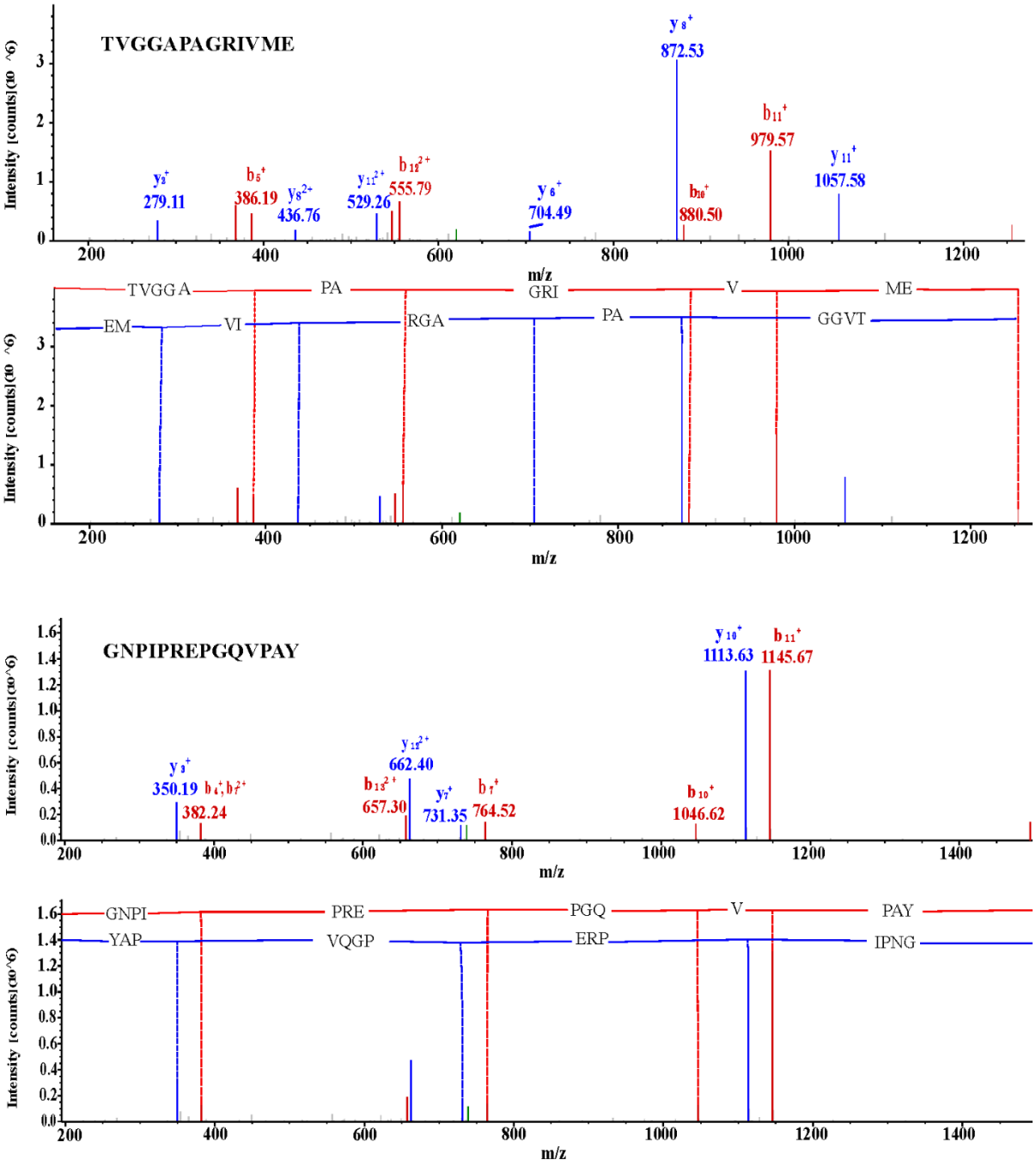

Figure 3. Identification of the molecular mass and amino acid sequence of the F_7 peptide using LC-ESI/MS/MS spectrometer. Typical MS/MS chromatograms of the F_7 peptides and interpretation of selected MS/MS spectra for the fraction F_7.

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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 Fe^{2+}-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.

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

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چکیده

در این مطالعه، اثرات شرایط هیدورلیز (زمان، نسبت آنزیم به سوبسترا، دما) بر فعالیت آنتی‌اکسیدانی پروتئین جوانه گندم هیدرولیز شده با آلکالاز مورد بررسی قرار گرفت. و با روش سطح پاسخ بهینه سازی شد. پارامترهای بهینه هیدرولیز در دمای ۵۲/۲۸ درجه سانتی‌گراد، زمان ۲۳۳ دقیقه و نسبت آنزیم به سوبسترا ۱/۴۶٪ یافت شد. پروفایل آمینواسید پروتئین‌های هیدرولیز شده و دست نخورده نشان داد که هیدرولیزات پروتئین جوانه گندم درصد بالاتری از اسیدآمینه‌های هیدروفوبیک را نسبت به پروتئین دست نخورده دارند. سپس فرکشن‌ها در نقطه بهینه با استفاده از RP-HPLC جدا شدند و فرکسیون با بالاترین فعالیت مهار رادیکال ABTS با استفاده از LC-ESI/MS/MS شناسایی شد. نتایج تعیین توالی نشان داد که پپتیدهای خالص شده دارای توالی TVGGAPAGRIVME (۱۲۵۷/۶۶ دالتون) و GNPIPREPGQVPAY (۱۴۹۴/۷۷ دالتون) می‌باشند.

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