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Research Article

Effect of Low-Frequency Ultrasonic-Assisted Enzymolysis on the Physicochemical and Antioxidant Properties of Corn Protein Hydrolysates

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The aim of this study was to investigate the effect of low-frequency ultrasound on the enzymolysis of corn protein. A L_9 (3^4) orthogonal design was used to optimize ultrasound pretreatment conditions. Degree hydrolysis (DH), conversion rate of protein (CR), and DPPH IC_{50} were selected as analytical indicators. Under the optimal ultrasound conditions (5 W/L power, 2 s/2 s on/off time, 50°C temperature, and 25 min time), the DH, CR, and radical (DPPH$^{\bullet}$, $^{\bullet}$OH) scavenging capacities were significantly increased. Molecular weight distribution and amino acid profile analysis showed that ultrasound pretreatment enhanced the formation of short-chain peptides with molecular weight of 200–3000 Da, especially the peptides containing hydrophobic amino acids. Moreover, 40 potential antioxidant peptides were purified by C18 semipreparative column and identified by UPLC-ESI-MS. The results suggest that the optimal ultrasonic-assisted enzymolysis technology could be useful for preparation of antioxidant peptides from corn.

1. Introduction

Corn, the third most widely cultivated cereal in the world, supplies approximately 42 million tons of protein per annum. China is the second largest country for production and consumption of corn [1]. Corn gluten meal (CGM), a main byproduct of the corn wet-milling process, contains 60–71% (w/w) protein [2]. Currently, CGM is mainly used as feedstuff or disposed due to low water solubility and severely imbalanced amino acid profile [3]. But on the other hand, CGM contains a high proportion of hydrophobic amino acids (leucine, alanine, phenylalanine, etc.) [4], which makes it become a potential source for peptides with biological activities such as antioxidant activity [5], antihypertensive effect [6], facilitating alcohol effect [7], and antitumor efficacy [8], while the poor solubility of corn protein and the special structure have created barrier for enzymes to cleave the protein, which seriously limited the functional peptides' release and reduced the corn protein's bioavailability. Therefore, developing a more efficient technique to overcome these problems is of great interest to food scientists all over the world.

Several kinds of new technologies have used extensively in food industry and its related fields, such as high pressure treatment, microwave assist, ultrasonication, heating at ambient and moderate pressures, and super-high frequency electromagnetic field, which could increase the extraction yield, achieve higher quality, protect environment, and so forth; our team has been devoted to the research of application of the ultrasound technology in food physical processing for many years. Its mechanism is attributed to the thermal, cavitation, and mechanical efficacies and they can enhance mass

transfer and increase the contact frequency between substrate and enzyme or change the substrate configuration [9, 10]. Ultrasound pretreated-assisted enzymolysis could induce protein unfolding and enhance proteolysis through increased exposure of susceptible peptide bonds [11] that enhance the efficiency of enzymatic protein hydrolysis for bioactive peptides production. Recent studies have shown that ultrasound pretreatment could facilitate release of angiotensin converting enzyme-inhibitory peptides from corn protein [2, 12] and wheat gluten [13], as well as antioxidant peptides from peanut protein [14] and wheat gluten [15]. In consideration of the ultrasonic mechanism and the characteristic of corn protein, more scientific studies are required to investigate the effect of ultrasound pretreatment on the physicochemical and antioxidant properties of corn protein hydrolysates.

Oxygen free radicals are very reactive molecules that can react with every cellular component and cause functional and morphologic disturbances in cells [16]. The bioactive peptides derived from food can play a significant role in an oxidative systems, protecting the human body from free radicals and retarding the progress of many chronic diseases, such as brain disorders, cancer, obesity, and cardiovascular diseases [17, 18]. Several studies have reported the antioxidant activity of corn protein hydrolysates [1, 3, 19], and only a few antioxidant peptides have been identified [5]. Thus, there are much more antioxidant peptides that need to be purified and determined.

In the present study, the optimum ultrasound pretreatment factors were developed by using orthogonal L_9 $(3)^4$ tests. The effects of ultrasound pretreatment on DH, protein conversion rate of corn protein and hydrolysates' antioxidant activities, molecular weights, and amino acid composition were evaluated. Furthermore, the antioxidant peptides from the corn protein hydrolysates with highest antioxidant activity were identified by mass spectrometry.

2. Materials and Methods

2.1. Materials and Chemicals. CGM, with 65.2% protein, was purchased from Yishui Earth Corn Development Co., Ltd. (Shandong, China). The concentrated corn protein with 92.5% protein purity was prepared according to a previous study [20]. Alcalase 2.4 L with an activity of 23,400 U/ml was purchased from Novozymes Co., Ltd. (Tianjin, China). All other chemicals used were of analytical grade.

2.2. Ultrasound Pretreatment of Corn Protein. Prior to the enzymolysis reaction, the corn protein was pretreated by the multifrequency energy-gathered ultrasound equipment. This apparatus was developed by our research team and manufactured by Meibo Biotechnology Co., Ltd (Zhenjiang, Jiangsu, China). Before ultrasound treatment, the corn protein dispersions (2.0%, w/v) were adjusted to pH 9.0 with 1.0 M NaOH. Then the protein dispersions were processed at a constant frequency of 28 kHz. The probe (2.5 cm in diameter) was dipped into the reaction solution to a depth of 0.5 cm and sonication was done at different sonication times ranging from 15 to 25 min and powers ranging between 45 and 65 W/L. Three kinds of ultrasound pulse modes of on time/off time (2 s/2 s, 3 s/2 s, and 4 s/2 s) were used.

2.3. Enzymatic Hydrolysis of Corn Protein. The enzymatic hydrolysis was carried out immediately after low-frequency energy-gathered ultrasound (LFEU) pretreatment. After 10 min preheating at 50°C, Alcalase (E/S = 2500 U/g) was added to initial the reaction and the pH was maintained at 9.0 by continuously adding 0.5 M NaOH during the enzymolysis process. The enzymolysis time was 60 min and the reaction was terminated by boiling the mixtures for 10 min. Then it was centrifuged at 5030g for 15 min to get the supernatant. The traditional enzymolysis (control) was conducted with a magnetic stirring apparatus under the same conditions, instead of ultrasound.

2.4. Determination of Degree of Hydrolysis (DH) and Protein Conversion Rate (CR). The DH of corn protein was determined using the pH-state method [4], which was calculated as follows:

$$\mathrm{DH}\,(\%) = \frac{h}{h_{\mathrm{tot}}} = \frac{N_b \times B \times 100}{\alpha \times M_p \times h_{\mathrm{tot}}}, \quad (1)$$

where B is the NaOH volume consumed (mL), N_b is the normality of the NaOH (mol/L), M_p is the protein weight (g), α is the average dissociation degree of α-NH_2 in substrate (0.99 for corn protein), and h_{tot} is the total number of peptide bonds in the protein substrate (9.2 mmol/g for corn protein).

The conversion rate of protein (CR) was calculated based on the following:

$$\mathrm{CR}\,(\%) = \frac{C \times V}{10M} \times 100\%, \quad (2)$$

where C is the concentration of corn protein hydrolysates (mg/mL), which was determined by Lowry method, using bovine serum albumin (BSA) as standard; V is the volume of the hydrolysates (mL); M is the mass of corn protein (mg).

2.5. Molecular Weight Distribution and Amino Acid Profile Analysis. The molecular weight distribution of the hydrolysate was analyzed by size exclusion chromatography with a TSK gel-G2000 SWXL column (7.8 mm × 30 cm, Tosoh). The elution was done using 45% acetonitrile plus 1% trifluoroacetic acid (TFA) with the flow rate of 0.5 mL/min for 65 min. The absorbance was monitored at 220 nm. Molecular weight of the peptide fractions was calculated and compared with the standards [21, 22].

The total amino acid profile of the sample was analyzed using an amino acid analyzer (RP-HPLC, Agilent 1100, US), after hydrolyzing under vacuum with 6 M HCl at 110°C for 24 h and 1% phenol (v/v), as described by Wang et al. [23] The free amino acid profile was measured by the analyzer after precipitation with 10% cold trichloroacetic acid for 2 h.

2.6. Radical Scavenging Activity. The DPPH radical scavenging activity was determined by the method described by Hu et al. [24] with slight modification. Two mL of sample was added to 2 mL of 0.2 mM ethanol solution of DPPH. After reacting for 30 min in the dark at 37°C, the absorbance of the solution

was monitored immediately at 517 nm. The scavenging rate was calculated as follows:

$$\text{Scavenging rate}\,(\%) = \left[1 - \frac{A_1}{A_0}\right] \times 100, \quad (3)$$

where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

The hydroxyl radical ($^{\bullet}$OH) scavenging activity was determined by the method reported previously, with slight modification [25]. Briefly, 0.5 ml of 9 mmol/L $FeSO_4$, 1 mL of 9 mmol/L salicylic acid in ethanol, 1 mL of 4.4 mmol/L H_2O_2, and 2 ml distilled water were sequentially added to 1 mL of sample. Absorbance of the mixture was measured at 510 nm after reacting for 30 min at 37°C. The $^{\bullet}$OH scavenging rate was calculated as follows:

$$^{\bullet}\text{OH scavenging rate}\,(\%) = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100, \quad (4)$$

where A_0 is the absorbance of the blank, A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample without H_2O_2.

2.7. Isolation of Antioxidant Peptides. The lyophilized corn peptides were dissolved in water contained 0.1% trifluoroacetic acid and subjected to an Agilent ZORBAX Eclipse XDB C18 semipreparative column (9.4 × 250 mm, 5 μm) and separated by a gradient of acetonitrile (2–15% in 0–20 min, 15–30% in 20–35 min, and 30–80% in 35–40 min) at 2.5 mL/min. Online UV absorbance was monitored at 220 nm. The elution fractions collected every minute were concentrated and freeze-dried. The antioxidant activity of each fraction was expressed as the DPPH radical scavenging rate per unit weight peptide (mg).

2.8. Identification of Antioxidant Peptides by UPLC-ESI-MS. The purified peptides were dissolved in 0.1% aqueous formic acid and identified using Waters ACQUITY UPLC coupled to SYNAPT Q-TOF MS with electrospray ionization. 10 μL sample was injected into a BEH130 C18 column (2.1 × 150 mm, 1.7 μm) and eluted with a linear gradient of acetonitrile (0–30% in 8 min and 30–80% in 8–15 min) at 0.2 mL/min. The amino acid sequence was analyzed using BioLynx software and confirmed by performing searches against manually restricted NCBI database with entries only from G. max (11, 1525 sequences, 03-2016) taxonomy [26]. To analyze the antioxidant activity, all identified peptides were searched against online available databases BioPep [27].

2.9. Statistical Analysis. All the tests were conducted in triplicate and data were presented as mean and standard deviations. The results obtained were subjected to one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$. All computations were done with SPSS 15.0.

3. Results and Discussion

3.1. Analysis of Orthogonal Design Experiment of LFEU Pretreatment. Over the past decades, ultrasound has been increasingly investigated for application of controlled release of peptides. High power ultrasound treatment for longer treatment time can generate high temperature and pressure conditions which can alter the native state of food protein or peptide [11]. It is necessary to optimize the treatment conditions for specific ultrasound applications to achieve optimum results. In the present study, L_9 (3^4) orthogonal design was used to optimize processing conditions to obtain peptides, which might possess potent high antioxidant activity. The full experimental design, with respect to the evaluation indicators (DH, DPPH IC_{50}, and CR) were presented in Table 1.

The results of the orthogonal test are shown in Tables 2 and 3. Fisher's F test, however, provided a decision at some confidence level so as to make sure whether these factors have significant effect on experimental indicators. Table 2 indicates that the F-test on all four factors was significant ($p < 0.05$) for DPPH IC_{50}. For the other two indicators, only factors A and C were significant. Results of the range analysis (Table 3) showed that the influential orders of the four factors to DPPH IC_{50}, DH, and CR were $A > D > C > B$, $A > C > D > B$, $A > C > B > D$, respectively. Based on the results of range analysis and ANOVA, the optimum pretreatment condition for DPPH IC_{50}, DH, and CR were $A_3D_2C_2B_1$, $A_3C_3D_2B_1$, and $A_3C_3B_3D_1$, respectively. Based on the frequent best levels obtained for the reference indicators of DPPH IC_{50}, DH, and CR (Table 1), the best pretreatment condition was $A_3B_1C_3D_2$, namely, 65 W/L ultrasound powers, 2 s/2 s ultrasound on/off time, 50°C ultrasound temperature, and 25 min ultrasound time. These corresponded to number 7 in Table 1, which achieved the highest DPPH IC_{50}, DH, and CR.

3.2. DH and CR of Corn Protein after Ultrasonic Pretreatment. Table 1 shows the change of DH and CR during enzymolysis process of corn protein for the different pretreatments. For the traditional hydrolysis, the DH was 19.28% and 51.58% for CR. Obviously, compared to traditional hydrolysis, ultrasonic pretreatment caused increase of 5.42% and 11.27% in DH and CR. The current study results are in agreement with the that of ultrasound-assisted enzymolysis of solid leather waste [28], zein [12], and soy protein isolates [29]. This enhancement in DH and CR could be attributed to the particle size reduction and change in molecular conformation of protein [2, 30].

3.3. Molecular Weight Distribution and Amino Acids Profile after Ultrasonic Pretreatment. The molecular weight distribution of the corn peptides obtained from traditional and LFEU assisted enzymolysis is presented in Figure 1. The ultrasound pretreatment (the best conditions) caused an increase of 11.84% for 200–1000 Da peptides fraction and 21.29% for the fraction of 1000–3000 Da peptides. Meanwhile, the fraction of <200 Da and >3000 Da peptides decreased by 15.61% and 46.5%, respectively. This increase in the amounts of small peptides fraction (200–3000 Da) after ultrasound pretreatment might be due to exposure of more hydrophobic cores buried inside the protein. Moreover, Alcalase is a typical endoprotease with a preference for sites containing hydrophobic residues [31]. You et al. [32] also reported that the peptides were absorbed by the body mainly in the form of tetrapeptide, tripeptide, and dipeptide, rather than mainly

Table 1: Factors and levels of the orthogonal design L_9 (3^4) of ultrasound pretreatment process and experimental results for corn protein hydrolysis (n = 3, mean ± SD); number 10 represents traditional hydrolysis.

Test number	Factors (levels)				Experimental results		
	(*A*) Ultrasound powers (W)	(*B*) Ultrasound on/off time	(*C*) Ultrasound temperature (°C)	(*D*) Ultrasound time (min)	DPPH IC_{50} (mg/ml)	Degree hydrolysis (%)	Conversion rate of protein (%)
1	45 (1)	2/2 (1)	30 (1)	15 (1)	3.125 ± 0.078	19.528 ± 0.402	51.451 ± 3.065
2	45 (1)	3/2 (2)	40 (2)	20 (2)	2.495 ± 0.092	20.720 ± 1.065	54.288 ± 2.539
3	45 (1)	4/2 (3)	50 (3)	25 (3)	2.643 ± 0.118	21.497 ± 2.102	56.615 ± 2.457
4	52 (2)	2/2 (1)	40 (2)	25 (3)	2.322 ± 0.045	22.266 ± 1.402	56.304 ± 4.258
5	52 (2)	3/2 (2)	50 (3)	15 (1)	2.647 ± 0.035	23.713 ± 0.645	58.067 ± 4.831
6	52 (2)	4/2 (3)	30 (1)	20 (2)	2.581 ± 0.184	20.755 ± 0.056	56.985 ± 3.112
7	65 (3)	2/2 (1)	50 (3)	20 (2)	2.035 ± 0.113	24.704 ± 0.495	62.85 ± 0.491
8	65 (3)	3/2 (2)	30 (1)	25 (3)	2.289 ± 0.045	21.640 ± 0.112	56.768 ± 0.328
9	65 (3)	4/2 (3)	40 (2)	15 (1)	2.354 ± 0.168	23.502 ± 0.682	60.288 ± 2.539
10	—	—	—	—	2.402 ± 0.098	19.275 ± 0.608	51.581 ± 2.654

Table 2: Analysis of variance (ANOVA) on indicator parameters obtained from the L_9 (3^4) orthogonal experiment (n = 3, Mean ± SD). *A*, ultrasound powers; *B*, ultrasound on/off time; *C*, ultrasound temperature; *D*, ultrasound time.

Indicators	Source	Sum of squares	df	Mean square	*F*	*p*	Sig.
DPPH IC_{50}	Model	170.874	9	18.986	3.073*E*3	<0.05	significant
	A	1.260	2	0.630	102.012	<0.05	significant
	B	0.511	2	0.245	39.902	<0.05	significant
	C	0.384	2	0.192	31.070	<0.05	significant
	D	0.604	2	0.302	48.863	<0.05	significant
	Error	159.653	18	8.870			
	Total	50178.870	27				
DH	Model	50019.217	9	5557.691	626.600	<0.05	significant
	A	154.079	2	77.040	8.686	<0.05	significant
	B	11.891	2	5.945	.670	0.524	
	C	76.142	2	38.071	4.292	<0.05	significant
	D	12.778	2	6.389	.720	0.500	
	Error	13.700	18	.761			
	Total	13191.364	27				
CR	Model	13177.664	9	1464.185	1.924*E*3	<0.05	significant
	A	33.400	2	16.700	21.942	<0.05	significant
	B	0.279	2	.139	.183	0.834	
	C	32.144	2	16.072	21.116	<0.05	significant
	D	0.905	2	.453	.595	0.562	
	Error	13.700	18	.761			
	Total	13191.364	27				

Note. Differences were considered significant at $p < 0.05$.

free amino acids. Therefore, the result indicated that the LFEU pretreatment helped peptides absorb rapidly and had a high bioactivity.

Table 4 shows the amino acid composition of corn protein hydrolysates obtained by control and LFEU assisted enzymolysis. The total AAs significantly increased after ultrasound pretreatment, which is in accordance with the influence of ultrasound on corn protein's DH and CR. The corn protein hydrolysates contained considerable amounts of hydrophobic AAs (429.95 μg/ml), which increased by 13.41% after being pretreated by ultrasound. However, the percentage of free hydrophobic AAs was barely changed. By comparison, we

Table 3: Range analysis (R) on indicator parameters obtained from the L_9 (3^4) orthogonal experiment (n = 3, mean ± SD).

Indicators	(A) Ultrasound powers (W)	(B) Ultrasound on/off time	(C) Ultrasound temperature (°C)	(D) Ultrasound time (min)
DPPH IC_{50} (mg/ml)				
K_1	2.754 ± 0.096	2.477 ± 0.236	2.665 ± 0.307	2.709 ± 0.281
K_2	2.517 ± 0.983	2.494 ± 0.172	2.39 ± 0.305	2.37 ± 0.389
K_3	2.226 ± 0.109	2.526 ± 0.47	2.442 ± 0.208	2.418 ± 0.208
R	0.528	0.049	0.275	0.339
Best level	A_3	B_2	C_2	D_2
Degree hydrolysis (%)				
K_1	20.615 ± 0.656	22.199 ± 3.299	20.674 ± 1.858	22.106 ± 1.729
K_2	22.245 ± 0.834	22.024 ± 1.822	22.163 ± 1.633	22. 281 ± 3.016
K_3	23.282 ± 0.763	21.918 ± 1.640	23.305 ± 2.642	21.801 ± 2.016
R	2.667	0.281	2.631	0.48
Best level	A_3	B_1	C_3	D_2
Conversion rate of protein (%)				
K_1	54.118 ± 2.687	56.868 ± 2.605	55.068 ± 2.168	58.041 ± 3.478
K_2	57.119 ± 4.067	56.374 ± 2.566	56.960 ± 3.112	56.602 ± 2.047
K_3	59.969 ± 1.119	57.963 ± 2.702	59.177 ± 2.593	56.562 ± 2.347
R	5.851	1.589	4.109	1.479
Best level	A_3	B_3	C_3	D_1

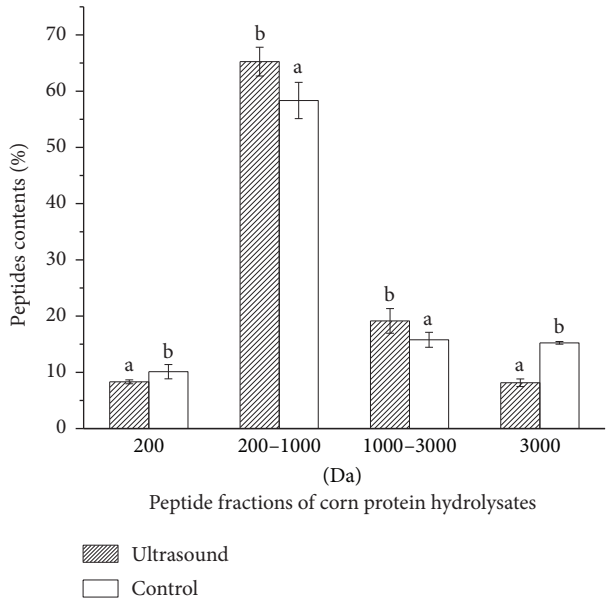

Figure 1: Molecular weights distribution of corn protein hydrolysates. [a,b]Values which are significantly different at $P < 0.05$.

found a large amount of increased hydrophobic AAs in form of peptides. This result also indicated that ultrasonic treatment to corn protein tended to produce more peptides with hydrophobic amino acid residues than traditional hydrolysis, which is in line with the finding reported by Jia et al. [13].

3.4. Antioxidant Activity of Corn Protein Hydrolysates after Ultrasonic Pretreatment. Antioxidant activity depends on many different factors, such as DH, CR, molecular weight distribution, and AAs composition. The results above showed that ultrasound pretreatments improved the DH and CR obviously and significantly ($p < 0.05$) changed the molecular weight distribution and amino acids composition of the corn peptides. Therefore, the radical (DPPH, $^{\bullet}OH$) scavenging capacities were analyzed to evaluate the antioxidant activity of corn peptides after ultrasonic pretreatment.

Table 4: Total and free amino acids composition of corn protein hydrolysates.

	Total amino acids				Free amino acids			
	Control		Ultrasound		Control		Ultrasound	
	mg/ml	%	mg/ml	%	mg/ml	%	mg/ml	%
Asp	44.83	6.01	37.21	4.50	0.14	0.26	0.18	0.36
Glu	143.46	19.22	195.12	23.60	5.60	10.12	5.50	11.08
Ser	34.77	4.66	31.42	3.80	0.88	1.59	0.67	1.35
His	24.96	3.34	21.50	2.60	0.36	0.66	0.31	0.62
Gly	23.25	3.12	19.84	2.40	3.99	7.23	3.37	6.78
Thr	28.67	3.84	23.98	2.90	0.36	0.69	0.36	0.72
Arg	30.53	4.09	28.94	3.50	0.28	0.52	0.31	0.62
Ala	59.67	8.00	81.02	9.80	14.13	25.60	11.48	23.15
Tyr	30.80	4.13	34.72	4.20	0.46	0.84	0.41	0.83
Cys	15.16	2.03	9.92	1.20	0.33	0.54	0.33	0.67
Val	35.46	4.75	28.94	3.50	1.46	2.66	2.03	4.09
Met	15.37	2.06	14.06	1.70	10.46	18.93	9.66	19.47
Phe	42.66	5.72	62.09	7.51	8.08	14.58	6.01	12.12
Ile	29.94	4.01	28.11	3.40	5.78	10.44	5.99	12.07
Leu	106.75	14.30	125.92	15.23	0.78	1.38	0.98	1.97
Lys	19.87	2.66	18.19	2.20	0.91	1.62	0.54	1.09
Pro	60.60	8.12	65.83	7.96	1.29	2.34	1.49	3.00
The total of hydrophobic amino acids (HAAs)	379.12	50.80	429.95	52.00	42.34	76.62	38.00	76.59
The total of amino acids (AAs)	746.76	100.0	826.79	100.00	55.24	100.00	49.61	100.00

Hydrophobic AAs: Ala, Val, Leu, Ile, Phe, Pro, Thr, and Met.

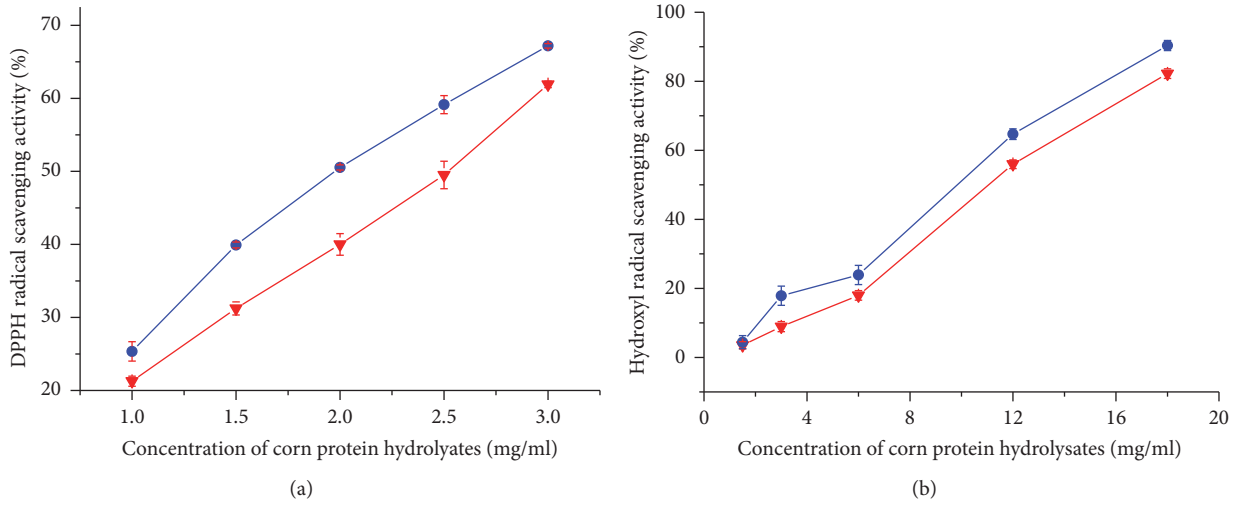

Figure 2: Antioxidant capacities of corn protein hydrolysates on DPPH radical scavenging activity (a) and hydroxyl radical scavenging activity (b). Ultrasonic (●); control (▼).

Generally, hydrolysates contain peptides or AAs, which were hydrogen donors that could react with radicals to convert them to more stable products, thereby terminating the radical chain reaction [33]. Figure 2 shows the changes of the antioxidant activity at various concentrations in traditional and LFEU assisted enzymolysis. As depicted, the corn protein hydrolysates showed dose-dependent antioxidant activity to varying extents. At any tested concentration, the DPPH radical and $^{\bullet}$OH scavenging activity of corn peptides prepared by ultrasound pretreatment were significantly higher than that of control. With respect to IC_{50}, the lower the value means, the higher the antioxidant activity and vice versa. The IC_{50} values of corn peptides were 2.41 and 9.98 mg/ml (control) 1.95 and 7.61 mg/ml (ultrasound pretreatment), respectively, for DPPH and $^{\bullet}$OH radical scavenging activities. The result suggested that LFEU assisted enzymolysis could significantly increase the antioxidant activity of corn protein hydrolysates. Additionally, comparing DPPH IC_{50} values to that of other

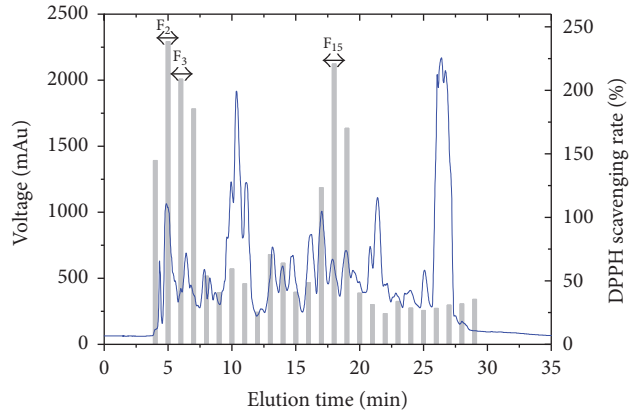

Figure 3: Chromatographic and antioxidant profiles of corn protein hydrolysates using Eclipse XDB C18 semipreparative column. Histogram, DPPH radical scavenging rate per unit weight peptide (mg).

protein hydrolysates, such as loach peptides (17.0 mg/mL) [34], albumen peptides (5.767 mg/mL), and soybean peptides (6.268 mg/mL) [35], corn peptides exhibited remarkable DPPH radical scavenging activity. It indicated that LFEU pretreatment might help in releasing the higher antioxidant peptides from corn protein hydrolysates, which could be used as source of antioxidant peptides for the further purification and identification.

3.5. Purification and Identification of Antioxidant Peptides. The hydrolysates from Alcalase-hydrolyzed corn protein after ultrasonic pretreatment were chromatographically fractionated by the C18 semipreparative column (Figure 3). As shown in Figure 3, three fractions displayed the strong DPPH radical scavenging activity, namely, F_2, F_3, and F_{15}. The fractions displaying the strong activity were collected and further purified and identified using UPLC-ESI-MS spectrometry. Identification of a large amount of peptide sequences in complex food protein hydrolysates is challenging. Therefore, all of the identified peptides were further searched against the sequences of corn (NCBI database) and 40 peptides were obtained with a probability of certainty of 100 (Table 5).

It has been reported that the majority of antioxidant peptides derived from food protein consist of 2 to 20 amino acids and the lower the molecular weight, the higher their chance to cross the intestinal barrier and exert biological effects [36]. Dávalos et al. [37] suggested that hydrolysates with higher proportion of low molecular weight peptides could access more easily the oxidant system and lead to high values of TEAC and DPPH radical scavengers. The number of amino acids ranged from 2 to 5 and the MW range of the identified antioxidant peptides was 200–500 Da, which corresponded to the above research findings.

Additionally, the antioxidant activity of peptides was highly dependent on their sequences and amino acid compositions. Statistical analyses found that the most popular AAs in our current study appeared to be Gly, Ala, Ser, Leu, Phe, Val, Pro, and His. The result is in agreement with the finding of Mendis et al. [38] who purified the peptides from jumbo squid skin gelatin and reported that the peptides containing Pro, Gly, Ala, Val, and Leu in the peptides sequence had strong antioxidant activity. Another frequent amino acid residue of the identified peptides was His, an important amino acid residue responsible for the radical scavenging activity of peptides due to their special structure characteristics (the imidazole group in His has the proton-donation ability) [39]. Furthermore, it has been reported that hydrophobic amino acids (Ala, Leu, Phe, Val, Met, and Pro in the identified peptides) have a significant effect on radical scavenging, which can enhance the presence of peptides at the water lipid interaction and facilitate the permeability to the lipid phase to scavenge the generated free radicals [40].

Sequenced peptides were checked through BIOPEP bioactive peptide databases. We found 7 peptides with significant antioxidant activity that have been reported in previous studies. Especially, LPH and LLPH were also found in corn peptides hydrolyzed by Alcalase [36]. Furthermore, there were 33 peptides, which still need to be identified and their antioxidant activity and physiological effects need to be validated after synthesizing.

4. Conclusion

In this study, an orthogonal design L_9 (3^4) was applied to ultrasonic-assisted enzymolysis in the preparation of corn antioxidant hydrolysates. Under the optimal ultrasound pretreatment, the radical (DPPH, $^{\bullet}OH$) scavenging capacities were significantly increased. The increase in DH, CR, short-chain peptides with molecular weight 200–3000 Da, and the peptides containing hydrophobic AAs coincided with the improvement in the antioxidant activity of corn protein hydrolysates. Furthermore, the potential antioxidant peptides were purified by C18 semipreparative column and identified by UPLC-ESI-MS. For the identified 40 peptides, except the 7 peptides that have been reported before, further studies are

Table 5: Amino acid sequence of purified peptide (F_2, F_3, and F_{15}) identified using UPLC-ESI-MS.

Fraction number	Peptide sequence	Calculated mass	Probability of certainty	Activity
F_2	SGV	261.28	100	Antioxidant
	FNV	378.44	100	
	AL	202.25	100	
	MT	250.32	100	
	SPL	315.37	100	
	LAH	339.40	100	
	PEA	315.33	100	
	YPQ	406.45	100	
	LDV	345.40	100	
	ENN	375.34	100	
	EDL	375.38	100	
	EPDE	488.46	100	
	LPF	375.47	100	Antioxidant
F_3	LLPH	478.60	100	Antioxidant
	LLPF	478.60	100	Antioxidant
	FLPF	512.62	100	Antioxidant
	VGA	245.29	100	
	PAAQ	385.43	100	
	AAV	259.30	100	
	LGA	259.30	100	
	AH	226.24	100	
	AHL	339.39	100	Antioxidant
	LAH	339.40	100	
	RLQ	415.50	100	
F_{15}	LGV	287.36	100	Antioxidant
	SHL	355.39	100	
	SPGA	330.34	100	
	NGGGA	374.35	100	
	PSAQ	401.43	100	
	VGSP	358.40	100	
	TNLA	417.47	100	
	ANLT	417.47	100	
	SPSP	385.43	100	
	ALSP	386.45	100	
	MM	280.41	100	
	SF	252.27	100	
	SH	242.24	100	
	SHQ	370.37	100	
	HSQ	370.38	100	
	SPL	315.37	100	

needed to validate their antioxidant activity and physiological effects after synthesizing.

Additional Points

Practical Application. The ultrasound technology has been widely applied to solve traditional enzymatic hydrolysis problems. The results of this study suggest that the low-frequency ultrasonic pretreatment could significantly increase DH, CR, and radical ($DPPH^{\bullet}$, $^{\bullet}OH$) scavenging capacity of corn derived protein hydrolysates. The optimal ultrasonic pretreatment parameters were attained by orthogonal design. This study could be useful for producing antioxidant peptides for foods and pharmaceuticals industries.

Disclosure

This article does not contain any studies involving human or animal subjects.

Conflicts of Interest

There are no conflicts of interest to declare.

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