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Antioxidative Activities and Peptide Compositions of Corn Protein Hydrolysates Pretreated by Different Ultrasonic Methods

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Abstract Three different ultrasonic methods were compared based on the production of antioxidative peptides from corn protein. The corn protein was sonicated with probe ultrasound (PU), flat plate ultrasound with sweeping frequency (FPUSF), and flat plate ultrasound with fixed frequency (FPUFF) before hydrolysis by acalase. Degree of hydrolysis (DH), antioxidative activities and peptide compositions were determined. The hydrolysates derived from PU pretreatment yielded the highest DH. However, lower Fe^{2+}-chelating activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and hydroxyl (OH) radical scavenging activities were observed for the corn protein hydrolysates (CPH) under PU pretreatment. The percentage of peptides with molecular weight (MW) of 500–180 Da increased with the increasing of DH. While the order of percentage of MW 2000–500 Da relative to ultrasonic method was in accord with Fe^{2+}-chelating activity and OH radical scavenging activity. Amino acid analysis indicated that CPH under FPUSF and FPUFF pretreatments, with higher hydrophobic amino acid contents, had stronger antioxidative activities.

Keywords: *ultrasound, corn protein, antioxidative activity, peptide composition*

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1. Introduction

Free radicals and reactive oxygen species (ROS) can cause oxidative damage to proteins, lipids, enzymes, and DNA molecules [1]. Apart from the deterioration of food quality attributes, oxidative damage may lead to many degenerative diseases, such as cancer, Alzheimer's disease and cardiovascular disease by cell degeneration [2,3]. Removal of free radicals and ROS is probably an effective way in preventing deterioration of foods and some deleterious diseases [4,5,6]. Antioxidants are substances that can delay the oxidation process by scavenging free radicals [7,8]. Some synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been commonly used to delay discoloration and deterioration of food products. However, the uses of synthetic antioxidants are limited because of health hazards [9]. So there is increasing interest in finding safe and natural antioxidants contained in dietary plants, which could enhance the body's antioxidative defenses through preventing the oxidative damage [10,11,12].

Corn protein, an abundant and low-cost by-product of corn starch and oil production industry, is rich in essential amino acids. Traditionally, this protein has been used for animal feed and fertilizer. Therefore, it is desirable to find new uses for corn protein. Zhou et al. [13] reported that corn is a potential protein resource for preparation of antioxidative peptides. Antioxidative peptides are often inactive within the sequence of the parent protein and can be released by hydrolysis with enzymes. During hydrolysis, the proteins are broken down and converted into marketable and acceptable forms which can be widely used in food rather than as animal feed or fertilizer [14,15]. Usually, the activity of bioactive peptides was dependent on molecular weight (MW) distribution and amino acid composition of the peptide fractions [16,17].

Ultrasound has attracted more and more attention in food science and technology such as ultrasound-assisted extraction [18], nanoemulsion preparation [19], altering enzyme activities [20] and accelerating the enzymatic hydrolysis [21]. Recently, the interest of food technologists has turned to the use of different ultrasonic equipments in improving functional properties of food materials. The ultrasonic irradiation pattern includes probe-type and plate-type. The frequency of the ultrasound can mainly be classified into two fields: fixed and sweeping. In our previous report we indicated probe-type ultrasonic pretreatment is an effective way to increase the ACE-inhibitory activity of hydrolysates of DWGP [22]. Zhu et al. [15] reported that the frequency could influence the antioxidative activities of wheat gluten

hydrolysate. There's hardly any report on the comparison about the effects of different ultrasonic irradiation pretreatments on the release of antioxidative peptides from corn protein.

For this reason, the objective of this work was to evaluate the effects of different ultrasonic equipments on the antioxidative activities of corn protein hydrolysates (CPH). The probe ultrasound (PU), flat plate ultrasound with sweeping frequency (FPUSF) and flat plate ultrasound with fixed frequency (FPUFF) were compared. The antioxidative activities of the hydrolysates were characterized by Fe2+-chelating activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl (OH) radicals scavenging activities. The compositions of amino acid and peptide size in the hydrolysates were also investigated.

2. Materials and Methods

2.1. Materials

Corn protein (protein content, 58.3%) supplied by Pizhou Fenda Starch Co., Ltd. (Jiangsu, China), was milled and sieved through a 250 μm mesh. Alcalase was purchased from Novozymes Biotechnology Co. Ltd (Tianjin, China). 3-(2-pyridyl)-5,6- bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine) and DPPH were obtained from Sigma (St. Louis, MO, USA). The other chemicals were of analytical grade.

2.2. Pretreatment of Corn Protein Using Probe Ultrasound (PU)

Sample solutions were sonicated in an ultrasonic reactor (Shangjia Biotechnology Co., wuxi, China; Model GA92-II) (Figure 1A) with a probe of 2.0 cm in diameter operating in a pulsed mode (on-time 2 s and off-time 2 s). A digital wattmeter was connected to the reactor to measure the output power of the system. Ten gram of corn protein powder was dispersed in 200 mL of distilled water. The suspension was put into a 250 mL beaker, and the beaker was placed in a water bath at initial temperature of 25±2°C for 10 min. The probe was submerged to a depth of 2.0 cm in the solution and sonication was done at 600 W and 22 kHz for 20 min.

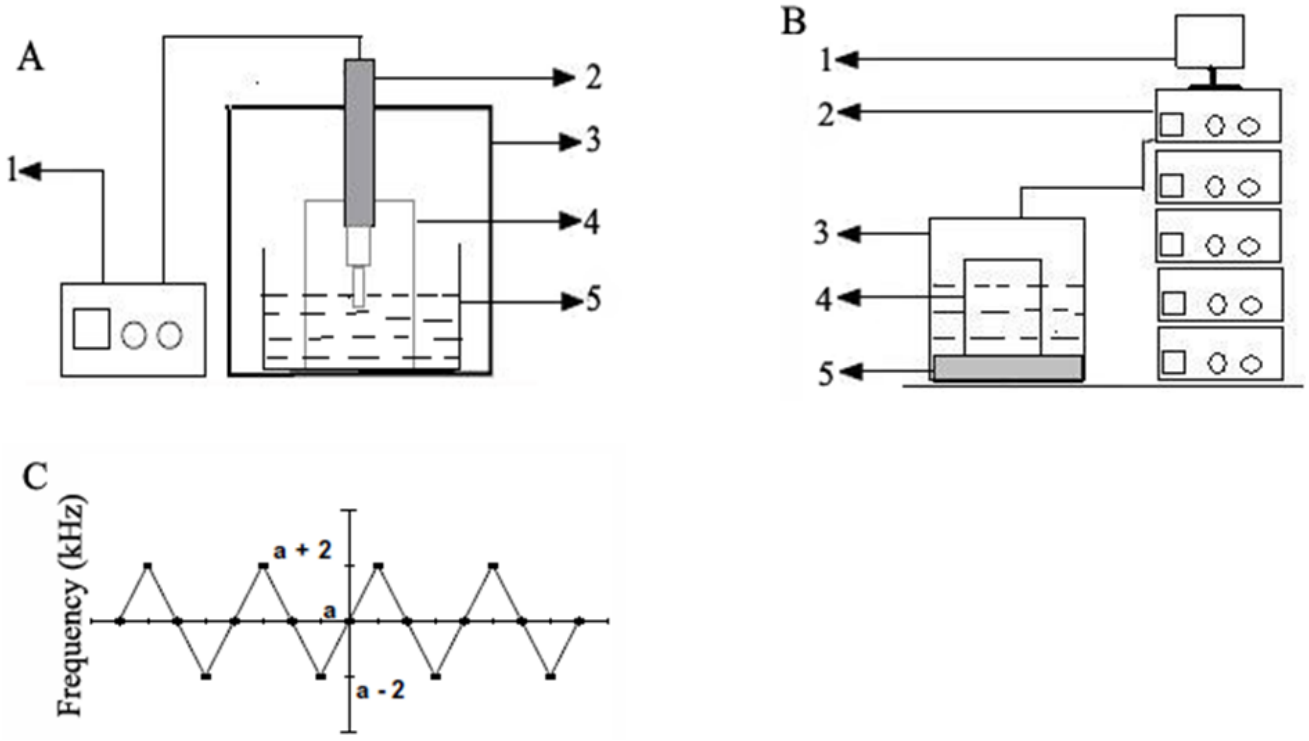

Figure 1. (A–C) (A) Schematic diagram of probe ultrasound pretreatment 1.Ultrasonic controller. 2. Probe ultrasound. 3. Acoustic chamber. 4. Sample pretreatment beaker. 5. Thermostatic water bath. (B) Schematic diagram of flat plate ultrasound pretreatment. 1. Computer. 2. Frequency controller. 3. Ultrasonic tank. 4. Sample pretreatment beaker. 5. Flat plate ultrasound. (C) Frequency variation curve of ultrasound for sweep model

2.3. Pretreatment of Corn Protein Using Flat Plate Ultrasound with Fixed Frequency (FPUFF)

A flat plate ultrasonic reactor, manufactured by Jiangsu University, was equipped with a ultrasonic bath as a power source for acoustic cavitation (Figure 1B). It has function modes of fixed frequency and sweeping frequency operation. The major units of the equipment were made up of a flat plate ultrasonic tank (35 cm×30 cm×50 cm) and a frequency controller. Ten gram of corn protein powder was dispersed in a 250 mL beaker with 200 mL of distilled water. The beaker was placed in an ultrasonic tank with 10 L of water at initial temperature of 25±2°C. Sample was treated at 22 kHz and 600 W for 20 min. The pulsed on-time and off-time were 2 s and 2 s, respectively.

2.4. Pretreatment of Corn Protein Using Flat Plate Ultrasound with Sweeping Frequency (FPUSF)

The equipment for this experiment was the same as the one for the FPUFF. Differing from fixed frequency,

sweeping frequency was used. The frequency is periodically increased from the lower frequency f-Δf to the upper frequency f+Δf, then decreased from f+Δf to f-Δf with the same linear speed in the form of an isosceles triangle, being expressed as f±Δf (Figure 1C). An increasing period plus a decreasing period is defined as the cycle time of the sweep frequency. In this experiment, the sweeping frequency was 22±2 kHz and the cycle time was 100 ms. The sample preparation and pretreatment were the same as the method in "Pretreatment of corn protein using flat plate ultrasound with fixed frequency (FPUFF)".

2.5. Preparation of Enzymatic Hydrolysates

After sonication, the treated protein solution was placed in 1000 mL beaker equipped with a stirrer and a pH electrode. After incubating at 50°C for 10 min, pH of the sample was adjusted to 8.0 with 1 M NaOH and the hydrolysis reaction was started by the addition of alcalase (E/S, 2000 U/g). The pH of mixture was kept constant during hydrolysis by addition of 0.5 M NaOH. The enzymatic hydrolysis was stopped by boiling for 10 min. The hydrolysate of corn protein without ultrasonic pretreatment was used as the control. Then the mixture of protein and enzyme was centrifuged at 5000 g for 15 min at 4°C. The supernatant was frozen, lyophilized and stored in a dryer before further analysis.

2.6. Assessment of Degree of Hydrolysis (DH)

DH was calculated from the amount of alkaline (NaOH) added to keep the pH constant during the hydrolysis as given below [23]:

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

Where B is the NaOH consumed (mL) to keep the pH, N_b is the concentration of the NaOH (mol/L), α is the average degree of dissociation of the α-NH_2 groups in protein substrate, M_p is the mass of hydrolysed protein (g), h_{tot} is the total number of peptide bonds in corn protein and was assumed to be 7.35 mmol/g determined by the amino acid composition.

2.7. Determination of Fe^{2+}-chelating Activity

The chelating activity on Fe^{2+} was determined by the method of Zhu *et al*. [6]. Four milliliter of sample at different concentrations was mixed with 0.1 mL of 1 mM $FeSO_4$ and 0.2 mL of 5 mM Ferrozine, and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the solution was then measured at 562 nm using a UV–visible spectrophotometer (Varian Inc., Palo Alto, USA; Model Cary 100). The control was prepared in the same manner except that distilled water was used instead of the sample. The percentage of inhibition was calculated according to the following equation:

$$Fe^{2+} - \text{Chelating activity} = \left(1 - A_1 / A_0\right) \times 100 \quad (2)$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample against blank solution containing the sample without Ferrozine.

2.8. DPPH Radical Scavenging Activity Assay

DPPH radical scavenging activity was measured according to the method of Huang and Mau [24] with a slight modification. Samples were dissolved in distilled water to obtain different concentrations. Then 2.0 mL of sample was mixed with 2.0 mL of 0.15 mM DPPH that was dissolved in 95% ethanol. After incubating at 25°C in the dark for 30 min, the absorbance of solutions was measured at 517 nm. For the control, distilled water was used instead of sample. DPPH radical scavenging activity was calculated as:

$$\text{Scavenging activity of DPPH } (\%) = \left(1 - A_1 / A_0\right) \times 100 \quad (3)$$

Where A_0 was the absorbance of the control and A_1 was the absorbance of sample against blank solution containing the sample without DPPH. EC_{50} value (mg/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

2.9. Hydroxyl Radical Scavenging Ability Assay

Hydroxyl (OH) radical scavenging activity was determined based on the method of Smirnoff and Cumbes [25] with some modifications. 2 mL of sample at various concentrations was mixed with 2 mL of 3 mM $FeSO_4$, and then the reaction was started by the addition of 2 mL of 3 mM H_2O_2. After incubating at 25°C for 10 min, 2 mL of 6 mM salicylic acid was added. After 15 min, the mixture was centrifuged at 8000 g for 5 min at 4°C. The absorbance of the supernatant was detected at 510 nm. In the control, sample was substituted with distilled water. Hydroxyl radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity of OH } (\%) = \left(1 - A_1 / A_0\right) \times 100 \quad (4)$$

Where A_0 was the absorbance of the control and A_1 was the absorbance of sample against blank solution containing the sample without salicylic acid.

2.10. Determination of Molecular Weight Distribution

The molecular weight distribution was determined using a Waters 600 Series HPLC (Water 600, Milford, MA, USA), which is equipped with a TSK-gel 2000-SWXL (Tosoh, Japan) column and a UV detector (Waters 2487, Milford, MA, USA) working at 220 nm. Elution was achieved at flow rate of 0.5 mL/min with 45% (v/v) acetonitrile aqueous solution containing 0.1% (v/v) trifluoroacetic acid. A molecular weight calibration curve was prepared by the following markers: cytochrome C (MW 12.5 kDa), bacitracin (MW 1450 Da), Gly-Gly-Tyr-Arg (MW 451 Da) and Gly-Gly-Gly (MW 189 Da).

2.11. Amino Acid Analysis

Hydrolysate of corn protein was hydrolyzed in 6 M HCl at 110°C for 24 h. Amino acid analysis of the hydrolysate was performed with a S-433D amino acid analyzer (Sykam Co., Germany) according to standard methods. The amino acid composition of the peptides was expressed

as percentage content of the total amino acids in the hydrolysate.

2.12. Statistical Analysis

The experimental results obtained were expressed as means ± SD of three replicates. Statistical analysis was carried out using the software SPSS (Version 19.0, SPSS Inc. Chicago, USA). Analysis of variance (ANOVA), followed by Duncan's multiple-range test, was performed to compare the significance level of $p < 0.05$.

3. Results and Discussion

3.1. Effects of Ultrasonic Methods on DH of Corn Protein

Figure 2 shows the relationship between DH and hydrolysis time of corn protein pretreated by different ultrasonic methods. It can be seen that all of the DH values of corn protein pretreated by ultrasound were higher than that of the control (without ultrasonic pretreatment) ($p < 0.05$). The maximum DH value of 9.17% was achieved at hydrolysis time of 25 min under PU, which was increased by 30.76% over the control. The hydrolysates of corn protein pretreated by PU possessed the highest DH values at any investigated time ($p<0.05$). This means that PU has the biggest energy in loosening the protein tissue and facilitating breakage of peptide bonds in corn protein during enzymatic hydrolysis [26]. Duncan's multiple-range test was used for comparison among effects of different ultrasonic methods. Results showed that there were remarkable differences in DH values among the control, PU, FPUSF and FPUFF at hydrolysis times of 10 min, 15 min, 20 min and 25 min. With respect to hydrolysis time of 5 min, no difference was detected between FPUSF and FPUFF.

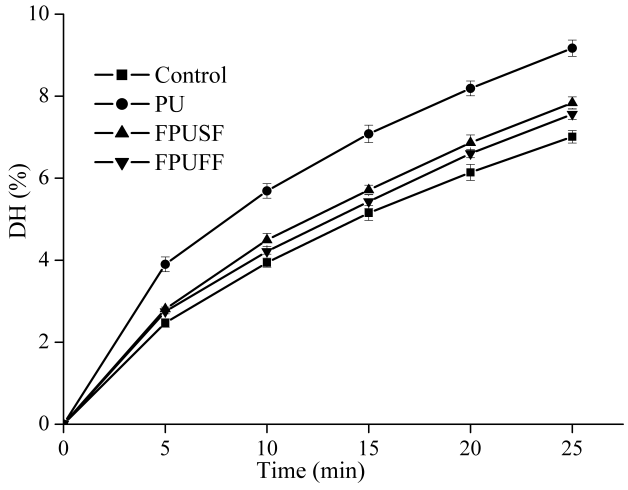

Figure 2. Enzymatic hydrolysis of corn protein under different ultrasonic methods

3.2. Fe^{2+}-chelating Activity

The transition-state metals, such as Fe^{2+} and Cu^{2+}, are catalysts to induce per-oxidation of polyunsaturated fatty acids [15, 27]. Therefore, chelation of transition metal ions by antioxidative peptide would retard the oxidation reaction. Different concentrations of CPH were used to determine the Fe^{2+}-chelating properties. As shown in Figure 3, their chelating activities increased with increasing concentrations used in the test ($p < 0.05$). But there was no linear correlation between DH and chelating activity. The hydrolysate of corn protein pretreated by FPUFF exhibited the highest chelating activity (75.18%) at concentration of 0.25 mg/mL, which was increased by 7.07% over the control ($p < 0.05$). Results of Duncan's multiple-range test showed that there was a significant difference between control and FPUSF, while there was no difference between the effects of FPUSF and FPUFF. The changes in the Fe^{2+}-chelating activity of corn protein showed that higher DH does not guarantee higher Fe^{2+}-chelating activity. A very high degree of hydrolysis may have enormously negative effects on the functional properties [28].

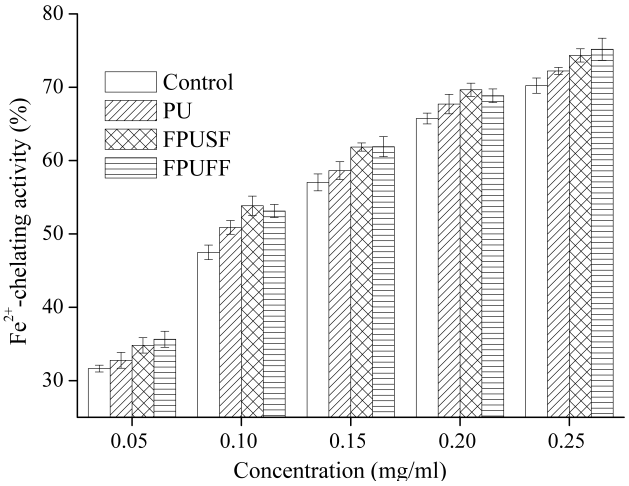

Figure 3. Fe^{2+}-chelating activity of corn protein hydrolysates under different ultrasonic methods

3.3. DPPH Radical Scavenging Activity

DPPH is a stable free radical, which has been widely used to investigate the antioxidative activity of some natural compounds [29]. In this study, the DPPH radical scavenging activity of each kind of hydrolysate showed a concentration dependent increase of antioxidative activity for concentrations up to 1.0 mg/mL ($p < 0.05$). The EC_{50} was determined using graphical extrapolation by plotting DPPH radical scavenging activity as a function of different hydrolysate concentrations. The EC_{50} values of corn protein hydrolysates with different ultrasonic pretreatment were shown in Figure 4. Hydrolysate of corn protein pretreated by PU with a higher EC_{50} had poorer DPPH radical scavenging activity, which was in accordance with the results of yellow stripe trevally reported by Klompong *et al*. [30]. PU is a kind of high energy ultrasound which will cause the destruction of pressurized micro-bubbles resulting in intense local heating and high pressure. The decline of Fe^{2+}-chelating activity and DPPH radical scavenging activity under PU may be due to the different amino acid composition or higher degradation of bioactive peptide caused by the irradiation as also observed by Kristinsson and Rasco [28]. There were remarkable differences in EC_{50} values among the control, PU and FPUSF, while no difference was observed between FPUSF and FPUFF as determined by Duncan's multiple-range test.

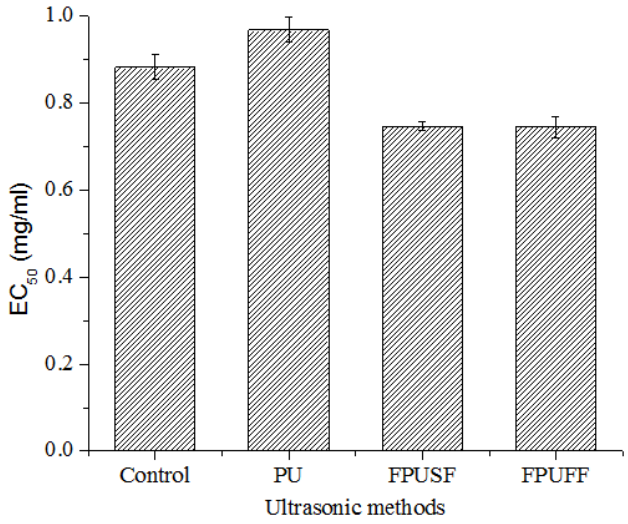

Figure 4. DPPH radical scavenging activity of corn protein hydrolysates under different ultrasonic methods

3.4. Hydroxyl Radical Scavenging Activity

The results in Figure 5 showed that hydroxyl radical scavenging activity of each kind of hydrolysate exhibited a significant increase with increasing amount ($p < 0.05$). At the highest concentration of 5 mg/mL, the hydroxyl radical scavenging activities of FPUSF and FPUFF were increased by 10.95%, and 7.52% over the control respectively ($p < 0.05$). There was no remarkable difference between FPUSF and FPUFF. This result illustrates that FPUSF and FPUFF pretreatments are both effective to increase the hydroxyl radical scavenging activity of CPH, which was in accordance with the results of Fe^{2+}-chelating activity and DPPH radical scavenging activity. However, the significant difference in hydroxyl radical scavenging activity between PU and control was not detected. Our results also showed that the hydrolysate of corn protein could not display total reducing power (data not shown). It can be concluded a higher Fe^{2+}-chelating activity could not guarantee any other kinds of antioxidative activities.

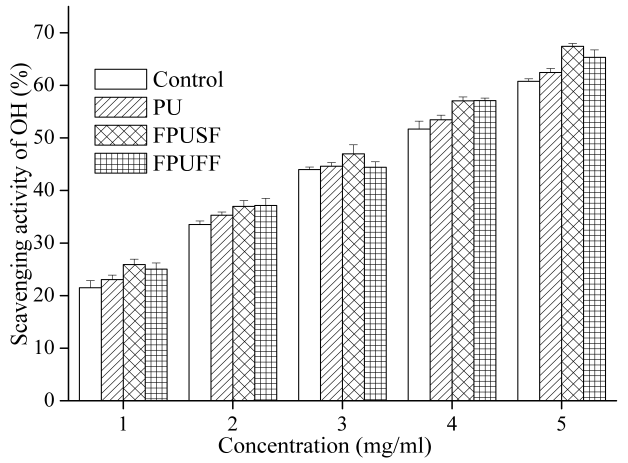

Figure 5. Hydroxyl radical scavenging activity of corn protein hydrolysates under different ultrasonic methods

3.5. Molecular Weight Distribution

In recent years, many reports had found that molecular weight of the peptides from the protein hydrolysate was one of the most important factors concerning desired functional properties [16,31]. The molecular weight distribution determined by HPLC was shown in Table 1. Relative percentage of each peptide was determined by its percentage area in HPLC profile. It was found that the percentage content of peptides with MW 500–180 Da increased with the increasing of DH, while those over MW 5000 Da decreased with the increasing of DH. The order of percentage content of MW 1000–500 Da relative to ultrasonic method was in accord with that of DPPH radical scavenging activity. While the order of percentage content of MW 2000–500 Da relative to ultrasound method was in accord with Fe^{2+}-chelating activity and OH radical scavenging activity. It can be concluded that peptides with MW of 2000–500 Da showed the strongest antioxidative activities. All these investigations confirmed the importance of MW in the antioxidative activities of peptides.

Table 1. Relative percent of the peptides in HPLC in total area (%)

Molecular weight	Control	PU	FPUSF	FPUFF
>5000	2.54	2.29	2.33	2.41
5000-3000	2.21	2.01	1.97	2.09
3000-2000	1.98	1.96	1.77	1.96
2000-1000	6.66	6.88	6.43	6.75
1000-500	15.07	14.85	15.47	15.46
500-180	54.23	56.04	54.93	54.75
<180	17.3	15.97	17.1	16.58

3.6. Composition of Amino Acid

Table 2. Amino acid composition of enzymatic hydrolysates produced from untreated (control) and ultrasonic pretreatments of corn protein (%)

Amino acid	Control	PU	FPUSF	FPUFF
Asp	5.2	4.8	5.1	5.2
Thr	3.3	3.0	3.1	3.1
Ser	5.1	4.8	5.0	5.1
Glu	17.7	16.9	17.7	18.1
Gly	2.9	2.7	2.7	2.8
Ala	8.0	7.4	7.8	7.8
Cys	5.5	5.6	5.1	4.2
Val	4.1	3.9	3.9	4.0
Met	2.5	5.2	4.6	4.8
Ile	3.1	3.6	3.7	3.6
Leu	14.6	13.9	14.8	14.5
Tyr	4.9	4.5	4.7	4.7
Phe	5.5	5.0	5.4	5.2
His	2.9	4.2	3.6	3.5
Lys	3.6	3.7	3.0	3.2
Arg	3.0	3.5	2.3	2.4
Pro	7.9	7.2	7.6	7.9
Hydrophobic amino acid[a]	45.8	46.3	47.8	47.6

[a] Hydrophobic amino acid: Ala, Val, Met, Leu, Ile, Phe and Pro.

Usually, ultrasound bio-processing could lead to the opening up of substrate surface to the action of enzyme, resulting in the different peptide profiles and amino acid compositions of the hydrolysates from the same protein

[21]. In order to determine the possible effect of the amino acid profile on antioxidant activities, the hydrolysates obtained were subjected to amino acid composition analysis. The amino acid compositions of the CPH pretreated by different ultrasonic methods were listed in Table 2. There was no tryptophan detected for all samples because of being decomposed by HCl during hydrolysis. In addition, all the hydrolysates were rich in glutamic acid/glutamine and leu. Hydrolysates of PU, FPUSF and FPUFF have more methionine than the control. Furthermore, the hydrophobic amino acid contents under FPUSF and FPUFF were higher (47.8% and 47.6%) than those under PU and control (46.3% and 45.8%). Therefore, in our results, higher hydrophobicity may result in the higher antioxidative activities of hydrolysates under FPUSF and FPUSF, which agreed closely with the result of Amadou *et al* [32].

4. Conclusion

In this investigation, the effect of three different ultrasonic methods on the DH values and antioxidative activities of CPH were studied. The results showed that all the ultrasonic methods could improve the DH of corn protein. When corn protein was pretreated by PU at 600 W for 20 min, DH was increased by 30.76% over the control at hydrolysis time of 25 min ($p < 0.05$). Hydrolysates under FPUSF pretreatment had the highest OH radical and DPPH radical scavenging activities, and also the higher Fe^{2+}-chelating activity compared with those under PU and control. There were no differences between FPUSF and FPUFF ($p > 0.05$). The percentage content of peptides with MW 2000–500 Da was found to be strongly correlated with their antioxidative activities. Under the same ultrasonic power of 600 W and ultrasonic time of 20 min, FPUSF and FPUFF are more effective in improving the antioxidative activities of CPH. In view of the demand for natural functional foods, and the non-polluting nature of ultrasound, the CPH obtained under FPUSF and FPUFF could be used in food systems as natural additives possessing antioxidative properties.

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