Hindawi
Journal of Food Biochemistry
Volume 2023, Article ID 4556548, 12 pages
https://doi.org/10.1155/2023/4556548

Research Article

Potential Antioxidant Activity of Novel Antioxidant Peptides from Protein Hydrolysate of Peony Seed Dreg in Chemical and H_2O_2-Induced RAW264.7 Cells

Cuiling Wu,[1] Chenxia Ren,[2] Lianyun Zhang,[3] Yingda Song,[4,5] and Huifang Gao[3]

[1]*Department of Biochemistry, Changzhi Medical College, Changzhi 046000, China*
[2]*Central Laboratory, Changzhi Medical College, Changzhi 046000, China*
[3]*First Clinical College, Changzhi Medical College, Changzhi 046000, China*
[4]*Fifth Clinical College, Shanxi Medical University, Taiyuan 030000, China*
[5]*Department of Thoracic Surgery, Shanxi Provincial People's Hospital, Taiyuan 030000, China*

Correspondence should be addressed to Cuiling Wu; clw928@163.com

Received 6 April 2023; Revised 23 September 2023; Accepted 25 September 2023; Published 16 October 2023

Academic Editor: Kandi Sridhar

Copyright © 2023 Cuiling Wu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peony seed dreg protein (PSDP) was hydrolyzed by proteases secreted by *Pseudoalteromonas* sp. CSN423. This hydrolysate exhibited excellent antioxidant capability after passing through a 3 kDa ultrafiltration membrane. The antioxidant peptides further purified by size-exclusion chromatography scavenged 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) ($73.0 \pm 1.46\%$) and hydroxyl radicals (•OH) ($81.17 \pm 3.69\%$), protected DNA from oxidative injury, and displayed an oxygen radical absorbance capacity (ORAC) of 4.04 ± 1.11 μmol TE/mg. Furthermore, the antioxidant peptide inhibited apoptosis by inhibiting the production of reactive oxygen species (ROS) and the decrease of mitochondrial membrane potential in H_2O_2-induced RAW264.7 cells. We identified four major peptides such as YFPF, ECCASLAPL, YVSLK, and YFEM by using mass spectrometry. YFPF showed a potent •OH scavenging capability of $75.30 \pm 1.08\%$. ECCASLAPL exhibited higher DPPH• scavenging capability ($87.20 \pm 1.03\%$) than glutathione (GSH) ($49.50 \pm 1.50\%$). Moreover, the peptide YVSLK exhibited the highest ORAC of 2.52 ± 1.20 μmol TE/mg of the identified peptides. This work highlights that the peptides from PSDP might have potential uses in cosmetics, functional foods, and drug development.

1. Introduction

Peroxyl radicals (ROO•$^-$), •OH, hydrogen peroxide (H_2O_2), and superoxide anion (O_2•$^-$) are typical ROS that play vital roles in the body, such as participating in intracellular signal transduction, energy supply, and anti-inflammatory functions [1]. Once the accumulation of ROS exceeds the capacity of the cellular free radical scavenging system, it leads to cellular oxidative stress and causes oxidative damage to various cellular components such as membrane lipids, proteins, and DNA [2], increasing the incidence of different diseases, including immune dysfunction [3], cancer [4], diabetes mellitus [5], neurodegenerative diseases [6, 7], and cardiovascular diseases [8, 9]. Additionally, ROS-mediated lipid peroxidation contributes to food deterioration, unacceptable tastes, and the generation of potentially toxic products [10]. Based on this, mitigating the ROS-induced oxidative damage via antioxidants is an effective treatment for free radical-related diseases, antiaging, and delaying food spoilage [11]. Instead of synthetic antioxidants with their potential health hazards, natural antioxidants have received widespread attention [12]. Antioxidant peptides show great advantages of low molecular weight, poor immunogenicity, excellent stability, and high absorption efficiency [13, 14]. Antioxidant peptides are encrypted sequences in the amino acid sequence of precursor proteins, usually inactive, and can be released after hydrolysis by proteases. It was reported that antioxidant peptides can slow oxidative damage by scavenging or converting free radicals into stable or less active forms [15].

Peony (*Paeonia suffruticosa Andr.*) is a multifunctional traditional plant with ornamental, edible, and medicinal

purposes [16], which has become an important cash crop in China [17]. Many studies have shown that the application promising of peony seed oil as function-rich edible oil is very extensive [18]; however, the protein-rich cold-pressed peony seed dreg, a byproduct of oil processing, was only been used as organic fertilizer, animal fodder, or discarded as waste due to storage inconvenience [19, 20]. To explore the potential of peony seed dreg as a source of novel antioxidant peptides, we prepared antioxidant peptides from PSDP hydrolyzed by *Pseudoalteromonas* CSN423 extracellular proteases and characterized the antioxidant properties of the peptides via chemical-based antioxidant analysis and protective effect on H_2O_2-induced RAW264.7 cells.

2. Materials and Methods

2.1. Materials. Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). Sephadex® G-25 was purchased from GE Healthcare Co. (Uppsala, Sweden). AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride), DPPH• (2, 2-diphenyl-1-picrylhydrazyl), Trolox (6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid), and fluorescein sodium salt were obtained from Sigma-Aldrich, Ltd. (St. Louis, MO, USA). 2′-7′-Dichlorofluorescin diacetate (DCFH-DA) was provided by Beyotime Biotech (Shanghai, China). Cell Counting Kit-8 (CCK-8) was provided by Dojindo (Kumamoto, Japan). Peony seed dreg was obtained from the Zhihua Peony Biotechnology Company (Shanxi, China).

2.2. Bacterial Culture. Pseudoalteromonas sp. CSN423 isolated from the Bohai Sea was inoculated into a medium comprising 0.5 g tryptone, 0.001 g $Fe_2\ (PO_4)_3$, 0.1 g yeast extract, and artificial seawater 100 ml (pH 7.5) and incubated at 18°C and shaken overnight at 200 rpm.

2.3. Protease Production by Pseudoalteromonas sp. CSN423. Pseudoalteromonas sp. CSN423 was cultured in a fermentation medium [21]. The fermentation supernatant was obtained by centrifugation (8000 × g, 4°C, 10 min) after 96 h of incubation and subsequently precipitated with 60% $(NH_4)_2SO_4$ at 4°C. The precipitate was redissolved and dialysed against Tris-HCl (20 mM, pH 8.8), then loaded onto a 5 ml HiTrap™ DEAE FF column (Cytiva Life Sciences, USA), and eluted with a linear gradient of 1 M NaCl from 0 to 100% concentration in 20 mM Tris-HCl (pH 8.8). Active fractions were collected and loaded onto a Superdex 75 column (Cytiva Life Sciences, USA) and equilibrated with 20 mM Tris-HCl (pH 8.8). The protease activities were detected using the Folin phenol method [21]. Total protein content was detected using the BCA method [22].

2.4. Extraction of Peony Seed Dreg Protein (PSDP). PSDP was prepared from cold-pressed peony seed dreg by the traditional alkali dissolution and acid precipitation method [23]. Defatted peony seed dreg was cracked and passed through an 80 mesh screen to obtain defatted peony seed dreg flour. The defatted peony seed dreg flour was dissolved in distilled water (1 : 20, *w/v*), and the pH was adjusted to 8.5. The slurry of peony seed dreg was stirred gently at 40°C for 4 hours, and the supernatant was recovered by centrifugation (8000 × g, 10 min, 4°C). The supernatant was adjusted to pH 3.6 with 1M HCl and then centrifuged at 8000 × g for 10 minutes at 4°C to collect the precipitate. The precipitate was redissolved in distilled water, and the pH was adjusted to 7.0. Finally, the solution was lyophilized.

2.5. Preparation of PSDP Hydrolytic Peptides. Hydrolysis of PSDP was performed with a substrate/enzyme ratio of 9 : 1 (*v/v*) at 40°C for 30, 60, 90, 120, 150, and 180 min. After digestion, the enzyme was inactivated at 90°C for 10 min. The indene triketone colorimetric method was used to evaluate the free amino acid content at different treatment times [24]. The hydrolysates were centrifuged at 12, 000 × g for 5 min. After that, the supernatants were recovered and filtered through a 3 kDa ultrafiltration membrane.

2.6. Amino Acid Analysis. The ultrafiltrated fraction with higher (>3 kDa) and lower molecular (<3 kDa) weights was designated as UF-1 and UF-2, respectively. UF-2 was further analyzed for its amino acid contents [25]. The amino acid analysis was evaluated after hydrolysis of UF-2 with 6 M HCl at 110°C for 22 h, and its amino acid profile was detected using an HPLC system. The methionine and cysteine contents were analyzed postperformic acid oxidation.

2.7. Purification of the Antioxidant Peptides. The fraction UF-2 (500 μg/ml, 1 ml) was loaded onto a Sephadex G-25 column (1.6 × 60 cm) and then washed with distilled water at 0.75 ml/min. The absorbance of the eluates at 220 nm was monitored, and the antioxidant activities were estimated.

2.8. Chemical-Based Analysis of Antioxidant Capability

2.8.1. •OH Scavenging Capability. The •OH scavenging capability was evaluated as previously described [17]. The $FeSO_4$•7 H_2O solution (60 μl, 6 mM), peptide solution (200 μl), and H_2O_2 (60 μl, 6 mM) were mixed to form a homogenous mixture. After that, 60 μl of salicylic acid solution (6 mM) was added to form a colored reaction product, followed by the addition of 420 μl of distilled water. The absorbance at 510 nm was detected after incubating at 37°C for 30 min. 500 μg/ml GSH was used as a standard reference. The scavenging capability of •OH was calculated using the following equation:

$$\bullet\text{OH scavenging capability (\%)} = \left[\frac{1-(A_s-B)}{A_b}\right] \times 100, \quad (1)$$

where A_s is the absorbance of the sample group, B is the reaction system without salicylic acid, and A_b is the absorbance of the blank group (the sample was replaced with distilled water).

2.8.2. DPPH• Scavenging Capability. The DPPH• scavenging capability was detected as previously described with a slight modification [26]. The reaction system comprised 100 μl DPPH• solution (100 μM) and 20 μl sample solution. After incubating the mixture at room temperature for 60 min in the dark, the absorbance at 517 nm was selected for the measurement of the scavenging capability. 500 μg/ml GSH was used as a reference standard. The DPPH• scavenging activity was calculated by the following equation:

$$\text{DPPH} \bullet \text{scavenging capability (\%)} = \left[\frac{(A_b - A_s)}{A_b}\right] \times 100, \quad (2)$$

where A_b is the absorbance of the blank group (the sample was replaced with distilled water) and A_s is the absorbance of the sample.

2.8.3. DNA Damage Protective Effect. The protective effect on ROS-induced DNA damage was carried out as previously described [27]. The reaction mixture contained 8 μl of 0.1 μg/μl pUC18 plasmid DNA, 2 μl of 2 mM $FeSO_4$ solution, 8 μl of 0.2 μg/μl sample solution, and 2 μL of 0.1 mM H_2O_2. The reaction mixtures were incubated at 37°C for 8 min and electrophoresed on 1% agarose gel.

2.8.4. ORAC Analysis. The reaction was prepared in 75 mM phosphate buffer (PBS, pH 7.4) as previously described [28]. A fluorescein sodium salt solution of 100 μl (96 nM) was mixed gently with 20 μl of sample and incubated at 37°C for 15 min. Thereafter, prewarmed AAPH (30 μl, 120 mM) was used as a free radical initiator. Fluorescence was recorded with excitation and emission wavelengths of 485 nm and 538 nm, respectively. PBS was used as a blank, and Trolox was used as a positive reference. The ORAC value was expressed as trolox equivalent (μmol TE/mg sample).

2.9. Cytotoxicity Assay on RAW264.7 Cells

2.9.1. The Effect of the Peptide Fraction F7 on Proliferation of RAW264.7 Cells. RAW264.7 cells (1.0×10^5 cfu/ml) were cultured on DMEM with 10% (*v/v*) FBS and treated with different F7 concentrations for 24 h. The cytotoxicity of F7 was analyzed by the CCK-8 kit. After all treatments, the supernatant was removed, 10 μl of CCK-8 diluted in 100 μl DMEM was added to it, and the absorbance at 450 nm was measured after 3 h incubation. The sample replaced with 0.01 M PBS was used as a blank, and the percentage of cell viability was calculated.

2.9.2. The Effect of H_2O_2 on Proliferation of RAW264.7 Cells. RAW264.7 cells were incubated with H_2O_2 (0, 100, 300, 500, and 700 μM) for 4 h, 8 h, and 12 h, respectively. Cell viability was evaluated by the CCK-8 kit. The H_2O_2 replaced with 0.01 M PBS was used as a blank, and the percentage of cell viability was calculated.

2.10. Detection of ROS Level. RAW 264.7 cells were incubated with the peptide fraction F7 for 24 h and then exposed to H_2O_2 (500 μM) for 8 h. After all treatments, the supernatant was removed and 100 μl of 10 μM DCFH-DA (prepared with DMEM) was added and incubated for 20 min. Excess DCFH-DA was rinsed away by PBS (0.01 M). Images of the RAW 264.7 cells were taken under a fluorescence microscope.

2.11. Analysis of Mitochondrial Membrane Potential ($\Delta\Psi m$). RAW264.7 cells (1.0×10^5 cfu/ml) were cultured in DMEM with 10% (*v/v*) FBS and treated with the peptide fraction F7 (50 μg/ml) for 24 h. H_2O_2 (500 μM) was then added, and the incubation continued for 8 h. The lipophilic cation JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl-benzimidazol-carbocyanine iodide) was used to assess the $\Delta\Psi$m. Briefly, after rinsing with PBS (0.01 M), the cells were stained with JC-1 in a serum-free medium at 37°C for 20 min. Subsequently, the supernatant was removed and cells were rinsed and resuspended into the dilution buffer for flow cytometry analysis.

2.12. Detection of Cell Apoptosis. Annexin V-FITC/PI apoptosis detection kit was used to assess the apoptosis in RAW 264.7 cells [29]. Briefly, cells (1.0×10^5 cfu/ml) were cultured in DMEM with 10% (*v/v*) FBS and treated with the peptide fraction F7 (50 μg/ml) for 24 h. H_2O_2 (500 μM) was then added, and the incubation continued for 8 h. Cells were subsequently harvested and rinsed twice with PBS (0.01 M). Annexin V-FITC and PI double staining were performed according to the manufacturer's protocol. Cell apoptosis was assessed by flow cytometric analysis.

2.13. Identification of the Antioxidant Fraction and the Synthesis of Peptides. The nano-RPLC-MS/MS was carried out using an Easy-nLC 1200 System coupled to a Q Exactive Plus System fitted with a nanospray (Thermo Scientific, Rockford, USA). Samples were separated with a column (Acclaim PepMap RSLC C18, 2 μm, 100 Å, 50 μm i.d. × 150 mm, NanoViper) using two solvent systems: buffer A (0.1% formic acid in deionized water) and buffer B (0.1% formic acid in acetonitrile). The gradient elution was conducted as follows: buffer B from 2% to 20% in 90 min, buffer B from 20% to 30% in 15 min, buffer B from 30% to 90% in 5 min, and 90% buffer B for 10 min. The raw MS/MS data were subjected to de novo sequencing using PEAKS software (version X, Bioinformatics Solutions Inc., Waterloo, Canada) [17].

Based on the above analysis, selected peptides were synthesized by the solid-phase peptide synthesis procedure by Sangon Biotech Co., Ltd (Shanghai, China). The purity of the synthesized peptides was over 95%.

2.14. Statistical Analysis. SPSS 26 software was used to organize and analyze the data. The quantitative data conforming to normal distribution were represented by mean ± standard deviation ($\bar{x} \pm s$). Comparison between the two groups was

statistically analyzed by *t*-test. Comparison between multiple groups was statistically analyzed by ANOVA, and multiple experimental groups were compared with one control group using Dunnett's *t-test.* $p < 0.05$ indicated statistically significant.

3. Results and Discussion

3.1. Preparation of Proteases. The protease E423 secreted by *Pseudoalteromonas* sp. CSN423 was isolated via anion exchange chromatography and gel filtration after precipitation with 60% ammonium sulfate. As shown in Table 1, the specific protease activity of the crude enzyme was 10.5 U/mg and it was up to 595.2 U/mg after the purification treatment. By comparison, Wang et al. [17] used commercial alkaline protease (20 U/mg) to hydrolyze *Paeonia ostii* seed meal protein to obtain five antioxidant peptides (FSAP, PVETVR, QEPLLR, EAAY, and VLRPPLS). The antioxidant peptide SMRKPPG was obtained by hydrolysis of *Paeonia* seed protein with alcalase (62 U/mg) [20]. The cleavage site of proteases from different bacteria varies widely [30]. Therefore, the sequence of antioxidant peptides prepared by E423 may also be different.

3.2. The Hydrolysis of PSDP. The hydrolysis of PSDP was effective and reproducible. As shown in Figure 1(a), PSDP can be efficiently digested by proteases produced by *Pseudoalteromonas* sp. CSN423 and the rate of hydrolysis reached close to a steady state after 120 minutes. The DPPH• scavenging capacity of UF-2 increased with the hydrolysis time. UF-1 showed a lower DPPH• scavenging activity than UF-2 (Figure 1(b)). It is suggested that the disruption of protein's native structure by enzymatic hydrolysis might lead to unfolding, thereby enhancing the accessibility of their functional groups to ROS. However, over-hydrolysis can result in the conversion of peptides to amino acids or the destruction of their active groups, and the product activity may also be decreased. Therefore, the degree of enzymatic hydrolysis must be strictly controlled [31, 32].

3.3. Amino Acid Analysis of UF-2. According to Table 2, the amino acid composition of UF-2 included seventeen kinds of amino acids. Hydrophobic amino acids, including leucine, alanine, glycine, valine, methionine, proline, phenylalanine, and isoleucine, accounted for 39.8% of the total amino acids. The aromatic amino acids tyrosine and phenylalanine accounted for 9.44%. Hydrophobic amino acids such as Ile, Val, and Leu could enhance the solubility of peptides in lipids, thus enhancing their antioxidant activity. Aromatic amino acids, such as Phe and Tyr, can act as proton donors to maintain ROS stability during the free radical scavenging process [33, 34].

3.4. The Antioxidant Peptide Purification. Figure 2 shows the column chromatographic profile of the UF-2, where eight major components were eluted. All the fractions displayed •OH and DPPH• scavenging activities with the highest activities observed in F7 ($81.17 \pm 3.69\%$ and $73.0 \pm 1.46\%$, respectively) (Figure 3). It is suggested that the fraction F7 may be the major contributor to the antioxidant activity of UF-2.

3.5. Detection of Antioxidant Activities

3.5.1. •OH Scavenging Activity. Hydroxyl radical is the most powerful oxidant that causes chronic diseases [35]. Scavenging hydroxyl free radicals is an effective method for organisms to defend against various diseases. The fraction F7 exhibited the highest •OH scavenging activity of $81.17 \pm 3.69\%$, possibly due to the contribution of hydrogen atoms or blocking the generation of oxygen free radicals, as shown in Figure 3(a).

3.5.2. DPPH• Scavenging Ability. DPPH• exhibits a characteristic absorption at 517 nm. After exposure to free radical scavengers, the absorbance value of DPPH• decreased rapidly. We estimated the DPPH• scavenging ability of F7 to be $73.0 \pm 1.46\%$, which was significantly higher than other isolated fractions (Figure 3(b)). It is suggested that F7 contained more effective antioxidant peptides that could convert DPPH• into a stable form and terminate the free radical chain reaction.

3.5.3. Inhibition of DNA Oxidative Damage. Plasmid DNA exhibited different structures and electrophoretic mobility in agarose gel due to the varied degrees of damage. •OH can cause oxidative stress-induced DNA strand breaks. The formation of open-circle DNA indicates that one of the phosphodiester bonds was cleaved, whereas the formation of linear DNA indicates further cleavage near the first break [36]. As shown in Figure 4, H_2O_2 decomposed to produce •OH, which damaged most of the supercoiled DNA in this reaction system (lane 3). However, the supercoiled DNA reappeared when the plasmid DNA was exposed to the fraction F7 (lanes 4 and 5). The fraction F7 may have donated a hydrogen atom or an electron to scavenge •OH or directly prevent the reaction of Fe^{2+} with H_2O_2 to protect the pUC18 plasmid DNA from oxidative damage.

3.5.4. The ORAC Assay of the Peptide Fraction F7. The ORAC assay was used to detect the oxygen radical scavenging capability of peptides, which is reflected by the area under the fluorescence decay curve. From the results in Figure 5(a), the peptide fraction F7 exhibited a significant effect in decreasing fluorescence decay, in a dose-dependent manner. The ORAC of the peptide fraction F7 was $4.04 \pm 1.11\ \mu$mol TE/mg, as shown in Figure 5(b).

3.6. Cytotoxicity Assay of the RAW264.7 Cells

3.6.1. The Effect of the Isolated Peptides and H_2O_2 on the Proliferation of RAW264.7 Cells. The cytoprotective effect of antioxidant peptides on damaged cells can directly reflect the tension caused by oxidation. As shown in Figure 6, we evaluated the cytotoxicity effect of the isolated peptide component F7 and

Table 1: Protease activity in the purification process of E423.

Purification stage	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)
Crude enzyme	367	3844	10.5
Ammonium sulfate precipitation	60.8	2492	41
Anion exchange	4.9	1641	334.9
Size exclusion	2.0	1190.4	595.2

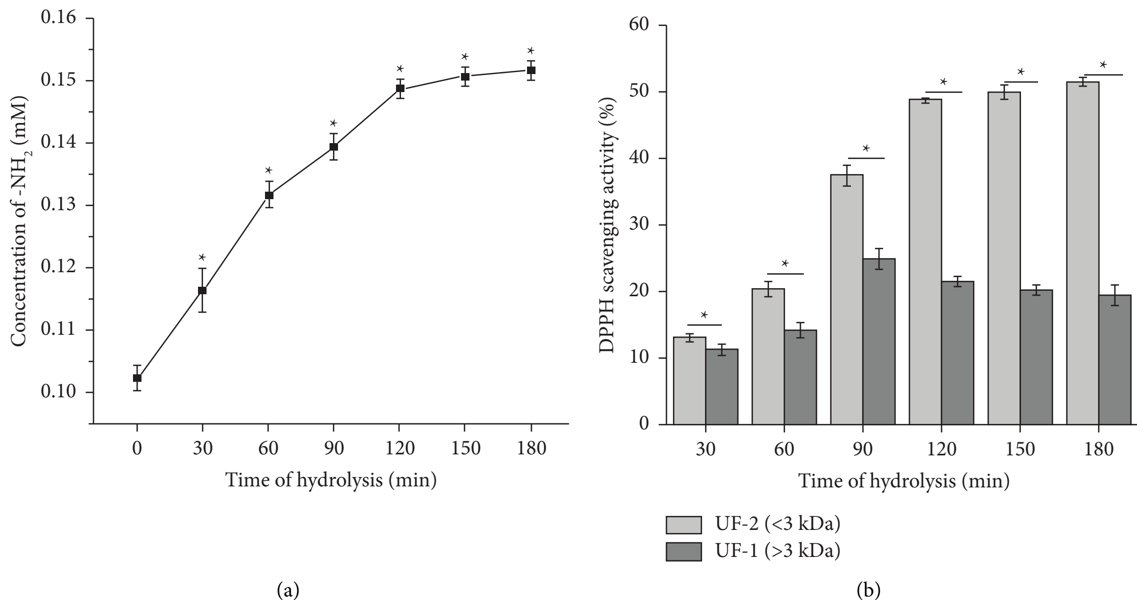

Figure 1: The free amino acid content of PSDP hydrolyzed for different times (a); analysis of the DPPH• scavenging activities of the UF-1 and the UF-2 (b). Changes were analyzed for statistical significance, which is indicated with asterisks; $^*p < 0.05$.

Table 2: Amino acid composition of the fraction UF-2.

Amino acid	UF-2 (g/100 g)
Aspartic acid (D)	9.79 ± 0.01
Alanine (A)	4.77 ± 0.07
Valine (V)	5.72 ± 0.11
Isoleucine (I)	3.78 ± 0.07
Tyrosine (Y)	4.24 ± 0.11
Histidine (H)	3.56 ± 0.04
Arginine (R)	6.58 ± 0.10
Glycine (G)	5.64 ± 0.08
Serine (S)	4.41 ± 0.10
Threonine (T)	2.54 ± 0.05
Cysteine (C)	3.96 ± 0.14
Methionine (M)	1.82 ± 0.09
Leucine (L)	7.92 ± 0.12
Phenylalanine (F)	5.2 ± 0.18
Lysine (K)	1.38 ± 0.05
Proline (P)	4.99 ± 0.15
Glutamic acid (E)/glutamine (Q)	20.21 ± 0.66

Results are expressed as the mean ± standard deviation ($n = 3$).

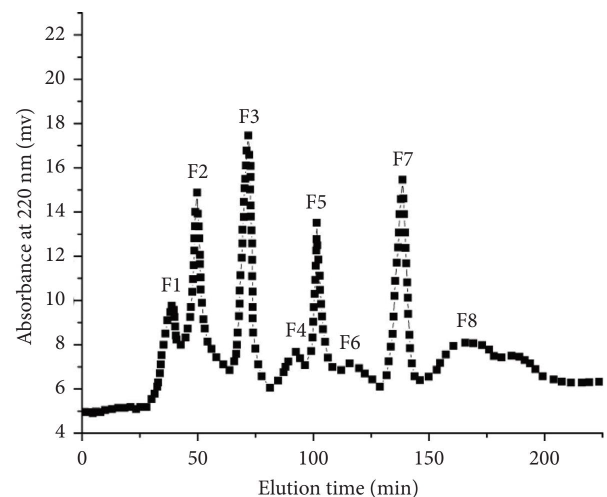

Figure 2: Size-exclusion chromatography of the fraction UF-2 on a Sephadex G-25 column. F1–F8 represent the eight isolated fractions, respectively.

H_2O_2 on the RAW264.7 cells. The cell viabilities of F7 at the concentrations of 20, 50, and 100 μg/ml were $103.25 \pm 2.48\%$, $108.02 \pm 1.67\%$, and $110.67 \pm 1.53\%$, respectively (Figure 6(a)). The results suggested that the component F7 is safe for RAW264.7 cells and, therefore, can evaluate the intracellular ROS scavenging ability of F7.

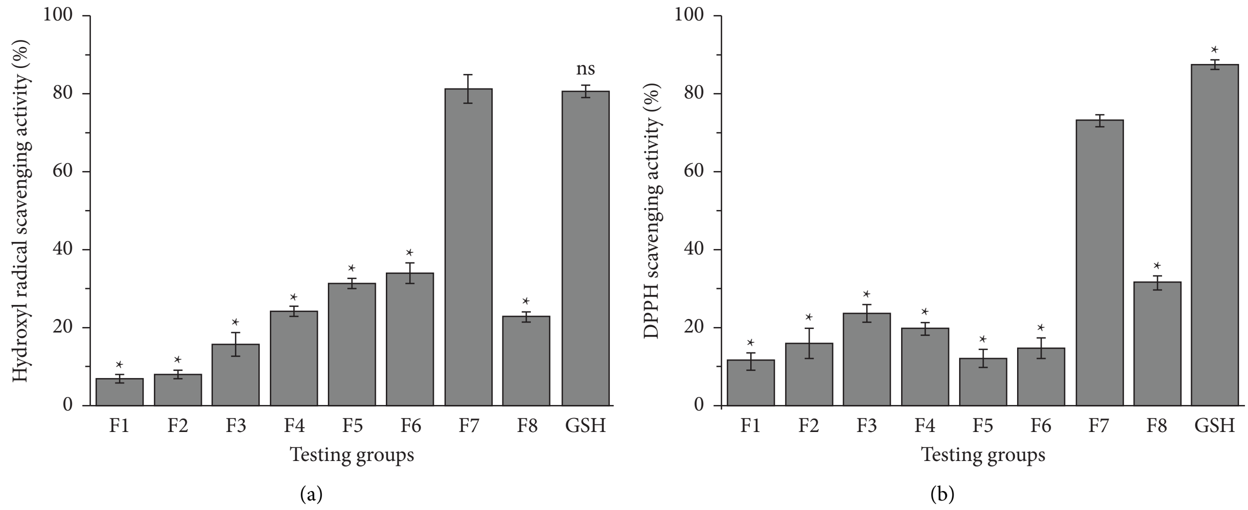

Figure 3: •OH and DPPH• scavenging activities of the F7 fraction. The concentration of both the isolated fractions F1–F8 and GSH was 500 μg/ml. Changes compared to F7 were analyzed for statistical significance, which is indicated with asterisks; $^*p < 0.05$, while nonsignificant differences are marked as ns.

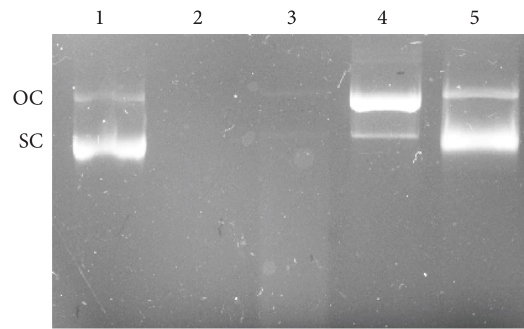

Figure 4: Effects of peptide fraction F7 on H_2O_2-mediated oxidative damage on plasmid DNA: lane 1, pUC18; lane 2, F7; lane 3, $FeSO_4 + H_2O_2$ + pUC18; lane 4, $FeSO_4 + H_2O_2$ + pUC18 + F7 (100 μg/ml); lane 5, $FeSO_4 + H_2O_2$ + pUC18 + F7 (200 μg/ml).

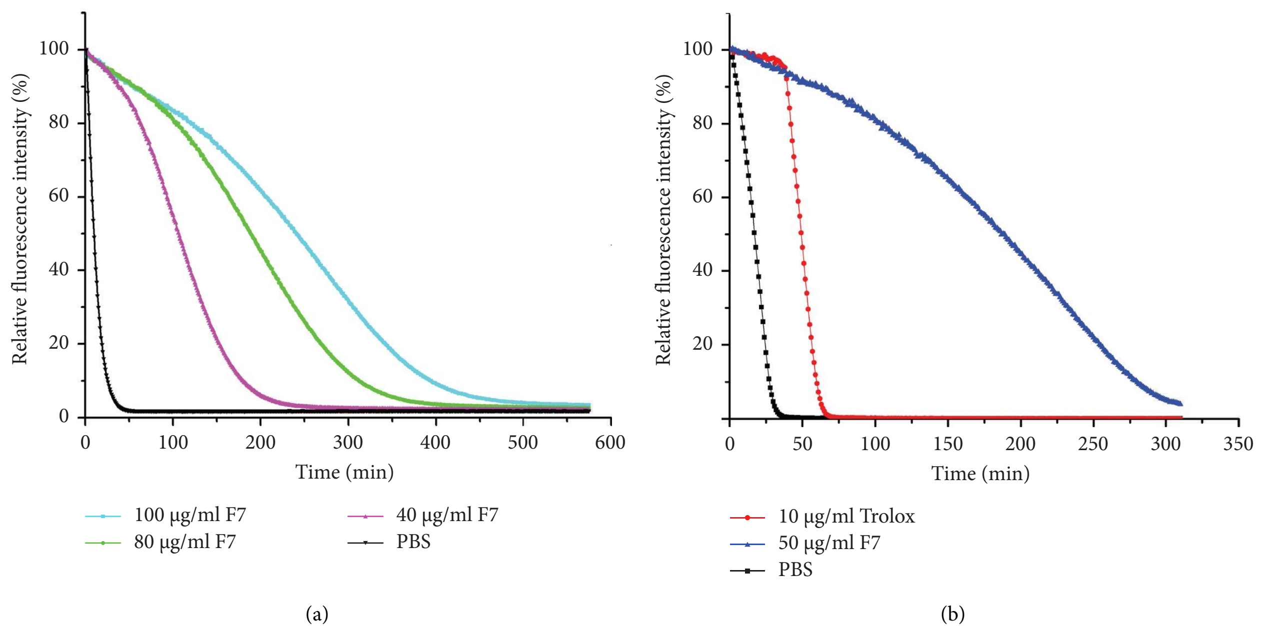

Figure 5: ORAC assay of F7 at different concentrations (a); comparison of the oxygen free radical scavenging activities of F7 and Trolox (b).

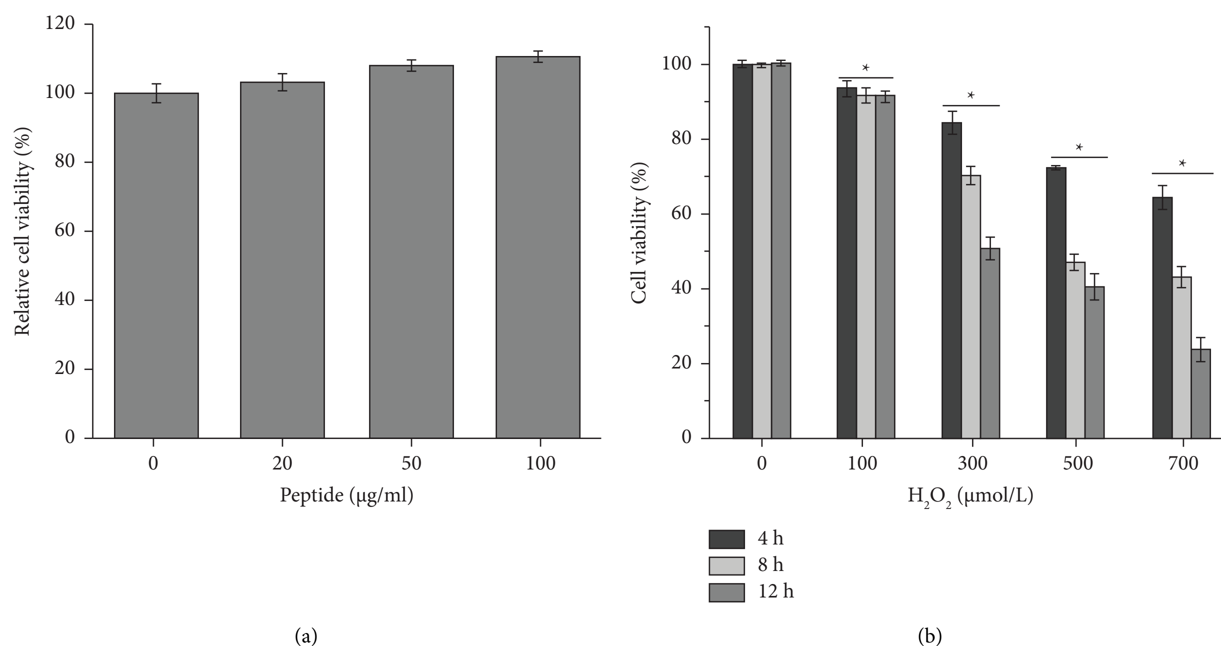

Figure 6: The effects of the peptide fraction F7 (a) and H_2O_2 (b) on RAW264.7 cell viabilities at different concentrations and treatment times.

With the increase in H_2O_2 concentrations and their treatment time, the viability of the RAW264.7 cells decreased significantly (Figure 6(b)). H_2O_2 at 300 μM significantly inhibited the RAW264.7 cells following 8 h incubation. When the concentration of H_2O_2 reached 500 μM and the treatment time was 8 h, the cell viability was 47% as compared with the control group. Hence, we established the experimental model cells by means of H_2O_2 (500 μM for 8 hours).

3.6.2. Intracellular Radical Scavenging Effects of the Isolated Peptide Component F7. Compared with the in vitro chemical analysis, the evaluation of the effect of peptides on oxidative injured cells could more directly reflect their ability to resist oxidative stress. To evaluate whether F7 has the capability of scavenging intracellular ROS, we labelled the RAW264.7 cells with DCFH-DA. The ROS content in cells was reflected by the fluorescence intensity. From the results in Figure 7, the ROS content significantly increased in cells treated with 500 μM H_2O_2, as compared with the untreated control cells. The fluorescence intensity decreased when F7 was added. Hence, the protective effect of F7 on H_2O_2-induced RAW264.7 cells may be due to the effective removal of excessive intracellular ROS.

3.6.3. Assessment of Mitochondrial Membrane Potential Changes. The JC-1 is selectively taken up by the mitochondria. In apoptotic cells, JC-1 is monomeric and its color changes from red to green as the $\Delta\Psi$m decreases. As shown in Figure 8, the number of C-gated cells reflects the $\Delta\Psi$m, with the greater number in the H_2O_2-induced group indicating a reduction in $\Delta\Psi$m of the RAW 264.7 cells ($p < 0.05$). Cells treated with the peptide fraction F7 had higher $\Delta\Psi$m than the H_2O_2-induced group ($p < 0.05$).

3.6.4. Inhibition of the Peptide Fraction F7 on Cell Apoptosis Induced by H_2O_2. Cell apoptosis was evaluated through the Annexin V-FITC/PI staining via flow cytometry. As shown in Figure 9, the apoptosis rate of the H_2O_2-induced cells was higher than that of the control group ($p < 0.05$), which can reach about 35%. The peptide fraction F7 significantly inhibited the cellular apoptosis ($p < 0.05$). In a state of oxidative stress, excess reactive oxygen species are produced, damaging a multitude of cellular functional molecules and further causing apoptosis due to reduced membrane potential, inactivation of antioxidant enzymes, and even transgeneration [37] The peptide fraction F7 has a cytoprotective effect on H_2O_2-induced RAW 264.7 cells, and its mechanism may be related to the increase of reduced membrane potential.

3.7. Identification and Verification of the Peptide Components of F7. The peptide fraction F7 was sequenced by nano-RPLC-MS/MS. We identified four novel antioxidant peptides YFPF, ECCASLAPL, YVSLK, and YFEM and considered them as the major contributors to the antioxidant activity of the fraction F7. We synthesized the peptides by the solid-phase method and assessed their antioxidant activities. As shown in Table 3, the YFPF peptide sequence showed 75.3 ± 1.08% hydroxyl radical scavenging at 500 μg/ml, as compared with a powerful antioxidant like GSH

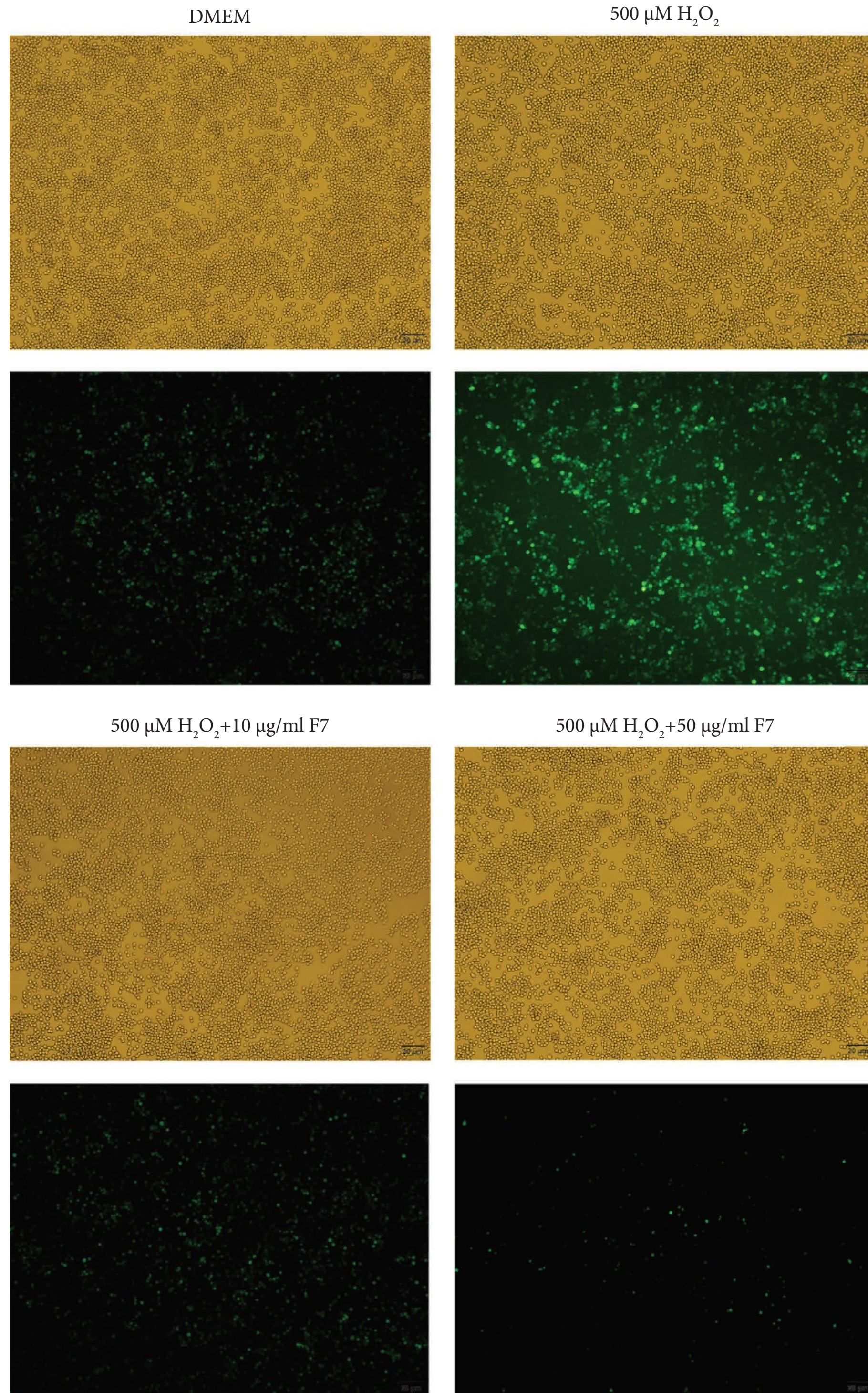

Figure 7: Intracellular ROS are indicated as green fluorescence by DCFH-DA in RAW264.7 cells.

(77.56 ± 1.40%). The hydroxyl radical scavenging ability of YFPF was higher than that of EAAY (61.9% ± 1.3%) and SMRKPPG (16.48%) at 1 mg/ml, which were isolated from peony seed dreg [17, 20]. The peptide ECCASLAPL exhibited a higher DPPH• scavenging ability than GSH at 200 μg/ml, which was 87.2 ± 1.03%. Among all the synthetic peptides, YVSLK exhibited the strongest ORAC value at 2.52 ± 1.20 μmol TE/mg.

The antioxidant activities of these peptides may be related to their molecular weight. The molecular weight of the peptides identified in this study is all less than 1000 Da, and they are more likely to react with reactive oxygen species due to their low steric resistance [38]. Moreover, the effect of the amino acid composition of the peptides on antioxidant activity has been stressed in the literature. A high proportion of hydrophobic amino acids was

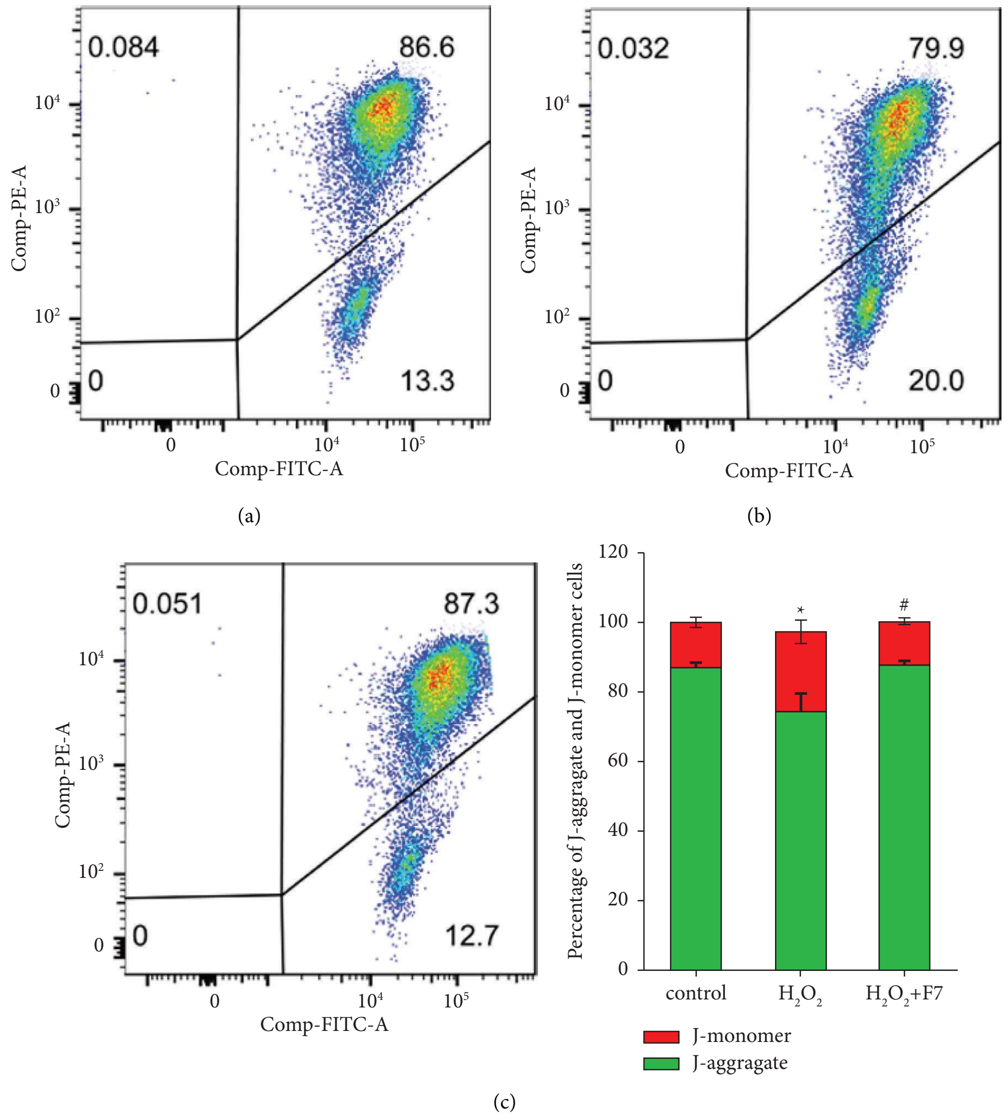

Figure 8: Analysis of ΔΨm in the RAW 264.7 cells. Bar graph represents the percentage distribution of cells with red fluorescence or green fluorescence. Changes compared to control were analyzed for statistical significance, which is indicated by $^{*}p < 0.05$. Changes compared to the H_2O_2-treated group were indicated by $^{\#}p < 0.05$.

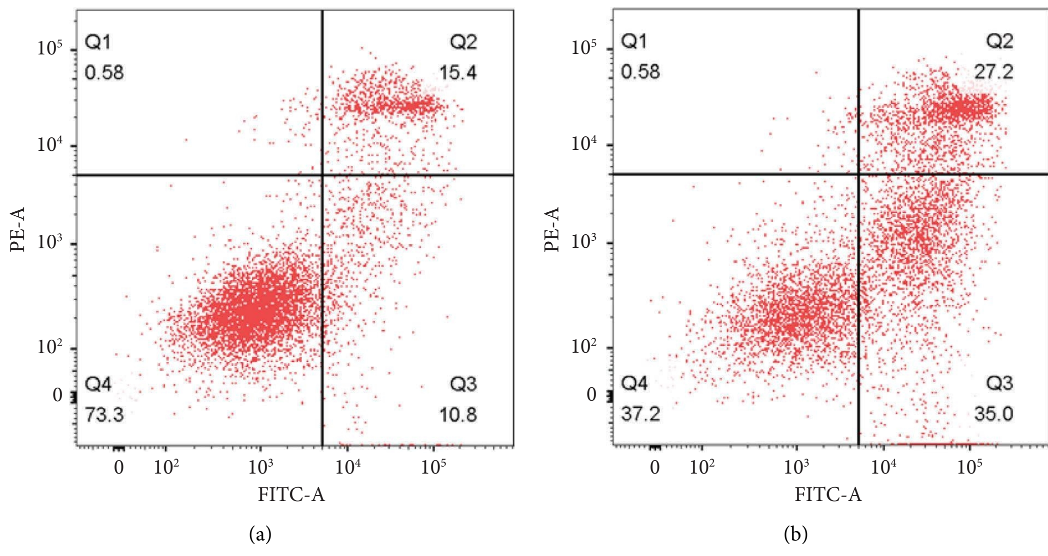

Figure 9: Continued.

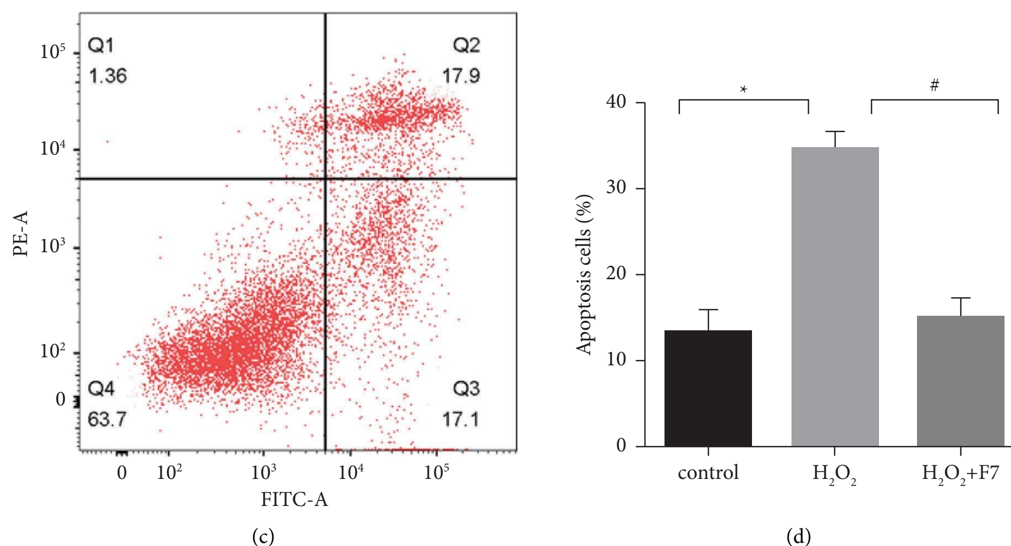

Figure 9: Flow cytometry analysis of the H_2O_2-induced cell apoptosis. Changes compared to control were analyzed for statistical significance, which is indicated by $^*p < 0.05$. Changes compared to the H_2O_2-treated group were indicated by $^{\#}p < 0.05$.

Table 3: The antioxidant activities of the synthesized peptides.

	YFPF	ECCASLAPL	YVSLK	YFEM	GSH
DPPH• scavenging activity (%)	$17.73 \pm 0.98^*$	$87.20 \pm 1.03^*$	—	$16.32 \pm 1.40^*$	49.50 ± 1.50
•OH scavenging activity (%)	$75.30 \pm 1.08^*$	$26.90 \pm 0.71^*$	$36.53 \pm 0.40^*$	$40.47 \pm 0.21^*$	77.56 ± 1.40
ORAC (μmol TE/mg)	1.50 ± 1.40	0.73 ± 1.50	2.52 ± 1.20	2.08 ± 1.20	—

The concentration of YFPF, YVSLK, and YFEM was 500 μg/ml, except in ORAC analysis, in which the concentration was 100 μg/ml. The concentration of ECCASLAPL was 500 μg/ml in the •OH scavenging activity analysis, 200 μg/ml in the DPPH• scavenging activity analysis, and 100 μg/ml in the ORAC analysis. The concentration of trolox was 25 μg/ml. Changes compared to GSH were analyzed for statistical significance, which is indicated with asterisks; $^*p < 0.05$.

considered as the key factor in the peptide ability to scavenge radicals [33]. The hydrophobic amino acids in YFEM and YFPF accounted for 50% and 75%, respectively. Hydrophobic amino acids can promote the presence of peptides at the water-lipid interface, facilitate the scavenging of radicals in the lipid phase, and promote their accessibility to hydrophobic targets, making it easier for the peptides to pass through the cell membranes [39, 40]. Since the aromatic amino acids can provide electrons for free radicals to convert them into stable molecules, we hypothesized that such a high •OH scavenging capacity of YFPF requires a high proportion of aromatic amino acids up to 75%. In addition to the presence of hydrophobic amino acids and aromatic residues, some peptides containing sulfur (Cys or Met) have been found to possess strong antioxidant properties. The thiol group in Cys and the thioether in Met are relatively easily oxidized. Cys is a hydrogen donor through the SH group or can lose an electron from its atom of sulfur [41]. Therefore, it is suggested that the higher DPPH• scavenging ability of ECCASLAPL may be related to the SH group of Cys.

4. Conclusions

In summary, peony seed dreg, the byproduct remaining after oil extractions with a low added value, has proven to be an ideal protein resource for preparing antioxidant peptides. The antioxidant peptide fraction F7 effectively protected the H_2O_2-induced RAW264.7 cells and is considered as a potential alternative peptide ingredient for synthetic antioxidants, which can be utilized by the nutraceutical, functional food, and cosmetics industries. The novel antioxidant peptides YFPF, ECCASLAPL, YVSLK, and YFEM were considered to be major contributors to the antioxidant activity of F7. However, further research on gastrointestinal stability, bioavailability, and allergenicity assessment is needed.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

C.W. and C.R. conceived and designed this study, wrote this article, and contributed equally to this work. L.Z., Y.S., and H.G. performed the experiments. H.G. provided the project administration, and Y.S. directed software processing. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31900038), Academic Leader of Changzhi Medical College (XS202002), and Scientific and Technological Innovation Programs of Higher Education Institutions in Shanxi (2020L0397).

References

[1] K. Jakubczyk, K. Dec, J. Kałduńska, D. Kawczuga, J. Kochman, and K. Janda, "Reactive oxygen species- sources, functions, oxidative damage," *Polski Merkuriusz Lekarski*, vol. 48, no. 284, pp. 124–127, 2020.

[2] Y. Zhu, F. Lao, X. Pan, and J. Wu, "Food protein-derived antioxidant peptides: molecular mechanism, stability and bioavailability," *Biomolecules*, vol. 12, no. 11, p. 1622, 2022.

[3] L. Zhu, J. Chen, X. Tang, and Y. L. Xiong, "Reducing, radical scavenging, and chelation properties of in vitro digests of alcalase-treated zein hydrolysate," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 8, pp. 2714–2721, 2008.

[4] L. Chen, L. Song, T. Li et al., "A new antiproliferative and antioxidant peptide isolated from Arca subcrenata," *Marine Drugs*, vol. 11, no. 6, pp. 1800–1814, 2013.

[5] C. B. Ahn, J. G. Kim, and J. Y. Je, "Purification and antioxidant properties of octapeptide from salmon byproduct protein hydrolysate by gastrointestinal digestion," *Food Chemistry*, vol. 147, pp. 78–83, 2014.

[6] B. Halliwell, "Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment," *Drugs and Aging*, vol. 18, no. 9, pp. 685–716, 2001.

[7] Y. Shen, B. Cao, N. R. Snyder, K. M. Woeppel, J. R. Eles, and X. T. Cui, "ROS responsive resveratrol delivery from LDLR peptide conjugated PLA-coated mesoporous silica nanoparticles across the blood-brain barrier," *Journal of Nanobiotechnology*, vol. 16, no. 1, p. 13, 2018.

[8] L. O. Santos, P. G. P. Silva, W. J. F. Lemos Junior, V. S. de Oliveira, and A. Anschau, "Glutathione production by *Saccharomyces cerevisiae*: current state and perspectives," *Applied Microbiology and Biotechnology*, vol. 106, no. 5-6, pp. 1879–1894, 2022.

[9] N. Meshginfar, A. Sadeghi Mahoonak, F. Hosseinian, M. Ghorbani, and A. Tsopmo, "Production of antioxidant peptide fractions from a by-product of tomato processing: mass spectrometry identification of peptides and stability to gastrointestinal digestion," *Journal of Food Science and Technology*, vol. 55, no. 9, pp. 3498–3507, 2018.

[10] S. K. Kim and I. Wijesekara, "Development and biological activities of marine-derived bioactive peptides: a review," *Journal of Functional Foods*, vol. 2, no. 1, pp. 1–9, 2010.

[11] W. W. Cai, X. M. Hu, Y. M. Wang, C. F. Chi, and B. Wang, "Bioactive peptides from skipjack tuna cardiac arterial bulbs: preparation, identification, antioxidant activity, and stability against thermal, pH, and simulated gastrointestinal digestion treatments," *Marine Drugs*, vol. 20, no. 10, p. 626, 2022.

[12] F. Shahidi and Y. Zhong, "Novel antioxidants in food quality preservation and health promotion," *European Journal of Lipid Science and Technology*, vol. 112, no. 9, pp. 930–940, 2010.

[13] B. H. Sarmadi and A. Ismail, "Antioxidative peptides from food proteins: a review," *Peptides*, vol. 31, no. 10, pp. 1949–1956, 2010.

[14] A. Sila and A. Bougatef, "Antioxidant peptides from marine by-products: isolation, identification and application in food systems. A review," *Journal of Functional Foods*, vol. 21, pp. 10–26, 2016.

[15] T. Maruthiah, G. Immanuel, and A. Palavesam, "Purification and characterization of halophilic organic solvent tolerant protease from marine Bacillus sp. APCMST-RS7 and its antioxidant potentials," *Proceedings of the National Academy of Sciences, India- Section B: Biological Sciences*, vol. 87, no. 1, pp. 207–216, 2017.

[16] Y. Wang, Y. Li, C. Wang, J. He, and H. Mo, "Antioxidant activity, functional properties, and cytoprotective effects on HepG2 cells of tree peony (Paeonia suffruticosa Andr.) seed protein hydrolysate as influenced by molecular weights fractionation," *Foods*, vol. 11, no. 17, p. 2592, 2022.

[17] M. Wang, C. Li, H. Li et al., "In vitro and in silico antioxidant activity of novel peptides prepared from Paeonia ostii 'feng dan' hydrolysate," *Antioxidants*, vol. 8, no. 10, p. 433, 2019.

[18] X. Wang, C. Li, M. D. M. Contreras, V. Verardo, A. M. Gómez-Caravaca, and C. Xing, "Integrated profiling of fatty acids, sterols and phenolic compounds in tree and herbaceous peony seed oils: marker screening for new resources of vegetable oil," *Foods*, vol. 9, no. 6, p. 770, 2020.

[19] C. Zhou, Y. Zhang, Y. Sheng et al., "Herbaceous peony (Paeonia lactiflora Pall.) as an alternative source of oleanolic and ursolic acids," *International Journal of Molecular Sciences*, vol. 12, no. 1, pp. 655–667, 2011.

[20] F. Zhang, J. Qu, K. Thakur, J. G. Zhang, A. Mocan, and Z. J. Wei, "Purification and identification of an antioxidative peptide from peony (Paeonia suffruticosa Andr.) seed dreg," *Food Chemistry*, vol. 285, pp. 266–274, 2019.

[21] C. Wu, D. Liu, X. Yang et al., "Improving production of protease from pseudoalteromonas sp. CSN423 by random mutagenesis," *Marine Biotechnology*, vol. 18, no. 5, pp. 610–618, 2016.

[22] S. Chutipongtanate, K. Watcharatanyatip, T. Homvises, K. Jaturongkakul, and V. Thongboonkerd, "Systematic comparisons of various spectrophotometric and colorimetric methods to measure concentrations of protein, peptide and amino acid: detectable limits, linear dynamic ranges, interferences, practicality and unit costs," *Talanta*, vol. 98, pp. 123–129, 2012.

[23] L. L. Gao, Y. Q. Li, Z. S. Wang, G. J. Sun, X. M. Qi, and H. Z. Mo, "Physicochemical characteristics and functionality of tree peony (Paeonia suffruticosa Andr.) seed protein," *Food Chemistry*, vol. 240, pp. 980–988, 2018.

[24] D. Liu, J. Huang, C. Wu et al., "Purification, characterization, and application for preparation of antioxidant peptides of extracellular protease from pseudoalteromonas sp. H2," *Molecules*, vol. 24, no. 18, p. 3373, 2019.

[25] B. A. Bidlingmeyer, S. A. Cohen, and T. L. Tarvin, "Rapid analysis of amino acids using pre-column derivatization," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 336, no. 1, pp. 93–104, 1984.

[26] L. S. Borquaye, G. Darko, E. Ocansey, and E. Ankomah, "Antimicrobial and antioxidant properties of the crude peptide extracts of Galatea paradoxa and Patella rustica," *SpringerPlus*, vol. 4, no. 1, p. 500, 2015.

[27] Z. J. Qian, W. K. Jung, H. G. Byun, and S. K. Kim, "Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, *Crassostrea gigas* against free radical induced DNA damage," *Bioresource Technology*, vol. 99, no. 9, pp. 3365–3371, 2008.

[28] A. Dávalos, C. Gómez-Cordovés, and B. Bartolomé, "Extending applicability of the oxygen radical absorbance

capacity (ORAC-fluorescein) assay," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 1, pp. 48–54, 2004.

[29] S. K. Suo, Y. Q. Zhao, Y. M. Wang, X. Y. Pan, C. F. Chi, and B. Wang, "Seventeen novel angiotensin converting enzyme (ACE) inhibitory peptides from the protein hydrolysate of *Mytilus edulis*: isolation, identification, molecular docking study, and protective function on HUVECs," *Food and Function*, vol. 13, no. 14, pp. 7831–7846, 2022.

[30] R. Wu, L. Chen, D. Liu et al., "Preparation of antioxidant peptides from salmon byproducts with bacterial extracellular proteases," *Marine Drugs*, vol. 15, no. 1, p. 4, 2017.

[31] A. T. Idowu and S. Benjakul, "Bitterness of fish protein hydrolysate and its debittering prospects," *Journal of Food Biochemistry*, vol. 43, no. 9, Article ID e12978, 2019.

[32] J. Venkatesan, S. Anil, S. K. Kim, and M. S. Shim, "Marine fish proteins and peptides for cosmeceuticals: a review," *Marine Drugs*, vol. 15, no. 5, p. 143, 2017.

[33] T. B. Zou, T. P. He, H. B. Li, H. W. Tang, and E. Q. Xia, "The structure-activity relationship of the antioxidant peptides from natural proteins," *Molecules*, vol. 21, no. 1, p. 72, 2016.

[34] Y. Li and J. Yu, "Research progress in structure-activity relationship of bioactive peptides," *Journal of Medicinal Food*, vol. 18, no. 2, pp. 147–156, 2015.

[35] H. Amitani, A. Asakawa, K. Cheng et al., "Hydrogen improves glycemic control in type1 diabetic animal model by promoting glucose uptake into skeletal muscle," *PLoS One*, vol. 8, no. 1, Article ID e53913, 2013.

[36] Y. Q. Zhao, L. Zeng, Z. S. Yang, F. F. Huang, G. F. Ding, and B. Wang, "Anti-fatigue effect by peptide fraction from protein hydrolysate of croceine croaker (pseudosciaena crocea) swim bladder through inhibiting the oxidative reactions including DNA damage," *Marine Drugs*, vol. 14, no. 12, p. 221, 2016.

[37] J. Kong, X. M. Hu, W. W. Cai, Y. M. Wang, C. F. Chi, and B. Wang, "Bioactive peptides from skipjack tuna cardiac arterial bulbs (II): protective function on UVB-irradiated HaCaT cells through antioxidant and anti-apoptotic mechanisms," *Marine Drugs*, vol. 21, no. 2, p. 105, 2023.

[38] T. Damgaard, R. Lametsch, and J. Otte, "Antioxidant capacity of hydrolyzed animal by-products and relation to amino acid composition and peptide size distribution," *Journal of Food Science and Technology*, vol. 52, no. 10, pp. 6511–6519, 2015.

[39] F. Zhao, C. Liu, L. Bordoni et al., "Advances on the antioxidant peptides from nuts: a narrow review," *Antioxidants*, vol. 11, no. 10, 2022.

[40] K. L. Sun, M. Gao, Y. Z. Wang, X. R. Li, P. Wang, and B. Wang, "Antioxidant peptides from protein hydrolysate of marine red algae eucheuma cottonii: preparation, identification, and cytoprotective mechanisms on H_2O_2 oxidative damaged HUVECs," *Frontiers in Microbiology*, vol. 13, Article ID 791248, 2022.

[41] I. D. Nwachukwu and R. E. Aluko, "Structural and functional properties of food protein-derived antioxidant peptides," *Journal of Food Biochemistry*, vol. 43, no. 1, Article ID e12761, 2019 Jan.