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Isolation and Identification of Antioxidant Peptides from Jinhua Ham

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Supporting Information

ABSTRACT: The antioxidant activities of the peptides extracted from Jinhua ham were evaluated on the basis of hydroxyl radical scavenging activity, DPPH radical scavenging activity, and Fe^{2+} chelating ability. The peptide extracts exhibited great hydroxyl radical scavenging activity and DPPH radical scavenging activity as well as Fe^{2+} chelating ability at the concentration of 1 mg/mL, which suggested the presence of peptides with antioxidant activity. The peptides were separated using size exclusion chromatography and reversed-phase HPLC. The fraction with highest DPPH radical scavenging activity was further purified and identified using liquid chromatography tandem matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (HPLC-MALDI-TOF/TOF-MS). The sequence of the antioxidant peptide was identified as Gly-Lys-Phe-Asn-Val. The assessment of fractions indicated that the hydrophobic fractions contributed more to free radical scavenging activities than the hydrophilic peptides. It was concluded that natural peptides extracted and isolated from the Jinhua ham by several chromatographic techniques have antioxidant activities.

KEYWORDS: *Jinhua ham, antioxidant peptides, antioxidant activity, HPLC-MALDI-TOF/TOF-MS*

INTRODUCTION

Dry-cured hams are important in the diets of many countries. Jinhua ham is a traditional Chinese meat product that gained much popularity all over the world from ancient China. The processing of typical Jinhua ham generally takes 8−10 months.

During the long ripening time, intense biochemical reactions take place; the proteolysis of sarcoplasmic and myofibrillar proteins is one of the main phenomena. Muscle proteins are hydrolyzed to some extent by internal enzymes to produce many small peptides and free amino acids, which eventually contribute to the unique flavor of Jinhua ham.[1] Besides flavor characteristics, these peptides may have special functions in human health. A large amount of peptides generated during the processing of ham may have a positive impact on body functions that may ultimately influence health.[2] These peptides are able to cross the digestive epithelial barrier and reach the blood vessels because they contain only a few amino acid residues. This special property allows them to reach peripheral organs and has beneficial effects on the organisms.[3]

People have gradually realized that fatigue and many human diseases are associated with reactive oxygen species. Reactive oxygen species are produced continuously through non-enzymatic and enzymatic reactions in vivo. At the same time, they can be removed by antioxidant enzymes and endogenous antioxidant agents under synergistic actions. However, when the body is suffering from aging, illness, or fatigue, the radical balance in the body could be damaged. The accumulation of free radicals could lead to the irreversible oxidative damage of organisms by attacking macromolecules and organelles of the body, accelerating organism aging and fatigue and inducing various diseases.[4,5]

Because antioxidant activity deficiency has been involved in the occurrence of many human diseases, more and more studies have been conducted to investigate the effects of antioxidants. Most antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which have been used extensively, are synthetic antioxidants. Although they have strong antioxidant activity, various potential side effects and toxic effects on the human enzyme system are reported.[6] Therefore, exploration for and development of safe and nontoxic natural antioxidants have been popular topics in recent decades.

Studies about antioxidant peptides from food protein sources are very extensive in recent years because of their safety and wide distribution properties. Compared to synthetic antioxidants, these peptides have relatively low molecular weight, simple structure, high activity, easy absorption, more stability in different situations, and no hazardous immunoreactions. Various studies have been conducted to investigate the antioxidant properties of natural peptides from plant or animal sources. For example, antioxidant activity has been found in soybeans,[7] whey proteins,[8] peanuts,[9] and rice.[10] Antioxidant peptides from animal resources are mainly extracted from fish,[11] loach,[12] eggs,[13] and meat.[14] Saiga et al.[15] reported that hydrolysates obtained from porcine myofibrillar proteins exhibited high levels of antioxidant activity, in which the peptide sequenced as Asp-Ala-Gln-Glu-Lys-Leu-Glu showed the highest level of activity. Lee et al.[16] digested duck processing byproducts using different enzymes to produce antioxidant peptides. The peptide sequence with antioxidant ability was identified as Asp-Val-Cys-Gly-Arg-Asp-Val-Asn-Gly-Tyr. However, little research has been conducted about antioxidant peptides generated from Jinhua ham. Therefore, the current study focused on the purification and identification of antioxidant peptides from Jinhua ham using consecutive

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chromatography and MALDI-TOF/TOF MS and then identified the antioxidant activities of these peptides.

MATERIALS AND METHODS

Materials. Jinhua hams were purchased from the supermarket of Jin Zi Wholesale Chain Co. (Jinhua, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and BHT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade, and other reagents were of analytical grade, purchased from Jiancheng Chemical Reagent Co. (Nanjing, China).

Processing of Jinhua Ham and Sample Preparation. Jinhua hams were processed according to a traditional method consisting of natural cooling, salting, soaking and washing, sun-drying, loft-aging, and postaging. The whole process takes 8 months in Zhejiang province, China. At the end of postaging, five hams were randomly selected as samples. M. biceps femoris was fully taken from the whole Jinhua ham, packed, and stored under −20 °C before analysis.

Peptide Extraction. The extraction was performed according to the method of Mora et al.[17] with slight modifications. Samples were denuded of extramuscular fat and connective tissues and minced, and then 25 g of M. biceps femoris muscle was homogenized with 100 mL of 0.01 N HCl in a polytron homogenizer (IKA T25 digital ultraturrox, IKA, Germany; 4 strokes, 10 s each at 22000 rpm with cooling in ice). The homogenate was centrifuged at 12000*g* for 20 min at 4 °C, and after filtration through glass wool, the supernatant was added to 3 volumes of ethanol to remove the protein fraction. The mixture was kept for 20 min at 4 °C and then centrifuged at 12000*g* for 20 min at 4 °C again, and the supernatant was dried in a rotatory evaporator. The dried extract was dissolved in 12.5 mL of 0.01 N HCl, filtered through a 0.45 μm nylon membrane filter (Millipore, Bedford, MA), and stored at −20 °C prior to use.

Measurement of Peptide Contents. The peptide contents of Jinhua ham were measured according to the method of Church et al.[18] with some modifications using *o*-phthaldialdehyde (OPA) spectrophotometric assay. OPA solution was made by dissolving 40 mg of OPA in 1 mL of methanol and mixed with 25 mL of 100 mM sodium tetrahydroborate, 2.5 mL of 20% (w/w) sodium dodecyl sulfate, and 100 μL of β-mercaptoethanol, and then the volume was adjusted to 50 mL with deionized water. This reagent was prepared daily. Then 100 μL of peptides, containing 5−100 μg of protein, was mixed with 2 mL of fresh OPA reagent and incubated for 2 min at ambient temperature. The absorbance at 340 nm was measured using a multifunctional microplate reader (model Spectral Max M2e). Casein tryptone was used as the standard to quantify the peptide contents.

$$\text{peptide content (\%)} = (\text{peptide contents/muscle contents}) \times 100\%$$

Measurement of Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity was determined using the method described by Li et al.[19] with slight modifications. Sample group was a mixture of 0.6 mL of 1,10-phenanthroline monohydrate (5 mmol/L) and 0.4 mL of phosphate buffer (0.2 M, pH 7.4), and then 0.6 mL of sample, 0.6 mL of EDTA (15 mmol/L), and 0.6 mL of $FeSO_4$ (5 mmol/L) were mixed thoroughly. After mixing, 0.8 mL of H_2O_2 (0.1%) was added, and the mixture was incubated at 37 °C for 60 min. Afterward, the mixture absorbance was measured at 536 nm with the multifunctional microplate reader (model Spectral Max M2e). The damaged group contained the same solutions as the sample group except deionized water was used instead of sample. The nondamaged group contained the same solution as the damaged group except deionized water was used instead of H_2O_2. Results were determined according to the equation

$$\text{hydroxyl radical scavenging activity (\%)} = (\text{As} - \text{Ad})/(\text{An} - \text{Ad}) \times 100\%$$

where As, Ad, and An represent the absorbance of the sample and the damaged and nondamaged groups, respectively. BHT was used as a control.

Measurement of DPPH Radical Scavenging Activity. The DPPH radical scavenging activity of peptides extracted from Jinhua ham was measured according to the method of Shimada et al.[20] The sample group was aliquots of samples mixed 1:1 (v/v) with 0.2 mmol/L DPPH (in 95% ethanol), the control group was aliquots of samples mixed 1:1 (v/v) with 95% ethanol, and the blank group was aliquots of 95% ethanol mixed 1:1 (v/v) with 0.2 mmol/L DPPH (in 95% ethanol). The mixture was shaken and kept for 30 min at room temperature protected from light. The reduction of DPPH free radicals was determined by measuring the absorbance at 517 nm with the multifunctional microplate reader (model Spectral Max M2e). The ability of the fractions to scavenge the DPPH free radicals was calculated according to the equation

$$\text{DPPH radical scavenging activity (\%)} = 1 - [(\text{As} - \text{Ac})/(\text{Ab} - \text{Ac})] \times 100\%$$

where As, Ac, and Ab represent the absorbance of the sample, the control, and the blank groups, respectively. BHT was used as a control.

Measurement of Fe^{2+} Chelating Ability. The ferrous (Fe^{2+}) chelating ability of extracted substance from Jinhua ham was measured as described by Dinis et al.[21] with slight modifications. Briefly, 1 mL of sample was mixed with 0.05 mL of $FeCl_2$ (2 mmol/L) and 0.2 mL of ferrozine (5 mmol/L). The mixture was vortexed and kept at room temperature for 10 min prior to measurement of the absorbance at 562 nm (As). Control (Ac) contained everything except using deionized water instead of sample. The chelating ability was calculated according to the equation

$$Fe^{2+}\ \text{chelating ability (\%)} = (\text{Ac} - \text{As})/\text{Ac} \times 100\%$$

where As and Ac represent the absorbance of the sample and the control groups. BHT was used as a control.

Peptide Separation by Size Exclusion Chromatography. Peptide extracted with a volume of 0.5 mL aliquot was subjected to size exclusion chromatography using a Sephadex column (5 × 5 cm) packed with Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden) to fractionate the peptides according to their molecular masses. Separation was performed at a constant flow rate of 0.5 mL/min with 0.01 N HCl at room temperature. Fractions were assayed using an ultraviolet detector (Amersham Biosciences) at 280 nm, and 1 mL was collected in every tube by an automatic fraction collector. Fractions were dried in a vacuum freeze-dryer for further analysis.

Fractionation of Antioxidant Peptides by RP-HPLC. The fraction with the highest DPPH radical scavenging activity was selected for further fractionation by reversed-phase high-performance liquid chromatography (RP-HPLC). The lyophilized sample was dissolved again in 1 mL of distilled water and injected into a HPLC system equipped with a BEH C18 column (1.7 μm, 2.1 × 100 mm, Waters Inc., Milford, MA, USA). The gradient elution was performed at a flow rate of 0.3 mL/min with eluent A as 0.1% formic acid and eluent B as 100% acetonitrile (ACN). The flow gradient was as follows: 0−10 min, 100% A; 10−22 min, 30−80% B; 22−23 min, 100% A. The peptide peaks were monitored at a UV wavelength of 280 nm. The peaks corresponding to peptides were collected as six fractions and freeze-dried. The antioxidant properties of these fractions were evaluated using the same methods as described above.

Identification of Antioxidant Peptides by LC-MS/MS. The fractionation of the peak with the highest DPPH radical scavenging activity from RP-HPLC fractionation was carried out using an Acquity (Waters Inc.) HPLC system equipped with a reversed phase BEH C18 analytical column (1.7 μm, 2.1 × 100 mm, Waters Inc.). The gradient elution was performed at a flow rate of 0.3 mL/min with eluent A as 0.1% formic acid and eluent B as 100% ACN. The flow gradient was as follows: 0−10 min, 100% A; 10−22 min, 30−80% B; 22−23 min, 100% A. The column temperature was maintained at 25 °C. The RP-HPLC system was connected to a MS/MS (MALDI SYNAPT Q-TOF MS, Waters Inc.). The MS/MS was used with multiple reaction

Table 1. Peptide Content and Antioxidant Activity of Peptides from Jinhua Ham

	peptide content (%)	concentration (mg/mL)	OH radical scavenging activity[a] (%)	DPPH radical scavenging activity[a] (%)	Fe^{2+}chelating ability[a] (%)
peptide	1.16 ± 0.12	1	54.00 ± 1.49a	77.39 ± 2.24a	63.20 ± 2.08b
BHT		1	75.85 ± 1.08b	88.52 ± 0.59b	10.59 ± 4.66a

[a]Different letters (a, b) in the same column indicate significant differences ($P < 0.05$).

measurement (MRM). For ionization, a matrix-assisted laser desorption ionization positive ion pattern (ESI+) was employed. TOF MS survey scan was recorded for mass range m/z 200−4000 followed by MS/MS scans of the three most intense peaks. Nitrogen was used as collision gas. Capillary voltage was set at 3.5 kV and cone voltage at 20 V. Source block temperature was set at 100 °C and desolvation temperature at 250 °C. Collision energy and detector voltage were 15 and 1600 V, respectively. The desolvation and cone gas flow were 500 and 50 L/h, respectively.

Statistical Analysis. The molecular masses of the isolated peptides were determined by mass spectrometry (Dionex, MSQ). The MS/MS information was input to a Mass Lynx4.1 (Waters, USA) data processing system to obtain accurate molecular masses and amino acid sequences of the peptides. Because many different amino acid combinations could correspond to a given mass, the matched peptides were further verified manually. SPSS 20.0 was used for the major data processing throughout this work. All results were expressed as the mean ± SD. Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Peptide Contents and Antioxidant Activities of Peptides. According to Zhou and Zhao,[1] nonprotein nitrogen accounts for 14−20% of total nitrogen, whereas nitrogen from the free amino acids and small peptides with molecular weight of <1 kDa accounts for >95% of the total nonprotein nitrogen at the end of Jinhua ham processing. In fact, a very high increase in the concentration of free amino acids at the end of the process in low-salt, typical Italy ham has been previously reported.[22] However, there is lack of information regarding the generated or hydrolyzed peptides during the process. The peptide content in this experiment is 1.16%. The high amount of peptides may contribute to the flavor and human health. Escudero et al.[23] have reported that antihypertensive and antioxidant fractionated peptide extract existed in Spanish ham.

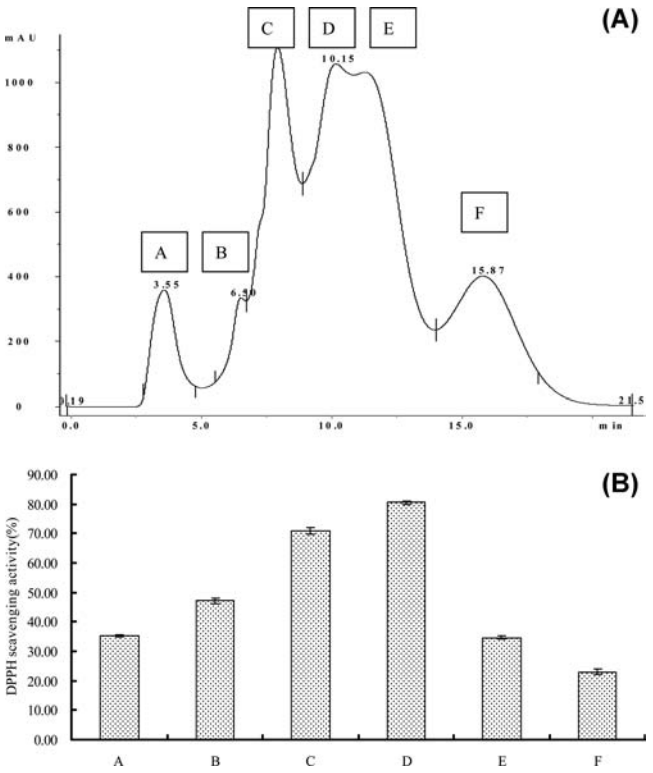

Figure 1. (A) Size exclusion chromatography of peptides from Jinhua ham; (B) DPPH scavenging activity of fractions obtained by size exclusion chromatography.

The methods based on electron transfer were usually used to measure the antioxidant activity of an antioxidant, which can change the color of the mixture when reduced. The degree of color change was correlated with the sample's antioxidant activity.[24] The hydroxyl radical scavenging activity ($OH^•$), the DPPH radical scavenging activity, and Fe^{2+} chelating ability were adopted in this assay to express the antioxidant activity of the peptides. Among the various reactive oxygen species, $OH^•$ is the most harmful one due to its reaction with biological macromolecules such as protein and DNA.[25] As demonstrated in Table 1, the $OH^•$ scavenging activity of peptides from Jinhua ham was approximately 50% at the concentration of 1 mg/mL, whereas BHT exhibited nearly 75% scavenging activity on hydroxyl radicals. This value is even higher than that of many other protein hydrolysates. In the hydrolysates of bullfrog skin, the effects of the Neutrase hydrolysate (42.3%), papain hydrolysate (34.5%), and pepsin hydrolysate (47.6%) on hydroxyl radicals at the concentration of 1.5 mg/mL were weaker than that of peptides extracted from Jinhua ham.[26] Ko et al.[27] reported that the peptide purified from a marine *Chlorella ellipsoidea* exhibited 50% $OH^•$ scavenging activity at the peptide concentration of 2.698 mg/mL. Thus, we can infer that Jinhua ham contained some antioxidant peptides which could convert free radicals to more stable products and terminate the radical chain reactions.

DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH accepts an electron or hydrogen radical to become a stable diamagnetic molecule, the radicals are scavenged and the absorbance is reduced.[20] Therefore, DPPH is often used as a substrate to evaluate the antioxidant activity of an antioxidant. As described in Table 1, the DPPH scavenging activity for BHT was almost 88% at the concentration of 1 mg/mL. Peptides extracted from Jinhua ham exhibited a comparable lower DPPH scavenging capacity, reaching a scavenging activity of 77% at the concentration of 1 mg/mL, comparable to that of tuna dark muscle hydrolysates at the concentration of 3 mg/mL.[28] In the hydrolysates of tuna dark muscle, the DPPH radical scavenging activity increased initially and peaked at 31.5% at 60 min of hydrolysis. Zhang et al.[14] reported rice protein hydrolysates by papain and chymotrypsin exhibited DPPH scavenging activities of 44.31 and 35.26% at a concentration of 1.5 mg/mL, respectively. It has been reported that the high level of DPPH free radical scavenging activity of protein hydrolysates was associated with a high amount of hydrophobic amino acids of peptides.[29] From the DPPH free radical scavenging activities assessment, we

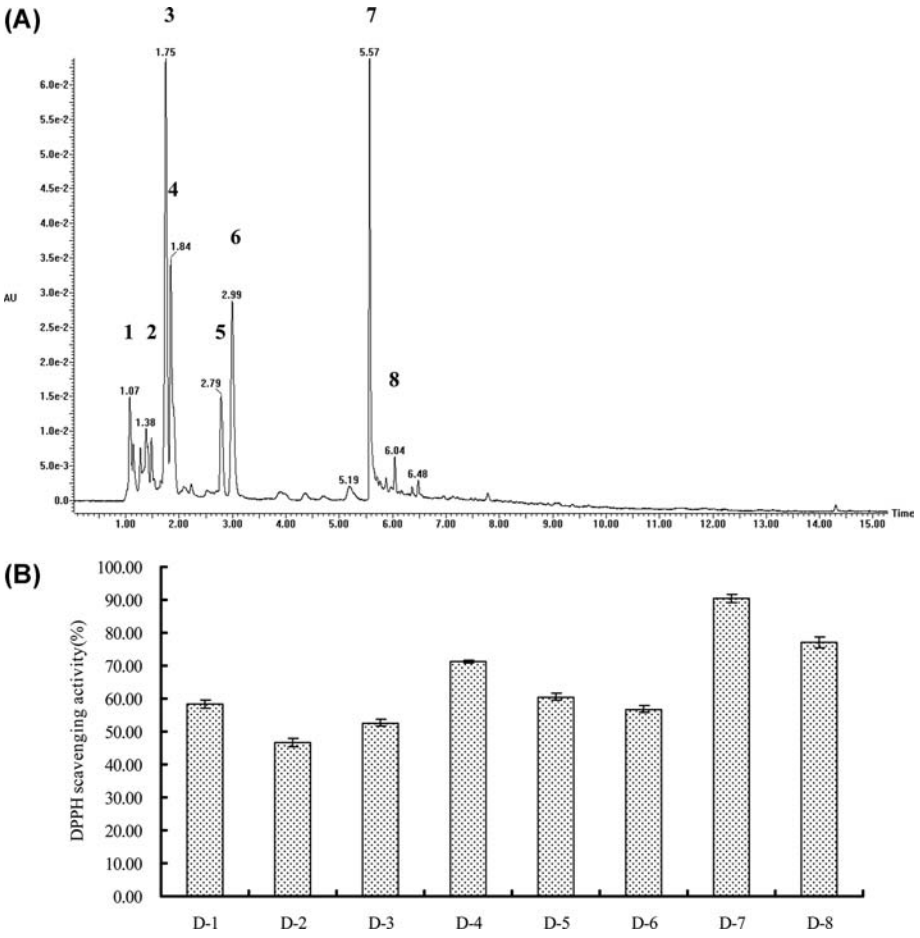

Figure 2. (A) Separation of fraction D by RP-HPLC; (B) DPPH scavenging activity of fractions obtained by RP-HPLC.

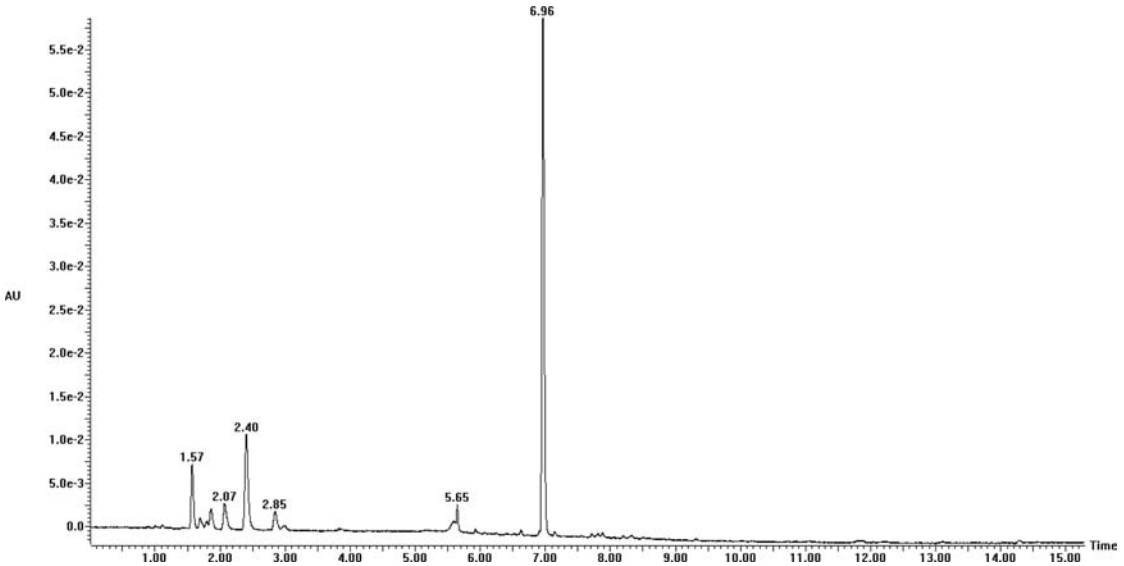

Figure 3. RP-HPLC-UV chromatograms of fraction D-7 obtained by two-step RP-HPLC.

deduced that some hydrophobic amino acids possibly exist in the antioxidant peptides.

Transition metals such as iron and copper ions are strong agents to generate free radicals, which can catalyze the generation of reactive oxygen species, such as hydroxyl radicals and superoxide radicals. The presence of metal ions will consume the antioxidants soon.[15] Because compounds interfering with the catalytic activity of metal ions could affect the prooxidative process, measuring the chelating ability of the compound is important for evaluating its antioxidant activity.[30] In Table 1, the Fe^{2+} chelating ability of peptides from Jinhua ham was higher than that of the BHT. High Fe^{2+} chelating ability in the peptides from Jinhua ham may be from the exposure of more acidic and basic amino acids by peptide

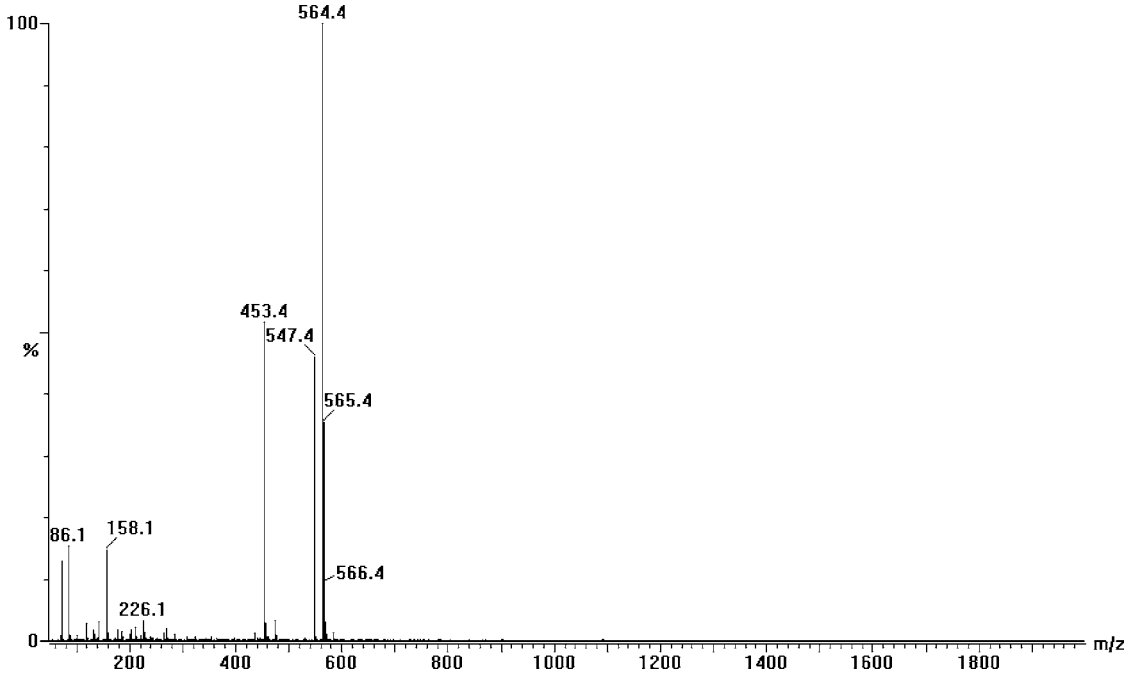

Figure 4. Mass spectrum of peak P1.

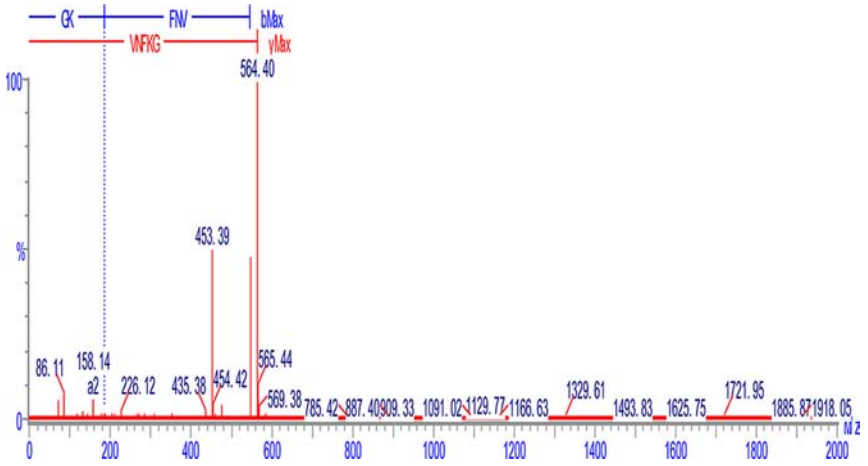

Figure 5. MS/MS spectrum of component at m/z 562.9.

cleavage as the carboxyl and amino groups in their side chains can bind Fe^{2+}.[14]

Antioxidant Peptides from Size Exclusion Chromatography. The Jinhua ham extracts were fractionated by size exclusion chromatography packed with SephadexG-25, and the result is shown in Figure 1A. According to their molecular weights, they were separated into six fractions, A, B, C, D, E, and F. The retention time of A is the shortest, representing the large-sized peptide fragments. On the contrary, fraction F corresponds to small-sized peptide fragments. All fractions were collected and lyophilized. Their antioxidant activities were measured and compared between different fractions at the concentration of 1 mg/mL (Figure 1B). Fraction D showed much greater ($P < 0.05$) DPPH radical scavenging activity compared to other fractions. The molecular weight of fraction D contains a certain range of molecular mass expressing the highest antioxidant activity. This result is consistent with previous research. Wu et al.[31] discovered that peptides with the molecular weight of 1400 Da have strong antioxidant activity. Jea et al.[32] found that peptides of Alaska cod bone with the molecular weight of <1 kDa have the strongest antioxidant activity.

Antioxidant Peptides Separated by RP-HPLC. RP-HPLC is the basic separation technology in the modern biological area. Because of its high speed, high sensitivity, and good reproducibility, it has become the common and effective method for the separation and purification of relative molecular weight of <5 kDa, especially for small-sized peptides with the molecular weight of <1 kDa. The method is based on the differences of the polarity between molecules, and a C18 column is usually used to separate the bioactive peptides. Fraction D through the size exclusion chromatography continued to be purified by RP-HPLC. Eight fractions were separated, D-1, D-2, D-3, D-4, D-5, D-6, D-7, and D-8, respectively (Figure 2A). Each fraction was pooled, lyophilized, and measured for DPPH radical scavenging activity (Figure 2B). Fraction D-7 showed the maximum DPPH scavenging activity with a value of 90.31%. The retention time is long,

suggesting that the target antioxidant peptide contains a hydrophobic amino acid. Fraction D-7 was further purified and identified by RP-HPLC online connection of the electrospray ion mass spectrometry (ESI-MS-MS) for amino acid sequencing.

Identification of Antioxidant Peptides by HPLC-MS-TOF. Mass spectrometry (MS) is the most important progress in the field of protein sequencing. MS has gradually become the preferred method for the identification of proteins and peptides in recent years because it can be used as the linking bridge between protein and sequence database. Some advanced MS techniques, such as electrospray ionization mass/mass spectrometry (ESI-MS/MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), have been more and more applied in the identification of bioactive peptides. Mora et al. identified 27 peptides generated from TnT during dry-cured processing using MALDI-TOF/TOF.[17] Different peptide fragments, according to their different masses and charges, can generate different tracks in the magnetic field. On that basis, MS can make use of the m/z ratio to separate them. MALDI and electrospray ionization technique (ESI) have provided a sensitive method for the analysis of peptide and protein structure determination,[33] making a revolutionary leap in the analysis of protein structure. ESI-MS is the soft ionization mass spectrometry method that has developed rapidly in recent years. Because of its high specificity, high sensitivity, and multistage MS features, it can simultaneously detect multiple components in the complex system. The fraction of D-7 with the highest DPPH scavenging activity was collected and subjected to HPLC tandem electrospray mass spectrometer for peptide sequence identification. Figure 3 illustrates the HPLC chromatogram of D-7 with one major peak and a plurality of low-absorbance peaks. By means of certain software, the mass spectrometer analyzes samples from HPLC automatically and predicts the structure of each component. Under the condition of this experiment, the information is not enough to identify the chemical structure of other peaks because their contents are relatively low, so the corresponding ion peaks in the MS/MS spectrum are too little and too weak. Thus, only the sequence analysis of the major peak was summarized. As shown in Figure 4, the patron ion of the major peak is at m/z 564.4. MS/MS result showed that the molecular weight of target peptide is 562.9 Da (Figure 5). This relative molecular mass was input to a Mass Lynx4.1 (Waters, USA) data processing system to obtain accurate molecular masses and amino acid sequences of the peptides, and the sequence was determined to be Gly-Lys-Phe-Asn-Val.

The targeted antioxidant peptide is a pentapeptide, which further proved that short-chain peptides with 2−10 amino acids have higher antioxidant activity than their parent proteins and polypeptides. Mora et al.[34] reported small peptides were released from muscle glycolytic enzymes during dry-cured ham processing, and 45 specific components resulting from the processing of dry-cured ham within the range of 1100−2600 Da were observed. During the long processing of Jinhua ham, Zhao et al.[35] have reported that proteins were hydrolyzed and small molecular products such as short-chain peptides with molecular weight <1 kDa and free amino acids were generated. In addition, in the long intense hydrolysis process, more active peptide segments were exposed and the effects of free radical scavenging activity and metal chelating ability were strengthened. In addition, the antioxidant activity of peptides is closely related to their amino acid sequence as well as hydrophobicity.[36] Studies reported that peptides containing one or several hydrophobic amino acids such as Val or Leu can increase the presence of the peptides at the water−lipid interface and therefore facilitate access to scavenge free radicals generated at the lipid phase.[37] In addition, metal-chelating amino acid residues such as Met, Glu, Gln, Lys, and Arg within the sequences of these peptides contributed to the superior Fe^{2+} chelating of the antioxidant peptides as well as their high radical scavenging potential. The peptide extracts exhibited great radical scavenging activity, and as the purifcation steps went on, a small increase was shown in the DPPH radical scavenging activity. Therefore, it has been postulated that the overall antioxidant activity may be ascribed to the integrative effects of these actions rather than to the individual actions of peptides. With regard to the high DPPH radical scavenging activity, Gly-Lys-Phe-Asn-Val could be the main peptide that plays a key role in the antioxidant activities of peptides extracted from Jinhua ham. In this study, the search for such a beneficial component led to a new antioxidant peptide from Jinhua ham. The results indicate that it is feasible that natural antioxidant peptides could be produced in Jinhua ham.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DPPH, 1,1-diphenyl-2-picrylhydrazyl; TFA, trifluoroacetic acid; $OH^{\bullet}$, hydroxyl radicals; BHT, butylated hydroxytoluene; RP-HPLC, reversed-phase high-performance liquid chromatography

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