JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Isolation and Characterization of an Oxygen Radical Absorbance Activity Peptide from Defatted Peanut Meal Hydrolysate and Its Antioxidant Properties

Lin Zheng, Guowan Su, Jiaoyan Ren, Longjian Gu, Lijun You, and Mouming Zhao*

College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, China

ABSTRACT: Defatted peanut meal hydrolysate (DPMH) was purified using ultrafiltration, gel filtration chromatography, and high-performance liquid chromatography. A tripeptide with strong oxygen radical absorbance capacity (ORAC) was isolated and identified as Tyr-Gly-Ser by ESI-MS/MS. It was then synthesized to measure its antioxidant properties in different systems. The ORAC value of Tyr-Gly-Ser was 3-fold higher than that of glutathione (GSH), and it displayed a stronger protective effect on linoleic acid peroxidation and H_2O_2-induced oxidative injury in rat pheochromocytoma line PC12 cells than GSH ($p < 0.05$). However, Tyr-Gly-Ser showed negligible DPPH radical scavenging activity, reducing power, and no metal chelating ability. The results suggested that Tyr-Gly-Ser displayed antioxidant activity via the hydrogen atom transfer mechanism, and the Tyr at the N-terminal was the hydrogen donor. The ORAC assay was recommended as a reliable and effective method to measure the antioxidant activity in the course of antioxidant peptide isolation.

KEYWORDS: *antioxidant peptide, peanut meal, hydrolysate, ORAC, radical scavenging activity, lipid peroxidation*

INTRODUCTION

Oxidative reactions could not only cause human disease but also cause lipid oxidation in food systems. Peptides derived from food proteins are potentially antioxidants by inactivating reactive oxygen species, scavenging free radicals, chelating prooxidative transition metals, and reducing hydroperoxides.[1] Antioxidant peptides usually contain 2−20 amino acid residues per molecule, and these peptides are inactive when in the parent peptide, being released during enzymatic proteolysis, microbial fermentation, gastrointestinal digestion, or food processing.[2] This could be due to an increase in the exposure of the amino acids, resulting in increased peptide-free radical reactions and the ability of the peptide to decrease the energy of the free radical.[1] In the past decade, many antioxidant peptides have been isolated from a variety of food proteins, such as soy protein,[3] hempseed protein,[4] royal jelly protein,[5] egg white protein,[6] Alaskan pollack frame protein,[7] grass carp muscle,[8] and loach protein.[9]

A wide range of methods, e.g., the DPPH radical scavenging activity assay, reducing power assay, oxygen radical absorbance capacity (ORAC) assay, and lipid peroxidation inhibition assay, have been commonly used to measure the in vitro antioxidant activity for these peptides. Since the mechanism for antioxidant action is different from one assay to another, a test sample may show different results depending on the assay used. Saito et al.[10] found that tripeptides containing two Tyr residues had high activity in preventing the peroxidation of linoleic acid, but it showed only moderate reducing activity, and the tripeptides containing Trp or Tyr residues at the C-terminal had strong radical scavenging activities but very weak peroxynitrite scavenging activity. Therefore, more than one assay should be used to measure the antioxidant activity of peptides. However, it is hard to balance the inconsistent results by different assays in the course of antioxidant peptide isolation. Thus, a commonly accepted and validated assay that can more closely reflect the in vivo antioxidant activity is necessary. The ORAC assay directly measures chain-breaking antioxidant capacity against peroxyl radicals, which is most relevant to the human body.[11] It is recommended to be one of the standardized methods for use in the routine quality control and measurement of the antioxidant capacity of dietary supplements and other botanicals.[12] However, there is still a need for more information to clarify if the antioxidant activity measured for peptide by ORAC value can be translated to its inhibition capacity of fatty acid autoxidation in an actual food system or/and its bioactivity in the human body.

Defatted peanut meal (DPM) is the main byproduct after the extraction of oil from peanut (*Arachis hypogaea* L.) seed, which contains 50−55% protein with high content of essential amino acid.[13] However, DPM is commonly used as animal feed and fertilizer at present. Peanut seeds are liable to be contaminated with aflatoxins, and the toxins are primarily retained in DPM after oil extraction processes. Moreover, DPM is often prepared from roasted peanut kernels during the oil extracting process, leading to high denaturation of its protein. Seifert et al.[14] designed a process to sequester aflatoxin from contaminated peanut meal by using a bentonite clay, Astra-Ben 20A, making it possible to apply peanut meal in enhanced feed and/or food applications. In recent years, some researchers have also worked on DPM to convert its protein into some antioxidant peptides by fermentation or enzyme hydrolysis.[13,15] However, there is still no information concerning the identification of any antioxidant peptides from DPM.

In our previous study, defatted peanut meal hydrolysate (DPMH) was found to possess good antioxidant activity by

Received: January 31, 2012
Revised: May 10, 2012
Accepted: May 11, 2012
Published: May 11, 2012

dx.doi.org/10.1021/jf3017173 | J. Agric. Food Chem. 2012, 60, 5431−5437

ORAC assay.[16] In this study, DPMH was purified using ultrafiltration, gel filtration chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC) to isolate the antioxidant peptide. The amino acid sequence of the purified peptide was determined by electron spray ionization tandem mass spectrometry (ESI-MS/MS). Furthermore, the peptide was synthesized and its antioxidant properties evaluated by different assays, including the ORAC value, DPPH radical scavenging activity, reducing power, metal ion chelating ability, protective effect on lipid peroxidation, and oxidative injury induced by H_2O_2 in PC12 cells.

MATERIALS AND METHODS

Materials. DPM was provided by Shandong Luhua Group Co., Ltd. (Yantai, China). DPM was generated from freshly prepared roasted peanut kernels during the oil pressing process. The peanut kernels were of high grade, and the aflatoxin concentration of DPM was 8.7 ppb, which is suitable for human consumption according to FDA regulations.[17]

1,1-Diphenyl-2-picrylhydrazyl (DPPH), glutathione (GSH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein disodium, 2,2′-azobis (2-methylpropionamide)-dihydrochloride (AAPH), glycine (Gly), ferrozine (the monosodium salt hydrate of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid), and Sephadex G-15 were purchased from Sigma-Aldrich (St. Louis, MO). Tyrosine (Tyr) and serine (Ser) were obtained from CapitalBio Corporation (Beijing, China). Linoleic acid was obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). The rat pheochromocytoma line PC12 cells were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cell culture media and all the other materials required for culturing the organisms were obtained from Gibco BRL, Life Technologies (Grand Island, NY). All other chemicals and reagents used were of analytical grade and obtained in China.

Preparation of Defatted Peanut Meal Hydrolysate (DPMH). DPMH was prepared as previously described.[16] It was centrifuged at 5,000*g* for 20 min at 4 °C, and the supernatant was collected, lyophilized, and stored at −20 °C until used.

Determination of Antioxidant Activities. Different in vitro methods were applied to evaluate the antioxidant activities of peptide.

DPPH Radical Scavenging Activity Assay. The DPPH radical scavenging activity was determined by the method of You et al.[9] Two milliliters of sample solution was mixed with 2.0 mL of 0.2 mM DPPH solution dissolved in 95% ethanol. The mixture was then shaken vigorously and kept for 30 min in the dark. The absorbance value was measured at 517 nm using a UV-2100 spectrophotometer (UNIC Equipment Co. Ltd., Shanghai, China). Ethanol instead of DPPH solution was used for the blank, while distilled water instead of sample solution was used for the control. The DPPH radical scavenging activity of the sample was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}] \times 100$$

where A_{sample}, A_{control}, and A_{blank} were the abosorbances of sample, control, and blank, respectively.

The plot of the scavenging activity against the concentration of sample was prepared, and the IC_{50} which was the effective concentration at which 50% of the DPPH radicals were scavenged was calculated by linear regression.

GSH is a naturally occurring antioxidant tripeptide that prevents damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides.[18] It was used as a positive control.

Reducing Power Assay. The reducing power was determined according to the method of Lu et al.[4] Briefly, 2.0 mL of sample was mixed with 2.0 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.0 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then, 2.0 mL of 10% trichloroacetic acid was added. After centrifugation at 3,000*g* for 10 min, 2.0 mL of the supernatant was collected and mixed with 2.0 mL of distilled water and 0.4 mL of 0.1% (w/v) $FeCl_3$. After keeping at room temperature for 10 min, we measured the absorbance spectrophotometrically at 700 nm. Higher absorbance indicated stronger reducing power. An equivalent volume of distilled water instead of the sample was used as the blank.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay was done according to Ou et al.[19] as modified by Davalos et al.[20] The reaction was done in 75 mM sodium phosphate buffer (pH 7.4), and the final assay mixture was 200 μL. Briefly, 20 μL of antioxidant and 120 μL of 70 nM fluorescein solution (final concentration) were placed in a well of a black 96-well microplate (Thermo Fisher Scientific, Waltham, MA). The mixture was preincubated for 15 min at 37 °C. Then, 60 μL of 12 mM AAPH solution (final concentration) was added rapidly using a multichannel pipet (Thermo Fisher Scientific, Waltham, MA). The plate was shaken for 30 s before the first reading, and the fluorescence was recorded using a Varioskan Flash Spectral Scan Multimode Plate Reader (Thermo Fisher Scientific, Waltham, MA) every minute for 100 min. The excitation and emission wavelengths were 485 and 520 nm, respectively. A blank using phosphate buffer instead of the antioxidant solution and calibration solutions using 1−6 μM Trolox (final concentration) as the antioxidant were used for each assay. Each sample was done at least in triplicate. Raw data were exported from the Fluostar Galaxy software to an Excel (Microsoft, Roselle, IL) spreadsheet for further calculations. Fluorescence measurements were normalized to the curve of the blank. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as follows:

$$\text{AUC} = 1 + \sum_{i=100}^{i=1} \frac{f_i}{f_0}$$

where f_0 is the initial fluorescence reading at 0 min, and f_i is the fluorescence reading at time *i*. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \text{AUC}_{\text{antioxidant}} - \text{AUC}_{\text{blank}}$$

The linear regression equation between the net AUC and the antioxidant concentration was calculated. The final ORAC values were expressed as μmol TE (Trolox equivalent)/g of antioxidant or μmol TE (Trolox equivalent)/μmol of antioxidant.

Metal Chelating Assay. Chelation of ferrous ions was measured according to the method of Pownall et al.[21] with slight modifications. A sample at a concentration of 5.0 mg/mL, 0.5 mL, was combined with 50 μL of 2 mM $FeCl_2$ solution and 2.35 mL of distilled water. Then, 0.1 mL of 5 mM ferrozine was added. After 10 min, the absorbance was measured at 562 nm. In the control, the peptide was replaced with distilled water. The ferrous chelating ability was calculated using the following equation:

$$\text{Ferrous ion chelating ability (\%)} = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100$$

Protective Effect on Lipid Peroxidation in a Linoleic Acid System. The protective effect on lipid peroxidation was measured in a linoleic acid model system according to the method of Je et al.[7] Briefly, a sample which was dissolved in 10 mL of 50 mM sodium phosphate buffer (pH 7.0) at a concentration of 0.5 mg/mL was added into 0.13 mL of linoleic acid dissolved in 10 mL of 99.5% ethanol. Then, the final volume was adjusted to 25 mL with distilled water. The mixed solution in a sealed screw-cap conical tube was incubated at 40 °C in the dark. The degree of linoleic acid oxidation was measured at 24-h intervals up to 7 days using the ferric thiocyanate method. Briefly, 0.1 mL of the reaction mixture was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 20 mM ferrous chloride in 3.5% HCl. After 3 min, the degree of color development, which represented linoleic acid oxidation, was measured at 500 nm.

Culture of PC12 Cells and the Viability Assay. PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum and 100 U/mL each of penicillin and

streptomycin in a fully humidified atmosphere in a 5% CO_2, 37 °C Excella Eco-70 incubator (New Brunswick Scientific, Edison, NJ). The culture medium was renewed after every three days. To determine the radical-induced cytotoxicity, cells were seeded in a 96-well plate at a concentration of 5×10^3 cells/well and preincubated with DMEM for 24 h. Cells were treated with various concentrations of antioxidant peptides and incubated for 12 h. Cellular oxidation was induced by treating the cells with 600 μM H_2O_2 for 2 h and then replacing with fresh DMEM. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method that assessed the ability of the succinate dehydrogenase in the cells to convert MTT into visible formazan crystals.[22] Cells in each well were rinsed with sodium phosphate buffer (pH 7.2), and the supernatant was discarded by the multichannel pipet, then 90 μL of DMEM and 10 μL of 0.5 mg/mL MTT (final concentration) were added and incubated at 37 °C in the dark for 4 h. Finally, the medium with MTT was replaced with 150 μL of dimethyl sulfoxide to solubilize the formazan crystals, and the absorption value was determined at 490 nm by using a Multiskan spectrum reader (Thermo Fisher Scientific, Vantaa, Finland). The relative cell viability was calculated by using the amount of MTT converted into formazan salt, and data were expressed as a percentage of the viability of the control culture.

Isolation and Purification of Antioxidant Peptides. DPMH was purified by the following steps.

Ultrafiltration. DPMH was separated into two fractions using an ultrafiltration membrane system (Millipore Co., Bedford, MA) using a 3 kDa molecular weight cutoff membrane, with DPMH-I being the fraction with MW > 3 kDa and DPMH-II being the fraction with MW < 3 kDa.

Gel Filtration Chromatography. The fraction DPMH-II was further purified using gel filtration chromatography on a Sephadex G-15 column previously equilibrated with distilled water. The eluted fractions were detected using an online STI UV 50199 spectrophotometer (Science Technology Co., Hangzhou, China) at 214 nm. The flow rate was 1 mL/min. Four fractions (F1, F2, F3, and F4) were obtained.

RP-HPLC. The fraction which had the highest antioxidant activity of all the fractions was further purified by RP-HPLC. The column used was a 150 mm × 10 mm i.d., 10 μm, XBridge pre-C18 (Waters, Milford, MA). The sample was eluted with a linear gradient of methanol containing 0.1% (v/v) trifluoroacetic acid from 5 to 50% [v/v] over 25 min at a flow rate of 2 mL/min. The elution peaks were monitored at 214 nm, and fractions were collected manually and lyophilized for the assay of antioxidant activity.

Peptide Identification. To identify the antioxidant peptide purified by RP-HPLC, its amino acid composition and sequence were determined.

Amino Acid Analysis. Amino acid composition was determined by an A300 auto amino acid analyzer (membra Pure, Bodenheim, Germany). Sample solution was filtrated through a 0.22 μm membrane filter and then directly injected into the analyzer for free amino acid determination. The total amino acid composition of the sample was determined after hydrolysis at 110 °C for 24 h with 6 M hydrochloric acid. Alkaline hydrolysis at 105 °C for 24 h with 4 M NaOH was also done for the determination of tryptophan level. The contents of different amino acids were expressed as mmol/g.

Amino Acid Sequence. The molecular mass and de novo peptide sequencing was done in the positive ion mode using both electrospray ionization−mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) using the quadrupole time-of-flight micromass spectrometer with capillary liquid chromatography capability and an electrospray ion source (Bruker Daltonics Inc., Billerica, MA). The sample was directly injected into the electrospray ion source. Spectra were recorded over the mass/charge (m/z) range of 100−1000. A precursor ion scan of the purified antioxidant fraction was used to identify the unique peptide mass, which was then fragmented using a low energy collision induced dissociation to determine the peptide fragment for de novo sequencing. The peptide sequencing was done by processing the MS/MS spectra using the computer program BioTools, version 3.0 (Bruker Daltonics Inc., Billerica, MA), and manual calculations.

Peptide Synthesis. The antioxidant peptide was synthesized using a high-efficiency solid-phase peptide synthesizer (Protein Technologies Inc., Tucson, AZ) by ChinaPeptides Co., Ltd. (Shanghai, China). The purity was verified by RP-HPLC-MS/MS. The synthesized peptide was stored at −20 °C until use.

Statistical Analysis. All sample determinations were done in triplicate, and the results are expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) using SPSS, version 13.0 (SPSS Inc., Chicago, IL). Significant differences were determined using the LSD range test ($p < 0.05$).

RESULTS AND DISCUSSION

Antioxidant Activities of DPMH. The antioxidant activities of DPMH were determined by different assays, and the results are shown in Table 1. The IC_{50} value of DPMH to scavenge the DPPH radical was 0.49 mg/mL, while the reducing power of DPMH at 1.0 mg/mL was 0.44. The ORAC value of DPMH was 1160 μmol TE/g. The DPPH free radical scavenging activity and reducing power are commonly thought to test the ability of donating electrons, i.e., to evaluate the antioxidant activity.[11] As for phenolic compounds, the DPPH assay is also considered to assess the hydrogen donating activity.[23] The ORAC assay is widely used to test the hydrogen atom donating activity.[11] It suggested that DPMH displayed potent antioxidant activities in different mechanisms, and it could be a good assay to isolate antioxidant peptide from DPMH.

Table 1. Antioxidant Activity of Defatted Peanut Meal Hydrolysate and Its Fractions from Ultrafiltration[a]

sample	reducing power[b] (A_{700})	DPPH (IC_{50}, mg/mL)	ORAC (μmol TE/g)
DPMH	0.44 ± 0.01 b	0.49 ± 0.01 c	1160 ± 26 b
DPMH-I (> 3 kDa)	0.69 ± 0.01 c	0.20 ± 0.01 b	1170 ± 29 b
DPMH-II (<3 kDa)	0.22 ± 0.01 a	1.19 ± 0.04 d	952 ± 69 a
GSH	2.03 ± 0.01 d	0.10 ± 0.01 a	1350 ± 47 c

[a]Different letters indicate significant differences between groups ($p < 0.05$). [b]The absorbance at 700 nm for samples at 1.0 mg/mL.

Isolation and Purification of Peptides. *Ultrafiltration.* As shown in Table 1, DPMH-I displayed stronger DPPH radical scavenging activity, reducing power, and ORAC value than DPMH-II ($p < 0.05$). The result was inconsistent with that of previous studies, which found that the peptides with MW < 3 kDa had higher antioxidant activity than other fractions.[8,9,24] In comparison with DPMH-II, DPMH-I was brown and flak, which had not been observed previously for protein hydrolysates. An absorbance at 420 nm (A_{420}) was measured to account for the brown pigment formation. The result showed that the A_{420} of DPMH-I at 1.0 mg/mL was 0.23, while that of DPMH-II was only 0.01, suggesting that the brown pigments (melanoidins) derived from the Maillard reaction product during the oil squeezing process was predominant in DPMH-I. Melanoidins were of interest not only because of their contribution to color formation but also for their strong antioxidant capacity.[25] This might be why the DPMH-I fraction showed higher antioxidant activity than DPMH-II. In addition, the protein yields of the two ultrafiltration fractions were 27.91% (DPMH-I) and 67.00% (DPMH-II), respectively.

The ORAC assay represented the hydrogen atom transfer reaction mechanism, which was most relevant to the human body as mentioned above. Therefore, it was feasible to adopt the ORAC assay to measure the antioxidant activity of the peptides at the following purification step.

Gel Filtration Chromatography. To purify antioxidant peptides, DPMH-II was initially separated into four fractions (F1−F4) by gel filtration chromatography on a Sephadex G-15 column (Figure 1). Each fraction was pooled and lyophilized,

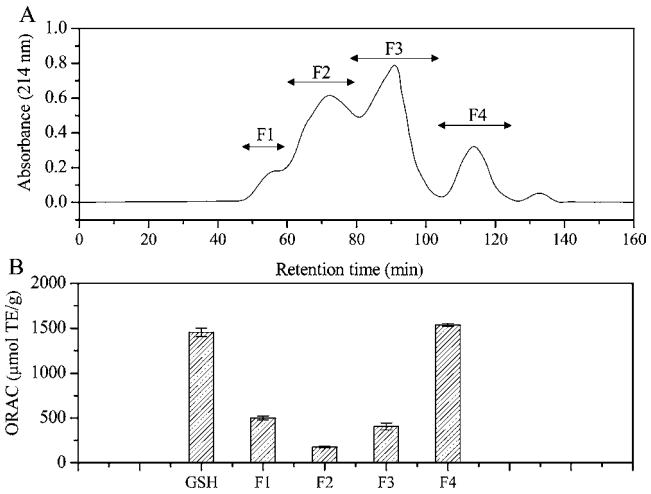

Figure 1. (A) Chromatography of DPMH-II separated by gel filtration on a Sephadex-G15 column. (B) ORAC value of each fraction.

and its ORAC value was evaluated. As shown in Figure 1, the ORAC value among the four fractions was in the order of F4 > F1 > F3 > F2. Except for F1, the ORAC value of the other three fractions increased as the retention time increased. Gel filtration chromatography separated the peptide on the basis of size. The fraction with longer retention time had lower molecular weight. It indicated that the ORAC value of peptide increased as the molecular weight decreased. F4 with the smallest molecular weight displayed the highest ORAC value with 1500 μmol TE/g, which was close to GSH. Light brown was observed in F1, probably due to the presence of some residual melanoidins from DPMH-II. Therefore, the ORAC value of F1 was relatively higher than that of F3 and F2.

RP-HPLC. Fraction F4 was further separated by RP-HPLC on an XBridge pre-C18 column. Five peaks were collected. The elution profile and the antioxidant activity of the peptides are shown in Figure 2. A clear difference for the ORAC value was observed among the five fractions, and peak P3 had the highest ORAC value with 4400 μmol TE/g, followed by P5, P1, P2, and P3, respectively.

Peptide Identification. *Amino Acid Analysis.* As shown in Table 2, there were in total seven amino acids in P3, including Ser, Gly, Ala, Ile, Leu, Tyr, and Phe. Among them, Ser, Gly, and Tyr were the main compositions, and the molar ratio of the main three amino acid residues was 1:1:1. There was also a small quantity of free amino acids (Ala, Ile, Leu, Phe, and Tyr) existing in P3.

Amino Acid Sequence. To identify the amino acid sequence, P3 was analyzed by ESI-MS for molecular mass determination and ESI-MS/MS for the characterization of peptides. The MS spectrum and the MS/MS spectrum of a single charged ion with *m/z* 326.5 are shown in Figure 3. The molecular mass of the antioxidant peptide was determined to be 325.5 Da.

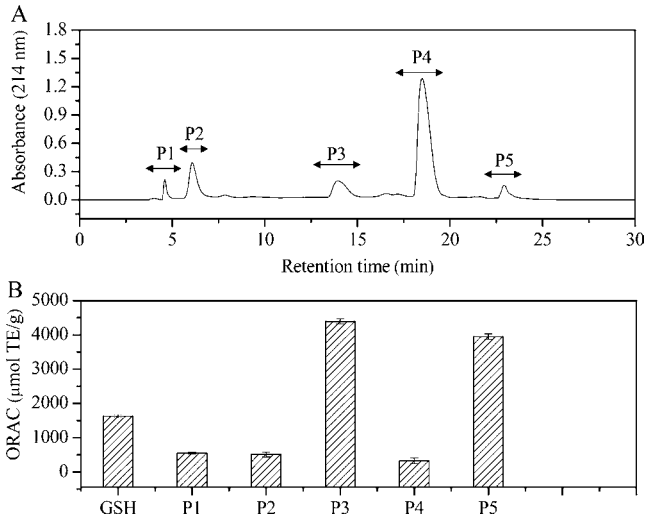

Figure 2. (A) Chromatography of the fraction F4 separated by semipreparing RP-HPLC. (B) ORAC value of each fraction.

Table 2. Free Amino Acid Composition and Total Amino Acid Composition of P3 (mmol/g)

	free amino acid composition	total amino acid composition
Ser	nd[a]	2.29 ± 0.05
Gly	nd	2.32 ± 0.05
Ala	0.16 ± 0.03	0.16 ± 0.04
Ile	0.17 ± 0.02	0.18 ± 0.05
Leu	0.08 ± 0.03	0.09 ± 0.03
Tyr	0.26 ± 0.05	2.56 ± 0.03
Phe	0.28 ± 0.04	0.27 ± 0.03

[a]nd, not determined.

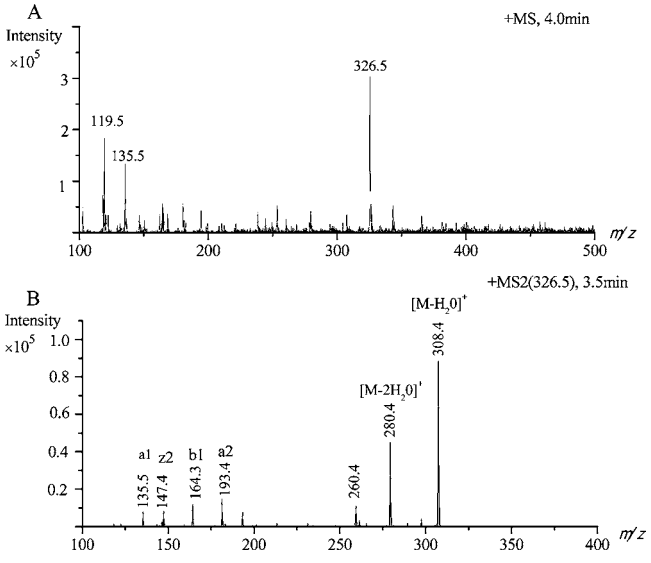

Figure 3. (A) Mass spectrum of the peak P3. (B) Collision induced fragmentation of *m/z* 325.5.

According to the manual calculation and data search, the purified peptide consisted of three amino acids, and the sequence was NH_2-Tyr-Gly-Ser-COOH (Tyr-Gly-Ser). The sequence of Tyr-Gly-Ser was located in f (229−231) of conarachin (SWISS-PROT accession number: Q647H1), which is a seed storage protein consisting of 662 amino acids.

dx.doi.org/10.1021/jf3017173 | J. Agric. Food Chem. 2012, 60, 5431−5437

Antioxidant Activities of Peptide. The purified antioxidant peptide, Tyr-Gly-Ser, was chemically synthesized using solid phase synthesis. The purity of the synthesized peptide was more than 95%, and the molecular weight was 325.3 Da (data not shown). Its antioxidant activity was further evaluated, including the ORAC value, DPPH radical scavenging activity, reducing power, metal ion chelating activity, protective effect on lipid peroxidation, and oxidative injury induced by H_2O_2 in PC12 cells.

ORAC Value. The ORAC value of Tyr-Gly-Ser was 1.82 μmol TE/μmol, which was 3-fold higher than that of GSH (0.45 μmol TE/μmol). Its constitutive amino acids were also determined for the ORAC value, Tyr (2.74 μmol TE/μmol), Gly (0.01 μmol TE/μmol), and Ser (<0.01 μmol TE/μmol). Tyr has been recognized as a good hydrogen donor due to the phenolic hydroxyl group.[26] Therefore, the peroxyl radical scavenging activity of Tyr-Gly-Ser could mainly be attributed to the presence of the Tyr residue donating a hydrogen atom from its phenolic hydroxyl group. However, the ORAC value of Tyr-Gly-Ser was lower than that of Tyr. In addition, the ORAC value of an equimolar amount of the three constitutive free amino acids mixture was 2.81 μmol TE/μmol, which was close to that of Tyr. It indicated that there was no antagonist effect on the Tyr from the other two free amino acids. Therefore, the peptide bond or the peptide conformation might have attenuated the antioxidant activity of the Tyr residue for Tyr-Gly-Ser. Similarly, Hernandez-Ledesma et al.[26] also observed that the ORAC value of Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile (2.62 μmol TE/μmol) derived from β-lactoglobulin was lower than that of the constitutive amino acid Trp (4.65 μmol TE/μmol) and an equimolar mixture of the constitutive amino acid mixture (4.31 μmol TE/μmol).

DPPH Radical Scavenging Activity and Reducing Power. Although Tyr-Gly-Ser had high ORAC value, Tyr-Gly-Ser displayed negligible DPPH radical scavenging activity and reducing power (data not shown). The DPPH radical scavenging activity of the phenolic compound was dependent on the number of hydroxyl groups bonded to the aromatic ring providing hydrogen atoms.[23,27] Though Tyr-Gly-Ser contained a phenolic hydroxyl group within the tyrosine residue, it had negligible DPPH radical scavenging activity. This could be due to having only one phenolic hydroxyl group, while at least two hydroxyl groups were required for the antioxidant activity of phenolic acids as suggested previously.[28] However, the position of the phenolic hydroxyl group and the other substituents could affect DPPH radical scavenging activity.[27] In Tyr-Gly-Ser, the Tyr residue at the N-terminal and the other two residues might play a negative role in scavenging the DPPH radical.

Metal Ion Chelating Activity. The ferrous ion chelation of Tyr-Gly-Ser and GSH were also determined. However, neither of them showed ferrous chelating activity. Pownall et al.[21] also did not observe metal chelating activity for GSH and suggested that the presence of Cys in GSH was not important in chelating metal ions. They also pointed out that the presence of aromatic rings in peptide fractions might be a contributing factor to higher metal chelation activities. However, Tyr-Gly-Ser containing the aromatic amino acid Tyr at the N-terminal did not show any metal chelating activity. Guo et al.[5] found that three dipeptides containing Tyr (Lys-Tyr, Arg-Tyr, and Tyr-Tyr) at the C-terminal among 12 small peptides derived from royal jelly protein had metal chelation activity but that there was no difference among them. For Tyr-Tyr, it had an extra Tyr residue at the N-terminal, but it did not show higher metal chelating ability in comparison with the other two dipeptides. For Tyr-Gly-Ser, it had a Tyr at the N-terminal and showed no metal chelating activity. These results suggested that an N-terminal Tyr residue was not important in chelating metal ions.

Protective Effect of Tyr-Gly-Ser on Lipid Peroxidation in a Linoleic Acid System. Tyr-Gly-Ser was further assessed for the ability to protect linoleic acid against peroxidation. As shown in Figure 4, the level of linoleic acid peroxides in the control

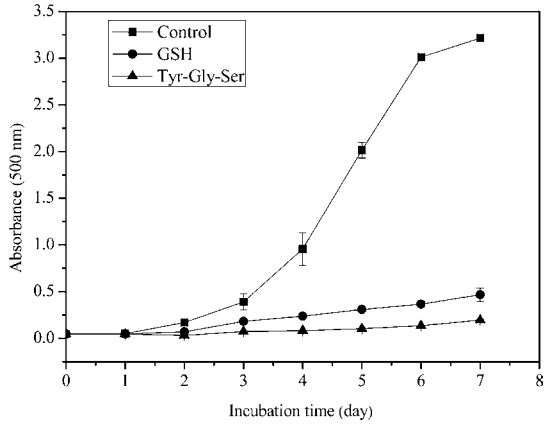

Figure 4. Protective effect of Tyr-Gly-Ser on lipid peroxidation in a linoleic acid system measured by the ferric thiocyanate method for 7 days.

increased slowly in the first 3 days, but increased rapidly from day 4, and reached the highest concentration by day 7. However, the oxidation of linoleic acid was markedly protected by the addition of GSH or Tyr-Gly-Ser over 7 days, the lipid peroxidation inhibitory ratio of Tyr-Gly-Ser was 91% at the seventh day, which was higher than that of GSH (83%). As far as Tyr-Gly-Ser was concerned, it contained two hydrophobic amino acids in its sequence (Tyr and Gly), which might increase the presence of the peptides at the water−lipid interface and thereby facilitate access to scavenge hydrophobic radical species or free radicals generated in the lipid phase.[11] Rajapakse et al.[29] found that the hydrophobic amino acids in the peptide (His-Phe-Gly-Asp-Pro-Phe-His) derived from fermented mussel sauce might contribute to its peroxidation inhibition. Mendis et al.[30] identified a radical-scavenging peptide (GRSP) from fish skin gelatin hydrolysate and also pointed out that the antioxidant activity of GRSP in the linoleic acid proxidation system was due to the hydrophobic amino acids of the residues. In addition, the Tyr in Tyr-Gly-Ser might interfere with the propagation cycle of lipid peroxidation by donating a hydrogen atom and thereby slowing radical mediated linoleic acid oxidation.

Protective Effect on Oxidative Injury Induced by H_2O_2 in PC12 Cells. To evaluate the antioxidant activity of Tyr-Gly-Ser within the cellular environment, cultured PC12 cells were pretreated with different concentrations of Tyr-Gly-Ser for 12 h, washed, and then exposed to H_2O_2 for 2 h. The ability of the Tyr-Gly-Ser to protect the cells against oxidative injury was assessed using the MTT assay. The untreated PC12 cells showed long fibriform shapes and were closely connected. After being exposed to H_2O_2, most of the cells were round-shaped, and cell viability was only 11% (Figure 5). However, when the cells were preincubated with Tyr-Gly-Ser, cell viability was enhanced in a dose dependent manner within the tested concentration range. At a low concentration of 50 μg/mL, Tyr-

dx.doi.org/10.1021/jf3017173 | J. Agric. Food Chem. 2012, 60, 5431−5437

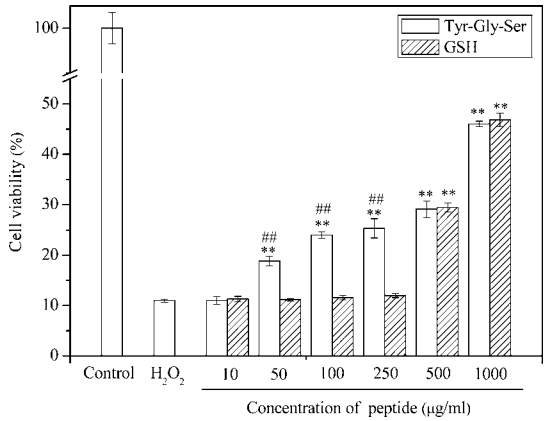

Figure 5. Protective effect of Tyr-Gly-Ser on the oxidative injury induced by H_2O_2 in PC12 cells. The control group represents the untreated PC12 cells; the H_2O_2 treated group represents cells incubated with 600 μM H_2O_2 for 2 h; the peptide treated groups represent cells pretreated with Tyr-Gly-Ser or GSH at various concentrations for 12 h and then treated with 600 μM H_2O_2 for 2 h. **Significant difference in cell viability between the H_2O_2 treated group and peptide treated groups ($p < 0.01$). ##Significant difference in cell viability between the Tyr-Gly-Ser treated groups and the GSH treated groups ($p < 0.01$).

Gly-Ser could significantly enhance cell viability ($p < 0.01$). Compared to GSH, Tyr-Gly-Ser was more effective against oxidative injury ($p < 0.01$) at the concentration ranging from 50 μg/mL to 250 μg/mL, while there was no significant difference between them when the concentration was 500 μg/mL or 1000 μg/mL ($p < 0.05$). These results showed that Tyr-Gly-Ser could protect PC12 cells from oxidant injury induced by H_2O_2 more effectively than GSH at a low concentration.

In conclusion, a tripeptide was isolated from defatted peanut meal hydrolysate and identified as Tyr-Gly-Ser. It displayed a 3-fold higher ORAC value than GSH. It was also shown to possess high antioxidant activity in protecting linoleic acid against peroxidation and protecting PC12 cells against oxidant injury induced by H_2O_2. However, the peptide showed negligible DPPH radical scavenging activity and reducing power, and no metal ion chelating activity. Present results suggest that different assays for evaluating antioxidant activity differ greatly. The ORAC assay is recommended to be a reliable and effective method to measure the antioxidant activity in the course of antioxidant peptide isolation. The Tyr at the N-terminal within the tripeptide is of great importance to the hydrogen atom donating activity. Further studies need to be done to clarify the different antioxidant mechanisms of Tyr at different positions within the peptide and the connection of ORAC assay to other in vitro biological assays and in vivo assays for assessing antioxidant activity.

AUTHOR INFORMATION

Corresponding Author

*Tel: +86 20 87113914. Fax: +86 20 87113914. E-mail: femmzhao@scut.edu.cn.

Funding

We gratefully acknowledge the National Natural Science Foundation of China (No. 31171783 and No. 31101222), Key Technology Foundation of Guangdong Province (2011A080403019), and Research Cooperation Project of Guangdong Province and the Ministry of Education (2011A090200078) for financial support.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Elias, R. J.; Kellerby, S. S.; Decker, E. A. Antioxidant activity of proteins and peptides. *Food Sci. Nutr.* **2008**, *48*, 430−441.

(2) Samaranayaka, A. G. P.; Li-Chan, E. C. Y. Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *J. Funct. Foods.* **2011**, *3*, 229−254.

(3) Chen, H. M.; Muramoto, K.; Yamauchi, F. Structural analysis of antioxidative peptides from soybean β-conglycinin. *J. Agric. Food Chem.* **1995**, *43*, 574−578.

(4) Lu, R. R.; Qian, P.; Sun, Z.; Zhou, X. H.; Chen, T. P.; He, J. F.; Zhang, H.; Wu, J. P. Hempseed protein derived antioxidative peptides: Purification, identification and protection from hydrogen peroxide-induced apoptosis in PC12 cells. *Food Chem.* **2010**, *123*, 1210−1218.

(5) Guo, H.; Kouzuma, Y.; Yonekura, M. Structures and properties of antioxidative peptides derived from royal jelly protein. *Food Chem.* **2009**, *113*, 238−245.

(6) Huang, W. Y.; Majumder, K.; Wu, J. Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interaction with phytochemicals. *Food Chem.* **2010**, *123*, 635−641.

(7) Je, J. Y.; Park, P. J.; Kim, S. K. Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Food Res. Int.* **2005**, *38*, 45−50.

(8) Ren, J.; Zhao, M.; Shi, J.; Wang, J.; Jiang, Y.; Cui, C.; Kakuda, Y.; Xue, S. J. Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chem.* **2008**, *108*, 727−736.

(9) You, L. J.; Zhao, M. M.; Regenstein, J. M.; Ren, J. Y. Purification and identification of antioxidative peptides from loach (*Misgurnus anguillicaudatus*) protein hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Res. Int.* **2010**, *43*, 1167−1173.

(10) Saito, K.; Jin, D. H.; Ogawa, T.; Muramoto, K.; Hatakeyama, E.; Yasuhara, T.; Nokihara, K. Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry. *J. Agric. Food Chem.* **2003**, *51*, 3668−3674.

(11) Huang, D. J.; Ou, B. X.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.

(12) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.

(13) Zhang, Y. W.; Zhang, H.; Wang, L.; Guo, X. N.; Qi, X. G.; Qian, H. F. Influence of the degree of hydrolysis (DH) on antioxidant properties and radical-scavenging activities of peanut peptides prepared from fermented peanut meal. *Eur. Food Res. Technol.* **2011**, *232*, 941−950.

(14) Seifert, L. E.; Davis, J. P.; Dorner, J. W.; Jaynes, W. F.; Zartman, R. E.; Sanders, T. H. Value-added processing of peanut meal: Aflatoxin sequestration during protein extraction. *J. Agric. Food Chem.* **2010**, *58*, 5625−5632.

(15) Hwang, J. Y.; Shyu, Y. S.; Wang, Y. T.; Hsu, C. K. Antioxidative properties of protein hydrolysate from defatted peanut kernels treated with Esperase. *LWT-Food Sci. Technol.* **2010**, *43*, 285−290.

(16) Su, G. W.; Zheng, L.; Cui, C.; Yang, B.; Ren, J. Y.; Zhao, M. M. Characterization of antioxidant activity and volatile compounds of Maillard reaction products derived from different peptide fractions of peanut hydrolysate. *Food Res. Int.* **2011**, *44*, 3250−3258.

(17) Administration, U. S. F. A. D. Guidance for industry: Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ChemicalContaminantsandPesticides/ucm077969.htm (accessed Apr 30, 2012).

(18) Pompella, A.; Visvikis, A.; Paolicchi, A.; Tata, D. V.; Casini, A. F. The changing faces of glutathione, a cellular protagonist. *Biochem. Pharmacol.* **2003**, *66*, 1499−503.
(19) Ou, B. X.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619−4626.
(20) Davalos, A.; Gomez-Cordoves, C.; Bartolome, B. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *J. Agric. Food Chem.* **2004**, *52*, 48−54.
(21) Pownall, T. L.; Udenigwe, C. C.; Aluko, R. E. Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) enzymatic protein hydrolysate fractions. *J. Agric. Food Chem.* **2010**, *58*, 4712−4718.
(22) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(23) Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615−622.
(24) Rajapakse, N.; Mendis, E.; Byun, H.-G.; Kim, S.-K. Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *J. Nutr. Biochem.* **2005**, *16*, 562−569.
(25) Bekedam, E. K.; Schols, H. A.; Van Boekel, M. A. J. S.; Smit, G. High molecular weight melanoidins from coffee brew. *J. Agric. Food Chem.* **2006**, *54*, 7658−7666.
(26) Hernandez-Ledesma, B.; Davalos, A.; Bartolome, B.; Amigo, L. Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *J. Agric. Food Chem.* **2005**, *53*, 588−593.
(27) Sroka, Z.; Cisowski, W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food Chem. Toxicol.* **2003**, *41*, 753−758.
(28) Dziedzic, S. Z.; Hudson, B. J. F. Hydroxy isoflavones as antioxidants for edible oils. *Food Chem.* **1983**, *11*, 161−166.
(29) Rajapakse, N.; Mendis, E.; Jung, W. K.; Je, J. Y.; Kim, S. K. Purification of radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Res. Int.* **2005**, *38*, 175−182.
(30) Mendis, E.; Rajaparkse, N.; Kim, S. K. Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *J. Agric. Food Chem.* **2005**, *53*, 581−587.