

Influence of the degree of hydrolysis (DH) on antioxidant properties and radical-scavenging activities of peanut peptides prepared from fermented peanut meal

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Abstract To study the influence of degree of hydrolysis (DH) on antioxidant properties of peanut peptides, the peanut meal was fermented by *Bacillus subtilis*. The fermentation time was 48, 72, and 96 h, respectively, to prepare peanut peptides at different degree of hydrolysis. The peanut peptides (11.18, 16.20, and 21.41% of DH, respectively) were extracted from fermentation liquid. The antioxidant properties of these peanut peptides were evaluated based on DPPH[•] radical (1,1-Diphenyl-2-picrylhydrazyl radical) scavenging activity, superoxide anion-scavenging activity, reducing power, metal-chelating activity, and inhibition of linoleic acid autooxidation. Peanut peptides (21.41% of DH) at 1 mg/mL exhibited 80.86 and 29.35% of scavenging concentration percent on DPPH[•] and superoxide anion radical, respectively. In addition, the reducing power of peanut peptides was 0.368 at 2 mg/mL, and they possessed 76.32% of Fe²⁺-chelation ability at 2 mg/mL and 63.75% of inhibition of linoleic acid autooxidation at 0.8 mg/mL. The antioxidant activities of the peanut peptides (21.41% of DH) were stronger compared with others (11.18 and 16.20% of DH), and this indicated the antioxidant activities of peanut peptides increased with increasing DH ($p < 0.05$). To know much about the peanut peptides, they were subjected to amino acid analysis and determination of molecular weight distribution. Some acidic amino acids and essential amino acids were found, and the average molecular weight distributions were concentrated in <1,400 Da (86.96%).

Combined with the results of the amino acid profiles, the peanut peptides were believed to have high nutritive value besides antioxidant activities.

Keywords Degree of hydrolysis · Antioxidant properties · Peanut meal · *Bacillus subtilis* · Fermentation · Molecular weight

Introduction

Free radicals can be defined as molecules with an unpaired electron in the outer orbit [1, 2]. They are generally unstable and very reactive [3]. Free radicals and reactive oxygen species (ROS) including superoxide anion radicals (O₂^{•-}), hydroxyl radicals (OH[•]), and hydrogen peroxide (H₂O₂) are highly reactive and potentially damaging transient chemical species formed in aerobic life [4]. The human body possesses many defensive mechanisms against oxidative stress, including antioxidant enzymes and non-enzyme compounds [5]. However, sometimes these repair mechanisms fail to keep pace with such deleterious effects [6–8]. As a result, the excess of free radicals can damage both the structure and function of a cell membrane in a chain reaction leading to degenerative diseases and conditions such as Alzheimer, aging process, cataracts, acute liver toxicity, cardiovascular disease, arteriosclerosis, nephritis, diabetes mellitus, rheumatism, inflammation process, and DNA damage that can lead to carcinogenesis [6].

Free radicals not only cause human disease but also cause lipid oxidation in food system. Oxidation of lipids, which is the main cause of quality deterioration in many food systems, may lead to the development of undesirable off-flavors and formation of some toxic compounds and may lower the quality and nutritional value of foods [9].

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Antioxidants can be defined as compounds which can inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [10]. Antioxidants can protect human body from free radicals and ROS damage [9]. The antioxidant activity of plasma has been shown to increase after consumption of food rich in antioxidants [11]. Antioxidants can protect food from lipid oxidation initiated by free radicals and ROS. In the food industry, synthetic antioxidants are often used because they are effective and cheaper than natural antioxidants. They can increase the shelf life of foods by 15–200%, allowing food to be transported and stored for long periods [12]. At the present time, the most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) [9]. However, use of such synthetic compounds has been related to health risks resulting in strict regulations over their use in food products [13]. Because of health concerns, people are looking for natural or origin-natural alternates for synthetic antioxidants.

In recent years, the use of natural protein hydrolysate as antioxidants has increasingly attracted particular interest. Many kinds of food proteins hydrolysates, including capelin protein hydrolysate, egg-yolk protein hydrolysate, Alaska Pollack frame protein hydrolysate, hoki frame protein hydrolysate, chickpea protein hydrolysate, grass carp muscle hydrolysate, alfalfa leaf protein hydrolysate, rice endosperm protein hydrolysate, rotifer protein hydrolysate, whey protein hydrolysate, and sardinelle protein hydrolysate, have been reported to possess antioxidant activity [14–24]. Peanut (*Arachis hypogaea*) is an important crop, which is grown in China and worldwide [25]. In 2007, Chinese export of peanut was about 673.4 million kilogram [26]. In China, out of the total production, 50% is used for peanut oil industry and the rest is consumed in other ways. Because of a large proportion of peanut consumed in the form of peanut oil, thousands of tons of peanut meals are generated every year. The peanut meal is an inexpensive and underutilized byproduct of the peanut oil industry, which contains 50–55% protein [25].

Bacillus subtilis, known also as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium commonly found in soil. *Bacillus subtilis* is an important starter culture for fermented soybean foods like Japanese natto, Indian kinema, Thai thuanao, and Chinese douchi [27]. Zhu et al. [28] have reported that *Bacillus subtilis* fermentations of soybean are characterized by extensive hydrolysis of soybean protein to amino acids, peptides, and ammonia, resulting in improvement of the functionality, such as antioxidant properties. In this study, we used *Bacillus subtilis* to ferment the peanut meal to prepare peanut peptides and determined the antioxidant activity of

peanut peptides, in order to find its potential value in food industry.

Materials and methods

Material and chemicals

Peanut meal was kindly provided by Luhua Group, Shandong Province, China. The peanut meal was ground to a fine powder in a mill. The powder, which passed through a 50-mesh sieve, was stored at 4 °C in a sealed plastic bag until use. DPPH and ferrozine were obtained from Sigma-Aldrich. All other chemicals used were of analytical grade made in China.

Microorganisms, culture condition, and inoculum

A freeze-dried culture of *Bacillus subtilis* was purchased from China Center of Industrial Culture Collection (CICC). The *B. subtilis* strain was rejuvenated and maintained on nutrient agar slope at 4 °C. The inoculum was prepared by adding a loop full of cells to 100 mL sterile nutrient broth (NB) medium, which contained 5 gL⁻¹ beef extract, 30 gL⁻¹ peptone, 5 gL⁻¹ NaCl, and 1 gL⁻¹ glucose at pH 7.2, and then the nutrient broth was incubated in an air bath shaker for 24 h at 30 °C. The cells from actively growing *Bacillus subtilis* were harvested by centrifugation at 4,000 rpm for 20 min at room temperature, resuspended in sterilized saline. After adjusting to a concentration of 10⁸ colony-forming units (cfu)/mL, the cell suspension was used to inoculate peanut meal for fermentation.

Fermentation of peanut meal and extraction of peanut peptides

Fermentation of peanut meal

Peanut meal (40 g), distilled water (800 mL), NaCl (4 g), and glucose (0.8 g) were placed into each of several 1,000-mL-capacity screw-capped bottles, adjusted pH value by adding 0.1 M NaOH solution to 7.2, and then sterilized at 115 °C for 30 min in an autoclave. After cooling to room temperature, the peanut meal was inoculated quickly with *Bacillus subtilis* inoculum prepared in Section of Microorganisms, culture condition, and inoculum.

The ratio of inoculation was 2% (v/v). The inoculated peanut meal was incubated at 32.6 °C with 140 rpm of agitation in an air bath shaker for fermentation. The fermentation time was 48, 72, and 96 h, respectively, in order to obtain peanut peptides at different degree of hydrolysis.

Extraction of peanut peptides

The fermented peanut meal was boiled for 10 min in an autoclave to passivate the enzyme activity and then centrifuged at 4,000 rpm for 20 min. Each supernatant was filtered through a 0.45- μ m membrane under vacuum. The filtrates were concentrated at 40 °C on a rotary evaporator and freeze-dried. Prior to use, the raw peanut peptides were kept at 4 °C.

Determination of degree of hydrolysis (DH)

The degree of hydrolysis (DH) of peanut peptides was determined according to ninhydrin colorimetry [29, 30] with tiny modification. Supernatant (0.2 mL) prepared in Section of Extraction of peanut peptides was placed into a 100-mL-capacity volumetric flask, added distilled water to 100 mL, and mixed thoroughly. One mL of diluted supernatant, 1 mL of distilled water, 3 mL of solution ninhydrin (1.2 g of ninhydrin, 15 mL of n-propanol, 30 mL of n-butyl alcohol, 60 mL of ethylene glycol and 9 mL of pH 4.5 acetic acid buffer/114 mL), and 0.1 mL of 1% (w/w) ascorbic acid were mixed in a clean glass tube, heated in boiling water for 15 min. Took the tube from boiling water, shook it, and cooled it to room temperature. Added 60% (v/v) ethanol to make sure that the total volume of mixture was 5 mL, mixed thoroughly. The absorbance of the mixture was read on a spectrophotometer at 580 nm, and distilled water was used to calibrate the spectrophotometer. With the help of the absorbance, the number of free amido (-NH₂) per mL of supernatant could be calculated via the calibration curve of ninhydrin colorimetry. The degree of hydrolysis (DH) was calculated as the following equation:

$$\text{DH (\%)} = (A_1 - A_2)/(A_3 - A_2) \times 100$$

A_1 —total free amido (mmol) in supernatant;

A_2 —originally existing free amido (mmol) in peanut meal;

A_3 —total free amido (mmol) of peanut meal hydrolyzed by 6 M HCl at 110 °C for 24 h.

Determination of DPPH[•] radical-scavenging capacity

The DPPH[•] radical-scavenging capacity of peanut peptides was determined according to the method [31, 32] with minor modification. Distilled water was used to prepare the solution of peanut peptides in all experiments. Samples (2 mL) at different concentrations were mixed with 2 mL of a 0.2 mM DPPH[•] in ethanol. The mixture was shaken immediately and allowed to stand at room temperature in the dark for 30 min. The absorbance was read against a blank control at 517 nm. Pure ethanol was used to calibrate

the spectrophotometer. SC% (Scavenging concentration percent) on DPPH[•] radical was calculated as given below:

$$\text{SC\%} = [1 - (A_{\text{sample}}/A_{\text{blank}})] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the sample), and A_{sample} is the absorbance of the tested sample. The sample concentration providing 50% scavenging concentration (SC₅₀) was calculated from the graph of scavenging percentage against sample concentration. BHT and reduced glutathione (GSH) were used as control. All tests were carried out thrice.

Superoxide anion-scavenging activity

The superoxide anion radical-scavenging effect was determined according to the method described by Xiao et al. [33]. The superoxide anion was produced by ammonium persulfate-N, N, N', N'-tetramethylethylenediamine (AP-TEMED). One mL of solution A (48 mL of 1 M HCl, 36.6 g Tris, and 0.23 mL of TEMED/100 mL) and 4 mL of solution B (AP, 0.14 g/100 mL) were mixed and reacted for 1 min, added 1 mL of peanut peptides sample (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL, respectively). Transferred 2-mL mixture to a clean tube, added 0.4 mL of 10 mM hydroxylammonium chloride, and reacted at 25 °C for 20 min. Then, added 2 mL of 17 mM 4-aminobenzenesulfonic acid and 2 mL of 7 mM α -aminonaphthalene to the tube above, mixed thoroughly, and reacted at 25 °C for 20 min. Extracted the mixture with the same volume n-butanol, transferred the n-butanol, and read at 530 nm on a spectrophotometer. SC% (Scavenging concentration percent) on superoxide anion was calculated as the following equation:

$$\text{SC\%} = [1 - (A_{\text{sample}}/A_{\text{blank}})] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the sample), and A_{sample} is the absorbance of the tested sample. All tests were carried out for three times.

Reducing power

The reducing power was determined according to the method described by Kaur et al. [34]. Two milliliter of peanut peptides of varying concentrations was mixed with 5 mL of phosphate buffer (200 mM, pH 6.6) and 5 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Afterward, a volume of 5 mL of 10% trichloroacetic acid (TCA) was added to the mixture and centrifuged at 3,000 rpm for 10 min. Two mL of supernatant was mixed with 1 mL of distilled water and 0.5 mL of FeCl₃ solution (0.1%, w/v), and the absorbance

was read at 700 nm in a spectrophotometer. Increased absorbance values indicate a higher reducing activity.

Metal-chelating capacity (MCC)

The metal-chelating capacity on ferrous ions was determined according to the method described by Giménez et al. [35] with tiny modification. In brief, a test sample of 3 mL was mixed with 50 µL of 2 mM FeCl₂·4H₂O, 750 µL distilled water, and 200 µL of 5 mM ferrozine. The mixture was vortexed and allowed to stand at room temperature for 10 min prior to measure the absorbance at 562 nm. Ethylene diamine tetraacetic acid (EDTA) and reduced glutathione (GSH) were used as control. The chelating ability, expressed as %, was calculated as equation given below:

$$\text{Chelating ability (\%)} = \left\{ \left[1 - \left(A_{\text{sample}} / A_{\text{control}} \right) \right] / A_{\text{max}} \right\} \times 100$$

where A_{sample} is the absorbance of test sample, A_{control} is the absorbance of the control, consisted of a mixture composed by 3 mL water, 50 µL of 2 mM FeCl₂, 750 µL distilled water, and 200 µL 5 mM ferrozine, and A_{max} is the absorbance of EDTA at maximum concentration tested. All tests were carried out three times.

Inhibition of linoleic acid autoxidation

The antioxidant activities of peanut peptides were determined by the method described by Osawa and Namiki [36] with some modifications. Two mL of sample (0.4, 0.6, and 1.0 mg/mL, respectively), 2 mL of 2.5% (v/v) linoleic acid prepared with pure ethanol, 4 mL of 0.05 M phosphate buffer (pH 7.0), and 2 mL of distilled water were placed into a glass tube sealed tightly with silicon rubber cap and kept at 40 °C in the dark for 1 week. The degree of linoleic acid oxidation was measured by the method described by Chen, Muramoto, and Yamauchi [37]. The sample solution (0.5 mL) incubated in the linoleic acid model system described above was mixed with 3.5 mL of 75% ethanol, 0.5 mL of 30% ammonium thiocyanate, and 0.5 mL 20 mM ferrous chloride dissolved in 1 M HCl. Mixed the solution thoroughly, and 3 min later, the absorbance was read on a spectrophotometer at 500 nm. Butylated hydroxytoluene (BHT) was also assayed at the same concentration as control.

Determination of amino acid composition and molecular weight distribution of peptides with 21.41% of DH

Sample of peanut peptides (150 mg) was subjected to acid hydrolysis with 8 mL of HCl (6 M) at 110 °C for 24 h

under nitrogen atmosphere. Reversed phase high-performance liquid chromatography analysis was carried out in an Agilent 1100 (Agilent Technologies Inc., USA) assembly system after precolumn derivatization with *o*-phthaldialdehyde (OPA). One µL of sample was injected into a Zorbax 80 A C18 column (4.6 × 180 mm, Agilent Technologies, Inc., USA) at 40 °C with detection at 338 nm. Mobile phase A was 7.35 mM sodium acetate/triethylamine/tetrahydrofuran (500/0.12/2.5, v/v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mM sodium acetate/methanol/acetonitrile (1/2/2, v/v/v). The amino acid composition was expressed as g of amino acid per 100 g of peanut peptides sample. Molecular weight distribution was determined using a Waters 600 high-performance liquid chromatography (HPLC) system, with TSK gel column (2000 SWXL, 300 mm × 7.8 mm), in combination with 2,487 UV detector and M32 work station, the wavelength of detection was at 220 nm. Elution was acetonitrile/water/trifluoroacetic acid (45/55/0.1) at the flow rate of 0.5 mL/min at 30 °C. The results were processed with Waters Empower 2 Software.

Statistical analysis

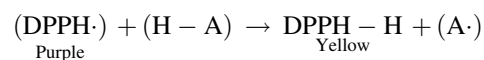
All the tests were carried out thrice, and the data were averaged. Standard deviation was also calculated. Duncan's new multiple-range test was used to determine the differences of means. *P* values < 0.05 were regarded as significant.

Results and discussion

Scavenging effect of DPPH[·] radical

DPPH[·] is a stable existing free radical, which shows maximal absorbance at 517 nm in ethanol. And DPPH[·] assay has been widely used to provide basic information on the antioxidant ability of extracts from plants, food material, or single compounds, because this method has been proved to be rapid and simple available.

This method depends on the decrease in the purple DPPH[·] by catching hydrogen from antioxidant (A–H) to a stable diamagnetic molecule (yellow-colored diphenyl picrylhydrazine) [5]. And the reaction between DPPH[·] and an antioxidant (H–A) can be described as follows:



The degree of color change from purple to yellow at different concentrations was spectrophotometrically measured at 517 nm. The degree of discoloration indicated the scavenging potential of the antioxidant compounds or

extracts in the term of hydrogen-donating ability [38]. In our DPPH test, the peanut peptides reduced the DPPH to a yellow-colored compound, apparently due to the DPPH radical catching an electron or hydrogen to become a stable diamagnetic molecule. The scavenging effect (Fig. 1) followed this order: 80.86% (21.41% of DH) > 61.82% (16.20% of DH) > 47.80% (11.18% of DH) at the concentration of 1 mg/mL. The SC% increased significantly ($p < 0.05$) with increasing DH. BHT and GSH were used as references. Figure 1 shows the scavenging effect clearly. The result suggested that peanut peptides of 21.41% of DH possibly possessed more active groups, which were electron or hydrogen donors and could react with free radicals to convert them to less harmful or unarmful products and break the radical chain reaction. In addition, some other components in peanut meal, such as phenolic compounds and flavonoids, and some bacterial metabolites excreted by *Bacillus subtilis* may partly contribute to the antioxidant properties.

Scavenging activity on superoxide anion radical

Superoxide anion radical is biologically quite toxic and is deployed by the immune system to kill invading microorganisms. It is an oxygen-centered radical with selective reactivity. It is also produced by a number of enzyme systems in autooxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen. The biological toxicity of superoxide is due to its capacity to inactivate iron–sulfur cluster-containing enzymes, which

are critical in a wide variety of metabolic pathways, thereby liberating free iron in the cell, which can undergo Fenton chemistry and generate the highly reactive hydroxyl radical. It can also reduce certain iron complex such as cytochrome c [9].

It has been reported that antioxidant of some peptides is effective mainly via scavenging of superoxide anion radicals [18]. In the present study, the superoxide anion radical-scavenging capacity of peanut peptides was assayed by the AP-TEMED system. The decline of absorbance at 530 nm reflected the elimination of superoxide anion radical. Figure 2 shows the inhibition percentage of superoxide anion radicals by peanut peptides, GSH and BHT. The scavenging effect followed this order: 29.35% (21.41% of DH) > 25.30% (16.20% of DH) > 20.28% (11.18% of DH), and this revealed that the scavenging effect increased significantly ($p < 0.05$) with increasing DH. It was postulated that some amino acid residuals and active groups which were possibly electron or hydrogen donors were released from protein secondary structure with increasing DH during the course of fermentation. Then, the special amino acid residuals and groups played the role of “radical-killer” to quench the radicals. Honestly, superoxide anion radical-scavenging activity of peanut peptides (29.35%) was lower than that of GSH (47.64%) and BHT (66.22%) at 1 mg/mL, but peanut peptides can be used in foods at higher concentrations to obtain higher scavenging effect, because their origin is natural and there is no risk of health. All these above indicated that the peanut peptides at 21.41% could be a potential source of natural antioxidant.

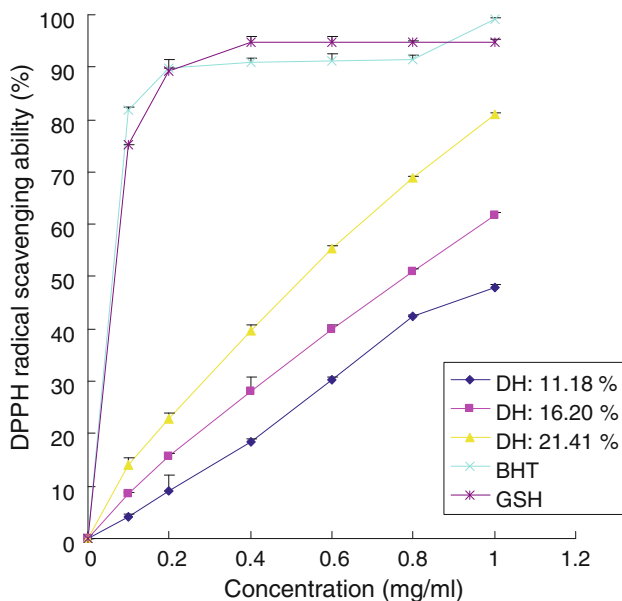


Fig. 1 DPPH radical-scavenging activities of peanut peptides at different degree of hydrolysis

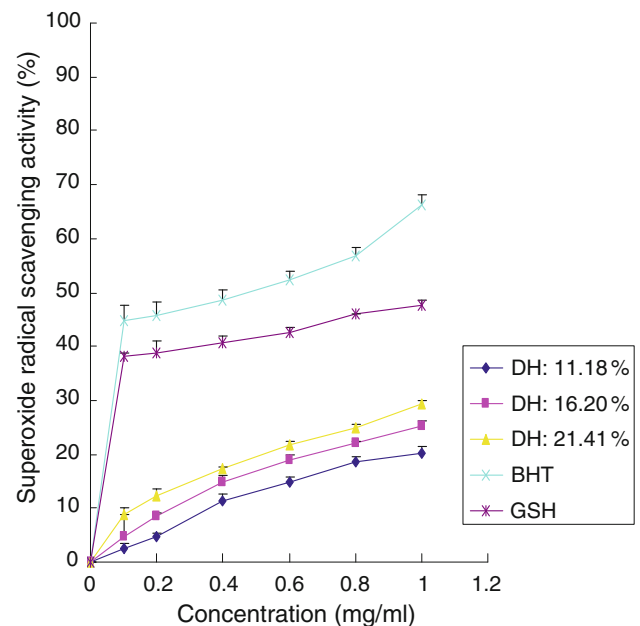


Fig. 2 Superoxide anion radical-scavenging effect of peanut peptides at different degree of hydrolysis

Reducing power

Samples with higher reducing power have better abilities to donate electron [20]. Reducing power assay is often used to evaluate the ability of natural antioxidants to donate electron or hydrogen [39]. It has been widely accepted that the higher the absorbance at 700 nm, the better the reducing power [40].

The reducing power of peanut peptides at different DH is shown in Fig. 3. In this test, the yellow color of the tested solution changes to various shades of green and blue, due to the reducing power of peanut peptides. The presence of reducers leads to the reduction in Fe^{3+} -ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration [41]. The higher absorbance indicated the stronger reducing power of peanut peptides at the same concentration. The reducing power followed this order: 0.368 (21.41% of DH) > 0.3317 (16.20% of DH) > 0.2533 (11.18% of DH) at 2 mg/mL. This suggested that DH influenced the reducing power of peanut peptides significantly ($p < 0.05$). A similar observation has been reported by Cumby et al. [42] on canola protein hydrolysate. As we know, *Bacillus subtilis* can produce many kinds of proteases in the course of fermentation, most of which are endopeptidase which can cleave peptide bonds like sharp knife at the interior of the polypeptide chain. Due to the action of endopeptidases produced by *Bacillus subtilis*, many small- and medium-sized oligopeptides or polypeptides were produced, and some of them took

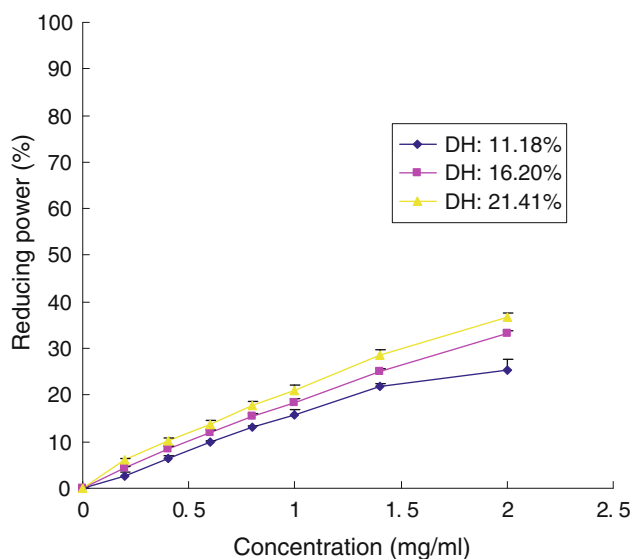


Fig. 3 Reducing power of peanut peptides at different degree of hydrolysis

possession of antioxidant activity. Because of the relationship between antioxidant activity and reducing power, the results indicated that the peanut peptides could be used as a potential alternate of synthetic antioxidants.

Metal-chelating capacity

It is known that transition metal ions are involved in many oxidation reactions in vivo. Ferrous ions (Fe^{2+}) can catalyze Haber–Weiss reaction and induce superoxide anion to more harmful hydroxyl radicals. Hydroxyl radicals react rapidly with the adjacent biomolecules and induce severe damage [20].

In our study, the metal-chelating activities were determined by the method described by Giménez et al. [35]. Figure 4 shows the chelating activities of peanut peptides and EDTA on ferrous ions. The order is given as following: 76.32% (21.41% of DH) > 58.92% (16.20% of DH) > 50% (11.18% of DH) at 2 mg/mL. The metal-chelating activity of peanut peptides increased with increasing DH ($p < 0.05$). Liu et al. [43] have reported a similar research result on the chelating activity of porcine plasma protein. Peanut peptides at 21.41% of DH showed much better metal-chelating activity (76.32% at 2 mg/mL) than that of GSH (because of its low activity, data not shown), which possessed negligible metal-chelating activity at the range of 0.2–2.0 mg/mL. When the peanut meal was fermented by *Bacillus subtilis*, DH went up with degradation of peanut proteins, which led to a higher metal ion binding power possibly due to an increased concentration of

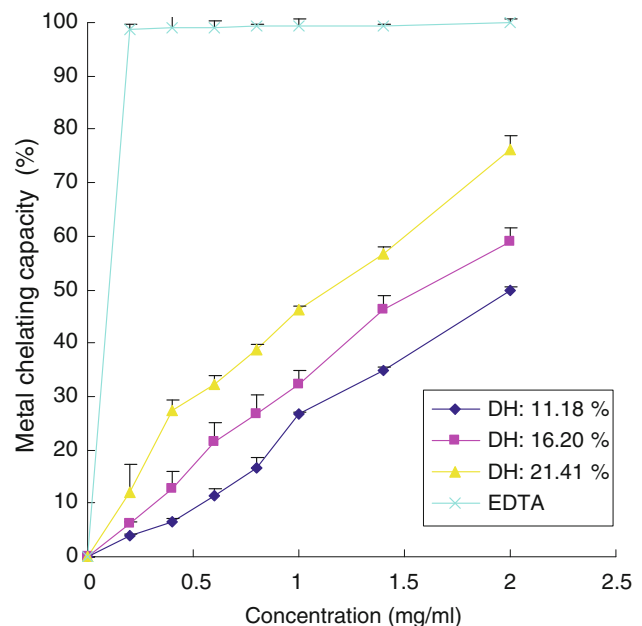


Fig. 4 Metal-chelating activity of peanut peptides at different degree of hydrolysis

carboxylic groups in branches of the acidic and basic amino acids, thus capturing prooxidative free metal ions from the hydroxyl radical system. Je et al. [44] have reported antioxidant peptide-containing acidic amino acids and pointed that the antioxidant activity is related to the metal ion binding activity of carboxylic groups in branches of the acidic amino acids. Histidine residues were often observed in sequences of peptide ion chelator, so we can further presume that the high metal-chelating power of peanut peptides at 21.41% was partly due to imidazole rings coming from histidine residues. The results suggested that DH influenced metal-chelating activities of peanut peptides significantly ($p < 0.05$), and these peanut peptides at 21.41% of DH were nice natural Fe^{2+} chelators.

Inhibition of linoleic acid autoxidation

Lipid oxidation is an important chemical change that lowers the nutritional value of food and shortens their shelf life. The primary and secondary products of lipid oxidation are detrimental to health. Consumption of these foods may cause a lot of adverse effects including toxicity to human body. In the body, excessive production of free radicals affects lipid cell membranes to produce lipid peroxides [45]. Lipid peroxides are likely involved in numerous pathological events; including inflammation, metabolic disorders, and cellular aging [46]. Therefore, the peanut peptides at different DH were further assayed for their antioxidant activity by evaluating their ability to protect linoleic acid against oxidation.

The antioxidant properties of peanut peptides were determined by the thiocyanate method and compared with BHT. As shown in Fig. 5, all the peanut peptides at different DH exhibited marked inhibition of linoleic acid oxidation, 63.75% (21.41% of DH) $>$ 49.27% (16.20% of DH) $>$ 42.17% (11.18% of DH) at the concentration of 0.8 mg/mL. Among these peanut peptides, the highest antioxidant activity was found in peanut peptides at 21.41% of DH, which showed a significant ($p < 0.05$) inhibition of linoleic acid peroxidation. Apparently, the inhibition effect increased with the increasing DH ($p < 0.05$). Rajapakse et al. [47] reported that lipid peroxidation proceeded via radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids. Presumably, at the beginning of fermentation, the peanut meal had compact structure because of the low DH. However, with the development of DH, we postulated that peptide cleavages led to the opening and exposure of some active amino acid residuals and branches, of which possessed excellent antioxidant properties and were made the “scapegoat” for lipids to react with radicals. Certainly, an inhibition of linoleic acid peroxidation (63.75%) showed by peanut peptides was lower than that of

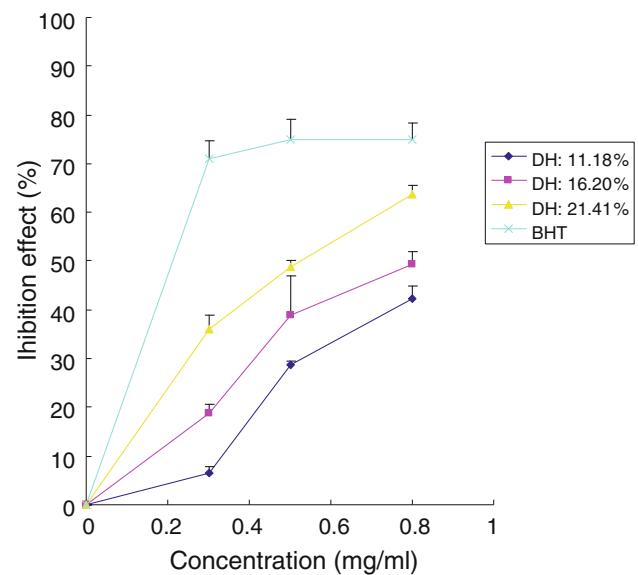


Fig. 5 Inhibition of linoleic acid autoxidation of peanut peptides at different degree of hydrolysis

BHT (75%) at 0.8 mg/mL, but they can be used at much higher concentrations than the synthetic antioxidants, due to the very restrictive toxicological parameters of the latter. Besides, the incorporation of peanut peptides to food stuffs could improve their nutritional value and supply people with extra amino acids.

Amino acid composition and molecular weight distribution of peptides

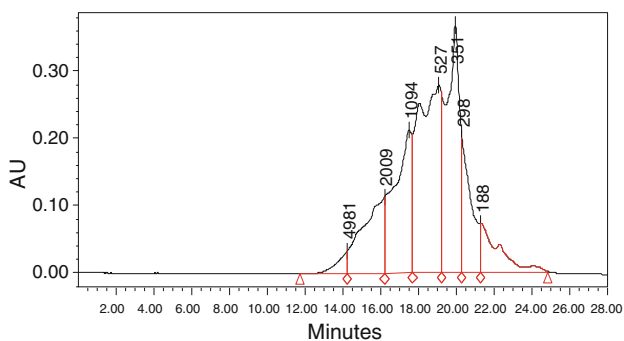
The peanut peptides with 21.41% of DH were subjected to amino acid composition analysis to determine the possible effect of the amino acid profile on antioxidant activity. The total amount of amino acid was 96.07 g per 100 g of sample. Table 1 shows the amino acid profiles of peanut peptides. Some amino acids, such as His, Cys, Tyr, and Leu, and their derivatives had been reported to exhibit antioxidant activity. Chen et al. [37] reported that peptide-containing His showed strong radical-scavenging activity due to its imidazole ring. It was found that the peanut peptides were rich in acidic amino acids, such as Asp and Glu. Saiga et al. [48] isolated an acidic peptide fraction which possessed high antioxidant activity. The isolated peptides contained mainly not only basic amino acids but also acidic amino acid residuals. Rajapakse et al. [47] and Je et al. [44] have reported antioxidant peptide-containing acidic amino acids and thought that they possessed antioxidant properties due to the acidic amino acid residues.

Molecular weight distribution profile of peanut peptides was assayed by high-performance size exclusion chromatography. The result was shown in Fig. 6. The average molecular weight distributions of peanut peptides were

Table 1 Amino acid composition of peanut peptides (g/100-g sample)

Amino acid	Amount	Amino acid	Amount
Aspartic acid	11.47	Glutamic acid	20.05
Serine	5.30	Histidine	2.41
Glycine	5.43	Threonine	2.98
Arginine	9.64	Tyrosine	4.76
Lysine	2.11	Cysteine	0.75
Valine	5.76	Methionine	1.63
Phenylalanine	10.19	Isoleucine	3.43
Leucine	7.21	Alanine	2.95
Proline	6.96	Total (g/100 g sample)	96.07

The peanut peptides with higher antioxidant activities are rich in Asp, Glu, Phe, Leu, Arg, and Pro. Each value is expressed as mean

**Fig. 6** Molecular weight distribution of peanut peptides at 21.41% DH. Their average molecular weight distributions were concentrated in <1,400 Da**Table 2** Molecular weight distribution of peanut peptides

Molecular weight	Peanut peptides (%)
>5,000	1.63
5,000–2,000	11.48
2,000–1,000	18.10
1,000–500	29.41
500–300	24.11
300–189	8.89
<189	6.38
Total (average molecular weight <1,400)	86.89

The average molecular weight distributions of peanut peptides with higher antioxidant activities were concentrated in <1,400 Da (86.89%). The data in Table 2 were calculated using GPC (gel permeation chromatography) option of Waters Empower 2 Software

concentrated in <1,400 Da (86.89%, Table 2), suggesting that peanut peptides contained a large proportion of low-molecular-weight peptides with 2–10 amino acid residuals. The data in table 2 were calculated using GPC (gel permeation chromatography) option of Waters Empower 2

Software. Many research papers have shown that the relationship had been established between the antioxidant activity of protein hydrolysates and their molecular weight distribution [4, 49, 50]. In our study, the result revealed that the peanut peptides with average molecular weight distribution <1,400 Da were probably related to the higher antioxidant activities. These findings suggested that antioxidant properties of the peanut peptides were dependent on their molecular weight distribution as well as amino acid composition. Two low-molecular-weight peptides with high antioxidant activities have been isolated and purified, and identification of their amino acid sequence and the relationship between their structure and antioxidant activities will be reported elsewhere.

Conclusions

In this study, using various assay systems, the antioxidant potential of peanut peptides with different DH was evaluated based on DPPH, superoxide anion-scavenging activity, reducing power, metal-chelating activity, and inhibition of linoleic acid autoxidation. The results clearly confirmed the relationship between DH and the free radical-scavenging activity and antioxidant properties of peanut peptides. Based on the discussion elsewhere, the peanut peptides (21.41% of DH) can be used for minimizing or preventing lipid oxidation in food products, retarding the formation of toxic oxidation compounds, keeping nutritional value, and prolonging the shelf life of foods. In addition to the functional characteristic of antioxidant activity, the peanut peptides can be used as protein intensifier in people's daily diet because of its rich in amino acids and low cost.

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