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Peptide Fractionation and Free Radical Scavenging Activity of Zein Hydrolysate

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Alcalase-treated zein hydrolysate (ZH) was separated by gel filtration, ultrafiltration, and reversed-phase HPLC, and the scavenging activities for 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), and superoxide anion ($O_2^{\bullet-}$) radicals of different peptide fractions were measured to establish the antioxidant efficacy. Results showed that the ability to stabilize water-soluble free radicals ($ABTS^{\bullet+}$) by ZH components was insensitive to the peptide size, whereas that against ethanol-soluble free radicals ($DPPH^{\bullet}$) and $O_2^{\bullet-}$ was molecular weight dependent. Antioxidative peptides of <1 kDa were further separated by HPLC into 30 components, of which those with great hydrophobicity exhibited strong $DPPH^{\bullet}$ and $O_2^{\bullet-}$ scavenging ability and those with intermediate hydrophobicity displayed the maximum $ABTS^{\bullet+}$ scavenging activity. Two dominant components (fractions 8 and 17) were further purified and identified by LC-PDA-ESI-MS to be Tyr-Ala and Leu-Met-Cys-His, respectively. The results demonstrated that the free radical scavenging activity of ZH depended on the radical species and was strongly related to the molecular weight and hydrophobicity of the constituting peptides.

KEYWORDS: Zein hydrolysate; peptides fractionation; gel filtration; free radical scavenging; antioxidant

INTRODUCTION

Lipid oxidation is one of the limiting factors for the quality and acceptability of foods because it leads to the development of undesirable off-flavor (rancidity), discoloration, and the formation of potentially toxic compounds. To curtail lipid oxidation, various antioxidant strategies have been developed, and the use of both synthetic and natural compounds capable of neutralizing reactive oxygen species represents the most common method. Yet, due to the potential health risks of synthetic antioxidants, plant extracts (e.g., tea polyphenols and rosemary extracts), tocopherols, ascorbate, and many other natural antioxidants have become attractive alternatives that are in high demand in the present food industry.

Over the past decade, peptides and protein hydrolysates prepared from common food proteins have emerged as a new source of natural antioxidants. These protein-derived compounds have demonstrated strong antioxidant efficacy in both model and in situ systems, including radical scavenging, reducing, and metal ion chelating activity (*1*). Reported proteins from which antioxidative peptides are produced include whey protein (*2*, *3*), egg protein (*4*), muscle protein (*5*), soybean protein (*6*), potato protein (*7*), maize zein (*8*), and buckwheat protein (*9*). To identify the prominent antioxidant components, studies have been carried out to fractionate and purify the active peptides (*6*, *9*−*16*). Despite these previous investigations, the relationship between the structure of the isolated peptides and their specific antioxidant activity has not been fully elucidated.

Zein, a byproduct obtained from corn starch processing, is an alcohol-soluble protein. Several recent studies, including the work conducted in our laboratory, have revealed remarkable antioxidant activity of zein following enzymatic hydrolysis (*8*, *17*, *18*). For example, zein hydrolysate (ZH) prepared with Alcalase can act as a hydrogen donor, a water-soluble radical quencher, and a transitional metal ion sequester (*8*). However, the effects of peptide size and structure and the constituents of ZH on the overall antioxidant activity of ZH have not been clearly defined.

In this study, we obtained peptide fractions from a previously identified antioxidative zein hydrolysate using gel filtration, ultrafiltration, and reversed-phase HPLC. The objectives were to determine the influence of molecular size and hydrophobicity of ZH peptides on the antioxidant activity and to identify the active peptide constituents present in the antioxidative ZH fractions.

MATERIALS AND METHODS

Materials and Chemicals. Zein (92% protein) was purchased from Freeman Industries LLC (Tuckahoe, NY). Alcalase (endoproteinase from *Bacillus licheniformis*, 2.4 AU/g) was obtained from Novozymes North America Inc. (Franklinton, NC). Bovine serum albumin, molecular weight

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markers (oxytocin, oxidized glutathione, reduced glutathione, cytochrome C, bacitracin, diaminoacetic acid−tyrosine−arginine, and triaminoacetic acid), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich, Inc. (Shanghai, China). All other chemicals and reagents (from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of analytical grade.

Preparation of ZH. The procedure described previously (*17*) was used to prepare antioxidative ZH. Briefly, the zein suspension (3%, w/v) was hydrolyzed with Alcalase (2%, w/w) at 50 °C and pH 9.0 for 4 h to obtain a hydrolysate that was established to be strongly antioxidative (*17*). The yellow pigments in ZH were removed through extraction with an equal volume of chloroform. The depigmented ZH solution was centrifuged at 1800*g* for 5 min. The supernatant was freeze-dried, sealed in plastic bags, and stored at 4 °C before use.

Molecular Mass (MM) Distribution Determined By Gel Filtration. The MM distribution in ZH was estimated using a Waters 600 Series HPLC (Water 600, Milford, MA) with a TSK-gel G2000 column (7.8 mm i.d. × 300 mm) and a UV detector (Waters 2487, Milford, MA) working at 220 nm (*8*). The mobile phase was acetonitrile/water/trifluoroacetic acid (45:55:0.1, v/v/v) at a flow rate of 1.0 mL/min. Cytochrome C (12500 Da), bacitracin (1450 Da), glycine−glycine−tyrosine−arginine (451 Da), and triglycine (189 Da) were used as the MM markers.

Peptide Fractionation. ZH was fractionated with an ultrafiltration system (Pellicon-2, Millipore, Bedford, MA). The ZH solution was pumped through 10, 5, 3, and 1 kDa Biomax membranes (BM) in the order of decreasing pore size on the BM. The > 10 kDa fraction (retentate from 10 kDa BM), 10−5 kDa fraction (retentate from 5 kDa BM), 5−3 kDa fraction (retentate from 3 kDa BM), 3−1 kDa hydrolysate (retentate from 1 kDa BM), and < 1 kDa hydrolysate (permeate from 1 kDa BM) were collected and freeze-dried.

Peptide Purification and Identification. The < 1 kDa ultrafiltration permeate was first purified on a reversed-phase HPLC system (Waters 1525, Milford, MA) with an ODS column (Hedera ODS-2, 20 mm i.d. × 250 mm) operated at ambient temperature. Peptides were eluted at a flow rate of 8.0 mL/min with various gradient mixtures composed of solvent A (5% methanol containing 0.2% formic acid) and solvent B (methanol). The gradient elution conditions were as follows: 100% solvent A for 5 min, then 0−30% solvent B over 50 min, 30−50% solvent B over 55 min, and 50−100% solvent B for 70 min. The elution profile was monitored using a UV detector (Waters 2487) set at 280 nm. Each fraction was collected and concentrated using a rotary evaporator before the antioxidant activity of the peptides was analyzed.

The reversed-phase HPLC fractions that exhibited strong radical scavenging activity (described later) were subjected to HPLC-MS analysis using a Waters Platform ZMD 4000 system consisting of a Micromass ZMD mass spectrometer (Micromass, Beverly, MA) and a Waters 2690 HPLC (Waters, Milford, MA) equipped with a photodiode detector. Data were collected and processed with MassLynx software version 3.1 (Micromass, a diversion of Waters Corp., Beverly, MA). The samples were injected by an autosampler and subsequently separated by a C18 column (Atlantis, 2.1 mm i.d. × 150 mm). The HPLC gradient was linear from 5 to 100% mobile phase B in 10 min using mobile phase A (H_2O, 0.1% formic acid) and mobile phase B (acetonitrile). The Waters Micromass ZMD mass spectrometer was operated using the electrospray ionization (ESI) source. All measurements were carried out using the positive ESI. A source temperature of 100 °C and a desolvation temperature of 250 °C were optimal. The capillary voltage was 3.88 kV, and the cone voltage was 60 V.

Determination of Radical Scavenging Activity (RSA). The RSA of ZH and its peptide fractions was tested in three systems: the DPPH$^{\bullet}$ system (for ethanol-soluble free radicals), the ABTS$^{\bullet+}$ system (for water-soluble free radicals), and the superoxide anion ($O_2^{\bullet-}$) system. For the testing of DPPH$^{\bullet}$ scavenging activity, the method of Brand-Williams et al. (*19*) was used. ZH samples were mixed 1:1 (v/v) with 0.1 mM DPPH in anhydrous ethanol. The mixture was shaken and left to stand at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. BHA (predissolved in 95% ethanol) and ascorbate (0.1 and 1 mg/mL, respectively) were also tested for comparison. The DPPH$^{\bullet}$ radical scavenging effect was expressed as the percentage inhibition of DPPH$^{\bullet}$.

For the testing of ABTS$^{+\bullet}$ scavenging activity, the radical cation (ABTS$^{\bullet+}$) was produced by reacting ABTS stock solution (7 mM) with 2.45 mM (final concentration) of potassium persulfate (*20*). The mixture was left in the dark at room temperature for 12 h before use. In the assay, ZH samples were mixed 1:4 (v/v) with diluted ABTS$^{\bullet+}$, and the absorbance reading (734 nm) was taken after 6 min. BHA (predissolved in 95% ethanol) and ascorbate (0.1 and 1 mg/mL, respectively) were also tested for comparison. Antioxidant activity was expressed as the percentage inhibition of ABTS$^{\bullet+}$.

For the $O_2^{\bullet-}$ scavenging activity measurement, the autoxidation of a pyrogallol method described by Marklund and Marklund (*21*) was followed. Briefly, 1.0 mL of ZH sample was mixed with 1.8 mL of 50 mM Tris-HCl buffer (pH 8.2). The mixture was incubated at 25 °C for 10 min, and then 0.1 mL of 10 mM pyrogallol (dissolved in 10 mM HCl) was added. The absorbance of the solution at 320 nm was measured up to 4 min. The oxidation rate of pyrogallol for samples was calculated as the slope of the absorbance line (ΔA_1). The autoxidation rate of pyrogallol for control was measured with 1.0 mL of double-distilled water (ΔA_0). For comparison, the $O_2^{\bullet-}$ scavenging activity of BHA and ascorbate (0.1 and 1 mg/mL for both) was also tested. The $O_2^{\bullet-}$ scavenging activity was calculated as $[(\Delta A_0 - \Delta A_1)/\Delta A_0] \times 100\%$.

Statistical Analysis. The entire experiments were performed with three independent trials (replications). All antioxidant assays were carried out with triplicate samples. Results were subjected to analysis of variance using the SAS (SAS Institute, Inc., Cary, NC), and significant differences ($P < 0.05$) between individual means were identified by the least significant difference (LSD) procedure. Data were presented as means with standard deviations.

RESULTS AND DISCUSSION

Radical Scavenging Activity of ZH. The neutralization of free radicals is one of the most important mechanisms by which peptides control oxidative reactions in foods (*1*). Because radicals produced by different sources have different reactivities, multiple methods are generally recommended for assessing the impact of the specific oxygen radicals in food systems. In the present study, three common testing methods for in vitro determination of antioxidant capacity were employed to evaluate the antioxidant capacity of ZH, namely, scavenging activity against DPPH$^{\bullet}$, ABTS$^{\bullet+}$, and $O_2^{\bullet-}$.

As shown in **Table 1**, ZH was capable of quenching all three types of radicals, and the activity was dose-dependent. ZH exhibited the strongest scavenging activity for ABTS$^{\bullet+}$. For example, at the 1 mg/mL application level, ZH inhibited 64.3% of ABTS$^{\bullet+}$, compared with 12.8% for DPPH$^{\bullet}$ and none for $O_2^{\bullet-}$. At 50 mg/mL, ZH completely neutralized ABTS$^{\bullet+}$ but had a substantially reduced efficacy against DPPH$^{\bullet}$ (73.5%) and $O_2^{\bullet-}$ (69.1%). The inhibitory effect of 10 mg/mL of ZH on ABTS$^{\bullet+}$ was stronger than that of 0.1 mg/mL of BHA and ascorbate. On the other hand, the scavenging activity of ZH for DPPH$^{\bullet}$ and $O_2^{\bullet-}$ was considerably weaker than that of BHA and ascorbate at a 0.1 mg/mL concentration level.

The difference between the radical scavenging capacity for DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ could be, in part, due to the difference of the radicals' solubility and diffusivity in the reaction medium. Although DPPH$^{\bullet}$ scavenging is a widely used method for the assessment of free radical scavenging activity of natural products, it has a notable limitation when used to interpret the role of hydrophilic antioxidants because DPPH$^{\bullet}$ can be dissolved only in organic media (especially in alcoholic media), not in an aqueous solution (*8*, *22*). A further disadvantage is that DPPH serves both as oxidizing substrate and as the reaction indicator molecule; therefore, the assay would easily lead to the problem of spectral interferences (*23*). In contrast, ABTS$^{\bullet+}$ can be solubilized in aqueous as well as organic media; thus, radical scavenging

activities of both hydrophilic and lipophilic compounds can be measured (*22*). The results, in agreement with our previous findings on zein hydrolysate (*8*), suggested that the $ABTS^{\bullet+}$ method was more sensitive than the $DPPH^{\bullet}$ assay for the measurement of antioxidant activity of water-soluble proteins and peptides in an aqueous solution. A similar observation was reported for fermented shrimp biowaste, which exhibited 40% scavenging activity for $DPPH^{\bullet}$ at a concentration of 1.0 mg/mL, compared with a 95% activity against $ABTS^{\bullet+}$ even at a concentration of 0.5 mg/mL (*24*). The same could be true for the lower $O_2^{\bullet-}$ scavenging activity due to limited solubility of the oxygen species in water.

Estimation of Molecular Mass Distribution. The HPLC with a TSK-gel G2000 column for the Alcalase ZH yielded three major fractions with estimated peak MMs of 640, 354, and 251 Da, along with four minor ones (**Figure 1**). These three major fractions, corresponding to penta-, tri-, and dipeptides, respectively (assuming an average MM of 120 Da for amino acids), made up 93.7% of the total ZH. Fraction 6 (115 Da) and fraction 7 (70 Da), which represented approximately 6% of the total ZH, were presumably free amino acids.

Scavenging Activity of Peptides From Ultrafiltration. To gain insight into the relative contributions of different peptides, ultrafiltration with membranes of different pore sizes (< 1, 1−3, 1−3, 3−5, 5−10, and > 10 kDa) was carried out to obtain peptide fractions of various sizes. Scavenging activities of different MM fractions and the original ZH against $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and $O_2^{\bullet-}$ were compared, and the results are shown in **Figure 2**. There were considerable variations between these MM fractions in stabilizing radicals. When the MM of peptides was lower than 5 kDa, the smaller the size of the peptides the greater the $DPPH^{\bullet}$ scavenging activity ($P < 0.05$), whereas the $O_2^{\bullet-}$ and $ABTS^{\bullet+}$ scavenging activities were not affected. In contrast, when the MM was greater than 5 kDa, both the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ quenching abilities increased ($P < 0.05$) with MM, whereas the $O_2^{\bullet-}$ neutralizing capability declined slightly with MM. However, despite their apparently strong $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activities, the ≥5 kDa peptides seemed to play a minimal role in the overall activity of ZH due to their minute quantity as indicated in **Figure 1**.

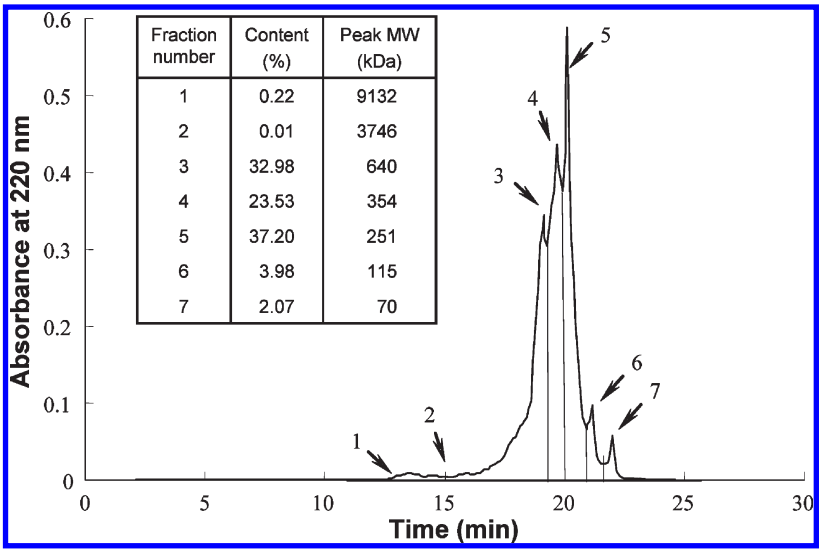
The results indicated that the ability to stabilize water-soluble free radicals ($ABTS^{\bullet+}$) by ZH components was insensitive to the peptide size, whereas that against ethanol-soluble free radicals ($DPPH^{\bullet}$) and $O_2^{\bullet-}$ was MM-dependent. During Alcalase hydrolysis, active fragments of zein protein are released, and the specific activity is dependent upon the proteases used or the peptide bonds cleaved (*8*, *17*). Wu et al. (*25*) reported that radical scavenging activity of the fractions of mackerel protein hydrolysate obtained by size exclusion chromatography was MM dependent, with a 1400 Da fraction possessing the highest $DPPH^{\bullet}$ scavenging activity. It is noteworthy that only a few individual MM fractions were as effective as their mother hydrolysate, that is, the ZH (**Figure 2**). This phenomenon has also been recorded for bullfrog skin protein hydrolysate (*15*), suggesting that the overall antioxidant activity of mixed zein hydrolysate was the result of a concerted action of small, intermediate, and large peptides.

Scavenging Activity of Peptides Separated by a Hydrophobic Column. Results from the HPLC gel filtration (**Figure 1**) and the ultrafiltration analysis (**Figure 2**) indicated that peptides of < 1 kDa, which made up the vast majority of the hydrolysate, were practically the determinant components for the radical scavenging activity in ZH. Hence, to further elucidate the radical scavenging role of the specific peptides in this prevalent MM fraction, the 1 kDa MM cutoff ultrafiltration permeate was subjected to further fractionation using a reversed-phase HPLC system with an ODS C18 column. Chromatography with the column produced more than 30 hydrophobicity-based subfractions shown as peaks (**Figure 3b**), of which 23 peaks were

Table 1. Free Radical Scavenging Activity of Alcalase-Hydrolyzed Zein (ZH) and Controls[a]

		scavenging activity (%)		
antioxidant	concn (mg/mL)	$ABTS^{\bullet+}$	$DPPH^{\bullet}$	$O_2^{\bullet-}$
ZH	50	≥100.0	73.5 ± 1.0	69.1 ± 1.4
	10	90.2 ± 0.5	46.0 ± 0.9	11.5 ± 0.3
	1	64.3 ± 0.9	12.8 ± 1.5	
BHA	1	96.3 ± 0.1	92.4 ± 0.6	≥100.0
	0.1	29.2 ± 0.3	55.5 ± 1.1	≥100.0
ascorbate	1	100.0	96.4 ± 0.7	96.3 ± 0.4
	0.1	76.9 ± 0.7	80.7 ± 1.3	13.9 ± 1.2

[a] Values represent means ± standard deviations from three replications ($n = 3$), each with duplicate measurements.



Fraction number	Content (%)	Peak MW (kDa)
1	0.22	9132
2	0.01	3746
3	32.98	640
4	23.53	354
5	37.20	251
6	3.98	115
7	2.07	70

Figure 1. Molecular mass distribution of zein hydrolysate (ZH) prepared with Alcalase (2%, w/w) at 50 °C and pH 9.0 for 4 h. (Inset) Relative peptide/amino acid content (percent) in major fractions of ZH. Peptide fractions are indicated by arrows.

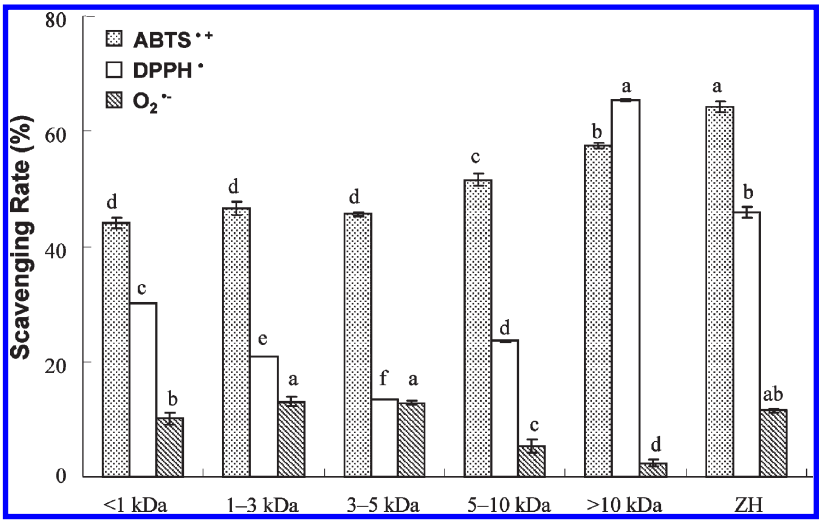


Figure 2. Scavenging activity of molecular weight peptide fractions of ZH against $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and $O_2^{\bullet-}$. The plots are means ± SD from three replications ($n = 3$), each with three measurements. Means without a common letter differ significantly ($P < 0.05$). The assay peptide concentrations were 1 mg/mL for $ABTS^{\bullet+}$ and 10 mg/mL for $DPPH^{\bullet}$ and $O_2^{\bullet-}$.

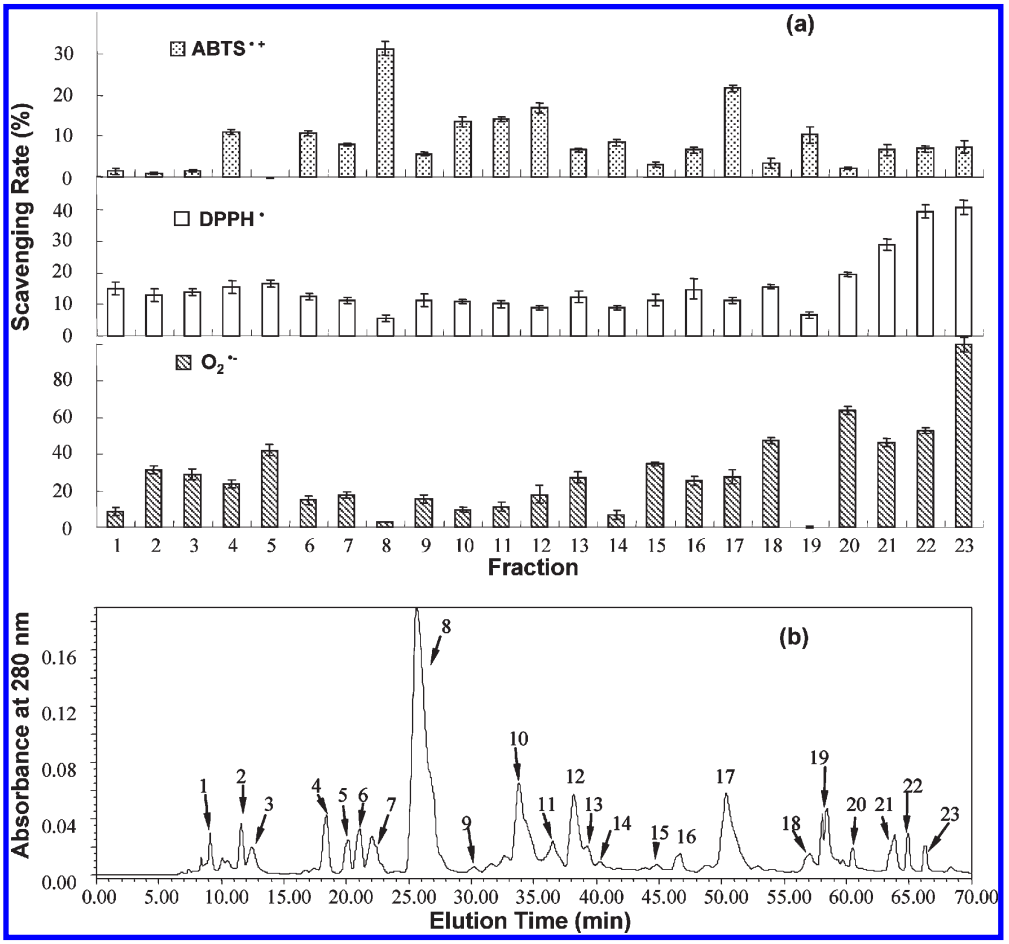


Figure 3. Scavenging activity of reversed-phase HPLC fractions of the 1 kDa MW cutoff ultrafiltration permeate against $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and $O_2^{\bullet-}$ (**a**) and the HPLC chromatogram (**b**). The results are plotted as the means ± SD from three replications ($n = 3$), each with three measurements. The assay peptide concentrations were 1 mg/mL for $ABTS^{\bullet+}$ and 10 mg/mL for $DPPH^{\bullet}$ and $O_2^{\bullet-}$. Peptide fractions are indicated by arrows.

collected and tested for scavenging activity against $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and $O_2^{\bullet-}$.

In reversed-phase HPLC, compounds are separated on the basis of their hydrophobic character; that is, peptides with large hydrophobicity values have longer elution times in a reversed-phase column. As shown in **Figure 3a**, the fractions with high hydrophobicity (long elution time) exhibited stronger $DPPH^{\bullet}$ and $O_2^{\bullet-}$ scavenging activities, whereas those with intermediate hydrophobicity displayed the maximum $ABTS^{\bullet+}$ scavenging activity. Fraction 23, which had the strongest hydrophobicity and the greatest scavenging activity for $O_2^{\bullet-}$ and $DPPH^{\bullet}$, inhibited nearly 40% $DPPH^{\bullet}$ and 100% $O_2^{\bullet-}$ at the concentration

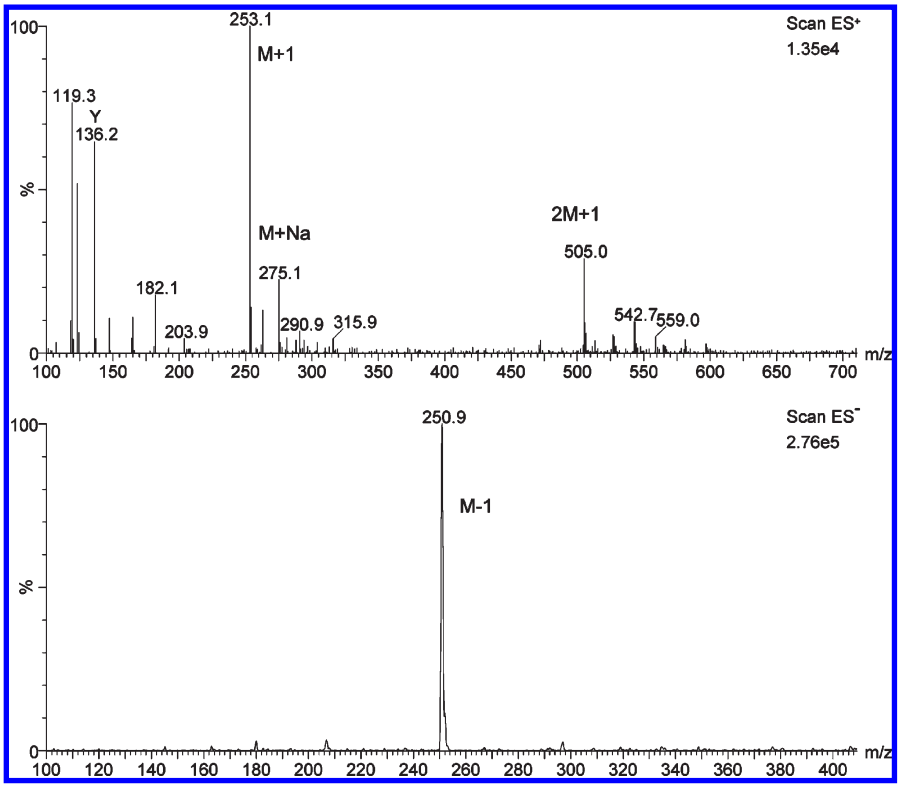


Figure 4. ESI-MS$^{\pm}$ fragmentation pattern of reversed-phase HPLC fraction 8.

of at 10 mg/mL. Fraction 8, the dominant fraction of all, which had an intermediate hydrophobicity and the strongest scavenging activity for ABTS$^{\bullet+}$ in the entire fraction range, inhibited ABTS$^{\bullet+}$ by 30%, followed by fraction 17, which inhibited ABTS$^{\bullet+}$ by >20%. The results demonstrated that free radical scavenging activity of zein hydrolysate was related not only to the size (MM) but also to the hydrophobicity of peptides. The strongly hydrophobic peptides in fraction 23 at the concentration of 10 mg/mL were equally capable of quenching $O_2^{\bullet-}$ when compared with 1 mg/mL BHA or ascorbate. On the other hand, peptides with an intermediate hydrophobicity, notably fraction 8, at a 1 mg/mL concentration level possessed a similar effect as 0.1 mg/mL of BHA for quenching ABTS$^{\bullet+}$.

The results also showed that the ability of purified peptides to scavenge DPPH radicals (ethanol-soluble) was a magnitude lower ($P < 0.05$) than their ability to scavenge water-soluble ABTS radicals (**Figure 3a**). The scavenging efficiency of fraction 8 for DPPH$^{\bullet}$ was roughly 60-fold less than for ABTS$^{\bullet+}$ on an equal peptide assay concentration basis. These results confirmed again that zein peptides had strong scavenging activity for water-soluble free radicals.

Amino Acid Sequence of Potent Radical Scavenging Peptides. Reversed-phase HPLC fractions 8, 17, and 23, which had relatively strong radical scavenging activity as indicated above, were further purified and subsequently subjected to LC-PDA-ESI-MS for peptide sequence identification. Due to multiple possibilities and poor matching with the known peptide segments in zein, the exact peptide sequence of fraction 23 was difficult to establish. However, the MassLynx software was able to idendify with 100% certainty two prominent peptides in fractions 8 and 17: Tyr-Ala and Leu-Met-Cys-His, respectively. The ESI-MS$^{\pm}$ fragmentation patterns of fractions 8 and 17 are shown in **Figures 4** and **5**.

It is well accepted that free radical scavenging activity of peptides depends on their structure, and a myriad of short peptides with strong antioxidant activity have been identified so far. Suetsuna et al. (*11*) isolated a radical scavenging hexapeptide from casein hydrolysate. The Glu-Leu sequence in the identified peptide, Tyr-Phe-Tyr-Pro-Glu-Leu, was thought to be critical for the activity. Medis et al. (*14*) purified a radical scavenging peptide with the sequence His-Gly-Pro-Leu-Gly-Pro-Leu from the hydrolysate of fish skin gelatin and suggested that the presence of His, Leu, Gly, and Pro played an important role in the activity. Chen et al. (*6*) studied the antioxidant activity of designed peptides based on the antioxidative peptide isolated from soybean protein hydrolysate (Leu-Leu-Pro-His-His), noting that His and Pro played an important role in the antioxidative behavior. The presence of a terminal Leu was also shown to be associated with the radical scavenging activity of buckwheat peptides (*9*).

A myriad of interpretations have been put forth to explain the antioxidant properties of peptides. For example, the antioxidative potency of peptides containing Leu has been attributed to its long aliphatic side-chain group that conceivably is capable of interaction with acyl chains of susceptible fatty acids (*14*). Histidine exhibits strong radical scavenging activity due to the decomposition of its imidazole ring (*27*). On the other hand, the radical scavenging activity of Tyr, as well as Trp, has been explained by the reactivity of the phenolic and indolic structures that function as hydrogen donors (*26*). Hernandez-Ledesma et al. (*3*) also indicated that the antioxidant activity of amino acid residues was affected by the specific conformation of peptides composed of these amino acids, namely, the amino acid sequence. Although it is not possible to establish a precise structure−activity relationship of zein peptides on the basis of the present data, it is reasonable to speculate that hydrophobic amino acids present in the peptide sequences contributed greatly to the DPPH radical scavenging activity, whereas hydrophilic amino acid residues were largely responsible for the ABTS$^{\bullet+}$ scavenging activity.

In summary, low MM peptides from Alcalase-treated zein hydrolysate had strong free radical scavenging activity. The ABTS assay was a more sensitive method for the measurement of antioxidant activity of water-soluble proteins and peptides in

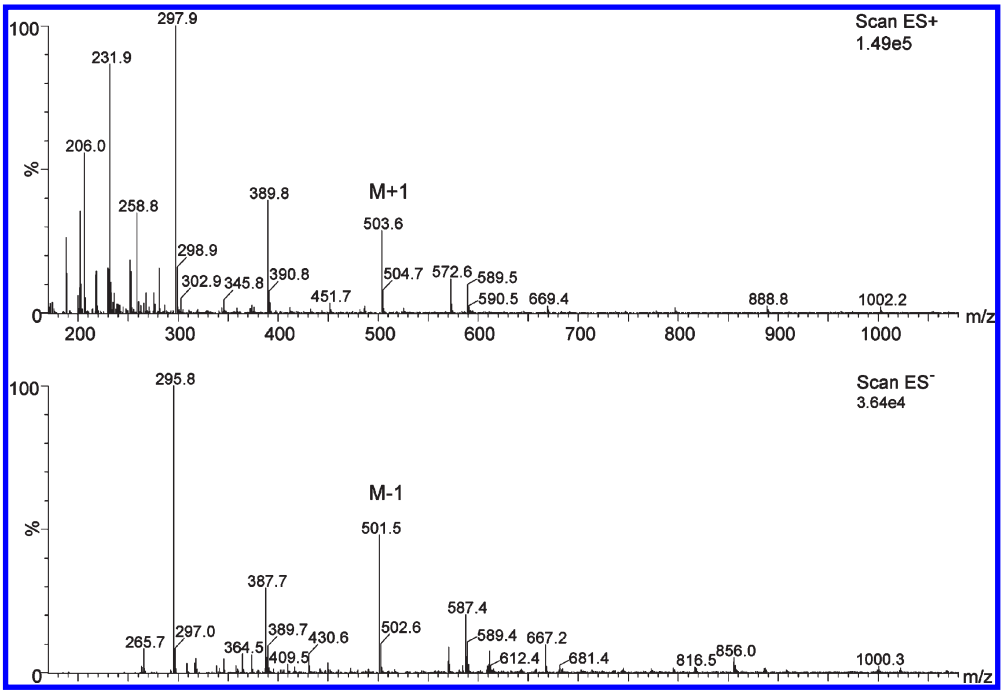


Figure 5. ESI-MS$^{\pm}$ fragmentation pattern of reversed-phase HPLC fraction 17.

an aqueous solution when compared with the DPPH method. The antioxidant activity of ZH was related not only to the molecular mass but also to the hydrophobicity of the constituting peptides. Results from this study indicated that it is feasible to produce natural antioxidants from zein by enzymatic hydrolysis and different separation and enrichment techniques.

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