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Reducing, Radical Scavenging, and Chelation Properties of in Vitro Digests of Alcalase-Treated Zein Hydrolysate

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The objective of the study was to assess the antioxidant potential of alcalase-treated zein hydrolysate (ZH) during a two-stage (1 h of pepsin → 0.5−2 h of pancreatin, 37 °C) in vitro digestion. Sephadex gel filtration and high-performance size exclusion chromatography were used to separate ZH into fractions. The amino acid composition, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\bullet}$) and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) free radical scavenging activity, reducing power, and Cu^{2+} chelation ability were tested to determine the antioxidant efficacy of ZH. Results showed that in vitro digests of ZH contained up to 16.5% free amino acids, with short peptides (<500 Da) making up the rest of the mass. The $ABTS^{+\bullet}$ scavenging activity of ZH was decreased by 27% ($P < 0.05$) after pepsin treatment but was fully recovered upon subsequent pancreatin digestion, while the $DPPH^{\bullet}$ scavenging activity of ZH was substantially less than $ABTS^{+\bullet}$ scavenging activity and showed a 7-fold reduction following pancreatin treatment. The reducing power of ZH increased 2-fold ($P < 0.05$) following pancreatin digestion when compared with nondigested ZH. The ability of ZH to sequester Cu^{2+} was reduced by pepsin digestion but was reestablished following pancreatin treatment. The antioxidant activity demonstrated by in vitro digests of ZH (1−8 mg/mL) was comparable to or exceeded ($P < 0.05$) that of 0.1 mg/mL of ascorbic acid or BHA. The results suggested that dietary zein alcalase hydrolysate may have the benefit to promote the health of the human digestive tract.

KEYWORDS: Zein protein hydrolysate; in vitro digestion; antioxidants; lipid oxidation

INTRODUCTION

It is increasingly recognized that free radical-mediated oxidation is involved in human health and in the quality and nutrition of food and food products. There is mounting evidence that links uncontrolled generation of reactive oxygen species (ROS) from free radical reactions to a variety of diseases, such as cancer, atherosclerosis, coronary heart disease, diabetes, neurological malfunctioning, and weakening of the immune system. These adverse health conditions have been attributed to oxidative damage to cell components, including membranes, lipoproteins, and nucleic acids (*1*–*4*). On the other hand, ROS negatively impact flavor, texture, nutritive value, and shelf life of food products and, under extreme conditions, produce toxins (*5*). Thus, antioxidants with a strong capability to inhibit oxidation both in food and in the human body are of high demand.

Because of potential health risks of synthetic antioxidants, natural antioxidants have garnered much attention in the food industry. For example, the antioxidants rosemarinic acid, catechin, tocopherols, ascorbate, and various phenolic extracts from plants are now widely used in processed foods. The search for natural antioxidants has extended beyond the above traditional sources. A number of studies have shown that peptides and protein hydrolysates of plant and animal origins possess significant antioxidant activity, for example, the hydrolysates of soy protein (*6, 7*), whey protein (*8, 9*), zein protein (*10*), potato protein (*11*), wheat protein (*12, 13*), gelatin (*14*), and egg albumin (*15*).

The antioxidant activity of hydrolysates is related to the amino acid composition and sequence, the size and configuration of peptides (*6, 16*). For example, peptides with a Leu-Leu-Pro-His-His sequence exhibited strong antioxidant activity (*16*). Furthermore, the presence of certain particular amino acid residues, notably His, Tyr, Met, and Pro, was significantly correlated with radical quenching activity of peptides (*9, 17, 18*). The study reported by Saiga et al. (*19*) on porcine myofibrillar proteins showed that short peptides rich in acidic amino acid residues (Asp and Glu) exhibited strong antioxidant activity, probably due to stabilizing prooxidative metal ions through charge interactions.

Zein, an alcohol-soluble protein (prolamine), is a byproduct obtained from corn starch processing. Several recent studies have shown that zein peptides produced by enzymatic treatments

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possessed bioactivities, notably antioxidant activity in emulsion systems (*10*), antihypertensive activity, and the ability to accelerate the metabolism of ethanol in vivo (*20*). In a preliminary investigation, we found that zein hydrolysate prepared with alcalase inhibited lipid oxidation in dry sausages.

While the results from previous studies have established zein hydrolysate and peptides as potential antioxidants for food applications, the degradation and possible antioxidative performance of zein hydrolysate in the digestive tract of the human body are unclear. A number of studies have linked ROS, generated in the digestion process, to gastrointestinal tract diseases. For example, dietary iron-induced hydroxyl radicals were shown to cause oxidative damage to digestive tract mucosa of experimental animals, leading to gastric ulcers and inflammatory intestinal disorders (*21, 22*). Similarly, nitric oxide radicals were found to inflict oxidative inflammation of the gastrointestinal system (*23*). Hence, the objective of the present study was to investigate whether the previously identified antioxidative zein hydrolysate (*10*) would retain, improve, or lose its efficacy when further degraded by the human digestive enzymes. A two-stage in vitro digestion model system was used to simulate the process of human gastrointestinal digestion, and the digests were evaluated for reducing, radical scavenging, and metal ion sequestering properties.

MATERIALS AND METHODS

Materials. Zein was purchased from Freeman Industries LLC (Tuckahoe, NY). The dry zein powder contained 92% protein on a dry weight basis. Alcalase (endoproteinase from *Bacillus licheniformis*, 2.4 AU/g) was obtained from Novozymes North America Inc. (Franklinton, NC). Pepsin, pancreatin (8 × USP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), butylated hydroxyanisol (BHA), L-leucine, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). All of other chemicals and reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), unless specified otherwise, and were of analytical grade.

Preparation of Zein Hydrolysate (ZH). The procedure described previously (*10*) was used to prepare ZH. Zein solution (3%, w/v) was hydrolyzed with alcalase at 50 °C for 4 h to obtain a hydrolysate that was previously shown to be strongly antioxidative (*11*). The enzyme: zein substrate ratio was 2:100 (w/w). The pH of the zein solution was maintained at 9.0 during hydrolysis with 1 M NaOH. After hydrolysis, the pH of the hydrolysate was brought to 7.0 with 1 M HCl, and the solution was then heated in a 95 °C water bath for 5 min to inactivate the enzyme. The yellow pigments in ZH were removed (decoloring) by mixing ZH with an equal volume of chloroform. The organic phase, which contained the extracted pigments, was removed. The ZH solution was centrifuged at 1800*g* for 5 min, and the supernatant was freeze-dried, sealed in plastic bags, and stored at 4 °C before use.

In Vitro Digestion. To mimic the in vivo digestion process, an in vitro digestion model system using enzymes similar to those in the upper gastrointestinal digestive tract of humans was used (*24, 25*). ZH solution (3% w/v, in Milli-Q water) was adjusted to pH 2.0 with 1 M HCl, and pepsin (4% w/w, protein basis) was added. After incubation at 37 °C for 1 h, the solution was adjusted to pH 5.3 with 0.9 M $NaHCO_3$. Pancreatin (4% w/w, protein basis) was then added, and the pH was adjusted to 7.5 with 1 M NaOH. The solution was incubated at 37 °C for 2 h and then submerged in boiling water (100 °C) for 10 min to terminate the digestion. The ZH digests were cooled to room temperature and centrifuged at 11000*g* for 15 min. The supernatant was lyophilized, sealed in plastic bags, and stored at 4 °C before use.

For the monitoring of antioxidant activity at intervals during digestion, aliquots of ZH digests were removed at 0, 60, 90, 120, 150, and 180 min during the in vitro digestion. These sampling times represented no digestion (i.e., the 4 h alcalase ZH), 1 h pepsin digest, and 0.5, 1, 1.5, and 2h pancreatin digests, respectively. The sample aliquots were heated in boiling water for 5 min to inactivate pepsin and pancreatin, cooled in an ice slurry, and then stored in a −20 °C freezer before use.

Degree of Hydrolysis (DH). The DH of ZH digests was determined using the TNBS method (*26, 27*) with some modifications. Samples (0.25 mL each) were pipetted into test tubes containing 2.0 mL of sodium phosphate buffer (0.2125 M, pH 8.2), and 2 mL of TNBS reagent (0.01%) was added, followed by mixing and incubation at 50 °C for 30 min in a covered water bath (to exclude light). At the end of the incubation, the reaction was terminated by the addition of 2 mL of 0.1 M Na_2SO_3 to each tube. The solutions were cooled to room temperature (20–25 °C), and the absorbance was measured at 420 nm.

L-Leucine (0–2.5 mM) was used to generate a standard curve. The contents of free amino groups in ZH digest samples were expressed as Leu amino equivalents, based on the equation of Leu standard curve generated. The DH values were calculated using the following formula:

$$\mathrm{DH} = (h/h_{\mathrm{tot}}) \times 100\%$$

where h is the number of peptide bonds broken and h_{tot} is the total number of bonds per unit weight with h_{tot} equaling 9.2 mmol/g of protein for zein (*28*).

Total Amino Acid Analysis. Triplicate samples of ZH were digested in sealed, evacuated glass tubes with 6 N HCl at 110 °C for 24 h. For each sample, the whole digest was transferred into a 25 mL volumetric flask and brought to volume (25 mL) with deionized water. After filtration through two layers of Whatman #2 paper, 1 mL of filtrate was transferred into a 25 mL beaker and evaporated to dryness in a vacuum drier. The dry sample was redissolved in 1 mL of HCl (pH 2.2) and centrifuged at 16000*g* for 15 min, and the supernatant was used for amino acid analysis. The analysis was performed with an Agilent HP1100 amino acid analyzer (Agilent Co., Palo Alto, CA) equipped with a C_{18} column (4.6 mm × 125 mm) for amino acid separation. Precolumn reaction with phthalic dicarboxaldehyde (OPA) yielded amino acid derivatives. The concentrations of the specific amino acids were determined from their respective absorption intensities, which were calibrated to the known concentrations of amino acid standards.

Free Amino Acid Analysis. The method described by Wu et al. (*29*) was used with slight modifications. ZH and digest samples were precipitated with 10% cold trichloroacetic acid for 2 h and then centrifuged at 11000*g* for 15 min. The pH of the supernatant was adjusted to 2.0, and the solution was passed through a microfiltration membrane (0.45 μm). The filtrate was subjected to RP-HPLC analysis (Agilent HP1100) after precolumn derivatizing with OPA as indicated above.

Peptide Fractionation. In vitro digests of ZH collected from different digestion times were subjected to high-performance size exclusion chromatography using a Waters HPLC system (Water 600, Milford, MA) and a TSK-Gel G2000 column (7.8 mm i.d. × 300 mm length). Samples were prepared with 0.1% of trifluoroacetic acid (TFA) in 45% aqueous acetonitrile. To establish molecular mass of peptides, the following molecular weight (MW) markers (Sigma-Aldrich, Inc., St. Louis, MO) were also run as follows: cytochrome C (12500 Da), bacitracin (1450 Da), glycine-glycine-tyrosine-arginine (451 Da), and triglycine (189 Da). The markers yielded a linear log MW vs elution time regression line ($r = 0.9945$). Aliquots of 10 μL samples were injected onto the column, and elution was performed isocratically in the same TFA−acetonitrile buffer at 30 °C with a flow rate of 0.5 mL/min over 30 min. Eluted peptides were detected at 220 nm, and the molecular mass was estimated based on the elution time against those of molecular weight markers. The relative content of each peptide fraction was expressed as the percentage area of its chromatogram peak.

Radical Scavenging Activity (RSA). The RSA of ZH in vitro digests was tested in two systems, that is, an $ABTS^{+\bullet}$ system (for water-soluble free radicals) and a $DPPH^{\bullet}$ system (for fat-soluble free radicals). For the testing of $ABTS^{+\bullet}$ radical scavenging effect, ABTS radical cation ($ABTS^{+\bullet}$) was produced first by reacting ABTS stocking solution (7 mM) with 2.45 mM (final concentration) potassium persulfate (*30*). The mixture was left in the dark at room temperature for 12 h before use. The $ABTS^{+\bullet}$ solution was diluted with 0.1 M sodium phosphate

Table 1. Antioxidant Activity of Alcalase-Hydrolyzed Zein (ZH)[a]

ZH concentration (mg/mL)	reducing power (A_{700})	ABTS scavenging activity (μM Trolox equiv)	DPPH scavenging activity (μM Trolox equiv)	Cu^{2+} chelating activity (%)
1	0.082 ± 0.006 d	1228 ± 12 d	23.97 ± 0.14	
5	0.232 ± 0.003 c	2276 ± 12 c	45.09 ± 0.11 a	27.47 ± 0.17 c
10	0.397 ± 0.002 b	2462 ± 4 b	43.79 ± 0.53 b	44.28 ± 1.17 b
16	0.652 ± 0.009 a	2522 ± 2 a	42.53 ± 0.16 b	49.57 ± 2.64 a

[a] Means (±standard deviations, $n = 3$; each with duplicate measurements) without a common letter differ significantly ($P < 0.05$).

buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. To test the ABTS scavenging activity, 20 μL of ZH digest sample solutions (1–16 mg/mL) was mixed with 2 mL of diluted $ABTS^{+\bullet}$, and the absorbance reading (734 nm) was taken after 6 min. BHA (predissolved in 95% ethanol) and ascorbate (0.01, 0.1, and 1 mg/mL for both) were also tested for comparison. A standard curve (linear) was prepared by reacting 20 μL of a series of concentrations of Trolox (0−3 mM) with 2 mL of diluted $ABTS^{+\bullet}$ solution. The degree of scavenging ability of individual samples, expressed as Trolox concentration equivalent and referred to as "Trolox equivalent antioxidant capacity (TEAC, μM)", was obtained from the Trolox standard curve based on the corresponding sample absorbance values.

For the testing of $DPPH^{\bullet}$ radical scavenging effect, 1.5 mL of ZH digest samples (1–16 mg/mL) was added to 1.5 mL of 0.1 mM $DPPH^{\bullet}$ dissolved in 95% ethanol. The mixture was shaken for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm (*29*). For comparison, BHA and ascorbate (0.01, 0.1, and 1 mg/mL for both) in lieu of ZH samples were tested. A standard curve (linear) was generated by reacting 1.5 mL of a series of concentrations of Trolox (0−50 μM) with 1.5 mL of diluted $DPPH^{\bullet}$ solution, and the sample antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC, μM) as described above.

Reducing Power. The procedure described by Wu et al. (*29*) was followed to measure the reducing power of ZH in vitro digests. Briefly, 2 mL of diluted samples (1–16 mg/mL) was mixed with 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, and then, 2 mL of 10% TCA was added. The mixture was centrifuged for 10 min at 3000*g*, and 2 mL of the supernatant was mixed with 2 mL of MilliQ water and 0.4 mL of 0.1% $FeCl_3$. After reaction for 10 min, the absorbance of the solution was read at 700 nm. For comparison, the reducing power of BHA and ascorbate (0.01, 0.1, and 1 mg/mL for both) in place of ZH samples was tested. An increased absorbance of the reaction mixture indicated increased reducing power.

Metal Chelating Activity. The ability of ZH in vitro digests to chelate prooxidative Cu^{2+} was investigated according to Wang and Xiong (*11*) with slight modifications. In the chelation test, 1 mL of 2 mM $CuSO_4$ was mixed with 1 mL of pyridine (pH 7.0) and 20 μL of 0.1% pyrocatechol violet. After the addition of 1 mL of digest samples (5–16 mg/mL), the disappearance of the blue color, due to dissociation of Cu^{2+}, was recorded by measuring the absorbance at 632 nm at 5 min of the reaction. For comparison, 0.1 and 1 mg/mL of ascorbate acid were also tested. The Cu^{2+} chelating activity of the ZH samples was calculated as:

$$[(\text{blank absorbance} - \text{sample absorbance})/\text{blank absorbance}] \times 100\%$$

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

RESULTS

ZH. The 4 h hydrolysate of zein exhibited strong, dose-dependent reducing, $ABTS^{+\bullet}$ scavenging, and Cu^{2+} chelating activity (**Table 1**). The result agreed with a previous finding that hydrolyzed zein was capable of inhibiting lipid oxidation in a liposome system (*10*). The values of these antioxidant activity indexes increased rapidly ($P < 0.05$) at low concentrations of ZH (<5 mg/mL), suggesting that ZH had a high efficiency in dilute solutions. While ZH also displayed scavenging activity against DPPH radicals, the efficacy was substantially lower (approximately 50-fold less; $P < 0.05$) than that against $ABTS^{+\bullet}$ at their respective assay concentrations, indicating that $DPPH^{\bullet}$ reacted poorly with zein peptides in this assay system.

The amino acid composition of ZH is indicated in **Table 2**. It was rich in Glu (27.2%), Leu (19.2%), Ala (9.5%), Pro (7.8%), Phe (7.2%), and Ser (5.6%), most of which reportedly have relation to antioxidant properties either in their free forms or as residues in proteins and peptides (*9, 18, 19, 31*). Although hydrophobic amino acids accounted for almost 50% of the total amino acids in ZH, the abundance of charged or polar N- and C-terminal groups in peptides produced would enable ZH to be readily soluble in aqueous solution.

Characteristics of In Vitro Digests. Nondigested ZH (from 4 h alcalase hydrolysis) had a DH of 25.2% (**Figure 1**). As reported previously, this ZH consisted almost completely of short peptides with molecular mass of <6 kDa (*10*). Alcalase is a nonspecific endopeptidase, and protein hydrolysates derived from alcalase hydrolysis were likely a mixture of extremely small peptides (*9, 32*). Sequential digestion with pepsin from 0 to 1 h and then with pancreatin for an additional 0.5 h increased the DH in a linear fashion (**Figure 1**). Further incubation with pancreatin (from 1 to 2 h) produced negligible increases of DH ($P < 0.05$). The 3 h in vitro digestion brought the total DH to 38.2%.

Consistent with the changes in DH and, as expected, the two-stage digestion substantially increased the content of free amino acids (FAAs), including a phase of exponential increases between 1 and 2.5 h during the digestion (**Figure 1**, inset). FAAs accounted for less than 0.5% of the total amino acids in ZH, that is, 4.86 mg/g (alcalase, 4 h), which consisted predominantly of those with nonionizable side chain groups (**Table 2**). The 1 h pepsin digestion did not raise the FAA content or their relative distribution, indicating that early digestion mainly effected peptide fragmentation. However, after 0.5 h digestion with pancreatin, FAAs increased 5-fold (30.61 mg/g) and the composition became more balanced in hydrophobic (e.g., 35% Phe) and hydrophilic (e.g., 26% Arg and His) amino acids. The concentrations of all FAAs continued to rise with further pancreatic digestion. While the percentage of Arg and His essentially stayed constant during the entire course of pancreatic digestion, that of Ile increased drastically and accounted for one-third of the total FAAs in the 2 h digests. Overall, the two-stage in vitro digestion increased the FAA content in ZH from 4.86 to 164.62 mg/mL or a 32-fold net production ($P < 0.05$). Thus, more than 80% of the final digest (i.e., end of 3 h) was still comprised of peptides, which contributed to the antioxidant activity in the upper digestive tract as described later.

Table 2. Changes in Free Amino Acid Content in Alcalase-Hydrolyzed Zein (ZH) during Sequential in Vitro Digestion

	total amino acid		free amino acid[b]											
	alcalase		alcalase		pepsin		pancreatin							
	4.0 h[a]		4.0 h		1.0 h		0.5 h		1.0 h		1.5 h		2.0 h	
amino acid	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%
Asp[c]	39.69	4.97	0.02	0.36	0.06	0.92	0.14	0.46	0.13	0.26	0.26	0.22	0.34	0.21
Glu[d]	217.07	27.17	0.55	11.35	0.70	11.13	1.46	4.77	1.95	3.88	4.41	3.75	6.57	3.99
Ser	44.96	5.63	0.08	1.65	0.10	1.60	0.42	1.38	0.52	1.02	1.28	1.09	1.85	1.12
His	10.03	1.26	0.01	0.14	0.05	0.72	3.94	12.86	6.64	13.21	15.00	12.78	19.57	11.89
Gly	14.69	1.84	0.48	9.89	0.62	9.94	0.76	2.48	1.01	2.01	2.26	1.93	3.08	1.87
Thr	17.79	2.23	0.03	0.64	0.04	0.63	0.41	1.34	0.80	1.58	2.10	1.79	3.34	2.03
Arg	10.15	1.27	0.03	0.52	0.05	0.74	4.17	13.61	6.58	13.09	14.95	12.73	20.70	12.57
Ala	76.09	9.53	1.26	26.00	1.32	21.05	2.43	7.93	3.84	7.64	9.32	7.94	14.45	8.78
Tyr	39.17	4.90	0.04	0.84	0.12	1.99	2.01	6.55	2.77	5.51	6.29	5.35	8.04	4.89
Cys	2.33	0.29	0.02	0.49	0.07	1.07	0.15	0.50	0.27	0.54	0.59	0.50	0.89	0.54
Val	24.23	3.03	0.03	0.66	0.02	0.39	0.09	0.30	0.11	0.22	0.22	0.19	0.22	0.13
Met	3.22	0.40	0.90	18.57	1.16	18.57	2.16	7.06	3.10	6.16	6.94	5.91	9.32	5.66
Phe	57.60	7.21	0.47	9.58	0.91	14.48	10.56	34.51	13.07	25.99	13.72	11.68	16.43	9.98
Ile	25.04	3.13	0.60	12.44	0.67	10.63	1.07	3.51	1.68	3.34	37.27	31.74	56.48	34.31
Leu	153.21	19.18	0.05	1.03	0.05	0.80	0.06	0.20	0.07	0.14	0.07	0.06	0.10	0.06
Lys	1.67	0.21	0.08	1.62	0.09	1.44	0.10	0.33	0.12	0.24	0.21	0.18	0.32	0.19
Pro	61.98	7.76	0.14	2.86	0.19	3.06	0.71	2.32	1.45	2.89	2.53	2.16	2.93	1.78
total	798.93	100	4.86	100.00	6.26	100.00	30.61	100.00	50.28	100.00	117.42	100.00	164.62	100.00

[a] Supernatant (1800*g* centrifugal force) of the 4 h alcalase hydrolysate before in vitro digestion. [b] Supernatants (11000*g* centrifugal force) of the in vitro digests except for the 4 h alcalase hydrolysate. [c] Aspartic acid + asparagine. [d] Glutamic acid + glutamine.

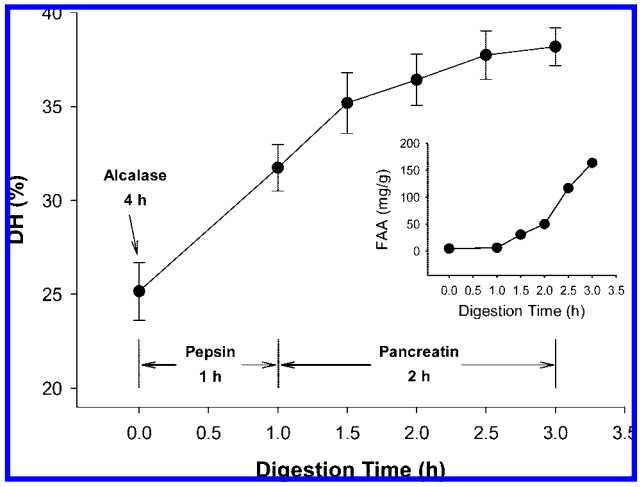

Figure 1. Changes in degree of hydrolysis (DH) during sequential in vitro digestion of alcalase-hydrolyzed zein. Inset: changes in free amino acids (FAAs) during digestion. Results are plotted as the means ± SD ($n = 3$; each with duplicate measurements).

Size exclusion high-performance liquid chromatography (HPLC) was run to separate peptide fractions and identify their MW distributions. The original ZH (alcalase, 4 h) yielded three fractions (**Figure 2a**), with peak molecular masses of 449, 338, and 257 Da. These fractions, representing 39.9, 23.6, and 35.9% of the total mass (**Table 3**), respectively, corresponded to tetra-, tri-, and dipeptides. The broad tailing of peak 1 indicated that a significant portion of the fraction consisted of larger peptides. Peptides with MW > 4833 Da made up 0.47% of the total eluted materials. Overall, peptides of >500 Da accounted for approximately 40% of the eluent, and those with less than 500 Da made up the rest (60%). No peptide/amino acids were detected in eluents collected before 15 and after 25 min.

In vitro digestion degraded oligopeptides into smaller peptides and amino acids. As shown in **Figure 2b**, pepsin digestion diminished peak 1 and augmented peak 3, changing the percentage of distribution to 33.0, 23.5, and 37.2% for fractions 1, 2, and 3, respectively (**Table 3**). The pepsin digestion also yielded two small, yet, well-resolved peaks (x and y), which were presumed to be small, free amino acids and possibly other organic materials that absorbed at 220 nm.

The change in the peptide/amino acid distribution in the in vitro digests manifested a dynamic process. While the percentage of larger peptides (fraction 1) continued to drop during pancreatic digestion, a new fraction (fraction 4) emerged after 0.5 h, which was apparently the result of further breakdown of fraction 3 (**Figure 2c−f**). At the end of the 2 h pancreatin digestion, fraction 1 (>500 Da) was reduced to 21.8% with no peptide of <4500 Da remaining, while the amount of peptides with <500 Da increased to 75% (**Table 3**). The peptide chromatograph results supported those of DH and the FAAs analysis.

Activity of In Vitro Digests. Strong scavenging activity for the water-soluble ABTS free radicals, expressed as TEAC, was demonstrated by ZH samples, confirming a previous report (*10*). However, following pepsin digestion, the TEAC value was reduced by 27% ($P < 0.05$) (**Figure 3a**). The subsequent digestion with pancreatin up to 1 h regained the lost $ABTS^{+\bullet}$ scavenging activity, and additional benefit was generated upon further pancreatin digestion up to 2 h. The ZH digests, with TEAC values of 1257–1875 μM at the 1 mg/mL assay concentration, were slightly less effective than 1 mg/mL of BHA (2400 μM TEAC) and ascorbate (2493 μM TEAC) for quenching $ABTS^{+\bullet}$ but were more potent than both standard antioxidants at 0.01 or 0.1 mg/mL concentrations.

Control and pepsin-digested ZH samples (8 mg/mL) were also able to stabilize DPPH radicals. However, in contrast to their effects on quenching ABTS radicals, the ability of ZH and the pepsin digest to scavenge DPPH radicals (ethanol-soluble) was a magnitude lower ($P < 0.05$) (**Figure 3b**). Namely, the TEAC values for $DPPH^{\bullet}$ were roughly 50-fold less than for $ABTS^{+\bullet}$. Moreover, sharply differing from the ABTS test result, the subsequent 0.5 h pancreatin digestion almost completely diminished the capability of ZH to stabilize $DPPH^{\bullet}$. The 7-fold loss of $DPPH^{\bullet}$ scavenging power was essentially uninfluenced by the length of digestion with pancreatin, as the TEAC remained at a marginal level. The TEAC values for BHA

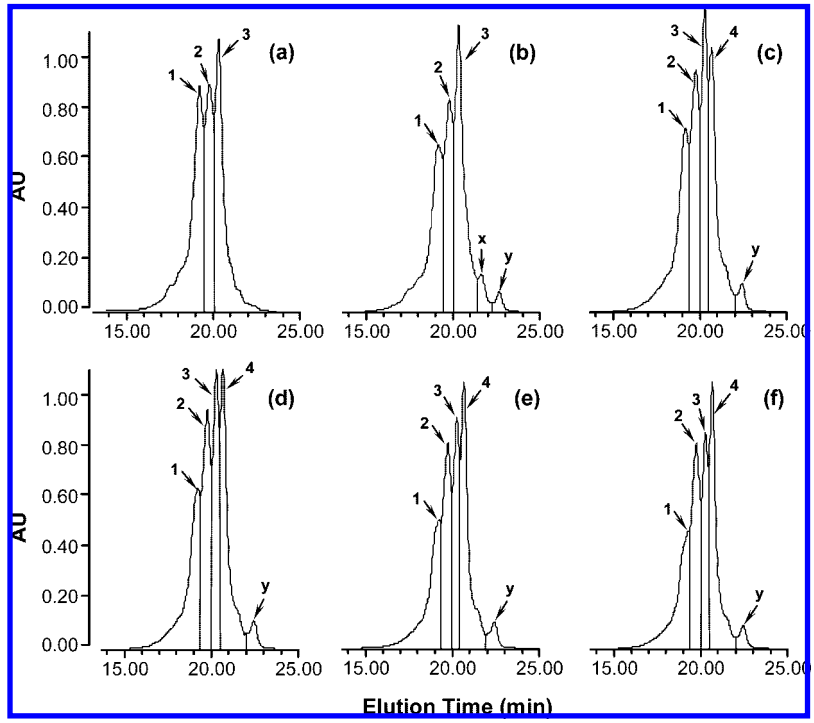

Figure 2. HPLC size exclusion chromatographs of in vitro digests of alcalase-hydrolyzed zein. (**a**) Alcalase, 4.0 h; (**b**) pepsin, 1.0 h; (**c**) pancreatin, 0.5 h; (**d**) pancreatin, 1.0 h; (**e**) pancreatin, 1.5 h; and (**f**) pancreatin, 2.0 h. Peptide fractions are indicated by arrows.

Table 3. Relative Peptide/Amino Acid Content (%) in Major Fractions of in Vitro Digests of Alcalase-Hydrolyzed Zein (ZH)

	fraction MW range (Da)	peak MW (Da)	alcalase 4.0 h	pepsin 1.0 h	pancreatin 0.5 h	pancreatin 1.0 h	pancreatin 1.5 h	pancreatin 2.0 h
1	>500	445−470	39.9	33.0	28.7	25.9	23.8	21.8
2	300–500	335−355	23.6	23.5	21.8	23.5	23.8	25.9
3	250–299	255−275	35.9	37.2	21.4	19.8	18.8	18.2
4	100–249	140−225			25.4	27.1	30.2	31.1

and ascorbate (0.01–1 mg/mL) in the DPPH$^{\bullet}$ assay system were also low, at 31–55 and 13–53 μM, respectively.

The reducing power of ZH increased slightly after pepsin treatment; however, the value doubled ($P < 0.05$) after subsequent 0.5 h digestion with pancreatin (**Figure 4a**). No significant increase was observed over the next 1.5 h. The reducing power of the final digest (2 h pancreatin; $A_{700} = 0.768$) was greater than that of 0.1 mg/mL of ascorbate ($A_{700} = 0.128$) and 0.01 mg/mL of BHA ($A_{700} = 0.162$) and was about 50% of that of 0.1 mg/mL of BHA ($A_{700} = 1.704$) ($P < 0.05$). The results established zein peptides and amino acids in the ZH in vitro digests, at the concentration tested (8 mg/mL), to be effective hydrogen or electron donors.

To further evaluate the effect of in vitro digestion on the antioxidant activity of ZH, the digested samples (8 mg/mL) were tested for Cu^{2+} chelation. As shown in **Figure 4b**, the chelating activity decreased abruptly upon pepsin digestion but recovered rapidly when further treated with pancreatin. The 0.5 h pancreatic treatment brought the chelation to the initial level, and no major change was observed with further digestion. The Cu^{2+} chelation during the course of in vitro digestion followed a very similar trend to that of ABTS$^{+\bullet}$ scavenging. At the end of digestion (3 h), the ZH samples had a Cu^{2+} chelating activity of 43%, which was superior to that of 0.1 mg/mL of ascorbate (19%) ($P < 0.05$) and equivalent to that of 1 mg/mL of the standard antioxidant (42%).

DISCUSSION

The antioxidant activity of hydrolyzed proteins and peptides in model food systems has been well-documented in the literature, but relatively few studies have been conducted to evaluate their activity and fate in the human upper digestive system. Because of the unique peptide bond specificity of digestive proteases, the products from pepsin and pancreatin (trypsin and chymotrypsin) digestion will depend on the characteristics of the ingested peptides and, in this study, the composition of alcalase-hydrolyzed zein. The results from the present study demonstrated that ZH, which possessed strong antioxidant activity in aqueous and emulsion systems (*10*), had a decreased or improved activity during the course of in vitro digestion, depending on the enzymes encountered and the duration of hydrolysis.

The increased reducing power of ZH digests can be attributed to a number of factors. With the increase of DH, electron-dense amino acid side residue chain groups, that is, polar or charged moieties, became more exposed. Furthermore, peptide scissions and an increased availability of FAAs during digestion provided an additional source of protons and electrons to maintain a high redox potential. These physicochemical changes can also explain the enhanced radical scavenging capacity of ZH digests. A number of studies have demonstrated a good correlation between certain amino acids residues or short peptides with radical scavenging ability of protein hydrolysates or peptides (*16, 33–36*). Peptides rich in His, Arg, Ala, Val, Met, and Leu were reported to exhibit strong antioxidant activity (*6, 9, 36*).

The different scavenging patterns for DPPH and ABTS radicals were likely related to the structure of peptides produced at different stages. DPPH$^{\bullet}$ is an oil-soluble free radical that becomes a stable product after accepting an electron or a hydrogen from an antioxidant. After the pepsin treatment (1 h), more hydrophobic amino residue side chain groups were expected to be exposed; therefore, peptides became more accessible by DPPH$^{\bullet}$. This would facilitate electron transfer from peptides, thereby stabilizing DPPH$^{\bullet}$. The conceivable structural

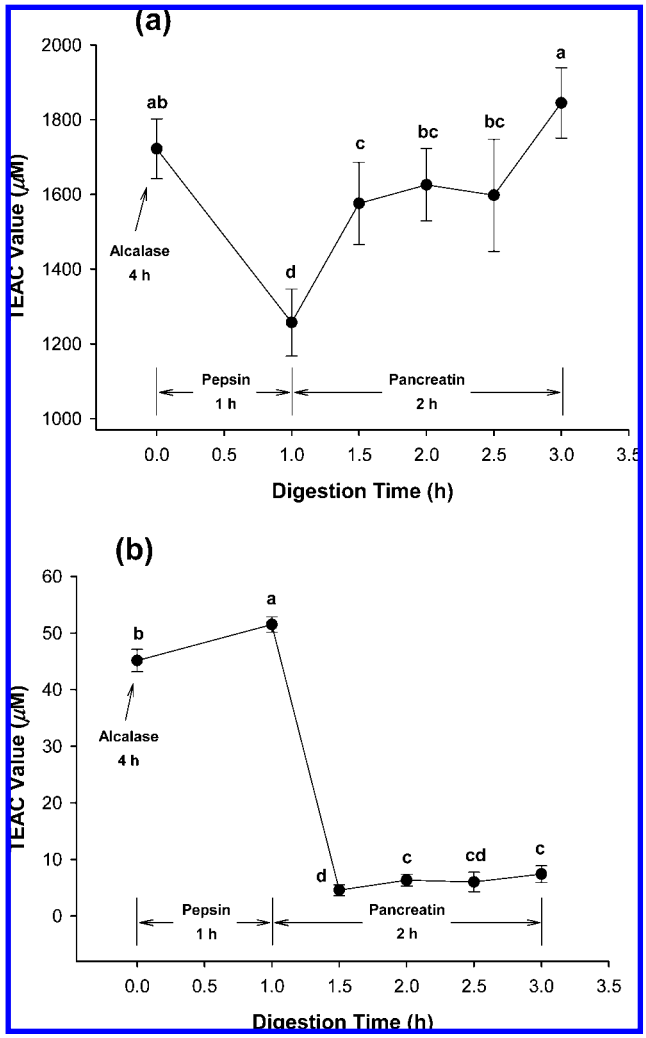

Figure 3. Changes in ABTS (**a**) and DPPH (**b**) radical scavenging activity of alcalase-hydrolyzed zein during sequential in vitro digestion. The activity was expressed as Trolox equivalent antioxidant capacity (TEAC), and the results are plotted as the means ± SD (n = 3, each with three measurements for ABTS or five measurements for DPPH). Means with different letters differ significantly (P < 0.05). Protein sample solutions were 1 mg/mL for ABTS and 8 mg/mL for DPPH.

changes resulting from pepsin digestion may also favor trapping of DPPH radicals, thus further enhancing the quenching by the ZH digest. Peptide structural changes at this stage would hinder the access by $ABTS^{+\bullet}$, a water-soluble radical. In our preliminary study, we attempted to establish the surface hydrophobicity of ZH digests using the fluorescent 1-anilinonaphthalene-8-sulfonic acid probe, but the result was inconclusive, most probably due to charge interaction of short peptides with the anionic probe that hindered an accurate measurement (*37*).

However, when the pepsin digest was further hydrolyzed with pancreatin, additional peptide bond cleavages would lead to the accumulation of shorter peptides (tri- and dipeptides) and amino acids, namely, the products became more hydrophilic. The digests with increased polarity (amino acids; small peptides) could readily react with water-soluble $ABTS^{+\bullet}$ but not with lipid-soluble $DPPH^{\bullet}$, and this would explain the complex scavenging behaviors of the protein digests in the two radical systems. Chen et al. (*6*) reported that peptides derived from the digests of soybean protein could not interact properly with hydrophobic peroxyl radicals due to the lack of hydrophobic patches in peptides. Mendis et al. (*38*) indicated that antioxidant

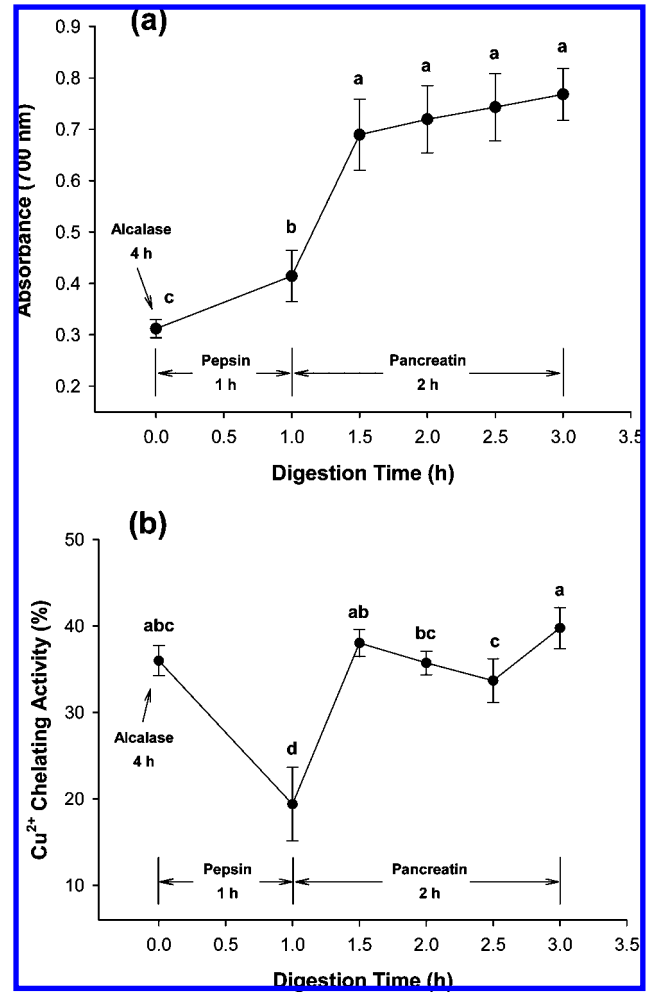

Figure 4. Changes in reducing power (**a**) and Cu^{2+} chelating activity (**b**) of alcalase-hydrolyzed zein during sequential in vitro digestion. Results are plotted as the means ± SD (n = 3, each with three measurements). Means with different letters differ significantly (P > 0.05). Protein sample solutions for both assays were 8 mg/mL.

reactivity of squid skin gelatin peptides was due to hydrophilic−hydrophobic partitioning in the sequence.

Of particular note was the remarkable difference in the overall capability to scavenge ABTS radicals and DPPH radicals by the same ZH samples, with or without in vitro digestion. The ZH samples tested in the $ABTS^{+\bullet}$ system, although at a lower concentration (1 mg/mL), had a Trolox equivalence of approximately 50 times that of ZH sample (8 mg/mL) tested in the $DPPH^{\bullet}$ system. The difference in efficacy can be attributed to solubility and diffusivity of radicals. A fat-soluble compound, $DPPH^{\bullet}$, although predissolved in ethanol prior to being dispersed in the aqueous assay solution, may not readily diffuse to the target peptides; therefore, its reactivity could be limited. In contrast, as a water-soluble radical species, $ABTS^{+\bullet}$ would readily reach peptides in the aqueous assay solution and, hence, react effectively with peptides in ZH. Even though DPPH is widely employed as a reactant radical in the study of antioxidant peptides in aqueous solutions (*12, 19, 29, 38*), caution must be taken when interpreting the assay results. Data obtained from the present study suggested that the DPPH assay was not an appropriate method for the measurement of antioxidant activity of water-soluble proteins and peptides in an aqueous solution.

The activity change for Cu^{2+} chelation and $ABTS^{+\bullet}$ scavenging followed a similar trend during in vitro digestion, suggesting that the two might be related. The structure–function relationship

for metal binding by peptides has been well-studied. For example, it was reported that a "cage structure" in metallothionein, which excludes surrounding water, would allow segments of the thiol protein to bind more Cu^{2+} than a structure that was loose (*39*). It was possible that treatment with pepsin disrupted the spatial structure of ZH peptides conducive to binding and trapping of Cu^{2+} and $ABTS^{+\bullet}$, resulting in reduced chelation capability. On the other hand, as high-affinity metal-binding groups became fully exposed or newly formed by pancreatic digestion, for example, the imidazole and carboxylic groups, electrostatic and ionic interactions with Cu^{2+} were probably enforced. Decker et al. (*40*) showed that the specific peptide structure and amino acid side chain groups played an important role not only in terminating free radical chain reactions but also in chelating transition metal ions. Peptides reported to stabilize prooxidative transitional metal ions include carnosine (*35, 41*), fragments of myofibrillar proteins (*19*), potato peptides (*11*), synthetic oligopeptides (*18*), and peptides rich in His (*42*).

The size of peptides is known to be a significant factor in the overall antioxidant activity of hydrolyzed proteins. We have previously shown that short peptides (MW 350–1500 Da) were responsible for the bulk of antioxidant activity in commercial whey protein hydrolysates in a liposome oxidizing system (*7*). Wu et al. (*29*) reported that a peptide from mackerel protein hydrolysate with MW of approximately 1400 Da possessed stronger antioxidant activity than higher MW peptides. Chen et al. (*43*) claimed that six peptides of soybean protein hydrolysate with 5–16 amino acid residues were highly effective in inhibiting the autoxidation of linoleic acid. In the present study, the final digest of ZH (2 h pancreatin), which exhibited the highest overall antioxidant potential, was comprised of almost 50% short peptides with three or more amino acids residues (MW 335–355 Da). It was not clear whether some of the remnant peptides could be absorbed, thereby exerting biological functions in the target cells, or otherwise perform additional functions in the lower gastrointestinal tract, such as bile acid binding, as demonstrated by other plant protein digests (*44*).

In conclusion, alcalase zein hydrolysate can act as a hydrogen donor, a water-soluble radical quencher, and a transitional metal ion sequester. The ABTS assay was a far more sensitive, thus, a superior, method to analyze the radical scavenging ability of water-soluble peptides, when compared with the DPPH method. These activities of zein hydrolysate were retained or improved following stepwise enzyme digestion simulating the human upper digestive tract. The peptide chain length, the high percentages of antioxidative amino acid residues present, and the concentration of free amino acids appeared to collectively contribute to the strong bioactivities of the protein digests. Therefore, the benefits of ZH could be extended beyond its role as a potential antioxidant to preserve food quality, because when ingested, it may help promote human health.

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NOTE ADDED AFTER PRINT PUBLICATION

A printing error occurred in Table 3. The paper was originally posted ASAP on April 1, 2008, and the corrected version was posted on May 7, 2008. An addition and correction is included in the May 28, 2008 (Vol. 56, No. 10), issue.

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