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# Antioxidative Activity of Protein Hydrolysates Prepared from Alkaline-Aided Channel Catfish Protein Isolates

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Antioxidative activity of hydrolyzed protein prepared from alkali-solubilized catfish protein isolates was studied. The isolates were hydrolyzed to 5, 15, and 30% degree of hydrolysis using the protease enzyme, Protamex. Hydrolyzed protein was separated into hydrolysates and soluble supernatants, and both of these fractions were studied for their metal chelating ability, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability, ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and their ability to inhibit the formation of thiobarbituric acid reactive substances (TBARS) in washed tilapia muscle containing tilapia hemolysate. Both hydrolysates and supernatants were characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results showed that DPPH radical scavenging ability and reducing power of catfish protein hydrolysates decreased, whereas the ORAC value, metal chelating ability, and ability to inhibit TBARS increased, with an increase in the degree of hydrolysis. Hydrolysate samples showed higher DPPH radical scavenging ability and $Fe^{3+}$ reducing ability, and supernatant samples had higher metal chelating ability. In general, low molecular weight (MW) peptides had high ORAC values and high metal chelating ability, and high MW peptides had a higher reducing power (FRAP) and were more effective in scavenging DPPH radicals. In a washed muscle model system, the ability of catfish protein hydrolysates and their corresponding supernatants to inhibit the formation of TBARS increased with an increase in the degree of hydrolysis.

**KEYWORDS: Enzyme hydrolysis; Protamex; fish protein hydrolysate; protein isolate; catfish; DPPH; FRAP; ORAC; TBARS; metal chelation**

## INTRODUCTION

During the past decade, consumers have begun to show increased preference for the use of natural antioxidants and additives in food products (*1*). Traditionally, natural food antioxidants were prepared from plants and plant by products (*2–4*). However, due to interest in the utilization of seafood byproducts, numerous researchers have started evaluating aquatic products as a source of natural antioxidants (*5, 6*). Fish muscle contains a large number of components that can be either pro-oxidative or antioxidative. For example, heme pigments present in the muscle tissue could promote lipid oxidation and oxidative rancidity (*7*), whereas components from the press juice of fish muscle has been shown to inhibit hemoglobin-mediated lipid oxidation (*8*). In addition, certain proteins and peptides found in muscle have demonstrated high antioxidative activity (*9*). Previous studies have shown that protein hydrolysates derived from fish muscle may have antioxidative effects (*5, 10*). However, one drawback in the preparation of antioxidants from fish and fish byproducts is the presence of unstable lipids and heme proteins (*9*). Fish products can be a rich source of polyunsaturated fatty acids and glycerides (*11*) and could potentially contaminate and decrease the antioxidant activity of protein hydrolysates. Heme proteins in the raw material can also become oxidized during the hydrolysis process, thereby promoting lipid oxidation. One way to overcome this problem is to prepare protein isolates using an alkaline solubilization technique (*12*). In brief, this technique involves solubilizing the myofibrillar and sarcoplasmic proteins of fish muscle at alkaline pH (around pH 11.0) to separate contaminants such as lipids, connective tissues, and bones from soluble proteins. Protein isolates can then be prepared by precipitating the myofibrillar and sarcoplasmic proteins at their isoelectric pH (around pH 5.5). Undeland et al. (*13*) had earlier shown that protein isolates prepared using an alkali solubilization technique have lower total lipid and phospholipid content. Hence, protein isolates prepared using the alkaline pH-shift method could yield a much purer protein substrate for enzyme hydrolysis. In our current study, we intended to evaluate the antioxidant properties of protein hydrolysates prepared from alkali-solubilized channel catfish

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(*Ictalurus punctatus*) protein isolates. Channel catfish is the most aquacultured species in the United States (*14*), and hence, exploring new and creative methods to utilize catfish would be a lucrative and environmentally responsible avenue. In the research work presented here, we have investigated the antioxidant activity of catfish protein hydrolysates using radical scavenging ability, metal chelating ability in aqueous solution, reducing power, oxygen radical absorbance capacity (ORAC), and the ability to delay the formation of thiobarbituric acid reactive substances (TBARS) in a washed muscle model system, in which oxidation was catalyzed using tilapia hemolysate.

## MATERIALS AND METHODS

**Materials.** Catfish and tilapia fillets were purchased from a local establishment within one day of harvest and transported to the laboratory in ice. Chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). All reagents were of ACS grade. Protamex, the enzyme used for protein hydrolysis, was a gift from Novozymes A/S Denmark.

**Preparation of Proteins Isolates Using the Alkali Solubilization Method.** Protein isolates were prepared from catfish fillets using an alkaline treatment as described in Hultin et al. (*12*). In brief, catfish fillets were minced in a Waring PRO Professional Meat Grinder, model MG800 (Waring Products, East Windsor, NJ) through a $^3/_{16}$-in.-diameter sieve. The mince was mixed with 9 parts of cold, deionized water and homogenized using a Waring commercial blender (model 51BL32, Waring Commercial, Torrington, CT) at high speed for 30 s. The pH of the homogenized sample was adjusted to 11.0 using 2 N sodium hydroxide, and the mixture was centrifuged at 10 000*g* for 20 min at 0−4 °C in a Sorvall RC-5B superspeed refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co. Newtown, CT). The supernatant consisting of soluble protein was filtered using a double-layered cheesecloth, and the pH of the filtrate was adjusted to 5.5 using 2 N HCl. The mixture was then centrifuged at 10 000*g* for 20 min at 0−4 °C. Protein isolate obtained as sediment was used for enzymatic hydrolysis. The amount of protein in the isolates was determined by the Biuret reaction (*15*).

**Enzymatic Hydrolysis of Catfish Protein Isolate.** Catfish protein isolates were hydrolyzed using the enzyme Protamex, a bacillus protease complex. Protamex is a commercial mixture of exopeptidases and endoproteases from multiple microbial sources. For enzymatic hydrolysis, catfish protein isolate was mixed with deionized water and homogenized for 1 min at maximum speed using a tissue homogenizer (Ultra Turrax T18, IKA, Germany). The protein concentration of the homogenate was adjusted to 3% w/v by using double-distilled water. The temperature and the pH of the homogenate were maintained at 22 °C and 7.5, respectively. The enzyme, Protamex, was then added to the protein homogenate at 1% w/w (based on the weight of homogenate), and the degree of hydrolysis (DH) was calculated using the equation (*16*)

$$\%\ \text{degree of hydrolysis (DH)} = \frac{BN_{\text{base}}}{\alpha h_{\text{tot}}\text{MW}} \times 100 \qquad (1)$$

where, $B$ = volume of base used, $N_{\text{base}}$ = normality of base used, $\alpha$ = degree of dissociation, MW = grams of protein (% N × 6.25), $h_{\text{tot}}$ = total number of peptide bonds per mass unit (7.502 meq/kg). The degree of dissociation ($\alpha$) was found by the following equation

$$\alpha = \frac{10^{\text{pH}-\text{p}K_a}}{1 + 10^{\text{pH}-\text{p}K_a}} \qquad (2)$$

where pH is the value at which enzyme hydrolysis was performed. The isolates were hydrolyzed to 5, 15, and 30% degree of hydrolysis (DH). Once the desired % DH was achieved, the homogenate was heated to 90 °C for 10 min to inactivate the enzymes. The samples were homogenized again and then transferred to freezer bags and stored at −20 °C until analysis.

Catfish protein hydrolysate was separated into two fractions, namely, soluble protein supernatant and precipitated protein hydrolysate, by centrifuging in an Eppendorf centrifuge (model 5415D, Eppendorf Int., Hamburg, Germany) at maximum speed of 16 000*g* for 10 min at 4 °C. The hydrolysates and supernatants were subjected to the Kjeldahl procedure to determine crude protein content, as outlined by the Official Methods of Analysis of the AOAC (*17*). On the basis of the Kjeldahl protein analyses, the hydrolysates and supernatants were adjusted to 0.15% protein concentration using deionized water and homogenized on speed 5 with a hand-held Tissue Tearor (Biospec Products Inc., Bartlettsville, OK) before all analyses.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).** The molecular weight of the protein hydrolysates and supernatants was determined using Tris−HCl, 4−20% linear gradient SDS gels (Bio-Rad Laboratories, Hercules, CA) according to the method of Laemmli (*18*) using 50 $\mu$g per lane protein concentration. Sigma (Sigma Chemical Co, St. Louis, MO) wide range markers with molecular weights 6.5−205 kDa was used as the protein molecular weight (MW) standard. After electrophoresis, the proteins were stained using Sigma Brilliant Blue perchloric acid staining solution. After the desired staining was achieved, gels were placed on an Epson Stylus CX5400 scanner and scanned. Images were analyzed using Scion Image 4.0.2 (Scion Co., Frederick, MD) software. The bands in the samples were compared with known bands of protein standards. Percentages of specific bands could be calculated on the basis of the area in the densitogram.

**Determination of Radical Scavenging Ability.** The radical scavenging ability of catfish protein hydrolysates and supernatant was determined using a combination of the methods of Bersuder et al. (*19*) and Saiga et al. (*20*). This assay measures the decomposition of the stable radical, α-,α-diphenyl-$\beta$-picrylhydrazyl (DPPH), which correlates directly with the ability of hydrolysates to act as free radical scavengers. The hydrolysates/supernatant or deionized water (blank) was vortexed with a mixture of ethanol (99%) and 0.02% DPPH and incubated at room temperature (~22 °C) for 30 min. After incubation, the sample was pipetted into a cuvette, and its absorbance was read at 517 nm using an Agilent diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). The residual radicals were calculated using the following equation:

$$\text{residual DPPH radicals (\%)} = 100 - \frac{\{(X + Y - Z) \times 100\}}{X} \qquad (3)$$

where X = absorbance of the DPPH blank, $Y$ = absorbance of the control sample, and $Z$ = absorbance of the DPPH sample.

**Ability to Chelate Metal Ions.** The metal chelating ability of catfish supernatant and hydrolysates was evaluated using the method of Saiga et al. (*20*) and Cheng et al. (*21*). The following three solutions were prepared for this assay: 0.1% pyridine (pH 7.0), 4 mM $CuSO_4$, and 20 mM pyrochatechol violet (PV). Ethylenediaminetetraacetic acid (EDTA) at 0.045% was used as the standard metal ion chelating agent. The hydrolysate, supernatant, or EDTA samples (1 mL) were combined with 2 mL of 0.1% pyridine (pH 7), 100 $\mu$L of 4 mM $CuSO_4$, 400 $\mu$L of deionized water, and 20 $\mu$L of 20 mM PV. The color change resulting from metal chelation was measured spectrophotometrically at 620 nm, and the results were reported as absorbance at 620 nm.

**Oxygen Radical Absorbance Capacity (ORAC) Assay.** The ORAC values of catfish hydrolysate supernatant fractions were measured using the methods of Ehlenfeldt et al. (*22*) and Cao et al. (*23*) at both 0.15 and 1.5% protein concentrations. The ORAC assay monitors the decay inhibition of fluorescein in the presence of AAPH 2,2′-azobis (2-amidinopropane) dihydrochloride, a peroxyl radical generator. The fluorescein decay rate was tracked by calculating the area under the decay curve at 2 min intervals for a total of 70 min using SoftMax Pro Standard Edition software (Molecular Devices Corp., Sunnyvale, CA), and the fluorescein decay products were quantified using a standard curve of Trolox. The results were expressed as micromoles of Trolox equivalents (TE) per gram of sample tested.

**Measurement of Reducing Power.** The reducing power of catfish hydrolysates and supernatant was measured using the modified method of Oyaizu (*24*) and Wu et al. (*10*). In brief, the method involves mixing 2 mL of protein samples or deionized water (control) with 2 mL each of 0.2 M phosphate buffer at pH 6.6 and 1% potassium ferricyanide solution. The entire mixture was heated at 50 °C for 20 min and mixed

with 2 mL of 10% trichloroacetic acid solution. The reaction was initiated by mixing 2 mL of incubated protein/TCA solution with 0.4 mL of 0.1% ferric chloride and 2 mL water. After 10 min of incubation at room temperature, the mixture was centrifuged at 1000*g*, and the absorbance of the supernatant was read at 700 nm. Higher absorbance values indicated greater reducing power.

**Preparation of Washed Tilapia Model System.** The ability of catfish protein hydrolysates to inhibit oxidation in muscle foods was tested using a washed tilapia model system. Tilapia hemolysate was used for catalyzing oxidation. The washed tilapia model system was prepared as in Richards et al. (*25*). Tilapia white muscle was separated from the fillets and was minced in a Waring PRO professional meat grinder. The mince was washed twice with deionized water at a 1:3 mince-to-water ratio (w/w) by stirring for 2 min, allowing the mixture to stand for 15 min at 4 °C, and then dewatering the mixture on a fiberglass screen. The washed mince was mixed with 50 mM sodium phosphate buffer of pH 6.5 (close to the postmortem pH of tilapia) at a 1:3 mince-to-buffer ratio and homogenized using a Biohomogenizer at maximum speed for 30 s. The mixture was then centrifuged at 15 000*g* for 20 min at 4 °C in a Sorvall refrigerated centrifuge, and the sediment was collected. The pH of the sediment was adjusted to 6.5, and the moisture content was adjusted to 87%. Streptomycin was added to the sediment at 300 ppm to prevent microbial spoilage, and the entire mixture was used for washed system studies.

For model system studies, the washed system as prepared above was mixed in a mortar using a pestle along with 12 $\mu$mol of tilapia hemolysate/kg of muscle tissue and 0.35% (i.e., 0.35 g protein for every 100 g of washed system) protein hydrolysates or supernatant. A sample of the washed system with no hydrolysate was used as a control. Twenty-gram samples were transferred to Petri dishes and flattened using a spatula. The samples were then stored at 4 °C for oxidation studies. All samples were prepared in duplicate.

**Preparation and Quantification of Tilapia Hemolysate.** A modified method of Fyhn et al. (*26*) was used for preparing tilapia hemolysate. Blood was collected from the caudal vein of live tilapia fish using a micro pipet and transferred into heparin solution (30 units/mL and 150 mM NaCl). Heparinized blood was washed with four volumes of ice-cold 1.7% NaCl in 1 mM Tris buffer, pH 8.0, and centrifuged at 1000*g* for 10 min at 4 °C to remove blood plasma. The red cells obtained were washed three times with 10 volumes of the above buffer and centrifuged at 1000*g*. Cells were then lysed in three volumes of ice-cold 1 mM Tris buffer, pH 8.0, for 1 h. One-tenth volume of 1 M NaCl was then added to aid stromal removal before centrifugation at 30000*g* for 15 min at 4 °C in a Sorvall refrigerated centrifuge. The hemolysate obtained as the supernatant was stored at −80 °C. The heme protein content in tilapia hemolysate was quantified using a Coomassie plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL). In brief, tilapia hemolysate was first diluted using phosphate buffer (pH 8.0), and 100 $\mu$L of this diluted sample was mixed with 3 mL of Coomassie reagent and incubated at room temperature for 10 min, and the absorbance was measured at 595 nm. Bovine serum albumin with a concentration range of 1−100 $\mu$g/ mL was used for the standard plot.

**Measurement of Thiobarbituric Acid Reactive Substances.** The method of Lemon (*27*) was modified according to Raghavan et al. (*28*) for measuring thiobarbituric acid reactive substances. A 1-g amount of the sample was extracted with 3 mL of 7.5% TCA solution by homogenization with a Biohomogenizer at high speed for 1 min. The samples were centrifuged at 2000 rpm in an Eppendorf Centrifuge 5702 (Brinkmann Instruments Inc., Westbury, NY) for 10 min. A 2-mL aliquot of the supernatant was mixed with 2 mL of 0.02 M TBA solution and heated in a boiling water bath for 40 min. The color developed was spectrophotometrically measured at 530 nm. A standard curve was plotted using tetraethoxypropane. Malonaldehyde concentration was calculated using an extinction coefficient of $1.6 \times 10^5$ $M^{-1}$ $cm^{-1}$.

**Statistical Analysis.** Several batches of catfish fillets were combined, and protein isolates were prepared using the alkaline solubilization method. Catfish protein hydrolysates were prepared in duplicate from the protein isolate batches. Each assay with the hydrolysates was then performed in at least triplicate. Results are expressed as the mean ± SD. Dunnet's multiple comparison test was used for comparing the effect of different treatments to the control. Analysis of variance was employed to examine the difference among treatments at the $P < 0.05$ level. Tukey's test was used to determine significant differences ($p < 0.05$) among samples by using the statistical analysis system (SAS).

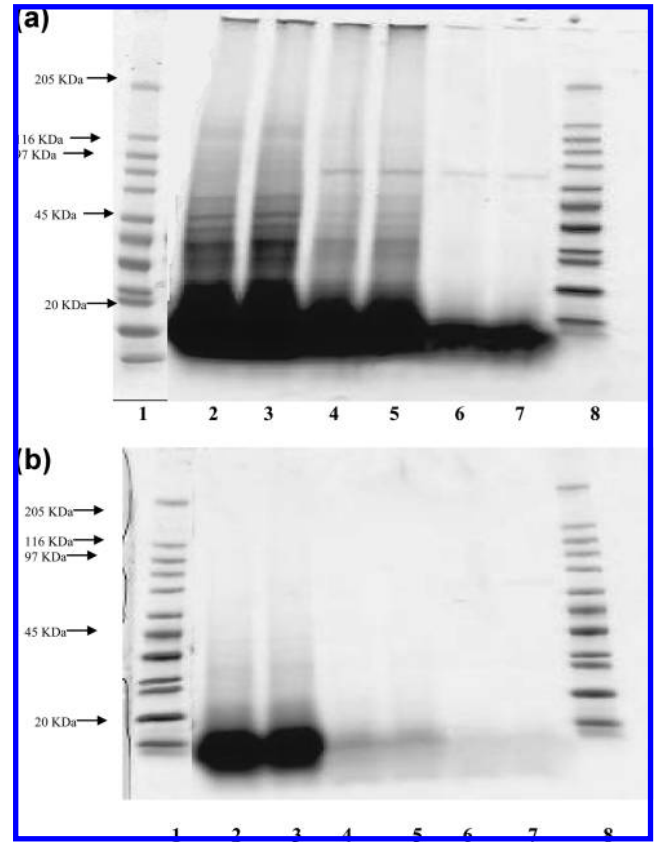


**Figure 1.** The protein composition of catfish protein (**a**) hydrolysates and (**b**) supernatants analyzed using precast Tris−HCl 4−20% linear gradient SDS-PAGE gel electrophoresis. Lanes 1 and 8 indicate molecular weight standards; lanes 2 and 3, 5% degree of hydrolysis (DH); lanes 4 and 5, 15% DH; lanes 6 and 7, 30% DH.

## RESULTS AND DISCUSSION

**SDS-PAGE Analysis of Catfish Protein Hydrolysate Fractions.** Catfish protein hydrolysates and their respective supernatant fractions were analyzed using SDS gel electrophoresis (**Figure 1a** and **b**). Standard MW markers (lanes 1 and 8) were used to determine the presence or absence of peptides of various molecular weights. Since enzyme hydrolysis breaks up myofibrillar proteins into a number of small peptides, absolute peptide identification was not possible using this method. Catfish hydrolysate showed a number of bands with a MW of 150 KDa or less. Even at 5% DH, myosin heavy chains (~200 KDa) were completely broken down into low-MW peptides, and the bands corresponding to the heavy chains were absent, whereas bands corresponding to light chains (~10−20 KDa) and actin (~42 KDa) could be seen for hydrolysate with 5% DH. A number of bands in the range of 20−45 KDa were also observed. These bands may indicate the hydrolytic products of myofibrillar proteins during alkali solubilization (*13*). The supernatant fraction of unhydrolyzed protein isolate (**Figure 1b**) showed prominent bands corresponding to actin and bands with molecular mass lower than 40 KDa. Among the catfish protein hydrolysates (**Figure 1a**), an increase in the degree of hydrolysis increased the amount of low-MW fractions, especially those with a MW less than 20 KDa. Similar trends have been reported by various other researchers for hydrolysates prepared from

salmon (*29*) and sardines (*30*). As the percent degree of hydrolysis increased from 5 to 30%, the number of bands corresponding to peptides of size 20−45 KDa decreased significantly ($p < 0.05$), indicating an extensive hydrolysis of catfish myofibrillar constituents. The highest MW peptide present in the hydrolysates was around 90 KDa (a light band was also visible around 116 KDa). The supernatant fractions (**Figure 1b**) had a significantly ($p < 0.05$) lower amount of high MW peptides than the hydrolysates (**Figure 1a**). Hydrolysis decreased the amount of high-MW peptides and increased the amount of low-MW peptides in the supernatant fraction. At 5% DH, the supernatant fractions showed a prominent band below the 20 KDa range. However, with an increase in the percent DH, the bands corresponding to the <20 KDa range disappeared, indicating extensive hydrolysis.

**Radical Scavenging Ability of Catfish Protein Hydrolysates.** One of the methods to evaluate the antioxidant property of fish protein hydrolysates is to determine their ability to scavenge free radicals. Free radicals are involved in initiating and propagating lipid oxidation, and hence, food antioxidants such as hydrolysates would play an important role in scavenging these radicals (*31*). Numerous methods are available to assay free radicals, such as the Trolox equivalent antioxidant capacity method, oxygen radical absorbance capacity, the ability to scavenge DPPH radicals, etc. Among these, some methods involve hydrogen atom transfer; others involve single electron transfer (SET), and a few others involve a combination of both the former and the latter. These methods have their own advantages and disadvantages (*32*). In our current study, we studied the ability of catfish protein hydrolysates to scavenge DPPH radicals. Scavenging of DPPH radicals involves primarily the SET mechanism, and DPPH radicals are not usually present in biological systems. However, this assay is quick and simple and is useful for fast screening of antioxidants. In our studies, both hydrolysate (**Figure 2a**) and supernatant samples (**Figure 2b**) showed a decrease in radical scavenging ability with an increase in the degree of hydrolysis. Among the hydrolysates (**Figure 2a**), samples with 5% DH had around 68% residual radicals, and hydrolysates with 15 and 30% DH had around 73 and 77% residual DPPH radicals, respectively. Among the supernatant samples (**Figure 2b**), those prepared with 5% DH had around 82% residual DPPH radicals, and samples prepared with 15 and 30% DH had around 90 and 93% residual DPPH radicals, respectively. The antioxidant ability of protein hydrolysates has been studied and reported by several researchers (*10, 33*) in test model systems. A number of researchers have shown an increase in DPPH radical scavenging ability with an increase in the degree of protein hydrolysis (*34, 35*). However, some researchers (*36*) have also reported a loss in DPPH radical scavenging activity with an increase in the hydrolysis of fish proteins. Hence, it seems that the radical scavenging ability of protein hydrolysates would depend on a variety of factors, such as the size of peptides, their composition, composition of free amino acids, DPPH test conditions, etc. (*10*). Our results suggest that partially hydrolyzed isolated catfish proteins are better scavengers of DPPH radicals than highly (15 and 30%) hydrolyzed proteins.

When compared side-by-side for the same protein concentration, the hydrolysate fractions were better free radical scavengers than their corresponding supernatant fractions. The SDS-gel electrophoresis data shows that the hydrolysates have higher MW peptide fractions (**Figure 1a**) than their corresponding supernatants (**Figure 1b**). In addition, the MW of the different hydrolysate and supernatant fractions decreased with an increase

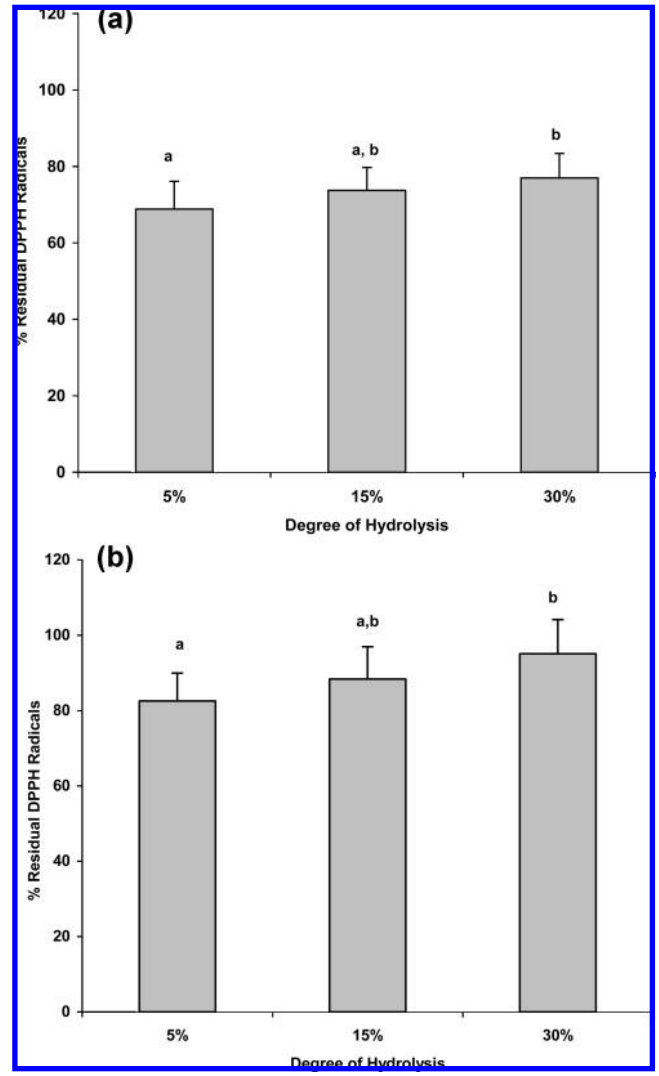


**Figure 2.** (**a**) DPPH radical scavenging activity of catfish protein hydrolysates at varying degrees of hydrolysis at 0.15% protein concentration. Lower percent residual DPPH radicals indicate higher scavenging ability. Treatments having different alphabets are significantly different ($p < 0.05$). (**b**) DPPH radical scavenging activity of catfish protein hydrolysate supernatants at varying degrees of hydrolysis at 0.15% protein concentration. Lower percent residual DPPH radicals indicate higher scavenging ability. Treatments having different alphabets are significantly different ($p < 0.05$)

in the degree of hydrolysis. These results could imply that that low-MW fractions are poorer radical scavengers as compared to high-MW fractions and that optimum radical scavenging ability was attained with around 5% DH for this system.

**Reducing Power of Catfish Protein Hydrolysates.** Another method of measuring the antioxidant potential of catfish protein hydrolysates is by using the ferric reducing antioxidant power (FRAP) assay. This SET assay uses antioxidants to reduce oxidants ($Fe^{3+}$ to $Fe^{2+}$) in a redox-linked colorimetric method (*37*). An increase in absorbance would indicate an increase in the reducing power of the samples. Among the several studies involving fish protein hydrolysates, some researchers have reported an increase in reducing power ability with an increase in the degree of protein hydrolysis (*38*), whereas others have reported a decrease in reducing ability (*36*). In our studies, all the catfish protein hydrolysate fractions (**Figure 3a**) showed a high reducing power as compared to the control (deionized water). Among the different hydrolysates, the reducing power

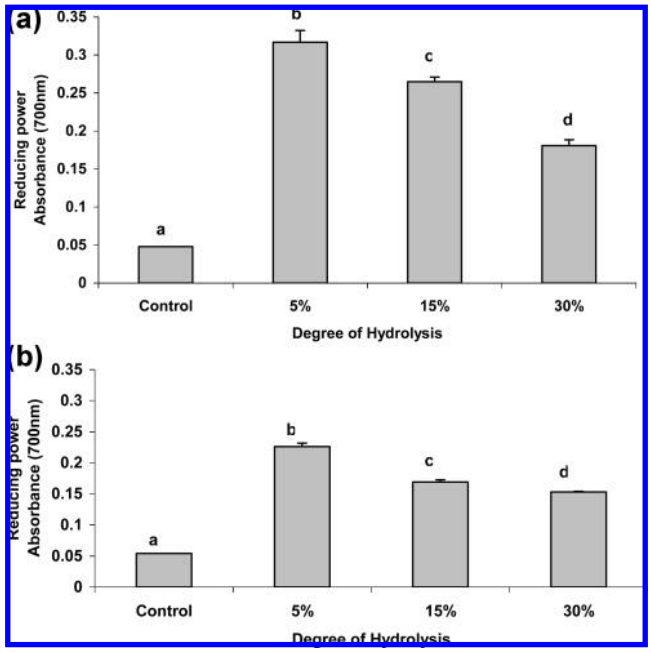


**Figure 3.** (**a**) Reducing power of catfish protein hydrolysates at varying degrees of hydrolysis at 0.15% protein concentration. Deionized water was used as a positive control. Higher absorbance indicates higher reducing power. Treatments having different alphabets are significantly different ($p < 0.05$) (**b**) Reducing power of catfish protein hydrolysate supernatants at varying degrees of hydrolysis at 0.15% protein concentration. Deionized water was used as a positive control. Higher absorbance indicates higher reducing power. Treatments having different alphabets are significantly different ($p < 0.05$).

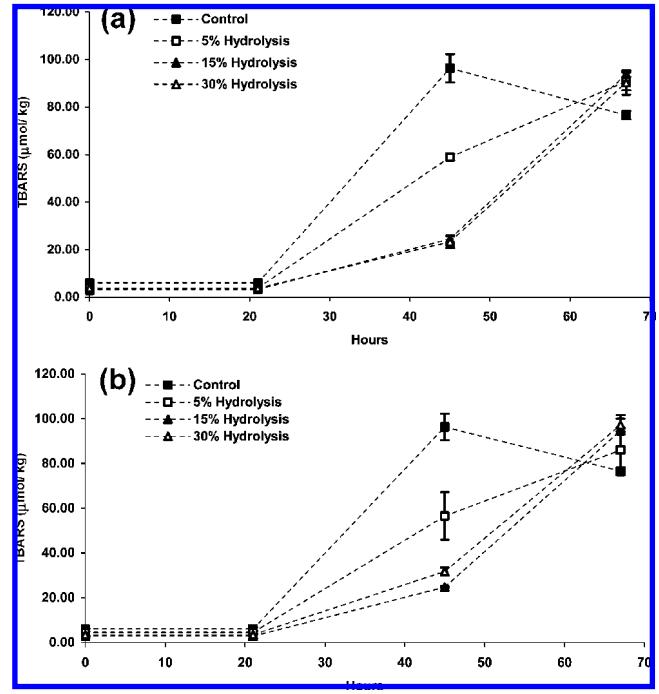


**Figure 4.** (**a**) The ability of catfish protein hydrolysates at varying degrees of hydrolysis to inhibit lipid oxidation in a washed tilapia muscle system containing tilapia hemoglobin (12 $\mu$M) as a catalyst. The control sample had no hydrolysate present. (**b**) The ability of catfish protein hydrolysate supernatant at varying degrees of hydrolysis to inhibit lipid oxidation in a washed tilapia muscle system containing tilapia hemoglobin (12 $\mu$M) as a catalyst. The control sample had no hydrolysate present.

decreased ($p < 0.05$) with an increase in the degree of hydrolysis. The reducing power of the hydrolysates decreased in the order, 5% DH > 15% DH > 30% DH > control. The supernatant fractions (**Figure 3b**) also showed a decrease in reducing power with an increase in the degree of hydrolysis. When supernatant fractions were compared with hydrolysate fractions, the hydrolysate samples had significantly ($p < 0.05$) higher reducing power than supernatant samples. These results (**Figure 3a** and **b**) indicate that reducing power, like DPPH radical scavenging ability, was dependent on the MW of the peptide fractions. In addition, low-MW fractions, such as highly hydrolyzed hydrolysates or supernatant samples, had lower reducing power.

**Ability to Inhibit Lipid Oxidation in a Model Washed-Muscle System.** The antioxidant ability of hydrolysates is usually tested in aqueous solutions using various chemical and bioassays. However, a meat or a muscle food product would contain, in addition to water, several other constituents, such as proteins, lipids, pro-oxidants such as heme pigments, and lipid- and water-soluble antioxidants. Hence, researchers have used washed-muscle model systems for simulating muscle foods and for testing various antioxidants (*8, 28, 35*). Oxidation in these model systems can be promoted by the external addition of fish hemolysate, and the substrate for lipid oxidation, such as triglycerides, can be added externally at desired levels (*28*). These model systems also contain membrane phospholipids (which are polyunsaturated and have a large surface area), which could initiate oxidation in muscle food products. In general, oxidation in muscle foods could be studied by measuring (a) lipid hydroperoxides or (b) the formation of TBARS. In earlier studies using a washed-muscle model system (*35*), we have found that the development of lipid hydroperoxides and TBARS follows a very similar pattern. Moreover, in a model system, we found that the development of TBARS was related closely to the development of "painty" odor and rancidity (*28*). Hence, in our current research, we focused on the ability of protein hydrolysates to control the formation of TBARS. The ability of catfish hydrolysates and the supernatants to inhibit lipid oxidation and the formation of TBARS was studied using a washed tilapia model system. Oxidation was catalyzed using tilapia hemoglobin. Oxidation curves were characterized by an initial lag phase (linear portion) followed by a slow or rapid increase in lipid oxidation. The intersection of the slope of a rapidly oxidizing phase and that of the lag phase was considered as the point where oxidation begins to occur. Both catfish protein hydrolysates (**Figure 4a**) and supernatants (**Figure 4b**) were effective ($p < 0.05$) in reducing lipid oxidation. The control with no added hydrolysates and samples with 5% DH hydrolysates started oxidizing in 20 h. Hydrolysates with 15 and 30% DH could inhibit oxidation up to 41 h. Among the supernatant fractions, supernatant with 5, 15, and 30% DH could inhibit oxidation for up to 20, 40, and 38 h, respectively. These results indicate that hydrolysates with a greater degree of hydrolysis were more effective in inhibiting lipid oxidation in a washed tilapia model system. However, these results were contradictory to the DPPH radical scavenging ability (**Figure 2a** and **b**) and the FRAP ability (**Figure 3a** and **b**) of the hydrolysates and supernatants. Discrepancies among the radical scavenging ability and ability of antioxidants to inhibit oxidation in a washed-muscle system have been reported earlier by other researchers (*35, 39*). One reason for this difference could be the physical state of the system in which different antioxidant assays were performed. TBARS were measured in a biological system( i.e., washed tilapia), whereas DPPH and FRAP were performed in an aqueous system. The washed system mimics well a muscle food system and gives a good indication of the fish protein hydroly-

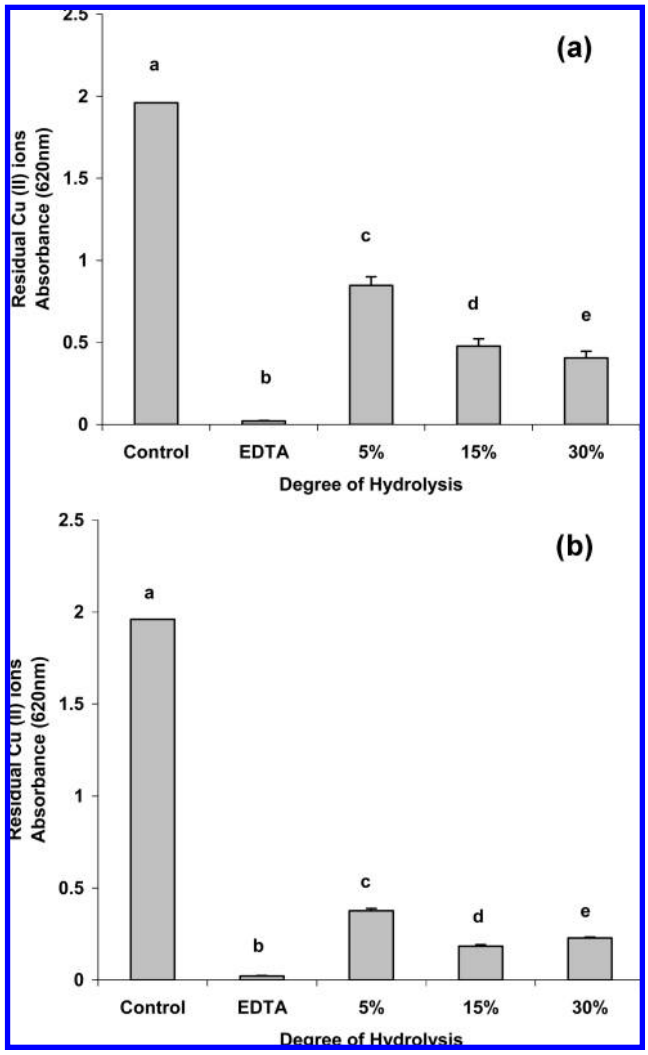


**Figure 5.** (**a**) Metal ion chelating activity of catfish protein hydrolysates at varying degrees of hydrolysis at 0.15% protein concentration. EDTA was added as a standard chelating agent. The control did not contain a metal chelating agent. Lower residual Cu (II) ions indicate higher chelation ability. Treatments having different alphabets are significantly different ($p < 0.05$). (**b**) Metal ion chelating activity of catfish protein hydrolysate supernatants at varying degrees of hydrolysis at 0.15% protein concentration. EDTA was added as a standard chelating agent. The control did not contain a metal chelating agent. Lower residual Cu (II) ions indicate higher chelation ability. Treatments having different alphabets are significantly different ($p < 0.05$)

sates' effectiveness in muscle foods. The mechanism of action of the antioxidants in various test systems (*40*) and partitioning of antioxidants into various phases of food systems (*41, 42*) could all affect the results of antioxidant assays.

**Metal Chelating Ability of Hydrolysates.** Transition metals such as $Fe^{2+}$ and $Cu^{2+}$ could promote lipid oxidation by catalyzing the breakdown of peroxides into free radicals (*43*). Hence, in our studies, we wanted to determine the ability of catfish protein hydrolysates and their supernatants to chelate $Cu^{2+}$ in an aqueous system. EDTA was used as a positive control. Our studies showed that both catfish hydrolysates (**Figure 5a**) and supernatants (**Figure 5b**) were significantly more ($p < 0.05$) effective in chelating $Cu^{2+}$ ions than the control without any hydrolysate. Among the catfish protein hydrolysates, the metal chelating ability increased in the order 5% DH < 15% DH < 30% DH (**Figure 5a**). For the supernatant samples, the metal chelating ability increased in the order 5% DH < 30% DH < 15% DH (**Figure 5b**). These results indicate that small-MW peptides obtained from catfish hydrolysates were more effective in chelating metal ions than large-MW peptides. Similar results were earlier reported by Thiansilakul et al., (*38*) who studied the antioxidative activity of protein hydrolysates from round scad muscle. In our current studies, the ability of the hydrolysates and supernatants to chelate $Cu^{2+}$ correlated well with their corresponding ability to inhibit the formation of TBARS. It is possible that one of the mechanisms of action of catfish protein hydrolysate to inhibit oxidation in washed tilapia muscle was through metal chelation. The heme-bound iron in hemoglobin, the catalyst in the washed tilapia muscle, could therefore have been chelated by the hydrolysates. Previous studies have speculated that part of the pro-oxidative activity of heme proteins could be due to heme released from the heme proteins into the hydrophobic and oxidatively susceptible cell membranes (*44*).

**Oxygen Radical Absorbance Capacity Assay (ORAC) on Catfish Hydrolysate Supernatant.** Lately, numerous researchers have started using the ORAC assay, an assay based on the HAT mechanism, to evaluate activities of various types of antioxidants (*23, 40, 45*). This assay monitors the decay inhibition of a fluorescent probe, fluorescein, in the presence of AAPH, a peroxyl radical generator. In our current study, we used the ORAC assay to evaluate the radical quenching ability of catfish supernatant samples at 0.15 and 1.5% protein concentrations. Only supernatant samples were tested, because the hydrolysates were not soluble enough for the ORAC assay. The results of this assay were expressed in micromoles of Trolox equivalents per gram of sample. Among the supernatant samples, 30% DH supernatants showed the highest ORAC value for both the 1.5% (**Figure 6a**) and 0.15% protein concentrations (**Figure 6b**). In general, the antioxidative ability assessed using the ORAC assay increased with an increase in the degree of protein hydrolysis. At 1.5% protein concentration, 5, 15, and 30% DH showed around 9, 13, and 16 $\mu$mol Trolox equiv/g of protein, $p < 0.05$ (**Figure 6a**). For 0.15% protein concentration, 5, 15, and 30% DH showed around 2.7, 3.0, and 3.5 $\mu$mol Trolox equiv/g of protein (**Figure 6b**).

The results from ORAC assay of catfish supernatants were similar to the results obtained from (a) the metal chelation assay (**Figures 5a** and **b**) and (b) the ability of the supernatants to inhibit the formation of TBARS (**Figures 4a** and **b**) in the washed tilapia system. However, the results from the ORAC assay on catfish supernatants was quite different from the results from the FRAP and DPPH assays. The FRAP assay is a single-electron-transfer-based reaction, whereas the ORAC assay involves a hydrogen atom transfer reaction, and DPPH involves predominantly a single electron transfer (*46*) mechanism. Hence, the difference in the mechanism of ORAC, FRAP, and DPPH assays could have resulted in the lack of correlations between these methods, as discussed widely by several researchers (*40, 46–48*). The results from our studies show that the ability of catfish protein hydrolysates and supernatants to inhibit oxidation in muscle food systems would be best represented by TBARS measurement, the metal chelation effect, and the ORAC assay, as opposed to the FRAP and DPPH assays.

## CONCLUSION

Catfish protein hydrolysates showed a high antioxidant ability in terms of scavenging DPPH radicals, an ability to chelate $Cu^{2+}$ ions, ferric reducing power, ORAC value, and an ability to inhibit TBARS in a washed tilapia model system. Both hydrolysate and supernatant samples showed a similar ability

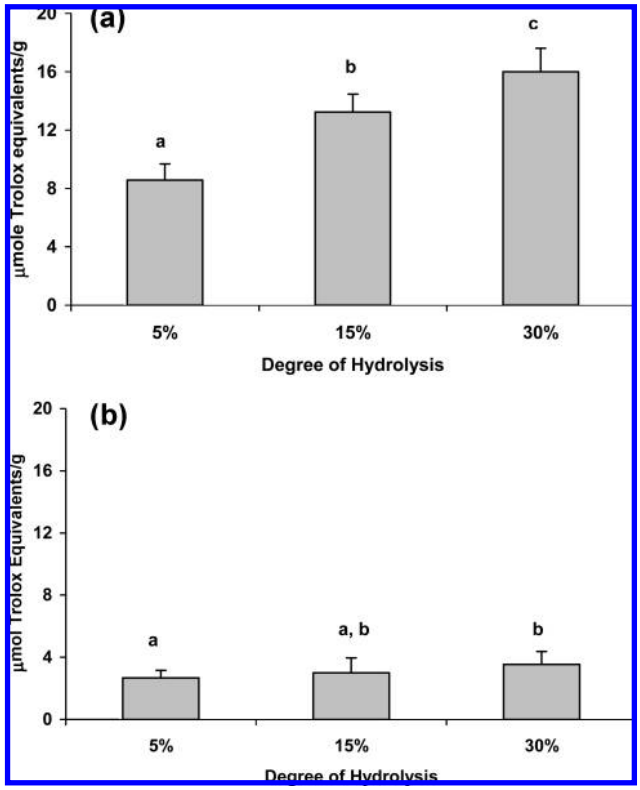


**Figure 6.** (**a**) The antioxidant activity (Trolox equivalence) of catfish protein hydrolysate supernatants at varying degrees of hydrolysis (1.5% protein concentration) as assessed by the ORAC method. Treatments having different alphabets are significantly different ($p < 0.05$). (**b**) The antioxidant activity (Trolox equivalence) of catfish protein hydrolysate supernatants at varying degrees of hydrolysis at (0.15% protein concentration) as assessed by the ORAC method. Treatments having different alphabets are significantly different ($p < 0.05$)

to inhibit TBARS. However, the ability to scavenge DPPH radicals and reduce $Fe^{3+}$ ions decreased with an increase in the degree of hydrolysis, whereas the ORAC values, metal chelating ability, and ability to inhibit TBARS increased with an increase in the degree of hydrolysis. Among hydrolysate and supernatants, hydrolysates showed a higher DPPH radical scavenging and reducing power and supernatants showed a high metal chelation ability. In supernatant samples, the ORAC value increased with an increase in the protein concentration. SDS-PAGE analysis showed that hydrolysates had a higher amount of high molecular weight peptides, and supernatant samples were higher in low molecular weight peptides. Hence, it could be concluded that high molecular weight peptides of alkali-treated catfish protein hydrolysates were better DPPH radical scavengers and reducing agents, and low molecular weight peptides were better metal chelators and showed high ORAC values. No significant difference between hydrolysates and supernatants were observed for their ability to inhibit TBARS formation in washed muscle model systems.

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