JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Radical Scavenging and Reducing Ability of Tilapia (*Oreochromis niloticus*) Protein Hydrolysates

SIVAKUMAR RAGHAVAN,[†] HORDUR G. KRISTINSSON,*[,†] AND CHRISTIAAN LEEUWENBURGH[‡]

Laboratory of Aquatic Food Biomolecular Research, Department of Food Science and Human Nutrition, University of Florida, Gainesville, Florida 32611, and Biochemistry and Aging Laboratory, Division of Biology of Aging, Department of Aging and Geriatrics, College of Medicine, Gainesville, Florida 32611

Enzymatically hydrolyzed fish protein hydrolysates could be used as a source of antioxidative nutraceuticals. In our current work, we have investigated alkali-solubilized tilapia (*Oreochromis niloticus*) protein hydrolysates for their ability to scavenge reactive oxygen species (ROS) and for their reducing power. Tilapia protein isolate was prepared by an alkaline solubilization technique and used as a substrate for enzyme hydrolysis. Cryotin, protease A 'Amano' 2, protease N 'Amano', Neutrase and Flavourzyme, were used separately to determine their effectiveness in hydrolyzing tilapia protein isolate. ROS scavenging ability was quantified using an isoluminol enhanced chemiluminescent assay in the presence of a) hydrogen peroxide or b) mononuclear cells isolated from human blood. Ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) of the hydrolysates using 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) or 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), were also investigated. Results showed that, in general, the TEAC, FRAP values and ROS scavenging ability of the hydrolysates increased with an increase in the degree of hydrolysis. Among the different hydrolysates, those prepared using Cryotin were most effective and Amano A2 hydrolysates were least effective in scavenging $ABTS^{\bullet+}$ and ROS generated by hydrogen peroxide. However, FRAP assay showed that hydrolysates prepared using Flavourzyme were most effective, and Amano N and Neutrase hydrolysates were least effective in reducing ferric ions. No significant difference was observed among the hydrolysates produced with different enzymes in their ability to scavenge ROS generated by phorbol myristate acetate stimulated mononuclear cells. These results shed light on the *in vitro* ROS scavenging ability of alkali solubilized tilapia protein hydrolysates, as well as potential nutraceutical use of these hydrolysates.

KEYWORDS: Reactive oxygen species, Mononuclear cells, H_2O_2, FRAP, TEAC, Enzyme hydrolysis, Tilapia isolate, Alkali solubilization

INTRODUCTION

Reactive oxygen species (ROS) are thought to play an important role in many disease conditions (*1*−*3*). In a normal aerobic cell, ROS usually exist in balance with biological antioxidants. However, disruption of this critical balance could result in oxidative stress, leading to many diseases. Oxidative stress could be alleviated by several food antioxidants (*4*), such as polyphenols (*5*) and carotenoids (*6*). Numerous researchers have studied the effect of food antioxidants and components on various pathological conditions (*7*−*10*). Hydrolysates obtained from the enzymatic hydrolysis of various food proteins are also known to possess antioxidant potential. During the past two decades, several studies have described the antioxidant properties of animal and plant protein hydrolysates (*11*−*14*). Recently, researchers have started focusing their attention on the nutraceutical properties of protein hydrolysates (*15*−*19*). One such source of protein hydrolysates is fish and fish byproducts of the aquatic industry. Investigators have reported various physiological and bioactive properties of fish protein hydrolysates, such as their role as immunomodulators (*20, 21*), effect on cholesterol lowering (*22*), role in glucose homeostasis (*23, 24*), role in controlling hypertension (*25*), and their antiproliferative and reparative properties (*26*).

Numerous factors could affect the antioxidant quality of fish protein hydrolysates. Among these, the nature and the quality of the raw material used for the preparation of hydrolysates could have a great impact on the functionality and antioxidant property of hydrolysates (*27*). One potential difficulty in the preparation of hydrolysates from muscle protein sources stems

* To whom correspondence should be addressed. Tel: 352-392-1991. Fax: 352-392-9467. E-mail: hordur@ufl.edu.
[†] University of Florida.
[‡] College of Medicine.

10.1021/jf8017194 CCC: $40.75
Published on Web 10/02/2008

from the presence of pro-oxidants such as heme and unstable oxidized lipid substrates. Contamination with these pro-oxidants could decrease the stability of protein hydrolysates and may limit their use. Earlier, Hultin et al. (*28*) had developed a new method, which involved solubilizing myofibrillar proteins at high (alkaline) pH and then recovering the solubilized proteins at pH 5.5 to obtain protein isolates with improved functional properties (*29*) and a low amount of unstable lipid substrates (*30*). In our current studies, we have used fish protein isolates prepared using the above-mentioned alkali process as a substrate for enzyme hydrolysis. We used five different enzymes for protein hydrolysis: (i) Cryotin-F, a mixture of trypsin, chymotrypsin, and elastase; (ii) Protease A "Amano" 2 (Amano A2), a mixture of endoprotease and exopeptidase from *Aspergillus oryzae*; (iii) Protease N "Amano" (Amano N), an endoprotease from *Bacillus subtilis*; (iv) Flavourzyme, a mixtures of endoprotease and exopeptidase from *A. oryzae*; and (v) Neutrase, an endoprotease prepared from *B. subtilis*. One of the objectives of our research is to determine the optimum degree of hydrolysis (DH) for the five (above-mentioned) enzymes, which would yield tilapia protein hydrolysates with optimum antioxidant efficacy.

The efficacy of an antioxidant could be determined using various assays (*31*). Choosing the right assay for a food or biological antioxidant, and subsequent interpretation of the assay results, has always been a challenge. In our current research, we wanted to look at the effect of tilapia fish protein hydrolysates on oxidative stress. Cellular model system studies using mononuclear cells prepared from human blood have been shown to be a sensitive in vitro method for testing antioxidant activity and oxidative stress (*32, 33*). Although these model system studies may not replicate the different types of chemical and physical conditions encountered by antioxidants in vivo, these in vitro studies using mononuclear cells have advantages in terms of cost, reproducibility, ability to control the environment, and time taken for studying antioxidant activities. Hence, our second objective was to study the efficacy of alkali-solubilized protein hydrolysates to scavenge oxygen species, especially superoxide, produced by human mononuclear cells. Mononuclear cells consisting of lymphocytes and monocytes were isolated from human blood (*33*), and these mononuclear cells were stimulated to produce ROS by using phorbol myristate acetate (PMA). ROS were then detected using an isoluminol-enhanced chemiluminescence (CL) method (*32*). The CL method could also be used for detecting other ROS such as H_2O_2, hydroxyl radicals, and alkoxyl radicals. Hence, in our studies, we also tested the ability of fish protein hydrolysates to quench CL due to exogenous H_2O_2 addition. In general, human cells function in a highly reduced state (*34*). Therefore, in addition to radical scavenging ability, the reducing potential of an antioxidant also plays an important role in physiological systems. In our current studies, we evaluated the electron transfer or reducing potential of tilapia protein hydrolysates using two assays, (i) the ability to reduce ferric to ferrous ions and (ii) a Trolox equivalent assay using 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radicals ($ABTS^{\bullet+}$). The results from these studies could shed light on the in vitro radical scavenging ability and reducing ability of alkali-solubilized protein hydrolysates prepared using different enzymes.

MATERIALS AND METHODS

Materials. Fillets of tilapia (*Oreochromis niloticus*) were purchased locally in Gainesville, FL, and transported to the laboratory on ice. Chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). All reagents were of ACS grade. Enzymes, Protease A "Amano" 2, and Protease N "Amano" were provided by Amano Enzyme USA Co. Ltd. (Elgin, IL). Flavourzyme and Neutrase samples were given by Novozymes A/S Denmark. Cryotin-F was a gift from North Ltd. (Reykjavik, Iceland).

Methods. *Preparation of Protein Isolates Using the Alkaline Method.* Protein isolates were prepared from tilapia white muscle using alkaline treatment as described in Hultin et al. (*28*). In brief, white muscle from tilapia was separated from the fillets and minced with 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 Biohomogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) at high speed for 1 min. The pH of the homogenized sample was adjusted to 11.0 using 2 N sodium hydroxide, and the mixture was incubated at 0−4 °C for 30 min. After incubation, the mixture was centrifuged at 10000*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 was filtered using a double-layered cheese cloth, and the pH of the filtrate was adjusted to 5.5 using 2 N HCl. The mixture was then centrifuged at 10000*g* for 20 min at 0−4 °C. The protein isolate obtained as sediment was used for enzymatic hydrolysis. The amount of protein in the isolates was determined by the Biuret reaction (*35*).

Enzymatic Hydrolysis of Protein Isolates. Enzymatic hydrolysis of tilapia protein isolates was performed using five different proteases: Amano A2, Amano N, Flavourzyme, Neutrase, and Cryotin-F. The optimum hydrolysis conditions used for these enzymes were as follows: Flavourzyme (50 °C, pH 7.0), Neutrase (55 °C, pH 7.0), Amano A2 (50 °C, pH 7.0), Amano N (55 °C, pH 7.0), and Cryotin (45 °C, pH 8.0). The protein isolate was added to deionized water and homogenized for 1 min at maximum speed using a Biohomogenizer. The concentration of the homogenate was then adjusted to 2% (w/v). The homogenate was equilibrated to the optimum pH and temperature condition corresponding to those enzymes used for hydrolysis. The enzyme preparation was then added to the homogenate, and the DH was calculated using the equation (*36*)

$$\mathrm{DH}\ (\%) = \frac{B \times N_{\mathrm{base}}}{\alpha \times h_{\mathrm{tot}} \times \mathrm{MW}} \times 100 \qquad (1)$$

where B = volume of base used, N_{base} = normality of base used, α = degree of dissociation, MW = amount of protein used, and h_{tot} = total number of peptide bonds per mass unit. The degree of dissociation (α) was determined by the equation

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{p}K_{\mathrm{a}}}}{1 + 10^{\mathrm{pH}-\mathrm{p}K_{\mathrm{a}}}}$$

where pH is the value at which enzyme hydrolysis was performed. The pK_a values were calculated according to Steinhardt et al. (*37*):

$$\mathrm{p}K_{\mathrm{a}} = 7.8 + \frac{(298 - T)}{298 \times T} \times 2400$$

Using each of the five proteases, tilapia protein isolates were hydrolyzed to achieve 7.5, 15, and 25% DH. Once the desired % DH was achieved, the homogenate was heated to 90 °C for 10 min to inactivate the enzymes, followed by cooling on ice. Hydrolyzed samples were stored at −20 °C until further use.

Determination of the Total Antioxidant Potential. The total antioxidant activity of alkali-solubilized tilapia protein hydrolysates was evaluated using the ABTS bisradical cation decolorization assay (*38*). This method is based on the ability of antioxidant molecules to quench the stable radical cation, $ABTS^{\bullet+}$, a blue-green chromophore with characteristic absorption at 734 nm, as compared with that of Trolox, a water-soluble vitamin E analogue. $ABTS^{\bullet+}$ was prepared by reacting 7 mM ABTS with 2.5 mM potassium persulfate in a 10 mM phosphate buffer (pH 7.4). The solution was kept in dark at room temperature for 12−16 h before use. The assay was standardized using Trolox, the synthetic antioxidant, and results were expressed as Trolox equivalents (Trolox equivalent antioxidant capacity, TEAC). Hydrolysate samples were prepared by vortexing a mixture of 2 mL of tilapia protein

hydrolysate and 5 mL of methanol for 1 min followed by centrifuging the mixture at 2000*g* for 10 min at 10 °C. The supernatant was used for analysis. The entire assay was run at 25 °C.

Determination of the Reducing Power. The reducing power of tilapia protein hydrolysates was determined according to the method of Oyaizu (*39*) with modifications. The antioxidant action of a reducing agent is based on its ability to donate an electron to Fe(III) ion to reduce it to Fe(II) ion. Protein hydrolysate extracts in phosphate buffer (0.2 M, pH 6.6) were mixed with 0.5 mL of methanol and 0.5 mL of potassium ferricyanide (1%), and the mixture was incubated at 50 °C for 30 min. Trichloroacetic acid solution (10%) was added to the mixture, which was then centrifuged at 600*g* for 10 min at room temperature. The supernatant was mixed with distilled water and ferric chloride solution (0.1%), and the absorbance was measured immediately at 700 nm.

Isolation of Mononuclear Cells from Whole Blood. Mononuclear cells were isolated using BD Vacutainer CPT cell preparation tubes with sodium heparin (Becton, Dickinson and Co., Franklin Lakes, NJ) according to manufacturer's directions. In brief, whole blood from healthy human donors was collected in the BD Vacutainer tubes. The contents of the tube were mixed immediately by gently inverting the tubes 8−10 times, and the blood samples were centrifuged at 1500*g* for 15 min at room temperature (18−25 °C). After centrifugation, the mononuclear cells were collected from the whitish layer beneath the blood plasma. The cells were washed twice with cold phosphate-buffered saline solution containing calcium and magnesium salts. The cells were finally suspended in Krebs−Ringer bicarbonate buffer supplemented with glucose, pH 7.3 (KRG buffer). The number of cells were counted using a hematocytometer, and the concentration was adjusted to 5×10^5 cells/mL. The cell percentage using BD Vacutainer method was reported as 79% lymphocytes and 12% monocytes (Data on file, Report no. R-88-99-QC-195, BD Vacutainer Systems, NJ). All human procedures were approved by the University of Florida Institutional Review Board.

CL Method. CL technique (*33*) was used for detecting ROS produced by (i) hydrogen peroxide (H_2O_2) and (ii) PMA-stimulated mononuclear cells. CL was detected using a LS-45 luminescence spectrometer (Perkin-Elmer Instruments, Waltham, MA). When H_2O_2 was used, the reaction mixture contained 60 μL of 0.1 M H_2O_2, 20 μL of 51.2 mM isoluminol, and 2.7 mL of Krebs−Ringer bicarbonate buffer solution with glucose (KRG). The reaction was started by the addition of 5 units (100 μL) of horseradish peroxidase (HRP). Protein hydrolysates diluted to a concentration of 0.035% were used for studying radical scavenging ability.

When mononuclear cells were used, the reaction mixture contained 200 μL of 51.2 mM isoluminol, 5 units of HRP, 2.1 mL of KRG buffer, and 200 μL of mononuclear cells. The reaction was activated by the addition of 300 μL of PMA (10^{-5} M). Protein hydrolysates were added at a concentration level of 2% for studying the radical scavenging ability. The CL was recorded at 25 °C until the maximum CL intensity was achieved. The area under the CL curve was calculated and compared with that of the control, which contained KRG buffer instead of protein hydrolysates. The ROS scavenging ability of the protein hydrolysates was measured by their ability to reduce the area under the CL curve.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Electrophoresis. SDS-PAGE was performed on the protein hydrolysates using a 10−20% tris-tricine gel (*40*) to characterize the hydrolysates based on their molecular weights. Around 50 μg of protein sample was loaded in each lane. The molecular weight of the hydrolysates was calculated with reference to the migration of SDS-PAGE wide range molecular weight standard (Sigma Aldrich, St. Louis, MO). After electrophoresis, the gels 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 based on the area in the densitogram.

Statistical Analysis. For the mononuclear cell assay, samples were collected from two ($n = 2$) different cell donors. Each hydrolysate sample was tested in duplicate on each mononuclear cell preparation.

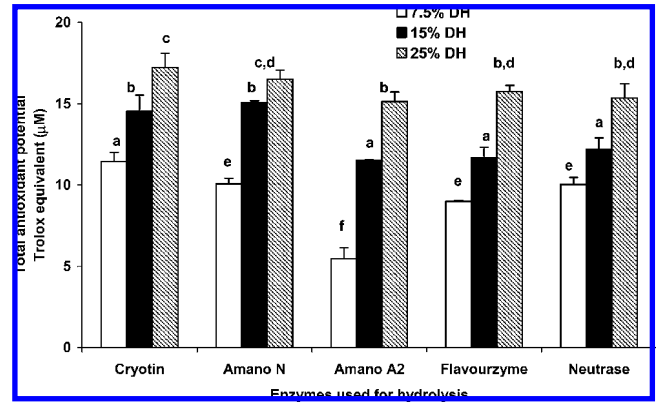

Figure 1. TEAC assay on tilapia protein hydrolysates. Tilapia protein isolates were prepared by solubilizing myofibrillar proteins at pH 11.0 and precipitating them at pH 5.5 (see the Methods section). Five different enzymes were used for hydrolyzing the protein isolates to 7.5, 15, and 25% DH (see the Methods section). The results were expressed as μM Trolox equivalents. Protein hydrolysates with different alphabets are significantly different ($p < 0.05$).

All other assays were conducted in triplicate ($n = 3$) on each hydrolysate sample. Statistical analyses were performed using JMP Statistical Discovery Software (version 5.0). Tukey's multiple comparison tests were used to compare the differences among the means. 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 differences among treatments at $P < 0.05$.

RESULTS

TEAC Assay. The TEAC assay is a colorimetric assay that evaluates the potential of an antioxidant to inhibit the formation of a colored radical cation $ABTS^{\bullet+}$, a blue-green chromophore with characteristic absorption at 734 nm. The $ABTS^{\bullet+}$ is generated by the oxidation of ABTS with potassium persulfate. The antioxidant activity was expressed as Trolox equivalents. In general, the total antioxidant potential of the hydrolysates increased significantly with an increase in % DH (**Figure 1**); that is, hydrolysates with 25% DH were more antioxidative than those with 15% DH, which in turn were better antioxidants than hydrolysates with 7.5% DH. At 7.5% DH, hydrolysates prepared using Amano A2 had the lowest TEAC value with a Trolox equivalent of 5 μM, while Cryotin hydrolysates showed the highest TEAC value with a Trolox equivalent of 11.4 μM. At 15% DH, hydrolysates prepared using Cryotin and Amano N (14.5 and 15.0 μM Trolox equivalents, respectively) showed significantly higher TEAC values than those prepared using Flavourzyme, Neutrase, and Amano A2 (~11.6 μM Trolox equivalents). At 25% DH, Amano A2 hydrolysates had the least Trolox equivalents, while Cryotin hydrolysates had significantly high Trolox equivalents. Among all of the hydrolysates, those prepared using Cryotin and Amano N protease showed the highest antioxidative potential, while Amano A2 protease showed the lowest antioxidant potential (**Figure 1**).

Reducing Power of Fish Protein Hydrolysates. One of the methods to determine the antioxidant capacity of fish protein hydrolysates is the measurement of ferric reducing antioxidant power (FRAP) or the reducing power assay. This method is based on the ability of an antioxidant (hydrolysates) to reduce Fe(III) to Fe(II) in a redox-linked colorimetric reaction, which involves one electron transfer. When the reducing power of various hydrolysates was compared in terms of enzymes used for hydrolysis, those prepared using Flavourzyme (with 7.5, 15, and 25% DH) showed a significantly high ($p < 0.05$) reducing

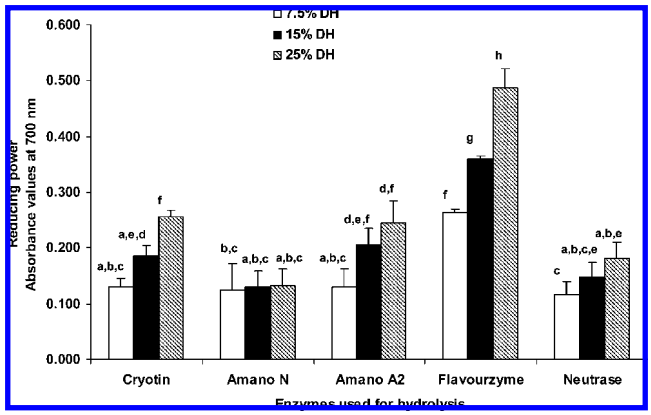

Figure 2. Reducing power of different protein hydrolysates was measured by the ability of hydrolysates to donate electrons and reduce Fe(III) to Fe(II) ions. The formation of Fe(II)/ferricyanide complex was measured as the absorbance at 700 nm. See the caption to **Figure 1** and the Methods section for the preparation of protein isolates and hydrolysates. Protein hydrolysates with different alphabets are significantly different ($p < 0.05$).

power, and Amano N hydrolysates showed a significantly low ($p < 0.05$) reducing power, as compared to Amano A, Cryotin, and Neutrase hydrolysates (**Figure 2**). In general, the reducing power of all enzyme hydrolysates increased with an increase in the DH. The reducing power of the Flavourzyme hydrolysates increased in the order 7.5 < 15 < 25% DH. For Amano A2, the increase in the reducing power followed in the order of 7.5 < 15 ~ 25% DH, while for Cryotin and Neutrase, the order was 7.5 ~ 15 < 25% DH and 7.5 ≤ 15 ≤ 25% DH, respectively. When Amano N was used for preparing hydrolysates, there was no significant difference ($p > 0.05$) among the reducing power of different hydrolysates (**Figure 2**).

Isoluminol-Enhanced CL Assay Using Hydrogen Peroxide. This assay is based on the detection of CL emitted by isoluminol in the presence of hydrogen peroxide and HRP. The mechanism involves the reaction of HRP with H_2O_2 to form an oxidized HRP, which in turn could react with the chromogenic substrate, isoluminol. In our studies, tilapia protein hydrolysates were added to scavenge the intermediate chromogenic radicals, which could result in a decrease in CL. All enzyme-hydrolyzed tilapia protein hydrolysates showed a significant ($p < 0.05$) decrease in isoluminol-enhanced peroxide CL (**Figure 3a**−**e**). The chemiluminescent area of the control was around 5.5×10^4, while all of the hydrolysates had a chemiluminescent area of less than 4.0×10^4. The ability of Cryotin hydrolysates to decrease CL increased in the order 7.5 < 15 ~ 25% DH (**Figure 3a**,**c**). When Flavourzyme, Neutrase, or Amano A2 was used, the antioxidant ability of the hydrolysates increased in the order 7.5 ~ 15 < 25% DH (**Figure 3b**,**d**,**e**). Among all of the protein hydrolysates, Cryotin hydrolysates with 15 and 25% DH and Flavourzyme hydrolysates with 25% DH showed a significantly ($p < 0.05$) high radical scavenging ability, while Amano A2 and Neutrase hydrolysates with 7.5 and 15% DH showed a significantly low radical scavenging ability.

Isoluminol-Enhanced CL Assay for the Detection of ROS. This assay was based on the production of ROS from the mononuclear cells of human blood upon stimulation by PMA and the subsequent detection of these ROS using the isoluminol-enhanced CL method. The antioxidant ability of tilapia protein hydrolysates was measured by their ability to scavenge ROS (or chromogenic radicals) as reflected by a reduction of CL signal. All of the hydrolysates showed a significant reduction ($p < 0.05$) in CL (**Figure 4a**−**e**) signal. Cryotin, Flavourzyme, and Amano A2 hydrolysates at 25% DH had the least chemiluminescent area of around 4×10^3 (and hence better scavengers of ROS), while Amano A2, Flavourzyme, and Cryotin hydrolysates at 7.5% DH level had the highest chemiluminescent area of around 1.1×10^4 (and hence poor ROS scavengers). The control had a chemiluminescent area of around 2×10^4. Hydrolysates prepared using Amano N and Neutrase showed no significant difference ($p > 0.05$) in ROS scavenging ability within themselves; that is, the scavenging abilities of hydrolysates with 7.5, 15, and 25% DH were similar (**Figure 4b**,**e**). However, for Cryotin, Amano A2, and Flavourzyme hydrolysates, the ROS scavenging ability increased in the order 7.5 ≤ 15 ≤ 25% DH (**Figure 4a**,**c**,**d**).

SDS−**PAGE of Tilapia Protein Hydrolysates.** Initial characterization of tilapia protein hydrolysates was done using SDS-PAGE. The molecular weight distribution and the pattern of peptides for different hydrolysates are shown in **Figure 5**. The tris-tricine gel showed that an increase in the DH decreased the amount of high molecular weight (HMW) peptides and increased the level of low molecular weight (LMW) peptides. At 7.5% DH, the amount of HMW (>30 kDa) decreased ($p < 0.05$) in the order Cryotin ~ Flavourzyme ≥ Amano *N* > Amano A2 > Neutrase. Cryotin and Flavourzyme showed a number of strong bands at 30 kDa, between 30 and 40 kDa, and a number of light bands at 50, 60, 80, and around 100 kDa. The corresponding bands for Amano N and Amano A2 were significantly less, and for Neutrase, they were virtually absent. At 15% DH, there was a significant decrease ($p < 0.05$) in the number of peptides with MW > 40 kDa. Cryotin and Flavourzyme showed a number of strong bands around 10, 15, and between 15 and 20 kDa. Amano N showed a number of strong bands between 3.5 and 10 kDa. Neutrase and Amano A2 showed a weak band around 10 kDa. At 25% DH, all of the protein hydrolysates showed a significant ($p < 0.05$) reduction in the number and the intensity of bands. The intensity of peptide bands in the SDS-PAGE gel decreased in the order 7.5 > 15 > 25% (**Figure 5**). This decrease could be due to the small size of the peptides (during 15 and 25% DH), which might have leaked out of the gel. Further studies are in progress to identify the specific type and sequence of peptides responsible for ROS scavenging activity.

DISCUSSION

ROS are responsible for numerous pathological conditions including cancer (*41*), cell injury (*42*), inflammation (*43*), Alzheimer's disease (*44*), intestinal diseases (*45*), and several other debilitating diseases. Hence, it is suggested that the consumption of antioxidant-rich diets would help alleviate diseases caused by ROS (*4*). In our current study, we wanted to evaluate the antioxidant potential of alkali-treated tilapia protein hydrolysates as a whole (prepared using different enzymes) and determine whether these hydrolysates have any commercial use as antioxidative nutraceuticals. We did not attempt at this point to fractionate these tilapia protein hydrolysates to identify individual peptides responsible for ROS scavenging or reducing activity. Numerous researchers have published results identifying individual fractions of peptides from various plant and animal hydrolysates, which are responsible for specific bioactivities (*46, 47*). Fractionation of peptides and identification of peptide sequence may provide information about the characteristics of peptides. However, we felt that a critical step toward commercializing fish protein hydrolysates as a nutraceutical would involve evaluation of the antioxidant functionality of the hydrolysate as a whole, rather than identify-

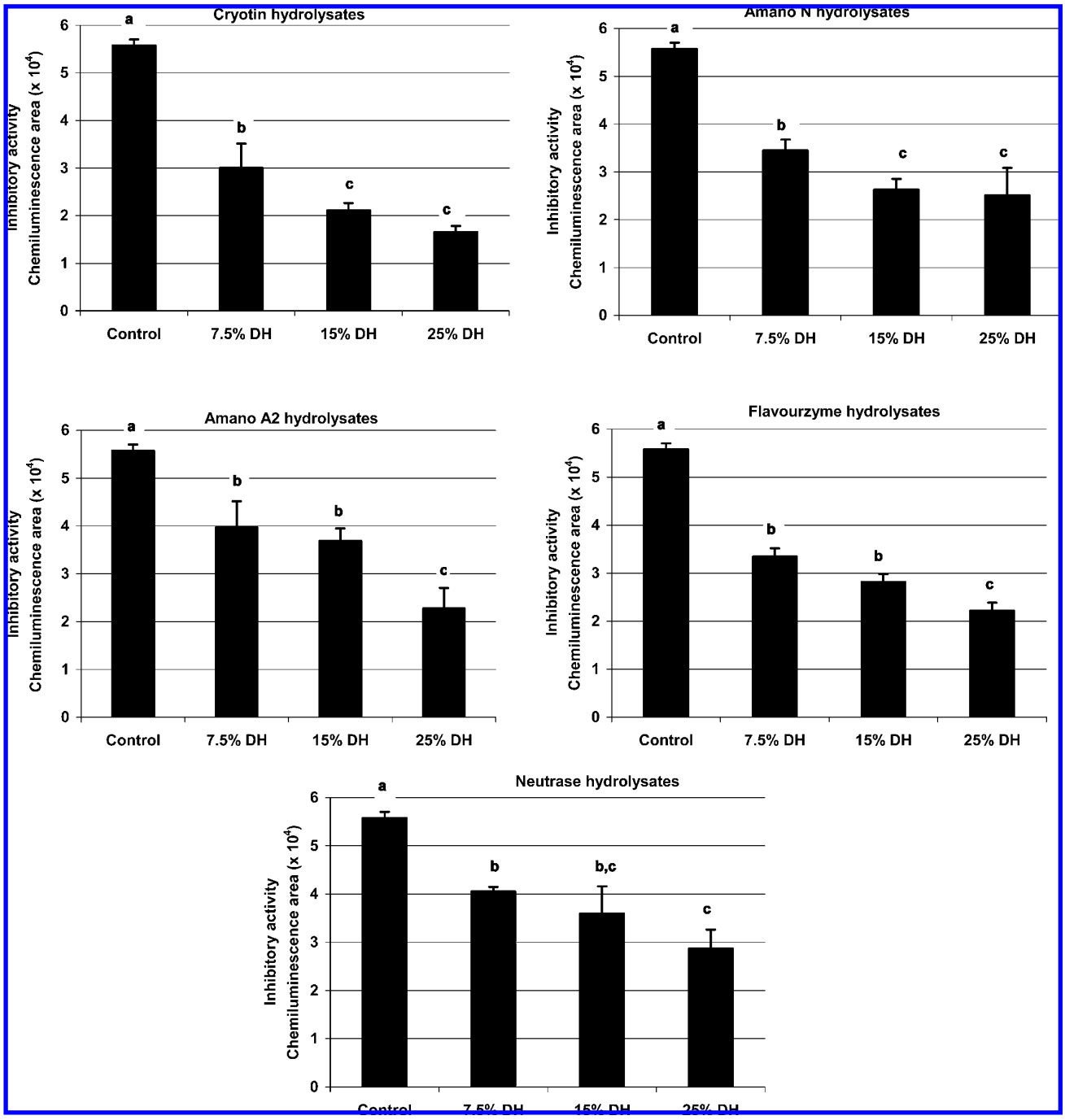

Figure 3. Chemiluminescent emission of the isoluminol−H_2O_2−HRP system at pH 7.3. See the caption to **Figure 1** and the Methods section for the preparation of protein isolates and hydrolysates. The chemiluminescent reaction was started after the addition of 5 units of HRP. Five different enzymes, Cryotin, Amano N, Amano A2, Flavourzyme, and Neutrase, were tested for scavenging radical species. In each figure, hydrolysates with different alphabets are significantly different ($p < 0.05$).

ing the various fractions of the hydrolysate responsible for antioxidant action. However, identifying, sequencing, characterizing, and evaluating individual peptides for various bioactivities could be important from both an academic stand point as well as for value addition. Hence, we intend in our future studies to identify the most antioxidative fractions and to determine ways to increase the yield of these specific fractions in a cost-effective and environmentally friendly manner.

Various in vitro (*31, 48*) and in vivo model systems (*49*) are being used for evaluating the potential of antioxidants. Among these, a cellular model system comprised of mononuclear cells and isoluminol/luminol was often used for antioxidant assays (*33, 50, 51*). This is due to the low cost, high speed, sufficient reliability, and relatively high reproducibility of these cell model systems as biomarkers of in vivo oxidation. Isoluminol is a chromogenic molecule, and the location of the amino group in the phthalate ring of isoluminol makes the molecule hydrophilic and membrane impermeable (*50*). Hence, isoluminol could be used for detecting extracellular H_2O_2 or peroxides secreted from the mononuclear cells. All of the protein hydrolysates (**Figure 4a**−**e**) showed a significant ($p < 0.05$) ability to scavenge ROS (generated by the stimulation of mononuclear cells) as compared to the control. The radical scavenging ability was indicated by a reduction in isoluminol CL. Cryotin, Flavourzyme, Amano A2, and Amano N hydrolysates at 7.5% DH (**Figure 4a**,**c**,**d**) showed the lowest ROS scavenging potential. Analysis of SDS-

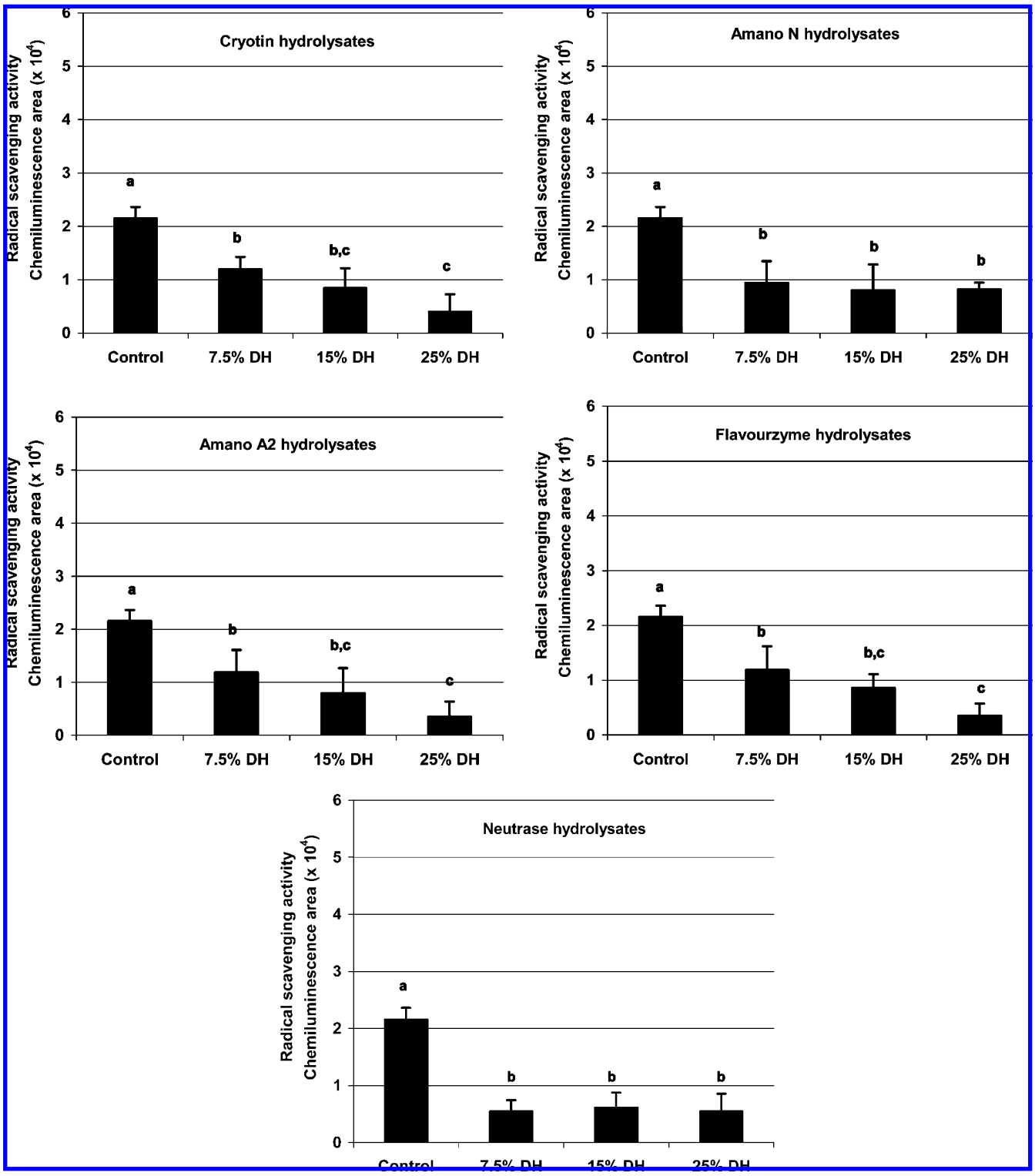

Figure 4. Effect of tilapia protein hydrolysates on PMA-induced chemiluminescent emission from freshly isolated mononuclear cells of human blood. The concentration of mononuclear cells used for chemiluminescent studies was 5×10^5 cells/ mL. See the caption to **Figure 1** and the Methods section for the preparation of protein isolates and hydrolysates. The chemiluminescent reaction was started after the addition of 5 units of HRP. Five different enzymes, Cryotin, Amano N, Amano A2, Flavourzyme, and Neutrase, were tested for scavenging radical species. In each figure, hydrolysates with different alphabets are significantly different ($p < 0.05$).

PAGE showed that these hydrolysates had the highest level of HMW peptides (>30 kDa). When tilapia protein isolate was hydrolyzed to 25% DH, the amount of HMW peptides decreased and LMW peptides increased ($p < 0.05$) significantly. These LMW peptides had a high ability to scavenge ROS generated by mononuclear cells. Hydrolysates prepared using Neutrase had a high level of LMW even at 7.5% DH (as compared to the other four enzymes), and Neutrase hydrolysates were effective scavengers of ROS. When H_2O_2 (chemical) was used instead of mononuclear cells, chemiluminescent analysis of H_2O_2 using peroxidase-isoluminol system showed an increase in antioxidant activity with an increase in % DH. Protein isolates hydrolyzed to 25% DH showed a significantly higher ($p < 0.05$) ability to scavenge radicals as compared to 7.5% DH (**Figure**

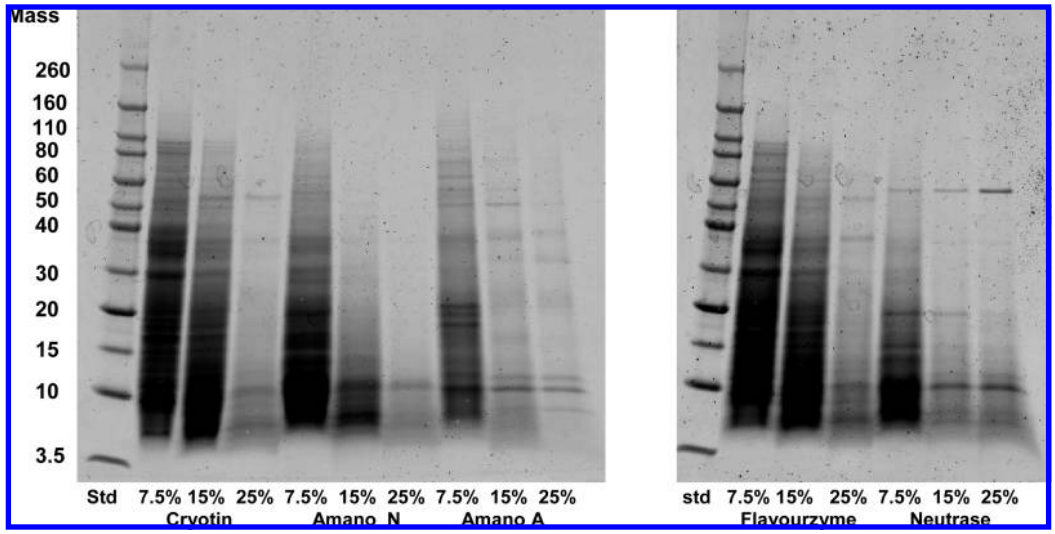

Figure 5. Commassie blue-stained SDS polyacrylamide gel of tilapia protein hydrolysates. Sample loaded on each lane, ~50 μg of protein. Samples loaded in the lanes correspond to 7.5, 15, and 25% degrees of hydrolysis using enzymes Cryotin, Amano N, Amano A, Flavourzyme, and Neutrase. The molecular weight standards (std) used were insulin B chain, 3.5 kDa; aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; BSA, 66.3 kDa; and phosphorylase B, 97.4 kDa.

3a−**e**). The radical scavenging ability of hydrolysates with 25% DH could be due to the presence of high amount of LMW peptides (**Figure 5**). Several researchers have reported the hydroxy radical scavenging ability of LMW peptides from casein (*52*), soy proteins (*53*), chick peas (*54*), and several other hydrolysates. The antioxidant activity has been attributed to the presence of free amino acids and small peptides, which can effectively scavenge radicals.

In general, the chemiluminescent area obtained using the H_2O_2−peroxidase system (**Figure 3a**−**e**) was higher than the corresponding area obtained using the mononuclear cells−peroxidase system (**Figure 4a**−**e**). For the H_2O_2−peroxidase system, the CL area was in the range of $2.0-5.0 \times 10^4$, while for mononuclear cells, the CL area was in the range of $0.5-2.0 \times 10^4$. Among the different enzymes, the hydrolysates prepared using Neutrase and Amano A2 (**Figure 3c**,**e**) were least effective, and Cryotin hydrolysates (**Figure 3a**) were most effective in scavenging ROS generated from H_2O_2. When mononuclear cells were used for ROS production, there was no significant difference ($p > 0.05$) among the activities of the five enzymes. However, Cryotin, Flavourzyme, and Amano A hydrolysates showed a difference in antioxidative activities with % DH. In the H_2O_2 −peroxidase system, exogenously added H_2O_2 was used as ROS, while in the cell-based assay, superoxide radical was primarily detected as ROS. Hence, the difference in the results between the two radical assays could be due to numerous factors including (i) the type of radical detected (peroxide or superoxide), (ii) the type of system (chemical or biological) used for ROS production, (iii) the location of the radical generating system, and (iv) how fast ROS are generated.

The TEAC assay is a based on both electron transfer as well as hydrogen atom transfer. The single electron transfer assay measures the ability of an antioxidant to reduce an oxidant, which in turn can be measured using a spectrophotometer as a change in color. In the TEAC assay, we generated and quantified $ABTS^{\bullet+}$ radical. TEAC studies have some disadvantages. The $ABTS^{\bullet+}$ radical is nonphysiological and hence may not truly represent an in vivo system. Also, the reaction may be susceptible to reducing agents with low redox potential. In spite of disadvantages, the TEAC assay is quick and easy and would give an overall estimate of the total antioxidant capacity and reducing power. The FRAP assay, which was developed by Benzie et al. (*55*), was originally used for measuring the reducing power in plasma. The FRAP or the reducing power assay is an electron transfer assay. In the FRAP assay, the protein hydrolysate reduces ferricyanide to ferrocyanide, which in turn combines with Fe^{3+} to produce a Prussian blue color complex (*56*). The pH of the solvent could play an important role in the FRAP assay. In acidic conditions, the electron-donating capability of an antioxidant could be suppressed due to protonation, while alkaline conditions could drastically enhance the electron transfer capability (*31*). Hence, we did the FRAP assay at a near neutral pH of 6.6 (physiological pH is around 6.8−7.0) using a phosphate buffer. Our results showed that an increase in % DH increased the reducing power of the hydrolysates (**Figure 2**). Also, we found that protein isolates hydrolyzed to 25% DH were significantly better antioxidants (with higher Trolox equivalents) than those hydrolyzed to 7.5% DH. Among the different enzymes used for hydrolyzing tilapia protein isolates, the total antioxidant potential (TEAC values) increased in the order, Amano A2 < Neutrase ~ Flavourzyme < Amano N ~ Cryotin hydrolysates (**Figure 1**). For the H_2O_2 CL assay, the increasing order of antioxidant activity was Neutrase ~ Amano A2 < Flavourzyme ~ Amano N < Cryotin hydrolysates (**Figure 3a**−**e**). The FRAP or reducing power of the hydrolysates increased in the order Amano N ~ Neutrase < Amano A2 ~ Cryotin < Flavourzyme (**Figure 2**). When mononuclear cells were used, there was no significant difference among the antioxidant activities of different enzymes (**Figure 4a**−**e**). Several factors could contribute to the difference in results between FRAP, TEAC, and ROS assays. One of them is the type of radical or substrate being analyzed (superoxide, peroxide, $ABTS^{\bullet+}$, or Fe^{3+} ion) and the different radical scavenging or reducing mechanisms involved. Another reason could be the time of incubation. During the FRAP assay, the total time of incubation (ferricyanide and hydrolysates) to reduce Fe^{3+} to Fe^{2+} was 30 min. The color developed during the reaction was measured immediately upon mixing ferrocyanide and Fe^{3+} solutions. A longer incubation time may increase the absorbance values. In the TEAC assay, the reagent solution was stored overnight with potassium persulfate to generate $ABTS^{\bullet+}$ radicals. In CL assays, the ROS generated was measured immediately. Moreover, ROS production by a biological or cell-based system could be affected to a greater extent

by the reaction medium and assay conditions. While doing the TEAC assay, the hydrolysates were mixed with methanol, and the supernatant fraction was used for determining the total antioxidant capacity. For the H_2O_2 CL assay, the protein hydrolysates were diluted using double-distilled water and were directly used for analysis. Hence, the difference in dilution and mode of dilution might also contribute to the difference in results of various assays.

In conclusion, the present study shows that alkali-solubilized tilapia protein hydrolysates could be used as effective scavengers of ROS generated using stimulated mononuclear cells or endogenous H_2O_2 addition. In general, the ability of the hydrolysates to scavenge ROS increased with an increase in the % DH; that is, LMW peptides were better scavengers of ROS as compared to HMW peptides. The high activity of LMW peptides as compared to HMW peptides could be due to the size of the peptides, its composition, or a combination of both. Antioxidant assays such as the TEAC assay and FRAP also showed a similar pattern. Among the different hydrolysates, those prepared using Cryotin were effective scavengers of ROS produced from H_2O_2 and $ABTS^{\bullet+}$, while no significant difference in the scavenging ability of hydrolysates was observed for ROS liberated by mononuclear cells. More detailed future studies using different hydrolysate fractions may shed light on individual peptides, their mechanism of action, and their ROS scavenging ability.

ABBREVIATIONS USED

DH, degree of hydrolysis; ROS, reactive oxygen species; ABTS, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid; TEAC, Trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant power.

ACKNOWLEDGMENT

We thank Dr. Tim Hofer, Dr. Emanuele Marzetti, and Dilber Dagdelen for their help with isolation of human mononuclear cells.

LITERATURE CITED

(1) Ames, B. N. Dietary carcinogens and anticarcinogens—Oxygen radicals and degenerative diseases. *Science* **1983**, *221* (4617), 1256–1264.

(2) Behrend, L.; Henderson, G.; Zwacka, R. M. Reactive oxygen species in oncogenic transformation. *Biochem. Soc. Trans.* **2003**, *31*, 1441–1444.

(3) Weitzman, S. A.; Gordon, L. I. Inflammation and cancer—Role of phagocyte-generated oxidants in carcinogenesis. *Blood* **1990**, *76* (4), 655–663.

(4) Aruoma, O. Free radicals, oxidative stress, and antioxidants in human health and disease. *J. Am. Oil Chem. Soc.* **1998**, *75* (2), 199–212.

(5) Urquiaga, I.; Leighton, F. Plant polyphenol antioxidants and oxidative stress. *Biol. Res.* **2000**, *33* (2), 55–64.

(6) Rao, A. V.; Agarwal, S. Role of lycopene as antioxidant carotenoid in the prevention of chronic diseases: A review. *Nutr. Res. (N.Y.)* **1999**, *19* (2), 305–323.

(7) Hertog, M. G. L.; Feskens, E. J. M. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* **1993**, *342* (8878), 1007.

(8) West, S.; Vitale, S.; Hallfrisch, J.; Munoz, B.; Muller, D.; Bressler, S.; Bressler, N. M. Are antioxidants or supplements protective for age-related macular degeneration. *Arch. Ophthalmol.* **1994**, *112* (2), 222–227.

(9) Bhathena, S. J.; Velasquez, M. T. Beneficial role of dietary phytoestrogens in obesity and diabetes. *Am. J. Clin. Nutr.* **2002**, *76* (6), 1191–1201.

(10) Borek, C. Dietary antioxidants and human cancer. *Integr. Cancer Ther.* **2004**, *3* (4), 333–341.

(11) Yamaguchi, N.; Naito, S.; Yokoo, Y.; Fujimaki, M. Application of protein hydrolysate to biscuit as antioxidant. *J. Jpn. Soc. Food Sci.* **1980**, *27* (2), 56–59.

(12) Jun, S. Y.; Park, P. J.; Jung, W. K.; Kim, S. K. Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *Eur. Food Res. Technol.* **2004**, *219* (1), 20–26.

(13) Yee, J. J.; Shipe, W. F.; Kinsella, J. E. Antioxidant effects of soy protein hydrolysates on copper-catalyzed methyl linoleate oxidation. *J. Food Sci.* **1980**, *45* (4), 1082–1083.

(14) Bishov, S. J.; Henick, A. S. Antioxidant effect of protein hydrolysates in a freeze-dried model system. *J. Food Sci.* **1972**, *37* (6), 873–875.

(15) Ariyoshi, Y. Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends Food Sci. Technol.* **1993**, *4* (5), 139–144.

(16) FitzGerald, R. J.; Meisel, H. Lactokinins: Whey protein-derived ACE inhibitory peptides. *Nahrung-Food* **1999**, *43* (3), 165–167.

(17) Kitts, D. D.; Weiler, K. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Curr. Pharm. Des.* **2003**, *9* (16), 1309–1323.

(18) Meisel, H.; FitzGerald, R. J. Biofunctional peptides from milk proteins: Mineral binding and cytomodulatory effects. *Curr. Pharm. Des.* **2003**, *9* (16), 1289–1295.

(19) Yamamoto, N.; Ejiri, M.; Mizuno, S. Biogenic peptides and their potential use. *Curr. Pharm. Des.* **2003**, *9* (16), 1345–1355.

(20) Bogwald, J.; Dalmo, R.; Leifson, R.; Stenberg, E.; Gildberg, A. The stimulatory effect of a muscle protein hydrolysate from Atlantic cod, *Gadus morhua L.*, on Atlantic salmon, *Salmo salar* L., head kidney leucocytes. *Fish Shellfish Immunol.* **1996**, *23*, 3–16.

(21) Gildberg, A.; Bogwald, J.; Johansen, A.; Stenberg, E. Isolation of acid peptide fractions from a fish protein hydrolysate with strong stimulatory effect on Atlantic salmon (*Salmo salar*) head kidney leucocytes. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1996**, *114* (1), 97–101.

(22) Bergeron, N.; Deshaies, Y.; Jacques, H. Dietary fish-protein modulates high-density-lipoprotein cholesterol and lipoprotein-lipase activity in rabbits. *J. Nutr.* **1992**, *122* (8), 1731–1737.

(23) Lavigne, C.; Marette, A.; Jacques, H. Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. *Am. J. Physiol. Endocrinol. Metab.* **2000**, *278* (3), E491–E500.

(24) Lavigne, C.; Tremblay, F.; Asselin, G.; Jacques, H.; Marette, A. Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. *Am. J. Physiol. Endocrinol. Metab.* **2001**, *281* (1), E62–E71.

(25) Kawasaki, T.; Seki, E.; Osajima, K.; Yoshida, M.; Asada, K.; Matsui, T.; Osajima, Y. Antihypertensive effect of Valyl-Tyrosine, a short chain peptide derived from sardine muscle hydrolysate, on mild hypertensive subjects. *J. Hum. Hypertens.* **2000**, *14* (8), 519–523.

(26) Picot, L.; Bordenave, S.; Didelot, S.; Fruitier-Arnaudin, I.; Sannier, F.; Thorkelsson, G.; Berge, J. P.; Guerard, F.; Chabeaud, A.; Piot, J. M. Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. *Process Biochem.* **2006**, *41* (5), 1217–1222.

(27) Kristinsson, H. G. The production, properties and utilization of fish protein hydrolysates. In *Food Biotechnology*, 2nd ed.; Shetty, K., Paliyath, G., Pometto, A., Levin, R. E., Eds.; CRC Press: New York, 2005; pp 1109−1133.

(28) Hultin, H. O.; Kelleher, S. D. High efficiency alkaline protein extraction. U.S. Patent 6,136,959, Oct 24, 2000.

(29) Kristinsson, H. G.; Hultin, H. O. Effect of low and high pH treatment on the functional properties of cod muscle proteins. *J. Agric. Food Chem.* **2003**, *51* (17), 5103–5110.

(30) Undeland, I.; Kelleher, S. D.; Hultin, H. O. Recovery of functional proteins from herring (*Clupea harengus*) light muscle by an acid

or alkaline solubilization process. *J. Agric. Food Chem.* **2002**, *50* (25), 7371–7379.
(31) Huang, D. J.; Ou, B. X.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53* (6), 1841–1856.
(32) Johansson, A.; Dahlgren, C. Characterization of the luminol-amplified light generating reaction induced in human-monocytes. *J. Biolumin. Chemilumin.* **1988**, *2* (4), 218–218.
(33) Gunnarsson, G.; Undeland, I.; Sannaveerappa, T.; Sandberg, A. S.; Lindgard, A.; Mattsson-Hulten, L.; Soussi, B. Inhibitory effect of known antioxidants and of press juice from herring (*Clupea harengus*) light muscle on the generation of free radicals in human monocytes. *J. Agric. Food Chem.* **2006**, *54* (21), 8212–8221.
(34) Halliwell, B. The antioxidant paradox. *Lancet* **2000**, *355* (9210), 1179–1180.
(35) Gornall, A. G.; Bardawill, C. J.; David, M. M. Determination of serum proteins by means of the Biuret reaction. *J. Biol. Chem.* **1949**, *177* (2), 751–766.
(36) Adler-Nissen, J. *Enzymatic Hydrolysis of Food Proteins*; Elsevier Applied Science Publishers: Oxford, United Kingdom, 1986; p 426.
(37) Steinhardt, H.; Beychok, S. Interaction of protein with hydrogen ions and other small ions and molecules. In *The Proteins*; Neurath, H., Ed.; Academic Press: NY, 1964; Vol. 2.
(38) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26* (9−10), 1231–1237.
(39) Oyaizu, M. Studies on products of browning reactions antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* **1986**, *44*, 307–315.
(40) Schagger, H.; Vonjagow, G. Tricine sodium dodecyl-sulfate polyacrylamide-gel electrophoresis for the separation of proteins in the range from 1-Kda to 100-Kda. *Anal. Biochem.* **1987**, *166* (2), 368–379.
(41) Feig, D. I.; Reid, T. M.; Loeb, L. A. Reactive oxygen species in tumorigenesis. *Cancer Res.* **1994**, *54* (7), S1890–S1894.
(42) Hensley, K.; Robinson, K. A.; Gabbita, S. P.; Salsman, S.; Floyd, R. A. Reactive oxygen species, cell signaling, and cell injury. *Free Radical Biol. Med.* **2000**, *28* (10), 1456–1462.
(43) Mullerpeddinghaus, R. Reactive oxygen species (ROS) and inflammation. *Dtsch. Tierarztl. Wochenschr.* **1989**, *96* (4), 210–212.
(44) Benzi, G.; Moretti, A. Are reactive oxygen species involved in Alzheimer's disease. *Neurobiol. Aging* **1995**, *16* (4), 661–674.
(45) Vandervliet, A.; Bast, A. Role of reactive oxygen species in intestinal-diseases. *Free Radical Biol. Med.* **1992**, *12* (6), 499–513.
(46) Jolles, P.; Levytoledano, S.; Fiat, A. M.; Soria, C.; Gillessen, D.; Thomaidis, A.; Dunn, F. W.; Caen, J. P. Analogy between fibrinogen and casein—Effect of an undecapeptide isolated from Kappa-casein on platelet function. *Eur. J. Biochem.* **1986**, *158* (2), 379–382.
(47) Maeno, M.; Yamamoto, N.; Takano, T. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **1996**, *79* (8), 1316–1321.
(48) Raghavan, S.; Hultin, H. O. Model system for testing the efficacy of antioxidants in muscle foods. *J. Agric. Food Chem.* **2005**, *53* (11), 4572–4577.
(49) Hill, M. F.; Singal, P. K. Antioxidant and oxidative stress changes during heart failure subsequent to myocardial infarction in rats. *Am. J. Pathol.* **1996**, *148* (1), 291–300.
(50) Lundqvist, H.; Dahlgren, C. Isoluminol-enhanced chemiluminescence: A sensitive method to study the release of superoxide anion from human neutrophils. *Free Radical Biol. Med.* **1996**, *20* (6), 785–792.
(51) Hulten, L. M.; Holmstrom, M.; Soussi, B. Harmful singlet oxygen can be helpful. *Free Radical Biol. Med.* **1999**, *27* (11−12), 1203–1207.
(52) Suetsuna, K.; Ukeda, H.; Ochi, H. Isolation and characterization of free radical scavenging activities peptides derived from casein. *J. Nutr. Biochem.* **2000**, *11* (3), 128–131.
(53) Moure, A.; Domínguez, H.; Parajó, J. C. Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochem.* **2006**, *41* (2), 447–456.
(54) Li, Y.; Jiang, B.; Zhang, T.; Mu, W.; Liu, J. Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food Chem.* **2008**, *106* (2), 444–450.
(55) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239* (1), 70–76.
(56) Berker, K. I.; Guclu, K.; Tor, I.; Apak, R. Comparative evaluation of Fe(III) reducing power-based antioxidant capacity assays in the presence of phenanthroline, batho-phenanthroline, tripyridyltriazine (FRAP), and ferricyanide reagents. *Talanta* **2007**, *72* (3), 1157–1165.

Received for review June 4, 2008. Revised manuscript received July 30, 2008. Accepted September 4, 2008. This work was supported by the Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture, by Grant 2004-35503-14119 of the U.S. Department of Agriculture NRI Competitive Grants Program.

JF8017194