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Antioxidative Efficacy of Alkali-Treated Tilapia Protein Hydrolysates: A Comparative Study of Five Enzymes

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The antioxidant activities of alkali-treated tilapia protein hydrolysates were determined by their ability to inhibit the formation of lipid hydroperoxides (PV) and thiobarbituric acid reactive substances (TBARS) in a washed muscle model system and by their ability to inhibit DPPH free radicals and chelate ferrous ion in an aqueous solution. Protein isolates were prepared from tilapia white muscle using alkali solubilization at pH 11.0 and reprecipitation at pH 5.5. Protein hydrolysates were prepared by hydrolyzing the isolates using five different enzymes, Cryotin F, Protease A Amano, Protease N Amano, Flavourzyme, and Neutrase, to 7.5, 15, and 25% degrees of hydrolysis (DH). All of the protein hydrolysates significantly ($p < 0.05$) inhibited the development of TBARS and PV. The antioxidant activity of the hydrolysates increased with the DH. Also, the antioxidant activity of the hydrolysates varied significantly ($p < 0.05$) among the different enzymes. The ability of different enzyme-catalyzed protein hydrolysates to scavenge DPPH radicals was not reflected in their ability to inhibit oxidation in a washed tilapia model system. In a washed muscle model system, the hydrolysates prepared using Cryotin F were most effective and the hydrolysates prepared using Flavourzyme and Neutrase were least effective in inhibiting the development of TBARS and PV, whereas in an aqueous solution, hydrolysates prepared using Flavourzyme were most effective in scavenging DPPH radicals and chelating ferrous ions. Enzymatic hydrolysis decreased the size of tilapia protein hydrolysates and, in general, tilapia protein hydrolysates with low molecular weights were better antioxidants than those with high molecular weights.

KEYWORDS: Tilapia protein hydrolysates; enzymes; antioxidant activity; alkali solubilization; DPPH; TBARS; peroxide value; iron chelation

INTRODUCTION

Oxidation in muscle foods leads to quality deterioration and loss in nutritional value (*1*). Antioxidants, both synthetic and natural, are usually added to muscle foods to prevent oxidation. Protein hydrolysates from a variety of animal and plant sources have been reported to possess antioxidant activity (*2, 3*). Due to safety and toxicity concerns related to the use of synthetic antioxidants (*4*), natural antioxidants such as protein hydrolysates have a huge market potential in the muscle foods industry. Numerous researchers have studied the antioxidative properties of aquatic product/byproduct hydrolysates such as Alaska pollack frames, herring byproduct, and capelin (*5–8*). One potential problem in the preparation of hydrolysates from muscle protein sources is the presence of pro-oxidants such as heme and unstable lipid substrates. Contamination with these pro-oxidants could decrease the stability of protein hydrolysates and may limit their use in food systems. A new method developed by Hultin et al. (*9*), which involved solubilizing myofibrillar proteins at high (alkaline) pH and then recovering the solubilized proteins at pH 5.5, yielded protein isolates with improved functional properties (*10*) and a low amount of unstable lipid substrates (*11*). In our current studies, we used protein isolates prepared using an alkali process as a substrate for enzyme hydrolysis. Five different enzymes were used for enzyme hydrolysis: Flavourzyme, Protease A Amano-2 (a mixture of endoprotease and exopeptidase), Neutrase, Protease N Amano (endoproteases), and Cryotin F (a mixture of trypsin, chymotrypsin, and elastase). The antioxidant ability of protein hydrolysates to inhibit oxidation in muscle foods was tested using a washed tilapia model system (*12*). Oxidation was catalyzed using tilapia hemolysate, and lipid oxidation was monitored by the measurement of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (PV).

Diphenylpicrylhydrazyl (DPPH) is very commonly used by researchers to evaluate the radical scavenging ability of antioxidants. Antioxidants with higher DPPH inhibition are said to possess higher antioxidant efficacy. In our studies, we wanted to evaluate DPPH inhibition by the protein hydrolysates and

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determine whether higher DPPH inhibition would correspond to higher antioxidant efficacy in a washed tilapia model system. Protein hydrolysates may also inhibit oxidation by their ability to chelate transition metal ions (*13*). Hence, in our current study, the ability of tilapia protein hydrolysates to chelate Fe^{2+} in an aqueous solution was also studied.

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, Bagsvaerd, Denmark. Cryotin F was a gift from North Ltd., Reykjavik, Iceland.

Methods. *Preparation of Protein Isolates Using Alkaline Method.* Protein isolates were prepared from tilapia white muscle using alkaline treatment as described in Hultin et al. (*9*). 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 cheesecloth, 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. Protein isolate obtained as sediment was used for enzymatic hydrolysis. The amount of protein in the isolates were determined by using the biuret reaction (*14*).

Enzymatic Hydrolysis of Protein Isolates. Enzymatic hydrolysis of tilapia protein hydrolysates was performed using five different proteases: Protease A Amano-2 (Amano A2), Protease N Amano (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 the enzyme used for hydrolysis. The enzyme preparation was then added to the homogenate, and the degree of hydrolysis (DH) was calculated using the equation (*15*)

$$\%\ \text{degree of hydrolysis (DH)} = \frac{B \times N_{\text{base}}}{\alpha \times h_{\text{total}} \times \text{MP}} \times 100 \quad (1)$$

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

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

where pH is the value at which enzyme hydrolysis was performed. The pK_a values were calculated according to the method of Steinhardt et al. (*16*):

$$\text{p}K_a = 7.8 + (298 - T)/298 \times T \times 2400 \quad (3)$$

Using each of the five proteases, tilapia protein isolates were hydrolyzed to achieve 7.5, 15, and 25% DH. The amount of enzyme used for preparing the hydrolysates was around 2.0–4.0% for Cryotin, 0.05–0.1% for Amano N and Amano A2, 0.3–0.6% for Flavourzyme, and around 0.5−1.2% for Neutrase. The mixtures were incubated for around 90–120 min to achieve the desired 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.

Preparation and Quantification of Tilapia Hemolysate. A modified method of Fyhn et al. (*17*) was used for preparing tilapia hemolysate. Blood was collected from the caudal vein of live tilapia fish using a micropipet and transferred into heparin solution (30 units/mL and 150 mM NaCl). Heparinized blood was washed with 4 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 3 volumes of ice-cold 1 mM Tris buffer, pH 8.0, for 1 h. A $^1/_{10}$ 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 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 μL of this diluted sample was mixed with 3 mL of Coomassie reagent and incubated at room temperature for 10 min; absorbance was measured at 595 nm. Bovine serum albumin was used for determining standard plots.

Preparation of Washed Tilapia Model System. Washed tilapia model system was prepared as in Richards et al. (*18*). Tilapia white muscle was separated from the fillets, and the white muscle 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 (pH 6.5) 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 15000*g* for 20 min at 4 °C in a Sorvall refrigerated centrifuge, and the sediment was collected. The pH of sediment was adjusted to 6.5 and mixed with 300 ppm of streptomycin, and the final 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 with 12 μmol of tilapia hemolysate and 0.35% (based on protein weight/100 g of washed system) hydrolysates. A sample of 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 10 °C for oxidation studies. All samples were prepared in duplicates.

Measurement of Thiobarbituric Acid Reactive Substances (TBARS). The method of Lemon (*19*) was modified according to Raghavan et al. (*12*) for measuring TBARS. 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 5702 centrifuge (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 $1.6 \times 10^5\ \text{M}^{-1}\ \text{cm}^{-1}$. All experiments were done in duplicates.

Measurement of Lipid Hydroperoxides. Lipid hydroperoxides were measured according to the method of Raghavan et al. (*12*). A 1 g amount of minced cod muscle was taken in a disposable glass tube, homogenized for 1 min with 10 mL of chloroform/methanol (1:1) using a Biohomogenizer, and mixed with 3 mL of 0.5 M NaCl. The mixture was first vortexed and then centrifuged at 2000 rpm in an Eppendorf centrifuge. The chloroform phase was removed, and a 2 mL volume of the chloroform phase was made to 10 mL using chloroform/methanol (1:1). Ammonium thiocyanate and ferrous chloride were prepared as in Shantha and Decker (*12*). A 25 μL aliquot of each reagent was added and vortexed for 10 s. The samples were incubated for 10 min at room

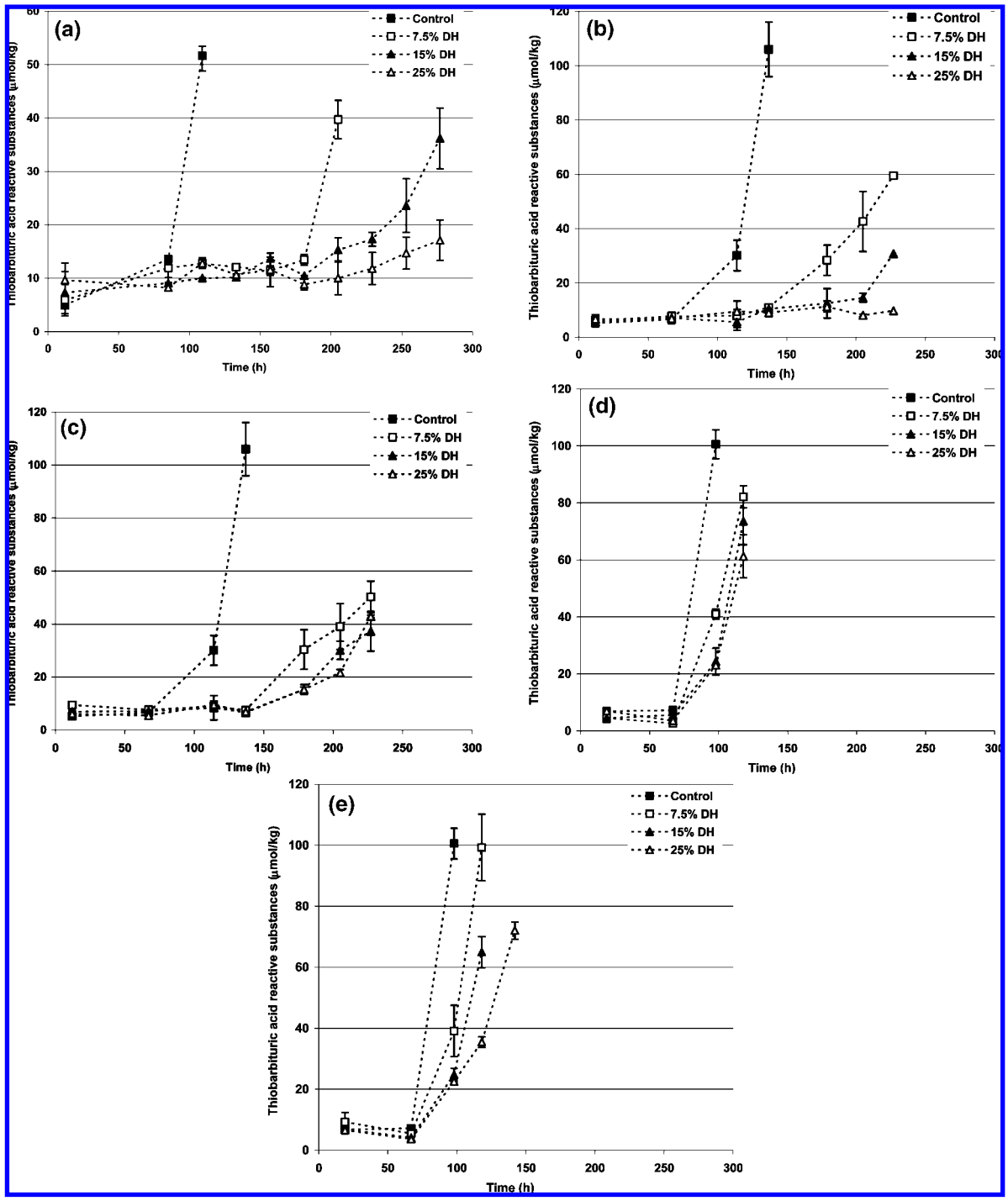

Figure 1. Ability of protein hydrolysates prepared using the enzymes Cryotin (**a**), Protease N Amano (**b**), Proease A Amano-2 (**c**), Flavourzyme (**d**), and Neutrase (**e**) to inhibit the development of thiobarbituric acid reactive substances. Three protein hydrolysates with 7.5, 15.0, and 25.0% degrees of hydrolysis (DH) were tested on a washed tilapia model system at 0.35% level based on the weight of model system. Oxidation was catalyzed using 12 μmol of tilapia hemolysate. A mixture of washed tilapia model system and 0.35% water was used as control. Samples were stored at 10 °C for storage studies. TBARS were measured as micromoles of malonaldehyde per kilogram of washed system.

temperature, and the absorbance was measured at 500 nm. A standard curve was prepared using cumene hydroperoxide.

Determination of Antioxidant Activity Using DPPH Radical Scavenging Method. The antioxidant activities of hydrolysates were measured in terms of hydrogen-donating or free radical scavenging ability, using the stable radical DPPH. All sample tubes used for DPPH assay were covered with aluminum foil to avoid degradation of DPPH by light. The hydrolysates were first mixed with methanol at a ratio of 1.5:8.5 (hydrolysate/methanol), and the mixture was centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was collected, and that sample was used for DPPH analysis. Three milliliters of hydrolysate sample was mixed with 150 μL of methanolic solution of DPPH (0.02%), and the mixture was incubated at room temperature for 30 min. A mixture of 3 mL of water/methanol (1.5:8:5 ratio) and 150 μL of DPPH was used as blank. A mixture of 3 mL of hydrolysate sample and 150 μL of methanol was used as control. After incubation, the decrease in absorbance was measured at 519 nm. The percent inhibition of DPPH was calculated according to the formula

$$\% \text{ inhibition} = [A_{\text{Blank}} - (A_{\text{Sample}} - A_{\text{Control}})]/A_{\text{Blank}} \times 100 \quad (4)$$

where A_{Blank} = absorbance of the blank, A_{Sample} = absorbance of the sample, and A_{Control} = absorbance of the control samples at 519 nm.

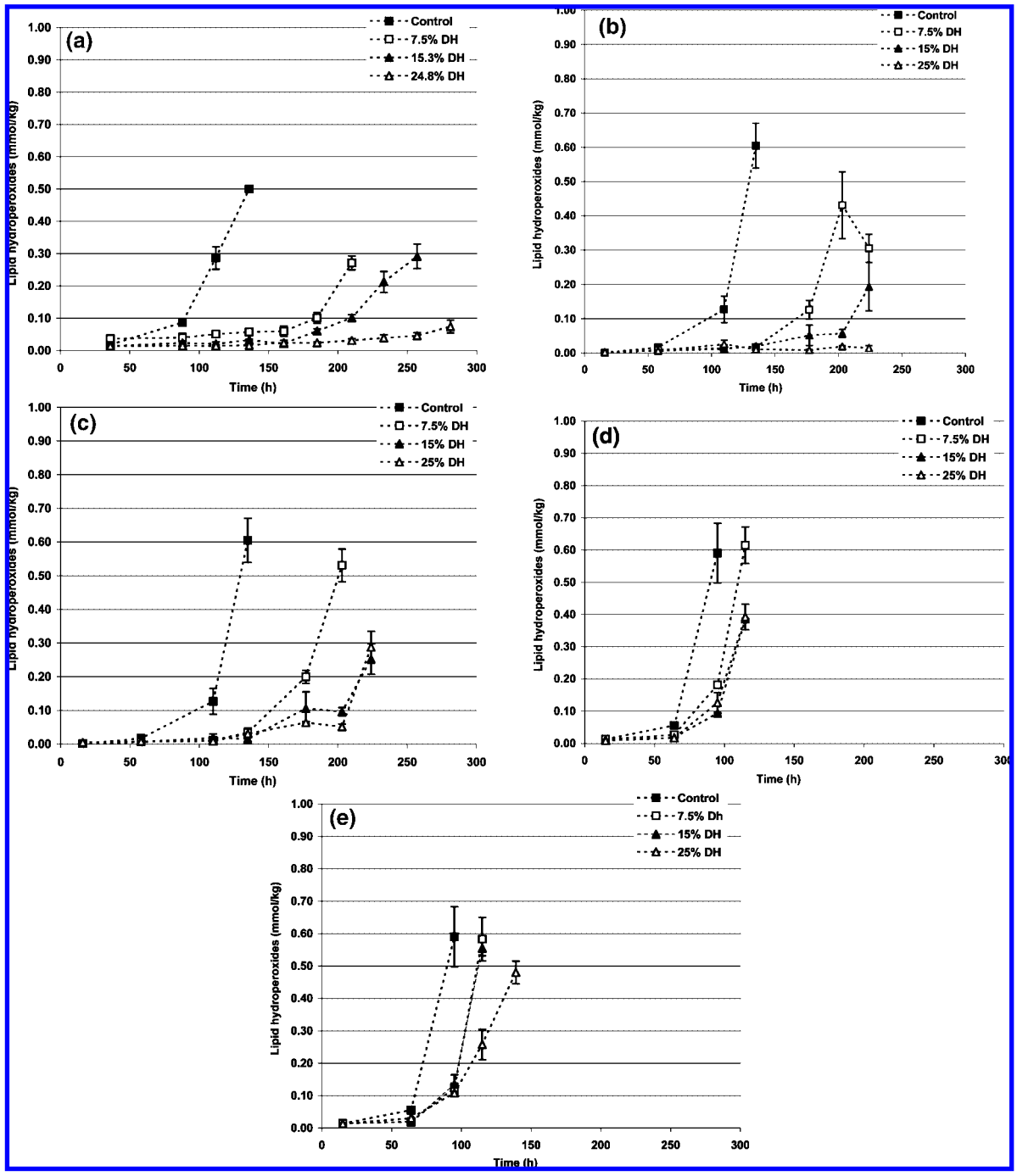

Figure 2. Ability of protein hydrolysates prepared using the enzymes Cryotin (**a**), Protease N Amano (**b**), Protease A Amano-2 (**c**), Flavourzyme (**d**), and Neutrase (**e**) to inhibit the development of lipid hydroperoxides. Three protein hydrolysates with 7.5, 15.0, and 25.0% degrees of hydrolysis (DH) were tested on a washed tilapia model system at 0.35% level based on the weight of model system. Oxidation was catalyzed using 12 μmol of tilapia hemolysate. A mixture of washed tilapia model system and 0.35% water was used as control. Samples were stored at 10 °C for storage studies. Lipid hydroperoxides were measured as millimoles of cumene hydroperoxide per kilogram of washed system.

Determination of Chelating Activity on Ferrous Iron (Fe^{2+}). The metal chelating activity was measured using a modified method of Boyer et al. (*20*). The hydrolysates (with 2% protein concentration) were first mixed with methanol at a ratio of 2.5:12.5 (hydrolysate/methanol), and the mixture was centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was collected and used for metal chelation analysis. Three milliliters of supernatant sample was mixed with 0.1 mL of 2 mM ferrous chloride ($FeCl_2$) and 0.2 mL of 5 mM ferrozine. The mixture was then incubated at room temperature for 30 min. A mixture of 3 mL of water/methanol (2.5:12:5 ratio), $FeCl_2$, and ferrozine was used as blank. Also, a mixture of 3 mL of supernatant sample with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of water was used as control. After 30 min of incubation, the absorbance was read at 561 nm. The percent metal chelating ability was calculated according to the formula

$$\%\ \text{chelating ability} = [A_{\text{Blank}} - (A_{\text{Sample}} - A_{\text{Control}})]/A_{\text{Blank}} \times 100 \quad (5)$$

where A_{Blank} = absorbance of the blank, A_{Sample} = absorbance of the sample, and A_{Control} = absorbance of the control samples at 561 nm.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed on the protein hydrolysates using a 10–20% tris−tricine gel (*21*) to characterize the hydrolysates on the basis of their molecular weights. 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).

Statistical Analysis. All samples and analysis were done in duplicates. Statistical analyses on the samples 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 difference among treatments at the $p < 0.05$ level.

RESULTS

Ability of Tilapia Protein Hydrolysates To Inhibit TBARS Development. Tilapia protein hydrolysates prepared using five different enzymes were tested for their ability to inhibit the development of TBARS in a washed muscle model system. The hydrolysates were added at 0.35% level (based on protein weight/100 g of washed muscle system). Oxidation was catalyzed using 12 μmol of tilapia hemolysate. The samples were stored at 10 °C, and oxidation was monitored by measuring TBARS. The antioxidative ability of the protein hydrolysates was measured by their ability to prolong the lag phase of TBARS development. Lag phase was calculated as the extension of the slope of rapidly oxidizing part of TBARS curve to where it intersects the low/no oxidation part of the curve. All of the protein hydrolysates, irrespective of the enzymes used for their preparation, could significantly ($p < 0.05$) inhibit TBARS development in a washed muscle system compared to the control treatment (**Figure 1**). However, the ability of different protein hydrolysates to prolong the lag phase of TBARS development was dependent on the type of enzyme used for hydrolysis. The ability of the hydrolysates to inhibit TBARS development decreased in the order Cryotin ≥ Amano N > Amano A2 > Neutrase ~ Flavourzyme. When Cryotin was used for hydrolyzing the alkali-treated protein isolates, the lag phase of TBARS development was increased by nearly 100 h for 7.5% DH and by 150 h for 15% DH compared to the control (**Figure 1a**). When Amano N was used, the increase in lag phase of TBARS development was nearly 50 h for 7.5% DH and 100 h for 15% DH compared to the control (**Figure 1b**). When Cryotin and Amano N protein hydrolysates with 25% DH were used, there was no increase in TBARS for up to 275 and 225 h, respectively. Analysis had to be discontinued beyond these times due to microbial spoilage. When Amano A2 was used, hydrolysates with 7.5% DH increased the lag phase by nearly 30 h, whereas 15 and 25% DH increased the lag phase by nearly 70 h, compared to control (**Figure 1c**). Hydrolysates prepared using Flavourzyme and Neutrase enzymes were least antioxidative in the washed muscle model systems. When Flavourzyme was used (**Figure 1d**), the hydrolysates with 7.5% DH increased the lag phase by 10 h, whereas hydrolysates with 15 and 25% DH increased the lag phase by nearly 24 h, compared to the control. For Neutrase, the increases in lag phase for 7.5, 15, and 25% DH were 15, 20, and 30 h, respectively, compared to the control (**Figure 1e**).

Ability of Tilapia Protein Hydrolysates to Inhibit the Development of Lipid Hydroperoxides. The antioxidative ability of tilapia protein hydrolysates prepared using five different enzymes was tested for their ability to inhibit the formation of lipid hydroperoxides (**Figure 2**). The hydrolysates were added at 0.35% level (based on protein weight/100 g of washed muscle system) to the washed tilapia model system. Sample preparation and storage conditions were identical to the TBARS assay. The ability of the hydrolysates to extend the lag phase compared to the control was used as a measure of antioxidative efficacy. Lag phase was calculated as in TBARS study. The ability of the hydrolysates to inhibit the development of lipid hydroperoxides decreased in the order Cryotin > Amano N ≥ Amano A2 > Neutrase ~ Flavourzyme. When Cryotin was used, the lag phase for hydroperoxide inhibition was around 90 h for 7.5% DH and 120 h for 15% DH, compared to the control. Hydrolysates with 25% DH did not show an increase in lipid hydroperoxides until 275 h of storage. Samples could not be analyzed beyond 275 h due to microbial spoilage (**Figure 2a**). When Amano N was used, hydrolysates with 7.5 and 15% DH extended the lag phase by 60 and 90 h, respectively, whereas hydrolysates with 25% DH did not show an increase in hydroperoxides until 225 h of storage, by which time the samples underwent microbial spoilage (**Figure 2b**). When hydrolysis was done using Amano A2, hydrolysates with 7.5% DH increased the lag phase by 60 h, whereas hydrolysates with 15 and 25% DH increased the lag phase by 90 h, compared to the control (**Figure 2c**). Hydrolysates prepared using Flavourzyme and Neutrase were least inhibitive of lipid hydroperoxide development in the washed muscle model system. Flavourzyme hydrolysates with 7.5% DH extended the lag phase by 15 h, whereas hydrolysates with 15 and 25% DH extended the lag phase by 20 h compared to the control. When Neutrase was used, hydrolysates with 7.5 and 15% DH increased the lag phase by 20 h, whereas 25% DH extended the lag phase by 15 h compared to the control.

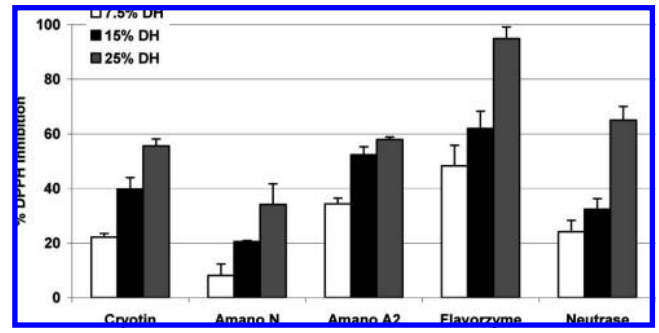

Figure 3. Ability of alkali-treated tilapia protein hydrolysates to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Protein hydrolysates with 7.5, 15.0 and 25.0% degrees of hydrolysis were prepared using the enzymes Cryotin, Protease N Amano, Protease A Amano-2, Flavourzyme, and Neutrase. Analyses were done in duplicates.

Determination of DPPH Radical Scavenging Ability of Tilapia Protein Hydrolysates. The different protein hydrolysates were evaluated for their ability to scavenge DPPH radicals. Percent inhibition of DPPH radicals was measured by a decrease in absorbance at 519 nm. Hydrolysates prepared using Flavourzyme showed a significantly high ($p < 0.05$) and hydrolysates prepared using Amano N showed a significantly low ($p < 0.05$) radical scavenging efficacy (**Figure 3**). Irrespective of the enzymes used, the ability of different hydrolysates to inhibit DPPH radical decreased in the order 25 > 15 > 7.5% DH. At 25% DH, the ability of hydrolysates to scavenge DPPH radicals decreased in the order Flavourzyme > Neutrase ~ Amano A2 ~ Cryotin > Amano N ($p < 0.05$). At 15% DH, the radical scavenging ability decreased in the order Flavourzyme ≥ Amano A2 ≥ Cryotin ≥ Neutrase ≥ Amano N. At 7.5% DH, the radical scavenging ability decreased in the order Flavourzyme ≥ Amano A2 ≥ Neutrase ~ Cryotin ≥ Amano N ($p < 0.05$).

Determination of Ferrous Ion (Fe^{2+}) Chelating Ability of Tilapia Protein Hydrolysates. The ability of different protein hydrolysates to chelate Fe^{2+} was studied. Among the different hydrolysates, Fe^{2+} chelating ability decreased in the order

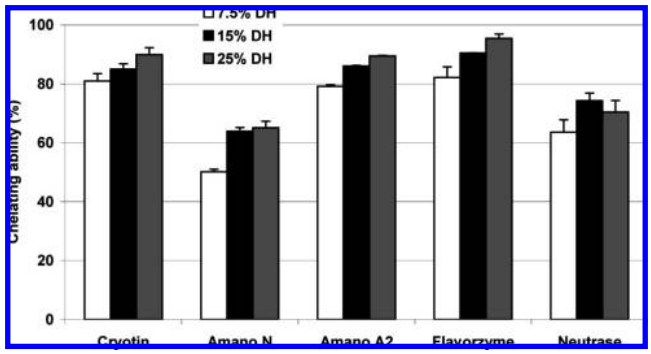

Figure 4. Antioxidant activity of alkali-treated tilapia protein hydrolysates measured by their ability to chelate Fe^{2+}. Protein hydrolysates with 7.5, 15.0, and 25.0% degrees of hydrolysis were prepared using the enzymes, Cryotin, Protease N Amano, Protease A Amano-2, Flavourzyme, and Neutrase. Analyses were done in duplicates.

Flavourzyme > Amano A2 ~ Cryotin > Neutrase > Amano N ($p < 0.05$) (**Figure 4**). In general, the ability to chelate metal increased in the order 7.5 < 15 < 25% DH. However, for Amano N and Neutrase, there was no significant difference ($p > 0.05$) between hydrolysates with 15 and 25% DH to chelate Fe^{2+}. For 25% DH, metal chelating ability decreased in the order Flavourzyme ≥ Amano A2 ~ Cryotin > Neutrase ≥ Amano N, whereas for 15 and 7.5% DH, the order was Flavourzyme ≥ Amano A2 ~ Cryotin > Neutrase > Amano N.

SDS-PAGE of Tilapia Protein Hydrolysates. The molecular mass distribution of tilapia protein hydrolysates was determined using SDS-PAGE. An increase in the degree of hydrolysis decreased the molecular mass of the hydrolysate fractions (**Figure 5**). All of the enzyme fractions showed distinct bands with molecular masses between 10 and 20 kDa for 7.5% DH. At 7.5% DH, the majority of the bands for Cryotin hydrolysates were between 15 and 20 kDa, whereas for Amano N hydrolysates, they were between 3.5 and 15 kDa. Flavourzyme and Amano A2 showed an even distribution of bands between 10 and 20 kDa. Also, hydrolysates obtained using Amano N and Neutrase at 7.5% DH showed a large number of <10 kDa low molecular mass peptides. At 25% DH, Cryotin hydrolysates showed two distinct bands around 10 and 12 kDa, whereas Amano N hydrolysates showed a weak band and Neutrase showed a strong band around 10 kDa. Flavourzyme and Amano N showed a number of weak bands between 3.5 and 10 kDa.

DISCUSSION

Enzyme hydrolysis of fish protein may yield peptides with improved functional and quality characteristics (*22*). Our interest was to evaluate the antioxidant potential of tilapia protein hydrolysates prepared using various commercial enzymes. Alkali solubilization is a new method that is used for preparing protein isolates with a low amount of unstable lipid substrates (*11*). In our research, we have used this solubilization technique to first prepare protein isolates from tilapia white muscle and then used the protein isolate as a substrate for enzyme hydrolysis. Antioxidant potential was evaluated by the ability of hydrolysates (a) to scavenge DPPH radicals, (b) to chelate Fe^{2+} (a potential pro-oxidant), and (c) to inhibit TBARS and lipid peroxide development in washed tilapia model system. Among the five enzymes, hydrolysates prepared using Cryotin showed maximum inhibition of TBARS and PV (**Figures 1a** and **2a**), whereas hydrolysates prepared using Flavourzyme showed maximum DPPH radical scavenging (**Figure 3**) and metal chelating (**Figure 4**) ability. The above results indicated that the ability of alkali-treated tilapia fish protein hydrolysates to scavenge DPPH radical was not reflected in the ability of the hydrolysates to inhibit the formation of TBARS and PV in washed tilapia model system. Similar results were reported by Lee et al. (*23*), who studied the ability of cranberry powder components to inhibit lipid oxidation in a washed muscle system. They found that a fraction of cranberry powder which was the most efficient scavenger of DPPH radical was a weak inhibitor of hemoglobin-mediated lipid oxidation in washed fish muscle system. In our current research, the observed difference between the DPPH radical scavenging ability and the ability of hydrolysates to inhibit TBARS and PV could be due to difference in the composition of the hydrolysates and the composition of the experiment medium as well as the physical location of antioxidants in different experiment systems. DPPH radical scavenging studies were performed in an aqueous medium, whereas the efficacy of hydrolysates to inhibit TBARS and PV were tested

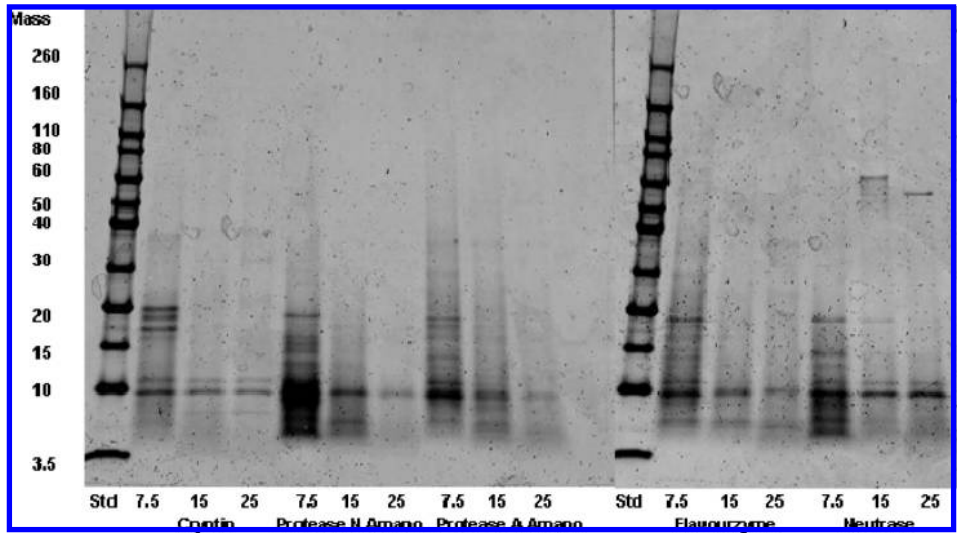

Figure 5. Commassie blue stained SDS−polyacrylamide gel of tilapia protein hydrolysates. Amount of sample loaded on each lane contained ~26 μg of protein. Samples loaded in the lanes correspond to 7.5, 15, and 25% degrees of hydrolysis using the enzymes Cryotin, Protease N Amano, Protease A Amano, Flavourzyme, and Neutrase. Molecular mass standards 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. Std corresponds to standard molecular mass marker.

in washed tilapia white muscle. Washed tilapia white muscle would contain water, protein, and membrane phospholipids as well as a small amount of neutral triacylglycerols. Hence, the ability of tilapia hydrolysates to inhibit TBARS and PV formation in a washed muscle system would depend on the ability to partition into the various components of muscle and how effectively the hydrolysates could scavenge lipid hydroperoxides in the muscle components into which it had partitioned. Hydrolysates prepared using Flavourzyme may act as effective DPPH radical scavengers. However, Flavourzyme hydrolysates may lack the ability to partition into the sites of lipid oxidation or where lipid hydroperoxides are produced.

The ability of hydrolysates to chelate Fe^{2+} decreased in the order Flavourzyme > Amano A2 ~ Cryotin > Neutrase > Amano N ($p < 0.05$) (**Figure 4**), whereas the ability to inhibit TBARS and PV decreased in the order Cryotin ≥ Amano *N* > Amano A2 > Neutrase ~ Flavourzyme (**Figures 1** and **2**). Metal chelating ability measured using the ferrozine assay was based on the ability of hydrolysates to chelate Fe^{2+} present in an aqueous solution of $FeCl_2$. On the other hand, lipid oxidation in the washed tilapia model system was catalyzed using tilapia hemolysate. Hemolysate catalyzes oxidation in muscle foods by the conversion of iron in heme protein from ferrous (Fe^{2+}) to ferric (Fe^{3+}) or met form. Met-heme protein could then interact with peroxides, resulting in the formation of compounds capable of initiating and propagating lipid oxidation (*24*). The ability of protein hydrolysates to chelate iron from the washed tilapia model system would hence depend on the availability of free iron from tilapia hemolysate. Therefore, the difference in the results between the ferrozine assay conducted in an aqueous medium and TBARS and PV measured in a washed tilapia model system could be due to the difference in the availability of free iron mechanism of oxidation as well as the physical conditions of the medium/model system itself.

Irrespective of the enzymes used, the ability of the hydrolysates to chelate Fe^{2+} and inhibit DPPH free radicals decreased in the order 25 > 15 > 7.5% DH. Also, the ability of the hydrolysates prepared using the three enzymes Cryotin-F, Amano A2, and Amano N to inhibit TBARS and PV development decreased in the order 25 > 15 > 7.5% DH. Thiansilakul et al. (*8*) found similar results while studying the antioxidative activities of protein hydrolysates prepared from scad muscle. They found that protein hydrolysates with a higher degree of hydrolysis (using the enzyme Flavourzyme) showed greater inhibition of DPPH radicals. When Alcalase was used for protein hydrolysis, they found an increase in the metal chelating activity with an increase in the % DH. In our study, SDS-PAGE analysis of tilapia hydrolysates showed that the molecular mass of the hydrolysates decreased progressively with an increase in the degree of hydrolysis (**Figure 5**), implying a greater amount of low molecular mass peptides due to enzymatic cleavage of tilapia protein. Hence, our results suggest that the low molecular mass peptides from the enzymatic hydrolysis of tilapia could have a higher DPPH radical scavenging and metal chelating ability and ability to inhibit TBARS and PV than high molecular mass peptides. However, Flavourzyme and Neutrase hydrolysates showed insignificant difference ($p > 0.05$) in TBARS and PV inhibiting powers (**Figures 1d**,**e** and **2d**,**e**). This discrepancy could be due to both the size and the type of peptides. Cryotin shows a large number of peptide bands in the 15–20 kDa range, whereas Amano N hydrolysates showed more peptides in the 3.5–10 kDa range. Flavourzyme showed an even distribution of peptide bands in 10–20 kDa range and Neutrase in 3.5–20 kDa range. Also, Flavourzyme and Amano A2 are a mixture of endoproteases and exopeptidases, whereas Neutrase and Amano N are endoproteases and Cryotin is a mixture of trypsin, chymotrypsin, and elastase. These differences in the size (molecular mass) of peptide fractions and the type of fractions could result in different antioxidant behaviors in test model systems. As our primary objective is to test the antioxidant ability of the hydrolysates as a whole and not to determine the individual peptides responsible for the antioxidative activity, we did not fractionate the hydrolysates for further analysis. However, from our study we were able to show that the antioxidant activity of peptides would differ with the type of enzyme used, degree of hydrolysis, and type of food model system in which the antioxidants are tested.

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