

## Influence of Ultrafiltration on Antioxidant Activity of Tilapia (*Oreochromis niloticus*) Protein Hydrolysate

<sup>1,2</sup>M.B.K. Foh, <sup>1</sup>J. Qixing, <sup>1</sup>I. Amadou and <sup>1</sup>W.S. Xia

<sup>1</sup>State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, No 1800, Lihu Road, Wuxi, 214122 Jiangsu, China

<sup>2</sup>School of Community Health and Clinical Sciences, Njala University, Kowama Campus, Bo, Sierra Leone, China

**Abstract:** The production of hot water dip hydrolysate (HWDH) from tilapia (*Oreochromis niloticus*) with DH of 25.43% improved its bioactivity. A pressure-driven ultrafiltration (UF) membrane separated HWDH based on molecular weight cut-offs (MWCO) of 1000, 3000, and 5,000 Da, to produce fractions F1-k, F3-k, F5-k respectively and 5k-R (retentate of 5,000 Da) with antioxidative activity. The UF produced permeates with smaller Mw distribution (177-3015 Da), with F1-k portraying lowest molecular weight range (180-2008) Da, whilst the retentate fraction ranged higher 179-8130 Da. The antioxidant efficacy of fractions with ABTS, DPPH, inhibition of linoleic acid autoxidation, Metal-chelating ability, and reducing power exhibited antioxidant activity (82.30%) for F1-k, within range of  $\alpha$ -tocopherol (87.04%) and BHT (89.71%) in linoleic acid oxidation system. Furthermore, F1-k exhibited higher ABTS, DPPH and Metal-chelating activity with a significant difference ( $p < 0.05$ ). The correlation between the smaller Mw size and antioxidant activity is superiorly exhibited in the F1-k fraction.

**Key words:** Antioxidant activity, hydrolysate, membrane, molecular weight cut-off, ultrafiltration

### INTRODUCTION

Enzymatic hydrolysis is a more desirable alternative to chemical treatment because chemical hydrolysis may oxidize cysteine and methionine, destroy some serine and threonine, and convert glutamine and asparagines to glutamate and aspartate, respectively, resulting to the reduction of protein quality and bioactive value (Kristinsson and Rasco, 2000). The relation between the structure of peptides and their activities is not known in detail but greatly depends on the Molecular Weight (MW) (Pihlanto, 2006).

Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavours, odours and potentially toxic reaction products (Lin and Liang, 2002). Furthermore, cancer, coronary heart disease and Alzheimers diseases are also reported to be caused in part by oxidation or free radical reactions in the body (Diaz *et al.*, 1997). In foods, oxidation also directly affects food quality, and commonly associated with changes of food flavor and texture. An antioxidant is defined as any substance that significantly delays or inhibits oxidation of a substance when present at low concentrations compared to that of an oxidisable substrate. Currently, synthetic antioxidants such as

Butylated Hydroxytoluene (BHT), Butylated Hydroxyanisole (BHA), Propyl Gallate (PG) and Tert-Butylhydroquinone (TBHQ) are used under strict regulations because of their toxic effects on human enzyme systems (Hatate *et al.*, 1990). In contrast, natural antioxidants have attracted more and more interests because of their safety and wide distribution properties in recent years (Hatate *et al.*, 1990; Nau *et al.*, 1995). Ultra Filtration (UF) Membrane technology has received tremendous importance for concentration, purification and fractionation of various products in diverse fields such as food, pharmaceutical, and biotechnological industries. The fractionation selectivity is mainly related to the peptides size according to their MWCO. Profitable production of bioactive peptides from food proteins has been limited by a lack of suitable large-scale technologies. Until now, membrane separation techniques have provided one of the best technologies available for the enrichment of peptides (Lewis, 1993). In the near future, such techniques are expected to solve problems related to community health and nutrition, economic, and environmental health. Utilization of membrane filtration technology will support the preparation of high quality bioactive hydrolysates from fish by-products and wastes. In this research, our experimental hydrolysate is fractionated sequentially using membranes fitted with a

5000, 3000 or 1000 Da nominal MWCO. Performances of the UF fractions, characterization of the peptides, amino acids composition, were investigated in conjunction with their antioxidant activities.

## MATERIALS AND METHODS

HWDH Sample was fractionated successfully using UF membrane with MWCO 5000, 3000, and 1000 Da. The fractionation process has been fed with rude extract obtained by dissolving 10 g/L of powder in pure water, centrifuged and filtered with watman filter paper No 1 to remove un-dissolved particles. Experiment carried out in UF membrane reactor (Micro lab, MSC300) pilot plant (Mosu Science Equipment Company Limited, Shanghai, China) with a maximum capacity of 500 mL (Launching tank 350 mL + dead volume of 150 mL). Connected to the pilot plant was a tubular organic membrane with diameter 80 mm, and the UF permeate used as feed solution. An UF membrane with nominal MWCO (5000, 3000 and 1000 Da) is a modified polyethersulphur with high dimensional stability. The old membrane was replaced with a new one for every 3 to 5 uses, whereas reuse was performed with the same that was chemically well-cleaned between every filtration. Alcalase 2.4 L from a strain of *Bacillus licheniformis*, was obtained from Novozymes China Inc. and stored at 4 °C for subsequent analysis. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl) 5,6-bis(4-phenyl-sulphonic acid) -1,2,4-triazine (Ferrozine), and 2,2-azino-bis (3 ethylbenzothiazoline -6-sulfonic acid) diammonium salt (ABTS), 2-thiobarbituric acid (TBA), ammonium thiocyanate, linoleic acid,  $\alpha$ -tocopherol were obtained from Sigma-Aldrich (Shanghai, China). All chemical reagents used for experiments were of highest analytical grade. The research was conducted from March to June, 2010 in the School of Food Science and Technology (SFST) and State Key Laboratory (SKL) of Jiangnan University, Wuxi, PR China.

**Robust ultrafiltration technique:** Selective permeable membranes are used to concentrate a protein solution according to their MWCO. The function of the membrane is to let the water and minute molecules pass through. The solution is directed against the membrane by mechanical pump or gas pressure and/or centrifugation. In this technique, pressure is applied to the solution to cause a bulk flow of water and dissolve low molecular weight solutes, through the membrane, while high molecular weight solutes are retained. Nonetheless, this special equipment required membranes that are having a unimolecular sieving layer. The sieving side is shiny and can be distinguished from the support side which is dull. Whether or not a particular molecule will pass through an ultrafiltration membrane is determined at the unimolecular sieving layer, and for proteins that are unable to pass through are rejected on the surface where they can easily

Table 1. Characteristics used in the preparation of sample for hydrolysis

| Enzyme               | Form         | pH  | T (°C) |
|----------------------|--------------|-----|--------|
| Alcalase 2.4L(AU/g)* | Liquid/grain | 8.0 | 55     |

\*AU (Anson units) is the amount of enzyme that under standard conditions digests hemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same color with the FILON reagent as one milliequivalent of tyrosine released per minute.

be removed. The pressure exerted on the solution causes a flow of solvent through the membrane but immediately flow commences, a phenomenon known as concentration polarisation occurs. A secondary membrane layer of retained protein is formed constituting the major resistance to flow and thus determines the flow rate. Resistance to flow increases and the flow rate drops when there is increase in thickness of the Polarized Concentrate (PC), to the original value. Since the resistance is determined by the thickness of the PC, reducing this thickness will result in an increased flow rate. One way of decreasing the thickness of the PC is to stir the solution and thus increase the effective rate of back diffusion. This is the purpose of the magnetic stirrer bar.

**Preparations of hot water dip protein hydrolysate (HWDH) and Ultrafiltrate fraction:** HWDH is produced from Fresh Nile tilapia by the method described (Foh *et al.*, 2010), using Alcalase 2.4 L, based on optimum hydrolysis conditions in Table 1. One hundred grams of HWD tilapia meat was weighed into a vessel immersed in a water bath maintained at an appropriate temperature and 300 mL of distilled water was added to make a suspension. The suspension was adjusted to an optimal pH condition and pre heated for 15 min. An enzymes substrate ratio (1.5%) was added with continuous stirring, the hydrolysis process was monitored for 120 min. After hydrolysis, the enzymes were inactivated by placing in boiling water for 15 min. The hydrolysate was allowed to cool down and centrifuged at 7,500 for 15 min at 4 °C using a D-3756 Osterode am Harz model 4515 centrifuge (Sigma, Hamburg, Germany). The hydrolysate was lyophilized, prepared for fractionation using UF membrane with MWCO (5000, 3000, and 1000 Da). The process is designated triple-stage UF when the 5000, 3000 or 1000 Da MWCO membranes are used successively. On the other hand, the first stage UF is carried out by fitting the 5000 Da MWCO filtration membrane. Permeate from 5000 Da MWCO membrane was successively processed in the membrane reactor fitted with 3000 Da MWCO membrane, and further permeate processed using a 1000 Da MWCO membrane. To aid in limiting Polar concentration of the protein in solution, a higher recirculation velocity via a magnetic stirrer is applied to membrane surface deposit (Cheison and Wang, 2003) hence a controlled permeate flow. The retentate is recycled by diluting with water that is introduced into the buffer tank or between two successive ultrafiltration modules. A back pressure (50 pound

force per square inch) was applied on the initial sample solution to achieve a controlled permeate flow rate. Sample ultrafiltrates and retentate of 5000 Da MWCO membrane were collected, and lyophilized for further analysis. All the experiments were performed in triplicate and the results are the average of three values.

**Degree of hydrolysis (DH) determination:** The degree of hydrolysis is defined as the percent ratio of the number of peptide bonds broken ( $h$ ) to the total number of bonds per unit weight ( $h_{tot}$ ). Reactions were monitored by measuring the extent of proteolytic degradation by means of the DH according to the pH-stat method described by Adler-Nissen (1986). In each case, was calculated from the amount of base consumed as described by Van DER Plancken *et al.*, (2003), as given below:

$$DH(\%) = \frac{V_b \times N_b}{\alpha \times mP \times h_{tot}} \times 100$$

Where  $V_b$  is base consumption in mL;  $N_b$  is normality of the base;  $\alpha$  is average degree of dissociation of the  $\alpha$ -NH<sub>2</sub> groups;  $mP$  is mass of protein ( $N \times 6.25$ ) in g; and  $h_{tot}$  is total number of peptide bonds in the protein substrate. All the experiments were performed in triplicate and the results are the average of three values.

**Determination of molecular weight:** The molecular weight distribution profile of the samples was determined using a Waters™ 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA). A TSK gel, 2000SWXL (7.8 00 mm) column was used with 10% acetonitrile + 0.1% TFA in HPLC grade water as the mobile phase. The calibration curve was obtained by running bovine carbonic anhydrase (29,000 Da), horse heart cytochrome C (12,400 Da), bovine insulin (5800 Da), bacitracin (1450 Da), Gly-Gly-Tyr-Arg (451 kDa) and Gly-Gly-Gly (189 Da). The results were obtained and processed with the aid of Millennium 32 Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

**Reducing power:** The reducing power activity of the partially purified ultrafiltrate fractions was measured spectrophotometrically according to the method of Oyaizu (1986) with slight modifications. The antioxidant action of a reducing agent is based on its ability to donate an electron to Fe (III) ion reducing it to Fe (II) ion. To the different concentrations of the sample (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg) was added 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 30 min. Then Trichloroacetic acid (2.5 mL, 10%) was added to the reaction mixture and centrifuged at 600xg for 12 min at

room temperature. A volume of 2.5 mL each from the supernatant was mixed with distilled water and ferric chloride solution (0.1%). After a 10 min reaction time, the absorbance was measured immediately at 700 nm. Triplicate tests were conducted for each sample.

**Inhibition of linoleic acid autoxidation:** The antioxidant activities of Ultrafiltrate fractions were measured based on the method of Osawa and Namiki (2001) with some modifications. One milligram of sample was dissolved in 1.5 mL of 0.1 M phosphate buffer (pH 7.0), and added to 1 mL of linoleic acid (50 mM) dissolved with ethanol (99.5%), incubated in a conical flask with a screw cap at 39 °C in a dark room for 7 days and the degree of oxidation was evaluated by measuring the ferric thiocyanate values. The degree of oxidation was evaluated by measuring the ferric thiocyanate values according to the method described by Mitsuda *et al.*, (1966). The sample solution (100) incubated in the linoleic acid model system described above (Osawa and Namiki, 2001) was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% (w/v) ammonium thiocyanate, and 0.1 mL of 0.02 M ferrous chloride dissolved in 1 M HCl. After 3 min, the degree of color development that represents linoleic acid oxidation was measured spectrophotometrically at 500 nm. The antioxidant activities of Butylated Hydroxytoluene (BHT) and  $\alpha$ -tocopherol were also assayed at the same concentration for comparison purposes. Percentage Inhibition of linoleic acid autoxidation was calculated using the equation below:

$$\text{Inhibition (\%)} = \left( \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \right) \times 100 \quad (2)$$

**Synergistic effects of ultrafiltrate fractions:** Ultrafiltrate fractions were measured synergistically as follows: in brief, a sample (1.0 mg) and  $\alpha$ -tocopherol (0.13 mg) were dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0), and added to a solution of 0.13 mL of linoleic acid and 10 mL of 99.5% ethanol. Then the total volume was adjusted to 25 mL with distilled water. The solution was incubated in a conical flask with a screw cap at 39 °C in a dark room, and the degree of oxidation was evaluated by measuring the ferric thiocyanate values.

**Metal-chelating activity:** Metal-chelating activity of sample fractions was assessed using the method of Decker and Welch (1990). 1 mL of peptide solution (5 mg/mL) was first mixed with 3.7 mL of distilled water. Then it was reacted with a solution containing 0.1 mL 2 mM FeCl<sub>2</sub> and 0.2 mL of 5 mM Ferrozine. After 10 min, the absorbance of the reaction mixture was measured at 562

nm. The Metal-chelating ability of the fractions was calculated as a percentage applying the equation;

Metal-chelating ability (%) =

$$\left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100 \quad (3)$$

**DPPH radical scavenging activity assay:** The method of Wu *et al.* (2003) with some modification was employed to spectrophotometrically quantify the scavenging effect on 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical. Samples from UF fractions were dissolved in distilled water to obtain a concentration of 40 mg protein per mL. Then 2.0 mL of sample was mixed with 2.0 mL of 0.15 mM DPPH that was dissolved in 95% ethanol. The mixture was then shaken vigorously using a mixer (QT-1 Mixer, Tianchen Technological Co. Ltd., Shanghai, China) and kept in the dark for 30-32 min. The absorbance of the resultant solution was recorded at 517 nm. The scavenging activity was calculated using the following equation:

DPPH (%) =

$$\left(\frac{\text{absorbance of DPPH} - \text{absorbance of sample}}{\text{absorbance of DPPH blank}}\right) \times 100 \quad (4)$$

Where the DPPH blank is the value of 2 mL of 95% ethanol mixed with DPPH solution, the DPPH sample is the value of 2 mL of sample solution mixed with DPPH solution, and the control sample is the value of 2 mL of sample solution mixed with 2 ml of 95% ethanol.

**ABTS radical scavenging activity assay:** ABTS radical scavenging activity of Samples obtained from UF fractions were assessed by the method described by Re *et al.* (1999), with modification. A stock solution of ABTS radicals was prepared by mixing 5.0 mL of 7 mM ABTS solution with 88 of 140 mM potassium persulfate, and keeping in the dark at room temperature for 12-16 h. An aliquot of stock solution was diluted with Phosphate buffer, PB (5 mM, pH 7.4) containing 0.15 M NaCl in order to prepare the working solution of ABTS radicals to an absorbance of 0.70 .02 at 734 nm. A 65 aliquot of the fractions dissolved in the same phosphate buffer (66.67/mL final assay concentration) or only buffer (for the control) was mixed with 910 μL of ABTS radical working solution, incubated for 15 min at room temperature in the dark, and then absorbance was measured at 734 nm. The percent reduction of ABTS<sup>+</sup> to ABTS was calculated according to the following equation:

$$\text{ABTS (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100 \quad (5)$$

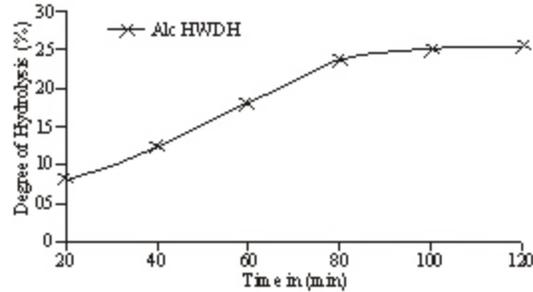


Fig. 1: Effect of time on the degree of hydrolysis (DH) of tilapia (*Oreochromis niloticus*) fish protein hydrolysates (TFPH). HWDH: Hot water dip hydrolysate Value represent the mean ± standard deviation (SD) of three determinations (Alc-Alcalase 2.4 L)

**Statistical analysis:** Data and Statistical Analysis of variance (ANOVA) was performed and differences in mean values were evaluated by Tukey's test at  $P < 0.05$  using SPSS version 18.0 (SPSS Inc, Chicago, IL, USA).

## RESULTS AND DISCUSSION

**Degree of Hydrolysis (DH):** The DH is an important factor highly related with the hydrolysis process yield (Shahidi *et al.*, 1994). There was an initial rapid increase in DH with increased time relating to the frequency of addition and volume of NaOH used to maintain pH, thereby, indicating very high cleavage of peptides occurring within the first hour of hydrolysis. Alcalase 2.4 L showed high value in terms of DH (Fig. 1). The result is in corroboration with results reported on enzymatic hydrolyses of fish protein substrates (Guerard *et al.*, 2002; Sathivel *et al.*, 2003).

**Membrane filtration:** A very complicated phenomenon caused by many chemical and physical factors occur during microfiltration of protein solutions containing both soluble and insoluble proteinaceous solids (Belfort *et al.*, 1994; Marshall *et al.*, 1993), consequently causing permeate fluxes to decline dramatically due to membrane fouling. Because of the inherent amphoteric hydrophobicity of protein molecules, interactions between proteins and membranes may occur. Depending on the particle size, proteins may enter the membrane pores, deposit and form a gel layer on the surface, thus significantly increasing the filtration resistance. During the three-stage UF process, four different fractions (permeates; F1-k, F3-k and F5-k, retentate; 5k-R) were obtained using MWCO 1000, 3000, and 5000 Da membrane. The values of the fractions (permeates; F1-k, F3-k and F5-k, retentate; 5k-R) and its impact on the peptidic population in terms of Mw, amino acids

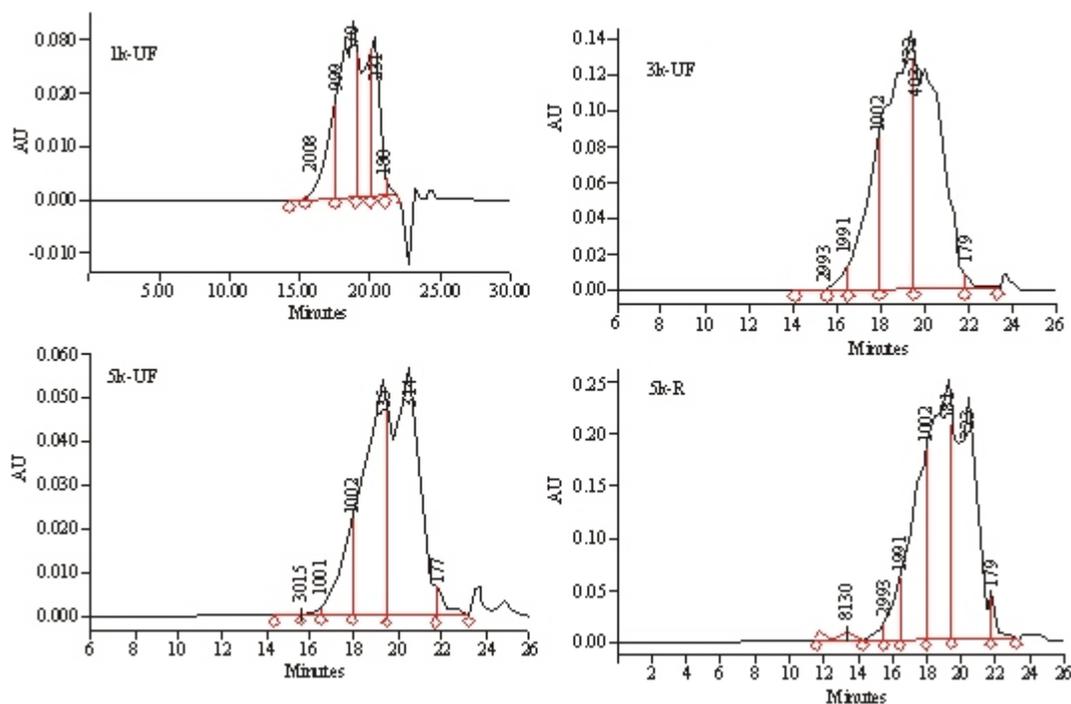


Fig. 2: Molecular weight distribution profile of the Ultrafiltrate fractions (F1-k, F3-k, F5-k and retentate 5k-R) using MWCO filtration membranes 1000, 3000 and 5000 Da

composition and antioxidative properties are discussed below. The ultrafiltrates (F1-k, F3-k and F5-k), and the retentate of 5000 Da (5k-R) were enriched with peptides within the prescribed MWCO of the ultrafiltration membrane used. Fish protein hydrolysates are characterized with good nutritional and natural bioactive compounds essential for food consumers.

**Molecular weight distribution:** The Gel Permeation Chromatography (GPC) using an HPLC system was used to study molecular weight distribution profiles of the HWDH fractions obtained using ultrafiltration membrane according to their MWCO; 1000, 3000 and 5000 Da. From the chromatographic data (Fig. 2), it was observed that all hydrolysate fractions were composed of low molecular weight peptides. Many studies have reported that low molecular weight peptides were more potent as bioactive peptides (Suetsuna *et al.*, 2000; Chen *et al.*, 1998). Our study was in accord with these and exhibited a significant antioxidative activity in the lower molecular weight peptide fraction (below 2000 Da). The reason that low molecular weight hydrolysates exhibited their antioxidant potential may be due to them acting as chain-breaking antioxidants by inhibiting radical-mediated peroxidation of linoleic acid. (Rajapakse *et al.*, 2005) According to these results, the hydrolysates below 2000 Da possibly contained peptides that acted as electron donors to form more stable products and terminate radical chain reactions. The high proportion of

peptides with molecular weights below 2000 Da suggests that they may exhibit biological activities. The molecular size distribution profiles shows the chromatographic data of the fractions (permeates; F1-k, F3-k and F5-k, retentate; 5k-R) were composed of peptides with molecular weight ranges (<2010, 2436-4363, 2436-4814, 5016-19611 Da) respectively calculated according to the equation:

$$\text{Log Mol Wt} = 6.70 - 0.2.14T \text{ with } R^2 = 0.9953 \quad (6)$$

In Fig. 2, results revealed that permeate fractions F1-K, F3-K and F5-k portrayed molecular weight distributions ranging between 177-3015 Da, whereas the retentate fraction 5K-R manifested (5016-19611 Da) the highest molecular weight distribution. These findings are in concurrence with observations from other studies where the functional properties of antioxidative peptides are highly influenced by molecular weight distribution profile (Klompong *et al.*, 2007; Kim *et al.*, 2007).

**Reducing power:** The reducing power of the HWDH fractions, increased with corresponding increase in sample concentration. The reducing capacity of a given compound may serve as a significant indicator of its potential antioxidant activity. In this assay, the presence of antioxidants caused the reduction of the  $\text{Fe}^{3+}$ /ferricyanide complex to the ferrous form, and the yellow color of the test solution changed to various shades

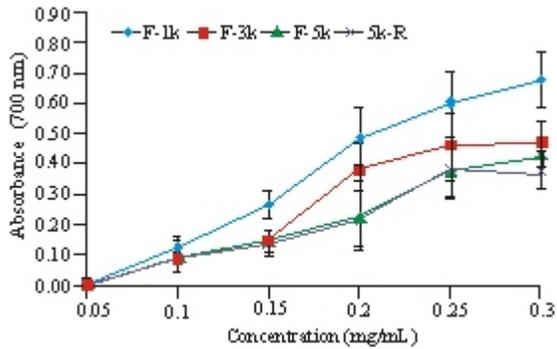


Fig. 3: Reducing power of the fractionated hydrolysates (HWDH) prepared using ultrafiltration membranes at different concentrations, measured by the ability to donate electrons and reduce  $Fe^{3+}$  to  $Fe^{2+}$  ions. The formation of a  $Fe^{2+}$ /ferricyanide complex was measured as the absorbance at 700 nm. Data were expressed as means with standard deviations (n=3)

of green and blue depending on the reducing power of each compound. The  $Fe^{2+}$  was then monitored by measuring the formation of Perl Prussian blue at 700 nm (Ferreira *et al.*, 2007). As shown in Fig. 3, the reducing power of sample fractions of different concentrations (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 mg/mL) exhibited a dose-dependent effect. It was also found in Fig. 3 that peptides of permeate fraction F1-k showed a significantly high ( $p < 0.05$ ) reducing power, followed by permeate fractions F3-k, F5-k, retentate; 5k-R, respectively. According to the result, the permeate fractions (F1-k, F3-k, F5-k), with molecular weight below 5000 Da contained peptides with a stronger electron donors capacity to form more stable products and terminate radical chain reactions than that of retentate fraction (5k-R) above 5000 Da. A similar observation has been reported by Je *et al.* (2007). A strong linear relationship was observed between the reducing power (y) and the sample concentration (x). The results also indicate that the reducing power correlates well with concentration of hydrolysates with different molecular weights.

**Inhibition of linoleic acid autoxidation and Synergistic effects on Ultrafiltrate fractions:** In this study, the sample fractions were investigated for Inhibition of linoleic acid autoxidation and also compared with that of  $\alpha$ -tocopherol and BHT. Peroxidation of fatty acids can cause deleterious effects in foods by forming complex mixture of secondary breakdown products of lipid peroxides. Toxicity to mammalian cells can be a consequence upon further intake of these foods. The antioxidative activity of the fractionated hydrolysates is shown in Fig. 4a, b. F1-k with molecular weight (MW) of below 2000 Da showed the highest antioxidative activity, which exhibited about 80% inhibition of linoleic acid peroxidation (Table 2). In addition, the synergistic

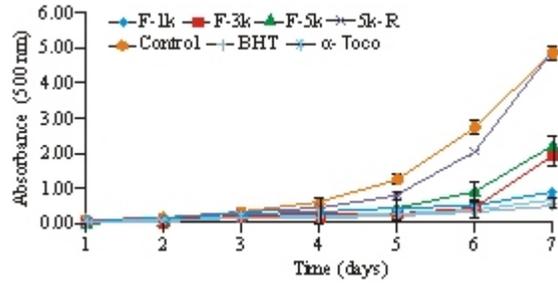


Fig. 4a: Antioxidative activities of fractionated Ultrafiltrate and retentate hydrolysates in linoleic acid autoxidation system measured by the ferric thiocyanate method. Butylated hydroxytoluene and  $\alpha$ -tocopherol were used as positive controls. Data were expressed as means with standard deviations (n=3)

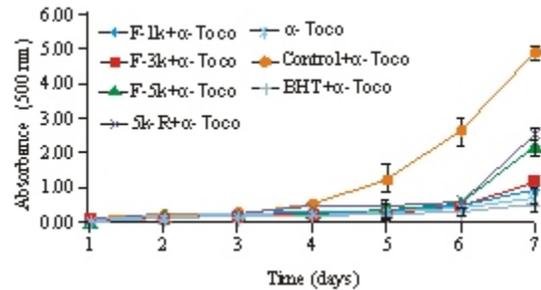


Fig. 4b. Synergistic effect of  $\alpha$ -tocopherol and hydrolysates of Ultrafiltrate and retentate in linoleic acid autoxidation system measured by the ferric thiocyanate method. Data were expressed as means with standard deviations (n=3)

antioxidative effect of the fractionated hydrolysates with the nonpeptidic antioxidant,  $\alpha$ -tocopherol and BHT, were studied. In Fig. 4b, the synergistic antioxidative effects of the hydrolysate fractions with the nonpeptidic antioxidant  $\alpha$ -tocopherol showed the fractions (permeates; F1-k, F3-k and F5-k, retentate; 5k-R), exhibited synergistic effects with  $\alpha$ -tocopherol and BHT. In this study, the fractions (permeates; F1-k, F3-k and F5-k, retentate; 5k-R) had both antioxidative and a synergistic effect with  $\alpha$ -tocopherol using the linoleic acid in water/ethanol system. The highest synergistic antioxidative effects was reported for permeate fraction F1-k and  $\alpha$ -tocopherol combined with a significant difference ( $p < 0.05$ ). The synergistic effects of nonpeptidic antioxidants on antioxidative activity have previously been demonstrated with the hydrolysates of a vegetable protein, yeast protein, Alaska Pollack skin gelatin hydrolysates, and bovine serum albumin (Kim *et al.*, 2007), reported that the hydrolysates of soybean protein showed a strong synergistic effect with nonpeptidic antioxidants. This study was in concurrence with our finding and exhibited a significant antioxidative activity in the lower molecular weight peptide fraction F1-

Table 2.: Percentage antioxidative activity of Ultrafiltrate fractions with and without Synergistic effects of  $\alpha$ -tocopherol on day seven measured by the ferric thiocyanate method in linoleic acid autoxidation system

| Fractions            | % Inhibition with $\alpha$ -tocopherol | Fractions      | % Inhibition without $\alpha$ -tocopherol |
|----------------------|--|----------------|---|
| F-1K+ $\alpha$ -Toco | 80.25                                  | F-1K           | 82.30                                     |
| F-3K+ $\alpha$ -Toco | 75.51                                  | F-3K           | 61.11                                     |
| F-5K+ $\alpha$ -Toco | 54.53                                  | F-5K           | 54.53                                     |
| 5K-R+ $\alpha$ -Toco | 47.74                                  | 5K-R           | 0.34                                      |
| $\alpha$ -Toco       | 84.98                                  | $\alpha$ -Toco | 87.04                                     |
| Control              | 0.00                                   | Control        | 0.00                                      |
| BHT                  | 89.71                                  | BHT            | 89.71                                     |

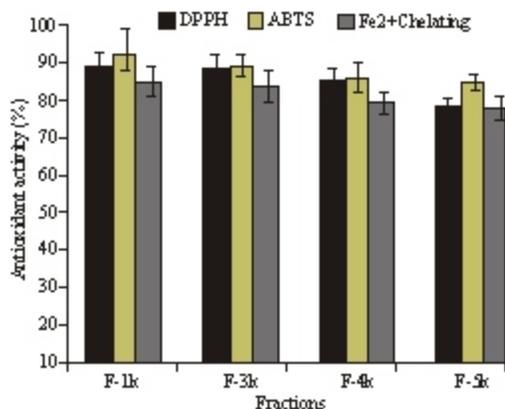


Fig. 5: Antioxidant activity of HWDH Ultrafiltrate fractions. Value represent the mean  $\pm$  standard deviation (SD) of n = 3 duplicate assays

k, than was for F3-k, F5-k and 5k-R fractions (Fig. 4a, b). A probable reason that low molecular weight hydrolysates exhibited their antioxidant potential is due to them acting as chain-breaking antioxidants by inhibiting radical-mediated peroxidation of linoleic acid (Rajapakse *et al.*, 2005).

**DPPH radical scavenging activity assay:** DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Prior *et al.*, 2005); therefore, DPPH is often used as a substrate to evaluate the antioxidative activity of an antioxidant. Results shown in Fig. 5 depicts the DPPH scavenging activity of fraction F1-k exhibited an excellent DPPH radical-scavenging activity (88.47%). Using the sample concentration (0.5 mg/mL) with varying molecular weights for F3-k, F5-k and retentate 5k-R, also showed good DPPH radical-scavenging activities with a significant difference ( $p < 0.05$ ). Our research findings corroborate with other facts (Deeslie and Cheryan, 1991).

**ABTS radical scavenging activity assay:** The ABTS radical assay is a widely used method of screening for antioxidant activity and is reported as a decolorization assay applicable to both lipophilic and hydrophilic compounds. A Reduction of the color indicated reduction of ABTS radical, and Protein hydrolysates from many sources have been found to possess antioxidative activity

(Miliauskasa *et al.*, 2004). The assay results of the different HWDH fractions (F1-k, F3-k and F5-k) and a retentate 5k-R) exhibited good ABTS radical scavenging ability with a significant difference ( $p < 0.05$ ) as shown in Fig. 5. The fractions possess high ABTS radical scavenging ability ( $p < 0.05$ ) and could reduce more than 84% of the ABTS radicals in the assay media at 66.67  $\mu$ g/mL sample concentration. From the result, F1-k exhibited the highest ABTS radical scavenging ability (92.27%), followed by F3-k (89.20%), F5-k (85.97%) and 5k-R (84.47%), respectively (Fig. 5). However, the antioxidative scavenging quality is closely related to the hydrolysis conditions, the amino acid composition and lower molecular weight. Similarly, high antioxidative scavenging quality is shown in HWDH fractions from DPPH and metal chelating activities, exhibiting similar results reported by (Klompong *et al.* 2007).

**Metal-chelating activity:** The result from Fig. 5 shows that HWDH fractions (F1-k, F3-k, F5-k and 5k-R) interacted with iron ion - Ferrozine forming complexes with  $Fe^{2+}$  ion. In the presence of chelating agents, the complex formation is disrupted resulting to a decrease in the red colour of the complex. Measurement of colour reduction therefore makes possible the estimation of the metal chelating activity of the coexisting chelator (Yamaguchi *et al.*, 2000). The result from Fig. 5 shows that sample fraction F1-k exhibited a superior chelating activity (84.70%) than F3-k, F5-k and 5k-R (83.60, 79.03 and 77.30%), with a significant difference ( $p < 0.05$ ) respectively. This result also corroborates a linear relationship with the increased DH, lower molecular weight distribution, and high peptide solubility that showed higher metal chelating activity (Saiga *et al.*, 2003). The assay result indicated that fraction F1-k had an effective capacity for iron binding, signifying that its action as an antioxidant may be related to its iron binding capacity. Nonetheless, this fraction can be a potential antioxidant source.

## CONCLUSION

In conclusion, HWDH from tilapia was found to be an effective antioxidant in different *in vitro* assays, including Inhibition of linoleic acid autoxidation, reducing power, ABTS radical scavenging activity, Metal-chelating activity and DPPH radical-scavenging activity. The different HWDH fractions obtained by UF membranes exhibited appreciable levels of antioxidant and free radicals scavenging activities. Results revealed that F1-k has superior antioxidant activities. However, the hydrolysis conditions, DH, and high proportion of lower molecular weight peptides, were found to be closely correlated to the antioxidative activity that needs further investigation.

## ACKNOWLEDGMENT

Authors wish to thank the earmarked fund for Modern Agro-industry Technology Research System (NYCYTX-49-22), PCSIRT0627 and 111project-B07029 for providing financial support to carry out this research. We also extend our profound thanks and appreciation to the governments of China and Sierra Leone.

## REFERENCES

- Adler-Nissen, J., 1986. Enzymic Hydrolysis of Food Proteins. Elsevier Applied Science Publishers, New York, NY, USA.
- Belfort, G., R.H. Davis and A.L. Zydney, 1994. The behavior of suspensions and macromolecular solutions in cross flow microfiltration. *J. Membr. Sci.*, 96: 1-58.
- Cheison, S.C. and Z. Wang, 2003. Bioactive milk peptides: redefining the food-drug interphase review. *Afr. J. Food, Agric. Nutr. Dev.*, 3: 29-38.
- Chen, H.M., K. Muramoto, F. Yamauchi, K. Fujimoto and K. Nokihara, 1998. Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *J. Agric. Food Chem.*, 46: 49-53.
- Decker, E.A. and B. Welch, 1990. Role of ferritin as a lipid oxidation catalyst in muscle food. *J. Agric. Food Chem.*, 38: 674-677.
- Deeslie, W.D. and M. Cheryan, 1991. Fractionation of soy protein hydrolysates using ultrafiltration membranes. *J. Food Sci.*, 57: 411-413.
- Diaz, M.N., B. Frei, J.A. Vita and J.F. Keaney, 1997. Antioxidants and atherosclerotic heart disease. *N. Engl. J. Med.*, 337: 408-416.
- Ferreira, I.C.F.R., P. Baptista, M. Vilas-Boas and L. Barros, 2007. Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. *Food Chem.*, 100: 1511-1516.
- Foh, M.B.K., I. Amadou, B.M. Foh, M.T. Kamara and W.S. Xia, 2010. Functionality and Antioxidant Properties of Tilapia (*Oreochromis niloticus*) as Influenced by the degree of Hydrolysis. *Int. J. Mol. Sci.*, 11: 1851-1869.
- Guerard, F., L. Guimas and A. Binet, 2002. Production of tuna waste hydrolysates by a commercial neutral protease preparation. *J. Mol. Catal. B: Enzym.*, 19: 489-498.
- Hatate, H., Y. Nagata and M. Kochi, 1990. Antioxidative effect of bovine serum albumin hydrolyzates and their synergistic effect with antioxidants. *Yukagaku*, 39: 42-46.
- Je, J.Y., Z.J. Qian, H.G. Byun and S.K. Kim, 2007. Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochem.*, 42: 840-846.
- Kim, S.Y., J.Y. Je and S.K. Kim, 2007. Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *J. Nutr. Biochem.*, 18: 31-38.
- Klompong, V., S. Benjakul, D. Kantachote and F. Shahidi, 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chem.*, 102: 1317-1327.
- Kristinsson, H.G. and B.A. Rasco, 2000. Fish protein hydrolysates: production, biochemical, and functional properties. *Crit. Rev. Food. Sci. Nutr.*, 40: 43-81.
- Lewis, N.G., 1993. Plant Phenolics. In: Alscher, R.G. and J.L. Hess (Eds.), *Antioxidants in Higher Plants*. Boca Raton, FL, CRC Press, pp: 135-160.
- Lin, C.C. and J.H. Liang, 2002. Effect of antioxidants on the oxidative stability of chicken breast meat in a dispersion system. *J. Food Sci.*, 67: 530-533.
- Marshall, A.D., P.A. Munro and G. Tragardh, 1993. The effect of protein fouling in microfiltration and ultrafiltration on permeate flux, protein retention and selectivity: A review. *J. Membr. Sci.*, 91: 65-108.
- Miliauskasa, G., P.R. Venskutonisa and T.A. Van Beekb, 2004. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chem.*, 85: 231-237.
- Mitsuda, H., K. Yasumoto and K. Iwami, 1966. Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyoto Shokuryo*, 19: 210-214.
- Nau, F., F.L. Kerherv, J. Leonil and G. Daufin, 1995. Selective separation of tryptic beta-casein peptides through ultrafiltration membranes: Influence of ionic interactions. *Biotechnol. Bioeng.*, 46: 246-253.
- Osawa, T. and M. Namiki, 2001. Natural antioxidants isolated from eucalyptus leaf waxes. *J. Agric. Food Chem.*, 49: 2992-2997.
- Oyaizu, M., 1986. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.*, 44: 307-315.
- Pihlanto, A., 2006. Antioxidative peptides derived from milk proteins. *Int. Dairy J.*, 16: 1306-1314.
- Prior, R.L., X. Wu and K. Schaich, 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.*, 53: 4290-4302.
- Rajapakse, N., E. Mendis, H.G. Byun and S.K. Kim, 2005. Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *J. Nutr. Biochem.*, 16: 562-569.
- Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Bio. Med.*, 26: 1231-1237.

- Saiga, A., S. Tanabe and T. Nishimura, 2003. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *J. Agric. Food Chem.*, 51: 3661-3667.
- Sathivel, S., P.J. Bechtel, J. Babbitt, S. Smiley, C. Crapo and K.D. Reppond, 2003. Biochemical and functional properties of herring (*Clupea harengus*) byproduct hydrolysates. *J. Food Sci.*, 68: 2196-2200.
- Shahidi, F., 1994. Seafood Processing Byproducts. In: Shahidi, F. and J.R. Botta (Eds.), *Seafoods: Chemistry, Processing Technology and Quality*, 1st Edn., Blackie, London, U.K., pp: 321-324.
- Suetsuna, K., H. Ukeda and H. Ochi, 2000. Isolation and characterization of free radical scavenging activities peptides derived from casein. *J. Nutr. Biochem.*, 11: 128-131.
- Van der Plancken, I., M. Van Remoortere, I. Indrawati, A. Van Loey and M.E. Hendrickx, 2003. Heat induced changes in the susceptibility of egg white proteins to enzymatic hydrolysis: A kinetic study. *J. Agric. Food Chem.*, 51: 3819-3823.
- Wu, H.C., H.M. Chen and C.Y. Shiau, 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Res. Int.*, 36: 949-957.
- Yamaguchi, F., T. Ariga, Y. Yoshimira and H. Nakazawa, 2000. Antioxidant and antiglycation of carcinol from *Garcinia indica* fruit rind. *J. Agric. Food Chem.*, 48: 180-185.