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Antioxidant activity and functional properties of fractionated cobia skin gelatin hydrolysate at different molecular weight

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Abstract

This study investigated the antioxidant activity and functional properties of fractionated cobia skin gelatin hydrolysate (CSGH) at different molecular weights (10, 5 and 3 kDa). Antioxidant activities studied included reducing power, ferrous ion chelation, DPPH (1, 1- diphenyl-2-picrylhydrazyl) radical scavenging, and superoxide anion scavenging. Functional properties studied included emulsifying and foaming properties as well as fat and water binding capacity. Results showed significant differences ($p < 0.05$) between the chelating activity of ferrous and superoxide anions for 5 kDa and other fractions, while the highest DPPH radical scavenging activity was presented by the 3 kDa fraction. The 10 kDa fraction had the highest fat binding capacity (28 ± 2%) while functional properties were not affected by lower molecular weight. Lower outcomes obtained for functional properties from smaller molecular weights may have been due to enzymatic hydrolysis and the fractionation process to which the hydrolysate was subjected. Hence, varied CSGH molecular weights appeared to benefit different functions.

Introduction

Although fish gelatin has been studied since the 1950s, most gelatin studies refer to mammalian products. Only in recent years has more intensive research on fish gelatin appeared in the literature (Karim and Bhat, 2009). Most gelatins are widely produced from pig skin as well as bovine skin and bone. But increasing demand for industrial gelatin has encouraged gelatin production from other sources such as fish bone, scales and skin especially to meet requirements of suppliers and consumers for *halal* gelatins. Moreover, *halal* gelatins are extensively used for food and various pharmaceutical products as well as biomaterial based packaging and as edible films, and also products in the photographic industry (Amiza and Siti Aishah, 2011).

Protein hydrolysate is a value added product that is derived from animal and plant sources via hydrolysis of protein utilizing the method of Vijay *et al.* (2010) via acid, alkaline, enzymatic, and fermentation treatments. A variety of enzymes such as alcalase, bromelain, flavourzyme, nutrase, pepsine, trypsine and papain have been applied to prepare protein hydrolysates with enhanced functional and antioxidant properties (Galla, 2012). Due to several potentials including antioxidant and functional properties for various applications, research on gelatin hydrolysates has attracted much interest recently and many investigators have reported on the excellent functional properties of fish gelatin hydrolysates; including Gimenez *et al.* (2009), who conducted a study on the functional properties of gelatin hydrolysate produced from the skins of sole and squid.

The molecular weight (MW) of hydrolysed gelatin influences antioxidant activity as the functional properties and peptides of low molecular weight are thought to have stronger antioxidant properties (Chen *et al*., 2007). Qin *et al.* (2011) reported a high percentage of DPPH radical scavenging due to the low molecular weight of purple sea urchin gonad hydrolysate. Qin *et al.* (2011) also reported higher reducing power for purple sea urchin gelatin due to low molecular weight (< 3kDa).

Cobia *(Rachycentron canadum)* is a warm water, fast growing pelagic marine species with global habitats (Liao *et al*., 2004) in warm, temperate, subtropical, and tropical waters (Briggs, 1960). In Malaysia, it is known as Haruan tasik *(Rachycentridae)*. Taiwan was among the first to produce cobia on a small scale during the 1970s as the fast growing cobia made it compatible for commercial production (Nhu *et al*., 2011). Subsequently, increases in fish products from cobia resulted in more waste materials with high protein content, which then gave researchers cause to seek out additional value added products. According to Kristinsson and Rasco (2000), more than 50% of excess materials such as head, skin and bone from overall fish catches were

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unutilized as food yet contributed nearly thirty-two million tonnes of waste annually, thus contributing to environmental pollution.

Hence, this study aimed to investigate fractionated cobia skin gelatin hydrolysate (CSGH) of different molecular weights (10, 5 and 3 kDa) for (i) antioxidant activity (i.e. reducing power, ferrous ion chelation, DPPH radical scavenging, and superoxide anion scavenging); and (ii) functional properties (emulsifying and foaming properties; fat and water binding capacity).

Materials and Methods

Materials

Cobia *(Rachycentron canadum)* fish were purchased from a local supplier in Langkawi, Kedah, Malaysia and chilled in ice while transported to the laboratory. The commercial food grade enzyme, Alcalase® 2.4L was purchased from Novo Industries A/S Bagsvaerd, Denmark. Alcalase® is a serine bacterial endoprotease derived from *Bacillus licheniformis* with proteolytic activity of 2.4 Anson units/g. All other chemical reagents used were of analytical grade.

Preparation of test samples

Cobia was filleted, beheaded, gutted and cleaned manually under tap water to remove impurities. The cleaned fish skin was cut into smaller pieces of about 1 x 1 cm^2 and then stored at -28°C.

Extraction of cobia skin gelatin

Cleaned cobia skin was subjected to gelatin extraction following the method of Muyonga et al. (2004) with some modification. Approximately 30 g of cobia skin was mixed with 120 ml of 0.15 mol acetic acid for 1 h at 4°C. The acetic acid was then drained and the skins were rinsed twice with 150 ml of distilled water and then mixed with distilled water at ratio of 1:6, (skin/water) and stirred for 3 h in a water bath shaker at 60°C. The extracts were then filtered with muslin cloth before being freeze-dried to produce 'gelatin powder'. The yield was calculated as follow:

$$\text{Yield (\%)} = \frac{\text{Dry weight of gelatin (g)} \times 100}{\text{Wet weight of skins (g)}}$$

Production of cobia skin gelatin hydrolysate (CSGH)

Extracted gelatin samples were subjected to enzymatic hydrolysis using Alcalase® as described by Gimenez *et al.* (2009) with slight modification under pH-stat controlled conditions (pH, temperature, enzyme concentration and time) as follows. Approximately 16.5 g of cobia skin gelatin was dissolved in distilled water (126.5 ml) and enzymatically hydrolysed with 1% Alcalase® at 50°C for 3 h at constant pH 8 using 1 N NaOH. The solution was heated at 90°C for 10 min to inactivate the enzyme and then centrifuged at 3800 rpm for 15 min at 4°C. The supernatant was collected and then fractionated to different molecular weights (3, 5 and 10 kDa) using the ultrafiltration membrane (Vivaspin 20, Sartorius stedim biotech, Goettingen, Germany). The Gelatin hydrolysate was passed through a 10 kDa molecular weight cut- off (MWCO) membrane, with the supernatant filtered in 5 kDa and 3 kDa MWCO membranes, respectively. These three CSGH fractions were freeze-dried and stored at -80°C.

Reducing power

The ability of CSGH was determined to reduce iron (III) according to the method of Yildirim *et al.* (2001). Each sample of 500 µl CSGH (3, 5 and 10 kDa) at different concentrations (0.5, 1.0, 5.0, 10 and 20 mg/ml) was mixed with 1.25 ml of 0.2 M phosphate buffer (pH, 6.6) and 1.25 ml of 1% potassium ferricyanide. These mixtures were then incubated at 50ºC for 30 min followed by the addition of 1.25 ml of 10% (w/v) trichloroacetic acid. They were then centrifuged at 10,000 rpm for 10 min after which 1.25 ml of supernatant was mixed with 1.25 ml of distilled water and 250 µl of 0.1% (w/v) ferric chloride. Allowing ten minutes for reaction, the absorbance spectrum for each resulting solution was then measured at 700 nm with the synthetic antioxidant BHT used as reference. Increased absorbance was understood as increased reducing power and three sample preparations were used for each assay and then averaged.

Chelating effects on the ferrous ion

The chelating effects of ferrous ions for each CSGH fraction (3, 5 and 10 kDa) was determined according to the method described by Dinis *et al.* (1994). Briefly, 500 µl of each of CSGH fraction at different concentrations (0.5, 1.0, 5.0, 10 and 20 mg/ml) were mixed with 1.6 ml of distilled water and 0.05 ml of $FeCl_2$. Reactions were initiated by the addition of 0.1 ml of ferrozine (5 mM) after 15 min, after which each mixture was vigorously shaken and left at room temperature for ten minutes. Absorbance of the Fe^{2+} ferrozine complex was then measured at 562 nm using a UV-Visible spectrophotometer for each tested sample. Chelating antioxidant activity for Fe^{2+} was calculated as follows:

$$\text{Chelating effect (\%)} = \frac{A_{control} - A_{sample} \times 100}{A_{control}}$$

where $A_{control}$ is absorbance of the control reaction;
A_{sample} is absorbance of the tested CSGH fraction.

Butylated hydroxytoluene (BHT) was used as a positive control and was prepared in the same manner except that distilled water was used instead of a trial sample. Each test used three different samples for each assay and results were averaged. IC_{50} values were also determined.

DPPH radical-scavenging activities

The ability of CSGH to scavenge free radicals was analysed by using a synthetic free radical compound [1, 1- diphenyl-2-picrylhydrazyl (DPPH)], according to the method described by Bersuder *et al*. (1998). Approximately 500 µl of each CSGH fraction (3, 5 and 10 kDa) was mixed with 500 µl of 99.5% ethanol and 125 µl (0.02%, w/v) of DPPH. The mixture was shaken vigorously using vortex and then incubated in the dark for 60 min. Absorbance was measured at 517 nm using a UV-Visible spectrophotometer for each fraction's assay with three samples and then averaged. DPPH radical-scavenging activities was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = \frac{A_{control} - A_{sample} \times 100}{A_{control}}$$

where $A_{control}$ is absorbance of the control reaction;
A_{sample} is absorbance of CSGH.

The IC_{50}, defined as the amount of antioxidant necessary to inhibit DPPH radical formation by 50% was also determined. The synthetic antioxidant reagent BHT was used as a positive control. Lower absorbance by the reaction mixture indicated higher DPPH radical-scavenging activity.

Scavenging effects on superoxide anions

The scavenging activity of CSGH toward superoxide anion radicals was determined by following the method of Liu *et al*. (1997). Superoxide anions were generated in a non-enzymatic phenazine-methosulfate-nicotinamide-adenine dinucleotide (PMSNADH) system by reactions with PMS, NADH, and oxygen. The assay measured the reduction of nitroblue tetrazolium (NBT). Superoxide anions were generated in 3 ml of Tris-HCL buffer (100 mM, pH 7.4) containing 750 µl of NBT (300 µM) solution; 750 µl of NADH (936 µM) solution; and 300 µl of different concentrations (25–175 µg/ml) of acetone. Butylated hydroxytoluene (BHT) was used as a positive control. Reaction was initiated by adding 750 µl of PMS (120 µM) to the mixture. After five min of incubation at room temperature, absorbance was measured at 560 nm in a spectrophotometer against a blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percent NBT decolourization of the sample was calculated as follows:

$$\text{Inhibition (\%)} = (A_0 - A_1/A_0) \times 100$$

where A_0 is absorba nce of the negative control;
A_1 is absorbance of the reaction mixture.

IC_{50} for each CSGH sample was calculated and compared with BHT (positive control). As described previously, each antioxidant activity assay was measured for three different samples of each molecular weight fraction and results averaged.

Determination of functional properties

Emulsifying properties

The emulsion activity index (EAI) and the emulsion stability index (ESI) for CSGH was determined according to the method described by Pearce and Kinsella (1978) with slight modification. 5 ml of each CSGH solution (3, 5 and 10 kDa) at a concentration of 10 mg/ml was mixed with 5 ml oil and then homogenized at 18,000 rpm for 1 min in a homogenizer. The emulsion was then centrifuged for 5 min at 1180 rpm to determine the emulsifying activity index (EAI). The height of the emulsifier layer and the total content height were then measured.

$$\text{EAI (\%)} = \frac{\text{Height of emulsified layer} \times 100}{\text{Height of the total content}}$$

To determine the emulsifying stability index (ESI), each sample was homogenized and heated in a water bath at 55°C for 30 minutes. The sample was then centrifuged for 5 min at 2000 rpm after which the two layered emulsion was measured. Again, each assay was measured in triplicate for each molecular fraction and then averaged.

$$\text{ESI (\%)} = \frac{\text{Height of emulsified layer after heating} \times 100}{\text{Height of the total content before heating}}$$

Foaming properties

The foaming capacity index (FCI) for each CSGH sample was measured according to the method

described by Shahidi *et al.* (1995). About 1 g of each CSGH fraction (3, 5 and 10 kDa) was added to 50 ml of distilled water. The solution was homogenised in a 100 ml cylinder at 16,000 rpm (Ultraturrax T25) to incorporate air for 1 minute. Total volume was measured at 0, 0.5, 1, 5, 10, 20 and 30 min after whipping, respectively. The foaming capacity index was expressed as expansion from time 0 min and calculated as follows:

$$\text{Foam capacity index (\%)} = [(A-B)/B)] \text{ X } 100$$

where A is volume after whipping (ml);
B is volume before whipping (ml).

All determinations were taken from a mean of three separate sample measurements for each molecular fraction.

Fat binding capacity

Following the method of Shahidi *et al.* (1995), the fat binding capacity of all three fractionated CSGH hydrolysates was determined by placing 0.5 g of each in a weighted (tube with CSGH) centrifuge tube, then adding 10 ml of palm oil before a 30 s vortex and final centrifugation at 4000 rpm for 25 minutes. The supernatant (upper phase) was removed and fat binding was determined by averaging the difference in weight for three samples of each fraction.

Water binding capacity

Water Binding Capacity was determined by centrifugation as described by Diniz and Martin (1997). About 0.5 g of each CSGH fraction was dissolved in 10 ml of water in centrifuge tubes and then dispersed with a vortex mixer for 30 s. The dispersion was allowed to stand at room temperature for 30 min before centrifuging at 4840 rpm for 25 min. The supernatant was filtered with Whatman No.1 filter paper and the volume recovered was measured. The difference between the initial volume of distilled water added to the CSGH and the volume of the supernatant was determined. Results were reported as ml of water absorbed per gram of CSGH.

Results and Discussion

Extraction of cobia skin gelatin

The yield of extracted cobia skin gelatin was 12.33%, a value that agreed with Karim and Bhat (2009) whose yield of extracted fish gelatin ranged from 6–19%. Gelatin yields have been reported with wide ranging variance among fish species, mainly due to differences in collagen content and skin and skin matrix compositions (Jongjareonrak *et al.*, 2010). The yield of cobia skin gelatin obtained was similar to yields from sin croaker (12.3%) (Cheow *et al*., 2007); and higher than yields reported for bigeye snapper skin (4%) (Binsi *et al.*, 2007); shortfin scad (7.25%) (Cheow *et al*., 2007); rainbow trout (9.36%) (Tabarestani *et al.*, 2010); and striped catfish (11.17%) (Jamilah *et al*., 2011). However, it was lower than those reported for off-armed giant catfish (20.1%) (Jongjareonrak *et al.*, 2010); and grass carp (19.83%) (Kasankala *et al.*, 2007). Higher yield is most likely due to the higher collagen content in the skin of cobia, while lower yields may be due to collagen leaching from skin and gelatin degradation during extraction (Jamilah and Harvinder, 2002).

Cobia skin gelatin hydrolysate (CSGH)

The cobia skin gelatin hydrolysates (CSGH) prepared were fractionated by ultrafiltration (10, 5 and 3 kDa) yielding 21.21%, 13.82% and 5.94%, respectively per fraction. These yields decreased per drop in molecular weight with the lowest yield (5.94% for the 3 kDa fraction) due to the smallest peptide produced and losses during filtration. Furthermore, various other parameters significantly affect yield during the process of hydrolysis (Zhang *et al.*, 2012). Lin *et al.* (2012) reported yields for squid skin gelatin fractions (6-10 kDa, 2-6 kDa, <2 kDa) of 15.7%, 12% and 14.7%, respectively. Differences in yield depend on peptide chains, pre-treatment, extraction time, temperature and pH during the process of hydrolysis. By way of contrast, yields obtained for rapeseed protein hydrolysate fractions (5-10 kDa, 3-5 kDa and 1-3 kDa) were 10.46%, 14.42% and 21.81%, respectively, and yet were increased with decreasing molecular weight fractions. A study by Lee *et al.,* (2012), found that the yield for duck skin gelatin hydrolysate from alkaline pre-treatment was higher (98.3%) than that obtained from acid pre-treatment (95.38%).

Antioxidant activities

Due to the variety of oxidation processes and reactions, the use of a single method to determine the antioxidant activity of gelatin hydrolysate cannot provide a clear idea of its antioxidant potential. Therefore, this activity was assessed via different analyses involving reducing power, chelating effects on ferrous ion, DPPH radical antioxidative activity, and superoxide anion scavenging.

Reducing power

Potassium ferricyanide is widely used to measure the reducing power of a hydrolysate fraction's antioxidant activity for which higher absorbance indicates higher reducing power. Figure 1 presents the trend observed for different molecular weights and concentrations of CSGH absorbance values. Reducing power for all CSGH fractions increased with increased concentration (0.5 to 20 mg/ml).

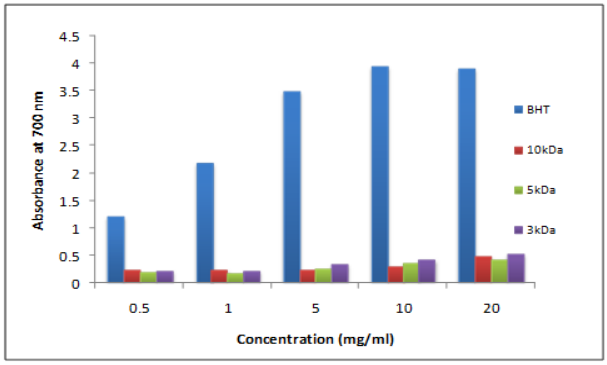

Figure 1. Reducing power for different CSGH fractions (10, 5 and 3 kDa) and BHT at different concentrations (0.5–20 mg/ml)

The reducing power for the 3 kDa fraction was higher in absorbance than that of 5 kDa and 10 kDa, which had increased from 0.22 nm to 0.44 nm, respectively, at concentrations of 0.5 and 20 mg/ml. However, all hydrolysate fractions showed lower reducing power than did BHT for all concentrations. A previous study by Nazeer and Anila Kulandai (2012) found that the reducing power of muscle and skin protein hydrolysate from the giant kingfish increased (0.05 to 0.17 nm) with increased concentrations (1 to 5 mg/ml). The graph also shows a significant difference ($p < 0.05$) for all CSGH fractions (10, 5 and 3 kDa) from the positive control, BHT. However, no significant differences ($p > 0.05$) were observed between CSGH fractions, although we recorded slight increases in reducing power for 5 and 3 kDa fractions. The highest reducing power accordingly increased due to the length of the sample's peptide content indicating that lower peptide chains had higher electron reduction potential. The present study's results also agreed with those of Qin *et al.* (2011) who found that lower molecular weight (1-3 kDa) purple sea urchin gelatin hydrosylate had higher reducing power (0.562%) than did 5–10 kDa fractions (0.48%).

Chelating effect on the ferrous ion

Results for CSGH scavenging of the ferrous ion for each fraction (10, 5 and 3 kDa) and also for BHT (1 mg/ml) are shown in Figure 2. At 5 kDa, CSGH exhibited the highest percentage of ferrous chelating activity (90.69%) and was significantly different

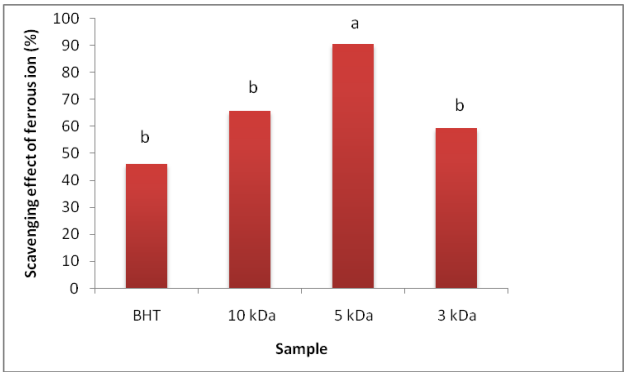

Figure 2. Scavenging effect on ferrous ion (%) for different CSGH fractions and BHT solution (concentration: 1 mg/ml)

($p < 0.05$) from BHT (45.96%). The chelation reaction begins when ferrozine quantitatively forms a complex with Fe^{2+} (Ktari *et al.*, 2012). A study by Taheri, *et al.* (2013) of different salted herring brine hydrolysate fractions showed that lower molecular weight increased chelating activity more than higher molecular weight. Furthermore, we recorded IC_{50} for 10 kDa, 5 kDa and 3 kDa CSGH fractions at 0.86 mg/ml, 1.73 mg/ml and 2.01 mg/ml, respectively. These results demonstrated that higher molecular weight CSGH had greater ability to chelate the ferrous ion, thus agreeing with He *et al.* (2012) on the IC_{50} of rapeseed protein hydrolysate, who reported that higher molecular weight (5-10 kDa) fractions exhibited stronger chelating capacity while lower weight fractions (<3 kDa) showed weaker Fe^{2+} chelating activity.

DPPH radical-scavenging activities

The analysis of DPPH radical-scavenging for each CSGH fraction (10, 5 and 3 kDa) was conducted using BHT as the positive control. Cobia skin gelatin hydrolysate (CSGH) inhibition of the DPPH radical increased with higher concentrations of both CSGH trial fractions and BHT (data not shown). Figure 3 presents a comparison of DPPH inhibition for each CSGH fraction with BHT (5 mg/ml).

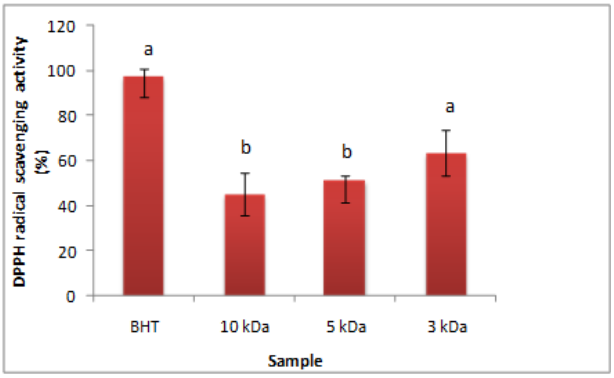

Figure 3. DPPH radical scavenging activity for different CSGH fractions (10, 5 and 3 kDa) and BHT solution (concentration: 5 mg/ml)

The 3KDa fraction had the highest level of DPPH radical inhibition of all three CSGH fractions, yet BHT presented the highest DPPH radical inhibition for all tested samples. The IC_{50} value is widely used to measure antiradical efficiency as it represents the hydrolysate concentration required to inhibit 50% of the free radical (DPPH). IC_{50} results for the 10 kDa, 5 kDa and 3 kDa fractions were 4.42, 3.71 and 3.58 mg/ml, respectively. Thus, the 3kDa fraction had higher scavenging activity compared to other fractions was also associated with a lower IC_{50} value (Sun *et al.*, 2011). This finding agreed with He *et al.* (2012) who reported an IC_{50} value for rapeseed protein hydrolysate (<1 kDa peptide fractions) of 0.45–0.6 mg/ml. Results obtained demonstrated that CSGH contains hydrogen donors that react with free radicals and converts them to more stable products and thus, terminate radical chain reactions. Nevertheless, BHT remained the better DPPH radical scavenger.

Scavenging effects on superoxide anion

Superoxide anions are the most common of in vivo free radicals generated in a variety of biological systems, either by autoxidation or via enzymatic activity (Lee *et al.*, 2001). At a concentration of 1 mg/ml for each solution tested, Figure 4 shows percentages for CSGH superoxide anion scavenging effects for each fraction with BHT as the positive control.

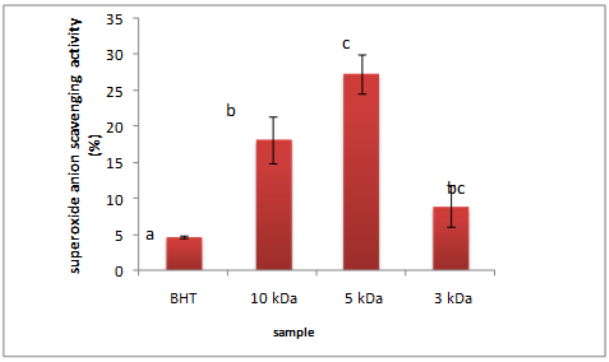

Figure 4. Superoxide anion scavenging activity for different CSGH fractions (10, 5 and 3 kDa) and BHT solution (concentration: 1 mg/ml)

Surprisingly, the 5 kDa fraction showed the highest percentage of activity (27.31%) and was significantly different ($p < 0.05$) from both BHT (4.66%) and10 kDa (18.14%). All three CSGH fractions showed higher superoxide radical scavenging activity than did BHT. The 3 kDa fraction had the lowest scavenging effect of all fractions, likely due to changes in amino acids composition during the hydrolysis process. Chi *et al.* (2013) reported strong antioxidant activity in the presence of peptide sequences containing Tyrosine, Proline, Methionin, Histidine, Lysine and Tyrosine.

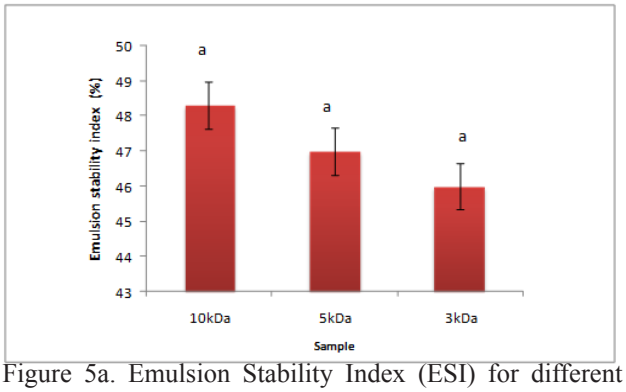

Figure 5a. Emulsion Stability Index (ESI) for different CSGH fractions (10, 5 and 3 kDa). Results presented as means ± SD

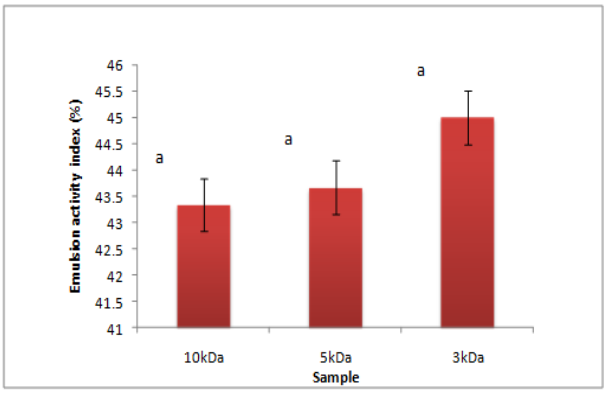

Figure 5b. Emulsion Activity Index (EAI) for different CSGH fractions (10, 5 and 3 kDa). Results presented as means ± SD

By way of contrast, a study by Onuh *et al.* (2013), found no significance differences in superoxide anion scavenging for different molecular weights of chicken skin protein hydrolysate (20.13, 22.31 and 21.06%) for 10, 5 and 3 kDa molecular fractions, respectively.

Emulsifying properties

The emulsion stability indexes (ESI) for CSGH fractions are shown in Figure 5a. There was no significant differences ($p > 0.05$) and the ESI for all samples decreased as the molecular weight of the hydrolysate decreased. This finding may arise from a decrease in peptide size as a result of fractionation in agreement with Taheri (2013), who reported the ESI for herring brain fish hydrolysate decreased at lower molecular weights because of reduced peptide size. In order to exhibit better emulsifying activity, proteins and/or peptides must migrate rapidly to the water/oil interface where they quickly unfold and rearrange the interface (Kotlar *et al.*, 2013). Although peptides with lower molecular weights can migrate rapidly to the interface, the hydrophobic/hydrophilic balance of smaller peptides is insufficient for the stabilization of emulsions (Deng *et al.*, 2011). Hence, smaller molecular weight hydrolysate fractions result

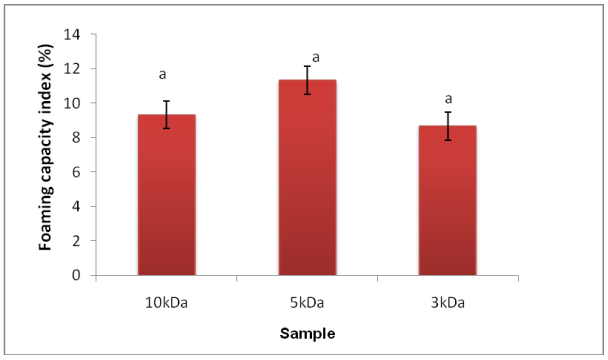

Figure 6. Foaming Capacity Index (FSI) for different CSGH fractions (10, 5 and 3 kDa). Results presented as means ± SD

in less emulsion stability due to a reduction in the hydrophobic/hydrophilic balance.

Similarly, the emulsion activity index (EAI) for all three CSGH fractions (Figure 6b) was also determined and observed no significant differences ($p > 0.05$) (43, 43.7, and 45%), for 10 kDa, 5 kDa and 3 kDa, respectively. Emulsion activity index (EAI) increased as fractional molecular weight decreased and the hydrolysis process appeared to improve the EAI of CSGH (from 43 to 45%) for both 10kDa to 3 kDa fractions. In conclusion, the 3kDa fraction had higher surface hydrophobicity. Partial hydrolysis alters the protein structure and function of the hydrolysate resulting in higher solubility and smaller molecular size which may then facilitate diffusion at the oil/water interface. Thus, exposed hydrophobic groups enhance interactions between proteins and lipids (Wu *et al.*, 1998).

Foaming capacity index

Foam formation requires the ability of a protein to be quickly adsorbed at the water/air interface, thereby lowering surface tension. Figure 6 shows the Foam Capacity Index for all three CSGH fractions. Of these, fraction 5 kDa had the highest index. Nevertheless, there was no significant differences ($p > 0.05$) in indexes for all fractions, which were 9.33, 2.31 and 6.43%, respectively. Smaller CSGH peptides allowed for quick adsorption at the air/water interface, thus lowering surface tension. However, the 3 kDa fraction's smaller peptides could not maintain a well-ordered molecular orientation at the interface due to hydrolysis and filtering, its peptide sequence was shorter. Hence, the adsorption rate, together with the ability to unfold and reorient at the interface, remain the most important factors for foam formation as also reported by Muhamyankaka *et al.* (2013). Foam stability chiefly depends on the extent of protein–protein interactions within the matrix of films surrounding air bubbles as well as protein flexibility and structure (Tsumura *et al*., 2005). Thus, the 5 kDa fraction exhibited superior foam stability, largely because it contained larger peptides than did 3 kDa, which then formed flexible films around air bubbles as evidenced in its higher foam stability index.

Fat binding capacity

Figure 7 presents percentages for the fat binding capacity of the tested CSGH fractions.

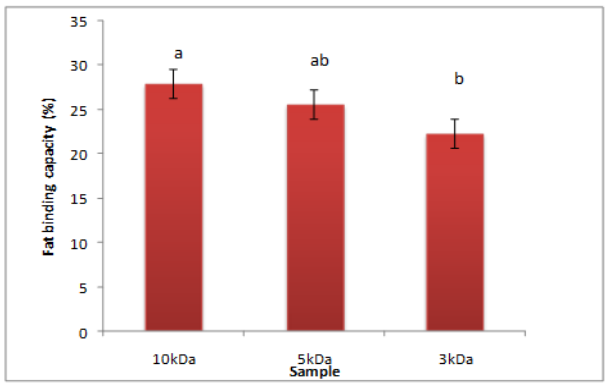

Figure 7. Fat Binding Capacity for different CSGH fractions (10, 5 and 3 kDa). Results presented as means ± SD

The 10 kDa fraction had the highest fat binding capacity (28%) compared to 5 kDa (25.67%) and 3 kDa (22.33%) ($p < 0.05$), and no significant difference between 5 kDa and 3 kDa fractions. These findings indicated that CSGH fat binding capacity decreased with lower molecular weights of the hydrolysate. The 3 kDa fraction had the lowest fat binding capacity which was likely due to the hydrolytic degradation of the CSGH structure. Fat binding capacity is mainly related to the degree of exposure of hydrophobic residues within CSGH so that hydrolysates containing larger amounts of lipids absorb more fat due to a positive relationship between fat absorption and the amount of phospholipids in the sample. A study by Wasswa *et al.* (2007) reported that lower degree hydrolysis yielded higher fat binding capacity (5.02%) for grass carp skin protein hydrolysate. Several factors have been reported that may affect the ability of hydrolysates to bind fat such as the bulk density of the protein (Jing, 1976); the degree of hydrolysis (Sorgentini and Wagner, 2002); and enzyme–substrate specificity (Chobert, 1988). These variables may well affect the unfolding of the protein structure, for example, as well as the degree of hydrophobic groups' exposure that allow for the physical entrapment of oil.

Water binding capacity

Figure 8 shows percentages for the water binding capacity of the CSGH fractions tested. We observed

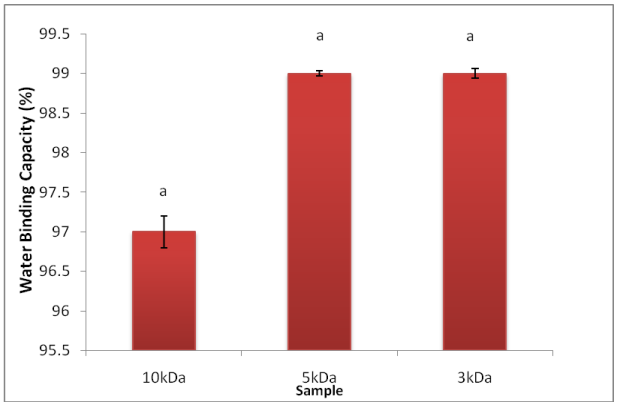

Figure 8. Water Binding Capacity for different CSGH fractions (10, 5 and 3 kDa). Results presented as means ± SD

no significant differences between 10, 5 and 3 kDa at 97%, 99% and 99%, respectively. These results indicated that smaller molecular weight hydrolysate fractions had higher water binding capacity. The greater water binding capacity was probably due to lower molecular weight peptides resulting from additional enzymatic hydrolysis as smaller peptide fragments have higher hydrophilic functional groups (Cumby *et al*., 2007). Kristinsson and Rasco (2000b) stated that fish protein hydrolysates had better water holding capacity and were more beneficial in certain food formulations. The trend for water binding capacity in this study was also similar to that observed for grass carp skin hydrolysates by Wasswa *et al.* (2007) where samples increased water binding capacity per degree of hydrolysis for the extracted gel. However, intrinsic factors affecting water binding in food proteins also include amino acid composition, protein conformation and surface hydrophobicity/ polarity (Barbut, 1999).

Conclusion

In conclusion, the molecular weight of CSGH fractions plays a significant role in antioxidant activity, with lower molecular weight fractions possessing higher antioxidant activity. These activities include the chelation of ferrous ion and superoxide anion scavenging, although BHT (positive control) showed the highest antioxidant activity overall. However, this study establishes that the lower molecular weight of smaller fractions does not necessarily influence the functional properties of CSGH.

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