Int Aquat Res (2020) 12:116-126
https://doi.org/10.22034/IAR(20).2020.1892203.1006

ORIGINAL RESEARCH

Characterization of antioxidant and surface-active properties of gelatin hydrolysates obtained from croaker fish skin

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Received: 25 November 2019 / Accepted: 6 April 2020 / Published online: 16 May 2020

Abstract In this study, antioxidant and surface-active properties of gelatin hydrolysates (GH) were mainly evaluated. Gelatin obtained from croaker fish (*Johnius dussumieri*) skin was hydrolysed by the application of visceral proteases extracted from gastrointestinal (GI) tract of two freshwater fish species rohu (*Labeo rohita*) and catla (*Catla catla*). The resultant GH was designated as 'gelatin hydrolysate-rohu protease' (GH-RP) and 'gelatin hydrolysate-catla protease' (GH-CP). Surface-active properties (foaming and emulsion) of GH-RP were significantly higher than GH-CP at concentrations studied ($P < 0.05$). The GH obtained by enzymatic hydrolysis treatment was fractionated using ultrafiltration and a total number of five fractions were collected unfractionated (UNF), >10 kDa, 5-10 kDa, 3-5 kDa, 1-3 kDa and <1 kDa. The IC_{50} value of DPPH radical scavenging activity of fraction 1-3 kDa from GH-RP and fraction <1 kDa from GH-CP showed the value of 0.55 mg/ml and 0.56 mg/ml respectively. The ferric reducing power assay results indicate that fraction from <1 kDa from GH-RP and GH-CP showed a significantly higher value than the other fractions ($P < 0.05$). The fractions of 1-3 kDa from GH-RP and <1 kDa from GH-CP showed the highest ABTS radical scavenging activity of 594.84 and 558.46 Trolox µM/mg respectively. Among all, the fractions <1 kDa from GH-RP and 3-5 kDa from GH-CP had shown higher antioxidant activity determined using β-carotene – linoleic acid model system. The results of the present study indicate that the peptides have good antioxidant and functional properties.

Keywords Croaker fish skin gelatin hydrolysates (CFS-GH) . Antioxidant activity . Foaming and emulsion properties

Introduction

With the increase in rapid demand for fish and fishery product, the supply side is becoming a limiting factor. Global fish production during the year 2018 was estimated to be 170.9 million tons (M.T) (FAO 2018). The perishable nature of fish has compounded the problem in the supply of nutritious and safe fish. Capture fish and production from the marine sector has almost become stagnant and contributes about 93.4 M.T. However, the fish production from culture practices both in marine and freshwater has shown rapid development and contributes more than 56% of total fish production (Idowu et al. 2019). The perishable nature of fish and shellfish coupled within sufficient infrastructure to handle the catch has led to huge post-harvest losses.

Processing leads to the generation of a large biomass of fish waste (e.g., heads, skin, viscera, bones, fins and scales) which is generally discarded (Karim and Bhat 2009). The digestive tract or the viscera constitutes 5–8% of the fish weight. This generated processing waste without the appropriate treatment or management leads to pollution and disposal problems. Nevertheless, this contains notable quantities of high

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value protein and essential amino acids (Karoud et al. 2019). Hence, there is a need to develop effective post harvesting technologies to minimize post harvest losses and environmental pollution, and at the same time evolves technologies for efficient utilization of fish processing waste. It will be of interest to prepare gelatin and gelatin hydrolysates (GH) from fish processing waste like skin using visceral enzymes. The advantages of promoting GH production will enable utilization of fish processing waste into a high-value product.

Gelatin is derived from the fibrous protein collagen, which is the principal constituent of animal skin, bone, and connective tissue. Gelatin possesses a characteristic melt-in-the-mouth property that makes it useful in a wide range of applications in the food and pharmaceutical industries. It has many applications including thickening, water holding, colloid stabilization, crystallization control, film formation, whipping and emulsification (Gómez-Guillén et al. 2010).

The visceral mass comprising of liver, pancreas, pyloric-cecae, and stomach can be a source of different enzymes, especially proteolytic enzymes. The application of proteolytic enzymes can be in the detergent industry, food processing sector or in the production of protein hydrolysates. The proteolytic enzymes associated with visceral mass include pepsin, trypsin, chymotrypsin, and pancreatin (Kristinsson and Rasco 2000).

Enzymatic hydrolysis is one of the most easily controlled techniques for producing bioactive peptides which improves the functional and biological properties of proteins. Hydrolysis breaks proteins down into smaller peptide chains containing 2-20 amino acids called 'bioactive peptides', producing an amino acid source for various physiological functions in the human body (Halim et al. 2016). A growing body of scientific evidence shows that bioactive peptides and protein hydrolysates from different proteins including fish and fish processing waste may promote human health. Depending on the amino acid sequence of peptides, they may be involved in various biological functions such as antioxidant, antihypertensive, antimicrobial, anticancer, antiproliferative and immunomodulatory properties with promising health benefits in terms of nutritional or pharmaceutical properties (Da Rocha et al. 2018). The nature of proteolytic enzymes used for the production of protein hydrolysates will determine its bioactive properties.

Over the decades, several researchers have suggested that bioactive peptides from marine sources could be promising functional components for pharmaceuticals, nutraceuticals and food (Chalamaiah et al. 2012). The hydrolysate prepared from gelatin (obtained from fish processing waste or/and bovine and porcine sources) will be an ideal substrate to prepare the gelatin hydrolysate (GH). The utilization of fish processing waste especially skins and visceral mass for the production of gelatin hydrolysate (GH) has received attention. In the present investigation, the fish processing waste like skin and visceral mass were made use to prepare gelatin hydrolysate (GH). The gelatin was obtained from the skin of croaker fish (*Johnius dussumieri*). The prepared gelatin was hydrolysed using the crude visceral proteases from gastrointestinal (GI) tract of two freshwater fish namely rohu (*Labeo rohita*) and catla (*Catla catla*). The resultant gelatin hydrolysates were fractionated using Ultrafiltration. Antioxidant and surface-active properties (foaming and emulsifying) of fractionated hydrolysates have been assessed.

Materials and methods

Chemicals

2,2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS), 2, 2 Diphenyl-1-picrylhydrazyl (DPPH), iron (II) chloride, linoleic acid, β –Carotene, potassium persulfate, tween-80. All other chemicals and reagents used in the present study were either analytical grade reagent (AR) or guaranteed grade reagent (GR).

Fish

Two species of Indian major carps (IMC) *Catla catla, Labeo rohita* and marine fish croaker (*Johnius dussumieri*) were used in the present study. The fish were purchased from KFDC, Mangalore, Karnataka, India in iced condition, in the ratio of 1:1 (fish: ice) and transported to the laboratory.

Preparation of gelatin hydrolysates (GH)

Gelatin hydrolysates (GH) was prepared by using gelatin extracted from the skin of croaker (*Johnius dussumieri*) by the application of proteolytic enzymes extracted from gastrointestinal (GI) tract of two

freshwater fish species rohu (*Labeo rohita*) and catla (*Catla catla*). Gelatin was extracted from the skin of croaker fish (*Johnius dussumieri*) as described by the following method of Kumar et al. (2017). Proteolytic enzyme extract was prepared from two freshwater fish species rohu (*Labeo rohita*) and catla (*Catla catla*), according to the method as described by Phanturat et al. (2010), with slight modification.

The gelatin powder was dissolved in distilled water at 2.5% (w/v), adjusted to the appropriate pH and temperature of each enzyme catla proteases (10.5; 60 ˚C) and rohu proteases (8.5; 60 ˚C) with 1 M NaOH or 1 M HCl. Crude enzyme was added to the mixture at enzyme/substrate ratio of 10 U/mg and incubated for 30 min. Enzymatic hydrolysis was stopped by heating the solutions at 90 ˚C for 10 min to inactivate enzymes. The mixture was centrifuged at 5000 ×g for 10 min to separate soluble and insoluble fractions. Finally, the soluble fractions were freeze-dried and referred to as GH-RP (gelatin hydrolysate-rohu protease) and GH-CP (gelatin hydrolysate- catla protease). Under optimised hydrolysis conditions, degree of hydrolysis (DH) % for catla proteases was found to be 28.48%, whereas for rohu proteases 31.07%, respectively (data not shown).

Yield and proximate composition of gelatin hydrolysates

The yield of gelatin hydrolysates extracted was calculated and expressed as follows:

$$\text{Yield}(\%) = \frac{\text{Weight of freezedried hydrolysate}(\text{g})}{\text{Weight of the dried skin}(\text{g})} \text{ X100}$$

Proximate composition of skin and gelatin were analyzed by measuring moisture, ash, protein and fat contents according to AOAC official methods (AOAC 2005).

Surface-active properties of gelatin hydrolysates (GH)

Foaming properties

The foaming properties include foaming capacity and foam stability. The foaming properties were determined according to the method as described by Sathe and Salunkhe (1981). The protein concentration used for GH was (0.1%, 0.5% and 1%). Different concentrations protein (GH) solution (20 ml) were whipped at a speed of 13,500 rpm using Ultra Turrax homogenizer (Ultra Turrax, T 25, Janke & Kunkel GMBH & Co., KG Staufen, Germany) to incorporate the air for 2 min at room temperature. The whipped sample was immediately transferred into a 100 ml measuring cylinder and volume was recorded. The foaming capacity was calculated using the following formula:

$$\text{Foaming capacity} = \frac{(\text{Volume after whipping}) - (\text{volume before whipping})}{\text{Volume before whipping}} \text{ X100}$$

The whipped sample was allowed to stand at 20 ˚C for 30 min and the volume of whipped sample was then recorded. Foam stability was calculated as follows:

$$\text{Foaming stability} = \frac{(\text{Volume after standing}) - (\text{volume before whipping})}{\text{Volume before whipping}} \text{ X100}$$

Emulsion properties

The emulsion properties measured include emulsion activity index (EAI) and emulsion stability index (ESI). The emulsion properties were determined according to thmethod as described by Pearce and Kinsella (1978). The protein concentration used for GH was 0.1%, 0.5% and 1%. Different concentrations of protein (GH) solution (30 ml) and refined sunflower oil (10 ml) was mixed and homogenized in the Ultra Turrax homogenizer at 20,500 rpm for 1 min. An aliquot of the emulsion (50 µl) was pipetted out from the bottom of the container at 0 and after 10 min and mixed with 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the solution was measured at 500 nm using double beam spectrophotometer. The absorbance was measured immediately 0 min (A_0) and after 10 min (A_{10}) of emulsion formation. The readings were used to calculate the emulsion activity index (EAI) and emulsion stability index (ESI) as follows:

$$\text{EAI} = 2\text{T } \Phi\text{C}$$

Where, T - turbidity ($T = 2.303A_{500} / l$; A_{500} is absorbance at 500 nm; l is path length); ϕ - oil volume fraction (0.25); C - protein concentration.

$$EAI\left(m^2 / g\right) = \frac{2 \text{ x} 2.303 \text{ x } Abs_{500nm}}{0.25 \text{ x gelatin weight}\left(g\right)}$$

$$ESI \text{ (min)} = \frac{A_0 x \Delta t}{\Delta A}$$

Where, ΔA = Absorbance at 0 min (A_0) – Absorbance after 10 min (A_{10}) and Δt = 10 min

Ultrafiltration

The gelatin hydrolysates were fractionated based on molecular weight using an Ultrafiltration system (GE, Watson Marlow, Quixstand Benchtop System, Sweden). Ultrafiltration system contains peristaltic recirculation pump, the reservoir (1 L capacity) and stainless-steel system stand. Ultrafiltration hollow fiber cartridges (30 cm L) polysulfone membrane with a different range of NWCO (Nominal molecular weight cut-off (10 kDa NWCO), 5 kDa NWCO, 3 kDa NWCO and 1 kDa) was used for the fractionation of GH. The chamber was pressurized during the filtration process. Trans-membrane pressure (TMP) depends on the membrane specifically. The TMP maintained for 10 kDa NWCO, 5 kDa NWCO, 3 kDa NWCO and 1 kDa membranes was 25 psig, 20 psig, 15 psig and 10 psig. A known volume of hydrolysate solution (10 mg/ml) was passed through 10 kDa NWCO membrane where two fractions were collected; retentate (>10 kDa) and permeate (5-10 kDa). The permeate (5-10 kDa) was passed through 5 kDa NWCO membrane to collect permeate (3-5 kDa). This was repeated serially for, 3 kDa NWCO and 1 kDa NWCO membranes to obtain retentate fractions, 1-3 kDa and <1 kDa. The total number of fractions obtained include unfractionated (UNF), >10 kDa, 5-10 kDa, 3-5 kDa, 1-3 kDa and <1 kDa.

Antioxidant properties of gelatin hydrolysates (GH)
Diphenyl-1-picryl hydrazyl activity (DPPH) assay

The DPPH free radical scavenging activities of fractionated and un-fractionated samples were determined according to the method described by Yen and Wu (1999). A 1.5 ml of GH fractionated solution was added to 1.5 ml of 0.1 mM DPPH in 99.50% ethanol and mixed thoroughly by vortex using cyclo - mixer at high speed. The solution was incubated at room temperature in dark for 30 min. The absorbance was measured at 517 nm using double beam spectrophotometer. Lower the absorbance of the reaction mixture indicated higher free radical scavenging activity. DPPH radical scavenging activity was calculated as:

$$\text{DPPH free radical scavenging activity}\left(\%\right) = 1 - \frac{Abs_{Sample}}{Abs_{Control}} \text{ x100}$$

The IC_{50} value was defined as an inhibitory concentration of the sample at which 50% of activity is inhibited.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing power was determined by the method as described by Oyaizu (1986). An aliquot of 1 ml GH fractionated sample was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium ferri-cyanide. The mixture was incubated at 50 ˚C for 30 min and the reaction was stopped by addition of 2.5 ml of 10% (w/v) trichloroacetic acid. Finally, 2.5 ml solution from the mixture was drawn and mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride solution. The solution was incubated for 10 min and absorbance was measured at 700 nm using double beam spectrophotometer. The Higher absorbance of the reaction mixture indicated higher reducing power.

ABTS [2, 2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid)] radical scavenging activity

For ABTS activity assay, the method as described by Re et al. (1999) with some modifications. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 50 ml methanol to obtain an absorbance of 0.70 ± 0.023 at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. The GH fractions (150 µl) were allowed to react with 2850 µl of the ABTS solution for 2 h in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. ABTS radical scavenging activity was calculated as:

$$\text{ABTS radical scavenging activty}(\%) = 1 - \frac{\text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \text{ x100}$$

β-Carotene - Linoleic acid bleaching model system

β-Carotene- Linoleic acid bleaching assay was performed according to the method as described by Koleva et al. (2002). A stock solution of β-carotene/linoleic acid mixture was prepared by dissolving 0.5 mg of β-carotene, 20 µl of linoleic acid and 200 µl of tween-80 in 1 ml of chloroform. The mixture was kept at 40 ˚C till chloroform is evaporated and then 100 ml of double-distilled water was added. The resulting mixture was vigorously stirred to form an emulsion. Aliquots of (2.5 ml) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 ml of hydrolysate solution. After one-hour incubation at 50 ˚C, the absorbance of each sample was measured at 470 nm. The tube without sample used as control.

$$\text{Antioxidant activty}(\%) = 1 - \frac{\text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \text{ x100}$$

Statistical analysis

One-way ANOVA was used to analyse the data. The experiments were carried out in triplicates independently. Significant difference between the means of triplicates was determined by Duncan's multiple comparison test using statistical software IBM SPSS.2 (SPSS Inc, Illinois, USA).

Results

The protein content of GH-RP (78.23%) was marginally higher than that of GH-CP (76.86%). The high protein content of gelatin hydrolysates demonstrates its potential use as protein supplements for human nutrition. The moisture content GH-RP and GH-CP were found to be 10.06 ± 0.04 and 9.34 ± 0.16 respectively (Table 1). The fat content of two GH samples was less than 1%. It is likely that the process used for GH had no effect on fat content on the final product. The ash content of GH-RP accounted for 9.62%, while that of GH-CP is around 11%.

Foaming capacity and stability of GH-RP and GH-CP samples at different concentrations are given in Fig. 1. The foaming capacity of GH-RP and GH-CP at a concentration of 1% was found to be higher as compared to other two concentrations. The foaming stability of these hydrolysates was found to be higher at 1% concentration than at 0.5 and 1%. The foaming capacity of GH-RP was significantly higher than GH-CP at concentrations studied ($P < 0.05$). Similarly, the foaming stability of GH-RP was significantly higher than GH-CP at concentrations studied ($P < 0.05$). The results indicated both foaming capacity and foaming stability concentration dependent.

Table 1 Proximate composition of gelatin hydrolysates (Mean ± Standard deviation values from triplicates)

Proximate composition (%)	GH-RP	GH-CP
Yield	31.78 ± 0.06	38.98 ± 0.97
Crude protein	78.23 ± 0.63	76.86 ± 0.51
Moisture	10.06 ± 0.04	9.34 ± 0.16
Fat	0.81 ± 0.01	0.58 ± 0.02
Ash	9.62 ± 0.13	11.01±0.39

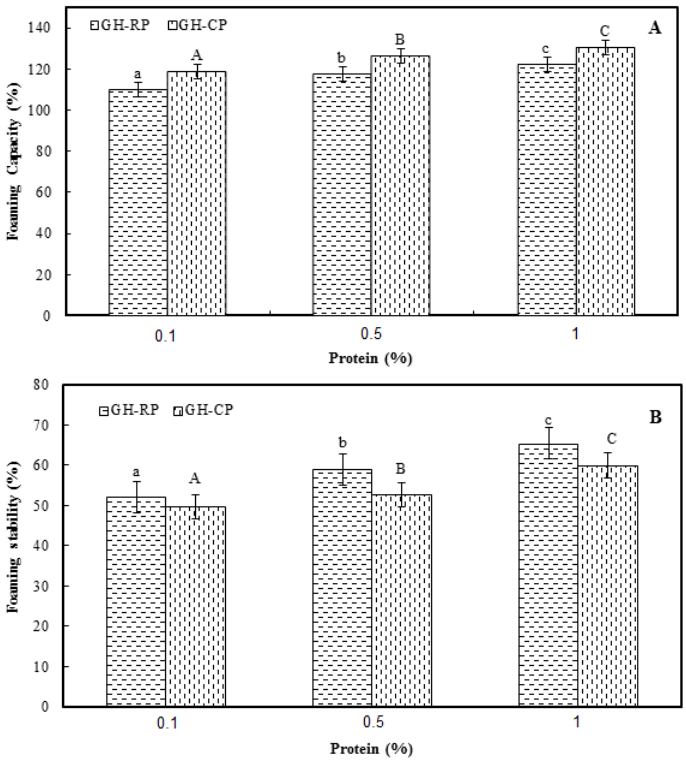

Fig. 1 Foaming properties of GH-RP and GH-CP hydrolysate samples (A) Foaming capacity (B) Foaming stability. Different capital and small letters on the error bars indicate that the results are significantly different ($P < 0.05$).

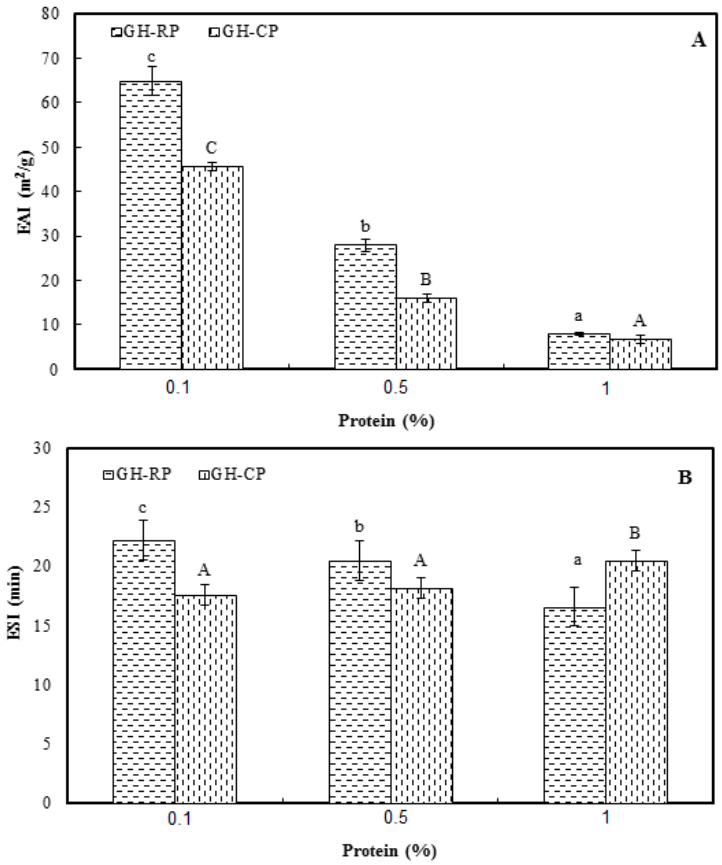

Fig. 2 Emulsion properties of GH-RP and GH-CP hydrolysate samples (A) Emulsion activity index (EAI) (B) Emulsion stability index (ESI). Different capital and small letters on the error bars indicate that the results are significantly different ($P < 0.05$).

Emulsion activity index (EAI) and emulsion stability index (ESI) of GH-RP and GH-CP as a function of concentration is shown in Fig. 2. A higher EAI and ESI were recorded at a concentration of 0.1% in GH-RP samples. The EAI of GH-CP samples were higher at a protein concentration of 0.1% while ESI was higher at 1%. There was a significant difference in EAI values between GH-RP and GH-CP samples at all concentrations studied ($P < 0.05$). This indicates that GH-RP had higher EAI properties than GH-CP samples. The ESI of GH-CP samples were different from that of GH-RP samples. A higher ESI value was recorded at a concentration of 1%. It should be pointed out that GH-CP samples showed a lower value of ESI than that of GH-RP at any given concentration.

The DPPH radical scavenging ability of different fractions from GH-RP and GH-CP as a function of protein concentration has been assayed. The beneficial effects of bioactive peptides are well known in scavenging free radicals and reactive oxygen species or in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Harnedy and FitzGerald 2012). The IC_{50} value of DPPH scavenging activity of fraction 1-3 kDa from GH-RP and fraction <1 kDa from GH-CP showed the value of 0.55 mg/ml and 0.56 mg/ml respectively which was not significantly different ($P > 0.05$) (Fig. 3).

The ferric reducing power assay of different fractions is presented in Fig. 4. The results indicate that fraction from <1 kDa from GH-RP and GH-CP showed a significantly higher value than the other fractions ($P < 0.05$). It has been reported that there exists a direct correlation between antioxidant activity and reducing power. Samples with higher reducing power have better abilities to donate electrons. FRAP is usually applied to quantify the ability of compound to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex (Binsan et al. 2008). Free radicals form stable substances by accepting donated electron and the free radical chain reactions are thus interrupted (Yen and Chen 1995).

ABTS assay is generally used for both lipophilic and hydrophilic compounds and this assay is based on

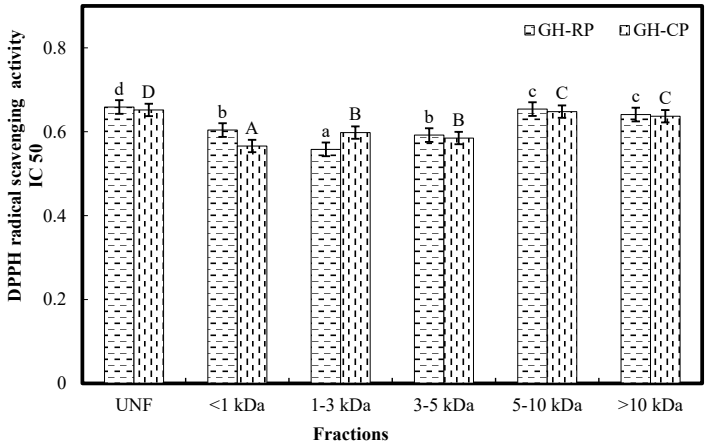

Fig. 3 Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity of GH-RP and GH-CP hydrolysate fractions. Different capital and small letters on the error bars indicate that the results are significantly different ($P < 0.05$).

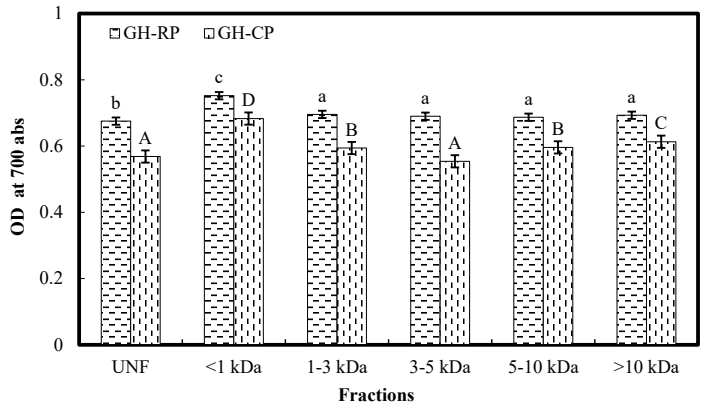

Fig. 4 Ferric reducing antioxidant power (FRAP) assay of GH-RP and GH-CP hydrolysate fractions. Different capital and small letters on the error bars indicate that the results are significantly different ($P < 0.05$).

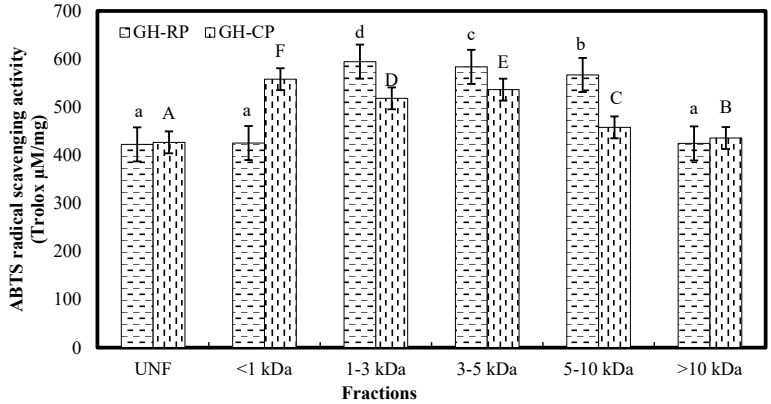

Fig. 5 ABTS radical scavenging activity of GH-RP and GH-CP hydrolysate fractions. Different capital and small letters on the error bars indicate that the results are significantly different ($P < 0.05$).

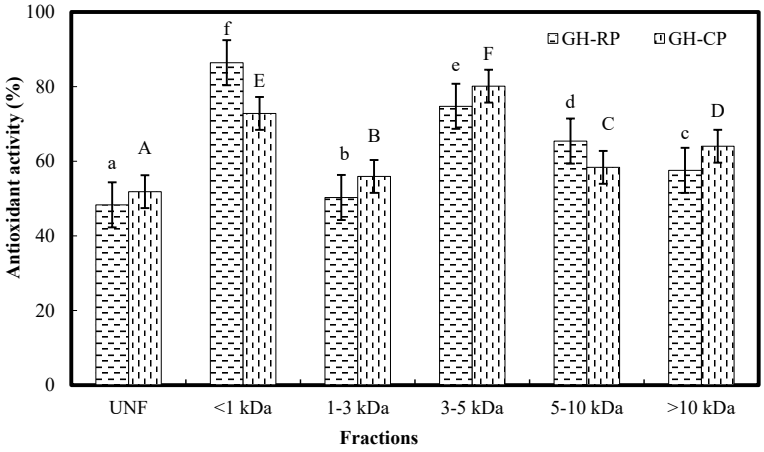

Fig. 6 β-carotene- Linoleic acid bleaching model system of GH-RP and GH-CP hydrolysate fractions. Different capital and small letters on the error bars indicate that the results are significantly different ($P < 0.05$).

the ability of antioxidants to inhibit the oxidation reactions of ABTS (Re et al. 1999). The ABTS radical scavenging ability was expressed in µM of Trolox/mg of sample (Fig. 5). The fractions of 1-3 kDa from GH-RP and <1 kDa from GH-CP showed the highest scavenging activity of 594.84 and 558.46 Trolox µM/mg respectively. ABTS activity is widely used to determine antioxidant activity of hydrogen donating compounds and chain breaking antioxidants (Binsan et al. 2008).

The antioxidant activity of different fractions of GH-RP and GH-CP using β-carotene – linoleic acid model system was determined (Fig. 6). The discoloration of β-carotene is widely used to measure the antioxidant activity of bioactive peptides, because β-carotene is extremely susceptible to free radical mediated oxidation of linoleic acid. Among all, the fractions <1 kDa from GH-RP and 3-5 kDa from GH-CP had shown higher antioxidant activity. The results indicate that the ability of peptides as an antioxidant is different in GH-RP and GH-CP. This could be possible due to nature of enzyme and DH achieved.

Discussion

The protein content of the protein hydrolysates depends on the solubilisation, hydrolysis of proteins and the removal of undigested insoluble fractionated non-protein substances after the hydrolysis (Idowu et al. 2019). Most of the earlier studies demonstrated that protein hydrolysates from various fish protein hydrolysate contain moisture below 10% (Chalamaiah et al. 2012). However, it is not uncommon to find in literature variation in the ash content of GH/FPH (fish protein hydrolysate) in the range of 0.45–20% in GH obtained using different enzymes. The ash content generally represents various minerals in different proportions. The fat and ash content of the protein hydrolysates obtained from Hake (*Merluccius merluccius*) heads was in

the range of 0.49–1.58% and 8.6–12.7%, whereas for the protein hydrolysate from salmon frames it was 6.03 – 6.34% fat, 9.81–11.09% ash, respectively (Karoud et al. 2019; Idowu et al. 2019). The common minerals found in cuttle fish skin gelatin hydrolysates obtained by using different enzymes were Ca, Na, P, Mg and others (Jridi et al. 2014).

Foam formation requires the ability of the protein to quickly adsorb at the air-water interface, thereby lowering surface tension. Food foams consist of air droplets dispersed in and enveloped by a liquid containing a soluble surfactant lowering the surface and interfacial tension of the liquid (Kinsella and Melachouris 1976). The amphiphilic nature of proteins/hydrolysates makes this possible the hydrophobic portion of the protein extends into the air and the hydrophilic portion into the aqueous phase. Very few studies have been performed on foaming properties of fish protein hydrolysates. The foaming stability depends on the nature of proteins/peptides in not allowing the liquid and air to collapse. It has been reported in whey proteins hydrolysates to a limited degree increased the foaming ability but reduced foaming stability (Kuehler and Stine 1974). This was attributed to more air being incorporated into the solution of smaller peptides, but the smaller peptides do not have the strength to require stable foam. It was concluded that there was a connection between the DH and foaming properties. The peptides with low molecular weight peptides (~1 kDa) were unable to maintain a well-ordered, interface orientation of the molecule which leads to poor foaming properties (Kristinsson and Rasco 2000). Nevertheless, transportation, penetration and rearrangement of molecules at the air-water interface govern foaming properties (Halim et al. 2016). Foaming stability mainly depends on the extent of protein-protein interactions within the matrix of films surrounding the air bubbles and as well as the flexibility of protein or peptide structure (Mutilangi et al. 1996; Klompong et al. 2007).

EAI estimates the ability of the protein to aid in the formation and stabilization of a newly-created emulsion by giving units of area of the interface that is stabilized per unit weight of protein (Giménez et al. 2009). In the present study, both EAI and ESI decreased with increase in protein concentration. A decrease in emulsifying ability with increasing protein concentration has been already reported for other fish proteins such as round scad protein hydrolysates (Thiansilakul et al. 2007). This phenomenon may be due to an increase in protein–protein interactions with increasing concentration, resulting in a lower protein concentration at the oil–water interface. Furthermore, solubility, molecular size and amino acid sequence are the factors contribute more, whereas the other factors such as degree of hydrolysis (DH), types of enzymes used, environmental pH and the extraction solvent affects the emulsifying properties (Halim et al. 2016). In the present study, it can be noticed that the DH of GH-RP is comparably higher than GH-CP. It was stated that though the smaller peptides have high stability and rapid diffusion property, and can be easily adsorbed at the interface, they are not effective in reducing the interfacial tension as they cannot unfold and reorient at the interface like higher molecular weight peptides (Kristinsson and Rasco 2000). A positive correlation between has been found in between surface activity and peptide length. The dependence of emulsifying activity on the protein concentration may be also explained by adsorption kinetics. At low protein concentrations, protein adsorption at the interface is diffusion-controlled. However, at high concentrations, the activation energy barrier does not allow protein migration to take place in a diffusion-dependent manner, leading to the accumulation of proteins in the aqueous phase (Kinsella and Melachouris 1976). Protein hydrolysates are surface active and promote oil-in-water emulsions because they have hydrophilic and hydrophobic functional groups and are water soluble (Wilding et al. 1984).

The IC_{50} values for DPPH radical scavenging activity of skin gelatin protein hydrolysates of tilapia (3.66 mg/ml) cartilage collagen hydrolysate (5.38 mg/ml) and thornback ray (1.98 mg/ml) (Zhang et al. 2012; Li et al. 2013; Lassoued et al. 2015). The IC_{50} values of DPPH radical scavenging in the present study are much lower than reported in the literature indicating higher ability to scavenge free radicals. In the present study, among all different fractions, it is low molecular weight peptides from GH-RP and GH-CP has shown higher DPPH radical scavenging activity. It has been reported that low molecular weight peptides can easily react with free radicals and exhibits more antioxidant activity (Ranathunga et al. 2006). Smaller peptides can arise only if higher DH is achieved. It is well known that the degree of hydrolysis is inverse to the peptide size, which can be attributed to high DPPH radical scavenging activity. Li et al. (2013) reported that the peptides responsible for scavenging activity act as electron donors and converts free radicals to more stable products and terminate radical chain reaction**.** The results of Da Rocha et al. (2018) suggest that the observed potent DPPH radical scavenging activity could be due to non-aromatic amino acids. Overall, the

difference in the amino acid composition and peptide length may leads to the difference in DPPH activities of bioactive peptide molecules, and the residues with high hydrophobic amino acids reported to have high radical scavenging activity (Karoud et al. 2019).

The reducing power of thornback ray gelatin hydrolysates prepared using different enzymes was found to be in the range 0.25–0.96 (Lassoued et al. 2015). The results of present work are comparable to thornback ray gelatin hydrolysate. The DH is known to affect ferric reducing power, higher FRAP values corresponded with an increase in the degree of hydrolysis (Raghavan et al. 2008; Kittiphattanabawon et al. 2012). Furthermore, the differences in reducing power might be due to increased availability of hydrogen ions because of peptide cleavages and hydrophobic content (Gómez-Guillén et al. 2010). Furthermore, the carboxyl and amino groups of the amino acid side chains such as Glu, Asp, His, Lys, and Arg can interact with metal ions and inactivate their pro-oxidant activity. The greater ferric reducing power leads to the greater efficacy in prevention and retardation of propagating in lipid oxidation reaction (Da Rocha et al. 2018).

ABTS radical scavenging activity of GH-RP and GH-CP hydrolysate fractions showed a higher value than the pacific hake hydrolysates fractions (Cheung et al. 2012). The radical scavenging activity 1-3 kDa from GH-RP was comparatively higher than <1 kDa from GH-CP. It has been reported that the type of enzyme, substrate and peptide composition might also influence the ABTS radical scavenging activity. Nevertheless, ABTS radical scavenging activities of 1-3 kDa from GH-RP and <1 kDa from GH-CP were reflecting similar ability in quenching ABTS radicals.

The antioxidant activity of gelatin hydrolysate obtained from thornback ray and cuttlefish skin gelatin hydrolysates were found to be less than that of present study using same model system (Lassoued et al. 2015; Jridi et al. 2014). The difference in antioxidant activities might be due to extensive hydrolysis. The antioxidant activity of peptides or protein in the free radical mediated lipid peroxidation system depends not only on the amino acid composition but also on the molecular size, chemical properties and electron transferring ability of amino acid residues in the sequence (Lassoued et al. 2015).

Conclusion

The aim of the present study was to investigate the preparation of potential bioactive and functional peptides from croaker skin gelatin. The findings revealed that gelatin hydrolysates had good surface-active properties. Both the hydrolysates showed a good foaming and emulsion properties at low concentrations. The resultant emulsions and foam made with GH-RP hydrolysate was stable. The gelatin hydrolysates were found to be an effective antioxidant, which was characterised in different *in vitro* assays. A dose-dependent effect between protein hydrolysates concentration and antioxidant activity was found. The bioconversion of marine biomass can be considered as one of the promising opportunities for the production of valuable functional and bioactive peptides.

List of abbreviations

GH	Gelatin hydrolysate
GH-RP	Gelatin hydrolysate - Rohu protease
GH-CP	Gelatin hydrolysate - Catla protease
DH	Degree of Hydrolysis
UNF	Unfractionated
DPPH	Diphenyl-1-picryl hydrazyl activity
ABTS	2, 2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid)

Conflict of interest The authors declare no conflicts of interest.

Acknowledgments University Grants Commission fellowship, Government of India, to the first author and financial support provided by European Union, Brussels under FP-7, SECUREFISH (Grant No.289282) for conducting the research work is gratefully acknowledged.

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