International Food Research Journal 28(4): 827 - 838 (August 2021)
Journal homepage: *http://www.ifrj.upm.edu.my*

Antioxidant activity and functional properties of grey mullet (*Mugil cephalus*) protein hydrolysates

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Article history

Received: 31 January 2020
Received in revised form: 30 December 2020
Accepted: 25 March 2021

Keywords

antioxidant activity, enzymatic hydrolysis, functional property, grey mullet, protein hydrolysate

Abstract

Grey mullet protein hydrolysate possessed high antioxidant activity and was prepared using neutrase. The hydrolysis conditions were optimised by the response surface methodology (RSM): enzyme to substrate ratio 5.8:1 (U of enzyme/mg of substrate), pH 7.3, 51°C, and 3.5 h. The hydrolysate obtained under the optimal conditions (HGM) possessed superior radical scavenging activity (IC_{50} values: 0.786 mg/mL for DPPH; 1.294 mg/mL for superoxide anion radical) to that of unhydrolysed grey mullet protein (GM). HGM also possessed better ferric-reducing power and higher ferrous ion-chelating activity. Electrophoretic analysis indicated that HGM mainly consisted of peptides with molecular weights below 10 kDa. The amino acid composition indicated a high nutritional value for HGM. The solubility, foamability, and emulsion activity index of HGM were improved as compared to those of GM. These results indicated that HGM has potential as a bioactive ingredient in the formulation of functional foods.

Introduction

Over the past few decades, the demand for functional foods has increased dramatically. Among the many claimed functions of functional foods, antioxidant properties have received much attention as this property may provide protection from injuries induced by reactive oxygen species (ROS). Elevated levels of ROS have been associated with cancer, diabetes, and some inflammatory and neurodegenerative disorders such as Alzheimer disease (de Jesus Raposo *et al.,* 2015). Many synthetic antioxidants such as butylated tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) are currently used by the food industry. However, due to the potential health risks associated with long term use of these synthetic antioxidants (Sabeena Farvin *et al.,* 2014), the search for safe and cheap natural antioxidants is timely. Recently, peptide natural antioxidants and hydrolysates of dietary proteins have attracted growing interest because of their safety, low molecular weights, and high nutritional value (Hattori *et al.,* 1998). Studies have shown that protein hydrolysates from various animal and plant sources obtained by controlled enzymatic hydrolysis can scavenge free radicals by inactivating the reactive intermediates and chelating pro-oxidative transition metals, both of which lead to oxidative stress (Ryan *et al.,* 2011; Sabeena Farvin *et al.,* 2014). Protein hydrolysates are beneficial for human consumption due to their nutritional properties. They generally possess a balanced amino acid composition, and are readily digested (Kristinsson and Rasco, 2000). To date, the antioxidant activities of enzymatic hydrolysates from various animal and plant sources have been investigated using *in vitro* antioxidant evaluation systems (Zhong *et al.,* 2011; Hmidet *et al.,* 2011; Žilić *et al.,* 2012; Tanzadehpanah *et al.,* 2012; Zheng *et al.,* 2019). Fish protein sources have gained much interest as potential antioxidant peptides particularly due to the availability of large quantities and underutilised species. Studies have also indicated that the hydrolysates of fish protein possess antioxidant properties (Vilailak *et al.,* 2008; Girgih *et al.,* 2013).

The grey mullet (*Mugil cephalus*), one of the most important commercial fishes in the world, has been widely cultured in the South and East China Sea. To the best of our knowledge, although there are

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DOI: https://doi.org/10.47836/ifrj.28.4.19

many reports relating to the nutritional composition and growth habit of grey mullet, there is little information regarding deep processing and value-added utilisation of grey mullet. The objective of the present work was, therefore, to establish an efficient enzymatic method for the preparation of grey mullet protein hydrolysate which possesses potent antioxidant activity. This will provide a basis for the development of grey mullet protein hydrolysate as a functional protein ingredient. The amino acid composition and the functional properties of grey mullet protein hydrolysate were also analysed and reported.

Materials and methods

Materials and reagents

Fresh grey mullet purchased from a local market (Hangzhou, China) was headed, gutted, washed, and sliced. The fish fillets were minced to a homogenate using a high-speed tissue homogeniser. The homogenate of grey mullet muscle was defatted by mixing with ethyl acetate in a ratio of 1:2 (w/v), followed by stirring at 55°C for 2 h. Following filtration under reduced pressure to remove ethyl acetate, the filter cake was lyophilised, ground, and later generating grey mullet protein powder (GM) which was stored at -20°C. The undefatted GM contained 62.9% protein (dry base) and 31.7% fat (dry base), while the defatted GM contained 86.9% protein (dry base) and 7.1% fat (dry base).

Neutrase (1.5 mg), protamex (500 mg), and flavourzyme (500 mg) were purchased from Novozymes Biotechnology Co. (Copenhagen, Denmark). Trypsin, papain, ethylene diamine tetraacetic acid (EDTA), and 1,1-diphenyl-2-picryl-hydrazy (DPPH) were purchased from Aladdin Chemical Reagents Co. (Shanghai, China). All other chemicals and solvents were of analytical grade.

Screening of the protease for the hydrolysis of GM

Hydrolysis of GM at a GM to water ratio of 1:20 (w/v) was performed in the presence of five different proteases (ratio of enzyme to substrate: 5 U of enzyme/mg of GM) at the respective optimal pH and temperature (trypsin: pH 7.5, 40°C; neutrase: pH 7.0, 50°C; protamex: pH 7.0, 45°C; flavourzyme: pH 7.0, 55°C; and papain: pH 7.0, 55°C) for 5 h. The pH value of the reaction solution was maintained constant with 1 M NaOH during the hydrolysis. The reaction solution was heated at 95°C for 15 min to deactivate the enzyme. After cooling, the suspension was centrifuged at 10,000 *g* for 10 min. The supernatant was then freeze-dried. The resulting hydrolysed protein was determined for the degree of hydrolysis (DH), DPPH radical scavenging activity, and soluble protein content.

$$DH\,(\%) = \frac{BN_b}{M_p \alpha h_{tot}} \times 100 \qquad \text{(Eq. 1)}$$

where, B = amount of alkali consumed (mL), N_b = normality concentration of alkali (normality/L), M_p = mass of the substrate (protein in g, N% × 6.25), $1/\alpha$ = calibration factor for the pH-stat, and h_{tot} = content of peptide bonds. For GM, h_{tot} = 7.84 mmol/g of protein.

Optimisation of reaction conditions for the hydrolysis of GM

Based on the results of the screening study, neutrase was selected for the hydrolysis of GM. Based on the results of single factor tests, the conditions for the hydrolysis of GM were further optimised by the response surface methodology (RSM). The RSM was conducted with four independent variables, including E/S ratio (U of enzyme/mg of substrate), hydrolysis time, pH, and temperature. Box-Behnken design (BBD) was used to survey the effects of these independent variables on the response dependent variable, namely DPPH radical scavenging activity (%) of the corresponding hydrolysates. This design permitted three distinct levels to be tested namely E/S ratio: 5.5, 6, and 6.5; hydrolysis time: 2, 3, and 4 h; temperature: 45, 50, and 55°C; and pH: 6.5, 7.0, and 7.5. The combination of these levels led to 29 experimental runs. The associated matrix of the experimental design and results are presented in Table 1.

Antioxidant activity assays

DPPH radical scavenging activity assay

The DPPH scavenging activity of GM and HGM was measured as previously described (Shao *et al.,* 2017) with slight modification. Briefly, sample solution (2 mL; 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL) in distilled water was mixed with DPPH solution (2 mL; 0.1 mM in ethanol). The resulting solution was shaken rigorously, and incubated in the dark at room temperature for 30 min. The absorbance of the solution was determined at 517 nm. Ascorbic acid served as positive control. The control was made by replacing DPPH ethanol solution with ethanol, while the blank was made by replacing sample solution with distilled water. The DPPH radical scavenging activity of the sample was calculated using Eq. 2:

Table 1. Experimental design and results of response surface methodology (RSM).

Run	Variable level				DPPH scavenging rate (%)	
	X_1	X_2	X_3	X_4	determined	predicted
1	50	7	2	6.5	53.12 ± 1.20	52.58
2	45	7.5	3	6	49.59 ± 0.76	47.58
3	50	7	3	6	57.77 ± 1.61	58
4	50	6.5	2	6	50.97 ± 2.33	50
5	45	7	3	6.5	46.98 ± 2.05	47.832
6	55	7	2	6	49.74 ± 1.48	50.06
7	45	6.5	3	6	44.23 ± 1.32	44.72
8	50	7	4	6.5	54.57 ± 1.26	54.6
9	55	6.5	3	6	50.94 ± 1.37	52.62
10	45	7	4	6	47.87 ± 0.92	47.42
11	50	7	3	6	58.49 ± 1.04	58
12	50	7	4	5.5	55.28 ± 1.73	55.48
13	55	7	3	5.5	53.71 ± 1.42	53.372
14	55	7	3	6.5	54.84 ± 3.23	55.228
15	50	7.5	2	6	48.49 ± 2.01	49.06
16	50	7.5	4	6	55.43 ± 1.17	56.92
17	50	7	2	5.5	48.54 ± 1.03	48.18
18	45	7	2	6	39.92 ± 2.49	40.96
19	50	7	3	6	56.28 ± 1.30	58
20	50	6.5	3	5.5	50.82 ± 1.84	50.55
21	50	6.5	3	6.5	57.35 ± 1.61	56.53
22	50	6.5	4	6	51.51 ± 1.72	51.46
23	45	7	3	5.5	46.04 ± 2.16	46.168
24	55	7	4	6	54.08 ± 1.52	52.92
25	50	7	3	6	58.63 ± 0.84	58
26	50	7.5	3	5.5	56.34 ± 1.42	57.03
27	50	7.5	3	6.5	54.44 ± 1.94	54.57
28	50	7	3	6	58.81 ± 1.25	58
29	55	7.5	3	6	55.11 ± 1.55	54.28

Four different variables, X_1, X_2, X_3 and X_4 represent temperature (°C), pH, hydrolysis time (h), and E/S ratio (U of enzyme/mg of substrate), respectively.

DPPH scavenging rate (%) = $[1 - (A_{sample} - A_{control}) / A_{blank}] \times 100$ (Eq. 2)

Superoxide anion radical scavenging activity assay

The superoxide anion radical scavenging activity of GM and HGM was measured according to a previously published method with modifications (Marklund and Marklund, 1974). A mixture of sample (2 mL) with different concentrations (0 - 2.5 mg/mL), Tris-HCl buffer (4.5 mL, pH 8.2, 100 mM), and distilled water (3 mL) were incubated at 25°C for 20 min. Pyrogallol solution (0.5 mL, 3 mM) pre-incubated at 25°C was then added to initiate the reaction. The rate of pyrogallol autoxidation was

reflected by the absorbance at 325 nm, which was recorded at 30 s intervals for 5 min using a spectrophotometer. A control solution was prepared using 10 mM HCl instead of the sample solution. The capability of scavenging superoxide anion radicals was calculated using Eq. 3:

$$O_2^{\bullet-}\text{scavenging activity}(\%) = \frac{\Delta A_0/\text{min} - \Delta A_s/\text{min}}{\Delta A_0/\text{min}} \times 100 \quad \text{(Eq. 3)}$$

where, ΔA_0/min = change in absorbance per minute of the control solution, and ΔA_s/min = change in absorbance per minute of the sample.

Ferric ion-reducing power assay

The ability of GM and HGM to reduce Fe^{3+} was measured according to a previously reported method (Chen *et al.,* 2016). Briefly, samples (2 mL; 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL) were mixed with phosphate buffer (2 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2 mL, 1%, w/v), then incubated at 50°C for 20 min. Trichloroacetic acid (2 mL, 10%, w/v) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The supernatant (2 mL) was mixed with distilled water (2 mL) and $FeCl_3$ (0.4 mL, 0.1%, w/v). The absorbance of the resulting solution was measured at 700 nm after 10 min. Vitamin C served as positive control.

Ferrous ion-chelating ability assay

The ability of GM and HGM to chelate ferrous ion was assessed using the method of Decker and Welch (1990). Sample solution (1 mL) was diluted with distilled water (1.85 mL), and added to a mixture of $FeCl_2$ (0.05 mL, 2 mM) and Ferrozine (0.1 mL, 5 mM). After 10 min, the absorbance of the reaction mixture was measured at 562 nm. Double distilled water served as control. The chelating ability was calculated using Eq. 4:

$$\text{Chelating ability}\ (\%) = [(A_c - A_s) / A_c] \times 100 \quad \text{(Eq. 4)}$$

where, A_s = absorbance of the sample at 562 nm, and A_c = absorbance of the control.

Determination of molecular weight distribution and amino acid composition

The molecular weight distribution of GM and HGM was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Schägger and Von, 1987) and tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (tricine-SDS-PAGE) (Schägger, 2006), respectively. GM and HGM (10 µL, 2 mg/mL), and molecular weight markers (10 µL) were loaded into separate wells. Electrophoresis was conducted at room temperature. After completion of electrophoresis, gels were fixed in glutaraldehyde (0.5%) for 20 min, stained with Coomassie Brilliant Blue G-250 (0.025%) for 30 min, and de-stained in acetic acid (10%) (Schägger and Von, 1987). The molecular weights of the proteins/peptides in hydrolysate were calculated from a standard curve derived from the relative migration rate and logarithmic relative molecular weight of protein markers (Zhou *et al.,* 2016).

The amino acid composition of GM and HGM was determined using a Hitachi L-8900 high speed amino acid analyser.

Determination of functional properties

Protein solubility

The solubility of GM and HGM was determined according to Marcet's method with slight modification (Marcet *et al.,* 2014). The sample solution (5 mL, 1 mg/mL) was adjusted to different pH values (2.0 - 10.0) with 1 M NaOH or 1 M HCl, and allowed to stand for 1 h. During this period, the pH value of the solution was checked every 20 min, and maintained constant. The resulting solution was centrifuged at 10,000 *g* for 30 min. The protein content of the supernatant and total protein in sample were determined using Coomassie Brilliant Blue method (Bradford, 1976). The protein solubility was calculated using Eq. 5:

$$\text{Protein solubility}\ (\%) = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100 \quad \text{(Eq. 5)}$$

Foamability and foam stability

The foamability of GM and HGM was determined referring to the reported methods with slight modification (Sathe and Salunkhe, 1981; Sha *et al.,* 2018). Briefly, 150 mg of sample was dissolved in deionised water (30 mL), adjusted pH to 7.0 with 1 M NaOH or 1 M HCl, and equilibrated by stirring at room temperature for 30 min. The mixture was dispersed with a DS-1 high speed dispersion machine at 15,000 rpm for 1 min. After standing for 2 min, the volume of foam was measured. The volume of the remaining foam was recorded again after 10, 20, and 30 min. The foamability and foam stability were calculated using Eq. 5 and Eq. 6, respectively:

$$\text{Foamability}\ (\%) = \frac{\text{volume of foam after dispersion}}{\text{volume of starting solution}} \times 100 \quad \text{(Eq. 6)}$$

$$\text{Foam stability}\,(\%) = \frac{\text{volume of foam after certain time}}{\text{initial volume of foam}} \times 100 \quad \text{(Eq. 7)}$$

Emulsifying properties

The emulsifying activity index (EAI) and emulsifying stability index (ESI) of GM and HGM were determined according to the previously reported methods with slight modifications (Pearce and Kinsella, 1978; Zhang *et al.,* 2014). Briefly, sample solution (30 mL, 1% (w/w)) with different pH (2.0 - 10.0) was mixed with bean oil (10 mL) and homogenised with a DS-1 high speed dispersion machine at 10,000 rpm for 1 min. Aliquots (50 µL) were pipetted at the bottom of the solution, and immediately mixed with SDS solution (5 mL, 0.1%). The absorbance of the resulting solution was recorded at 500 nm using 0.1% SDS as blank. After standing for 10 min, another aliquot (50 µL) was pipetted to measure its absorbance at 500 nm. The EAI and ESI were calsuclated using Eq. 8 and Eq. 9, respectively:

$$\text{EAI}\left(\frac{\text{m}^2}{\text{g}}\right) = \frac{2 \times 2.303 \times \text{A}_\text{t}}{0.25 \times \text{protein amount}\,(\text{g})} \quad \text{(Eq. 8)}$$

$$\text{ESI (min)} = A_0 \times \Delta_T/\Delta_A \quad \text{(Eq. 9)}$$

where, A_t = absorbance at different time, A_0 = absorbance at 0 min, A_T = absorbance at 10 min, and $\Delta_A = A_0 - A_T$; thus Δ_T = 10 min.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple-range tests. $p < 0.05$ was considered as a statistically significant difference. Design-Expert (version 8.0.5, State-Ease Inc., Minneapolis, MN, USA) was used for the experimental design and regression analysis of the experimental data.

Results and discussion

Selection of protease for the preparation of HGM

The antioxidant activity of protein hydrolysates depends on the type of protein, specificity of the enzyme, hydrolysis conditions, and the degree of hydrolysis. In the present work, five enzymes namely trypsin, neutrase, protamex, flavourzyme, and papain were screened for the hydrolysis of GM using the degree of hydrolysis (DH), DPPH radical scavenging activity, and soluble protein content of HGM as the indexes. It was found that papain was the most efficient in hydrolysing GM with a DH of 14.2%, and neutrase (13.5%) was the next. However, HGM prepared by neutrase had the strongest DPPH scavenging activity (49.9%), and the highest soluble protein content (3.3 mg/mL), while the corresponding values for papain were 37.7% and 2.75 mg/mL, respectively. Thus, neutrase was selected for the subsequent hydrolysis of GM. These findings are similar to that of Zhang *et al.* (2009) who reported that the hydrolysis of defatted rice endosperm protein by five enzymes (alcalase, chymotrypsin, neutrase, papain, and flavorase) for the production of antioxidant peptides, and found that neutrase was the optimum enzyme for this process.

Optimisation of hydrolysis parameters of GM

The DPPH radical scavenging activity of the hydrolysates obtained under different reaction conditions was found to vary appreciably ranging from 39.9 to 58.8% (Table 1). When the hydrolysis temperature, time, pH, and E/S ratio were at moderate levels, the hydrolysates possessed a relatively high DPPH radical scavenging activity of over 56% (runs 3, 11, 19, 25, and 28).

Analysis of variance (ANOVA) was used to determine the adequacy and significance of the quadratic model. This model presented a high determination coefficient (R^2 = 0.9656), high adjusted determination coefficient (R^2_{adj} = 0.9312), and low coefficients of variation (CV = 2.34%), thus indicating a high degree of correlation between the experimental and predicted values, and acceptable reliability of the experimental values.

The *F*-value of this quadratic polynomial model was high (28.07), while the corresponding *p*-value was low (< 0.0001), thus also indicating that the model was extremely significant. Both the *F*-value and *p*-value of lack-of-fit in the regression model were larger than 0.05, thus implying that the lack-of-fit was not significant for the response surface model, thereby confirming the validity of the model. The empirical relationship between DPPH radical scavenging activity and the four factors could be represented by a second-order polynomial equation as follows:

$$Y\,(\%) = -1645.79 + 26.44X_1 + 166.33X_2 + 29.15X_3 + 127.30X_4 - 0.12X_1X_2 - 0.18X_1X_3 + 0.02X_1X_4 + 3.20X_2X_3 - 8.43X_2X_4 - 2.65X_3X_4 - 0.24X_1^2 - 8.37X_2^2 - 4.05X_3^2 - 4.96X_4^2$$

where, Y represents DPPH radical scavenging activity, and X_1, X_2, X_3, and X_4 represent reaction temperature (°C), pH, reaction time (h), and E/S ratio (U/mg), respectively.

To visualise the effect of the four independent variables on the dependent variable, 3D surface responses were generated by varying two of the independent variables within the experimental range while holding the other two constants at the central point (Figures 1a - 1f). Among the interaction terms (X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4), the influence of X_2X_3 (temperature and time), X_2X_4 (temperature and pH), and X_3X_4 (time and pH) were found to be significant ($p < 0.05$).

The variation of DPPH radical scavenging activity with temperature and pH under the conditions of reaction time of 3 h and E/S ratio of 6 is presented in Figure 1a. The DPPH radical scavenging activity first increased and then decreased with increasing temperature from 45 to 55°C. High activities (> 58%) were obtained at around 51°C. A similar but weaker trend was observed by increasing the pH value from 6.5 to 7.5. The temperature was found to have the main impact on the response value; but, the interaction of temperature and pH was not significant ($p > 0.05$). The effects of temperature and time on the response at pH 7.0 and E/S ratio of 6 are presented in Figure 1b. At a fixed temperature, the DPPH scavenging activity increased with increasing hydrolysis time until about 3.5 h, and then slightly decreased. Both the changes of hydrolysis temperature and time had a major impact on the response value. Figure 1c demonstrates the effects of temperature and E/S ratio on the response, which indicates that the interaction of temperature and E/S ratio was not significant ($p > 0.05$). Figure 1d shows the effect of pH and hydrolysis time on the response at temperature 50°C and E/S ratio of 6. The DPPH scavenging activity increased with increasing pH up to about 7.3, and then decreased at the fixed time. The most obvious trend was observed at around 3.5 h. Figure 1e illustrates the effect of pH and E/S ratio on the response at 50°C for 3 h. When the pH was lower than 7.3, the DPPH radical scavenging activity increased with increasing E/S ratio. When the pH was over 7.3, the DPPH scavenging activity decreased with increasing E/S ratio. Maximum DPPH scavenging activity was obtained at pH 7.3 and E/S ratio of 5.8. These results are in agreement with the preceding ANOVA analysis. Figure 1f presents the effect of hydrolysis time and E/S ratio on the response at 50°C and pH 7.0. When the hydrolysis time was less than 3 h, the DPPH scavenging activity was found to increase with increasing E/S ratio. The change of E/S ratio had little influence on DPPH scavenging activity at the hydrolysis time of around 3.5 h. The effect of hydrolysis time on DPPH scavenging activity was greater than that of the E/S ratio.

According to the data analysis by software (Design-Expert 8.0), the highest DPPH scavenging

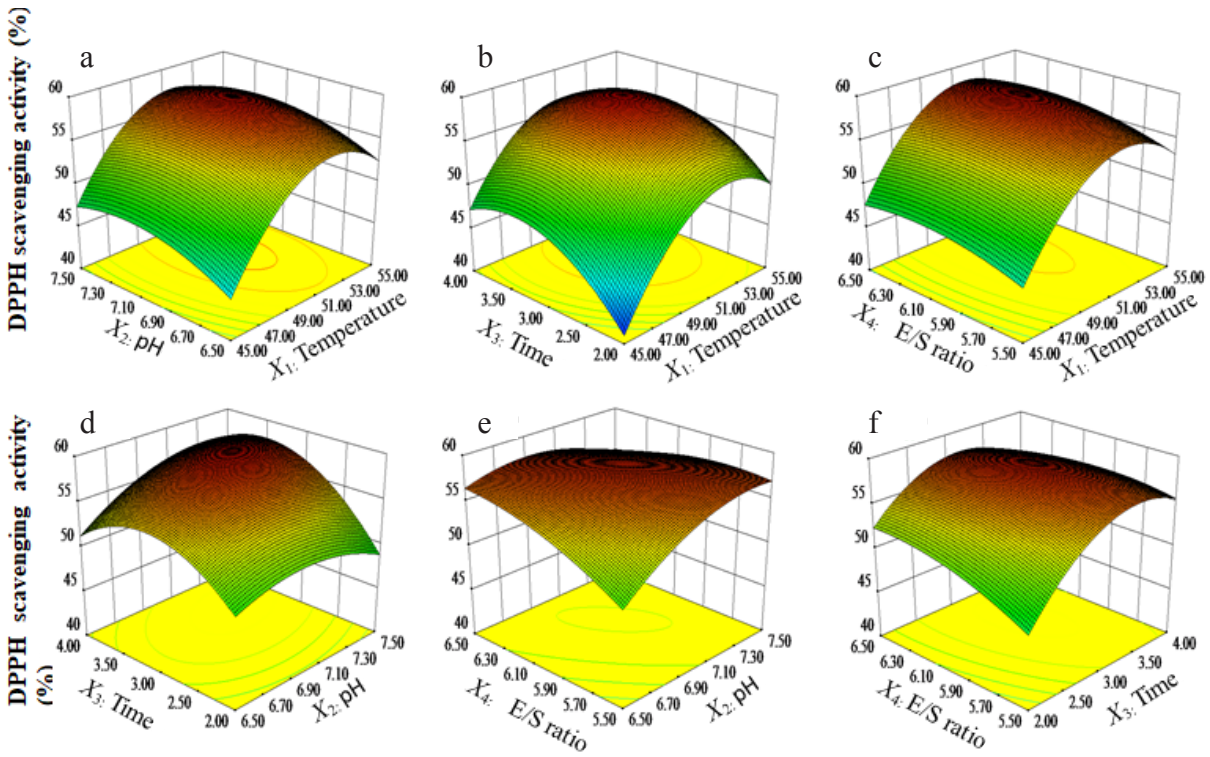

Figure 1. 3D response surface (a-f) demonstrating the effects of hydrolysis temperature, time, pH, and E/S ratio on DPPH scavenging activity of grey mullet protein hydrolysate.

activity (59.16%) was obtained under the following conditions namely enzyme to substrate ratio, 5.8:1 (U of enzyme/mg of substrate); pH, 7.3; temperature, 51°C; and time, 3.5 h. The result was verified by carrying out the experiments under the optimised conditions in triplicate, leading to the experimental value of 60.19 ± 0.57%.

Antioxidant activity

DPPH radical scavenging activity

DPPH is a stable free radical which shows maximum absorbance at 517 nm. It has been widely used in the evaluation of the ability of antioxidants to scavenge radicals. The DPPH-scavenging effect of both HGM and GM increased with increasing concentration (Figure 2a). HGM exhibited a potent DPPH-scavenging effect with an IC_{50} value of 0.786 mg/mL, which was stronger than that of GM, but weaker than vitamin C (V_C; control). The DPPH radical scavenging activity of HGM obtained in the present work is even higher than many reported antioxidant peptides from protein hydrolysates of blue mussel, monkfish, and scalloped hammerhead; for instance, YPPAK (IC_{50} value = 2.62 mg/mL) (Wang *et al.,* 2013), EWPAQ (IC_{50} value = 2.408 mg/mL), FLHRP (IC_{50} value = 3.751 mg/mL), and LMGQW (IC_{50} value = 1.399 mg/mL) (Chi *et al.,* 2014). These results suggested that proteins/peptides in HGM could serve as efficient hydrogen donor towards DPPH radicals.

Superoxide anion radical scavenging activity

The superoxide anion radical is generated in biological systems by the normal catalytic reaction of several enzymes, and the oxidation of haemoglobin. Although its reactivity is relatively weak, it can decompose to form stronger forms of ROS. Thus, it is valuable to assess the scavenging ability of HGM on the superoxide anion radical. The superoxide radical scavenging ability of GM and HGM was found to exhibit a dose-dependent manner within the tested concentration range (0.25 - 2.5 mg/mL) (Figure 2b). At 1 mg/mL, the superoxide anion radical-scavenging rate of HGM was measured as 42.5%, which was higher than that of GM (17.2%), but weaker than V_C (88.6%). Overall, HGM possessed moderate scavenging activity against the superoxide anion radical (IC_{50} value = 1.294 mg/mL) as compared to

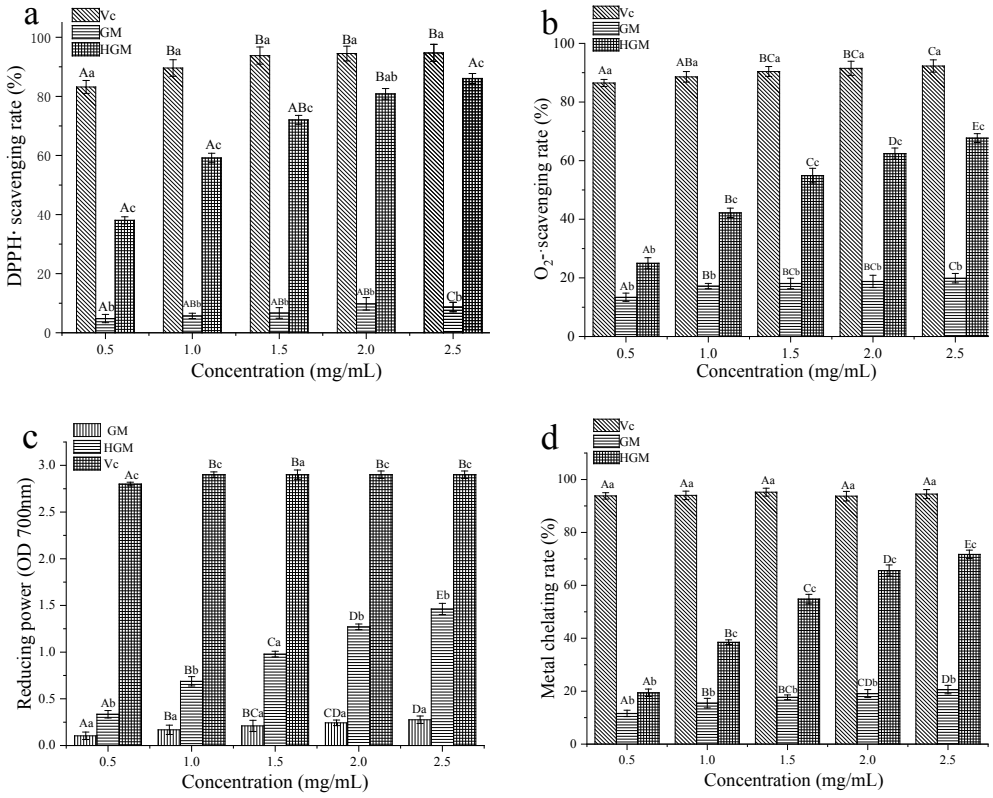

Figure 2. The antioxidant activities of grey mullet homogenate (GM), grey mullet protein hydrolysates (HGM), and vitamin C (V_C; control): (a) DPPH scavenging activity, (b) superoxide radical scavenging activity, (c) reducing power, and (d) metal-chelating ability. Values are means of triplicates (n = 3) with error bars indicating standard deviations (± SD). Different uppercase letters indicate significant difference between the activities of the same sample at different concentrations ($p < 0.05$). Different lowercase letters indicate significant difference between the activities of the different samples at the same concentration ($p < 0.05$).

the peptide NADFGLNGLEGLA (IC_{50} = 0.864 mg/mL) (Rajapakse *et al.,* 2005).

Total antioxidant ability

Ferric ion-reducing power was employed to assess the total antioxidant ability of the protein hydrolysate. In the presence of reducing agents, $[Fe(CN)_6]^{3-}$ is reduced to $[Fe(CN)_6]^{4-}$, which reacts with ferric ion to form $Fe_4[Fe(CN)_6]_3$ (Prussian blue). Thus, the formation of Fe(II) can be monitored by measuring the absorbance at 700 nm. Higher absorbance values correspond to stronger ferric ion-reducing power. As shown in Figure 2c, the reducing power of HGM was greatly improved as compared to that of GM. At a concentration of 2.5 mg/mL, the absorbance of the reaction solution in the presence of HGM reached 1.462, which was five times higher than that for the same concentration of GM (0.276). But HGM was still weaker than V_C. The superior ferric ion-reducing power of HGM to GM was likely to be associated with the iron-chelating ability and electron-donating property of the peptides in HGM.

Ferrous ion-chelating activity

In the presence of molecular oxygen, the redox cycle between the two most stable oxidation states of iron (Fe(II) and Fe(III)) generates ROS such as the toxic hydroxyl radical (Zhou *et al.,* 2012). Thus, the iron-chelating ability of peptides in hydrolysates is related to their antioxidant property. The ferrous ion-chelating ability of HGM and GM was found to be dose-dependent (Figure 2d). HGM exhibited a much stronger ferrous ion-chelating ability than GM, but weaker than EDTA, a well-known hexadentate chelator. Acidic and basic amino acids with carboxyl and amino groups on the side chains play an important role in metal ion chelation (Wiriyaphan *et al.,* 2015). Presumably, the enhanced Fe^{2+} binding ability of HGM can be attributed to an increased concentration of carboxylic groups and amino groups after hydrolysis. This premise is supported by the direct relationship between soluble protein/peptide concentration and the increase in the chelating ability (Sathivel *et al.,* 2003).

Molecular weight distribution and amino acid composition

The distribution of the molecular weights of GM and HGM was analysed by SDS-PAGE and tricine-SDS-PAGE. The molecular weights of most GM proteins were higher than 31 kDa, and these main proteins were not found in HGM. This result supported the fact that most GM proteins were partially degraded into peptides. The tricine-SDS-PAGE result demonstrated that the molecular weights of HGM were mainly distributed below 6.5 kDa. In the tricine-SDS-PAGE pattern of HGM, the colour of the band with molecular weight 6.5 kDa was intense.

The amino acid composition in protein hydrolysates has been demonstrated to play an important role in their antioxidant activities (Udenigwe and Aluko, 2011). For example, hydrophobic amino acids (HAA) act as antioxidants by increasing the solubility of peptides in lipids, thereby facilitating superior interaction with the free radicals that cause oxidative damage (Rajapakse *et al.,* 2005). The amino acid composition of GM and its enzymatic hydrolysates HGM is presented in Table 2. The HAA in HGM comprised about 39.34% of total amino acids, which was higher than that of GM (37.64%). Meanwhile, the high content of lysine and glutamic acid could enhance scavenging of superoxide anion radicals (Udenigwe and Aluko, 2011). The total content of essential amino acids (EAA) and EAA/NEAA (the ratio of essential and non-essential amino acid) in HGM were higher than those in GM, thus indicating the improvement of nutritional value after hydrolysis. The total content of EAA and EAA/NEAA in both HGM and GM were found to be higher than the Food and Agriculture Organization of the United Nations (FAO, 1991) recommendations for animal proteins, thus indicating that HGM is of high nutritional quality and could be used as a protein source in human diet.

Functional properties

Protein solubility

Solubility is an important indicator for the evaluation of the functionality and potential industrial application of protein ingredients, as protein-water interactions influence their foaming and emulsifying properties (Hall *et al.,* 2017). The solubility of HGM was investigated under different pH conditions in comparison with GM (Figure 3a). HGM was significantly more soluble over a wide pH range as compared to GM ($p < 0.05$). The solubility of HGM was found to be less affected by the change of pH. At pH 2.0, the solubility of HGM was 92.7%. With an increase of the pH from 2.0 to 10.0, the solubility of HGM only changed slightly. The lowest solubility of HGM was observed at pH 4.0, being 89.2%. This is possibly because the protein hydrolysate mainly consists of peptides with relatively low molecular weight, possessing a large number of hydrophilic groups which form hydrogen

Table 2. Amino acid composition of grey mullet homogenate (GM) and grey mullet protein hydrolysate (HGM) in g/100 g.

Amino acid	GM	HGM	FAO (1991)
Aspartic acid	9.08	9.1	
Threonine	4.84	4.83	3.4
Serine	5.3	5.17	
Glutamic acid	13.75	12.17	
Glycine	9.15	7.85	
Alanine	10.11	8.74	
Cysteine	0.39	0.92	
Valine	5.64	6.23	3.5
Methionine	2.51	1.73	
Isoleucine	4.29	5.24	2.8
Leucine	8.71	8.89	6.6
Tyrosine	2.36	2.99	1.1
Phenylalanine	3.47	4.28	6.3*
Lysine	9.88	8.79	5.8
Histidine	2.39	2.21	1.9
Arginine	4.34	4.53	
Proline	0.16	0.32	
EAA	39.34	39.99	
NEAA	57.03	54	
EAA/NEAA	0.69	0.74	0.60
HAA	37.64	39.34	

EAA = combined total of essential amino acid, NEAA = combined total of non-essential amino acids, and HAA = combined total of hydrophobic amino acids: alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine, and cysteine. *phenylalanine + tyrosine.

bonds with water. In contrast, the solubility of GM was strongly pH-dependent. The maximum solubility (48.6%) of GM was observed at pH 2.0. With an increase of pH from 2.0 to 6.0, the solubility of GM decreased to the lowest value of 15.4%, thereafter the solubility of GM increased with increasing pH. This is due to a lower solubility resulting when the pH is close to the isoelectric point.

Foaming property

Foam is a colloidal system formed by insoluble gas dispersed in a liquid phase (Khaled *et al.,* 2014). As shown in Figure 3b, the foamability of HGM was greatly improved as compared to that of GM. Under the identical conditions, the foamability of GM was 74.1%, while HGM was 121.7%. Theimprovement in foamability of HGM could be attributed to the structural change after hydrolysis, exposing surface-stabilising residues which can interact with the air-water interface. In addition, due to the superior solubility and lower viscosity, the proteins/peptides in HGM solution may have greater diffusion rate and flexibility, thus increasing the initial stabilisation of the interface which in turn improves the foamability (Liceaga-Gesualdo and Li-Chan, 1999).

Although hydrolysis significantly increased the initial foam capacity of hydrolysates ($p < 0.05$), the foaming stability of HGM was found to be slightly lower than that of GM ($p > 0.05$) (Figure 3b).

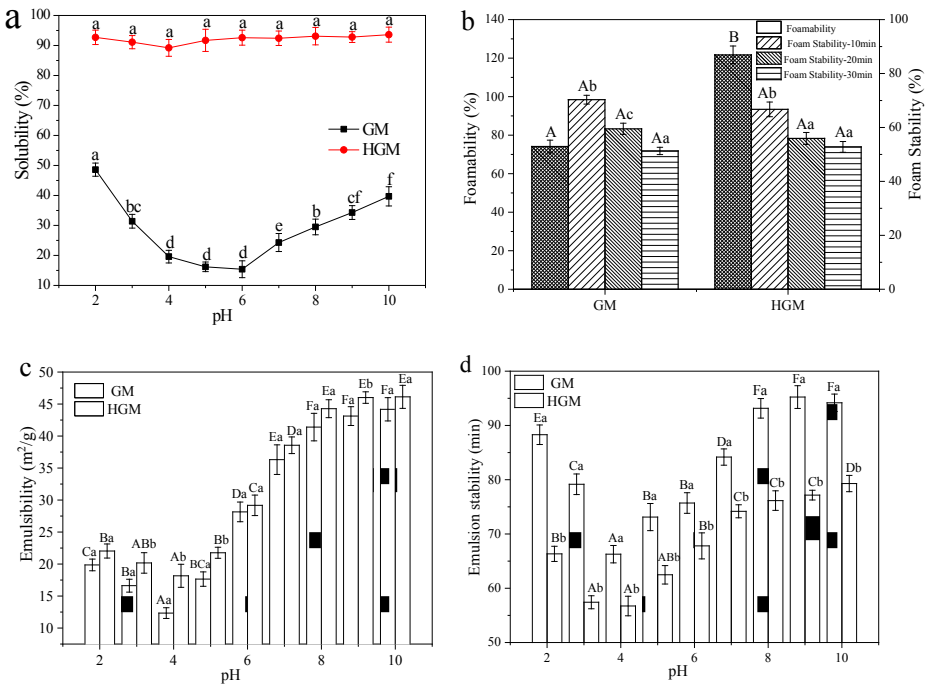

Figure 3. Functional properties of grey mullet homogenate (GM) and grey mullet protein hydrolysates (HGM): (a) solubility, (b) foamability and foaming stability, (c) emulsibility, and (d) emulsifying stability. Different uppercase indicate significant difference of results between groups ($p < 0.05$). Different lowercase indicate significant difference of results within each group ($p < 0.05$).

This finding agrees with those in the published reports (Liceaga-Gesualdo and Li-Chan, 1999; Hall *et al.,* 2017). After 10 min, the foaming stability of HGM was 66.7%, which was slightly lower than that of GM (70.3%). With increasing time, their foaming stability became similar. This could be due to the fact that the molecular weight of the protein/peptide influences the strength of the interfacial layer and the formation of networks (Hall *et al.,* 2017).

Emulsifying property

Good emulsifying property of protein ingredients is required in the production of many foods. A higher emulsifying activity index (EAI) value means a better adsorption ability of the protein/peptide at the oil-water interface. As shown in Figure 3c, under a wide range of pH conditions (2.0 - 10.0), the EAI value of HGM was slightly higher than that of GM. Although the difference was significant at pH 3.0 - 5.0 ($p < 0.05$), the difference was not significant at pH values 2.0 and 6.0 - 10.0 ($p > 0.05$). The EAI value of both HGM and GM exhibited a pH-dependent manner, decreasing with increase of pH (from 2.0 to 4.0), then increasing rapidly with pH values up to 8.0, after which there was no obvious increase in EAI. The maximum EAI value of HGM was observed at pH 9.0, being 45.9 m^2/g, and EAI value for GM under similar conditions was 42.7 m^2/g. The improved EAI value of HGM could be attributed to the generation of surface-stabilising residues by hydrolysis, which increases hydrophobic interactions, thereby facilitating emulsion formation (Jung *et al.,* 2005). However, the emulsifying stability (ESI) of HGM was found to be significantly lower than that of GM under the tested pH conditions ($p < 0.05$) (Figure 3d). This result is in agreement with previous reports (Hall *et al.,* 2017). Peptides with a relatively low molecular weight possess lower viscosity and interfacial tension than proteins, thereby reducing the emulsifying stability.

Conclusions

An efficient and convenient protocol for the preparation of grey mullet protein hydrolysates (HGM) with potent antioxidant activity was developed by using neutrase as a hydrolytic enzyme, and employing the response surface methodology for the optimisation of hydrolysis parameters. The HGM was mainly made up of peptides with molecular weights below 6.5 kDa. The HGM has a potential application as an ingredient for functional foods and as a nutraceutical due to its potent antioxidant activity, good functional properties, and excellent nutritional value. Since the antioxidant property of polypeptides is not only influenced by amino acid composition, but also by the specific amino acid sequences, the purification and identification of the

specific peptides in HGM responsible for its antioxidant ability are warranted.

Acknowledgement

The present work was financially supported by Key Research and Development Program, Science Technology Department of Zhejiang Province of China (No. 2018C02048), and Food Science and Engineering - the most important discipline of Zhejiang Province of China (No. 2017SIAR216). The authors thank Professor Robert Hider from King's College London for the English editing.

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