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Impact of microwave-assisted enzymatic hydrolysis on functional and antioxidant properties of rainbow trout *Oncorhynchus mykiss* by-products

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Abstract Fishery by-products can be better utilized following enzymatic hydrolysis treatment to produce fish protein hydrolysates (FPH) with potentially enhanced interface-stabilizing properties (e.g. functionality). The production of FPH could be accelerated through the application of rapid heating methods [e.g. microwave-assisted heating (MW)] rather than slower conventional heating (CH) treatments. The objective of this study was to investigate the effects of microwave heating during enzymatic hydrolysis on the functionality and antioxidant properties of FPH. Trout by-products were hydrolyzed with Alcalase at an enzyme substrate ratio (E:S) of 0.5, 1.7, and 3.0% (w/v), respectively, for 3, 5 and 15 min using a microwave system (1200 W, 20% power with 50% duty cycle at 50–55 °C) and a conventional heating method (water bath at 50 °C). The degree of hydrolysis and protein solubility was higher ($P < 0.05$) for the MW-FPH than for the CH-FPH. MW-FPH at 5 min (0.5% E:S) demonstrated higher ($P < 0.05$) emulsifying activity and emulsion stability than CH-FPH with the same treatment. Foam capacity and stability were also greater ($P < 0.05$) for MW-FPH samples that were treated 15 min by microwave-assisted heating (0.5% E:S) when compared to CH. Overall, MW-FPH exhibited higher ($P < 0.05$) 2,2-diphenyl-1-picryhydrazyl and ferric ion reducing capacity than CH-FPH. We therefore conclude that microwave-assisted hydrolysis is an alternative method to produce FPH with improved solubility, emulsifying activity, foaming properties and antioxidant activity.

Keywords Fish by-products · Proteins · Microwave-assisted hydrolysis · Functionality · Antioxidant activity

Introduction

According to a report from the U.S. Department of Agriculture, the total sale amount and value of farmed rainbow trout *Oncorhynchus mykiss* in the USA was over 1.8 million pounds and 96 million dollars in 2015, respectively. The state of Indiana alone produces roughly 1.5 million pounds of farmed fish per year from about 40 farmers, estimated to have a value of $15 million (U.S. Department of Agriculture Economic: http://www.ers.usda.gov/data-products/aquaculture-data.aspx/). About 30% of this total mass is generated as fish by-products such as frame/bones, skin, head, tail, fin and viscera depending on the type of fish and processing methods [1]. Fish by-products obtained during processing typically end up being used as compost, animal feed and fish waste. To minimize by-products being discarded to landfills, there should be an alternative way to reuse these natural resources that could potentially supplement human nutritional needs [2]. By-products may have significant value if proteins can be recovered from the fish and used in food products. However, fish meat that remains attached to the bones (frame), skins and head is typically difficult to recover using mechanical processing.

Fish protein hydrolysates (FPH) are defined as modified proteins from edible, unused fish materials that have undergone hydrolysis, resulting in smaller peptides and ionizable molecules. Hydrolysis techniques have improved over the past 50 years, driven by the efforts of aquatic industries to

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use fish processing by-products in the most cost-efficient way possible. By applying protein hydrolysis techniques, companies are able to add value to waste products that otherwise would be released back into the environment [3]. Enzymatic hydrolysis is more commonly used to produce FPH by utilizing proteolytic enzymes that hydrolyze peptide bonds at specific sites, producing small polypeptides (hydrolysates) that are capable of modifying and even improving the functional properties of proteins [4].

Studies have shown that FPH derived from enzymatic hydrolysis have several functional properties, such as high solubility, emulsifying capacity and stability and foaming capacity and stability [5–8]. The individual characteristics of hydrolysates will differ based on the type of enzyme, pH, hydrolysis temperature and time as well the species of fish being used [9]. Of the commercial enzymes currently available, Alcalase™, an alkaline endopeptidase enzyme produced from *Bacillus licheniformis,* is the most commonly used enzyme to produce hydrolysates with high functional properties, protein content and nutritional value [10, 11]. This commercially available food-grade enzyme has been widely used to produce FPH due to its high thermostability and high optimal pH [5, 12]. Alcalase is one of the most cost-effective enzymes when compared to other alkaline proteases used to hydrolyze muscle proteins [13].

Overall, FPH can be used in the food industry as flavor enhancers, milk replacers, beverage stabilizers and protein supplements [3]. In the past decade, there has also been an increased interest in using FPH as antioxidants. Studies have shown that protein hydrolysates prepared from various fish protein sources, such as yellowfin sole *Limanda aspera* frame, Alaska pollock *Theragra chalcogramma* frame, round scad muscle *Decapterus maruadsi* and Pacific hake *Merluccius productus*, exhibit strong antioxidant capacity [6, 14–16].

One of the limitations of the FPH production process is the extended hydrolysis time required when conventional heating methods (i.e. water bath) are used. However, this limitation can potentially be overcome by using microwave radiation as an alternative heating source. Due to its penetration capacity and dependence on the dielectric properties of the medium, microwave radiation can heat up susceptible materials much more rapidly than conventional heating methods. In particular, the heating of protein is achieved by both the absorption of microwave energy by rotation of bipolar water molecules and translation of the ionic components of the proteins [17]. During this process, the polar molecules are associated with the changing magnetic field of the microwave and convert the absorbed energy into heat, which then accelerates chemical, biological and physical processes [18]. The energy transmission in microwave radiation occurs primarily from dielectric losses, while in conventional heating the heat energy is transferred by conduction and convection [19]. Recently, Singh et al. [20] used a molecular dynamic modeling approach to demonstrate that the exposure of soybean hydrophobic protein to low-intensity electric fields causes the proteins to reorient under the field without significant changes in protein structure, whereas exposure to high-intensity electric fields resulted in protein unfolding and loss of the majority of the helical structures within the protein. Moreover, hydrolysates from microwave treatments are typically obtained in minutes, as opposed to hours with conventional heating methods [21, 22]. Microwave radiation has also been used in combination with high pressure for rapid enzymatic hydrolysis of β-lactoglobulin, resulting in the production of smaller peptides within minutes [23]. To our knowledge, research is lacking on the hydrolysis of rainbow trout by-products with the assistance of microwave heating to produce FPH. The objective of this study was to study the effects of microwave-assisted hydrolysis on antioxidant and functional properties of rainbow trout proteins.

Materials and methods

Materials

Fresh rainbow trout *Oncorhynchus mykiss* fish frames were obtained from Bell Aquaculture™ (Redkey, IN). The fish frames were transported to the Food Science department at Purdue University on ice and immediately frozen at −20 °C until used. The enzyme used was Alcalase™ 2.4 L (Novozyme, Franklinton, NC) with a declared activity of 2.4 Anson Units per gram. All chemicals used in this research were reagent grade unless otherwise specified. Chemicals and materials were obtained from VWR International (Radnor, PA), Sigma-Aldrich (St. Louis, MO) and Thermo Fisher Scientific (Waltham, MA).

Production of FPH

Fish protein hydrolysates were produced as described previously by Liceaga-Gesualdo and Li-Chan [5] with some modifications. Fish frames were thawed at 4 °C overnight and washed once using distilled water. Skin and fins were removed from the frames and the frames then cut into smaller pieces before being homogenized at medium speed for 2 min at ambient temperature in a commercial blender (Waring Commercial, Stanford, CT) in distilled water [1:2 (w/v) fish weight to distilled water]. A preliminary experiment was carried out to evaluate different enzyme concentrations and hydrolysis times on protein functionality. Based on data from these preliminary trials, the best treatment conditions were selected as follows: (1) Alcalase was added to the fish slurry at 0.5, 1.7 and 3.0% (w/v)

enzyme/substrate ratio (E:S), respectively, based on the protein content of the slurry (73% dry basis); (2) samples were heated using a microwave system (FISO Technologies Inc., Quebec City, QC, Canada) for 3, 5, and 15 min, respectively. For the microwave heating treatments, 20% power with 50% duty cycle at 1200 W was used, and the temperature was maintained at 50–55 °C during hydrolysis for optimal enzyme activity using an optical fiber probe in the microwave vessel. Similarly, a conventional heating (CH) method (using a water bath) was applied for 3, 5, and 15 min, respectively. For the CH treatments, temperature of the slurry was brought to 50 °C before Alcalase was added. The temperature of the slurry was measured using a thermocouple and maintained at 50–55 °C during hydrolysis. After hydrolysis, samples were pasteurized at 90 °C for 15 min to inactivate the enzyme. Samples were cooled and centrifuged (17,700 *g*) for 15 min at 4 °C (Avanti J-26S Centrifuge; Beckman-Coulter Inc., Brea, CA). The supernatant was collected, freeze-dried and stored in 50-ml capacity polypropylene tubes at −20 °C until further use.

Proximate composition

Moisture and ash contents were determined using standard Association of Official Analytical Chemists (AOAC) methods 950.46(b) and 920.153, respectively [24]. Total crude fat content was determined using a Soxhlet extraction method (AOAC 960.39), and total crude protein content was determined using standard AOAC method 984.13 (A–D) [24]. Total protein content was reported as (%N) using the standard conversion factor 6.25 [25].

Degree of hydrolysis

Degree of hydrolysis (DH) was measured using trinitrobenzene sulfonic acid (TNBS) as described previously with some modifications [5, 25]. Triplicate aliquots (1 ml) from each hydrolysis treatment were mixed with 1 ml of 24% trichloroacetic acid (TCA) and centrifuged (12,100 *g*) for 5 min. Supernatant (0.2 ml) was mixed with 2 ml of 0.2 M sodium borate buffer (pH 9.2) and 1 ml of 4 mM of TNBS. The solution was vortexed followed by incubation in the dark at room temperature for 30 min. Then, 1 ml of 2.0 M NaH_2PO_4 with 18 mM Na_2SO_3 was added. Absorbance was measured at 420 nm using an UV–Visible spectrophotometer (Beckman, Irvine, CA). Degree of hydrolysis (%DH) was defined as the ratio of the number of peptide bonds broken (h) to the total number of bonds per unit weight (h_{tot}), expressed as a percentage, using the following Eq. (1):

$$\%\text{Degree of hydrolysis (DH)} = \left[\frac{h}{h_{tot}}\right] \times 100 \quad (1)$$

The total number of peptide bonds (h_{tot}) is expected to be 8.6 m_{eq}/g in fish protein [26]. Hydrolysis equivalents (h) will be dependent on the free amino groups present as determined using the TNBS method.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the FPH prepared by both conventional and microwave-assisted heating (MW) as described previously with some modifications [5, 27]. The protein concentration of the samples was determined using the bicinchoninic acid (BCA) protein assay according to the manufacturer's protocol using bovine serum albumin as the standard (Thermo Fisher Scientific, Rockford, IL). Samples were diluted to final protein concentration of 8 mg/ml using a sample buffer consisting of 2 µl 2-mercaptoethanol, 20 µl 10% SDS, and 2 µl 1% bromophenol blue (tracking dye) in 0.0625 M Tris/HCl (pH 6.8). Samples were heated in a boiling water bath for 10 min and centrifuged at 13,000 *g* for 1 min. The samples were then loaded onto a PhastSystem™ electrophoresis unit (VWR–GE Healthcare, Chicago, IL) using a PhastGel™ gradient 10–15% polyacrylamide gel. The gel was run at 500 V, 5.0 mA and 3.0 W for 1 V hour (Vhr) at 15 °C. The gel was stained with Coomassie blue (0.1% Phastgel blue R solution in 30% methanol and 10% acetic acid), destained (30% methanol, 10% acetic acid) and preserved (10% acetic acid, 5% glycerol). Wide-range molecular weight standards (Sigma-Aldrich) were used for molecular weight estimations.

Amino acid composition

Amino acid composition of the CH-FPH and MW-FPH was performed in the Bindley Bioscience Center Metabolomics Laboratory, Purdue University. Samples were precipitated with TCA and mixed at 1:1 (v/v) supernatant to acetonitrile. Analysis was performed using an Agilent 6460 QQQ liquid chromatography/tandem mass spectrometry system with HILIC chromatography column (Agilent Technologies, Santa Clara, CA), and amino acid standards (Pierce, Rockford, IL) were used for calibration.

Protein solubility

Protein solubility (PS) was determined as reported by Chobert et al. [28] with some modifications. Lyophilized FPH (0.25 g) was dissolved in 10 ml of the chosen buffer solution and diluted 16.7-fold to prepare solutions at pH 3, 7 and 9; the buffer solutions used were 0.1 M acetate

buffer (pH 3), 0.1 M sodium phosphate (pH 7) and 0.1 M Tris/HCl buffer (pH 9), respectively. Protein content was determined using the BCA protein assay as outlined by the manufacturer, with bovine serum albumin as the standard before and after centrifugation at 4000*g* for 30 min at 20 °C, according to Eq. (2):

$$\%\text{Peptide solubility} = \frac{\text{Protein content after centrifugation}}{\text{Protein content before centrifugation}} \times 100\% \quad (2)$$

Analysis was conducted in triplicate, and the results were reported as percentage of protein solubility.

Emulsifying activity index

The emulsifying activity index (EAI) was determined using to the spectroturbidimetric procedure of Pearce and Kinsella [29] as modified by Liceaga-Gesualdo and Li-Chan [5]. A 3-ml aliquot of 0.5% (w/v) FPH in 0.1 M phosphate buffer (pH 7.0) was placed in a micro-chamber containing 1 ml of 100% pure canola oil (Crisco, Orrvile, OH) and the suspension homogenized at 18,000 rpm for 1 min using a Sorvall Omni Mixer with microattachment assembly (Omni International, Kennesaw, GA). Aliquots were pipetted immediately and at subsequent timed intervals from the emulsion and diluted 200-fold into test tubes containing 0.3% (w/v) SDS solution. Test tubes were inverted six times in order to obtain a homogenous mixture, and absorbance was read at 500 nm using an UV–Visible spectrophotometer (Beckman). The EAI was expressed using Eq. (3):

$$\text{EAI} = 2T/\phi c \quad (3)$$

where T = turbidity = 2.3A/L (A = absorbance at 500 nm at time zero, L = light path in meters), ϕ = oil phase volume (=0.25) and c = concentration of solids (0.5%) in the aqueous phase. Emulsion stability (ES) was determined by measuring the absorbance at 500 nm of the aliquots of the emulsion taken at 30, 60, 120 min after formation of the emulsion. EAI and ES are expressed as meters-squared/grams (m^2/g). Analysis was conducted in triplicate.

Foaming properties

Foaming capacity (FC) and foam stability (FS) were determined by the homogenization method as described previously, as modified by Liceaga-Gesualdo and Li-Chan [5]. Lyophilized FPH (0.6 g) was dissolved in 20 ml of 0.1 M sodium phosphate buffer. The mixture was homogenized at 18,000 rpm for 1 min using an Omni Mixer homogenizer (Omni International) with a 20 × 195-mm saw tooth generator probe. The mixture was then poured into a 50-ml graduated cylinder and the total volume measured. FC was expressed as the percentage volume increase after homogenization. FS was calculated as the volume of foam remaining after inactive periods of 15 and 45 min, respectively, and expressed as a percentage. The analysis was conducted in triplicate.

Surface hydrophobicity

Surface hydrophobicity was determined using the fluorescent probe propionyl-2-dimethlaminonaphthalen (PRODAN) as described previously with some modifications [27, 30, 31]. Duplicate FPH solution samples were prepared in 0.1 M Tris buffer/0.6 M NaCl (pH 7.5) with final protein concentrations of 0, 0.05, 0.10, 0.15, 0.20 and 0.25 mg/ml. FPH samples were analyzed with PRODAN stock solution concentration (0.032% w/v) in high-performance liquid chromatography-grade methanol and determined from its absorbance at 360 nm, using the molar absorption coefficient of $1.8 \times 10^4\ M^{-1}\ cm^{-1}$. Each FPH solution (4 ml) was added to 10 µl of PRODAN stock solution, mixed by inversion and incubated in the dark for 15 min. The net relative fluorescence intensity (RFI) was measured in the dark using a fluorescence spectrophotometer (model LS 55; PerkinElmer Inc., Waltham, MA) with excitation and emission slit widths set at 5 and 5 nm, respectively, an excitation wavelength of 365 nm and emission scans from 400 to 650 nm. The net RFI was calculated based on the difference between the RFI of the sample with PRODAN and the RFI of protein blank samples, plotted against protein concentrations to determine slope, which was interpreted as surface hydrophobicity (S_o).

Antioxidant activity

Radical scavenging activity of 2,2-diphenyl-1-picryhydrazyl

2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity of the FPH was determined as outlined by Klompong et al. [32] as modified by Bougatef et al. [33]. FPH solutions (4 ml) at different concentrations (0.5, 1.5, 2.5, 3.5 and 40 mg FPH/ml) were mixed with 1 ml of 0.2 mM DPPH solution. The mixtures were incubated in the dark at room temperature for 30 min. After incubation, absorbance at 517 nm was measured using an UV–Visible spectrophotometer (Beckmann). Distilled water was used to replace of the FPH solution as the control for the assay ($Abs_{assay\ ctl}$). Sample controls ($Abs_{sample\ ctl}$) were also prepared for each sample by mixing 4 ml of sample solution with 1 ml of 99.5% ethanol solution. DPPH radical scavenging capacity of the sample was calculated using Eq. (4):

%DPPH radical scavenging activity

$$= \left[\frac{\text{Abs}_{\text{assay ctl}} - \left(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample ctl}}\right)}{\text{Abs}_{\text{assay ctl}}}\right] \times 100 \quad (4)$$

The absorbance of the sample was inversely correlated with its DPPH radical scavenging activity, i.e. the lower the absorbance of the sample, the higher its DPPH radical scavenging activity. The synthetic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as positive controls.

Ferric ion reducing antioxidant capacity

The ferric ion reducing antioxidant capacity (FIRC) of FPH samples was determined according to Samaranayaka and Li-Chan [15]. FPH (18 mg) was dissolved in 2 ml of 0.2 M phosphate buffer (pH 6.6). The solution was then further mixed with 2 ml of the same buffer and 2 ml of 1% (w/v) potassium ferricyanide to a final FPH concentration of 3 mg/ml. The solution was incubated at 50 °C for 20 min. After the incubation, 2 ml of 10% TCA was added and mixed thoroughly. The solution (2 ml) was then mixed with 2 ml of double-distilled water and 0.4 ml of 0.1% (w/v) ferric chloride. After a 10-min incubation in the dark at room temperature, absorbance at 700 nm was measured as an indication of reducing power of the sample. Synthetic antioxidants BHA and BHT were used as positive controls. In this assay, a higher absorbance indicates a higher reduction capacity.

Statistical analysis

All sample analyses were completed in triplicate, unless otherwise stated. Analysis of variance using a general linear model with Tukey's pairwise comparison of means ($P < 0.05$) was used to determine statistical significance of observed difference among means. The statistical software program MINITAB® Version 16.0 (Minitab Inc, State College, PA) was used.

Results

Proximate composition

The control (slurry) had a protein content of 73% (dry basis). Overall, all FPH were found to have high protein content (88% on a dry basis). The lipid content was 2.1% (dry basis), while the ash content was high (7.3% dry basis) considering that fish frames/bones were used to prepare the hydrolysates.

Degree of hydrolysis

The DH of FPH produced from different enzyme:substrate ratios (E:S) treated at different reaction times for both microwave and conventional heating are shown in Table 1. When E:S and hydrolysis time increased, DH increased, ranging from 3 to 12% and from 11 to 23% for CH-FPH and MW-FPH, respectively. Overall, MW-FPH showed higher ($P < 0.05$) DH than CH-FPH at the same hydrolysis times (3–15 min) and E:S (0.5–3%).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The electrophoretic pattern (Fig. 1) revealed the molecular weights of FPH from the conventional heating and microwave treatments. Hydrolysis for 5 min (0.5% E:S) of both the CH-FPH and MW-FPH showed bands with molecular weights of <66 kDa. Hydrolysis for for 15 min (0.5% E:S) of the CH-FPH showed bands with molecular weights of <66 kDa, but the same treatment of MW-FPH showed predominant bands of <31 kDa indicating further hydrolysis (Fig. 1a, lanes 4, 5). Hydrolysis for 3 min (1.7% E:S) and 5 min (3.0% *E*:*S*) of the CH- and MW-FPH (Fig. 1b) showed a thick band of <14.4 kDa.

Protein solubility

The solubility of CH- and MW-FPH at pH 3, 7 and 9 is shown in Fig. 2. Overall, both CH- and MW-FPH displayed high solubility across all treatments and pH conditions. At pH 7, MW-FPH hydrolyzed for 15 min (0.5% E:S) and 3 min (1.7% E:S) showed higher solubility ($P < 0.05$) than CH-FPH at the same treatment conditions (Fig. 2a).

Table 1 Degree of hydrolysis of fish protein hydrolysates obtained from conventional heating and microwave-assisted heating with the alkaline endopeptidase enzyme Alcalase

Treatments (time/E:S)	Degree of hydrolysis (%)	
	CH-FPH	MW-FPH
5 min/0.5%	3 ± 0.62f	11 ± 0.33d,*
15 min/0.5%	8 ± 0.14e	18 ± 1.02c,*
3 min/1.7%	12 ± 0.69d	22 ± 0.14b,*
5 min/3%	9 ± 0.17e	23 ± 0.50a,*

Values in table are presented as the mean of three replicates ± standard deviation (SD). The asterisks indicate significant differences at $P < 0.05$ between conventional heating (CH) and microwave-assisted heating (MW) treatments (rows). Different lowercase letters indicate significant differences at $P < 0.05$ in DH across all treatments (columns)

E:S enzyme:substrate ratio, *FPH* fish protein hydrolysates

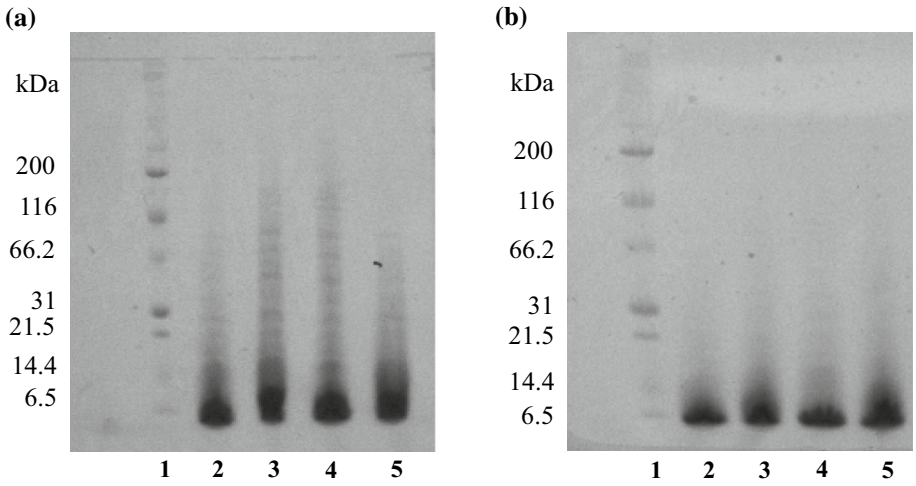

Fig. 1 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fish protein hydrolysates (FPH) produced from conventional heating (CH) and microwave-assisted heating (MW) **a** *Lanes*: *1* protein molecular weight markers, *2*, *3* CH-FPH and MW-FPH hydrolyzed for 5 min [0.5% enzyme:substrate ratio (E:S)], respectively, *4*, *5* CH-FPH and MW-FPH hydrolyzed for 15 min (0.5% E:S), respectively. **b** *Lanes 1* protein molecular weight markers, *2*, *3* CH-FPH and MW-FPH hydrolyzed for 3 min (1.7% E:S), respectively, *4*, *5* CH-FPH and MW-FPH hydrolyzed for 5 min (3.0% E:S), respectively

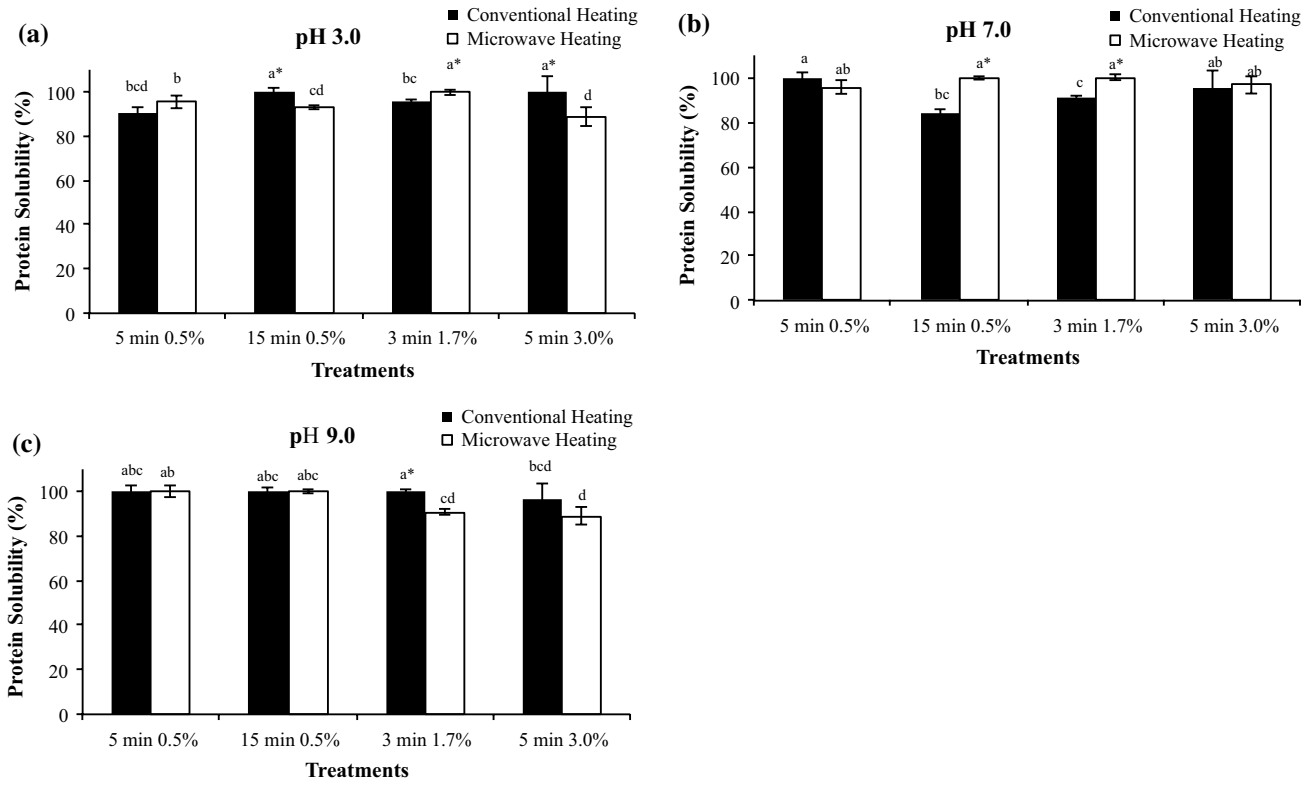

Fig. 2 Protein solubility of FPH produced from CH and MW at pH 3 (**a**), pH 7 (**b**) and pH 9 (**c**). Results are given as mean values of three replicates. *Asterisk* indicates significant differences ($P < 0.05$) between CH and MW treatments. *Different letters* indicate significant differences ($P < 0.05$) in solubility across all treatments

Table 2 Surface hydrophobicity of fish protein hydrolysates from conventional heating and microwave-assisted heating with the alkaline endopeptidase enzyme Alcalase

Treatments (time/E:S)	Surface hydrophobicity (slope of RFI vs. % protein)	
	CH-FPH	MW-FPH
5 min/0.5%	20.9 ± 0.18a,*	6.1 ± 0.01d
15 min/0.5%	16.2 ± 0.04b,*	5.8 ± 0.01de
3 min/1.7%	8.9 ± 0.01c,*	5.1 ± 0.004def
5 min/3.0%	4.7 ± 0.01ef	3.9 ± 0.002f

Values in table are presented as the mean of two replicates ± SD. The asterisks indicate significant differences at $P < 0.05$ between CH and MW treatments (rows). Different lowercase letters indicate significant differences at $P < 0.05$ in surface hydrophobicity across all treatments (columns)

RFI relative fluorescence intensity

Likewise, at pH 3, MW-FPH hydrolyzed for 3 min (1.7% E:S) also showed a higher ($P < 0.05$) solubility than CH-FPH. No differences were seen for the other hydrolysis treatments at pH 3 and pH 9.

Surface hydrophobicity

The surface hydrophobicity values (S_o), represent the number of hydrophobic groups on the surface of protein. The S_o of CH-FPH and MW-FPH was measured using PRODAN as the fluorescent probe, and the results are given in Table 2. The results revealed that most CH-FPH treatments (5 min/0.5% E:S, 15 min/0.5% E:S and 3 min/1.7% E:S) had significantly higher ($P < 0.05$) S_o values than the MW-FPH treatments, indicating higher surface hydrophobicity for CH-FPH compared to MW-FPH.

Emulsifying properties

The EAI at 0 min and ES at 30, 60 and 120 min of both CH-FPH and MW-FPH are shown in Fig. 3. Across all treatments, MW-FPH presented a higher ($P < 0.05$) EAI than CH-FPH. MW-FPH hydrolyzed for 5 min (0.5% E:S) produced the highest EAI, followed by hydrolysis for 15 min (0.5% E:S). For the MW-FPH treatments, with increasing E:S ratio and hydrolysis time, there was a decreasing trend on emulsion capacity. In terms of ES, of all the MW-FPH

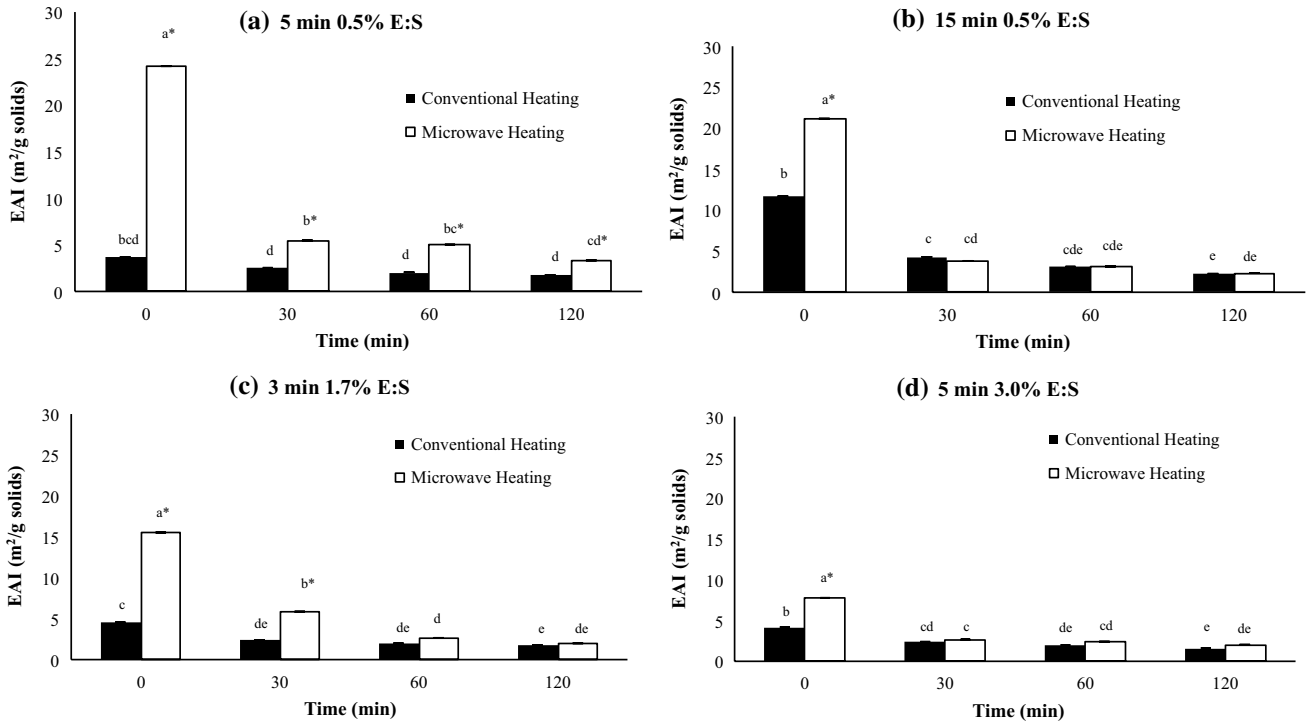

Fig. 3 Emulsion activity index (*EAI*) (time 0 min) and emulsion stability [ES; foam stability at 30, 60, 120 min] of FPH from CH and MW. Results are mean values from three replicates using treatments hydrolyzed for 5 min with 0.5% (E:S) (**a**), 15 min with 0.5% (E:S) (**b**), 3 min with 1.7% (E:S) (**c**) and 5 min with 3.0% (E:S) (**d**). *Asterisk* indicates significant differences ($P < 0.05$) between CH and MW treatments. *Different letters* indicate significant differences ($P < 0.05$) in ES over time across all treatments

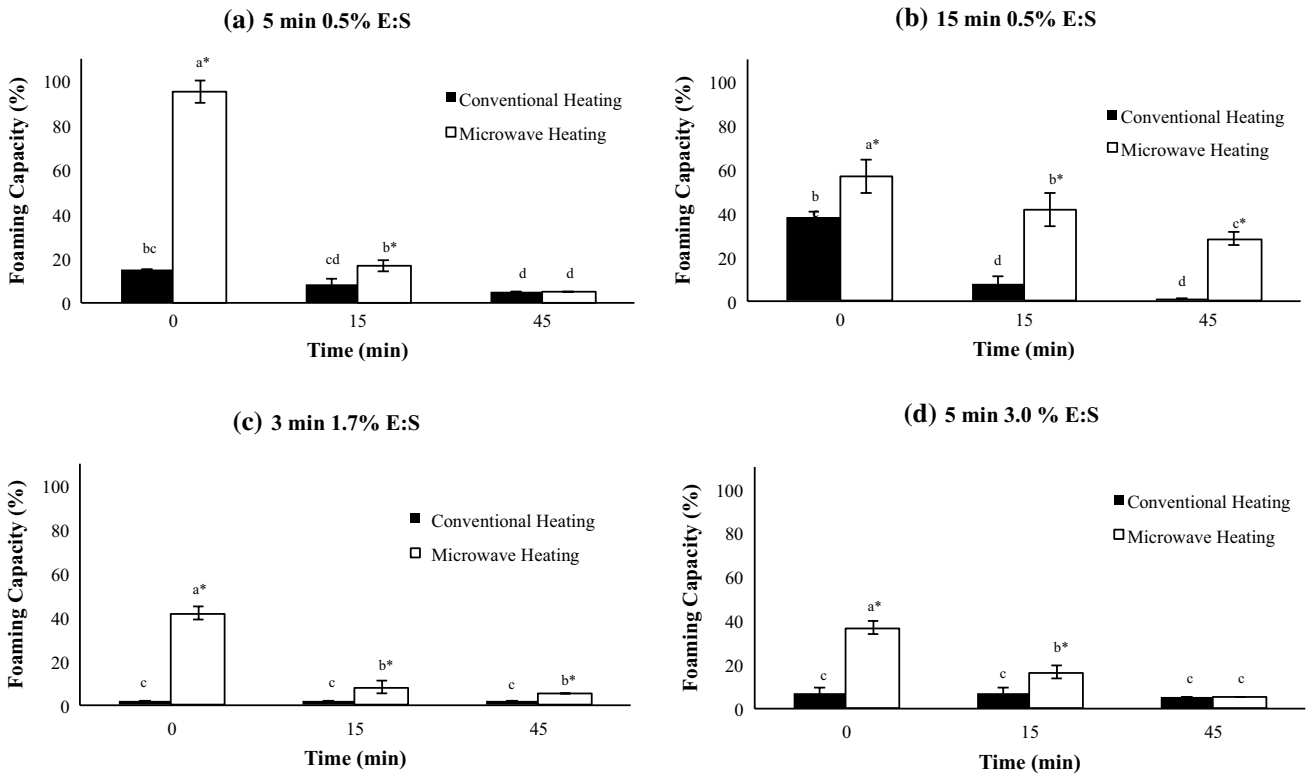

Fig. 4 Foaming capacity (measured at time 0 min) and foam stability (FS) (measured after 15 and 45 min) of FPH from CH and MW. Results represent mean values from 3 replicate treatments hydrolyzed for 5 min with 0.5% (E:S) (**a**), 15 min with 0.5% (E:S) (**b**), 3 min with 1.7% (E:S) (**c**) and 5 min with 3.0% (E:S) (**d**). *Asterisk* indicates significant differences ($P < 0.05$) between CH and MW treatments. *Different letters* indicate significant differences ($P < 0.05$) in foam stability over time across all treatments

treatments, only MW-FPH at 5 min (0.5% E:S) was more stable, for up to 120 min, than CH-FPH ($P < 0.05$; Fig. 3a).

Foaming properties

Results for FC (at 0 min) and FS (at 15 and 45 min) from CH-FPH and MW-FPH are given in Fig. 4. MW-FPH showed higher ($P < 0.05$) FC than CH-FPH across all treatments. MW-FPH treatments had significantly higher ($P < 0.05$) FS over time than CH-FPH. Overall, MW-FPH (15 min, 0.5% *E*:*S*) showed higher ($P < 0.05$) FC and FS than CH-FPH.

Free amino acid composition

The free amino acid composition of CH-FPH and MW-FPH is shown in Table 3. All essential amino acids were present in the treatments of both CH-FPH and MW-FPH, with relatively high amounts of leucine (20–28%). CH-FPH and MW-FPH treatments showed the presence of hydrophobic amino acids, such as leucine and alanine.

Antioxidant activity of FPH

2,2-Diphenyl-1-picryhydrazyl

A summary of results for DPPH radical scavenging for both CH-FPH and MW-FPH at 40 mg/ml concentration is given in Table 4. MW-FPH showed significantly higher ($P < 0.05$) DPPH radical scavenging activity than CH-FPH with increasing time and E:S ratio (increasing DH). Figure 5 shows the DPPH radical scavenging activity of both CH and MW treatments at lower protein concentrations (3.5, 2.5, 1.5 and 0.5 mg/ml). As expected, there was an increase in DPPH radical scavenging properties for most treatments with increasing sample concentration. In particular, the MW-FPH treatment for 5 min (0.5% E:S) and 15 min (0.5% E:S) had significantly higher ($P < 0.05$)

Table 3 Free amino acid composition of fish protein hydrolysates derived from conventional heating and microwave-assisted heating with the alkaline endopeptidase enzyme Alcalase

Amino acid	Treatments (time/E:S)							
	5 min/0.5% E:S		15 min/0.5% E:S		3 min/1.7% E:S		5 min/3% E:S	
	CH	MW	CH	MW	CH	MW	CH	MW
Trp[a]	0.6	0.5	0.6	0.6	0.6	0.6	0.7	0.6
Phe[a]	5.5	4.6	4.8	5.7	5.8	5.8	6.0	5.8
Leu[a]	22.7	21.8	20.2	27.1	27.5	27.9	27.3	28.7
Met[a]	2.7	2.4	2.3	3.0	3.3	2.9	3.2	3.1
Tyr	2.4	1.6	2.1	2.0	2.8	2.0	2.9	2.3
Ile[a]	6.1	6.7	6.3	7.3	4.8	6.0	5.4	5.7
Pro	2.2	1.8	2.3	1.3	1.2	0.8	1.4	0.7
Val[a]	3.7	4.3	3.7	4.3	3.4	3.9	3.5	4.0
Ala	12.9	14.4	14.7	13.0	11.5	12.4	11.4	12.0
Thr[a]	2.3	1.9	2.3	2.3	2.1	2.4	2.6	2.3
Glu	6.7	5.9	6.8	5.8	5.9	5.4	5.9	5.1
Gly	8.1	9.8	9.9	6.8	6.3	7.1	6.8	6.5
Asp	1.8	1.8	1.8	1.3	1.6	1.1	1.5	1.2
Ser	4.2	4.1	4.3	3.6	3.7	3.8	4.1	3.7
Asn	0.9	0.8	0.9	1.1	1.0	1.2	1.2	1.0
Cys-Cys[b]	0	0	0	0	0	0	0	0
Gln	1.7	1.6	1.6	1.9	1.7	1.7	1.8	1.7
His[a]	5.4	6.9	5.7	4.6	6.9	6.8	5.2	7.1
Lys[a]	6.3	5.7	6.2	4.9	5.9	4.8	5.3	4.8
Arg	3.6	3.4	3.4	3.3	3.9	3.3	3.7	3.5

Values in table are presented as the percentage of free amino acids in FPH

[a] Essential amino acids

[b] The value for cysteine/cysteine is probably underestimated as no pre-derivatization was performed.

Table 4 Antioxidant properties of rainbow trout fish protein hydrolysates produced from conventional heating and microwave-assisted hydrolysis

Treatments	DPPH radical scavenging (%)[a]		Ferric ion reduction capacity (Abs_{700})[b]	
	CH-FPH	MW-FPH	CH-FPH	MW-FPH
5 min 0.5%	1d	55d*	0.770bc	0.719c
15 min 0.5%	1d	71a*	0.742c	0.732c
3 min 1.7%	1d	50c*	0.796b	0.996a*
5 min 3.0%	72a	72a	0.745bc	0.959a*
BHT[c]	98		0.91	
BHA[c]	98		0.84	

The asterisks indicate significant differences at $P < 0.05$ between CH and MW treatments (rows). Different lowercase letters indicate significant differences at $P < 0.05$ in antioxidant activity across all treatments (columns)

[a] DPPH (2,2-diphenyl-1-picryhydrazyl) radical scavenging activity (%) of CH-FPH and MW-FPH at sample concentration of 40 mg/ml

[b] Ferric ion reduction capacity of CH-FPH and MW-FPH (at 3 mg/ml) measured as absorbance at 700 nm (Abs_{700})

[c] BHT (butylated hydroxytoluene) and butylated hydroxyanisole (BHA) at a sample concentration of 0.045 mg/ml

DPPH radical scavenging activity than the CH-FPH treatment under similar conditions.

Ferric ion reducing antioxidant capacity

As shown in Table 4, MW-FPH treatments from 3 min (1.7% E:S) and 5 min (3% E:S) had significantly higher ($P < 0.05$) FIRC reducing power than comparable CH-FPH treatments. MW-FPH treatments from 5 min (0.5% E:S) and 15 min (0.5% E:S) had no significant differences ($P > 0.05$) in FIRC reducing power when compared to comparable CH-FPH treatments.

Discussion

The DH results indicate the extent of peptide bond cleavage (higher DH) in the presence of higher amounts of enzymes and use of MW-assisted hydrolysis, with the protein cleaved by the enzyme into free amino acids and smaller peptides [34]. Protein hydrolysates with high DH values are reported to contain functional and antioxidant peptides [7].

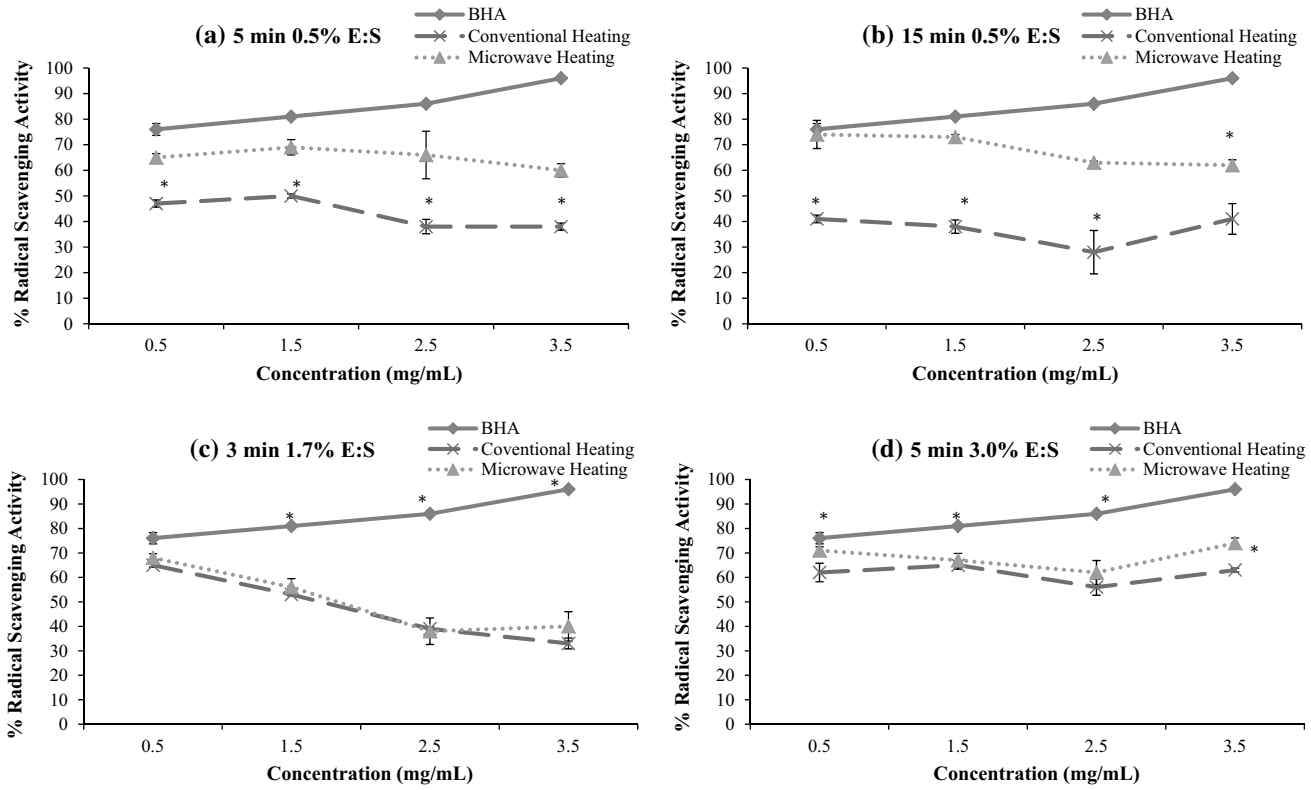

Fig. 5 2,2-Diphenyl-1-picryhydrazyl radical scavenging activity of butylated hydroxyanisole (*BHA*) and FPH from CH and MW treatments. Results are mean values of three replicates at FPH concentrations of 0.5, 1.5, 2.5 and 3.5 mg/ml using treatments hydrolyzed for 5 min with 0.5% (E:S) (**a**), 15 min with 0.5% (E:S) (**b**), 3 min with 1.7% (E:S) (**c**) and 5 min with 3.0% (E:S) (**d**). *Asterisk* indicates significant differences ($P < 0.05$) among BHA, CH and MW treatments

The difference in the DH observed between CH-FPH and MW-FPH (Table 1) could be due to the heating method applied. Conventional heating uses both conduction and convection heating, which delays heat transfer and produces a steady rate of hydrolysis by slower unfolding of the protein [22, 35]. In CH-FPH, the DH was higher with increased amounts of enzyme substrate and hydrolysis time. However, in the CH-FPH treatment (1.7% E:S, 3 min hydrolysis) there was a 12% DH whereas in the CH-FPH treatment (3% E:S, 5 min hydrolysis) the DH was 9%. We believe that this difference is caused by the enzyme at 3% concentration in the CH treatment saturating the substrate faster when more amount of enzyme is used. According to Williams et al. [36], increasing the proportion of the protease can negatively affect the hydrolysis due to the enzyme essentially producing a "limited digestion" where all, or most of, the potentially susceptible peptide bonds have been hydrolyzed. During proteolysis, a condensation reaction process can take place where a kinetically driven reversal occurs when high product (peptide) concentrations are used, producing new high molecular weight proteins [36]. In the case of the microwave treatments (MW-FPH), the increase in DH can be a result of microwave heating inducing a modification of the flexibility of the enzyme, consequently changing or accelerating the enzymatic properties [37]. Studies have shown that when the microwave-assisted hydrolysis was applied, a higher DH was achieved due to the rapid heat transfer accelerating the proteolytic cleavage of proteins [21, 22]. Microwave-assisted hydrolysis induces rapid heating from polar constituents within the protein that enables dipole rotation and ionic drifting [38]. The rapid heat transfer allows the protein to become unfolded faster, thus increasing the rate of hydrolysis [39].

Similarly, alkaline protease milk protein hydrolysates that were pretreated with microwave heating for 8 min provided higher DH, with a 184.9% increase compared to the control (non-pretreatment milk protein) DH of 13.3%. The pretreatment with microwave heating caused the peptide bonds to become more susceptible to enzyme hydrolysis due to an increased initial rate of hydrolysis and the catalytic efficiency of the enzymes, thereby allowing folding and unfolding to occur within the protein [39, 40]. Other studies have shown that combining microwave radiation with high pressure-generated rapid enzymatic hydrolysis of β-lactoglobulin produces smaller peptides in minutes as opposed to hours when conventional heating is used [21,

23]. These results are comparable to our results of high DH (smaller peptides) in the MW-FPH.

SDS-PAGE revealed that the hydrolysates had different peptide profiles (Fig. 1a, b). As observed in the gels, by increasing the enzyme substrate ratio to 1.7 and 3%, the number of larger molecular weight bands disappeared into hydrolysate fractions, with a thick band at <14.4 kDa. In the case of a lower E:S ratio (0.5%), numerous bands of varying molecular weights were still visible in the gel. Other studies have shown that peptides of the ideal molecular size that are able to provide good foaming and emulsifying properties are derived from limited hydrolysis that generates larger peptides; in contrast, extensive hydrolysis results in smaller peptides which reduces these functional properties [7, 41, 42]. Another study has reported that protein hydrolysates with a molecular weight of <13 kDa can have antioxidant activity [14]. Overall, our SDS-PAGE results indicate that enzymatic hydrolysis occurred in rainbow trout frames treated by either conventional heating or microwave-assisted heating.

Solubility is an important functional property in protein as it has a great influence on other functional properties, such emulsion and foaming activity. Our results show that at pH 7, MW-FPH treatments hydrolyzed for 15 min (0.5% E:S) and 3 min (1.7% E:S) exhibited higher solubility than CH-FPH at similar treatments (Fig. 2b). In addition, MW-FPH hydrolyzed 3 min (1.7% E:S) showed a higher solubility at pH 3 than did CH-PH (Fig. 2a). The high solubility in the FPH corresponds with the hydrolysis, where smaller peptides have increased polar and ionizable groups for enhanced interaction in water [43]. Overall, the MW-FPH treatment (3 min, 1.7% E:S) was considered to be the best treatment for solubility since higher solubility was observed at both pH 3 and 7. Below pH 4, carboxyl groups tend to shift towards unionized forms, which reduces peptide affinity to water molecules [44]. Changes in pH also affect the overall net charge of hydrolysates, which in turn influences repulsive and attractive forces [45]. Improved solubility of the hydrolyzed protein can be attributed to a general opening up of the enzymatic hydrolysis, where degradation of the protein units leads to increased repulsive interactions between peptides and hydrogen bonding with water molecules [44]. Unfolding of the protein molecules occurred in both CH-FPH and MW-FPH where both nonpolar and polar amino acid groups buried inside the protein molecules were exposed on the surface of the protein molecule. Polar amino acids are able to interact with the water molecules to form hydrogen bonds and electrostatic interactions, which result in increased solubility [45]. Other factors, such as small molecular size and the presence of hydrophilic groups, may have had an effect on protein solubility. Alcalase hydrolysis generated hydrophilic groups during cleavage which produced electrostatic repulsion between peptides, resulting in increased protein solubility [46]. Uluko et al. reported that microwave heating as a pretreatment prior to enzymatic hydrolysis increases the solubility of milk protein by generating smaller peptides [40]; these authors also showed a strong correlation between DH and protein solubility when the pretreatment time increased. Similar results of high solubility at pH 7 and 9 have been reported for FPH of salmon *Salmosalar*, yellow stripe trevally *Selaroides leptolepis* and blue whiting *Micromesistius poutassou* [32, 45, 47, 48]. The high protein solubility of MW-FPH indicates potential applications in beverage industry and formulated food systems.

The number of hydrophobic groups on the surface of the protein, as indicated by surface hydrophobicity (S_o), was measured to determine if the proteins were surface active. The increase in enzyme concentration (E:S) and hydrolysis time, which correlated with increasing DH of the FPH treatments (CH and MW), led to a decrease in surface hydrophobic (Table 2). One explanation for this result is that the protein molecules may have undergone protein aggregation due to the hydrophobic interactions or sulfhydryl group/disulfide bond interchange reactions, which would decrease the S_o [49]. Microwave radiation can accelerate the rate of protein unfolding due to faster heating rates and increase the chances of collision between partially unfolded molecules, leading to protein aggregation [39]. Furthermore, microwave radiation is known to induce modifications of the proteolytic enzyme's flexibility due to exposure to the electromagnetic field, thus altering the hydrophobicity of the protein [37, 50]. Additionally, the decrease in S_o as a result of increasing E:S and hydrolysis time can be a result of an increase in the number of hydrophilic groups released, as compared to the number of hydrophobic groups released, from the peptide after hydrolysis [51]. Although the free amino acid composition (Table 3) of the FPH in both the conventional heating and microwave-assisted heating treatments indicates that hydrophobic amino acids were present, a decreasing S_o was seen across all treatments, suggesting that the hydrophobic groups may not be surface active but rather present in free form. Similar patterns were found in sardine *Sardina pilchardus* hydrolysates using Alcalase where the S_o of FPH decreased with increasing DH [41]. A study in scallop *Patinopecten yessoensis* protein hydrolysates also revealed that the surface hydrophobicity of protein hydrolysis decreased with increasing DH due to enzymatic cleavage of hydrophobic clusters [48].

MW-FPH treatments were able to form an emulsion (Fig. 3). This was seen across all treatments where MW-FPH had a higher EAI than CH-FPH. Specifically, MW-FPH hydrolyzed 5 min (0.5% E:S) provided the highest emulsion capacity and stability. The conformational changes and protein–protein interactions occurring within the MW-FPH as well as the rapid diffusion of smaller

peptides to the interface influenced the formation and stabilization of the emulsion by forming a proteinaceous interfacial layer around oil droplets [37, 45, 52]. A study by Nalinanon et al. [53] reported that muscle hydrolysates of ornate threadfin bream *Nemipterus hexodon* with increasing DH were able to assemble at the interface but could not stabilize the interface tension over time due to a lack of amphiphilic properties.

Similar observations were seen for foaming capacity, where the lower molecular weight peptides (higher DH) derived from microwave-assisted hydrolysis increased the rate of diffusion to the interface, thus improving the protein's foaming capacity (Fig. 4). As expected, the small peptides were able to diffuse more rapidly to the air–water interface and encapsulate air bubbles, thereby developing a foam [54]. MW-FPH 15 min (0.5% E:S) also generated a stable foam over time due to the interaction of peptides within the film matrix that surrounds the air bubbles and/or due to the flexibility of the peptide structure [8]. Modifications of protein structure caused by microwave radiation allowed higher protein unfolding and protein–protein interaction that assisted in the formation of a multilayer cohesive protein film at the interface, preventing coalescence and maintaining stability over time [55]. Additionally, microwave radiation is known to have an effect on the composition and net charge of the peptide due to modifications in the flexibility of the protein structure, which in turn impacts the air–water interface [43, 52]. Nguyen et al. reported that using microwave-intensified enzymatic deproteinization on Australian rock lobster shell showed high FC and FS over time [43], and Khaled et al. found that protein hydrolysates from sardinelle *Sardinella aurita* had higher FC and FS due to increased DH [56]. However, hydrolysates from yellow stripe trevally had low FC and FS although a higher DH was obtained [32]. Different protein composition and amino acid sequence in different fish may be a reason for this observed variability in FC of the FPH.

Regarding free amino acids (Table 3), all essential amino acids were present in both the CH-FPH and MW-FPH from all treatments. Foh et al. also reported FPH from various fish species with high amounts of essential amino acids [57]. Therefore, it would appear that these FPH could be used as dietary protein supplements within food formulations to create nutritive products. Additionally, CH-FPH and MW-FPH treatments showed the presence of hydrophobic amino acids, such as leucine and alanine. Hydrophobic amino acids are important because their effect on the functional properties of proteins, such as increased solubility in lipids and antioxidant activity. Specifically, hydrophobic amino acids and histidine are known to have antioxidative properties due to their inhibition of lipid oxidation through chelating and lipid trapping of the imidazole ring [58]. Histidine is an aromatic compound and consequently capable of stabilizing free radicals by donating an electron [59]. Several other amino acids, such as tyrosine, methionine, histidine and lysine, have also shown antioxidant activity [60]. Therefore, our results suggest that FPH from rainbow trout by-products contain hydrophobic amino acids and histidine that could impart antioxidant activity.

Hydrolysates with antioxidant capacity must be able to donate hydrogen to reactive oxygen species in order to stabilize them. The radical scavenging activity of a hydrolysate depends on the number of bioactive peptides released that in turn is determined largely by the hydrolysis conditions used (e.g. substrate, proteolytic enzyme(s) used, pH, temperature, E:S ratio and hydrolysis time) [61]. The radical scavenging ability of MW-FPH could be attributed to the increase in solubility of the smaller peptides (higher DH) and presence of hydrophobic amino acids. Studies have shown that changes in the size, level and composition of the free amino acids and small peptides influence the anti-oxidative activity [62]. For example, pea seeds and chickpea protein hydrolysates have high radical scavenging properties due to their increased concentration of hydrophobic amino acids [63, 64]. Similarly, the presence of aromatic amino acids such as tyrosine and phenylalanine in yellowfin sole *Limanda aspera* and grass carp muscle *Ctenopharyngodon idella* hydrolysates contribute to their radical scavenging activity [14, 65]. The DPPH radical scavenging activity of round scad muscle *Decapterus punctatus* and Atlantic cod *Gadus morhua* backbone protein hydrolysates was found to increase with increasing DH [6, 66]. Peptide concentration can also influence the scavenging capacity of the hydrolysate. A study using Atlantic cod hydrolyzed with commercial proteases showed that the DPPH radical scavenging activity of the peptide fractions obtained was concentration dependent, with greater scavenging capacity at higher concentrations of peptides [67]. In our study, when 40 mg/ml of FPH was tested for DPPH scavenging activity (Table 4), the MW-FPH treatments showed >50% DPPH scavenging activity compared to CH-FPH. Conversely, when lower FPH concentrations were tested (0.5–3.5 mg/ml; Fig. 5), the MW-FPH treatments still showed improved DPPH activity compared to the CH-FPH treatments. An interesting observation is that the CH-FPH was not able to display DPPH activity at 40 mg/ml, but did at the lower concentrations. We speculate that when the higher concentration was used, the lack of scavenging activity could be due to poor solubility of the CH-FPH in the aqueous medium of the DPPH assay. The same was not observed in the 40 mg/ml MW-FPH, where higher DH and protein conformation changes caused by the microwave-assisted hydrolysis resulted in improved solubility during the assay. Additional studies are needed to evaluate the metal chelating capacity of the peptides at different concentrations.

In the case of the ferric ion reducing capacity (Table 4), the microwave radiation treatments with higher DH (3 min 1.7% E:S and 5 min 3.0% E:S) were capable of chelating the ions and forming more stable products. It is known that peptide size plays an important role in the chelating capacity of bioactive peptides. Chelating activity is dependent on the molecular weight, structure, amino acid composition and steric effects of the peptides [68, 69]. Additionally, it is plausible that microwave heating altered the structure of the protein to generate active amino acids within the hydrolysates, thereby contributing to high DH and the ability to react with free radicals to form stable products compared to conventional heating [70]. The same trend of increasing DH with higher reducing power was also seen for black scabbardfish *Aphanopus carbo* [71] and mackerel *Scomber austriasicus* hydrolysates [62]. For MW-FPH, the trend of reducing power was similar to the DPPH radical scavenging activities where higher DH in the MW-FPH treatments exhibited higher antioxidant properties. Several studies have also reported that the reducing power increased with increased amount of sample [72, 73]. Other research on antioxidant properties, such as that on sardinelle *sardinella aurita* by-products [74], revealed stronger reducing power capabilities with higher DH.

In conclusion, the results of our study show that rainbow trout (by-products) hydrolysates can be successfully prepared using microwave-assisted enzymatic hydrolysis. Functional properties (protein solubility, emulsifying activity, foaming capacity and foaming stability) of the microwave-assisted hydrolysates were equal to or improved compared to hydrolysates derived from conventional heating methods (water bath). The combination of microwave-assisted hydrolysis with a low enzyme concentration (such as 0.5% E:S) was sufficient to prepare functional hydrolysates within a short period of time (15 min). However, in terms of antioxidant activity, the microwave-assisted hydrolysis required a higher enzyme concentration (3% E:S) to generate antioxidant peptides. The results from this study indicate that MW-FPH could be a potential source of antioxidants. In addition, these MW-FPH by-products have the potential to be used as value-added food ingredients where functional properties are sought after (e.g. protein beverage formulations, bread, emulsions, etc.). Ideally, a treatment that is optimized for both functional and antioxidant properties is desired. Further investigation on treatments using different enzymes at various hydrolysis times and concentrations should be explored for the identification of hydrolysates with the desired functional and antioxidant properties. Studies on amino acid distribution in FPH also need to be conducted to obtain insights into the specificity of microwave treatment in selective cleavage of the peptide bonds which will in turn aid in elucidating the mechanism behind the enhanced functional and antioxidant properties.

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References

1. Ghaly A, Ramakrishnan V, Brooks M, Budge S, Dave D (2013) Fish processing wastes as a potential source of proteins, amino acids and oils: a critical review. J Microb Biochem Technol 5:107–129
2. Torres J, Chen Y, Rodrigo-Garcia J, Jaczynski J, Shahidi F (2007) Recovery of by-products from seafood processing streams. In: Shahidi F (ed) Maximising value marine by-products. Woodhead Publ Ltd., Cambridge, pp 65–90
3. Kristinsson H, Rasco B (2000) Fish protein hydrolysates: production, biochemical, and functional properties. Crit Rev Food Sci Nutr 40(1):43–81
4. Haard N (2001) Enzymic modification proteins in food systems in chemical and functional properties of food proteins. CRC Press, Boca Raton, pp 155–190
5. Liceaga-Gesualdo A, Li-Chan E (1999) Functional properties of fish protein hydrolysate from herring (*Clupea harengus)*. J Food Sci 64(6):1000–1004
6. Thiansilakul Y, Benjakul S, Shahidi F (2007) Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). Food Chem 103(4):1385–1394
7. Pacheco-Aguilar R, Mazorra-Manzano M, Ramírez-Suárez J (2008) Functional properties of fish protein hydrolysates from Pacific whiting (*Merluccius productus*) muscle produced by a commercial protease. Food Chem 109(4):782–789
8. Giménez B, Alemán A, Montero P, Gómez-Guillén M (2009) Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. Food Chem 114(3):976–983
9. Pasupuleti V, Braun S (2008) State of the art manufacturing of protein hydrolysates. In: Paupuleti V, Demain AL (eds) Protein hydrolysates in biotechnology. Springer SBM, Dordrecht
10. Quaglia G, Orban E (1987) Influence of the degree of hydrolysis on the solubility of the protein hydrolysates from sardine (*Sardina pilchardus*). J Sci Food Agric 38(3):271–276
11. Benjakul S, Morrissey M (1997) Protein hydrolysates from Pacific whiting solid wastes. J Agric Food Chem 45(9):3423–3430
12. Adler-Nissen J (1986) Enzymic hydrolysis of food proteins. Elsevier, Amsterdam
13. Kristinsson H, Rasco B (2000) Kinetics of the hydrolysis of Atlantic salmon (*Salmo salar*) muscle proteins by alkaline proteases and a visceral serine protease mixture. Process Biochem 36(1):131–139
14. Jun S, Park P, Jung W, Kim S (2004) Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. Eur Food Res Technol 219(1):20–26
15. Samaranayaka A, Li-Chan E (2008) Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (*Merluccius productus*). Food Chem 107(2):768–776
16. Je J, Park P, Kim S (2005) Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. Food Rese Int 38(1):45–50
17. Ohlsson T, Bengtsson N (2001) Microwave technology and foods. Adv Food Nutr Res 43:65–140

18. Roy I, Mondal K, Gupta M (2003) Accelerating enzymatic hydrolysis of chitin by microwave pretreatment. Biotechnol Prog 19(6):1648–1653
19. de la Hoz A, Diaz-Ortiz A, Moreno A (2005) Microwaves in organic synthesis. Thermal and non-thermal microwave effects. Chem Soc Rev 34(2):164–178
20. Singh A, Orsat V, Raghavan V (2013) Soybean hydrophobic protein response to external electric field: a molecular modeling approach. Biomolecules 3(1):168–179
21. Izquierdo F, Peñas E, Baeza M, Gomez R (2008) Effects of combined microwave and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins. Int Dairy J 18(9):918–922
22. Pramanik B, Mirza U, Hain Y, Liu Y, Bartner P, Weber P, Bose A (2002) Microwave-enhanced enzyme reaction for protein mapping by mass spectrometry: a new approach to protein digestion in minutes. Protein Sci 11(11):2676–2687
23. Izquierdo F, Alli I, Gómez R, Ramaswamy H, Yaylayan V (2005) Effects of high pressure and microwave on pronase and α-chymotrypsin hydrolysis of β-lactoglobulin. Food Chem 92(4):713–719
24. Association of Official Analytical Chemists (AOAC) International (2005) In: Horwitz W (ed) Official methods of analysis of AOAC International, 18th edn. AOAC, Rockville
25. Adler-Nissen J (1979) Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J Agric Food Chem 27(6):1256–1262
26. Adler-Nissen J (1984) Control of the proteolytic reaction and of the level of bitterness in protein hydrolysis processes. J Chem Technol Biotechnol 34(3):215–222
27. Mueller J, Liceaga A (2016) Characterization and cryoprotection of invasive silver carp (*Hypophthalmicthys molitrix*) protein hydrolysates. J Aquat Food Prod Technol 25(1):131–143
28. Chobert J, Bertrand-Harb C, Nicolas M (1988) Solubility and emulsifying properties of caseins and whey proteins modified enzymically by trypsin. J Agric Food Chem 36(5):883–892
29. Pearce K, Kinsella J (1978) Emulsifying properties of proteins: evaluation of a turbidimetric technique. J Agric Food Chem 26(3):716–723
30. Alizadeh-Pasdar N, Li-Chan E (2000) Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. J Agric Food Chem 48(2):328–334
31. Cheung I, Liceaga A, Li-Chan E (2009) Pacific hake (*Merluccius productus*) hydrolysates as cryoprotective agents in frozen Pacific cod fillet mince. J Food Sci 74(8):588–594
32. Klompong V, Benjakul S, Kantachote D, Shahidi F (2007) Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem 102(4):1317–1327
33. Bougatef A, Hajji M, Balti R, Lassoued I, Triki-Ellouz Y, Nasri M (2009) Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases. Food Chem 114(4):1198–1205
34. Tanuja S, Aiswarya H, Zynudheen A, Joshy C (2014) Functional and antioxidative properties of fish protein hydrolysate (FPH) produced from the frame meat of striped catfish *Pangasianodon hypophthalmus* using alkaline protease alcalase. Indian J Fish 61(2):82–89
35. Mazumder S, Laskar D, Prajapati D, Roy M (2004) Microwave-induced enzyme-catalyzed chemoselective reduction of organic azides. Chem Biodivers 1(6):925–929
36. Williams R, Brownsell V, Andrews A (2001) Application of the plastein reaction to mycoprotein: I. Plastein Synth Food Chem 72(3):329–335
37. Rejasse B, Lamare S, Legoy M, Besson T (2007) Influence of microwave irradiation on enzymatic properties: applications in enzyme chemistry. J Enzym Inhib Med Chem 22(5):519–527
38. Ho K, Ferruzzi M, Liceaga A, San Martín-González M (2015) Microwave-assisted extraction of lycopene in tomato peels: effect of extraction conditions on all-trans and cis-isomer yields. LWT Food Sci Technol 62(1):160–168
39. Uluko H, Zhang S, Liu L, Tsakama M, Lu J, Lv J (2015) Effects of thermal, microwave, and ultrasound pretreatments on antioxidative capacity of enzymatic milk protein concentrate hydrolysates. J Funct Foods 18:1138–1146
40. Uluko H, Zhang S, Liu L, Chen J, Sun Y, Su Y, Lv J (2013) Effects of microwave and ultrasound pretreatments on enzymolysis of milk protein concentrate with different enzymes. Int J Food Sci Technol 48(11):2250–2257
41. Quaglia G, Orban E (1990) Influence of enzymatic hydrolysis on structure and emulsifying properties of sardine (*Sardina pilchardus*) protein hydrolysates. J Food Sci 55(6):1571–1573
42. Jeon Y, Byun H, Kim S (1999) Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. Process Biochem 35(5):471–478
43. Nguyen T, Zhang W, Barber A, Su P, He S (2016) Microwave-intensified enzymatic deproteinization of Australian rock lobster shells (*Jasus edwardsii*) for the efficient recovery of protein hydrolysate as food functional nutrients. Food Bioprocess Technol 9(4):628–636
44. Zhao Q, Xiong H, Selomulya C, Chen X, Zhong H, Wang S, Zhou Q (2012) Enzymatic hydrolysis of rice dreg protein: effects of enzyme type on the functional properties and antioxidant activities of recovered proteins. Food Chem 134(3):1360–1367
45. Gbogouri G, Linder M, Fanni J, Parmentier M (2004) Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. J Food Sci 69(8):615–622
46. Wu W, Hettiarachchy N, Qi M (1998) Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. J Am Oil Chem Soc 75(7):845–850
47. Geirsdottir M, Sigurgisladottir S, Hamaguchi P, Thorkelsson G, Johannsson R, Kristinsson H, Kristjansson M (2011) Enzymatic hydrolysis of blue whiting (*Micromesistius poutassou*); functional and bioactive properties. J Food Sci 76(1):14–20
48. Jin W, Wu H, Zhu B, Ran X (2012) Functional properties of gelation-like protein hydrolysates from scallop (*Patinopecten yessoensis*) male gonad. Eur Food Res Technol 234(5):863–872
49. Laligant A, Dumay E, Casas Valencia C, Cuq J, Cheftel J (1991) Surface hydrophobicity and aggregation of beta-lactoglobulin heated near neutral pH. J Agric Food Chem 39(12):2147–2155
50. de Pomerai D, Smith B, Dawe A, North K, Smith T, Archer D, Candido E (2003) Microwave radiation can alter protein conformation without bulk heating. FEBS Lett 543(1–3):93–97
51. Paraman I, Hettiarachchy N, Schaefer C, Beck M (2006) Physicochemical properties of rice endosperm proteins extracted by chemical and enzymatic methods. Cereal Chem 83(6):663–667
52. Ponne C, Bartels P (1995) Interaction of electromagnetic energy with biological material—relation to food processing. Radiat Phys Chem 45(4):591–607
53. Nalinanon S, Benjakul S, Kishimura H, Shahidi F (2011) Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. Food Chem 124(4):1354–1362
54. Wierenga P, Gruppen H (2010) New views on foams from protein solutions. Curr Opin Colloid Interface Sci 15(5):365–373
55. Ashraf S, Saeed S, Sayeed S, Ali R (2012) Impact of microwave treatment on the functionality of cereals and legumes. Int J Agric Biol 14(3):365–370

56. Khaled H, Ktari N, Ghorbel-Bellaaj O, Jridi M, Lassoued I, Nasri M (2014) Composition, functional properties and in vitro antioxidant activity of protein hydrolysates prepared from sardinelle (*Sardinella aurita*) muscle. J Food Sci Technol 51(4):622–633
57. Foh M, Amadou I, Foh B, Kamara M, Xia W (2010) Functionality and antioxidant properties of tilapia (*Oreochromis niloticus*) as influenced by the degree of hydrolysis. Int J Mol Sci 11(4):1851–1869
58. Pihlanto A (2006) Antioxidative peptides derived from milk proteins. Int Dairy J 16(11):1306–1314
59. Nazeer R, Kumar N (2011) Purification and identification of antioxidant peptide from black pomfret *Parastromateus niger* viscera protein hydrolysate. Food Sci Biotechnol 20(4):1087–1094
60. Chen H, Muramoto K, Yamauchi F, Nokihara K (1996) Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. J Agric Food Chem 44(9):2619–2623
61. Sila A, Bougatef A (2016) Antioxidant peptides from marine by-products: isolation identification and application in food systems. A Rev J Funct Foods 21:10–26
62. Wu H, Chen H, Shiau C (2003) Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Res Int 36(9):949–957
63. Pownall T, Udenigwe C, Aluko R (2010) Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L) enzymatic protein hydrolysate fractions. J Agric Food Chem 58(8):4712–4718
64. Li Y, Jiang B, Zhang T, Mu W, Liu J (2008) Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). Food Chem 106(2):444–450
65. Ren J, Zhao M, Shi J, Wang J, Jiang Y, Cui C, Xue S (2008) Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. Food Chem 108(2):727–736
66. Šližytė R, Mozuraitytė R, Martínez-Alvarez O, Falch E, Fouchereau-Peron M, Rustad T (2009) Functional bioactive and antioxidative properties of hydrolysates obtained from cod (*Gadus morhua*) backbones. Process Biochem 44(6):668–677
67. Farvin K, Andersen L, Nielsen H, Jacobsen C, Jakobsen G, Johansson I, Jessen F (2014) Antioxidant activity of Cod (*Gadus morhua*) protein hydrolysates: in vitro assays and evaluation in 5% fish oil-in-water emulsion. Food Chem 149:326–334
68. Yildirim A, Mavi A, Kara A (2001) Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. J Agric Food Chem 49(8):4083–4089
69. Guo L, Hou H, Li B, Zhang Z, Wang S, Zhao X (2013) Preparation, isolation and identification of iron-chelating peptides derived from Alaska pollock skin. Process Biochem 48(5):988–993
70. Tao Y, Shuguo S, Qinlu L, Meihu M, Feijun L, Junwen L (2013) Effects of microwave irradiation pre-treatment of egg white proteins on ant oxidative activity of their hydrolysates prepared with pepsin. Adv J Food Sci Technol 5(7):936–940
71. Batista I, Ramos C, Coutinho J, Bandarra N, Nunes M (2010) Characterization of protein hydrolysates and lipids obtained from black scabbardfish (*Aphanopus carbo*) by-products and antioxidative activity of the hydrolysates produced. Process Biochem 45(1):18–24
72. Zhu K, Zhou H, Qian H (2006) Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. Process Biochem 41(6):1296–1302
73. Nazeer R, Anila Kulandai K (2012) Evaluation of antioxidant activity of muscle and skin protein hydrolysates from giant kingfish, *Caranx ignobilis*. Int J Food Sci Technol 47(2):274–281
74. Bougatef A, Nedjar-Arroume N, Manni L, Ravallec R, Barkia A, Guillochon D, Nasri M (2010) Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. Food Chem 118(3):559–565