

Effect of Microwave Treatments on Antioxidant Activity and Antigenicity of Fish Frame Protein Hydrolysates

Sunantha Ketnawa¹ · Andrea M. Liceaga¹

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Abstract Protein solubility, protein recovery, antioxidant activity, and antigenicity of microwave pretreatment and/or microwave-assisted hydrolysis of trout frame protein hydrolysates was investigated. Treatments consisted of (1) microwave pretreatment at low temperature (55 °C) followed by conventional enzymatic hydrolysis or (2) high temperature (90 °C) followed by conventional enzymatic hydrolysis; (3) microwave pretreatment at high temperature (90 °C) followed by microwave-assisted enzymatic hydrolysis, and (4) no microwave pretreatment followed by microwave-assisted enzymatic hydrolysis. Compared to controls, microwave treatments significantly improved ($P < 0.05$) protein solubility, protein recovery, degree of hydrolysis, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) free radical scavenging radical scavenging activity. Decrease of antigenicity (55–93 %) was obtained in all microwave-treated samples. Based on the results of this study, the use of microwave pretreatment for 5 min at 90 °C, followed by conventional enzymatic hydrolysis with alcalase for 2–10 min was the best treatment condition to produce fish peptides with antioxidant activity and the lowest immunoreactivity. These peptides have potential to be applied as hypoallergenic ingredients in food formulations and nutraceuticals.

Keywords Microwave heating · Fish protein hydrolysates · Antioxidant activity · Antigenicity

✉ Andrea M. Liceaga
aliceaga@purdue.edu

¹ Department of Food Science, Purdue University, 745 Agriculture Mall Dr, West Lafayette, IN 47907, USA

Introduction

Microwaves heat food systems by the absorption of microwave energy, rotation of the bipolar water molecules, and oscillatory migration of the ionic components of the food (Ohlsson and Bengtsson 2001; Izquierdo et al. 2007). During microwave heating, polar molecules such as water align with the continuously changing microwave-generated electric field, leading to accelerated chemical, biological, and physical processes (Roy et al. 2003). Advantages of electromagnetic radiation are mainly the fast, volumetric heating subject to penetration depth, improved reaction yields, nutrient retention, and decreased reaction times compared to conventional heating (Wathey et al. 2002). In food proteins, microwave heating (MH) could be beneficial for supporting enzymatic proteolysis, shortening hydrolysis time while maintaining the amino acid quality (He et al. 2013). Pretreatment by MH is also a well-known strategy to enhance the accessibility of the bonds susceptible to hydrolysis which facilitates more enzyme-cutting sites and subsequent exposure to proteases. It is thought that microwaves assist protein unfolding and increased accessibility of enzymes to peptide bonds resulting in hydrolysates with good bioactivities (Yang et al. 2013). Moreover, MH was studied to evaluate its effect on the protein's structure, leading to the accessibility of sequential epitopes to proteases, thus lowering of allergenicity (Bannon 2004).

Several studies have published the effects of proteolysis in combination with MH to enhance the hydrolysis rate of proteins and to impart a decrease in residual immunoreactivity, compared to conventional heating. For example, the application of microwave energy at 800 W for 8 min increased the degree of hydrolysis and stabilized the solubility of whey protein hydrolysates (Uluko et al. 2015). The effects of MH on the enzymatic hydrolysis of a commercial whey protein

concentrate was found to increase the proteolytic effectiveness of all enzymes tested and more efficiently produce hypoallergenic dairy hydrolysates (Izquierdo et al. 2008). Lin et al. (2010) reported the efficacy of microwave-assisted acid pretreatment for preparing antioxidant peptides derived from enzymatic hydrolysis of bone collagen.

Fish frames are by-product (waste) material derived from the fish filleting process with little commercial value including fertilizers and animal feed, while the majority of these by-products are discarded to landfills. Fish frames are rich in protein and thus could have a significant value if these proteins are recovered from the fish and used in food products. Enzymatic hydrolysis of fish by-products is commonly used to produce smaller peptides and amino acids known as fish protein hydrolysates (FPH). FPH have high protein content with good quality amino acid composition (Chalamaiah et al. 2012). Recently, FPH have attracted much attention due to their desirable functional and bioactive properties (He et al. 2013). However, their potential allergenicity has deterred their application in food formulations. Parvalbumins, the major fish allergens, are calcium-binding muscle proteins of about 12 kDa, highly resistant to heat, chemical denaturation, and proteolytic enzymes (Kuehn et al. 2010). To date, application of MH during enzymatic hydrolysis of fish proteins remains largely unexplored, especially its effect on allergenicity and antioxidant properties of the hydrolysates.

In this study, we established preliminary conditions for obtaining trout frame protein hydrolysates using microwave energy. We investigated microwave's potential to accelerate enzymatic hydrolysis in order to improve the degree of hydrolysis, protein recovery, and antioxidant activities. Antigenicity of the hydrolysates was also investigated with the goal of developing alternative approaches to produce hypoallergenic antioxidant fish peptides.

Material and Methods

Raw Material Preparation

Fresh rainbow trout (*Oncorhynchus mykiss*) frames were obtained from Bell Aquaculture™ (Redkey, IN, USA). Fish frames were transported to the Food Science Department on ice, divided into 500 g portions in polyethylene bags, and immediately frozen at -20°C until used. Frames were thawed overnight at $4 \pm 2^{\circ}\text{C}$ before homogenization with distilled water (1:4 w/v) for 5 min using a stainless steel commercial blender (Waring CB15, Torrington, CT, USA). Fish frame slurry (FFS) was heated at 90°C for 15 min with constant stirring to inactivate autolytic enzymes. FFS was adjusted to pH 8.0 with 1 N NaOH or 1 N HCl for optimal enzyme activity.

Processing Microwave Pretreatment and Assisted Enzymatic Process

FFS from previous step (40 mL) were added to individual microwave vessels (70-mL capacity each). Vessels were capped with stoppers and lids with fixed-pressure release valves. A microwave unit with Xpress™ vessel carousel (MarsExpress™ CEM Co., Matthews, NC, USA) was used in the microwave heating experiments. The microwave system was programmed to reach 55 or 90°C within 10 min at a maximum power of 800 W, based on previously reported conditions by Uluko et al. (2015). Temperature in each vessel was monitored using a fiber optic probe. The power required to maintain the set temperature was monitored using an infrared sensor which allows for power settings to be automatically adjusted in order to prevent temperature fluctuations. Following pretreatment, sample temperature was brought down to 55°C and hydrolysis carried out using alcalase (EC 3.4.21.62 Sigma Chemicals, St. Louis, MO) at 3 %, (w/w) protein content in FFS using the following treatments. Based on preliminary trials, four major treatments were investigated using the microwave system: (1) pretreatment at low temperature (55°C) for 2, 4, 6, 8, and 10 min followed by conventional enzymatic hydrolysis in a waterbath at 55°C (CE) for 15 min (PLCE 2–10); (2) pretreatment at high temperature (90°C) for 5 min followed by CE for 2, 4, 6, 8, and 10 (PHCE 2–10); (3) pretreatment at high temperature 90°C for 5 min followed by microwave-assisted hydrolysis at 55°C (ME) for 2, 4, 6, 8 and 10 min (PHME 2–10); and (4) no pretreatment, only ME for 2, 4, 6, 8, and 10 min (NPME 2–10). Additionally, control sets were as follows: (1) FFS with no enzyme addition and no pretreatment followed by conventional heating for 10 min at 55°C (CFS); (2) FFS with no enzyme addition and pretreatment at high temperature followed by conventional heating for 10 min at 55°C (CPH); (3) FFS with no enzyme addition and pretreatment at low temperature followed by conventional heating for 10 min at 55°C (CPL); and (4) FFS with no pretreatment followed by conventional enzymatic hydrolysis for 10 min at 55°C (CCE).

Hydrolysis was terminated by heating the samples at 95°C for 15 min using a water bath. Samples were then centrifuged at $15,000\times g$ for 15 min (Avanti J-25; Beckman, California, USA). Supernatants were collected and filtered through double-layer cheese cloth, lyophilized, stored at -20°C , and referred to as fish protein hydrolysates (FPH) until analysis.

Degree of Hydrolysis

The extent of enzymatic hydrolysis was quantified using the trinitrobenzenesulfonic acid (TNBS) method adapted from Adler-Nissen (1986), modified by Liceaga-Gesualdo and Li-Chan (1999). Absorbance was read at 420 nm using a UV-Visible spectrophotometer (Beckmann,

Irvine, CA, USA). Degree of hydrolysis (DH) was calculated using Eq. [1]:

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

Hydrolysis equivalents (h) were calculated by the increase in released amino groups. For fish protein, h_{tot} was assumed to be 8.64 meq/g (Adler-Nissen 1986).

Determination of Protein Content and Recovery

Protein content of samples was determined by the bicinchoninic acid (BCA) protein assay according to the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA, USA) using bovine serum albumin as the standard.

Protein recovery was calculated using Eq. [2]:

$$\text{Protein recovery}(\%) = \frac{A}{B} \times 100 \quad ([2])$$

where A is the protein content of supernatant and B is total protein content in fish frame protein before hydrolysis.

Antioxidant Activity Determination

The 2,2'-Azino-Bis(3-Ethylbenzthiazoline-6-Sulphonic Acid) Free Radical Scavenging Assay

The 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) free radical scavenging (ABTS) radical scavenging capacity was determined according to Alemán et al. (2011) with slight modifications. ABTS radical stock solution (7 mM ABTS in potassium persulfate 2.45 mM), was kept in the dark at 25 °C for 16 h. Stock solution was diluted in Milli-Q water to obtain a working solution of ABTS radical with an absorbance of 0.700 ± 0.02 at 734 nm. A 6-μL sample aliquot (5 mg/mL) was mixed with 294 μL of ABTS working solution in a 96-well microplate and incubated in the dark at 30 °C for 10 min. Absorbance at 734 nm was read (Multiskan™ GO, ThermoScientific, Rockford, IL, USA). Lower absorbance represents a higher ABTS scavenging activity. Results were expressed as increment of activity (%) of micromole TROLOX equivalent antioxidant capacity (μmol TE) per milligram of protein in the FPH compared to the control (CFS).

Reducing Power

The reducing power (RP) was measured according to Zhang et al. (2008) with modifications. Samples (250 μL) were dissolved in 0.2 M sodium phosphate buffer (pH 6.6) or blank (250 μL of buffer), mixed with 250 μL of the same buffer followed by addition of 250 μL of 1 % (w/v) potassium ferricyanide solution. Final peptide concentration in the assay

mixture (1 mg/mL) was heated to 50 °C and incubated 20 min after which 250 μL of 10 % (w/v) aqueous trichloroacetic (TCA) was added. Thereafter, 250 μL of TCA mixture was combined with 25 μL of 0.1 % (w/v) ferric chloride and 125 μL of double-distilled water and allowed to stand at 25 °C for 10 min. Solution was centrifuged at 10,000×g and 125 μL of the supernatant transferred to a 96-well microplate for absorbance determination at 700 nm. Increased absorbance of the reaction mixture indicated increased RP. Results were expressed as increment of activity (%) of micromol TROLOX equivalent antioxidant capacity (μmol TE)/mg of protein in the FPH compared to the control (CFS).

Metal Chelating Activity

Metal chelating activity (MCA) was measured according to Alemán et al. (2011). Briefly, 800 μL of sample (0.125 mg/mL) was mixed with 10 μL of 2 mM FeCl₂ and 20 μL of 5 mM ferrozine. Mixture was kept at 25 °C for 10 min prior to measuring the absorbance at 562 nm using a UV-Visible spectrophotometer. Results were expressed as increment of activity (%) of micromole EDTA equivalents/mg of protein in the FPH compared to the control (CFS).

Sodium Dodecyl Sulfate Polyacrylamide Gel

Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Laemmli 1970) was conducted using a PhastSystem™ (GE-Healthcare NJ, USA) with precast gradient high-density PhastGel® mini-gels. Samples were mixed with buffer (4 % SDS, 20 % glycerol, 0.125 M Tris-HCl, and 10 % β-mercaptoethanol, pH 6.8) in a 1:1 (v/v) ratio and boiled in water for 5 min. Aliquots (3 μL) of 2-μg final protein concentration were loaded onto the gel. Electrophoresis was run at 80 V for 30 min. Before staining, gel was fixed with 5 % glutaraldehyde for 2 h followed by overnight staining in 0.1 % (w/v) Coomassie brilliant blue R-250, 45 % methanol, and 10 % acetic acid. Gel was destained with 10 % methanol, 10 % acetic acid, and preserved in 5 % glycerol and 10 % acetic acid. Extra-low molecular weight standards (Sigma Aldrich, MO, USA) were used for band molecular weight approximations.

Enzyme-Linked Immunosorbent Assay

Sample allergenicity was analyzed using the Sandwich-enzyme-linked immunosorbent assay (ELISA) procedure. A 96-well flat-bottom clear polystyrene microtiter plate (Fisher Scientific, Waltham, MA, USA) was coated overnight at 4 °C with 100 μL/well of purified polyclonal parvalbumin antibody (5 μg/mL in 0.2 M bicarbonate buffer pH 9.4). Microplate was washed with wash buffer (25 mM Tris, 0.15 M NaCl, pH 7.2 containing 0.05 % Tween 20, Tris buffer saline; TBS). Wells were blocked with 300 μL/well of 2 %

BSA in wash buffer and incubated at 25 °C for 60 min. Standard diluent (2 % BSA in TBS) was used for all dilution steps. Blocked plate was washed and 100 µL/well biotinylated anti-codfish parvalbumin (10 µg/mL) added. Microplate was washed, followed by addition (100 µL/well) of codfish parvalbumin protein standards, assay buffer blanks, and sample extracts diluted 1:500. Microplate was incubated for 60 min at 25 °C. After washing, bound parvalbumins were detected by incubation for 60 min at 25 °C with streptavidin-horseradish peroxidase conjugated at a concentration of 200 ng/mL (100 µL/well). After an additional wash, 100 µL/well 3,3',5,5'-tetramethylbenzidine (TMB) was added and incubated for 15 min at 25 °C. Reaction was stopped by adding 50 µL/well of 2 M H₂SO₄. Absorbance was read at 450 nm. Results were expressed as allergen content (ppm) based on a codfish parvalbumin standard curve.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) with Duncan's multiple range test using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was established at $\alpha = 0.05$. Experiments were performed in triplicate.

Results and Discussions

Degree of Hydrolysis

The effect of microwave pretreatment on degree of hydrolysis (DH) was dependent on the hydrolysis time (Fig. 1). Using low temperature microwave pretreatment (55 °C) at different times (2 to 10 min) followed by conventional hydrolysis (PLCE2-10), increased DH to ~50% when compared to the controls CFS (14 % DH), CPH (13 % DH), CPL (13 DH), and CCE (29 % DH). However, there was no significant difference ($P > 0.05$) within same PLCE treatments (different pretreatment times). We can assume that the time given for microwave pretreatment did not play an important role in DH and instead, DH was more influenced (as expected) by the length of hydrolysis time. Nevertheless, microwave pretreatment itself can influence DH by shortening the time required for enzymatic hydrolysis. In PHCE, having high temperature microwave pretreatment (90 °C) but different conventional hydrolysis times (2–10 min), the DH significantly increased ($P < 0.05$) with longer hydrolysis times (42–47 %DH). The importance of hydrolysis time was highlighted again in the treatments that had no microwave pretreatment followed by microwave-assisted hydrolysis (NPME). When increasing hydrolysis time from 2 to 10 min in the microwave-assisted hydrolysis treatments, a higher %DH was also obtained ($P < 0.05$). This DH increase can be a result of microwave heating inducing a modification on the flexibility

of the enzyme, consequently changing or accelerating the enzymatic properties (Rejasse et al. 2007). In LPCE and PHCE, microwave pretreatment will increase the rate of protein hydrolysis by first the unfolding protein when it is exposed to the microwave pretreatment (Uluko et al. 2015). Conventional heating itself can also lead to protein to unfolding and exposure to the enzyme reaction. However, conventional hydrolysis needs longer time to obtain higher DH. An interesting observation is that high temperature microwave pretreatment followed by microwave-assisted hydrolysis at 55 °C (PHME2-8), did not improve ($P > 0.05$) DH in the same manner as PHCE and NPME. Moreover, PHME2-8 showed a lower DH compared to control (CCE) except for PHME10. We can speculate that the protein was initially denatured by the high temperature of the microwave pretreatment, followed by aggregation of the protein structure when exposed again to microwave heating during the hydrolysis step. This would result in limited accessibility of the endo-protease (alcalase) to reach the substrate target sites. As hydrolysis time increased, it lead to increased chances of collision between partially unfolded molecules, leading to irreversible aggregation (Uluko et al. 2015). As discussed, this would also greatly depend on the length of enzymatic hydrolysis time used. A similar trend was observed in milk protein hydrolysates, where the highest increase in DH occurred when microwave pretreatment was applied for 8 min (Uluko et al. 2013). Using similar energy levels and temperature limits, but different exposure time, the difference in the results obtained in this study was thus attributed to the effect of the hydrolysis time only. It seems that both low- and high-temperature microwave pretreatments render the protein bonds susceptible to enzymatic hydrolysis. There is also evidence that microwave radiation may speed up rates of folding and unfolding for globular proteins (Bohr and Bohr 2000; Uluko et al. 2015). Izquierdo et al. (2008) showed that microwaves cause different biological and chemical effects depending on the field strength, frequencies and exposure duration.

Several factors affect the degree of hydrolysis, including the protein substrate used, proteolysis conditions, and enzyme specificity. Literature suggests using a combination of enzymes (e.g., alcalase and flavourzyme) with endo- and exopeptidase activity, rather than single enzymes, because single enzymes cannot produce sufficient hydrolysate in a reasonable period of time (Nchienzia et al. 2010). However, in our study, we have proved that by using microwave heating (either as pretreatment and/or microwave-assisted hydrolysis) we were able to increase the degree of hydrolysis in relative short amounts of hydrolysis time, therefore eliminating the need for using a combination of enzymes.

Protein Recovery

Microwave pretreatment and microwave-assisted hydrolysis improved protein solubility, leading to increased protein

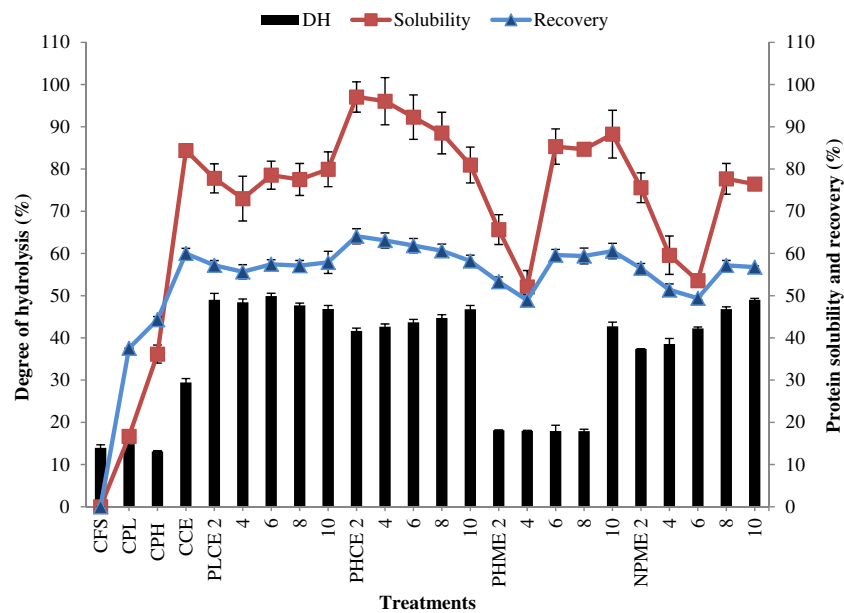


Fig. 1 Degree of hydrolysis (% bar), protein solubility (% line with square mark) and protein recovery (% line with triangle mark) of different treatments of fish frame protein hydrolysates. *CFS* fish frame protein slurry, incubated at 55 °C for 15 min followed by heating at 90 °C 15 min (water bath) with no enzyme added; *CPH* fish frame protein slurry, pretreated by microwave at 90 °C for 5 min with no enzyme added; *CPL* fish frame protein slurry, pretreated by microwave at 55 °C for 5 min with no enzyme added; *CCE* fish frame protein slurry pretreated by microwave at 55 °C for 10 min with enzyme addition; *PLCE* protein hydrolysates from fish frame pretreated by microwave at 55 °C for 2, 4, 6,

8, and 10 min followed by conventional enzymatic hydrolysis at 55 °C for 15 min (water bath); *PHCE* protein hydrolysates from fish frame pretreated by microwave at 90 °C for 5 min followed by conventional enzymatic hydrolysis at 55 °C (waterbath) for 2, 4, 6, 8, and 10 min; *PHME* protein hydrolysates from fish frame pretreated by microwave at 90 °C for 5 min followed by microwave-assisted enzymatic hydrolysis at 55 °C for 2, 4, 6, 8, and 10; *NPME* protein hydrolysates from fish frame treated with microwave-assisted enzymatic hydrolysis at 55 °C for 2, 4, 6, 8, and 10 min without pretreatment

recovery compared to that obtained by the conventional heating control (*CFS*). Protein recovery, ranging from 49 to 63 %, was observed for both pretreatments along with microwave-assisted hydrolysis (Fig. 1). The highest ($P < 0.05$) protein recovery was for *HPCE4* (63 %). Protein recovery for *PHME* and *NPME* was slightly lower but not significant compared to *PHCE2-10* ($P > 0.05$). However, when the samples were hydrolyzed for a longer time, the recovery increased although this increase was not statistically significant. In terms of solubility, *PHCE4* showed the highest ($P < 0.05$) value (96 %) (Fig. 1). However, solubility declined ($P > 0.05$) when the pretreated samples were hydrolyzed for longer time (e.g., 10 min). Low-temperature microwave pretreatment (*PLCE2-10*) had no effect on protein solubility, although it was still higher ($P < 0.05$) than the unhydrolyzed controls (*CFS*, *CPL*, and *CPH*). This supports the hypothesis that the combination of microwave pretreatment at high temperature (90 °C) followed by conventional enzymatic hydrolysis, was better for improving protein solubility than microwave-assisted enzymatic hydrolysis alone. A similar trend was reported for solubility of egg white protein hydrolysate pretreated with microwave radiation (Yang et al. 2013; Shuguo et al. 2013). Furthermore, Uluko et al. (2013) reported that microwave pretreatment of milk protein prior to enzymatic hydrolysis showed higher protein solubility when

pretreatment time was increased. Kristinsson and Rasco (2000) revealed that higher protein solubility was related to higher production yields of protein hydrolysates because lower molecular weight hydrolysates are more prone to form hydrogen bonds between water molecules and hydrophilic polar amino acid groups, thus increasing their solubility.

In this study, microwave pretreatment and conventional enzymatic hydrolysis for longer periods of time overall improved protein solubility and recovery. With microwave pretreatment, direct energy transfer from the electromagnetic field to the polar protein domains could induce modifications on the flexibility of protein structure; consequently, changing its structure and allowing the enzyme access to the target sites (Ponne and Bartels 1995). The presence of an electric field can influence biochemical reactions by changing molecular spacing and increasing inter-chain reactions (El Mecherfi et al. 2011). In comparison with conventional heating methods, heat is transferred from the outside to the inside of the samples during the hydrolysis; whereas microwave radiation heats samples from within, depending on the nature of the sample (Corsaro et al. 2006). Conventional heating methods can also have certain delays in heat transfer due to their superficial heating principle. Furthermore, a direct absorption of microwave energy by polar substrates of the enzyme could lead to higher reactivity of the functional groups involved in the

enzymatic reaction (Mazumder et al. 2004). The protein recovery and solubility results in this study indicate that using microwave pretreatments along with enzymatic hydrolysis can produce hydrolysates with protein functionality. Further studies to evaluate their functional properties are required.

Antioxidant Capacity

It is generally agreed upon that peptides possess substantially better antioxidant activities than their parent proteins, possibly due to increased accessibility of the functional side chain to the reactive species and the electron-dense peptide bonds generated by enzymatic hydrolysis (Udenigwe and Aluko 2011). Efforts have been investigated in pretreatment methods such as microwave irradiation (Uluko et al. 2015), high pressure (Garcia-Mora et al. 2015), ultrasound (Kangsantant et al. 2014), and sub-critical carbon dioxide (Asaduzzaman and Chun 2013) along with enzymatic hydrolysis to enhance proteins' antioxidative activity. In this study, the methods used for determination of antioxidant capacity are based on different mechanisms, thus leading to different results which are discussed below.

Antioxidant properties (ABTS, RP, and MCA) of different treatments compared to untreated controls (CPH, CPL, and CCE) are shown in Fig. 2. The percent increment reported was calculated compared to the initial ABTS activity of CFS (0.67 μmol TROLOX eq/mg protein in sample). Compared to the controls, there was a significant increase ($P < 0.05$) on ABTS activity (Fig. 2a) for high-temperature microwave pretreatments including PHCE (55–60 % ABTS) and PHME (34–62 % ABTS). In the case of low temperature pretreatment (PLCE), ABTS increased around 30–40 %; however, there was no significant difference ($P > 0.05$) when increasing the hydrolysis time of PLCE from 2 to 10 min. Asaduzzaman and Chun (2013) revealed that hydrolysates produced from mackerel (*Scomber japonicas*) skin using pressurized hydrothermal process increased ABTS up to 99 % when temperature and pressure were increased. Different increments in ABTS during high pretreatment temperature along with enzymatic hydrolysis can result from a wide variety of smaller peptides and free amino acids generated. Furthermore, changes in size, level, and composition of free amino acids and small peptides also affect the antioxidant activity (Wu et al. 2003; Guo et al. 2013).

The reducing power (RP) capacity for all treatments compared to controls is given in Fig. 2b. CFS showed initial RP activity as 0.007 μmol TROLOX eq/mg sample protein (data not shown). The increment of RP was primarily found in PHME and PHCE. However, an unstable trend for the RP with increasing hydrolysis time was found in HPME, with the highest RP increment being PHME4. Udenigwe et al. (2014) studied casein plastein prepared from casein hydrolysates and reported that peptide aggregation increased after

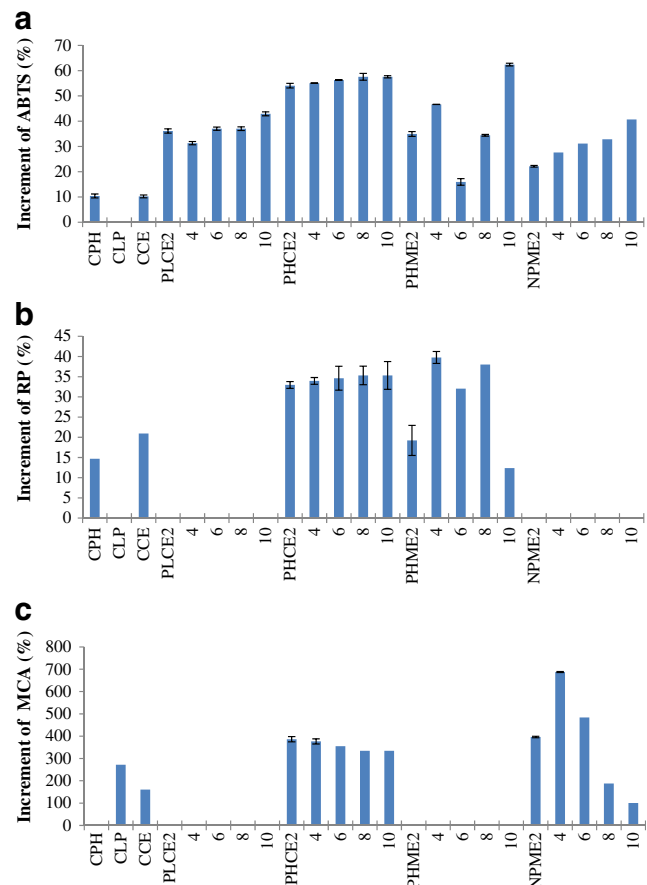


Fig. 2 Increment of **a** ABTS radical scavenging activity; **b** reducing power (RP); and **c** metal chelating activity (MCA) of different treatments of trout frame protein hydrolysates compared to the controls. Results were expressed as increment of activity (%) of micromole TROLOX equivalent antioxidant capacity (μmol TE)/milligram of protein in the FPH for **a** ABTS radical scavenging activity and **b** reducing power (RP) or of micromole EDTA equivalents per milligram of protein in the FPH for **c** metal chelating activity (MCA) by calculation compared to those of control (CFS). Treatment captions are described in Fig. 1. Sample concentrations for each assay where 5 mg/mL for ABTS, 1 mg/mL for RP, and 0.125 mg/mL for MCA

being heated at 100 °C. The Fe(III) reducing potential, however, decreased the Fe(II) chelating activity. In their study, the microwave pretreatment at 90 °C produced more RP activity. This can be explained by microwave irradiation at temperatures changing the conformation of the native protein structure allowing for the enzyme to access more target sites, liberating active peptides. However, when they used much higher temperatures (220 °C), the reducing power decreased Udenigwe et al. (2014). In another study, it was hypothesized that hydrolysates derived from thermal dried squid muscle could function by donating electrons to the free radicals, causing peptide decomposition into other organic compounds (Asaduzzaman and Chun 2013). Furthermore, the presence of more specific peptides or active amino acids in the hydrolysates with high DH, which react with free radicals to form more stable products, can also play a role (Yang et al. 2013). Moure et al.

(2006) demonstrated that protein size and concentration clearly influenced the reducing power. Higher RP can be attributed to the high content of electron or hydrogen-donating peptides (Je et al. 2009). It is known that the RP and ABTS assays use the single-electron transfer mechanism to reduce radicals (Samaranayaka and Li-Chan 2011). However, the RP assay measures the ability of antioxidants to reduce the ferric iron (FeIII) to ferrous iron (FeII) while the ABTS assay measures the ability of antioxidants to donate a proton and reduce the ABTS^{•+} radical. This can be the reason for the difference in the results obtained in our study.

Transitional metal ions, such as Fe²⁺ and Cu²⁺, can catalyze the generation of reactive oxygen species which oxidize unsaturated lipids. The chelating activity of peptides in hydrolysates could decrease lipid oxidation (Binsi et al. 2015). Therefore, the chelation of ferrous ions indirectly contributes to antioxidant activity to prevent oxidative reactions. The increment of MCA of all treatments was calculated compared to control (Fig. 2c). CFS had a MCA of 0.005 µmol EDTA/mg sample protein (data not shown). Compared to the CFS, an increment in MCA was observed for CLP (272 %), PHCE (249–376 %), and NPME (188–687 %). In the case of the no pretreatment, microwave-assisted hydrolysis (NPME), no increment was observed for RP (Fig. 2b) but it was observed for MCA (Fig. 2c). The highest MCA (687 %) was found in NPME hydrolyzed for 4 min, whereas high microwave pretreatment (PHCE) showed increments for both RP and MCA. Surprisingly, the low microwave pretreatment with no enzyme addition (CPL) also showed a 272 % increment on MCA (Fig. 2c). Whereas, PLCE with the same microwave pretreatment conditions as CPL, did not show any increments in MCA. We believe that the low-temperature microwave pretreatment caused an arrangement in the protein structure that exposed the protein's charged groups, enabling it to trap the metal ions. Presence of carboxyl groups in acidic amino acids sidechains and amino groups in basic amino sidechains are known to play a vital role in metal chelation (Farvin et al. 2014). The absence of MCA increment in PLCE could be due to the change in peptide size as a result of the enzymatic hydrolysis, as well as the low concentration tested. In a study with skipjack roe protein hydrolysates, MCA was reported at 20 mg/mL in the 50 %DH sample. The MCA decreased ($P < 0.05$) when compared to the 40 %DH sample at the same concentration (Intarasirisawat et al. 2012). In the present study, the combination of microwave pretreatment and enzymatic hydrolysis (PLCE) resulted in much smaller peptides evidenced also by the higher %DH in these samples, compared to the other treatments. These smaller size peptides are unable to form strong complexes with metal ions at the concentration tested in the assay (Aluko 2015; Xiong 2010). Additional studies evaluating the metal chelating capacity the peptides at different concentrations need to be further investigated.

In the case of PHME, no MCA increment was observed perhaps due to aggregation or rearrangement when microwave radiation was applied again during the enzymatic hydrolysis. It has been suggested that microwave pretreatment causes changes to the protein structure that prevent the formation of metal chelating peptides (Uluko et al. 2015). Thermal-induced aggregation of proteins has been reported by Zhao et al. (2009) with the resulting aggregates being resistant to enzymatic proteolysis that cause fewer active peptides to be released. This observation is corroborated by the lower DH also presented by PHME (Fig. 1). In this study, the metal chelating residues in fish frame peptides may have participated in stabilizing the hydrolysates by hydrophobic and electrostatic interactions, respectively, making them unavailable to chelate Fe(II) during the MCA assay (Udenigwe et al. 2014). In addition, the different MCA among treatments could be a direct relationship between soluble protein/peptide concentration and the increase in the chelation capability (Saiga et al. 2003). Yang et al. (2013) showed that microwave radiation of egg white protein hydrolysates resulted in a higher DH and the highest hydroxyl radical scavenging activity, superoxide anion scavenging, and reducing power activity in comparison to conventional heat pretreatment.

Molecular Weight Distribution by SDS-PAGE

Molecular weight profile of protein from different treatments was investigated by SDS-PAGE. Most major bands disappeared for PHCE and PLCE compared to PHME and NPME (data not shown). It seems that either using microwave pretreatment by high (90 °C) or low (55 °C) temperature, followed by conventional hydrolysis was able to break the size distribution of the protein into smaller peptide fragments. PLCE and PHCE, showed bands with molecular masses lower than 6.5 kDa. This indicates that microwave pretreatment along with enzymatic hydrolysis has the ability to produce low molecular weight peptides corresponding to the higher DH. Even though there was no significant difference in DH between PLCE and NPME, from the SDS-PAGE, we could see that PLCE showed smaller molecular weight peptides than NPME. As previously discussed, microwave pretreatment was able to unfold the protein structure allowing the enzyme to easily reach target sites (Quansah et al. 2013). Similar protein patterns were observed between PHME and NPME. However, NPME showed the presence of more small molecular weight bands than PHME, correlating with the higher DH in Fig. 1.

Antigenicity Properties

Antigenicity of fish proteins is caused by the presence of specific sequences in the allergen proteins (i.e., parvalbumins) called epitopes, which when recognized by the immune

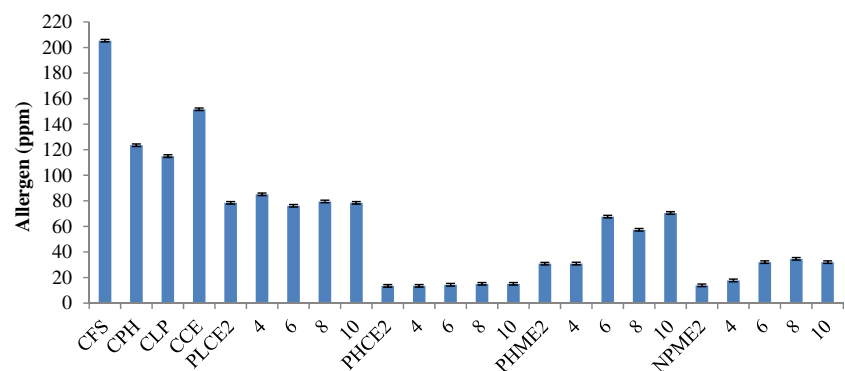
system, trigger allergic responses (Masilamani et al. 2012). This is of special importance when developing food products containing fish protein since parvalbumin retains its allergenicity even after heat treatment, hydrolysis, or exposure to extreme pH conditions. Trout and codfish have the highest amount of parvalbumins at 1.0–2.5 mg/g of fish meat (Kuehn et al. 2010). Unfortunately, there are limited clinical studies addressing the minimal provoking doses of fish in fish-allergic individuals. Taylor et al. (2002) and Koppelman et al. (2012) have indicated that most sensitive fish-allergic individuals react to the consumption of 5–10 mg of fish protein, corresponding to 25–67 mg of fish fillet based on the average of 20 % protein in fillets.

Enzymatic hydrolysis is expected to degrade some of the allergenic epitopes and therefore decrease the immunoreactivity of the native protein (Jiménez-Saiz et al. 2015; Masilamani et al. 2012). Several authors reported combined enzymatic and microwave treatments in bovine milk protein, compatible with retention of enzyme activity and reversibility of structural modifications of proteins, may have a practical relevance by decreasing their immunoreactivity (El Mecherfi et al. 2011). The assumption about how microwave treatments in combination with enzymatic hydrolysis can decrease protein allergenicity was investigated and confirmed by the allergen content (in ppm) values shown in Fig. 3. Microwave pretreatments and microwave-assisted hydrolysis significantly decreased ($P < 0.05$) the allergen level of the hydrolysates by more than 40 % compared to CFS (205 ppm). The lowest allergen content was observed in PHCE (13–15 ppm) and NPME (14–34 ppm), followed by PHME (30–70 ppm) and PLCE (75–85 ppm). The use of microwave radiation either in the form of high-temperature pretreatment (PHCE) or no pretreatment but microwave-assisted hydrolysis (NPME) can yield hydrolysates with decreased allergenicity. Buchanan et al. (1997) showed that when the stability of the major protein allergens was disrupted by reduction of disulfide bonds, the allergens were strikingly more sensitive to enzymatic digestion and lost their allergenicity. In our study, the combination of high pretreatment and

microwave-assisted hydrolysis (PHME) showed slightly higher allergenicity compared to PHCE and NPME. This can be explained as aforementioned that this particular treatment involved both a high temperature microwave pretreatment as well as microwave-assisted hydrolysis, which can lead to protein aggregation and changes in protein conformation that prevent the epitopes from being targeted. Although there are numerous protease digestion sites throughout the length of the protein, the aggregated structure might be so compact that potential cleavage sites are inaccessible until the protein is denatured. According to Lehrer et al. (1996), the majority of immunoreactive proteins contain intramolecular disulfide bonds and these bonds are believed to play a major role in their allergenicity. This observation allows us to assume that protein structure can be an important factor in an allergen's ability to resist denaturation. We will need to further explore the effect of this particular treatment to further understand the effect of microwaves on protein structure characteristics.

The combined enzymatic and microwave treatments, compatible with retention of enzyme activity and reversibility of structural modifications of proteins, may have a practical relevance for decreasing a protein's immunoreactivity. The unfolded proteins formed under microwave treatment could represent an ideal substrate for the action of proteases, making some epitopes more accessible to enzymatic hydrolysis. The decreased immunological reactivity of hydrolysates pretreated or derived from microwave-assisted hydrolysis was also reported for whey proteins hydrolysates. Proteolysis under microwave radiation of lactoglobulin hydrolysate by a mixture of trypsin/chymotrypsin inhibited IgE compared to peptic hydrolysates obtained under conventional heating (El Mecherfi et al. 2011). Izquierdo et al. (2008) also reported an important decrease in immunoreactivity of whey protein hydrolysates obtained by combining microwave radiation with pronase, papain, or alcalase in comparison to untreated samples and the enzymatic treatment alone. In light of the above, this study provides evidence that microwave pretreatment and/or microwave-assisted hydrolysis can help decrease the antigenicity of fish protein hydrolysates.

Fig. 3 Allergen content in different trout frame protein hydrolysates. Treatment captions are described in Fig. 1



Conclusions

This study established the benefit of using microwave pretreatments and/or microwave-assisted hydrolysis by offering shorter-time hydrolysis using a single proteolytic enzyme. The use of microwave radiation increased degree of hydrolysis, improved protein solubility, and protein recovery. Moreover, microwave-treated hydrolysates showed better antioxidant activity compared to controls. Significant decrease in immunochemical reactivity was found for all microwave-treated hydrolysates. Thus, microwave pretreatment and/or microwave-assisted hydrolysis can be an alternative method efficient tool for decreasing residual antigenicity of fish proteins. Based on the results of this study, the use of microwave pretreatment for 5 min at 90 °C, followed by conventional enzymatic hydrolysis with alcalase for 2–10 min was the best treatment condition to produce fish peptides with high antioxidant activity and lowest immunoreactivity. Although this study's results have been demonstrated at the laboratory scale, they can serve as a model for large-scale operations. Microwave-treated fish protein hydrolysates could be used as ingredients in the production of hypoallergenic food ingredients and nutraceuticals. Further studies on the effect of microwave energy on structure-function relationships of antioxidant peptides and their immunoreactivity are needed to corroborate this information. Research on the chain length, amino acid composition, and sequences of peptide fragments produced by microwave treatments with different proteolytic enzymes also needs to be explored.

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Compliance with Ethical Standards

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