PAPER

EFFECTS OF ULTRASOUND TREATMENT ON STRUCTURAL, CHEMICAL AND FUNCTIONAL PROPERTIES OF PROTEIN HYDROLYSATE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) BY-PRODUCTS

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ABSTRACT

In this study, the effects of ultrasound treatment on biochemical, physical, structural and functional properties of fish protein hydrolysate of rainbow trout (*Oncorhynchus mykiss*) by-products were investigated. Enzymatic hydrolysis was conducted by Alcalase 2.4 L, pH 8, 1 h at 60°C, and enzyme/substrate ratio at 0.5%. A probe-type ultrasound was used for ultrasound assisted hydrolysis (UH) process. Higher protein recovery was obtained in UH than in the conventional enzymatic hydrolysis (CH). The highest foaming capacities of CH and UH were measured as 137.5% and 152.5%, respectively ($p<0.05$). Overall, our data suggest that ultrasound treatment helps to improve the functional properties such as foaming capacity and stability.

Keywords: by-products, fish protein hydrolysate, *Oncorhynchus mykiss*, rainbow trout, ultrasound hydrolysis

1. INTRODUCTION

Seafood processing industry generates high amounts of by-products during the processing steps. These by-products include backbone, head, tail, viscera, blood and cut-offs. Pertaining to species and the process applied, the volumes of these by-products vary from 20 to 70% of the whole raw material. Such amount of by-products brings about pollution and create severe problems at disposal points.

Based on the present industrial practice, most of the by-products are either discarded or used for various feed applications by processing them into fish silage, fishmeal and oil (HSU, 2010). The frame has a high-value biochemical composition with potential for higher-value food applications (ARASON *et al*., 2009; KLOMKLAO and BENJAKUL, 2018). An increasing trend in industrial applications for the utilization of fishery by-products, is the manufacture of water-soluble fish protein hydrolysates (FPH). This will give an increased yield of solubilized proteins due to reduced molecular weight and an increase in the number of ionizable groups (KRISTINSSON and RASCO, 2000).

Besides utilization in the replacement of animal meals from other sources (LI *et al*., 2018), protein hydrolysates can be used as functional additives in the food processing industry with many functional properties, such as water holding, gelling and foaming capacities, fat absorption, emulsifying and also antioxidant and antimicrobial activities. However, alternative methodologies have become necessary for improving yield, functional properties and bioactivity in protein hydrolysates. The quality and functional characteristics of the obtained products vary with the usage of different enzymes and production conditions. It is therefore, necessary to test alternative innovative technologies that will enhance product quality. These technologies need to be safe, cheap and easy to apply. It should also have no toxic and side effects.

Power ultrasound is an emerging and promising technology that has been applied in a variety of fields (ARVANITOYANNIS *et al*., 2015). Recently, enhancement in peptide production by ultrasound has become a focus of research in the food industry. With ultrasonic pretreatment of substrates, the enzymatic hydrolysis of wheat germ protein can be significantly stimulated (ZANG *et al*., 2015). In addition, hydrolysis of food protein can also be enhanced using sonicated enzymes (KADAM *et al*., 2015). The ultrasound treatment seems to be useful in accelerating the release of peptides. Ultrasound treatment is regarded as safe, non-toxic and environmentally friendly. It is also considered to be more advantageous to other technologies and is covered by "green technologies" (KENTISH and ASHAKKUMAR, 2011). Food technologists focus on protein production using high-intensity ultrasound application to support enzymatic hydrolysis, and produce high-throughput peptides.

In this study, it was aimed to determine the effects of ultrasound treatment on biochemical, physical, structural, antioxidant and functional properties of the fish protein hydrolysate that was produced from rainbow trout by-products.

2. MATERIAL AND METHODS

2.1. Materials

A total of 45 (weighing 12 kg) individual fresh rainbow trout (with average length and weight being 29.36±1.72 cm and 264.83±53.42 g, respectively), obtained from a local fish farming company, were transferred to the laboratory in styrofoam box with ice. After evisceration, the by-products (head, backbone, fins, tail and skin) were separated by hand and used as raw material; total weights of by-products are presented in Table 1. To

minimize microbial contamination and internal enzyme activity, viscera was excluded. The food-grade Alcalase 2.4 L (AU/kg Sigma Aldrich, Novozymes, Bagswaerd, Denmark) was used. All chemical reagents used for the experimental analysis were of analytic grade. The hydrolysation process was done on the same day the raw material reached the laboratory.

Table 1. Total weights of by-products of rainbow trout.

Type	Total weight (g)
Head	2074,10
Tail and backbone	1226,11
Fins and skin	1298,93
Total By-products used as raw material	4599,14
Viscera (not used)	2194,51

2.2. Methods

2.2.1 Preparation of raw material

By-products were chopped with mincing machine (Super meat grinder, 5 mm; pore size), mixed with distilled water (1:1 w/w) and homogenized (200 rpm for 2 minutes) using WiseTis‹HG-15D (Daihan, Seoul, Korea).

2.2.2 Preparation of protein hydrolysis

Protein hydrolysates were prepared using the pH-stat method according to SATHIVEL *et al*. (2005) and KANGSANANT *et al*. (2004), with slight modifications. For maximum activity and stability of the enzyme, all reactions were conducted at pH 8 (adjusted with 1N NaOH) and a temperature of 60°C. The prepared homogenate was used as raw material, divided into two equal aliquots and then placed in glass examination vessels. Experiments were carried out in the shaking water bath (Wisebath, Wertheim, Germany) agitating at 200 rpm for CH (Conventional Enzymatic Hydrolysis) and UH (Ultrasonic-Assisted Enzymatic Hydrolysis) processes, using Alkalase (0.5% by weight of raw material). For ultrasound assisted system, a probe type ultrasound equipment (Sonics vibra cell, USA, tapered micro tip, 142 x 6 mm) was used and the probe was immersed into the experimental vessel with 40% ultrasonic amplitude, pulse duration of 10 s on- time; 20 s off-time. In both vessels, the temperature was increased to 60°C for enzyme activation and kept constant during the experiment. Hydrolysis was initiated by addition of enzyme and terminated after 60 min, the enzyme was inactivated by increasing the temperature to 90 ºC for 10 min. Coarse filtration was applied to heated suspensions using glass cotton and filter paper. Thereafter, filtrates (6000 g) were centrifuged in a refrigerated centrifuge (Universal 320 R, Hettich, Germany) at 4°C for 35 min. After centrifugation, 3 separate phases occurred in the separation funnel; bottom phase: insoluble protein, middle phase: soluble protein heavily liquid, and upper phase: lipid fraction light liquid. The middle layer was collected. The supernatants were stored in a freezer at -80ºC and dried in a freeze-dryer (Labconco Freezone 2.5 Benchtop Freeze Dryer, USA) for 48 hours. The resulting powdery hydrolysates were vacuum packed and stored in a freezer at -80°C until analysis. The hydrolysis process of CH and UH groups were done in duplicate. All the analysis for the CH and UH groups were performed in three parallels.

2.2.2.1 Yield of FPH

FPH yield was calculated following the method used by ILHAN and GÜLYAVUZ, 2003.
Yield of FPH (%) = [Weight of FPH (g)/Weight of by-products (g)] x100 (1)
Yield of protein (%) = [(wf × Pf)/ (wi × Pi)] x100 (2)
Where wf is the weight in grams of FPH, Pf is the protein content (%) of FPH, wi is the weight of by-products in grams and Pi is the protein content (%) of by-products (PIRES *et al*., 2012).

2.2.3 Determination of the degree of hydrolysis (DH)

DH was analyzed with pH-stat method described by WROLSTAD *et al*., 2005. About 10 g of freeze-dried sample was weighted; hydrolysis conditions of fish by-products were applied. The solution was stirred with the magnetic stirrer (Ika, RCT Basic, Germany) and pH was adjusted to 8.0 with 0,1 N NaOH for 60 min. NaOH consumption was reported every 5 min. Results were given as a percentage. The equation used in the calculation is given below;

DH (%) = B × Nb × 1/α × 1/Mp × 1/htot × 100 (3)
B: Amount of alkali consumed (ml)
Nb: Normality of the alkali; 0.5 N (= 0.5 mmol/ml)
Mp: The mass of substrate (protein (g), %N ×6.25)
1/α: The calibration factors for pH-stat
htot: The content of peptide bonds.

ADLER-NISSEN (1986) assumed htot as 8.6 mmolg^{-1} of protein and α as 1 for fish.

2.2.4 Determination of biochemical composition

Total crude lipid content was determined by Soxhlet extraction method and crude protein content was analyzed by Kjeldahl method. The total protein content was calculated as %N using the standard conversion factor of 6.25. (AOAC, 1990, method 2.507); moisture and ash contents were determined using AOAC 1990 method 985.14 and method 7.009, respectively.

2.2.5 Amino acid analysis

Total amino acid analyzes were carried out in Kazlıçeşme R and D Test Laboratory (AB-0513-T), an accredited laboratory in Istanbul, Turkey. After pre-column derivatization with HPLC (Agilent 1260 Infinity), Agilent Eclipse AAA method was modified using FLD/DAD detectors and determined by an in-house laboratory method. A 0.2 g sample was weighed and mixed with 5 ml of 6 N HCl and stored in the condenser for 24 h. Depending on the amount of amino acid, 0.6 g to 2 g of sample was transferred to 100 ml balloon flask, after addition of 5 ml norvaline standard, the flask volume was completed to 100 ml. Thereafter, 0.5 µl of the filtered sample was injected into the device and analyzed. OPA (Ortho Phthalaldehyde), FMOC (Fluorenylmethoxy Chloroformate) and Borate was used as the derivatizing agent.

2.2.5.1 HPLC conditions

Mobile phase A; 40 mN Na_2HPO_4 (pH 7.8) and Mobile Phase B; Asentonitrile/ Methanol/Water (45/45/10), a flow rate of 2 ml/min. ZORBAX Eclipse-AAA 4.6 * 150 mm (3.5 μm) was used as the column. The column temperature was set at 40°C. The injection volume of sample was 0.5 μl. DAD detector wave lengths were 338nm, 10nm bw; Ref: 390 nm, 20 nm bw (for OPA-amino acid) and 262 nm, 16 nm bw; Ref: 324 nm, 8 nm bw (for FMOC-amino acid).

2.2.6 Measurement of the color

The color was measured using a color meter (Konica Minolta (Specktropen CR10 Japan)). Three measurements were taken from the samples of CH and UH.
L * (brightness), a * (redness), b * (jaundice), W (whiteness), chroma and h hue angle /saturation degree;

$$W = 100 - [(100-L^*)^2 + a^{*2} + b^{*2})]^{1/2} \quad (5)$$

$$Chroma = (a^{*2} + b^{*2})^{1/2} \quad (6)$$

$$h = b^*/ a^* \quad (7)$$

(PIRES *et al*. (2012)

2.2.7 Determination of functional properties

2.2.7.1 Protein solubility

Protein solubilities of CH and UH were determined as reported by American Oil Chemists Society (AOCS) (1989). FPH's were dispersed in the water (10 g/l); pH of solutions were adjusted to 3, 5, 7 and 9 with 0.5 N NaOH or 0.5 N HCl for 45 min with constant stirring. The solutions were then centrifuged for 30 min at 2.800 *g*. N contents in 15 ml of supernatants were determined according to the Kjeldahl method;
Protein solubility (%) = protein content of the supernatant/total protein content.

2.2.7.2 Foaming capacity and foaming stability

Foaming capacity (FC) and foaming stability (FS) were performed according to WILDE and CLARK, 1996 and SHAHIDI *et al*., 1995, with slight modifications. Three g of FPH was mixed with 100 ml of distilled water, then transferred into a 250ml graduated cylinder. The mixture was homogenized at 11000 rpm for 1 min at room temperature. The total volume was measured at 0, 1st, 5th, 10th, 40th and 60th min. FC was expressed as foam expansion at 0 min, while FS was expressed as foam expansion at 60 min.

2.2.7.3 Oil binding capacity and water holding capacity

Oil binding capacity was determined by the protocol of SHAHIDI *et al*., 1995. Five hundred mg hydrolysate was put in a centrifuge tube and 10 ml of sunflower oil was added. After being thoroughly vortexed for 1 minute, it was centrifuged (Hettich Universal 320 R Refrigerated Centrifuge) at 4500 g for 30 min at a temperature of 4°C, thereafter the unconnected oil was discharged. The oil binding capacity was expressed as weight of fat (g) absorbed per gram of sample.
Water holding capacity was analyzed following the centrifuge method described by COBB and HYDER (1972), with slight modifications. Five hundred mg of FPH was weighted into a centrifuge tube and 20 ml distilled water was added. The mixture was vortexed for 30 s,

then put in a dark place at room temperature for 6 h. Thereafter, the tube at 2800 g was placed into the centrifuge for 30 min. Obtained supernatant was filtered from Whatman Paper No: 1 and the volume of liquid was weighted. The water holding capacity calculation was done by dividing the volume of the filtrate obtained from the initially used water volume by the amount of sample. The results are expressed as ml/g.

2.2.8 Scanning electron microscopy (SEM)

The surface morphology of CH and UH thin films was investigated using a JSM-6610 (JEOL) scanning electron microscope (SEM) equipped with an energy dispersive X-ray (EDX) analyzer operated at 20 kV acceleration voltages. Prior to the observation, the investigated specimens were coated with about 250 angstroms of gold by QUORUM-SC7620 sputter coater.

2.2.9 Antioxidant activity assay

To observe antioxidant capacities of the groups, copper (II) ion reducing antioxidant capacity (CUPRAC) and Fe (III) ion reducing antioxidant power methods were used. Radical scavenging activity was determined by ABTS·2,2'-azinobis-(3-etilbenzotiazolin-6-sülphonic acid) radical scavenging method.

2.2.9.1 Copper (II) ion reducing antioxidant capacity assay (CUPRAC)

The method is based on the reduction of copper (II)-neokuproine to copper (I)-neokuproine after addition of antioxidant solution to the medium (APAK *et al*., 2004; MENTESE *et al*., 2015). A total of 10 mM Cu(II) chlorure (Sigma Chemical Co, USA), 7.5 mMneokuproine (Sigma Chemical Co, USA), and 1 M ammonium acetate tampon solution at pH 7.0 (one mL each) were pipetted into the test tubes. About 20 μL sample solutions were added to the medium and vortexed. Final volume was completed to 4.1 ml and 1080 μl distilled water was added and again vortexed. The same procedure was applied for Trolox® standard. After incubation at room temperature for 50 min, absorbance was read at 450 nm (1601UV-Shimadzu, Australia). Using Trolox® curve (8 - 4 - 2 - 1 - 0.5 - 0.25 - 0.125 - 0.0625 mM Trolox®, (r^2=0.999)), Trolox® equivalent antioxidant capacity (mg TEAC/mg substance) per mg substance was calculated for each substance.

2.2.9.2 Iron (III) ion reducing antioxidant capacity assay (FRAP)

The method is based on the measurement of the absorbance of the complex, Fe^{2+} - TPTZ complex at 593 nm (BENZIE and STRAIN, 1999; CAN and BALTAS, 2016). Firstly, 300 mM acetate buffer at pH 3.6 was dissolved in 40 mM HCl, and 10 mM TPTZ (2,4,6-tris (2-pyridyl)-s-triazine and 20 mM of $FeCl_3.6H_2O$ solution were prepared. Freshly prepared solutions were mixed in a ratio of 10: 1: 1 and FRAP reactive was obtained. A total of 100 ml aliquots of samples and 3000 μl FRAP reactive were transferred to each sample tube and vortexed. The reaction mixture was incubated for 5 min at room temperature, and absorbance was read at 593 nm. The same treatments were carried out for $FeSO_4.7H_2O$ standard (r2 = 0.999) prepared at concentrations of 15.63 - 31.25 - 62.50 - 125 - 250 - 500 - 1000 µM, respectively. The absorbance of the test tubes, which were allowed to incubate for 5 min at room temperature, was measured at 593 nm (1601UV-Shimadzu, Australia) and the standard $FeSO_4.7H_2O$ curve was used to calculate the equivalent antioxidant capacity (mM $FeSO_4.7H_2O$/mg substances).

2.2.9.3 ABTS$^{\cdot+}$ cationic radical scavenging method

The radical scavenging activity of the ABTS$^{\cdot+}$ [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] groups were studied according to RE *et al*., 1999; YILMAZ *et al*., 2017. A 7 mM solution of ABTS in water was prepared and 10 ml of this solution was mixed with 2.45 mM and 5 ml potassium persulphate solution and allowed to incubate at room temperature for 18 hours to enable the formation of ABTS$^{\cdot+}$ cationic radical. The resulting radical solution was diluted with phosphate buffer (PBS) at pH 7.4, to give an absorbance of 0.700 ± 0.020 at 734 nm. 200 µL of the test compound (dissolved in DMSO) was added to 1800 µl of the radical solution, vortexed, and after 5 min, the absorbance was read on the UV-Visible spectrophotometer (1601 UV-Shimadzu, Australia) at a wavelength of 734 nm. The radical scavenge value of the groups was calculated from the following formula. The study consisted of three replications for each substance and standard.

$$\text{Radical scavenging } (\%) = [(OD_{control}-OD_{test})/(OD_{control})x100]$$

2.2.10 Statistical analysis

The obtained data were analyzed by analysis of variance (one way ANOVA) and when significant differences were found, comparisons among means were carried out using the Tukey and Mann Whitney U test (data not provided in the normality of assumptions) under the program called JMP 5.0.1 (SAS Institute. Inc. USA) and SPSS 18.0 (SPSS Inc., Chicago, IL) (SOKAL and ROHLF, 1987). A significance level of 95% ($p<0.05$) was used throughout the analysis.

3. RESULTS AND DISCUSSION

3.1. Yields of by-products, FPH's (CH and UH) and the protein of FPH

The yield of by-product was calculated as 38.32 % using the data shown in Table 1. The yields of CH and UH were calculated as 9.82% and 10.54%, respectively. Ultrasound application may have increased the yield because ultrasound have a positive effect on alcalase activity due to the ability to break down the molecular aggregates, giving enzymes the opportunity to yield higher accessibility for reaction and increasing activity, (MCCLEMENTS, 1995). MA *et al*. (2011) studied the mechanism of ultrasonic impact on protease activity and their results showed that ultrasound had an effect on the activity of alcalase. Protein yields of FPH's were also calculated as 57.13% for CH and 61.76% for UH. LIASET *et. al*. (2000) produced protein hydrolysate from Atlantic salmon frames without heads. They used alcalase for conventional enzymatic hydrolysis and FPH protein recovery was observed to be 61.8%. The higher protein recovery of the present study may be affected by different hydrolysis conditions.

3.2. Biochemical composition of by-products from rainbow trout

Biochemical composition of raw material (trout by-products), CH and UH, are shown in Table 2.

Table 2. Proximate composition of by-products, fish protein hydrolysates (CH) and (UH)

	Protein(%)	Lipid(%)	Moisture(%)	Ash(%)
By-products	14.82±0.18[a]	6.45±0.48[a]	72.19±1.38[a]	3.54±0.24[a]
(CH)	86.40±0.32[b]	0.05±0.01[b]	1.36±0.08[b]	6.25±0.40[b]
(UH)	86.75±0.28[b]	0.05±0.01[b]	2.10±0.18[c]	5.95±0.32[b]

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a,b) represent statistical differences amongst the groups ($p<0.05$).

Protein content in by-products was very low (14.82%). The CH and UH samples have high and similar protein contents. The high protein content of FPH is due to the characteristics of the hydrolysis process. During this process, proteins are solubilized and insoluble materials are removed by centrifugation (CHALAMAIAH *et al*., 2010). Lipid contents were the same for CH and UH and are very low (0.05%). This also may be as a result of characteristics of the hydrolysis process; the membranes tended to round up and form insoluble bubbles, which could cause the removal of membrane structural lipids. During the centrifugation stage after hydrolysis, these lipids separated into different layers and were removed from the medium. It is a desired feature for protein hydrolysates to have low lipid content (SHAHIDI *et al*., 1995). As estimated, the moisture content of by-products was high. Whereas, it was very low in CH and UH because FPHs were freeze-dried at the end of the hydrolysis process. The difference between CH and UH was significant ($p<0.05$). Since by-products have skin and bone parts, ash content was higher than trout flesh (average 1.21%) (TURCOMP, 2014), but it was higher in CH and UH than the by-products; this may be due to the increased salts by addition of alkali into the medium to adjust the pH 8 during the hydrolysis process (BENJAKUL and MORRISSEY, 1997). There was no significant difference in the ash contents of CH and UH.

3.3. Amino acids analysis

The functional differences in hydrolysates are closely related to the amino acid groups present in the structures. Table 3 shows the total amino acid contents in by-products, CH and UH, respectively.

Accordingly, values for by-products of amino acids were lower than all amino acid values of FPH ($p<0.05$). Amino acid contents of CH and UH were higher and close to each other. Total amino acid contents were calculated as 2.01g/100g for by-products, 80.54g/100g for CH and 82.65g/100g for UH. Generally, ultrasound application helps to open the surface of the substrate and increases the enzyme activity. As a result, it supports hydrolysis process. Glycine was the highest in all groups. Among the hydrophobic amino acids, (valine, methionine, leucine, isoleucine, alanine, tryptophan, phenylalanine and tyrosine), the value of leucine was the maximum and methionine was the minimum. Indicating the increased antioxidant activity, the sum of the hydrophobic amino acids was calculated as 22.98g/100g for CH and 23.72g/100g for UH. Differences for all amino acids of CH and UH were not significant, except valine *($p<0.05$)*. RAJAPAKSE *et al*., 2005 stated that hydrophobic amino acids, such as phenylalanine and glycine are highly soluble in lipids. Soluble amino acids have more capability to gain closer access to the radicals than neutral or hydrophilic amino acids.

Table 3. Total amino acid contents of by-products, CH and UH(g/100 g).

Amino Acid	By-product	CH	UH
Cysteine	0.00±0.00[a]	1.81±0.25[b]	1.94±0.01[b]
Aspartate	0.12±0.01[a]	6.80±0.06[b]	7.10±0.13[b]
Glutamate	0.09±0.01[a]	10.98±0.11[b]	11.45±0.28[b]
Aspargine	ND	ND	ND
Serine	0.11±0.01[a]	3.19±0.01[b]	3.24±0.17[b]
Glutamine	ND	ND	ND
Histidine	0.06±0.01[a]	1.93±0.00[b]	1.90±0.04[b]
Glycine	0.36±0.01[a]	12.05±0.16[b]	12.06±0.38[b]
Threonine	0.11±0.01[a]	3.00±0.01[b]	3.08±0.15[b]
Arginine	0.09±0.01[a]	5.59±0.06[b]	5.75±0.20[b]
Alanine	0.14±0.01[a]	5.70±0.06[b]	5.88±0.17[b]
Tyrosine	0.12±0.01[a]	1.95±0.05[b]	2.08±0.06[b]
Valine	0.14±0.01[a]	3.06±0.02[b]	3.21±0.03[c]
Methionine	0.11±0.01[a]	1.65±0.04[b]	1.74±0.02[b]
Norvaline	0.02±0.00[a]	0.01±0.01[a]	0.02±0.01[a]
Tryptophane	0.06±0.01[a]	0.33±0.06[b]	0.34±0.06[b]
Phenylalanine	0.08±0.00[a]	3.84±0.05[b]	3.99±0.11[b]
Isoleucine	0.07±0.01[a]	1.91±0.04[b]	2.05±0.08[b]
Leucine	0.07±0.01[a]	4.84±0.04[b]	5.13±0.11[b]
Lysine	0.09±0.01[a]	5.83±0.13[b]	5.89±0.08[b]
Hydroxyproline	0.13±0.01[a]	2.28±0.08[b]	2.07±0.16[b]
Sarcosine	ND	ND	ND
Proline	0.11±0.01[a]	3.89±0.05[b]	3.77±0.08[b]
Total	**2,01±0,04[a]**	**80,54±0,75[b]**	**82,65±0,60[b]**

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst the groups ($p<0.05$).

3.4. Measurement of the color

At the end of the hydrolysis, yellowish brown liquid mixtures were obtained in both vessels (CH and UH). The liquid mixtures had three layers; the bottom, including bones brown in color, the middle; a dark yellowish brown clear liquid, and the top dense brown liquid. After centrifugation, collected liquids (CH and UH) were bright dark yellow. The colors of freeze-dried powders of CH and UH were creamy yellow and L*, a*, and b* values are presented in Table 4.

Table 4. L*, a* and b* and W, c, h values of CH and UH.

	L*	a*	b*	W	c	h
CH	85.80±0.84[a]	2.90±0.51[a]	22.60±1.35[a]	73.15±0.78[a]	22.79±0.48[a]	7.80±0.46[a]
UH	83.90±0.60[a]	3.50±0.30[a]	24.30±0.30[a]	71.10±0.56[a]	24.10±0.30[a]	6.80±0.34[a]

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 10. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst the groups ($p<0.05$).

L*, a*, and b* values observed in CH were similar with UH. Using L*, a*, b* values whiteness, chroma and h values were calculated (Table 4).

3.5. DH

Fig. 1 illustrates the variation in the DH of CH and UH under experimental conditions, which showed a rapid increase in both groups due to many peptide bonds cleaved up to around 20th min regardless of ultrasound application. After this time, as fewer peptide bonds were available for cleavage, the reaction rate reduced for CH and UH. After about 35-40 min, a small decrease occurred in the DH of UH, which was significantly lower than DH of CH ($p<0.05$). This observation was not in accordance with the theory that ultrasound application would yield a higher DH than the conventional hydrolysis. KANGSANANT *et al*. (2014) produced enzymatic hydrolysate from Nile tilapia assisted by continuous ultrasound with 40W. The researchers observed that ultrasound assisted hydrolysis provoked a decrease in DH, but this decrease was not significant compared to other researches which focused on different food items. This may be due to the low intensity of ultrasound applied in the present study (HUANG *et. al*., 2015; ZHANG *et. al*., 2015).

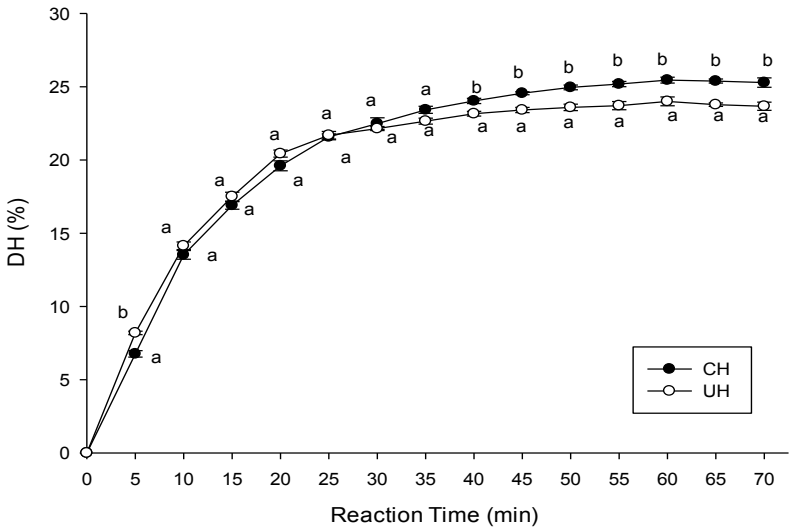

Figure 1. Evolution of DH during the hydrolysis of CH and UH.
CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different letters (a,b) represent statistical differences amongst the groups (p<0.05).

3.6. SEM analysis

To find out the structural effect of ultrasonic treatment on FPH, the microstructure of lyophilized CH and UH were observed by SEM under different magnifications. Fig. 2 illustrates the SEM images of CH and UH. As shown in Fig. 2, different microstructures were obtained in CH and UH. UH had larger aggregates plate-shaped morphology and a smooth surface structure, whereas CH had smaller aggregates both in the form of round structures and plate-shaped morphology. The differences might be due to the changes in application of ultrasound that led to the unfolding of UH molecules. As a result, higher hydrophobic groups might occur at the surface of the molecules and interaction of these

groups with each other formed larger structures. HU *et al*. (2013) found that treatments of different frequencies and times of ultrasound were effected on the structure of soy protein isolate dispersions. In their study, after ultrasound treatment, samples had larger and more heterogeneous structures. Also, they observed that longer ultrasound application might result in larger structure size. ZHOU *et al*. (2016) investigated the effects of heat, ultrasound and combinations of heat/ultrasound and ultrasound/heat on corn gluten meal hydrolysate. Researchers used 40 kHz frequency, on-time 10 s and off time 3 s, 40 min duration at 20°C. They observed that the control was in the form of massive texture, but the surface became incompact and porous after ultrasound pretreatment.

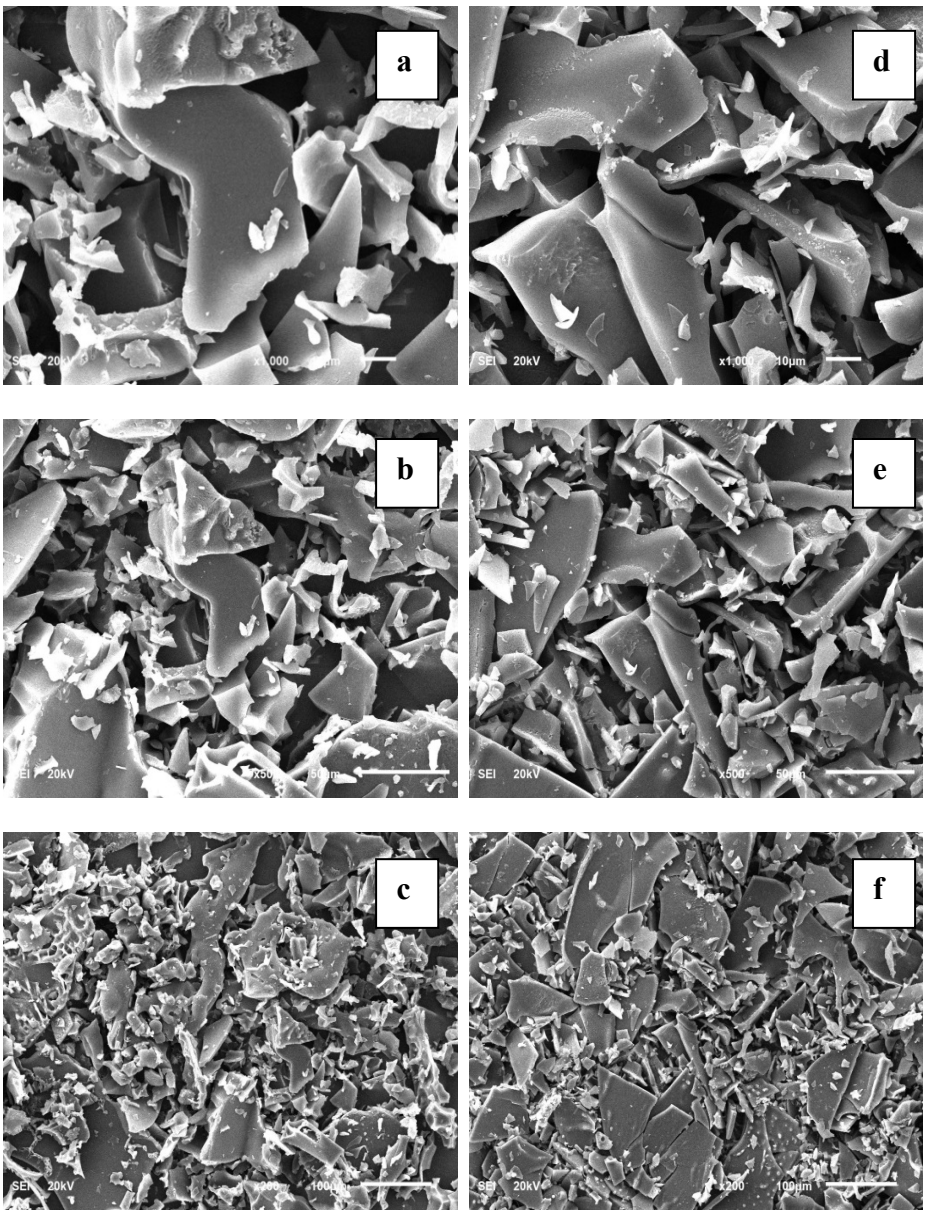

Figure 2. SEM analysis of CH (a,b,c) and UH (d,e,f).

In the present study, results are inconsistent with those emphasized studies. Different shapes may be due to the different ultrasonic conditions (pretreatment, ultrasonic-assisted hydrolysis and time, temperature, etc.) and the raw material used in the study.

3.7. Functional properties

In the food industry, proteins have a special attribution in food products due to their several significant functional characteristics. Among the functional properties, emulsifying, foaming, thickening and gelling capacities are often affected by their solubility (DAMODARAN, 1997). Soluble peptides obtained from enzymatic hydrolysis of proteins, can contribute to improving the emulsion and the foaming characteristics (RAYMUNDO *et al*., 2000). Ultrasound applications led to an improvement in the functional properties of different food items (BRYANT and MCCLEMENTS, 1999). But ultrasonic treatment conditions and variation in the rheological and thermos-physical properties of protein sources are considered effective on the functional properties of protein hydrolysates (AVAD *et al*., 2012).

3.7.1 Protein solubility

Protein solubility of CH and UH are shown in Fig. 3. It was low at acidic pH and gradually increased with increasing the pH, after neutral pH, it decreased again to pH 9. Both groups have the highest solubility at pH 7, and the lowest at pH 3. The differences between groups were significant, except pH 3 ($p<0.05$). As shown in Fig. 3, a parallel trend in CH and UH, ultrasound treatment was shown to improve the protein solubility.

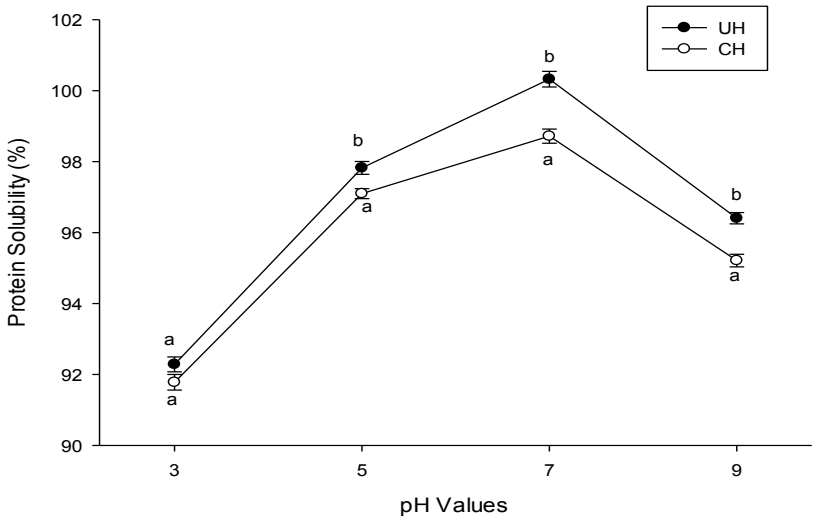

Figure 3. Protein solubility of CH and UH.

Ultrasound changes the conformation and structure of protein and hydrophilic amino acid residues directed towards water (ARZENI *et al*., 2011). This situation explains the case of higher solubility of UH than CH. Protein solubility is one of the most important representative factors in protein functionality. In the food industry, improvement in solubility led to a potential improvement in the functional properties of proteins (PELEGRINE and GASPARETTO, 2005).

3.7.2 Foaming capacity (FC) and foaming stability (FS)

FC and FS of CH and UH are shown in Fig. 4 a, b. The FC of UH in each duration was significantly higher than that of CH. The highest values for FC of CH and UH were measured accordingly as 137.5% and 152.5%, respectively. At the end of 60 min, it decreased to 11.0% in CH and 20.0% in UH. Diffusion of soluble proteins, rapid conformational change and reorganization of molecules at air-water interface are needed in the protein-based foam formation (NALINANON *et al*., 2011). Parallel to FC, FS of UH was also significantly higher than CH, this difference was significant ($p<0.05$) after the 5th to 60th min (Fig. 4b).

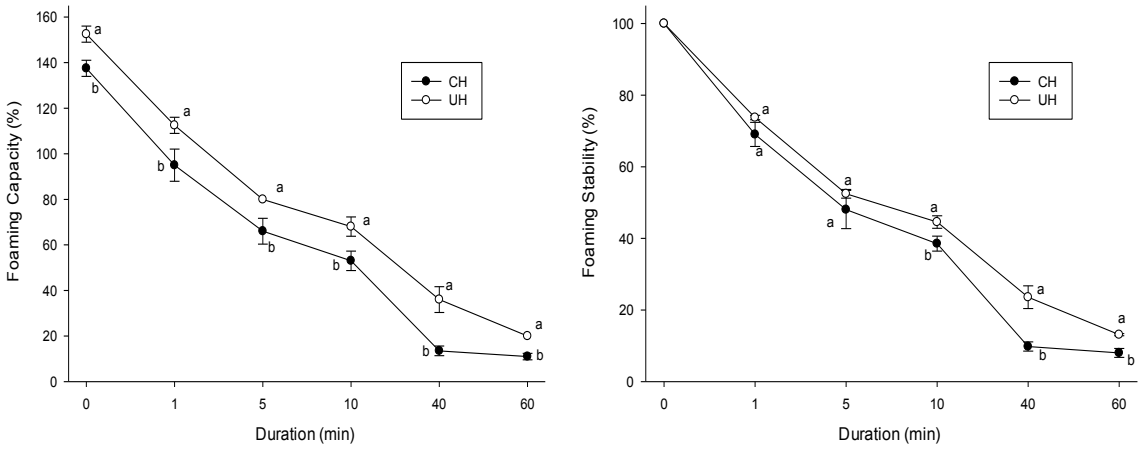

Figure 4. Foaming capacity (a) and stability (b) of CH and UH.
CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different letters (a,b) represent statistical differences amongst the groups (p<0.05).

It was reported that protein solubility has an important effect on functional properties of protein hydrolysates. In the present study, the results of FC and FS were in accordance with the solubility values. As the solubility increased with the ultrasound treatment, FC and FS increased. Researchers also reported that higher solubility results in higher foaming characteristics from different protein sources (SORIA-HERNÁNDEZ *et al*. 2015). JAMBRAK *et al*., (2009) illustrated that ultrasound application is an effective way to improve the physical properties of soy proteins. Anon, 2008 reported "The degree of hydrolyzation determines the functionality of the end products. Low degree of hydrolyzation results in highly functional foaming agents and high degree of hydrolyzation results in hydrolysed vegetable protein (HVP) which are used in soups and sauces as flavor enhancers".

3.7.3 Oil binding capacity (OBC) and water holding capacity (WHC)

OBC shows a major functionality of ingredients in the food industry. KRISTINSSON and RASCO (2000) stated oil binding capacity ranged from 2.86 to 7.07 mL of oil/g of protein for Atlantic salmon protein hydrolysates. The bulk density of the protein, the degree of hydrolysis and enzyme used in hydrolysis process affect this functionality. Water holding capacity is another important factor. It especially improves the textural properties of

foods. Different ingredients derived from proteins are used in muscle foods to improve water holding functions.
Data on OBC and WHC of CH and UH are presented in Table 5. UH has a better OBC than CH ($p<0.05$). On the contrary, WHC was lower in UH than CH, but this difference was not significant ($p>0.05$).

Table 5. Oil absorption and water holding capacity of CH and UH.

	CH	UH
Oil absorption capacity (g/g oil)	4.47±0.23[a]	6,36±0.40[b]
Water holding capacity (ml/g)	5.40±0.57[a]	4,70±0.14[a]

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a, b, c..) represent statistical differences amongst the groups ($p<0.05$).

3.8. Antioxidant activity

Different measurement methods are used for antioxidant capacity determination. Since only one experiment cannot give reasonable results, it was observed that the item act as antioxidant. Accordingly, antioxidant activities of CH and UH were measured using the methods; CUPRAC, FRAP and $ABTS^{\cdot+}$ Radical Scavenging Activities. Many studies have shown that all protein hydrolysates consist of peptides or smaller protein fractions that are hydrogen donor and could react with radicals to convert them to more steady products, thereby finalizing the radical reaction (KITTIPHATTANABAWON *et al*., 2012).

3.8.1 CUPRAC and FRAP Antioxidant Activity

CUPRAC method is easily used to measure total antioxidant capacities of both hydrophilic and lipophilic antioxidants (YAVAŞER, 2011). The results of the antioxidant activity obtained using the CUPRAC and the FRAP methods of the UH and CH are given in Table 6. Trolox equivalent antioxidant capacity (TEAC) values of the groups (according to the CUPRAC method) were calculated on the Trolox® standard and $FeSO_4.7H_2O$ standard.

Table 6. Antioxidant activities of UH and CH, (CUPRAC (mM Trolox/mg compound) and FRAP (mM FeSO4.7H$_2$O/mg compound) methods).

Compounds	TEAC Values (μM Trolox®/mg mixture)	FRAP Values (μM $FeSO_4.7H_2O$/mg mixture)
UH	244.89±0.020[a]	13.175±0.009[a]
CH	230.23±0.017[b]	12.161±0.003[b]

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst the groups ($p<0.05$).

TEAC method is based on electron transfer such as Trolox equivalent antioxidant capacity (SARMADI and ISMAIL, 2010). TEAC values for UH were significantly higher than CH ($p<0.05$). Reduction activities of UH and CH to iron (III) and iron (II) were calculated according to FRAP method. FRAP values of UH were higher than CH ($p<0.05$) (Table 6). In

both methods, higher antioxidant activities of UH samples might be due to change in the structures of fractions as the effect of ultrasound. JIANG *et al*. (2014) stated that ultrasonic treatment causes higher interactions of protein hydrophobic sites exposed to the surface of the molecules and buried inside the molecules.

3.8.2 $ABTS^{\cdot+}$ radical scavenging activity

The total radical scavenging capacities of CH and UH were determined using the $ABTS^{\cdot+}$ radical scavenging assay. $ABTS^{\cdot+}$ is generated by oxidation of ABTS with potassium per sulfate and is reduced in the presence of such as hydrogen or an electron donating antioxidant (BINSAN *et al*., 2008). The SC_{50} values for $ABTS^{\cdot+}$ radical scavenging activities of the CH and UH were presented in Table 6. The CH exhibited efficient radical scavenging activity when compared to UH, at the all final concentration (Table 7).
Increased compound concentrations caused an increase in radical scavenging ability. ABTS scavenging activity increased with increasing concentrations and it was stated that some amino acids like histidine, methionine, cysteine, phenylalanine and tyrosine might be effective in increasing the ABTS+ radicals scavenging activities (CHALAMAIAH *et al.* 2010). Aromatic amino acids in hydrolysates are capable of stabilizing free radicals by donating an electron. In the present study, total amounts of these amino acids were similar in CH and UH (11.18g/100g and 11.65g/100g, for CH and UH, respectively). Histidine shows capabilities of stabilizing free radicals by donating an electron and inhibiting lipid oxidation through chelating and lipid trapping of the imidazole ring. In the present study, histidine was higher in CH than UH. Lower SC_{50} values of CH display a higher radical scavenging effectiveness. The SC_{50} values for $ABTS^{\cdot+}$ method of CH and UH were found as 160.0 and 180.10 μg/ml, respectively (Table 7). In a study, ABTS scavenging activities were similar for control and ultrasound pretreated (91.2% and 92.7%) bighead carp hydrolysate, at a hydrolysate concentration of 30% (YANG *et al*., 2016).

Table 7. $ABTS^{\cdot+}$ radical scavenging activities at various final concentrations (%) and SC_{50} values of the UH and CH.

Compounds	ABTS$^{\cdot+}$ Method Radical Scavenging (%)						SC_{50} Values (μg/mL)
	1000 μg/mL	500 μg/mL	250 μg/mL	125 μg/mL	62.5 μg/mL	31.25 μg/mL	
UH	86.15±1.12^{a}	77.54±0.72^{a}	61.85±0.48^{a}	36.62±0.30^{a}	19.62±0.22^{a}	6.92±0.12^{a}	180.10±0.68^{a}
CH	87.08±0.90^{a}	79.08±0.50^{a}	64.31±0.56^{a}	42.58±0.42^{b}	24.46±0.28^{b}	9.85±0.08^{b}	160.00±0.45^{b}

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst the groups ($p<0.05$).

4. CONCLUSION

This research shows that FPH derived from trout by-products may have a potential utilization as a functional and nutritional ingredient in food systems with desirable properties. Ultrasound application improves protein solubility and it affects especially foaming capacity and stability, as well as oil absorption capacity of FPH. There were no significant differences observed in other functional properties. The SC_{50} value for $ABTS^{\bullet+}$ radical scavenging activity was gained by ultrasound treatment. Ultrasound assisted enzymatic hydrolysis of FPH can be used as a novel hydrolyzation process.

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