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Production of Hydrolyzed Protein from Beluga (*Huso huso*) Head: Effect of Ultrasound and Microwave on Antioxidant Properties

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ABSTRACT

This study aimed to evaluate the effects of ultrasound and microwave pretreatments on the degree of hydrolysis and antioxidant activity of hydrolyzed proteins derived from beluga sturgeon (*Huso huso*) head. Fish heads were pretreated using ultrasound (frequency 20 kHz, intensity 75 W) and microwave (frequency 2450 Hz, temperature 90 °C) for 5 and 10 minutes, followed by enzymatic hydrolysis with alcalase (2% enzyme concentration) at 55 °C and pH 8. The effect of different pretreatments on the degree of hydrolysis (DH) and antioxidant activity was subsequently investigated. Results indicated that both ultrasound and microwave pretreatments significantly increased DH values ($p < 0.05$). The minimum degree of hydrolysis was 34.48 ± 0.25% and the maximum was 50.42 ± 0.18%, corresponding to water bath and 10-minute microwave treatments, respectively. The highest and lowest antioxidant activities (DPPH and ABTS radical scavenging activity and iron ion reducing power) were observed in 5-minute ultrasound and water bath treatments, respectively. The IC_{50} values for DPPH and ABTS radical scavenging of the 5-minute ultrasound treatment were 2.97 ± 0.01 mg/mL and 3.95 ± 0.03 mg/mL, which were significantly lower than other treatments ($p < 0.05$). Also, in all samples, with increasing concentration, antioxidant activity increased significantly ($p < 0.05$). In general, it can be stated that ultrasound and microwave pretreatments have a favorable effect on the properties of hydrolyzed protein from beluga head, and different treatments can lead to the production of products with different characteristics.

1- Introduction

Aquatic organisms are considered rich sources of high-quality protein and essential fatty acids (such as omega-3) and play an important role in meeting human nutritional needs. Recently, the demand for seafood consumption has increased significantly, indicating substantial growth in the global aquaculture industry and seafood sectors [1 and 2]. Increased fish production has led to increased production of by-products from processing, but these wastes are rarely used for human consumption. After fish processing, large amounts of raw residues remain from processing, including vertebrae, trunk bones, head, skin, fins, scales, and viscera, which sometimes account for up to about 75% of the total weight of the fish. These by-products are excellent sources of proteins, lipids, minerals, polysaccharides, and carotenoids. However, the value of fish by-products can be increased using mild and environmentally friendly processes to produce high-quality products [3-5]. Evaluation of the chemical composition of waste from different fish species has shown that more than 50% of their dry weight is protein. Fish head is one of the major wastes and contains about 64% protein [6]. Fish proteins are a source of high-quality dietary proteins that are very important for human nutrition [7]. Therefore, different processing methods can be used to utilize these valuable compounds for maximum exploitation and recovery. Currently, there are many methods for extracting fish protein [8]. Although enzymatic hydrolysis is a common method for producing bioactive peptides, it still faces limitations such as low yield and long processing time [9 and 10]. In addition to the type of enzyme, hydrolysis reaction conditions also affect the biological activity of the hydrolyzed protein and the type of released peptides. Research has shown that pretreatment of proteins before enzymatic hydrolysis can help improve the release of bioactive peptides from various types of proteins. These methods include high hydrostatic pressure (HP) technology, ultrasound (US), microwave (MV), and pulsed electric field (PEF) [11-13]. Ultrasound, as high-frequency mechanical waves, has been employed as an auxiliary technique to improve the efficiency of enzymatic protein hydrolysis and the production of bioactive peptides. Compared to conventional enzymatic hydrolysis, ultrasound-assisted enzymatic hydrolysis significantly improves the degree of hydrolysis and the antioxidant capacity of the hydrolysate [14 and 15]. The use of ultrasound in enzymatic hydrolysis increases the enzymatic reaction rate and accelerates the conversion of proteins to target peptides through mechanical, cavitation, and thermal effects. Therefore, the use of moderate ultrasound-assisted enzymatic hydrolysis to break down protein structure and increase enzyme activity in the initial stages, followed by conventional enzymatic hydrolysis to continue the process, can be a more efficient and less energy-consuming method for preparing bioactive peptides [16]. Various studies have also shown that microwave pretreatment can affect the hydrolysis process, shortening hydrolysis time and preserving amino acid quality [17]. Microwave is a type of non-ionizing electromagnetic radiation that can penetrate through waves to reach target components. It is an effective and new tool for supplying energy to a chemical system, facilitating and enhancing the extraction of plant, animal, and marine proteins. This means that microwave energy can be used for the rapid and efficient extraction of target compounds without damaging them, leading to higher yield and purity [18, 19, and 20]. Hydrolyzed proteins have different antioxidant activities depending on the structure, size, and sequence of amino acids, and the protein source and hydrolysis conditions affect these characteristics [21]. Hydrolyzed protein is a mixture of different peptide fractions with a wide range of molecular weights and bioactive properties [22]. Peptides can be extracted from different parts of fish and have various applications [21]. Bioactive peptides show different functional characteristics such as

antimicrobial activity, immune system enhancement, angiotensin I-converting enzyme inhibition, renin inhibition, anticoagulant function, anti-cancer, anti-tumor, anti-diabetic properties, and also antioxidant activity [22-35]. The degree of hydrolysis is one of the critical parameters in examining the characteristics of hydrolyzed proteins, indicating the extent of peptide bond breakage and must be carefully controlled. This is because many properties of hydrolyzed protein, including antioxidant activity, free amino acid content, solubility, and molecular weight of the produced peptides, are highly dependent on the intensity and degree of hydrolysis [36]. Hydrolyzed proteins and peptides derived from various marine sources, including fish, mollusks, crustaceans, and aquatic processing waste, have significant antioxidant potential and can be used in functional foods, pharmaceutical industries, and nutraceuticals [22]. Considering that beluga sturgeon head is one of the wastes from processing that accounts for a high percentage of the initial weight, this study investigated its enzymatic hydrolysis using alcalase enzyme, and the effect of ultrasound and microwave pretreatments on the degree of hydrolysis and bioactive property (antioxidant activity) of the hydrolyzed protein was examined.

2- Materials and Methods

2.1. Raw Material Preparation

Five beluga sturgeon heads were obtained from the Qarahburun Sturgeon Farming Center located in Chapkerud region (Mazandaran province, Iran). They were transported frozen in polystyrene boxes containing ice to the processing laboratory of Sari Agricultural Sciences and Natural Resources University in the shortest possible time. The fish heads were then washed and rinsed with cold water, ground in a frozen state using a semi-industrial meat grinder, packaged in polyethylene bags, and stored at -20°C until the start of the experiments.

2.2. Production of Hydrolyzed Protein

Sample without pretreatment: To prepare the samples, 50 grams of ground beluga head were first thawed at 4 ± 0.2°C and mixed with distilled water at a ratio of 1:2 (W/V) and homogenized with a homogenizer (IKA T25-Digital Ultra-Turrax) for 2 minutes. The containers containing the sample were then placed in a water bath (Memmert wub 29, Germany) at 90°C for 15 minutes to inactivate internal enzymes. Alcalase enzyme concentration of 2%, temperature of 55°C, and pH 8 were considered [37]. After adding alcalase enzyme, the containers were placed in a shaker incubator, and the hydrolysis time was also considered to be 120 minutes. Finally, to stop the enzymatic reaction, the containers were placed in a 90°C water bath for 10 minutes. After cooling to room temperature, the samples were centrifuged for 30 minutes at 6000 g using a refrigerated centrifuge (Sigma, 3-30KS, United States). The supernatant was separated using a pipette and stored at -20°C. Part of the hydrolyzed protein sample was dried using a freeze dryer (Vaco 2 Zirbus, Germany) and stored at -20°C for related analyses.

Microwave Pretreatment: After going through the preparation steps mentioned for the control sample, the samples were subjected to microwave treatment before adding alcalase enzyme. For this purpose, homogenized samples in microwave-safe containers with lids were treated with microwave at a temperature of 90°C and frequency of 2450 Hz for 5 and 10 minutes [38]. The remaining hydrolysis steps were performed as described above.

Ultrasound Pretreatment: For ultrasound pretreatment, homogenized samples were also treated with ultrasound at a frequency of 20 kHz, intensity of 70 W, for 5 and 10 minutes before adding the enzyme [39 and 40]. The remaining hydrolysis steps were performed as described for the control treatment.

Thus, a total of 5 treatments were considered:

Control treatment: Sample prepared by the conventional method without pretreatment.

Treatment 1: Sample treated with microwave for 5 minutes.

Treatment 2: Sample treated with microwave for 10 minutes.
Treatment 3: Sample treated with ultrasound for 5 minutes.
Treatment 4: Sample treated with ultrasound for 10 minutes.

2.3. Soluble Protein Assay

The amount of soluble protein was measured by the Lowry method [41]. The standard curve for protein assay was also plotted using bovine serum albumin (0.1-1 mg/mL) as the standard protein (Figure 1). The absorbance of the samples was read using a spectrophotometer (Spectrophotometer UV-M51 UV/Vis, Italy) at a wavelength of 750 nm.

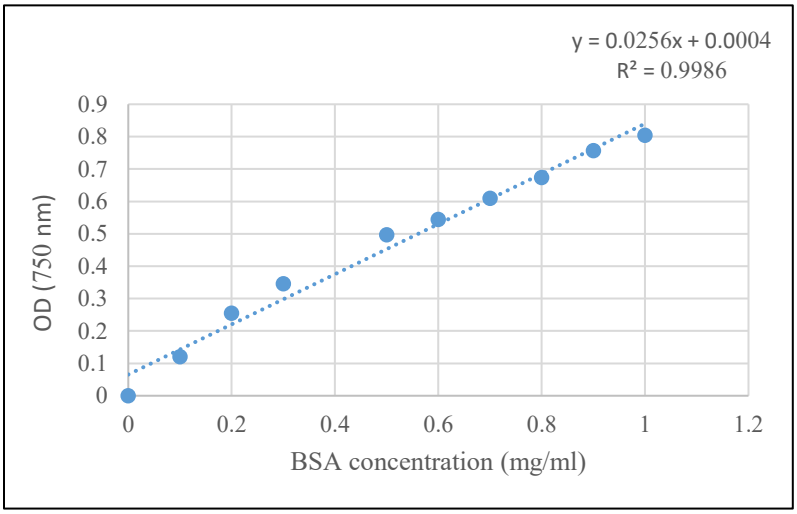

Figure 1- Standard curve of protein assay using bovine serum albumin (BSA)

2.4. Determination of Degree of Hydrolysis (DH)

The degree of hydrolysis was measured based on the method of Hoyle and Merritt (1994). For this purpose, 500 µL of the protein hydrolysate was mixed with 500 µL of 20% trichloroacetic acid and then centrifuged at 8000 g for 10 minutes. The amount of protein in the soluble phase was determined by the Lowry method, and the degree of hydrolysis was calculated using the following equation [42]:

Degree of hydrolysis (%) = (Amount of nitrogen in 10% trichloroacetic acid solution / Amount of nitrogen in the sample) × 100

2.5. Assay of Antioxidant Activity of Hydrolyzed Protein

2.5.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging activity

For this test, equal volumes of sample solution at different concentrations were mixed with 0.1 mM DPPH solution in methanol. The resulting mixture was shaken well and placed in the dark at 32°C for 30 minutes, and finally the absorbance of the mixture was read using a spectrophotometer (Spectrophotometr UV-M51 UV/Vis, Italy) at a wavelength of 517 nm. The percentage of radical inhibition was calculated according to the following **equation** [43]:

% DPPH inhibition = (Ab - As) / Ab × 100

Ab: Control absorbance; As: Sample absorbance

As = Absorbance of the read sample – Absorbance of the sample color (1 mL sample + 1 mL methanol)

2.5.2. Assay of ABTS Radical Scavenging Activity

The ABTS radical scavenging activity was measured using the method of Alemán et al.

(2011). A 7 mM ABTS solution in 2.45 mM potassium persulfate was prepared and kept at room temperature in the dark for 16 hours. After the specified time, dilution with distilled water was performed until reaching an absorbance of 0.7 ± 0.02 at a wavelength of 734 nm. Then, 20 µL of the sample was mixed with 980 µL of the diluted ABTS solution and incubated for 10 minutes in a dark place at 30°C. After the specified time, the absorbance of the samples was read at 734 nm by a spectrophotometer (Spectrophotometr UV-M51 UV/Vis, Italy). The percentage of ABTS radical inhibition was calculated using the following equation [44]:

% Inhibition = (Control absorbance – Sample absorbance) / Control absorbance × 100

2.5.3. Ferric Ion Reducing Power

Based on the method of Oyaizu (1986), 0.5 mL of protein hydrolysate at different concentrations was mixed with 2.5 mL of 0.2 M phosphate buffer (pH=6.6) and 2.5 mL of 1% potassium ferricyanide solution. The obtained mixture was then incubated for 20 minutes at 50°C, and subsequently, 2.5 mL of 10% trichloroacetic acid was added to it. This solution was then centrifuged for 10 minutes at 3000 rpm. 2.5 mL of the supernatant was taken and combined with 2.5 mL of distilled water and 2.5 mL of 0.1% ferric chloride solution. The absorbance of the resulting solution was read at a wavelength of 700 nm using a spectrophotometer (Spectrophotometr UV-M51 UV/Vis, Italy). A higher absorbance value indicates higher reducing power [45].

2.6. Statistical Analysis of Data

Data analysis was performed using SPSS 17 software. This research was conducted in a completely randomized design. After checking the normality of the data using the Shapiro-Wilk test, one-way analysis of variance was used to compare the effect of different treatments on the degree of hydrolysis and antioxidant performance of the hydrolyzed protein, and Duncan's test at the 5% level was used to examine significant differences between means. All experiments were performed with three replications, and Excel software was also used for drawing graphs.

3-Results

3.1. Soluble Protein

The results of investigating the effect of 5-minute microwave (MWH5), 10-minute microwave (MWH10), 5-minute ultrasound (USH5), 10-minute ultrasound (USH10), and water bath (HTH) pretreatments on the soluble protein concentration of hydrolyzed protein from beluga head are shown in Table 1. Statistical analysis of the data showed that there were significant differences between MWH10, USH5, USH10, and HTH treatments ($p<0.05$). However, no significant difference was observed between the MWH5 treatment and USH5 and HTH treatments. The maximum protein concentration was 49.37 ± 0.21 mg/mL and the minimum was 40.48 ± 1.27 mg/mL, corresponding to USH10 and HTH treatments, respectively.

Table 1- Soluble protein concentration of Beluga head protein hydrolysates

Pretreatment	Protein concentration (mg/ml)
HTH	40.48 ± 1.27^{d}
USH5	43.32 ± 1.25^{c}
USH10	49.37 ± 0.21^{a}
MWH5	42.25 ± 1.08^{cd}
MWH10	47.37 ± 1.03^{b}

Data are expressed as mean ± standard deviation (n=3). Means followed by different letters are significantly different ($p<0.05$).

HTH: Control Treatment, USH5: 5 min Ultrasound Treatment, USH10: 10 min Ultrasound Treatment, MWH5: 5 min Microwave treatment & MWH10: 10 min Microwave treatment.

3.2. Degree of Hydrolysis

The results of investigating the effect of 10-minute microwave (MWH10), 5-minute microwave (MWH5), 10-minute ultrasound (USH10), 5-minute ultrasound (USH5), and water bath (HTH) treatments on the degree of hydrolysis of hydrolyzed protein from beluga head are shown in Table 2. Statistical analysis of the data showed that there were significant differences between different treatments ($p<0.05$). As seen in the table, the minimum degree of hydrolysis was 34.48 ± 0.25% and the maximum was 50.42 ± 0.18%, corresponding to HTH and MWH10 treatments, respectively.

Table 2- Degree of hydrolysis (DH) of Beluga head protein hydrolysate

Pretreatment	DH%
HTH	34.48 ± 0.25^{e}
USH5	38.55 ± 0.38^{d}
USH10	43.51 ± 0.20^{b}
MWH5	41.78 ± 0.13^{c}
MWH10	50.42 ± 0.18^{c}

Data are expressed as mean ± standard deviation (n=3). Means followed by different letters are significantly different ($p<0.05$).

HTH: Control Treatment, USH5: 5 min Ultrasound Treatment, USH10: 10 min Ultrasound Treatment, MWH5: 5 min Microwave treatment & MWH10: 10 min Microwave treatment.

3.3. Antioxidant Activity

3.3.1. Effect of Different Treatments on DPPH Free Radical Scavenging Ability

The results of measuring the DPPH free radical scavenging power of hydrolyzed proteins from beluga head obtained from 10-minute microwave (MWH10), 5-minute microwave (MWH5), 10-minute ultrasound (USH10), 5-minute ultrasound (USH5), and water bath (HTH) treatments at concentrations of 0.5, 1, 2.5, and 5 mg/mL are shown in Table 3. According to the results, at each concentration, a significant difference was observed between treatments ($p<0.05$). At all measured concentrations, the highest and lowest DPPH free radical scavenging power belonged to the USH5 and HTH treatments, respectively. Also, comparison of the IC_{50} values of the treatments (Table 4) showed that there was a significant difference between treatments, and the IC_{50} value of the USH5 treatment (2.97 ± 0.01 mg/mL) was significantly lower than other treatments ($p<0.05$), indicating the highest DPPH free radical scavenging activity in this treatment. The highest IC_{50} value and the lowest DPPH free radical scavenging ability also belonged to the HTH treatment. As seen in Table 4, there was no significant difference between MWH5 and MWH10 treatments.

Table 3- DPPH free radical scavenging activity (%) of Beluga head protein hydrolysate

Concentration (mg/ml)	0.5	1	2.5	5
HTH	11.29 ± 0.31^{d}	14.34 ± 0.6^{d}	33.26 ± 0.35^{c}	68.49 ± 0.57^{d}
USH5	15.48 ± 0.51^{a}	19.32 ± 0.30^{a}	45.57 ± 0.45^{a}	78.15 ± 0.67^{a}
USH10	14.37 ± 0.44^{b}	16.53 ± 0.44^{b}	39.64 ± 1.14^{ab}	74.7 ± 0.46^{b}

MWH5	12.89 ± 0.35^{c}	15.4 ± 0.34^{c}	34.53 ± 0.54^{c}	70.24 ± 0.75^{c}
MWH10	12.42 ± 0.57^{c}	14.76 ± 0.25^{cd}	33.75 ± 0.54^{c}	70.58 ± 0.6^{c}

Data are expressed as mean ± standard deviation (n=3). Means followed by different letters are significantly different ($p<0.05$).
HTH: Control Treatment, USH5: 5 min Ultrasound Treatment, USH10: 10 min Ultrasound Treatment, MWH5: 5 min Microwave treatment & MWH10: 10 min Microwave treatment.

Table 4- The ability of Beluga head protein hydrolysate to inhibit 50% of DPPH free radical (IC_{50})

Treatment	IC_{50}
HTH	3.64 ± 0.02^{a}
USH5	2.97 ± 0.01^{c}
USH10	3.64 ± 0.02^{a}
MWH5	3.53 ± 0.04^{b}
MWH10	3.54 ± 0.03^{b}

Data are expressed as mean ± standard deviation (n=3). Means followed by different letters are significantly different ($p<0.05$).
HTH: Control Treatment, USH5: 5 min Ultrasound Treatment, USH10: 10 min Ultrasound Treatment, MWH5: 5 min Microwave treatment & MWH10: 10 min Microwave treatment.

3.3.2. Iron Ion Reducing power

The results of investigating the reducing power of hydrolyzed proteins from beluga head obtained from 10-minute microwave (MWH10), 5-minute microwave (MWH5), 10-minute ultrasound (USH10), 5-minute ultrasound (USH5), and water bath (HTH) treatments at concentrations of 0.125, 0.25, 0.5, 1, and 2.5 mg/mL are shown in Table 5. As can be seen, significant differences were observed between different treatments at concentrations of 0.25, 0.5, 1, and 2.5 mg/mL ($p<0.05$). The highest value at 2.5 mg/mL concentration was 1.86 ± 0.2 mg/mL and the lowest value at 0.125 mg/mL concentration was 0.21 ± 0.013 mg/mL, corresponding to USH5 and HTH treatments, respectively.

Table 5- Iron ion reducing power of Beluga head protein hydrolysate

Concentration (mg/ml)	0.125	0.25	0.5	1	2.5
HTH	0.21 ± 0.013^{e}	0.45 ± 0.014^{e}	0.46 ± 0.014^{e}	0.63 ± 0.018^{e}	1.47 ± 0.11^{e}
USH5	0.44 ± 0.023^{a}	0.62 ± 0.018^{a}	0.78 ± 0.016^{a}	0.95 ± 0.027^{a}	1.86 ± 0.2^{a}
USH10	0.37 ± 0.022^{b}	0.55 ± 0.018^{b}	0.68 ± 0.01^{b}	0.88 ± 0.02^{b}	1.76 ± 0.19^{b}
MWH5	0.32 ± 0.014^{c}	0.42 ± 0.016^{c}	0.6 ± 0.014^{c}	0.78 ± 0.017^{c}	1.64 ± 0.25^{c}
MWH10	0.26 ± 0.017^{d}	0.39 ± 0.018^{d}	0.52 ± 0.016^{d}	0.72 ± 0.016^{d}	1.52 ± 0.11^{d}

Data are expressed as mean ± standard deviation (n=3). Means followed by different letters are significantly different ($p<0.05$).

HTH: Control Treatment, USH5: 5 min Ultrasound Treatment, USH10: 10 min Ultrasound Treatment, MWH5: 5 min Microwave treatment & MWH10: 10 min Microwave treatment.

3.3.3. ABTS Free Radical Scavenging Ability

Table 6 shows the investigation of changes in ABTS radical scavenging ability at different concentrations of hydrolyzed protein from beluga head obtained from different treatments. According to the results, significant differences were observed between different treatments at all investigated concentrations ($p<0.05$). At all measured concentrations, the highest and lowest percentages of ABTS radical scavenging ability were obtained in USH5 and HTH treatments, respectively. The IC_{50} values of the hydrolyzed proteins were also calculated using the inhibition percentages at different concentrations, and as can be seen in Table 7, there was a significant difference between different treatments ($p<0.05$). The highest IC_{50} value (4.94 ± 0.06 mg/mL) and the lowest (3.95 ± 0.03 mg/mL) were obtained for HTH and USH5 treatments, respectively. Also, there was no significant difference between the IC_{50} of HTH and MWH10 treatments ($p>0.05$).

Table 6- ABTS free radical scavenging activity (%) of Beluga head protein hydrolysate

Concentration (mg/ml)	0.25	0.5	1	2.5	5
HTH	8 ± 0.12^{c}	10.34 ± 0.26^{c}	11.86 ± 0.15^{b}	23.43 ± 0.36^{d}	52.5 ± 0.61^{d}
USH5	9.85 ± 0.76^{a}	12.82 ± 0.45^{a}	13.65 ± 0.68^{a}	30.39 ± 0.60^{a}	63.72 ± 0.56^{a}
USH10	9.1 ± 0.45^{ab}	12.15 ± 0.27^{ab}	13.1 ± 0.93^{a}	27.7 ± 0.33^{ab}	57.96 ± 0.45^{b}
MWH5	8.86 ± 0.75^{abc}	11.14 ± 0.57^{bc}	12.86 ± 0.63^{ab}	25.87 ± 1.35^{c}	55.29 ± 0.93^{c}
MWH10	8.44 ± 0.26^{bc}	10.66 ± 0.18^{c}	12.53 ± 0.25^{ab}	24.09 ± 0.36^{d}	55.45 ± 0.45^{c}

Data are expressed as mean ± standard deviation (n=3). Means followed by different letters are significantly different ($p<0.05$).

HTH: Control Treatment, USH5: 5 min Ultrasound Treatment, USH10: 10 min Ultrasound Treatment, MWH5: 5 min Microwave treatment & MWH10: 10 min Microwave treatment.

Table 7- The ability of Beluga head protein hydrolysate to inhibit 50% of ABTS radical (IC_{50})

Treatment	IC_{50} (mg/ml)
HTH	4.94 ± 0.06^{a}
USH5	3.95 ± 0.03^{d}
USH10	4.38 ± 0.04^{c}
MWH5	4.63 ± 0.1^{b}
MWH10	4.84 ± 0.1^{b}

Data are expressed as mean ± standard deviation (n=3). Means followed by different letters are significantly different ($p<0.05$).

HTH: Control Treatment, USH5: 5 min Ultrasound Treatment, USH10: 10 min Ultrasound Treatment, MWH5: 5 min Microwave treatment & MWH10: 10 min Microwave treatment.

4- Discussion

The present study was conducted with the aim of investigating the degree of hydrolysis and antioxidant activity of hydrolyzed protein from beluga head under water bath, microwave, and ultrasound treatments.

The degree of hydrolysis indicates the reduction in the number of peptide bonds during the hydrolysis process [46] and is the most direct index for examining the characteristics of the hydrolyzed product. Studies have shown that when technologies such as microwave and ultrasound are used during or before enzymatic hydrolysis, the degree of hydrolysis increases [47], which was also confirmed in this study. In ultrasound-assisted enzymatic hydrolysis, the cavitation phenomenon causes partial denaturation of the protein and releases hydrophilic groups, ultimately facilitating enzyme-substrate binding, which can increase the rate of peptide bond breakage and consequently increase the overall efficiency of the enzymatic hydrolysis process [47]. There is also evidence that microwave radiation may increase the rate of unfolding of globular protein structures [48 and 49]. Izquierdo et al. (2008) stated that microwaves create different effects depending on the intensity, frequency, and duration of exposure [50]. In the present study, the microwave-pretreated sample showed a better effect compared to ultrasound and water bath samples. The increased degree of hydrolysis in the 10-minute microwave treatment compared to other treatments can also be the result of the effects of microwave waves, which cause protein structure unfolding and improved enzyme access, thereby accelerating enzymatic hydrolysis [51]. In the study by Bruno et al. (2019), ultrasound, microwave, and water bath pretreatments also showed different effects on the degree of hydrolysis of hydrolyzed protein from *Labeo rohita* head [40]. In that research, the use of microwave pretreatment, regardless of the enzyme type, increased the degree of hydrolysis compared to the control treatment. Also, the ultrasound-treated sample showed a better effect than the non-pretreated sample, which is similar to the present research. Uluko et al. (2013) also reported the effect of US and MW pretreatments on increasing the degree of hydrolysis of milk protein [52]. In the study by Zou et al. (2015) on porcine brain, the ultrasound-pretreated sample also showed a better degree of hydrolysis compared to the non-pretreated sample [53]. Wu et al. (2018), in a study on whey protein, also reported a higher degree of hydrolysis for the ultrasound-treated sample compared to the non-pretreated sample [54] and stated that this result may be due to the effect of ultrasound on protein structure, which makes more enzymatic binding sites available to the enzyme [49 and 55]. In the study by Ketnawa and Ligeaga (2017) on rainbow trout, a better degree of hydrolysis was reported for the sample treated with microwave compared to the non-pretreated sample [38]. These researchers stated that microwave pretreatment can affect the degree of hydrolysis by shortening the time required for enzymatic hydrolysis [51]. Also, Yang et al. (2016) in a study on bighead carp reported a higher degree of hydrolysis for the ultrasound-treated sample compared to the non-pretreated sample [56] and stated that ultrasound pretreatment can improve the efficiency of enzymatic hydrolysis, which may be related to the mechanical shocks caused by the collapse of micro-bubbles during the cavitation phenomenon, which opens the surface of solid substrates towards the active sites of enzymes [57].

DPPH is an unstable compound that converts to a stable molecule by accepting an electron or hydrogen [34]. This compound changes color from purple to yellow by taking an electron from the antioxidant. The more

antioxidant added, the more DPPH is consumed and the purple color shifts more towards yellow [43]. The antioxidant activity of hydrolyzed protein is not dependent on just one mechanism. Hydrolyzed proteins contain different peptide sequences with different mechanisms of action. Some antioxidant peptides are more effective as radical quenchers or inhibitors, and others are metal reducers [40]. The radical scavenging activity of a hydrolyzed protein depends on the number of released bioactive peptides, which in turn is mainly determined by the hydrolysis conditions used, such as the type of substrate, the type of proteolytic enzyme used, pretreatment conditions, pH, temperature, enzyme-to-substrate ratio, and hydrolysis time [58]. In the present study, the percentage of DPPH radical inhibition increased with increasing concentration of hydrolyzed protein, and the highest amount was observed in the 5-minute ultrasound treatment, indicating that ultrasound pretreatment probably led to the production of large amounts of hydrogen-donating peptides that can react with DPPH free radicals and convert them to more stable compounds [40]. In the study by Bruno et al. (2019) on *Labeo rohita* head, the ultrasound-pretreated sample also showed better DPPH inhibition activity compared to the control treatment [40]. In the study by Kangsanant et al. (2014) on tilapia, the ultrasound-pretreated sample also showed better DPPH inhibition effect compared to other samples [39]. Also, in the study by Zou et al. (2013) on pork, the ultrasound-pretreated sample showed better DPPH inhibition effect compared to the non-pretreated sample. These researchers stated that the ultrasound-treated sample probably contains small peptides with relatively low molecular weight that act as hydrogen donors and convert free radicals into more stable products [54]. Also, Yang et al. (2016) in a study on bighead carp also reported better DPPH radical inhibition activity for the ultrasound-treated sample compared to non-treated and microwave-treated samples [56]. Also, in the present study, the percentage of DPPH radical inhibition for MWH5 and MWH10 treatments at 5 mg/mL concentration is almost similar to the study by Nguyen et al. (2017) [59]. In the study by Bruno et al. (2019) on hydrolyzed protein from Labeo rohita head and Uluko et al. (2014), the percentage of DPPH radical inhibition of the microwave-treated sample was also reported to be better than the non-treated sample [13 and 14]. The DPPH radical scavenging ability of the microwave treatment can be attributed to increased solubility of smaller peptides (higher DH) and the probable presence of a high percentage of hydrophobic amino acids. Studies have shown that changes in the size, number, surface, and composition of free amino acids and small peptides during hydrolysis time affect antioxidant activity [53]. The results of Chabeaud et al. (2009) showed that hydrolysis conditions directly affect the level of enzyme activity and the production of antioxidant peptides [60]. Also, peptide concentration can affect free radical scavenging capacity [60]. In the present research, the degree of hydrolysis of ultrasound and microwave pretreated samples is higher than non-pretreated samples, and considering the higher activity of these samples in scavenging DPPH free radicals, the significant effect of ultrasound and microwave pretreatments on increasing the ability of hydrolyzed protein to inhibit DPPH free radicals can be observed.

The reducing power assessment method is often used to examine the ability of an antioxidant to donate electrons [61]. Various studies have stated that there is a direct relationship between the level of antioxidant activity and the reducing power of a bioactive compound. In this method, the ability of hydrolyzed proteins to reduce ferric ion to ferrous ion is evaluated [37]. Changes in size, structure, number of amino acids and peptides over time and increasing degree of hydrolysis affect iron ion reduction [62]. In the present study, hydrolyzed proteins from ultrasound and microwave pretreatments had the best iron ion reducing activity at all concentrations. In the research by Zou et al. (2013) on porcine brain, the ultrasound-pretreated sample

showed better reducing effect compared to the non-treated sample. The iron reduction results in this study showed that ultrasound treatment leads to better electron-donating ability, which is involved in antioxidant activity [54]. In the study by Kangsanant (2014) on tilapia, the best iron ion reduction value was also reported in the ultrasound-treated sample compared to the non-treated sample [39]. Moure et al. (2006) stated that the size and concentration of protein clearly affect iron ion reducing power [63]. Higher iron ion reduction can be attributed to the high content of electron or hydrogen-donating peptides [25]. In the study by Nguyen et al. (2017) on rainbow trout, the sample treated with microwave showed better reducing effect compared to the non-treated sample [59]. The results of this study indicated that peptide size plays an important role in the reducing capacity of bioactive peptides. In general, reducing activity depends on the molecular weight, structure, amino acid composition, and spatial conformation of peptides [64 and 65]. In the study by Bruno et al. (2019) on *Labeo rohita* head, samples from microwave and ultrasound pretreatments also had the highest iron ion reducing activity. Overall, ultrasound and microwave pretreatments improved the antioxidant activity of hydrolyzed protein from *Labeo rohita* head. These researchers stated that the improved antioxidant activity in samples from ultrasound and microwave treatments is likely due to the unfolding of protein structure or its rearrangement, leading to the availability of hydrophobic parts for the protease enzyme. These changes ultimately lead to increased hydrolysis rate and the production of large amounts of peptides with high antioxidant activity [40]. In the study by Ketnawa and Ligeaga (2017) on rainbow trout, better iron reducing effect was also reported for the microwave-pretreated sample compared to the non-pretreated sample. In this research, it was stated that this result can be linked to microwave radiation, which alters protein structure and allows the enzyme to access more target sites and release active peptides [38].

Another indicator for examining antioxidant activity is assessing ABTS free radical scavenging power. The ABTS radical is an unstable radical that is easily inhibited by an antioxidant [66]. The ABTS radical scavenging activity assay can be used for both lipophilic and hydrophilic compounds and is widely used as a method for assessing antioxidant activity [67]. In the present study, the best ABTS radical scavenging activity at all measured concentrations was observed in the USH5 treatment, and the highest ABTS radical scavenging in this treatment was obtained at 5 mg/mL concentration. The study by Jeevitha et al. (2014) on the antioxidant performance of hydrolyzed protein from sardine (*Sardinella longiceps*) also showed that with increasing concentration up to 5 mg/mL, ABTS radical scavenging ability increases [66]. In the study by Zou et al. (2013) on hydrolyzed protein from porcine brain, the ultrasound-treated sample also showed better ABTS radical inhibition effect compared to the control sample. These researchers attributed this observation to a high percentage of low molecular weight peptides and the presence of large amounts of hydrophilic and hydrophobic peptides [54]. In the study by Kangsanant et al. (2014) on hydrolyzed protein from tilapia, ABTS radical scavenging activity for ultrasound pretreatment at 70 W intensity was higher than non-pretreated samples [39]. Also, increasing pretreatment time reduced ABTS radical scavenging activity under all treatment conditions, which is similar to the results of the present research. In the study by Ketnawa et al. (2017) on rainbow trout, the percentage of ABTS radical inhibition was reported for the microwave-pretreated sample [38], and similar to the present research, the treatment from microwave performed better than the control sample. In the study by Zheng et al. (2021) on bovine bone, ultrasound and microwave-treated samples showed better ABTS radical inhibition percentage compared to the non-treated sample [68]. These researchers stated the probable reason for the better performance of the microwave-treated

sample compared to the control treatment as follows: microwave waves may have unfolded the secondary structure of proteins and caused changes in the hydrophobic region of proteins, leading to the production of peptides with favorable antioxidant function [69] and also leading to an increased degree of hydrolysis and the production of smaller peptides [70]. In the study by Yang et al. (2016) on bighead carp, similar to the present research, better ABTS inhibition activity was reported for the ultrasound-treated sample compared to the non-treated and microwave-treated samples [56].

5- Conclusion

In the present research, the results of degree of hydrolysis evaluation showed that ultrasound and microwave pretreatments had high efficiency in producing hydrolyzed protein with a suitable degree of hydrolysis. The results of investigating the antioxidant activity of hydrolyzed protein from beluga head showed that 5- and 10-minute ultrasound treatments had a significant effect on antioxidant activity compared to other treatments, followed by the 10-minute microwave treatment. The hydrolyzed protein from the 5-minute ultrasound treatment had the lowest IC_{50} value and the highest free radical scavenging ability compared to other treatments. Overall, these findings indicate that the use of novel ultrasound and microwave technologies can effectively influence the degree of hydrolysis and antioxidant activity of protein hydrolysates, and different pretreatment can lead to the production of products with different functional characteristics.

Data Availability

The data used to support the finding of this study are available from the corresponding author upon request.

Conflict Of Interest

The authors have no conflicts interest to report.

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تولید پروتئین هیدرولیزشده از سر فیل‌ماهی (*Huso huso*): اثر اولتراسوند و مایکروویو بر خصوصیات آنتی‌اکسیدانی

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چکیده

این مطالعه با هدف ارزیابی تاثیر پیش‌تیمارهای اولتراسوند و مایکروویو بر درجه هیدرولیز و فعالیت آنتی‌اکسیدانی پروتئین هیدرولیز شده سر فیل‌ماهی (*Huso huso*) انجام شد. سر ماهی پس از پیش‌تیمار با اولتراسوند (فرکانس ۲۰ کیلوهرتز وشدت ۷۵ وات) و مایکروویو (فرکانس ۲۴۵۰ هرتز و دمای ۹۰ درجه سانتی‌گراد) در دو زمان ۵ و ۱۰ دقیقه، با آنزیم آلکالاز با غلظت ۲ درصد، دمای ۵۵ درجه سانتی‌گراد و pH ۸ هیدرولیز شد و تاثیر پیش تیمارهای مختلف بر درجه هیدرولیز و فعالیت آنتی‌اکسیدانی مورد ارزیابی قرار گرفت. نتایج نشان داد که تحت تیمار با اولتراسوند و مایکروویو، درجه هیدرولیز به صورت معناداری افزایش پیدا کرد ($p< ۰/۰۵$) و. کمترین مقدار درجه هیدرولیز ۰/۲۵ ± ۳۴/۴۸ درصد و بیش‌ترین مقدار آن ۰/۱۸ ± ۵۰/۴۲ درصد بود که به ترتیب مربوط به تیمارهای حمام‌آبی و مایکروویو ۱۰ دقیقه می‌باشد. بیشترین و کمترین اثر آنتی‌اکسیدانی (توانایی مهار رادیکال‌های DPPH و ABTS و قدرت احیاکنندگی یون آهن) پروتئین هیدرولیز شده به ترتیب مربوط به تیمار اولتراسوند ۵ دقیقه و حمام‌آبی بود. مقادیر IC_{50} این تیمار در مهار رادیکال‌های DPPH و ABTS به ترتیب ۰/۰۱± ۲/۹۷ میلی‌گرم بر میلی‌لیتر و ۰/۰۳± ۳/۹۵ میلی‌گرم بر میلی‌لیتر بدست آمد که به طور معنی‌داری کمتر از سایر تیمارها بوده است ($p< ۰/۰۵$). همچنین در همه نمونه‌ها با افزایش غلظت، فعالیت آنتی‌اکسیدانی به صورت معنی‌داری افزایش پیدا کرد ($p< ۰/۰۵$). در مجموع می‌توان بیان نمود که پیش‌تیمارهای اولتراسوند و مایکروویو اثر مطلوبی بر خصوصیات پروتئین هیدرولیز شده سر فیل ماهی داشته و تیمارهای مختلف می‌توانند منجر به تولید محصولاتی با خصوصیات متفاوت گردند.