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The effect of microwave pretreatment on degree of hydrolysis and antioxidant activity of Beluga (*Huso huso*) viscera protein hydrolysate

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ABSTRACT

The purpose of this study was to investigate the effect of microwave pretreatment on the degree of hydrolysis and antioxidant activity of Beluga (*Huso huso*)visceraproteinhydrolysate. For this purpose, thesampleswere hydrolyzed after two conditions of pretreatment including no microwave treatment or after ten minutes of microwavetreatment (frequency 2450 Hz and temperature 90 °C), by Alcalase enzyme with a concentration of 2%, temperature of 55°C and pH 8,and then the degree of hydrolysis and antioxidant activity of the produced samples were evaluated. According to the results,the degree of hydrolysis after microwave treatment was significantly higher than the sample without microwave treatment ($p<0.05$). Also, the sample produced after microwave treatment showed higher antioxidant activity (DPPH and ABTS radicals scavenging activity and Fe reductioncapacity) compared to the control treatment ($p<0.05$). The IC_{50} values of this treatment in DPPH and ABTS radicals scavenging were obtained as 1.25 mg/ml and 1.63 mg/ml, respectively, which was significantly lower than the control treatment ($p<0.05$). Also, in both samples, antioxidant activity increased significantly with increasing concentration ($p<0.05$). In general, it can be stated that 10 minutes of microwave pretreatment at 90 °C has a favorable effect on the properties of Beluga viscera protein hydrolysate which can indicatethe applicability of this technology in the production process of fish protein hydrolysate.

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1. Introduction

In recent years, due to the growing trend of the world's population and the increase in the need for protein in the diet, the attention of researchers and industry has been drawn to marine resources, which has led to more consumption of aquatic life, especially fish [1]. The increase in aquatic production has led to an increase in the production of by-products from processing, but these wastes are rarely used for human consumption [2]. After processing fish, a large amount of raw residues from processing, including the spine, bones of the fish trunk, head, skin, fins, scales, and innards are left behind, sometimes up to 75% of the total weight of the fish [1]. These side products are rich in protein and fat and can be used to produce valuable compounds such as fish oil, biofuel, enzymes, omega fatty acids, proteins and bioactive peptides [3]. The evaluation of the chemical composition of wastes of different fish species has shown that more than 50% of their dry weight is protein [4]. The inside of fish, which is one of their most important wastes, is rich in protein [5]. Therefore, different processing methods can be used to use these valuable compounds in order to maximize exploitation and recycling [6]. One of these processing methods is the use of the hydrolysis process [7]. Fish protein hydrolysis is a suitable and economical strategy to obtain valuable products [8]. Hydrolyzed protein is a mixture of different peptide fractions with a diverse range of molecular weights and bioactive properties [9]. The properties of bioactive peptides depend on their size and molecular weight, as well as their amino acid composition and sequence, and sometimes the produced peptides have different multiple properties, such as antimicrobial function, strengthening the immune system, inhibition of angiotensin I converting enzyme, renin inhibition, anticoagulant function, properties They are anti-cancer, anti-tumor, anti-Alzheimer, anti-diabetic and also have antioxidant activity [10-12]. Various studies have shown that pretreatment of proteins before enzymatic hydrolysis improves the release of bioactive peptides from different types of proteins. These methods include the technologies of high hydrostatic pressure (HPP), ultrasound (US), microwave (MV), water bath (HT), pulsed electric field (PEF), etc. [13-19]. Microwave waves actually refer to electromagnetic waves with a wavelength from 1 mm to 1 meter and a frequency from 300 MHz to 300 GHz [20]. Recently, microwave-assisted extraction has been reported as an efficient technique for the extraction of bioactive compounds with high yield, low use of solvents, and reduced extraction time [21]. Microwaves have also been used for pretreatment of biological materials to improve the speed of enzymatic reactions [22]. This process increases the unfolding of the protein structure and increases the access of enzymes to the peptide bonds and finally leads to the production of hydrolyzed protein with functional properties and appropriate bioactive performance [23]. According to the stated contents, in the present research in order to exploit the inside of Filmahi (*H. house*) which is one of the wastes from the processing of this fish, the process of enzymatic hydrolysis with alcalase enzyme was used and also the effect of microwave pretreatment on the degree of hydrolysis and antioxidant activity of the hydrolyzed protein was investigated.

2- Materials and methods

2-1-Preparation of raw material

Five kilograms of elephant fish were procured from Qarabron sturgeon breeding center located in Chepkroud area and transported to the processing laboratory of Sari University of Agricultural Sciences and Natural Resources in the shortest possible time in Unilith boxes containing ice. Then, after washing and rinsing with cold water, the insides were ground using an industrial meat grinder and packed in polyethylene bags and kept in a freezer at -20 degrees Celsius until the next tests.

2-2-Production of hydrolyzed protein

2-2-1-Sample without pretreatment

In order to prepare the samples, first, 50 grams of ground fish internal samples were defrosted at a temperature of 4 ± 0.2 °C and mixed with distilled water at a ratio of 1:2 (W/V) and homogenized (IKA T25-Digital Ultra-Turrax).) were homogenized for 2 minutes. Then, the containers

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containing the samples were placed in a water bath (Memmert wub 29, Germany) with a temperature of 90°C for 15 minutes in order to deactivate the internal enzymes. Alcalase enzyme concentration (Alcalase 2.4 L FG, Novozymes) 2%, temperature 55 degrees Celsius and pH 8 were considered. After adding the alcalase enzyme, the container containing the sample was placed in a shaker incubator and the duration of hydrolysis was set to 120 minutes. Finally, to stop the enzymatic reaction, the containers containing the samples were placed in a 90°C water bath for 10 minutes. After cooling down to room temperature, the samples were centrifuged for 30 minutes at 6000 g using a refrigerated centrifuge (Sigma, 3-30KS**,** United States), were centrifuged. The supernatant part was separated by a sampler and after freezing, it was dried with a freeze dryer (Vaco 2 Zirbus, Germany) and stored in a freezer at -20 degrees Celsius for relevant analyzes [24].

2-2-2-pretreatment with microwave

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, the homogenized samples were microwaved for 10 minutes at a temperature of 90 degrees Celsius and a frequency of 2450 Hz. The rest of the hydrolysis steps were performed according to the method mentioned above [18].

2-3- Soluble protein measurement

The amount of soluble protein was measured according to Lori et al.'s method [25] and using bovine serum albumin (0.1-1 mg/ml) as a standard protein and reading the absorbance of the samples using a microplate absorbance reading device at a wavelength of 750 nm. .

2-4-determining the degree of hydrolysis

In order to measure the degree of hydrolysis, 500 microliters of hydrolyzed protein was mixed with 500 microliters of 20% trichloroacetic acid and then centrifuged at g8000 for 10 minutes. The amount of protein in the solution phase was determined by Lowry's method and finally the degree of hydrolysis was calculated with the following equation [26]:

degree of hydrolysis (%)

100× The amount of nitrogen in 10% trichloroacetic acid solution=

The amount of nitrogen in the sample

2-5-measurement of antioxidant activity of hydrolyzed protein

2-5-1-Free radical inhibition power of 2 and 2-diphenyl-1-picrylhydrazyl (DPPH)

To perform this test, a specific volume of the sample was mixed with the same amount of 0.1 mM DPPH solution in methanol at different concentrations. Then the resulting mixture was shaken well and placed in the dark for 30 minutes at ambient temperature and finally the absorption number of the mixture was read by a spectrophotometer (Spectrophotometer UV-M51 UV/Vis, Italy) at a wavelength of 517 nm. The percentage of radical inhibition was calculated according to the following equation [27]:

(Ab-As/Ab) × 100= percentage of DPPH inhibition

Ab: control absorbance As: sample absorbance

As = absorbance of the read sample – absorbance of the color of the sample (1 ml of sample + 1 ml of methanol)

2-5-2-measurement of ABTS radical inhibitory activity

The method of Alemán et al. (2011) was used to measure ABTS radical scavenging activity [28]. A solution of 7 mM ABTS in 2.45 mM potassium persulfate was prepared and kept for 16 hours at room temperature and in a dark place. It was done at a wavelength of 734 nm. Then, 20 microliters of hydrolyzed elephant fish internal protein samples in different concentrations were mixed with 980 microliters of diluted ABTS solution and incubated for 10 minutes in a dark place at a temperature of 30 degrees Celsius. After the desired time, the absorption of the samples at 734 nm was read by a spectrophotometer (Spectrophotometer UV-M51 UV/Vis, Italy) and the percentage of ABTS radical inhibition was calculated with the following equation:

ABTS radical inhibition=

100x absorption of the control sample/absorption of the sample-absorption of the control sample

- In the control sample, distilled water was used instead of hydrolyzed protein, and the other steps were similar to other treatments.

2-5-3- Reducing power of trivalent iron ion (ferric ion)

In order to measure the iron ion reduction activity according to the method of Oyaizu (1986), 0.5 ml

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of hydrolyzed internal protein of elephant fish in different concentrations with 2.5 ml of 1% potassium ferricinide and 2.5 ml of 0.2 M phosphate buffer (pH=6.6)) was mixed [29]. Then it was incubated for 20 minutes at 50 degrees Celsius, and then 2.5 ml of 10% trichloroacetic acid solution was added to it. Then the resulting mixture was centrifuged at 3000 rpm for 10 minutes. Next, it was removed from the upper phase and mixed with an equal volume of distilled water and 0.1% ferric chloride solution. Finally, the absorbance of the resulting solution was read at a wavelength of 700 nm with a spectrophotometer (Spectrophotometer UV-M51 UV/Vis, Italy).

6-2-method of statistical data analysis

Data analysis was done with SPSS 17 software. After checking the normality of the data using the Shapiro-Wilk test, unpaired t-analysis was used to compare the effect of different treatments on the degree of hydrolysis and the antioxidant performance of the hydrolyzed protein, and the significant difference between the means was checked at the 5% level. All experiments were done with three repetitions and Excel software was used to draw graphs.

3-Results

3-1- Soluble protein

The results of investigating the effect of microwave (MW) and water bath (HT) treatment on the soluble protein concentration of internal hydrolyzed protein of elephant fish are shown in Table 1. The statistical analysis of the data showed that there was a significant difference between the microwave treatment and the water bath(05/0>p) And its amount in microwave treatment was 43.32 ± 0.25 mg per milliliter.

Table 1 Soluble protein concentration of the proteinhydrolysate obtained from Beluga viscera

Pretreatment	Protein concentration (mg/ml)
HT	36.22±1.27
MW	*43.32±1.25

*indicate significant difference(p <0.05)

HT: Control Treatment, MW: Microwave treatment

2-3- degree of hydrolysis

The results of investigating the effect of microwave (MW) and water bath (HT) treatments on the degree of hydrolyzation of internal hydrolyzed protein of elephant fish inTable 2 shows. The statistical analysis of the data showed that there was a significant difference between microwave and water bath treatments (p<0.05). As can be seen in the table, its maximum amount19/0 ±06/56 was the percentage observed in MW treatment.

Table 2 Degree of hydrolysis (DH) of Beluga viscera protein hydrolysate

Pretreatment	DH (%)
HT	31.20±0.32
MW	56.06±0.19*

*indicate significant difference(p <0.05)

HT: Control Treatment, MW: Microwave treatment

3-3- Antioxidant activity

3-3-1- The effect of microwave and water bath treatments on the ability to remove DPPH free radical

The results of measuring the DPPH free radical inhibition power of internal hydrolyzed proteins of elephant fish from microwave (MW) and water bath (HT) treatments in concentrations of 0.5, 1, 1.5 and 2.5 mg/ml are shown in Table 3. It can be seen. According to the results, a significant difference between the treatments was observed in each concentration (p<0.05). In all measured concentrations, the highest DPPH free radical inhibitory power is related to MW treatment. Also compare IC values$_{50}$ The treatments (Table 4) showed that there was a significant difference between the two treatment samples and the value of IC_{50}MW treatment (1.25 ± 0.01 mg/ml) and was lower than HT treatment (p<0.05), which indicates higher activity in DPPH free radical inhibition.

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

Concentration (mg/ml)	0.5	1	1.5	2.5
HT	10.73±0.57	22.17±0.7	40.43±0.72	64.01±1.38
MW	14.57±0.49*	34.07±0.22*	50.88±0.37*	73.26±1.25*

* indicate significant difference($p < 0.05$)
HT: Control Treatment, MW: Microwave treatment

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

Treatment	IC_{50}
HT	1.78±0.09*
MW	1.25±0.01

* indicate significant difference($p < 0.05$)
HT: Control Treatment, MW: Microwave treatment

3-3-2- Ability to regenerate iron ion

The results of investigating the iron ion reducing power of hydrolyzed proteinsInside the elephant fishThe results of microwave (MW) and water bath (HT) treatments in concentrations of 0.5, 1, 1.5, 2 and 2.5 mg/ml can be seen in Table 5. According to the results, a significant difference between the treatments was observed in each concentration ($p<0.05$). In all measured concentrations, the highest amount of iron ion reduction was related to MW treatment.

Table 5 Iron ion reducing power of Beluga viscera protein hydrolysate

Concentration (mg/ml)	0.5	1	1.5	2	2.5
HT	0.093±0.004	0.184±0.009	0.322±0.014	0.541±0.018	0.727±0.017
MW	0.12±0.008*	0.236±0.008*	0.434±0.012*	0.726±0.02*	1.03±0.037*

* indicate significant difference($p < 0.05$)
HT: Control Treatment, MW: Microwave treatment

3-3-3- ABTS free radical removal ability

Table 6 shows the ability of internal hydrolyzed protein of elephant fish from MW microwave and HT water bath treatments to remove ABTS radical. According to the results, a significant difference was observed between MW and HT treatments in all investigated concentrations ($p<0.05$).. In all measured concentrations, MW treatment showed a higher ABTS radical removal percentage. IC value$_{50}$ Hydrolyzed proteins were also calculated using inhibition percentages in different concentrations and as can be seen in Table 7, there was a significant difference between MW and HT treatments.(05/0>p).

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

2.5	1.5	1	0.5	Concentration (mg/ml)
59.58±1.74	27.84±0.31	318.16±0.3	10.6±0.45	HT
72.12±1.63*	35.76±0.47*	25.46±0.66*	15.1±0.41*	MW

* indicate significant difference($p < 0.05$)
HT: Control Treatment, MW: Microwave treatment

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

IC_{50}	Treatment
1.74±0.01*	HT
1.63±0.04	MW

* indicate significant difference($p < 0.05$)
HT: Control Treatment, MW: Microwave treatment

4- Discussion

The present study was conducted with the aim of investigating the degree of hydrolysis and antioxidant activity of hydrolyzed internal protein of elephant fish under water bath and microwave treatment. The degree of hydrolysis indicates the percentage of reduction in the number of peptide bonds during the hydrolysis

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process [30] and is the primary indicator of investigating the characteristics of the hydrolyzed product. Studies have shown that when technologies such as microwaves are used during or before enzymatic hydrolysis, the degree of hydrolysis increases, which has been confirmed in this study. Izquierdo et al. (2008) have stated that microwaves depending on the intensity, frequency and The duration of exposure to waves produces different effects [31]. In the present study, the microwave treated sample showed a better effect than the water bath sample. The increase in the degree of hydrolysis in the microwave treatment compared to the water bath treatment can be the result of the effects of microwaves, which open the protein structure and improve the accessibility of the enzyme, thereby accelerating enzymatic hydrolysis [32]. In the study of Ketnawa and Ligeaga[18] on rainbow trout (*O. mykiss*) a better degree of hydrolysis has been reported for the microwave treated sample than the water bath sample. Researchers have stated that microwave pretreatment can affect the degree of hydrolysis by shortening the time required for enzymatic hydrolysis [32]. Uluko et al. [33] also investigated the effect of ultrasound and microwave pretreatments on the degree of hydrolysis of milk protein and reported that the treatment Microwave had the highest degree of hydrolysis compared to ultrasound and water bath treatment.

DPPH free radical is an unstable compound that becomes a stable molecule by receiving an electron or hydrogen [12]. This compound changes color from purple to yellow by taking an electron from the antioxidant compound [27]. The antioxidant activity of hydrolyzed protein does not depend on a single mechanism. Hydrolyzed proteins contain different peptide sequences with different mechanisms of action. Some antioxidant peptides are more effective as radical absorbers or inhibitors, and others are metal reducers [34]. The radical scavenging activity of a hydrolyzed protein depends on the number of broken 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. [35]. In the present study, the percentage of DPPH radical inhibitory activity depends on the concentration and increased with the increase in hydrolyzed protein concentration, and the highest amount was observed in microwave treatment (73.26% ± 1.25 at a concentration of 2.5 mg/ml), which is almost similar to It is with the study of Nguyen et al. (2017) that the amount of DPPH inhibitory activity for 15 minutes microwave treatment was reported as 71% [16]. In Bruno et al.'s (2019) study on hydrolyzed fish head protein*Labeorohita* and Uluko et al. (2014) on milk protein, DPPH radical inhibition percentage of the sample treated with microwaves was reported to be better than the untreated sample [34, 36]. The ability to inhibit the DPPH radical of microwave treatment can be attributed to the increased solubility of smaller peptides (higher degree of hydrolysis) and the possible presence of a high percentage of hydrophobic amino acids in the structure of the peptides. Studies have shown that changes in the size, number, surface and composition of free amino acids and small peptides during hydrolysis affect antioxidant activity [37].

The reducing power evaluation method is often used to check the ability of an antioxidant to donate electrons [38]. It has been stated in various studies that there is a direct relationship between the amount of antioxidant activity and the reducing power of a bioactive compound. In this method, the ability of hydrolyzed proteins to reduce ferric ion to ferro ion is evaluated [24]. Changes in the size, structure, number of amino acids and peptides due to the passage of time and increasing the degree of dehydration have an effect on the reduction of iron ions [39]. In the present study, as seen in the results section, the hydrolyzed protein obtained from microwave treatment had better iron ion reduction activity in all concentrations. In the study of Neguyen et al. (2017) on rainbow trout (*O. mykiss*) the sample treated with microwave showed a better regenerative effect than the untreated sample [16]. The results of this study determined that peptide size plays an important role in the reducing capacity of bioactive peptides. In general, the reducing activity depends on the molecular weight, structure, amino acid composition and steric shape of the peptides [40,41]. In the study of Ketnawa et al. (2016) on rainbow trout (*O. mykiss*) also reported a better

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iron reduction effect for the sample pretreated with microwave than the sample without pretreatment [18]. In this study, it was stated that this result can be related to microwave radiation, which changes the protein structure and allows the enzyme to access more target sites and release active peptides. In the study of Bruno et al. (2019) on Indian carp (*L. rohita*) also, the sample obtained from microwave treatment had the highest iron ion reduction activity compared to the control sample [34]. In general, microwave treatment improved the antioxidant activity of Indian carp head hydrolyzed protein. These researchers stated that the improvement of antioxidant activity in the samples obtained from microwave treatment is most likely due to the unfolding of the protein structure or its rearrangement, which led to the availability of hydrophobic parts for the protease enzyme. These changes ultimately lead to an increase in the rate of hydrolysis and the production of large amounts of peptides with high antioxidant activity.

Among the other indicators of antioxidant activity is the ability to inhibit ABTS free radicals. ABTS radical is an unstable radical that is easily inhibited by an antioxidant [42]. The ABTS radical scavenging activity assay can be used for both lipophilic and hydrophilic compounds and is widely used as an antioxidant activity assay [43]. In the present study, the best ABTS radical removal activity was observed in all measured concentrations in microwave treatment. In the study of Ketnawa et al. (2016) on rainbow trout (*O. mykiss*) ABTS radical inhibition percentage was reported for the sample with microwave pretreatment (55-60%) [18] and as in the present study, the microwave treatment performed better than the control sample. In Zheng et al.'s (2021) study on cow bone, samples treated with microwaves showed a better ABTS radical inhibition percentage than untreated samples [44]. The researchers stated the possible reason for the better performance of the sample treated with microwaves compared to the control treatment, that the secondary structure of proteins may have been opened by microwaves and changes in the hydrophobic region of proteins have been created, which led to the production of peptides with favorable antioxidant activity. becomes [45].

5. Conclusion

In the current study, the results of the evaluation of the degree of hydrolysis showed that microwave pretreatment was highly effective in producing hydrolyzed protein with the appropriate degree of hydrolysis. The results of investigating the antioxidant activity of the internal hydrolyzed protein of elephant fish (*H.huso*) showed that microwave treatment had a significant effect on antioxidant activity, so that the sample treated with microwave showed higher DPPH and ABTS radical inhibitory activity as well as higher iron ion reduction activity than the control treatment. Comparison of IC values$_{50}$ It also indicated the better performance of microwave treatment in such a way that 50% free radical inhibition was observed in a lower concentration of this hydrolyzed protein compared to the control treatment.

6- Appreciation and thanks

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7- Resources

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تاثیر پیش‌تیمار مایکروویو بر درجه هیدرولیز و فعالیت آنتی‌اکسیدانی پروتئین هیدرولیز شده اندرونه فیل‌ماهی (*Husohuso*)

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چکیده

هدف از پژوهش حاضر بررسی تاثیر پیش‌تیمار مایکروویو بر درجه هیدرولیز و فعالیت آنتی‌اکسیدانی پروتئین هیدرولیز شده اندرونه فیل ماهی (*Huso huso*) بود. بدین منظور، اندرونه ماهی با دو شرایط شامل بدون تیمار با مایکروویو و یا پس از ده دقیقه تیمار با مایکروویو (فرکانس ۲۴۵۰ هرتز و دمای ۹۰ درجه سانتی‌گراد)، توسط آنزیم آلکالاز با غلظت ۲ درصد، دمای ۵۵ درجه سانتی‌گراد و pH ۸ هیدرولیز گردید و سپس درجه هیدرولیز و فعالیت آنتی-اکسیدانی نمونه‌های تولید شده مورد ارزیابی قرار گرفت. بر اساس نتایج به‌دست آمده، درجه هیدرولیز تیمار تحت مایکروویو نسبت به نمونه بدون تیمار به صورت معناداری بالاتر بود($p<۰/۰۵$). همچنین نمونه تولید شده تحت تیمار با مایکروویو فعالیت آنتی‌اکسیدانی (توانایی مهار رادیکال‌های DPPH و ABTS و قدرت کاهندگی یون آهن) بالاتری نسبت به تیمار کنترل نشان داد ($p<۰/۰۵$). مقادیرIC_{50} این تیمار در مهار رادیکال‌های DPPH و ABTS به ترتیب ۱/۲۵ میلی‌گرم بر میلی‌لیتر و۱/۶۳ میلی‌گرم بر میلی‌لیتر بدست آمد که کمتر از تیمار کنترل بود($p<۰/۰۵$). همچنین در هر دو نمونه با افزایش غلظت، فعالیت آنتی‌اکسیدانی به صورت معناداری افزایش پیدا کرد ($p<۰/۰۵$). در مجموع می‌توان بیان نمود که پیش‌تیمار مایکروویو به مدت ۱۰ دقیقه در دمای ۹۰ درجه سانتی‌گراد اثر مطلوبی بر خصوصیات پروتئین هیدرولیز شده اندرونه فیل ماهی داشته که می‌تواند بیانگر قابلیت کاربرد این فن‌آوری در فرآیند تولید پروتئین هیدرولیز شده ماهی باشد.