Open Access

SCHOLARENA

Journal of Food Technology and Food Chemistry

RESEARCH ARTICLE

The Influence of Sonication Pre-Treatment on Physicochemical, Structural and Antioxidant Characteristics of Bighead Carp (Hypophthalmichthys Nobilis) Protein Hydrolysate Obtained Using Protamex

Alahmad Kamal*

State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Collaborative Innovation Center of Food Safety and Quality Control in Jiangsu Province, Jiangnan University, Wuxi 214122, China

***Corresponding Author**: Alahmad Kamal, State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Collaborative Innovation Center of Food Safety and Quality Control in Jiangsu Province, Jiangnan University, Wuxi 214122, China.

Citation: Alahmad Kamal (2026) The Influence of Sonication Pre-Treatment on Physicochemical, Structural and Antioxidant Characteristics of Bighead Carp (Hypophthalmichthys Nobilis) Protein Hydrolysate Obtained Using Protamex. J Food Technol Food Chem 8: 101

Abstract

The impact of ultrasonication pre-treatment under two sonication times 7.5 and 15 min using protamex protease on the degree of hydrolysis (DH), the content of total amino acids and the structural characteristics of bighead carp protein hydrolysates was investigated. DHs values were higher in sonicated hydrolysates samples than in non-sonicated sample. A significant increase in amino acid content from 79% in non-sonicated hydrolysate to 85% in sonicated hydrolysates. Hydrolysates solubility up to 94%, and molecular mass (≤1,000 Da) up to 95% after sonication pre-treatment. FTIR observed an evident change in protein secondary structure in sonicated samples with high wavelengths. The sonication process significantly influenced the antioxidant activity. The highest ABTS values were recorded after the ultrasonication process for protamex hydrolysates. The fractions derived from sonicated hydrolysates of bighead carp could serve as potent natural antioxidants, presenting a promising option to synthetic additives in both pharmaceutical and food industries.

Keyword: Protein hydrolysate, Protamex, Ultrasonication, Secondary structure, Antioxidants, bioactive peptides.

Introduction

Carp fish, native to several regions of China, were first identified and categorized there before being introduced to other parts of the world, including North America. In particular, the Ohio River has become a key location for carp populations. By 2019, global carp harvests had surpassed 3,500 kilotons, highlighted the species' widespread cultivation and increased impact on both local ecosystems and commercial fishing industries [1]. Carp fish are generally characterized by their silver-toned backs and sides, with their bellies varying from pale grey to creamy white. In East Asia, they remain a cost-effective and widely consumed option in local markets. Despite this, the focus during both production and consumption tends to be on the quality of bighead carp, which is prioritized throughout the harvesting and distribution process. Producers are particularly attentive to factors like size, texture, and flavor, as these attributes significantly influence market value and consumer preference [2]. Additionally, fish bighead d flavor, as these attributes significantly influence market value and consumer preference [2]. Additionally, fish bighead carp weighing between 8 and 14 pounds, are preferred for culinary use due to their ideal size for processing and consumption. However, both extremely large and small specimens tend to suffer from reduced market demand and lower prices. Freshwater fish proteins are particularly rich in essential amino acids, which contribute to their notable antioxidant and antibacterial properties. This unique composition enhances their ability to neutralize free radicals and inhibit microbial growth, making them more effective in combating oxidative stress and bacterial infections compared to many other protein sources [3]. In the context of utilizing fish resources in the food industry, it's essential to examine the specific roles of bioactive compounds like peptides, amino acids, and essential fatty acids.

These components are not just nutritional, but they offer functional benefits that can improve food quality, flavor, and even preservation. By optimizing the extraction and incorporation of these compounds from seafood, it's possible to unlock their full potential, leading to more value-added products in both the health food sector and broader food production [4]. There are several methods for obtaining protein hydrolysates, each with its own advantages and limitations. Techniques like microwave-assisted processing and ultrasound treatment have gained attention for their potential to speed up hydrolysis, while traditional methods rely heavily on the use of concentrated acids or alkalis to break down larger peptide chains. These conventional approaches aim to generate smaller peptides and amino acids by manipulating temperature and pressure [5]. When compared to alternative hydrolysis techniques, enzymatic hydrolysis of seafood protein demonstrates distinct advantages in terms of efficiency and specificity. This method not only yields higher-quality protein with better functional properties but also aligns more closely with the growing demand for natural, sustainable processes in the food industry. Moreover, enzymatic hydrolysis also generates bioactive peptides, which might serve as anti-biological agents, and antimicrobials, contributing to the regulation and enhancement of the immune system and overall body function [3]. Alkalase and Flavourzyme are among the most widely used proteases in hydrolysate production, primarily because of their efficiency in generating bioactive peptides. These enzymes are particularly valued for their ability to release peptides with diverse functional properties, such as antihypertensive, antioxidative, and anticancer activities [6]. The growing interest in processes like Neutrase and Protamex stems from their ability to produce a diverse range of peptides, offering more nuanced outcomes compared to traditional enzymes like Alcalase and Flavourzyme. However, while these proteases show promise, their effectiveness can vary depending on the substrate and process conditions [7]. Protamex, an endopeptidase derived from Bacillus species, exhibits a notable ability to cleave peptide bonds, with a particular affinity for hydrophobic amino acids. This enzyme's broad specificity allows it to act on a wide range of peptide substrates, making it a versatile tool in protein hydrolysis applications [8].

Efforts to improve the biological activity of fish-based hydrolysates have been limited, with only a handful of studies exploring the modification of their structural properties through advanced processing techniques. These include methods like pulsed electric fields, high-pressure processing, and ultrasound. Ultrasonication has demonstrated a significant ability to disrupt protein molecular structures, effectively reducing the protein’s average size. This disruption enhances proteolysis by increasing the ac-

cessibility of peptide bonds, making them more susceptible to cleavage by proteases [6]. Generally, the ultrasonic technique is a non-thermal process that enhances the release of peptide bonds by altering protein conformation. The ultrasound process was reported to influence the structural properties of hydrolysate products, in terms of the changes in unfolded along with α-helix and β-sheet compositions, in addition to changes in chemical bonds of peptide structure after the hydrolysis process [9].

Additionally, several previous studies have shown that applying ultrasound pre-treatment alongside with enzymolysis improved the DH in fish protein hydrolysates (FPH) obtained from rainbow trout fish (Oncorhynchus mykiss), while also enhancing certain functional properties of the hydrolysates [10]. Free radicals are implicated in a range of severe health conditions, such as cancer, diabetes, chronic inflammation, and neurodegenerative disorders. In their study, Tan et al. demonstrated that peptides derived from enzymatic hydrolysis exhibit both antioxidant and antimicrobial activities. These outcomes emphasize the potential of protein hydrolysates and its peptides not just as therapeutic agents, but as preventive tools. However, while the antioxidant and antimicrobial properties of these peptides are promising, more rigorous research is needed to fully assess their efficacy in mitigating the long-term effects of diseases like cancer and neurodegeneration [11].

It is essential to critically assess how ultrasonication treatment influences the physicochemical properties and secondary structure of protein hydrolysates along with antioxidant activity. Furthermore, testing bioactive peptides through ABTS scavenging activity, represents a key area of investigation in this field. This stepwise approach not only enhances the comprehensive understanding of the bioactivity of protein hydrolysates but also refines the methods for optimizing their functional properties in practical applications especially in pharmaceutical and food sector.

Materials

The fish samples were obtained from the aquatic Fish Institute of Technology in Jiangsu Province, China. The samples were transported in a specialized container to the Food Processing and Aquatic Products Laboratory at the School of Food Science and engineering. According to the Aquatic Products protocol, raw fish was processed and stored at or below −20 until further experiments.

Chemicals and Solutions

Protamex, a compound proteinase from Bacillus licheniformis (activity 100 U/mg, pH 7.0, reaction temperature 50 °C), was sourced from Wuxi Decheng Lebang Biotechnology Co., Ltd., located at 99 Jinxin Road, Binhu District, Wuxi City. The enzyme was stored at 4-5 °C. Alcaloides, acids, ABTS and other solutions and chemicals were ordered from Sigma-Aldrich (Shanghai-China). Other reagents and solutions were of analytical grade.

Ultrasonication Treatment of Fish Muscle Substrate

The protein hydrolysate was prepared by optimizing key parameters, including hydrolysis time (6 hours), pH, temperature, and the ideal enzyme concentration, which showed the maximum DH for Protamex enzyme. Briefly, sodium phosphate buffer was mixed with minced fish (1:1 liquid/solid) at Protamex optimal pH. The mixture was put through the ultrasonication treatment in the sonication processor (JIUPIN-1200E; Ultrasonic power/Ultrasonic horn: 1200 W/20 mm, Wuxi, China), equipped with a 20 mm diameter ultrasonic probe operating at 360 W power and a frequency of 20 kHz for sonication times of 7.5- and 15-min. Ice was added to maintain a low temperature during the sonication process. Once sonication was complete, the samples were prepared for enzymatic hydrolysis. The upper layer was collected then subsequently freeze-dried under vacuum at −60 °C, and eventually, the hydrolysates were maintained at −22 °C until further utilizing.

Preparation of FPH

The hydrolysis of sonicated and non-sonicated bighead carp samples was carried out using protamex according to the Noman protocol [12], with modifications to temperature, pH, enzyme concentration, and hydrolysis duration as outlined in Table 1. (50) mM of sodium phosphate buffer was prepared to mix with the targeted samples to maintain a stable pH for protamex enzyme during hydrolysis. The hydrolysis process was conducted in a 500 mL Jacketed vessel with continuous stirring. The vessels were connected to a circulating water bath to maintain a consistent reaction temperature. To inactivate the protease enzyme activity, the hydrolysis samples were heated to 90°C for 25 minutes in the water bath. Following this, the hydrolysis mixture was centrifuged at 8000 rpm for 20 minutes at 5°C. The supernatant was carefully separated, collected, and freeze-dried under vacuum at -65°C. The resulting hydrolysates were stored at -20°C for later use.

Measuring Degree of Hydrolysis

The degree of hydrolysis (DH) was determined by calculating the percentage of free amino groups in the hydrolysates, which was quantified through a titration process [12] with minor changes. 40 to 50 mL of protein water was mixed with 2 mL of 0.1 N hydrochloric acid. Then, the hydrolysate was adjusted to 7.0 using NaOH. Following this, about 13 mL of formaldehyde (38–40% w/w) was added to the solution and allowed to react for 10–12 min. 0.1 N of NaOH was added until the pH reached 8.5. The volume of NaOH used was noted and utilised to calculate the quantity of free amino groups. Following this, the total nitrogen content was measured using the Kjeldahl method [13]. The following two equations were used to calculate the DH and the free amino groups:

$$\text{Free amino groups } (\%) = \frac{V \times C \times 14.007}{M \times 1000} \times 100 \qquad [1]$$

$$\text{DH}(\%) = \frac{\%\text{ free amino groups}}{\%\text{ total nitrogen}} \times 100 \qquad [2]$$

Where the consumed volume of NaOH (mL), C = (0.1 M) the concentration of NaOH, Mw = the molecular weight of hydrolysate (g/mol).

According to Chabanian [14], the hydrolysate's yield was determined, and the results were reported according to the equation below:

$$\text{Yield } (\%) = \frac{\text{weight of Hydrolysate product (g)}}{\text{weight of Raw materials (g)}} \times 100 \qquad [3]$$

Amino Acid Determination

A 6 M hydrochloric acid (HCl) solution was diluted to digest 100 to 120 mg of hydrolysates for 21 to 23 hours at 110 to 130°C in an air oven. After digestion, 4.9 mL of 0.1 M sodium hydroxide (NaOH) and 25 mL of distilled water were then added to the hydrolysates.

Resulting solution was filtered through Whatman paper and centrifuged at 8000 rpm for 25 min. The amino acid composition was analyzed using a high-performance liquid chromatography (HPLC) system (model 1100, Agilent Technologies, CA, USA) equipped with a Zorbax SB-Aq C18 column maintained at a temperature of 38 to 39 °C. Detection occurred at 340 nm, with a constant flow rate of 1 mL/min. The mobile phases (A) consisted of 7.8 mM hydrochloric acid, acetonitrile, and trifluoroacetic acid (HCl/acetonitrile/TFA), and mobile phase (B) was a mixture of 7.8 mM acetonitrile, methanol, and sodium acetate (Na_2H-PO_4). Elution was conducted using a gradient system. The total concentrations were expressed as grams per 100 grams of protein hydrolysates.

The Analysis of Molecular Mass Distribution

The molecular mass distribution of the protein hydrolysates produced using protease following ultrasound-assisted pre-treatment was performed, with a slight modification based on the protocol of Li et al. [15]. A HSKgel 2000 SWXL column (300 × 7.8 mm) from Tosoh (Tokyo, Japan) was connected to the HPLC system (Waters 1525, MA, USA). The mobile phase used was a mixture of acetonitrile, water, and trifluoroacetic acid (TCA) in a ratio of 45:55:0.1 (v/v/v). Prior to injection, the samples were dissolved in the mobile phase, centrifuged for 18 min at 8000 rpm. The detection was performed at 220 nm with the column maintained at 38 °C and a flow rate of 0.7 mL/min. Different molecular standards were conducted to create the calibration curve for molecular mass distribution.

FTIR Analysis

FTIR analysis (PerkinElmer Technology, USA) was utilized to examine the changes in chemical bonding between the hydrolysates. The FTIR spectra of both sonicated and non-sonicated hydrolysates samples were recorded within the wave range of 400–4000 cm^{-1}, with a spectral resolution of 4 cm^{-1} to assess the influence of ultrasonication pre-treatment on the protein's secondary structure. A small quantity of the samples was carefully placed on the target crystal for FTIR analysis, and the pressure arm was gently applied to position the hydrolysates on the crystal. The FTIR measurements were recorded in triplicate.

Thermal Characterization Analysis

Following the protocol outlined by Alahmad et al. [5], fish protein hydrolysates (FPH) were analyzed using a differential scanning calorimeter (DSC-2000-V24, Build 120, TA Instruments, USA). A small amount of the hydrolysate product (15 ± 5 mg) was placed in aluminum pans, compressed, and sealed, then gradually heated at a rate of 10 °C/min within a Temperature range of 10 to 225 °C. The samples were maintained at 25 °C for 10 minutes before being cooled to 0 °C. The control sample was an empty aluminum pan.

Protein Secondary Structure

To examine the secondary structural changes in both sonicated and non-sonicated FPH samples, CD spectroscopy was employed using a Chirascan spectrometer (Applied Photophysics Ltd, Surrey, UK) with various accumulations in the far UV range (190–260 nm). In short, the phosphate buffer was mixed with the hydrolysates at pH 6.8, with different concentrations (0.2 to 0.6 mg/mL), and placed in a jagged cell with an optical path length of approximately 0.2 mm. The secondary structure components of the hydrolysates, including β-strand, α-helix, unordered coil, and β-turn were recorded. The software Selcon3 was utilized to calculate the percentage values, which were obtained in triplicate.

Solubility of FPH

The solubility of FPH in both sonicated and non-sonicated samples was investigated using a modified version of the protocol described by [16]. In this method, 20 mL of purified water was mixed with 100 to 150 mg of hydrolysates, and the pH was adjusted from 2.0 to 10 by adding 2 M HCl or 0.2 M NaOH. The mixture was incubated at 33 °C with continuous stirring at 250 rpm for 30 min, followed by centrifugation at 8500 g for 20 min. The protein content in the hydrolysates and supernatant was measured using the Kjeldahl protocol, and the following equation was used to calculate the solubility:

$$\text{Solubility } (\%) = \frac{\text{protein content in the supernatant}}{\text{protein content in FPH}} \times 100 \qquad [4]$$

The Analysis of Antioxidant Activity Via $ABTS^+$

The $ABTS^+$ assay was conducted according to the protocol [17], with slight changes. Potassium persulfate ($K_2S_2O_8$) solution (2.8 mM) and the $ABTS^+$ working solution (7.6 mM) were prepared, and equal volumes were mixed to prepare the final mix-

ture. The absorbance of the solution was adjusted to 0.75 ± 0.03 at 734 nm. To 5 mL concentration of the hydrolysate solution was mixed with 3.7 mL of $ABTS^+$ working solution and incubated in the dark for 20 min. A control sample was prepared using distilled water instead of the hydrolysate solution. The absorbance of the sample at 730 nm was measured using a UV spectrophotometer, and the $ABTS^+$ activity was calculated using the below equation:

$$ABTS(\%) = \left[1 - \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right)\right] \times 100 \qquad [5]$$

A control = the control absorbance without hydrolysates samples; A sample = the absorbance of FPH.

IC50 values were reported according to the hydrolysate concentration against the inhibition activity (Origin Excel (2013) using Excel (2013)).

Statistical Analysis

All experiments were conducted in triplicate (n = 3), and the results are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to assess significant differences, and Duncan's test was applied to compare the means across different groups. Statistical analysis was performed using SPSS version 19 (SPSS, Chicago, USA) with a significance level set at $p \leq 0.05$.

Results and Discussion

Influence Of Ultrasonication Pre-Treatment on the DH

The degree of hydrolysis (DH) provides a quantitative measure of the extent to which protein molecules have been degraded, offering insights into the efficiency of proteolysis. In our study, fish muscle was hydrolyzed using protease enzyme, (ES) enzymatic substrate ratios ranging from 1% to 5%, as illustrated in Figure 1. Raising the ES ratio from 1% to 5% resulted in a noticeable increase in the DH. However, further increasing the protease concentration to 5% did not yield a significant improvement in DH. This could be attributed to the aggregation of enzymes, which might have obstructed the active sites, thereby limiting the enzyme's ability to effectively catalyze the protein substrate during hydrolysis [12]. The optimal enzymatic hydrolysis conditions were determined to be a pH of 7 with an optimum temperature of 50°C for protease enzyme. The hydrolysis time was kept constant at 6 hours, based on a previous study (not published data). Under these optimal conditions, the highest degree of hydrolysis (DH) achieved was 24.21% for protease. Increased heat and protease activity resulted in cleaving peptide bonds. Protease is an endo-protease. It acts by hydrolyzing peptide bonds within proteins, breaking them down into smaller peptides. Furthermore, 4% of enzyme concentration of protease, and 6 h hydrolysis time were selected for further study under ultrasonication pre-treatment prior to enzymatic hydrolysis. The degree of hydrolysis (DH) values influenced by ultrasonication pre-treatment with proteases are shown in Table 2. The DH observed a value of 22.41% without ultrasound pre-treatment, which contrasts with the lower values reported by Norman [18], who used alcalase on Chinese sturgeon fish skin substrate. This discrepancy suggests that the combination of the specific substrate and enzymatic treatment in our study may yield higher hydrolysis efficiencies, suggesting that the peptide bonds in bighead carp fish are particularly susceptible to enzymatic hydrolysis by protease enzymes. Ultrasonication pre-treatment led to a marked improvement in the degree of hydrolysis (DH) relative to untreated samples. Specifically, after 7.5 and 15 minutes of ultrasound treatment, the DH increased from 22.41% to 30.04% and 29.65%, respectively. This suggests that ultrasound treatment enhances hydrolysis.

Moreover, the increase in the DH of the sonicated hydrolysates might be due to the impact of ultrasonication cavitation. This process induces the formation of intermolecular bonds between peptide molecules, thereby enhancing enzyme-mediated cleavage of peptide bonds [19]. Ultrasound generates cavitation, leading to microscopic bubbles forming and collapsing in the liquid

medium. The bubble collapses create intense local turbulence, microscopic vortices, and microjets. These hydrodynamic phenomena significantly enhance mass transfer by increasing the contact and mixing between the protein substrate and the enzyme. The improved mass transfer allows for better accessibility of the protein molecules to the enzymatic sites and facilitates hydrolysis [20]. The physical agitation caused by ultrasound can lead to the unfolding of protein molecules, exposing more susceptible sites for enzymatic attack [21]. The enhanced mass transfer by ultrasonication and removal of the diffusion boundary layer allows for more efficient enzyme-substrate interaction, leading to an increase in the degree of hydrolysis [22].

However, in some cases, the sonication does not increase the degree of hydrolysis up to a high level, even after a long time [23]. Moreover, the duration of sonication can impact the degree of hydrolysis. If the sonication duration is long, it could cause more mechanical stress on the proteins and peptides. This can lead to the small unfolding of proteins, which might hinder the accessibility of enzymes to the protein substrates, thus limiting the increase of DH [24]. Based on a previous study, when the degree of hydrolysis (DH) exceeds 10%, the hydrolyzed products can be utilized in nutritional and pharmaceutical applications. Our study confirmed that the DH surpassed 10%, suggesting that the hydrolysates obtained using ficin and Flavourzyme enzymes have the potential to enhance functional and nutritional properties in the food industry. [25]

Yield

The yield values from our present research are demonstrated in Table 2. Initially, at 0 minute of sonication, the yield of FPH was 17.30%. Following the sonication pre-treatment, the yields increased significantly, with values of 25.69%, at 7.5 minutes of sonication for protease enzyme. A notable variation in yield was observed across different degrees of hydrolysis (DH), highlighting the essential role of ultrasound pre-treatment in enhancing and increasing protein hydrolysate yields. The results exceeded those obtained in Chinese sturgeon fish using papain and alcalase enzymes, which were achieved under optimal enzymatic hydrolysis conditions [12]. The yield of hydrolysate products refers to the efficiency of the protease in breaking down proteins during hydrolysis, as well as the functional properties of the resulting hydrolysates. These products serve as protein additives in food, supporting various food and pharmaceutical industries, particularly those requiring high yields during the hydrolysis process [26].

Amino Acid Profile

Amino acids content of sonicated and non-sonicated protein hydrolysates are given in Table 3. The amino acid content for Pro (0 min sonication), Pro (7.5 min sonication), and Pro (15 min sonication) was 79.54, 85.12, and 84.24 g/100 g protein, respectively. The values differed significantly ($p < 0.05$) compared to non-sonicated and non-sonicated hydrolysate products. After sonication pre-treatment, the (DH) increased, leading to a higher concentration of TAA. This might be related to the peptides formed during enzymatic hydrolysis along with the notable influence as well as significant impact of ultrasonication in enhancing the hydrolysis process, which ultimately improved the final concentration of TAA. The primary amino acids identified in the hydrolysates across all samples subjected to protease enzymatic hydrolysis were leucine, aspartic acid, lysine, and glutamic acid. The amino acid levels found in our study exceeded those reported by Norman et al. [18]. Moreover, high and adequate amounts of essential amino acids (EAA) are likely important for the synthesis process. In other words, the quality and type of protein are closely linked to its amino acid composition. Furthermore, sonication pre-treatment combined with enzymatic processes may enhance EAA content in hydrolysates compared to non-treated substrates [16].

The Analysis of Molecular Weight

Molecular mass profiles of sonicated and non-sonicated hydrolysates, obtained using protease enzyme, are shown in Fig. 2. Compared to the non-sonicated samples, the sonicated hydrolysates exhibited smaller peptide molecular weights. This may be due to the combined influence of enzymolysis, ultrasound cavitation, and the sonication parameters (360 W power and 20 kHz

frequency), which likely aided in the decomposition of huge protein molecules into smaller fractions. The percentage of peptides with molecular mass < 1000 Da were 94.18%, 94.57%, and 95.07% for Prot-(0 min US), Prot-(7.5 min US), and Prot-(15 min US), respectively. Our findings show higher molecular mass values compared to those reported by Norman, who used alcalase hydrolysis on Chinese sturgeon at different time intervals [18]. Research by Romin, Stoytse, and others has shown that different proteases can be utilized to produce protein hydrolysates from fish substrates. The molecular weights of large peptides, originally above 6000 Da, can be reduced to peptides with molecular masses ranging from 150 to 500 Da [27].

Analysis of Circular Dichroism Spectra

Table 4 illustrates the effect of the enzymolysis process and ultrasonication pre-treatment with proteases on the protein's secondary structure. The software analysis (CDSel3) was applied to calculate and monitor the different amendments of protein structural components (β-sheet, α-helix, unordered and β-turn) on sonicated and non-sonicated hydrolysates. After the enzymatic hydrolysis using proteases, the α-helix and unordered had a remarkable increase compared with β-turn and β-sheet components which had a slight increase in all hydrolysate samples. Our study observed that the sonication process will enzymatically hydrolysis altered the protein structure, which may be due to peptide unfolding or protein precipitation during the hydrolysis process [26]. Ultrasonication entails exposing the protein solution to high-frequency sound waves, which causes the formation and collapse of tiny bubbles in a process called cavitation [28]. The secondary structure could be affected by ultrasonic treatment, resulting in the disruption of intricate folding patterns and the formation of new conformations. The β-turn and unordered components values were higher than the results recorded by [26], using flavourzyme and alcalase in legume protein substrate (o-heli, P-Sheets, and β-Turn). The major secondary structure elements reflect repeating, organized arrangements of peptide chains, incorporating hydrogen atoms, amino acid residues from the peptide backbone [29]. Overall, enzymatic hydrolysis using proteases, as well as ultrasonic treatment, have the potential to alter protein secondary structure. These modifications can impact protein's functional properties and physiological effects, making them useful in various applications such as food processing, biotechnology, and pharmaceutical industries. However, it is essential to note that the specific impact on protein secondary structure will depend on factors such as protein composition, the treatment conditions, and the duration of exposure to these processes.

Solubility of FPH

The hydrolysate's solubility is a key functional property in food systems. To assess this, we measured solubility across a range of pH values (2 to 10) for both sonicated and non-sonicated samples. The results, shown in Figure 3, highlight significant variations in solubility depending on the pH and the sonication treatment. In the food industry, hydrolysate's solubility plays a key role in improving emulsification, oil retention, and water absorption, which are critical to the functional performance of food ingredients. Among these properties, solubility stands out as particularly vital, as it directly influences the behavior of peptide fractions and protein molecules. A hydrolysate's solubility not only affects its integration into food systems but also has a profound impact on its ability to perform other essential functions, such as enhancing texture and stability. Without optimal solubility, the potential benefits of hydrolysates in these applications can be significantly compromised [30]. The solubility values varied between 83% and 94% across both sonicated and non-sonicated samples at different pH levels. The maximum solubility observed in this study was 94.51%, achieved with protamex after a 7.5-minute sonication time. The observed high solubility in fish hydrolysates is likely a result of both enzymatic hydrolysis and the effects of ultrasound cavitation, especially in sonicated hydrolysates. Ultrasound-induced cavitation appears to facilitate the breakdown and fragmentation of large protein molecules into smaller peptides, a process that significantly enhances the solubility properties of the hydrolysates. This increase in solubility could improve the functionality of these hydrolysates when incorporated into food products, making them more versatile in food processing applications [31]. The less values of solubility were observed at pH (4.5 to 5.5), and at this level, the interaction between protein molecules and water is fragile; this is called the isoelectric point in protein molecules. Moreover, reducing or

increasing pH far from the isoelectric point level could enhance solubility because of the remarkable interference and interaction between water and peptide fractions [32]. Based on previous publications, Norman and others reported less solubility recorded values compared with our current results in fish hydrolysates using alcalase enzyme [18], while 86% to 93.5% protein solubility was studied and reported by Kamal and his colleagues [5].

Differential Scanning Calorimeter measurement

A comparative analysis of the Differential Scanning Calorimeter of hydrolysate's products produced through enzymatic hydrolysis using protamex enzyme, both with and without sonication are presented in Figure 4. The data highlight distinct variations in thermal behavior, suggesting that sonication influences the stability and heat response of the hydrolysate. The samples were subjected to a temperature range of (0 to 250 °C) and a steady flow rate of 10 °C/min. A slight thermal degradation was observed at the temperature ranging from 130°C to 160°C in all hydrolysate's samples obtained using protamex enzyme. Furthermore, through the DSC process, thermal degradation with a sharp point was observed at 185°C for non-sonicated sample using protamex enzyme, while the sharp degradation for Prot-(7.5 min US) and Prot-(15 min US) were at 175°C. Based on the observed data, the endothermic peaks of the hydrolysates appear to be influenced by multiple factors, including the specific parameters of the sonication process (such as intensity and duration) and the enzymolysis conditions. Kamal and others reported higher endothermic peaks from 160°C to 168°C using ficin with various hydrolysis times in bighead carp fish [5]. The study revealed that during the DSC process, thermal curves were overshadowed due to the overlapping of endothermic transitions, making it difficult to distinguish individual phase changes. This indicates that both sonication and hydrolysis influenced the temperature-dependent behavior and thermodynamic properties of the hydrolysate products, potentially altering their stability and structural integrity. Differential thermal calorimetry was investigated in different protein sources, and the results ranged from 110 to 160°C [33].

FTIR Spectra of Fish Protein Hydrolysates

FTIR is a commonly employed analytical method for analysing hydrolysate products by investigating their structural characteristics, especially concerning peptide bonds. The technique identifies key spectral features, notably Amide bands I, II, and III, which correspond to distinct vibrational modes of proteins. Amide bands result from the diverse amino acid sequences present in peptides formed during hydrolysis. In protein molecules, these bands exhibit eight to nine distinct infrared absorption peaks, each corresponding to specific vibrational modes of peptide bonds. The complexity of these absorption patterns reflects variations in molecular structure, making them crucial for protein characterization and analysis [34]. The analysis of protein structure relies on examining the functional and chemical characteristics of Amide I, II, and III bands, particularly in relation to shifts in their absorption peaks. Such structural variations can significantly influence the protein's stability and functionality, making precise spectroscopic analysis crucial for understanding protein dynamics [26]. The FTIR results indicated that the bands corresponded to amide bonds associated with amino acid content. FTIR absorbance values of sonicated and non-sonicated hydrolysates were demonstrated in Figure 5. The results showed that the enzymolysis influenced protein secondary structure in all hydrolysate samples with or without sonication.

treatment. Based on the findings, the absorption peak area of non-sonicated hydrolyzate sample using protease was recorded at [3.61 cm^{-1} (amide I), 1.525 cm^{-1} (amide II), and 1.383 cm^{-1} (amide III), while, for Prot- (7.5 min US) were [1.615 cm^{-1} (amide I), 1.533 cm^{-1} (amide II) and 1.39 cm^{-1} (amide III) and finally for Prot- (15 min US) showed 1.629 cm^{-1} (amide I), 1.536 cm^{-1} (amide II) and 1.395 cm^{-1} (amide III). The higher wavelength after sonication-pretreatment at amide II and III bands may be related to the cavitation and ultrasound which occurred during the ultrasound process, which is explained by stretching N–H and C–N groups. The results of this investigation were consistent with those reported by Noman in sturgeon hydrolysates using alcalase and papain protease [34], however, the results were higher than those reported by Fadimu and his team, who used

alcalase and flavourzyme on lupin-isolated protein [26]. Key factors such as the specific type of protease used, reaction temperature, and sonication parameters—particularly the intensity of acoustic cavitation—serve as an important function in reshaping the structural integrity of the hydrolysates. These influences are not merely incidental but actively dictate the extent and nature of secondary structure modifications, underscoring the complexity of protein breakdown under varying processing conditions [35]. Amide III bands are commonly analyzed to assess protein structure based on spectral regions. While both amide I and amide III contribute to structural characterization, their roles differ: amide III is more closely associated with polypeptide backbone vibrations, whereas amide I is primarily influenced by C=O stretching. The application of sonication pre-treatment induced subtle alterations in the protein's secondary structure, suggesting a potential improvement in the bioactivity and functionality of protein hydrolysates [36].

Antioxidant Activity of Protein Hydrolysates

The analysis of ABTS scavenging activity provides a reliable method for assessing antioxidant activity by measuring the ability of a substance to donate hydrogen ions (H+). Its effectiveness lies in its consistency, as it quantifies antioxidant capacity based on the efficiency of electron or hydrogen ion donation [37]. The ABTS data of sonicated and non-sonicated enzymatic hydrolysis products were demonstrated in figure 6. Based on our study findings, increasing the concentration of hydrolysate products from 1 to 5 mg/mL increased ABTS+ values in all targeted samples. The results of our study align with previous research, particularly the work by Chalamaiah et al., who observed that elevating the concentration of hydrolysates in Labeo rohita protein hydrolysates enhances ABTS scavenging activity [14]. Similar findings were reported by Noman using ultrasound and microwave-assisted along with enzymatic hydrolysis to produce hydrolysates from Chinese sturgeon fish [38]. Furthermore, a lower IC_{50} value typically suggests a stronger antioxidant activity of the hydrolysates, implying its higher efficacy in radical scavenging. The IC_{50} records of both sonicated and non-sonicated protein hydrolysates obtained using protamex enzyme were 2.91, 1.78, and 2.33 mg/mL for Prot-(0 min US), Prot-(7.5 min US) and Prot-(15 min US) respectively. According to our findings, the lowest IC_{50} was 1.78 mg/mL in hydrolysate under sonication pre-treatment for 7.5 min using protamex, which was observed with the highest $ABTS^{+}$ activity. The variation in $ABTS^{+}$ values across sonicated and non-sonicated samples can likely be attributed to several factors, including the peptide's molecular weight and size. Additionally, the type of protease used, along with the specific conditions and duration of the sonication pre-treatment, play a significant role in shaping the resulting peptide profile. The amino acid composition formed during enzymatic hydrolysis also seems to be a crucial determinant, as it can directly influence the antioxidant potential. The interplay between these factors warrants further investigation to clarify their individual contributions to the observed differences [39].

Conclusion

The impact of combining sonication with enzymatic hydrolysis using protamex enzyme, was examined in terms of the DH, amino acid content, yield, molecular mass analysis, as well as structural and thermal characteristics. Additionally, the study explored the antioxidant activity. The results indicated that sonication significantly enhanced both the hydrolysate yield, and the DHs compared to non-sonicated sample. Additionally, the sonication treatment consistently elevated the amino acid content across all processed samples, suggesting a more efficient breakdown of proteins. Ultrasound treatment induced notable amendments in the hydrolysate's secondary structure, as revealed through FTIR and circular dichroism analysis. These alterations were not only detectable but also suggest a specific modulation of protein conformation. Antioxidant activity determined using ABTS recorded high activity at 7.5 min sonication time in the hydrolysate. The fractions derived from ultrasonicated protein hydrolysate demonstrated notable antioxidant activity, effectively scavenging free radicals. This finding highlights their potential for use in both pharmaceutical and food industries, offering a promising avenue for developing antioxidant-rich products. However, further studies are needed to evaluate their stability, bioavailability, and overall efficacy in practical applications.

References

1. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, et al. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine, 26: 1231–37.

2. Chalamaiah M, Dinesh Kumar B, Hemalatha R, Jyothirmayi T, et al. (2012) Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities. Food Chemistry, 135: 3020–38.

3. Noman A, Xu Y, Al-Bukhaiti WQ, Abed SM, Ali AH, et al. (2022)Influence of enzymatic hydrolysis conditions on physicochemical and functional properties of fish protein hydrolysates.Foods, 11: 1320.

4. Alahmad K, Xia W, Jiang Q, Xu Y, et al. (2022) Effect of enzymatic hydrolysis on bioactive properties of protein hydrolysates. Arabian Journal of Chemistry, 15: 104368.

5. Liu Y, Wang R, He Y, Feng C, Xia X, et al. (2024) Structural and functional changes in protein hydrolysates under different processing conditions. International Journal of Biological Macromolecules, 131632.

6. Penas E, Préstamo G, Gomez R, et al. (2006) High pressure and enzymatic hydrolysis effects on soybean protein.Food Chemistry, 97: 569–75.

7. Tang W, Zhang H, Wang L, Qian H, Qi X, et al. (2010) Targeted separation and antioxidant activity of peptides.Journal of Food Biochemistry, 34: 132–45.

8. Ibrahim Y, Kibret M, et al. (2020) Evaluation of physicochemical and antioxidant properties of protein hydrolysates. Frontiers in Nutrition, 7: 1511698.

9. Chen XL, Peng M, Li J, Tang BL, et al. (2017) Preparation and functional evaluation of marine protein hydrolysates. Scientific Reports, 7: 15971.

10. Fadimu GJ, et al. (2023) Structural and functional properties of lupin protein hydrolysates.Molecules, 28: 519.