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Impact of Enzymatic Hydrolysis Using Two Proteases on Functional, Structural, and Antioxidant Properties of Protein Hydrolysates Derived from Fresh and Microwave-dried Substrates of Bighead Carp (Hypophthalmichthys Nobilis)

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Abstract In our study, fish protein hydrolystes (FPH) were produced from bighead carp using ficin and flavourzyme enzymes with fresh and dried substrates. Under optimum conditions for two proteases, the hydrolysates obtained from fresh substrate showed a significant variation in different characteristics compared with hydrolysates obtained using microwave-dried substrate. The best E/S ratio was 3% with DH 16.85% and 4% with DH 22.51% (fresh substrate) for ficin and flavourzyme respectively, whereas, the best ratio of (dried substrate) was 4% with DH 13.92% and 4% with 17.31% for ficin and flavourzyme enzymes respectively. The yield of FPH was recorded 16.11% and 17.39% (fresh substrate) using ficin and flavourzyme respectively. Moreover, more peptides with low molecular weight were formed during hydrolysis which could enhance the functional properties of FPH in particular solubility property which ranged from 84% to 95%. However, FTIR demonstrated that enzymatic hydrolysis with type of substrate had an influence on the secondary structure of the protein by observing a slight change in wavelength of amide band. Furthermore, DDPH radical scavenging and hydroxyl radical scavenging were determined and showed high activity values of the hydrolysates products. The present study revealed that the physicochemical, structural and antioxidants properties of protein hydrolysates under hydrolysis of two proteases could be a great source of protein and possess a potential applications in food factory.

Keywords: *ficin, flavourzyme bighead carp, enzymolysis, amino acids, FTIR, solubility, SEM, antioxidants properties*

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1. Introduction

Fish carp were found and reported early in different parts of China and was spread to various areas including North America such as Ohio and Tippecanoe Rivers. The global harvest of carp fish was recorded over 3300 kilotons in 2018 [1]. In general, carp fish appearance silver on the back and sides, and then the bellies have grey to creamy color. Fish carp is popular and affordable ingredient in East Asia, and thus the quality of bighead carp has the priority concern for producers and consumers in term of fish planting, harvest and marketing [2]. Environmentally, carp fish could clean and filter the lakes and freshwater territories by using their rakers, in addition to feed on different and wide types of microorganisms like algae, insects and some freshwater plants. However, carp fish have a large tolerance in huge challenging conditions, which can be gathered in groups and defeat against environmental risks [3]. Moreover, bighead carp size between 7 to 15 pounds is preferable for consumption but huge or little size might affect the cost of fish market. Mainly, protein in fish is remarkably digestible with sufficient amounts of essential amino acids and peptides that are lack in other meat sources. On another word, freshwater fish proteins contain remarkable values of antioxidants and antimicrobial due to sufficient quantities of amino acids [4]. Obviously, there is a huge need to use fish resources with more focus and attention on ingredients including peptides, amino acids and some important fatty acids in seafood resources, abundant fatty

acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and good source of vitamins, D3, E, together with vitamin (B) complex including (B2, B6 and B12) [5]. Drying of fish is important, because it preserves fish by inactivating enzymes and removing high amount of the moisture which can stop bacterial and mold from growth and spoiled fish products [6]. Fish can be dried or smoked which extend the shelf life and improve the flavor with the texture and make the final product more preferable for consumption. Drying is a technique of simultaneous heat and mass transfer. Fish drying methods vary among and within countries depending on the species used and the type of product desired. Fish may be dried to various degrees with water content in the final ranging from about 10% to 30%.

Different fish species have different protein contents, ranging from 8% to 22%, leading to 50%–65% of energy produced in various carp fish species [7]. However, drying process is important to stop or reduce some undesirable changes in fish. Additionally, traditional drying techniques include solar drying, hot air drying, smoking and microwave drying are widely used in food drying techniques. Dried fish product is a tasty and suitable food and can be used in various food applications. Microwave technique provides a high heating with efficient energy, safe, harmless and easy to control. Microwaves heat material simultaneously inside and outside, which can applied high rates of evaporation. Hence, it is an efficient process for meat and fish because it needs less time and energy and improve final product with nutritive quality [8]. To produce protein hydrolysates, wide various techniques could be applied such as Ultrasonic sonication, microwave assisted technique and using organic or chemical solvent to obtain the hydrolystes. The conventional pathway to obtain protein hydrolysates; by using concentrated acid or alkaline to separates big peptide molecules at the optimum temperature and pressure to small derived peptides and desired amino acids [9]. The process of enzymatic hydrolysis targeting seafood protein had remarkable potential apply in different food industries in terms of comparison with other hydrolysis methods. The enzymatic hydrolysis might provide prospective nutritional and functional products in terms of wide uses in the food applications. The current process of hydrolysis could lead to enhance and release various and desirable properties of freshwater and dried fish protein. Additionally, the enzymatic hydrolysis release and provide small active peptides that effectively might be used as antimicrobial, anti-cancer and antioxidants, to improve and regulate the body functions along with immune system [4]. Various areas of food, pharmacy and cosmetic used active fractions of hydrolyzed peptides in their industries. The modification of enzymatic process of proteins utilizing selected protease enzymes to cleave big peptides into small fractions was hugely applied in the food industry and many researches were investigated about functional and morphological properties of hydrolysates over past 15 years [10]. Exo- and endo- proteases were applied to obtain FPH including Ficin, Papain, bromelain, trypsin, alcalase, protamex, validase, neutrase and flavourzyme [9]. The advantage of protease enzymes that can be produced from different sources such as microorganisms, plants and animals. Protease enzymes used in synthesis, catalysis and different applications of food biotechnology in term of producing active peptides, provide essential amino acids, improve the solubility and digestibility in food ingredients [11]. Ficin enzyme obtained from (Ficus carica) tree was described as a protease that catalysis ends of cysteine which, is similar to papain bromelain, and, cathepsin. These enzymes belong to the family of cysteine proteases and have the exact mechanism of catalysis and the same cleaving peptide bonds. Ficin can produce hydrolysates from different protein sources and apply in food industries, such as enhancing texture and tenderness in fish products. Ficin has also been used as milk clotting, a rennet starter, and a processing path for fermented cereals [12]. Flavourzyme is a fungal enzyme and commercially produced by Aspergillus oryzae with remarkable properties in term of generate amino acids sequences and active fractions peptide, and have a clear influence on some bio active compounds such as antioxidants and antimicrobial. Flavourzyme is a mixture of exo- and endo-peptidase which cleaves the peptide bonds within and at the ends of protein molecules sides [13]. The best conditions of hydrolysis to achieve and produce high-quality products of FPH is using protease enzymes, compared with other hydrolysis methods. Thus, in term to enhance the characteristics of FPH and maintain the nutritional properties in food applications, hydrolysis by enzymes might be an appropriate and applicable technique. The type of substrate, pH of the reaction, the temperature, specific concentration of enzymes and reaction time are the most important factors which influence the final product of FPH. Furthermore, the optimum conditions of mentioned factors can smooth the enzymatic process and lead to high quality production of FPH [14]. However, more factors play important roles in term of FPH production such as the specificity of protease enzyme, molecular weight, degree of hydrolysis, protein source, peptide sequences and the technique applied to obtain the protein hydrolysates could also influence the nutritional and biological values of final FPH [15]. To enhance the functional characteristics and improve the solubility of protein in different seafood sources then the process of enzymatic hydrolysis could be applied to obtain a desirable FPH. Emulsification properties, foaming capacity, oil and water holding capacity, anticancer, antimicrobials and antioxidants values could be enhanced and increased through hydrolysis process [16]. FPH possess different bioactive compounds including antioxidants which can be applied against different sicknesses or using as food additives' to improve the immune system, thus different researches indicated that the exist of antioxidants in FPH products might increase the body defense against heart and cancer diseases. The production of FPH with high activity of antioxidants play a remarkable role in which improve the body functions and apply in various areas of food industries, thus study these properties in FPH could give an overall comprehensive about functional and food additives [17].

2. Materials and Methods

2.1. Materials

Fresh Bighead carp fish with 3 to 5 kg and 40 to 50 cm length, age is between 3 to 4 years. To ensure the quality and freshness of the fish, Fresh bighead carp (Hypophthalmichthys nobilis) was obtained from a research center (Yangtze River aquatic products science and technology industry Co., Ltd in Wuxi, Jiangsu, China). The fish were caught from one location and handled according to standard procedures for storage and transportation (washing, how the fish is aligned in the tub, and icing).

2.2. Samples Preparation

The targeted fish were cleaned with tap water, and the head and bones were removed. The flesh was chipped into small slices length 4 to 5 cm, width 4 to 5 cm with thickness 2 cm, to obtain fish fillet. The raw fresh samples were ground, homogenized, and kept frozen at -20°C until further experiment. Drying was carried out using a microwave oven (ORW1.0S-5Z, 100-1000W, 380 V, /40 cm × 37 cm × 22 cm/ Nanjing Aorun Microwave Technology Co., Ltd.) according to Wu and Mao [18], with some modification, the sample dried at 2450 MHz and 360 W for 10 min. The dried bighead carp fish fillets were then blended and homogenized using a high-speed blender (25000 rpm).

2.3. Enzyme and Chemicals

Ficin enzyme from Ficus carica (enzyme activity 400 to 1000 MCU/mg, PH 6.0, temperature 40 °C), Flavourzyme from Aspergillus oryzae with approximate optimum conditions (activity 30 U/mg, pH 6.0, temperature reaction 50°C) purchased from (Wuxi Decheng Lebang Biotechnology Co., Ltd) located at (99 Jinxi Road, Binhu District, Wuxi City) and then all protease enzymes were maintained at 4 °C until use. Solutions and reagents, including alkaloids and acids, are provided by Sinopharm Chemical Company (Wuxi, China). DPPH and other reagents were purchased from Sigma-Aldrich (Shanghai-China). Other chemical components were at analytical grade.

2.4. Protein Hydrolysates Preparation

Fish protein hydrolysates of bighead carp were produced using Ficin and flavourzyme enzyme following the procedure explained by Noman [19] with modifications under Enzyme/substrate ratio (%) temperature, pH and hydrolysis time conditions given in Table 1. The raw fish was minced and microwave dried fillets were blended and homogenized then mixed with 50Mm (sodium phosphate) buffer to keep a firm pH for each enzyme during the time of hydrolysis process. The hydrolysis reaction was accomplished in a jacketed vessel (500) mL, with stirring during hydrolysis time, and to control the temperature, the circulated water bath (Blue pard Technical. Co., Shanghai, China) was connected the jacketed vessels. To deactivate ficin and flavourzyme enzyme activity, hydrolysis samples were heated up to 75 and 85°C for 20 to 30 min in the water bath for ficin and flavourzyme respectively. The hydrolysis was left at room temperature and then centrifuged for 20 minutes at a speed of 8000 rpm at 4°C. Eventually, the upper layer (supernatant) was collected and then freeze-dried at -55°C vacuum, and then the hydrolysate products for the three enzymes were kept at -18 °C for subsequent experiments.

Table 1. The enzymatic Hydrolysis conditions for obtaining protein hydrolysates from bighead carp *(Hypophthalmichthys nobilis)* using Ficin and flavourzyme enzymes

Hydrolysis conditions	Ficin	Flavourzyme
Enzyme/substrate ratio (%)	1, 2, 3, 4, 5	1, 2, 3, 4, 5
Reaction pH	6	6
Temperature (°C)	40	50
Inactivation temperature (°C)	75	85
Inactivation time (min)	20	30
Hydrolysis time for hydrolysate production (h)	6	6

2.5. Determination of Degree of Hydrolysis

The (DH) was reported as a percentage of free amino group cleaved from hydrolysates samples, which was determined through formal titration following the procedure [19], with slight modification. A detected amount of protein hydrolysates samples (1.5 to 1.7) g was added to 45 to 50 ml purified water. Then the mixture was calibrated to pH 7.0 by adding 0.1N NaOH, and then 10 mL of formaldehyde 37% (v/v) was added and kept for 8 min at room temperature. Using 0.1 N of NaOH standard solution, the titration was carried out to the endpoint at pH 8.5. The amount of free amino group was then calculated using the consumed volume of NaOH. The Kjeldahl technique determined the total nitrogen (TN) [20]. The degree of hydrolysis and the free amino groups were calculated as follows:

$$\begin{aligned} &Free\ amino\ groups\ (\%) \\ &= (V \times C \times 14.007 / M \times 1000) \times 100 \end{aligned} \quad (1)$$

$$\begin{aligned} &Degree\ of\ hydrolysis\ (DH) \\ &= (\%\ Free\ amino\ groups / \%\ total\ nitrogen) \times 100 \end{aligned} \quad (2)$$

Which, V= volume consumed of NaOH (mL); C= the concentration of NaOH (0.1M); M= the quantity of hydrolysate in the sample (g).

2.6. Yield

The yield of FPH was calculated by the weight of FPH obtained as a percentage of the fish muscles (substrate) was used in the enzymatic hydrolysis according to Romadhoni [21], and was given in the following equation:

$$Yield\ (\%) = \frac{weight\ of\ FPH\ (g)}{weight\ of\ Raw\ materials\ (g)} \times 100 \quad (3)$$

2.7. Water Activity

Water activity was carried out using a lab instrument (Novasina AG, 100-240V, Switzerland) at 25 ℃ with accurate of 0.001. After the calibration, the FPH powders

were placed in a sample chamber and kept until equilibrium was reached. Each sample was carried out on triplicate.

2.8. Colour Measurement

A Hunter Lab digital colorimeter (UltraScan PRO, D65, Virginia, USA) was used to scale the color of the protein hydrolysate product. The processed samples were located at the port of the colorimeter. The values of color evaluation L*, a*, and b* were measured; a* represents redness to green; b* represents yellowness to blue; L* represents lightness; while (ΔE) was expressed as the total color difference.

2.9. Amino Acid Determination

In brief, 100 mg of protein hydrolysate samples were digested by using 8 mL of (6 M hydrochloride-HCl) at 110 °C, under a nitrogen atmosphere in an air oven for 20 to 23 h. After that, under room temperature, the processed samples were cooled, then added 4.8 mL of (10 M NaOH). The sample was washed using purified water up to 25 mL. The samples were filtered using Whatman filter paper and centrifuged for 15 min at 10,000. The Amino acid was analyzed and separated using (HPLC, model 1100, Agilent Technologies, CA, USA), the reverse phase mode was (180 mm × 4.6, Agilent) with Zorbax 80 A and C18 column at 40°C, and the detection value was 338 nm with a constant flow rate 1 ml/min. The mobile phase (A) was 7.35 mM/L sodium acetate/triethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v) and to adjust pH to 7.2, acetic acid was used. The mobile phase (B) was 7.35 mM/L sodium acetate/methanol/acetonitrile (1:2:2, v/v/v) with pH 7.2. The analysis of amino acids was expressed as a gram of amino acid per 100 g of the protein sample.

2.10. Molecular Weight Distribution Profile

The analysis of molecular weight distribution of bighead carp fish muscles and the protein hydrolysate obtained by enzyme hydrolysis were investigated according to the guidance of Liu, Lin, Lu, and Cai [22] with minor modification. High-performance liquid chromatography using (Waters-1525, MA, USA) system with column TSKgel 2000 SWXL (300 × 7.8 mm) manufactured (Tosoh, Tokyo, Japan) was equilibrated with mobile phase acetonitrile/water/trifluoroacetic acid (TCA) 45/55/0.1 (v/v). The samples before injection were dissolved in mobile phase buffer and centrifuged for 20 min at 8500 rpm before filtration using 0.45 µm filtrate membrane. Finally, the samples were eluted at 0.5 mL/min flow rate and monitored at 220 nm, the temperature of the column was 30°C. Cytochrome C (12,384 Da), bacitracin (1422 Da), Gly-Gly-Try-Arg (451 Da), and Gly-Gly-Gly (189 Da) were utilized as standards to prepare the calibration curve of the molecular mass analysis.

2.11. Scanning Electron Microscopy

The morphological properties of the hydrolysates products were implemented using a scanning electron microscope analysis (Hitachi-High-Tech's-SU1510-Minato-Ku, Tokyo, Japan) instrument. The treated samples were coated before being loaded into the SEM machine, and the images were then observed under accelerating volatge 1.0 KV by secondary electron image. The image was scanned using 10.20 mm Ricoh Camera with 600x magnification.

2.12. Fourier Transform Infrared Spectroscopy

The difference in chemical bonding of the hydrolysates was measured using FTIR (PerkinElmer Technology, USA). In short, the FTIR spectrum of the samples which obtained under different degree of hydrolysis and a total of 32 scans were carried out at a spectral resolution of 4 cm^{-1}, with each spectrum wavenumber range of 400-4000 cm^{-1}. The transform infrared spectroscopy was applied to evaluate the impact of enzymatic hydrolysis on the secondary protein structure of hydrolysates. Different absorption peaks were studied and analyzed. To carry out the FTIR spectrum, a tiny amount of hydrolysates products were placed smoothly on the targeted crystal and then the pressure tool was pushed to fix the products on the diamond crystal lightly. FTIR records were in triplicate and the average of each sample was calculated.

2.13. Functional Properties Analysis of FPH

2.13.1. Solubility

The solubility of protein hydrolysate obtained by enzymatic hydrolysis was evaluated based on the procedure recorded by Jain and Anal [23] with slight changes. 20 mL purified water with (200 mg) of hydrolysate were mixed, and then the pH solution was adjusted from 2 to 10 by utilizing 0.1 M NaOH or 0.1 M HCl. The solutions were incubated at 30°C with stirring 150 rpm for 30 min and then centrifuged at 8000 g for 20 min. The content of protein in both the supernatant and the hydrolysate samples were determined using the Kjeldahl process and the following equation calculated the solubility:

$$Solubility\ \% = \frac{protein\ content\ in\ the\ supernatant}{Total\ protein\ content\ in\ the\ sample} \times 100 \quad (4)$$

2.13.2. Emulsifying Properties

The determination of emulsification properties of FPH under various degrees of hydrolysis, including emulsifying stability index (ESI) and emulsifying activity index (EAI), was according to Noman, Xia, and others [24] with minor modification. Different concentrations of protein hydrolysates (0.1%, 0.5% and 1% protein, 6 mL) solutions were mixed with 10 mL of soybean oil and homogenized (ULTRA-TURRAX, T 18 Digital, IKA, Germany) at 22000 rpm for 60 seconds. Then after emulsion formation, 50 µL was collected from the bottom of the samples and diluted with 5mL of 0.1 % sodium dodecyl sulfate solution. The absorbance of the solutions was detected at 500 nm utilizing UV 1000 spectrophotometer after 0 and 10 min. The following equations were used to calculate EAI and ESI:

$$EAI\ \left(m^2/g\right) = 2 \times 2.303 \times A_{500} \times DF / I\Phi C \quad (5)$$

$$ESI\left(min\right) = A_{500} \times 10 / \Delta A \quad (6)$$

A_{500} = Absorption value at 500 nm, Φ = oil volume fraction (0.25), I = path length of the cuvette (m), DF = dilution factor, C = the concentration of protein in aqueous phase (g/m^3), $\Delta A = A_0 - A_{10}$

2.14. Antioxidant Properties of FPH

2.14.1. DPPH Radical Scavenging Activity

The DPPH radical-scavenging activity of hydrolysates was measured according to the method described by [25] with modifications. In short, FPH hydrolysates powder was diluted in various concentration from (1 to 20 mg/mL) and then, 1.70 ml of methanolic DPPH solution (60 µmol) was added to 100 µL of the hydrolysates samples in a 24-well microplate. The mixture then was incubated in the dark for 30 min at room temperature with the control samples (DPPH solution was added to 100 µL of distillated water). The microplate reader was used to read the absorbance at 517 nm. The absorbance values were converted to DPPH scavenging activity (%) using the following equation:

$$\text{DPPH scavenging activity } \% = \left[\left(A_0 - A_1\right) / A_0\right] \times 100 \quad (7)$$

Where: A0 is the control absorbance, and A1 is the sample absorbance.
IC50 values were calculated based on the concentration of protein against the percentage of inhibition (50%) by using Excel (2013).

2.14.2. Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity was determined according to the method described by [26] with some changes. In 24 microplates 100 µl of hydrolysates samples at different concentration (1-20 mg/ml) and 200 µl of $FeSO_4$ (2 mM), 200 µl of 9 mM salicylic acid in 96% ethanol, and 200 µl of H_2O_2 (0.03% v/v), and then incubated at 37°C for 75 min. The absorbance was carried out at 536 nm against a blank (distilled water instead of hydrolysates solutions).

2.15. Statistical Analysis

The samples were analyzed in triplicate (n=3), and the data were provided as mean value ± standard deviation (±SD). The results were subjected to a one-way analysis of variance (ANOVA) to show the significant differences. Duncan's was performed to analyze multiple ranges between means utilizing SPSS ver.19 (SPSS. Chicago, USA), and the significance level was defined at $p \leq 0.05$.

3. Results and Discussion

3.1. Production of Protein Hydrolysates

In the current work, the influence of Ficin and flavourzyme enzymatic hydrolysis conditions including E/S% ratio, time of hydrolysis and type of substrate (fresh and microwave dried bighead carp) on the degree of hydrolysis DH was demonstrated in Figure 1. The optimum pH was 6 for both enzymes ficin and flavourzyme, and the optimum temperature was 40°C and 50°C for ficin and flavourzyme during enzymatic hydrolysis respectively. Experimentally, the effect of the E/S ratio on DH was investigated at various levels according to the protein content in the substrate (fresh and microwave dried bighead carp) 1%, 2%, 3%, 4%, and 5%. However, at 1% (w/w) enzyme concentration, the DH was 13.56% and 15.84% (fresh substrate) for ficin and flavourzyme enzyme respectively, while DH recoded 7.64% and 11.07% (dried substrate) for ficin and flavourzyme respectively, that might be inadequate catalytic sites and then improve the degree of hydrolysis values. Furthermore, the best E/S ratio was 3% with DH 16.85% and 4% with DH 22.51% (fresh substrate) for ficin and flavourzyme respectively, whereas, the best ratio of (dried substrate) was 4% with DH 13.92% and 4% with 17.31% for ficin and flavourzyme enzymes respectively, which observed a significant difference compared to the low concentrations of both enzymes.

The higher concentration of proteases enzymes at 4% and 5% for ficin and flavourzyme respectively, presented no significant variance in DH values; this likely due to the enzyme aggregation which leads to inhibited the active sites to catalytic protein in the substrate (fresh and dried) during the hydrolysis process [19]. Moreover, the degree of hydrolysis was studied and measured at seven times from (1 h to 7 h) under the optimum conditions (E/S%, pH and temperature). At the first hour, the enzymatic hydrolysis process was remarkably fast and the DH was 13.21% and 16.42% (fresh substrate) for ficin and flavourzyme respectively, while the DH was lower significantly using (dried substrate) with DH 9.67% and 11.73% using ficin and flavourzyme respectively. At the second and third hour continued with high rate of hydrolysis, furthermore, the rate of hydrolysis was slow from 3 h to 7 h, the highest DH was recorded at 6 h for both substrates and enzymes, which was 20.32% and 25.13% (fresh substrate) for ficin and flavourzyme respectively, while the DH was 17.80% and 19.21% (dried substrate) using ficin and flavourzyme enzymes respectively, no significant variation of DH between 6 h and 7 h and then the hydrolysis process was terminated at 6 h. Ovissipour and others declared that the reduce in hydrolysis rate after 2 to 3 hours due to the reduction of peptides molecules in the substrate which impact on the hydrolysis process [27]. The degree of hydrolysis at 6 h for fresh and microwave dried substrates under enzymatic hydrolysis for ficin and flavourzyme enzymes were chose for further study in this research.

3.2. Yield Determination

The yield results in the current study was presented in Table 2. The yield of FPH was recorded 16.11% and 17.39% (fresh substrate) using ficin and flavourzyme respectively, while the other values were 12.29 % and 14.07 % (microwave dried substrate) under using ficin and flavourzyme in enzymatic hydrolysis respectively. There was a significant difference between yield values at

different substrates and obviously fresh substrate was recorded high yield value compare with microwave dried substrate for both protease enzymes due to easy catalyze the peptide bonds in the fresh substrate and formed small peptides compared to dried fish substrate which is hard to attack and catalyze the peptide bonds in reaction with active site of protease enzymes. The observed results were slimier to those reported by Noman in Chinese sturgeon fish with papain enzyme [19], and higher than those achieved under optimal condition of using alcalase enzyme [24].

3.3. Water Activity

The water activity concept refers to food stability in term of molecular mobility and water existing. a_w plays an important role in biological process and chemical reactions in food products. The values of water activity are presented in Table 2. According to the current research, the best value of water activity was 0.25 and 0.31 (fresh substrate) using ficin and flavourzyme respectively, while increased to 0.39 and 0.34 (microwave dried substrate) under ficin and flavourzyme hydrolysis process respectively. Moreover, The value of aw between 0.50 to 0.60 could increase the food hardness and give low flavour quality, on the other hand increasing aw from 0.70 to 0.85 could motivate chemical reaction and supply a good environment for the growth of Bactria and fungi [28]. However, our results showed lower value of a_w and which might provide a remarkable stability and increase the food shelf life.

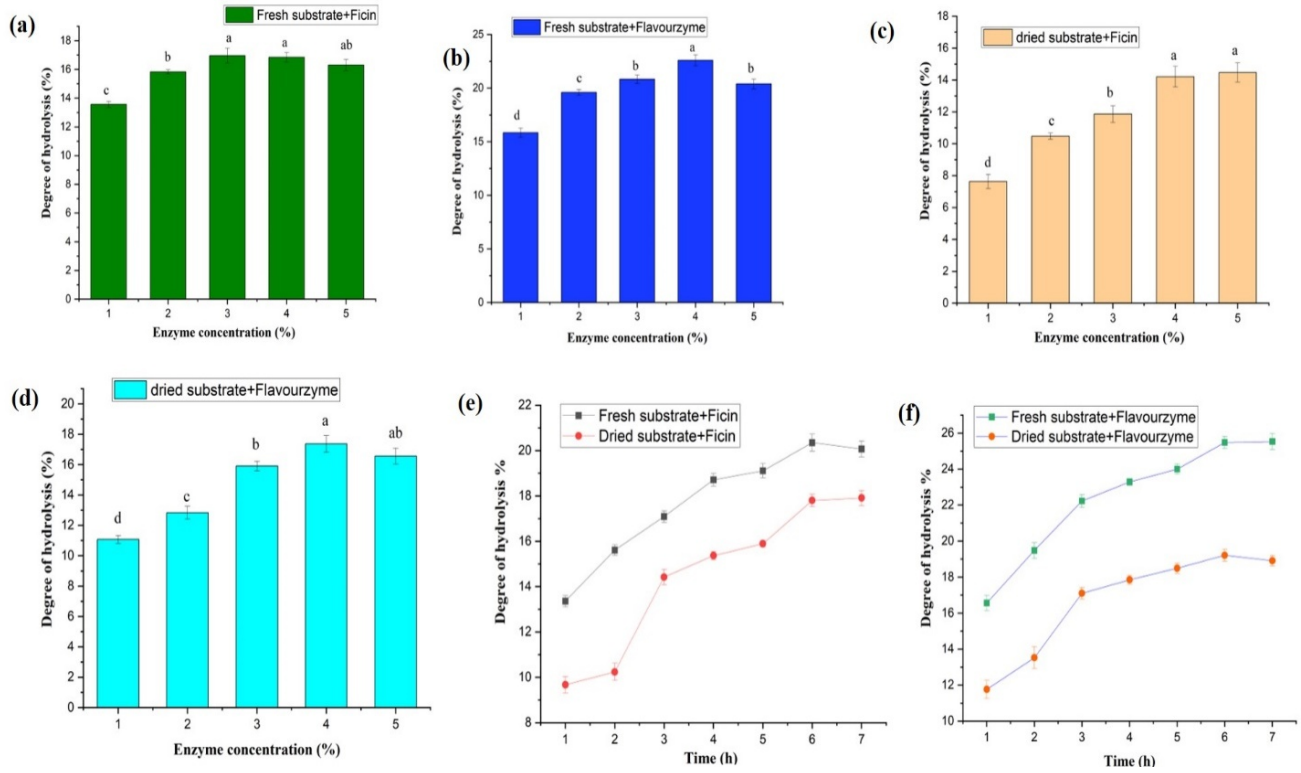


Figure 1. Influence of the enzymatic hydrolysis conditions: (a) E/S ratio [ficin+fresh substrate], (b) E/S ratio [flavourzyme+fresh substrate], (c) E/S ratio [ficin+dried substrate], (d) E/S ratio [flavourzyme+dried substrate], (e) time of hydrolysis reaction [fresh substrate+ficin & dried substrate+ficin], (f) time of hydrolysis reaction [fresh substrate+flavourzyme & dried substrate+flavourzyme] on the degree of hydrolysis (DH). The values represent mean± SD (n= 3), and various letters indicate the values are significantly different (P≤0.05)

Table 2. Protein content, Color measurement, yield and water activity of protein hydrolysates (FPH) prepared using ficin and flavourzyme enzymes and two substrates (fresh substrate and microwave-dried substrate) under the optimum conditions (*n* = 3, mean ±SD)

	Degree of hydrolysis			
Parameters	20.32%	17.80%	25.13%	19.21%
Hydrolysate Yield	16.11±0.35^{b}	12.29±0.10^{d}	17.39±0.46^{a}	14.07±0.33^{c}
Protein	84.91±0.67^{b}	75.54±1.07^{d}	87.68±0.59^{a}	78.72±0.86^{c}
Water activity	0.31±0.01^{a}	0.39±0.00^{a}	0.25±0.02^{a}	0.34±0.01^{a}
Color measurement				
L*	86.12±0.22^{a}	82.08±0.28^{c}	83.57±0.45^{b}	80.45±0.76^{d}
a*	0.37±0.08^{c}	3.66±0.14^{a}	2.63±0.39^{b}	3.82±0.04^{a}
b*	13.24±0.31^{c}	20.98±0.40^{a}	17.27±0.64^{b}	21.50±0.18^{a}
ΔE	19.40±0.55^{a}	12.11±0.62^{c}	17.96±0.44^{b}	12.31±0.35^{c}

Different letters indicate significantly different values (P ≤ 0.05). All values represent mean of triplicate determinations, mean ±SD (n=3). L*, a*, b*, ΔE values are standards of color measurements. L* refers to lightness, ranging from 0 to 100, a* refers to (green to red), and b* refers to (blue to yellow).

3.4. Color Determination

Potentially, Color factor is one of the most important factors in food application. The marketing of food products depends on the color to give the consumers a sufficient impression about flavour tenderness, and quality of the products. The color values (L*, a*, b* and ΔE) are presented in Table 2. The hydrolystes samples for both enzymes ficin and flavourzyme were obviously tend to whiteness with significant difference (P≤0.05) between fresh and microwave dried substrates. However, yellowness and redness values were higher in the hydrolysates using dried substrates compared to fresh substrate protein hydrolysates which might attribute to the type of substrate and the effect of microwave process on fish bighead carp during drying technique. Additionally, the changes in color values may attribute to the protease type (ficin or flavourzyme) in our current study, also using high temperature to terminate the enzymatic hydrolysis process could improve the browning reaction induced by different amino groups during the process [29].

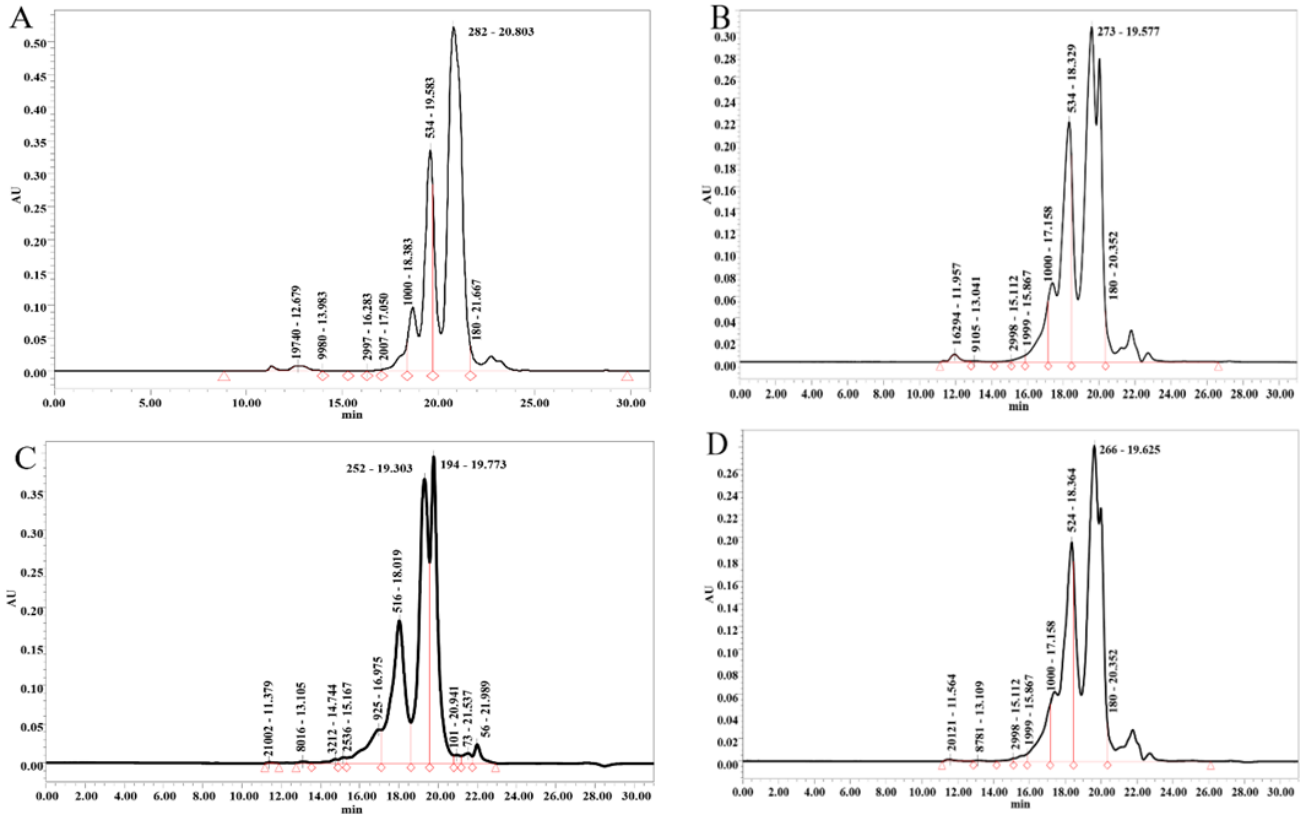
3.5. Amino Acid Analysis

The data of amino acids profile in fish protein hydrolysates (fresh substrate and microwave-dried substrate) produced using flavourzyme and ficin are displayed in Table 3. Under different degree of hydrolysis 20.32%, 17.80%, 25.13% and 19.21%, total amino acids were recorded 82.65, 73.84, 84.09 and 76.56 g/100 g protein. The values of total amino acids content were significantly different ($p< 0.05$). Generally, increasing the hydrolysis process time leads to increase the DH which had an impact to increase total amino acids contents in hydrolystes samples. The increasing might attribute to the peptides molecules which exist in protein hydrolystes and then led to boost the concentration of TAA. Additionally, TAA was higher in fresh fish substrate compared with microwave-dried substrate in the hydrolysates using ficin and flavourzyme respectively. Generally 17 amino acids were found in hydrolystes samples with 10 essential amino acids and 7 non-essential amino acids, Leucine, Lysine, Arginine, Aspartic acid and Glutamic acid were found in high amounts compared to other amino acids. These results were in track with those reported by Noman in Chinese sturgeon fish using Alcalse enzyme [24]. The high amounts of essential amino acids in FPH could be used as protein additives to support some meals and food products in food industries. In the our study, the difference of amino acids content under different degree of hydrolysis in comparison with those reported in previous studies might attribute to various impacts such as fish species, season fishing, and sexual type of raw materials (fresh fish and microwave-dried fish in our investigation) along with fish age and feeding system and finally type of protease enzyme used in hydrolysis process [19].

Table 3. Amino acids composition of bighead carp protein hydrolysates (g/100g protein) obtained using ficin and flavourzyme enzymes with two substrates (fresh fish and microwave-dried fish)

Amino acids	Hydrolysates			
(EAAs)	20.32%	17.80%	25.13%	19.21%
Histidine	2.64±0.09^{b}	2.13±0.02^{c}	2.97±0.08^{a}	3.21±0.12^{a}
Threonine	3.51±0.07^{b}	2.64±0.04^{c}	4.93±0.06^{a}	3.68±0.11^{b}
Valine	4.19±0.10^{b}	3.59±0.07^{c}	4.86±0.13^{a}	5.11±0.22^{a}
Methionine	3.04±0.15^{a}	2.34±0.04^{c}	2.78±0.24^{b}	2.15±0.03^{d}
Phenylalanine	2.89±0.17^{c}	2.81±0.11^{c}	4.43±0.29^{a}	3.39±0.18^{b}
Isoleucine	3.94 ±0.09^{b}	3.33 ±0.07^{c}	4.81±0.11^{a}	4.20±0.09^{b}
Leucine	6.43±0.05^{b}	6.16±0.11^{c}	6.63±0.17^{a}	5.89±0.31^{c}
Lysine	8.83±0.14^{a}	7.91±0.19^{b}	7.53±0.30^{c}	7.84±0.10^{b}
Arginine	5.42±0.03^{b}	4.83±0.16^{d}	6.81±0.09^{a}	5.32±0.24^{c}
Tyrosine	2.87±0.11^{a}	2.12±0.03^{b}	2.85±0.14^{a}	2.05±0.02^{c}
(NAAs)				
Cystenie	0.31±0.01^{a}	0.22±0.00^{b}	0.29±0.05^{a}	0.33±0.02^{a}
Aspartic acid	10.70±0.27^{a}	9.82±0.31^{b}	9.21±0.15^{d}	9.52±0.21^{c}
Glutamic acid	14.06±0.18^{a}	12.67±0.06^{b}	12.54±0.26^{b}	11.72±0.11^{c}
Serine	2.84±0.12^{b}	3.07±0.10^{a}	2.19±0.11^{d}	2.45±0.18^{c}
Glycine	3.82±0.20^{a}	3.35±0.08^{c}	3.55±0.17^{b}	2.97±0.10^{d}
Proline	2.41±0.15^{c}	2.67±0.19^{a}	2.54±0.08^{b}	2.61±0.14^{a}
Alanine	4.93±0.11^{a}	4.18±0.17^{b}	5.09±0.11^{a}	4.12±0.04^{b}
TEAA	43.58±0.47^{b}	37.88±0.53^{d}	48.47±0.86^{a}	42.84±0.72^{c}
TNAA	39.07±0.54^{a}	35.96±0.18^{b}	35.41±0.61^{c}	33.72±0.48^{d}
TAA	82.65±0.59^{b}	73.84±0.36^{d}	84.09±0.75^{a}	76.56±1.02^{c}
TEAA/TAA%	52.73±0.22	51.32±0.91	57.64±0.38	54.89±0.62

Different letters refer that the values are significantly different ($P \leq 0.05$), the values represent mean of triplicate determinations, mean ±SD (n=3). TAA total amino acids, TEAA total essential amino acids, TNAA total non-essential amino acids, TEAA/TAA% total essential amino acids to total amino acids



Molecular weight (Da)	Content %			
	DH 20.32% (A)	DH 17.80% (B)	DH 25.13% (C)	DH 19.21% (D)
> 10000	1.39	0.74	0.60	0.22
> 6000	0.13	0.12	0.21	0.17
> 3000	0.05	0.11	0.32	0.43
> 2000	0.09	0.47	0.25	0.66
< 1500	7.11	4.92	5.94	4.63
< 1000	94.19	86.17	95.07	89.25

Figure 2. Molecular weight distribution of the hydrolysates products derived from fish bighead carp using ficin and flavourzyme: (A) DH 20.32 %; (B) DH 17.80 %; (C) DH 25.13 % and (D) DH 19.21%

3.6. Molecular Weight Distribution

The analysis of the molecular weight profile of fish protein hydrolysates obtained from Chinese bighead carp using Ficin and flavourzyme enzymes with two substrates (fresh substrate and microwave dried substrate) is given in Figure 2. The current results observed that protein hydrolysates obtained using two different protease at different DHs possess small peptides. However, that could be related to the enzymatic hydrolysis process and its influence on the high molecular mass peptides. The peptides of various molecular weights were found in the protein hydrolysates obtained at 20.32% and 25.13% (fresh substrate) using ficin and flavourzyme respectively, while the DH values recorded 17.80% and 19.21% (microwave-dried substrate) using flavourzyme respectively. The molecular weight distribution <1000 Da showed 94.19% and 95.07% (fresh substrate) using ficin and flavourzyme in enzymatic hydrolysis respectively. Moreover, the molecular mass <1000 Da results showed 86.17% and 89.25% (microwave-dried substrate) using ficin and flavourzyme respectively. Furthermore, the fresh fish substrate showed better results in molecular weight distribution compared with microwave-dried substrate. The values of molecular mass in our study were higher than those reported by Noman in using Chinese sturgeon and alcalse enzyme at different hydrolysis times [24]. Rommi, Stizyte and others were studied and approved that under different protease enzymes could obtain protein hydrolysates from fish substrates and the number of substantial molecular weight could be reduced from >6000 Da to molecular mass peptides ranged (150 to 500 Da) [30].

3.7. Scanning Electron Microscopy Analysis

The microstructural analysis using a scanning electron microscope (SEM) was employed to investigate changes in surface morphology of protein hydrolystes prepared from ficin and flavourzyme enzymes using two substrates (fresh and microwave dried). The SEM images of the treated samples are presented in Figure 3. Based on the observed findings, after the hydrolysis process the SEM images showed that, the large molecular peptides catalytic into small peptides, and then the reducing of the particle size in (A, B, C and D) treated samples of protein hydrolysates products. Furthermore, the SEM images in micrographs of the targeted samples revealed that some particles have rectangular and circular shapes with different angles, which might be related to the influence of the enzymatic process along with water evaporation of hydrolystes during the freeze-drying process [9]. The

hydrolysates obtained from microwave dried substrate for both enzymes showed a complicated structure with varying sizes and irregular fragments. While, the hydrolysates produced from fresh substrate by using ficin and flavourzyme observed smooth structure with less irregular fragments. The Changes in the morphology of hydrolysates could influence their functionality in food application. Moreover, the observed results in this research was approximately similar to those reported by Elavarasan and others who found that large molecular peptides turn to degrade during enzymatic hydrolysis process into small peptides weight and finally, decrease the size of the targeted samples during the enzymatic hydrolysis process [31].

3.8. FTIR Spectra of Fish Protein Hydrolystes

Spectroscopy (FTIR) is a technical procedure to evaluate and determine the hydrolysates products and their structural properties in peptide bonds, represented by Amide bands I, II and III, which are the main bands of the protein in spectra of Fourier transform infrared. The amide bands defined as an accumulation of different amino acids sequences of peptides obtained during hydrolysis process, and these bands have eight to nine various and unique infrared absorbed bands in protein molecules [32]. The investigation of protein structure is related to chemical and functional groups in Amide I, II and III, along with absorption peaks changes. The amide bands are very delicate to any change in protein secondary structure such as stretching or bending in the functional amide groups which leads to variation in protein secondary structure [13]. FTIR results showed that the bands have given by amide bands which related to amino acids compositions. FTIR absorbance values of hydrolysates for ficin and flavourzyme were investigated and presented in Figure 4. From the observed data, the enzymatic hydrolysis had an influence on protein secondary structure in all hydrolysate samples. According to FTIR results, the absorption peak area of hydrolysate using fresh fish substrate with ficin enzyme was recorded at 1,576 cm^{-1} (amide I), 1,514 cm^{-1} (amide II), and 1,388 cm^{-1} (amide III), while, for the same substrate using flavourzyme recorded 1,575 cm^{-1} (amide I), 1,482 cm^{-1} (amide II) and 1,393 cm^{-1} (amide III). However, using microwave-dried substrate with ficin enzyme recorded absorption peaks at 1,529 cm^{-1} (amide I), 1,455 cm^{-1} (amide II), and 1,386 cm^{-1} (amide III), while, under flavourzyme enzymatic hydrolysis with microwave-dried substrate the values were 1,535 cm^{-1} (amide I), 1,463 cm^{-1} (amide II), and 1,381 cm^{-1} (amide III). The findings of our study were similar to those reported by Noman in sturgeon fish hydrolysis using papain and alcalse enzymes [32], but higher than the results reported by Fadimu and his colleagues by using flavourzyme and alcalse in lupin isolated protein [13]. Amide II and amide III bands absorption areas are strongly related to protein secondary structure, and the factors of enzymatic hydrolysis in particular protease type and the temperature of the reaction. Additionally, the type and quality of the substrate used for hydrolysis and the active sites of protease enzymes are playing the main role to release peptides and amino acids, which have the great impact and huge variance in infrared absorption peaks areas [33].

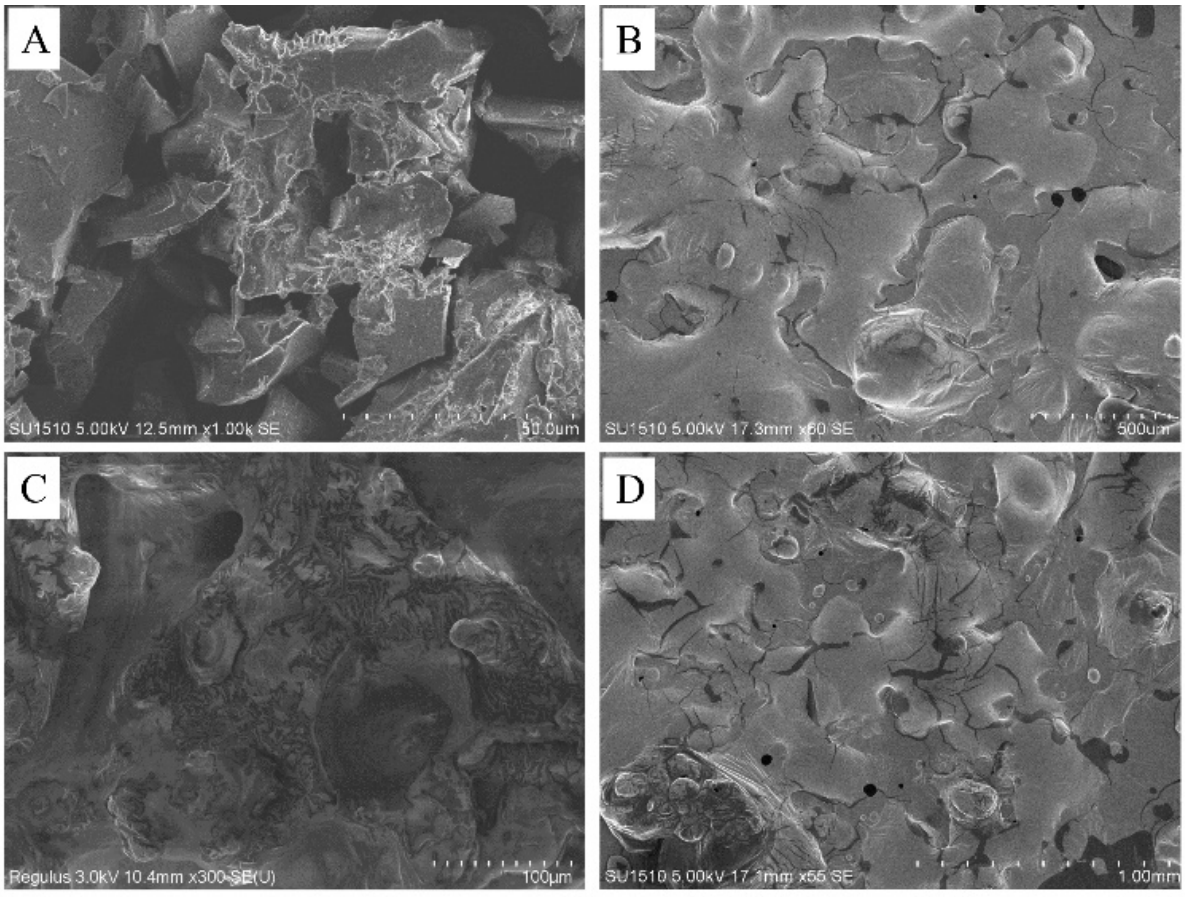


Figure 3. Scanning electron microscopy (SEM) of protein hydrolysates using ficin and flavourzyme enzyme and two substrates; (A) DH 20.32%, (B) DH 17.80%, (C) DH 25.13%, and (D) DH 19.21%

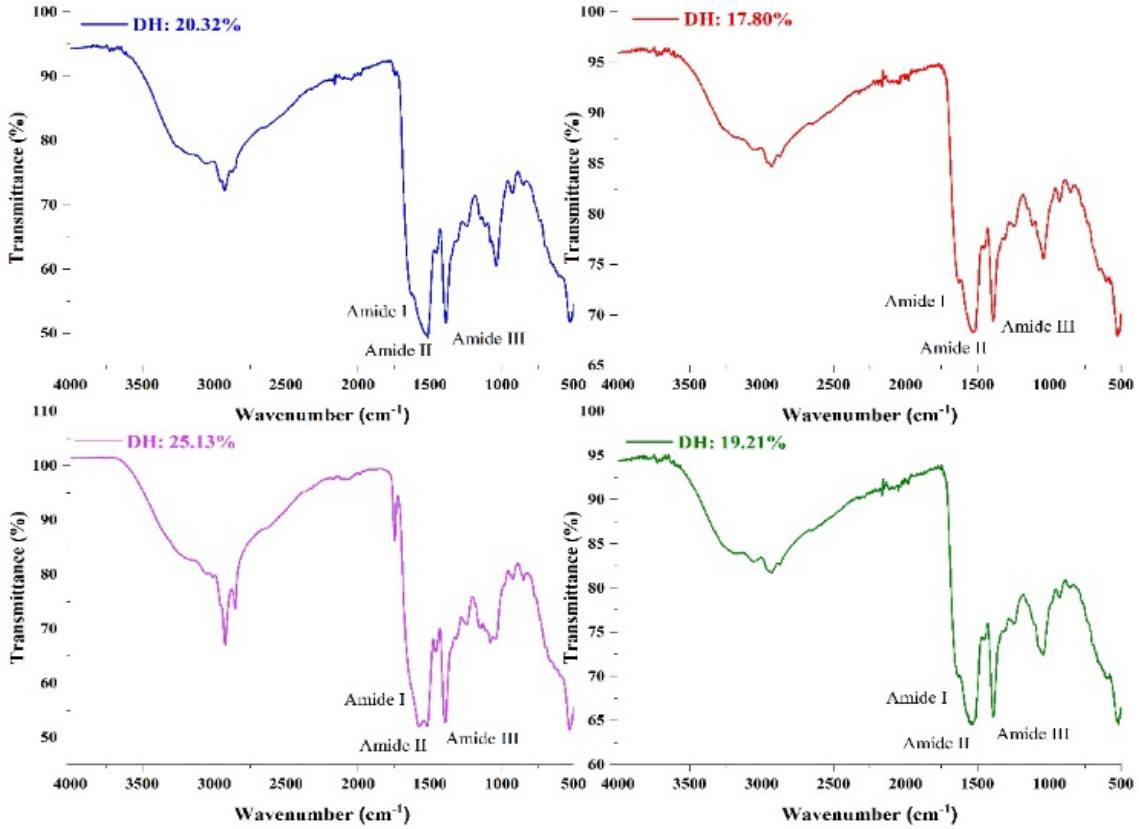


Figure 4. FTIR spectrum of FPH obtained at different degrees of hydrolysis using ficin and flavourzyme enzymes, different values on the graphs refer the changes in the amide bands I, II and III

3.9. Functional Properties of FPH

3.9.1. Protein Hydrolysate Solubility

The solubility of protein hydrolysates obtained from fresh and microwave-dried bighead carp fish substrate by using ficin and flavourzyme enzymes at various pH ranges (2 to 10) was displayed in Figure 5. Solubility property of hydrolysates products possess a remarkable influence on the various functions in protein and peptides fractions. Using hydrolystes products in food industry is very important to in the applications of foams and emulsification in term to boost and improve the functional properties of final products [34]. In the current study, the results showed remarkable solubility values of the hydrolysates samples averaged (80% to 96%) under different pH levels, the highest values recorded 94% to 96% in the hydrolysates obtained from fresh bighead carp substrate using ficin and flavourzyme respectively, while lower values were recorded between 80% to 88% in the hydrolysates produced from microwave-dried substrate for ficin and flavourzyme enzymes. The explanation of high solubility values in protein hydrolystes related with enzymatic hydrolysis which, fractionate big protein moleculars into small peptides with low molecular mass, and then increasing the solubility property in different hydrolystes products [35]. Protein molecules have isoelectric point at pH (4.5 to 5.5), and at the mentioned point protein hydrolystes showed less values of solubility due to weak interaction between protein molecules and water, however, increase or decrease pH value from the isoelectric point lead to improve and boost the solubility due to high interaction water to protein molecules [36]. According to previous studies, Noman and his colleagues reported lower values of protein solubility in FPH using alcalse enzyme with sturgeon fish muscles [24] compared with our results. Furthermore, Li and his colleagues also determined the solubility of hydrolystes using protease enzyme and the results were almost similar to the values in our study [37]. Finally, the difference in solubility values might be related to the type of substrate (fresh or dried fish substrate in our study), different size of peptide fractions formed during hydrolysis process and type of enzyme used in hydrolysis.

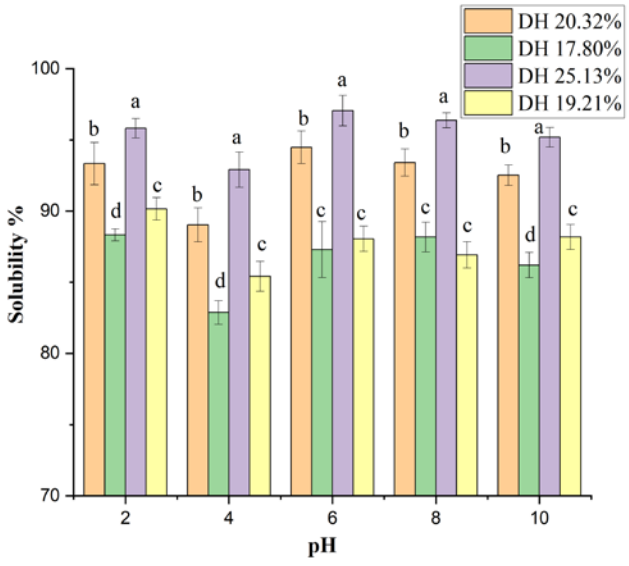


Figure 5. Protein solubility of hydrolysates obtained at different degrees of hydrolysis using ficin and flavourzyme enzymes at pH from (2-10). The values represent mean± SD (n= 3), and various letters indicate the values are significantly different (P≤0.05)

3.9.2. Emulsifying Properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of FPH prepared from Chinese bighead carp (fresh and microwave-dried substrates) using ficin and flavourzyme enzymes at different concentrations (0.1%, 0.5%, and 1%) are given in Table 4. The emulsion is the absorption of protein particles during the homogenizing process to the surface of composed oil droplets to build a shielding membrane to protect oil droplets from merging. EAI values at the hydrolysates concentration 0.1% were 74.22, 81.35, 62.94 and 78.49 m^2/g at DH 20.32%, 17.80%, 25.13% and 19.21% respectively. In addition, ESI values were significantly higher at concentration 0.1% compared to 0.5% and 1% protein concentrations at the various DH. The increase in protein concentration and DH observed a decrease in EAI and ESI records, an inverse correlation. These findings support by Nalinanon and other [38], who showed a similar influence in the relation between DH at different protein concentrations and (EAI&ESI) values from ornate threadfin bream using pepsin enzyme. Noman and his colleagues observed the same impact regarding the emulsifications and DH at various levels of hydrolysate concentrations [24]. The protein hydrolysates with small peptides or hydrophobic might improve emulsification stability [39]. Small peptides derived from enzymatic hydrolysis with low molecular mass might not be sufficiently amphiphilic to expose a significant emulsion property [40].

Table 4. Emulsifying characteristics of protein hydrolysate obtained from bighead carp fish using two substrates (fresh fish and microwave-dried fish), and two enzymes (Ficin and flavourzyme) (mean ±SD, *n*=3)

DH %	Concentration %	EAI (m^2/g)	ESI (min)
20.32%	0.1	74.22±2.01^a	38.52±1.13^a
	0.5	15.17±0.86^b	22.14±0.10^b
	1	9.05±0.75^c	12.78±0.64^c
17.80%	0.1	81.35±1.67^a	53.46±1.86^a
	0.5	20.73±0.38^b	26.93±0.88^b
	1	8.16±0.44^c	20.23±0.09^c
25.13%	0.1	62.94±1.57^a	29.89±0.73^a
	0.5	11.46±0.61^b	14.28±0.17^b
	1	6.27±0.12^c	10.58±0.34^c
19.21%	0.1	78.49±1.26^a	48.60±0.75^a
	0.5	16.07±0.25^b	22.35±0.41^b
	1	8.37±0.09^c	20.19±0.15^c

*Different letters in the same column of each concentration and degree of hydrolysis indicate significantly different values ($P \leq 0.05$).

3.10. Antioxidants Activities of FPH

3.10.1. DPPH Radical Scavenging Activity

The assay of scavenging capacity of FPH was determined and investigated using DPPH, with absorbance at 517 nm. In the DPPH assay violet color DPPH solution is reduced to yellow color because of contributing a hydrogen ion (H^+), which leads to reduce the absorbance [41]. According to the data, Figure 6.a demonstrated the DPPH radical scavenging activity values of FPH in fresh and microwave-dried bighead fish substrates using ficin and flavourzyme enzymes at different conversations from 1 to 20 mg/mL. The obtained hydrolysates observed that DPPH radical scavenging activity had a notable increase from 9.98 % and 14.08 % for fresh fish substrate to 62.74 % and 69.91% using ficin and flavourzyme enzyme respectively. However using microwave dried bighead carp substrate showed lower values of DPPH activity for ficin and flavourzyme respectively. These findings were higher than those reported Noman [24] in hydrolystes of sturgeon fish and in the track with the results found by Md. Serajul [42]. On the other hand, IC_{50} value is described as the concentration of protein hydrolytes that can scavenge 50% of DPPH free radical. The IC_{50} values of hydrolystes products produced at DH 20.32 %, 17.80 %, 25.13% and 19.21 % were 8.37, 11.07, 6.48 and 11.76 mg/mL respectively. Obviously, the hydrolystes produced at DH 25.13% and 20.32% had the highest values of activity to scavenge DPPH. Furthermore, the different values of radical scavenging activity of hydrolystes products often related to amino acid compositions, the sequences of different peptides and the size of peptides fractions which produced under several of DHs [43]. Finally, according to amino acids results showed in Table 3, of the hydrolystes samples, the different content of amino acids led to various values of IC_{50} in protein hydrolysates.

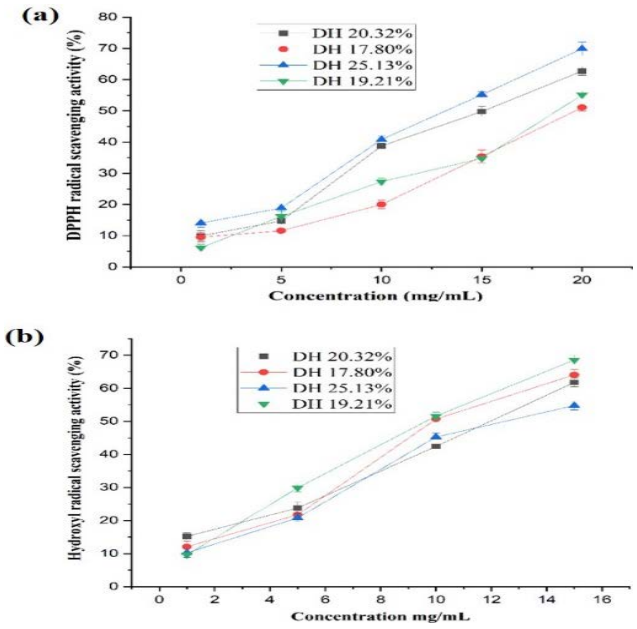


Figure 6. Antioxidant assays of FPH produced under the optimal parameters at different degree of hydrolysis, (a) DPPH radical scavenging activity, (b) Hydroxyl radical scavenging assay. The data were observed as mean±SD with triplicates

3.10.2. Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging assay is the most reactive oxygen and was evaluated by generating the hydroxyl radicals using ascorbic acid. The data of Hydroxyl radical scavenging activity are presented in Figure 6.b. Based on the results, a significant variation was observed between fresh and microwave dried substrates under ficin and flavourzyme enzymatic hydrolysis. The highest values recorded at lower degree of hydrolysis, whereas hydrolysates obtained from fresh fish substrate showed low values of Hydroxyl activity. Obviously, according to our study, the low degree of hydrolysis obtained the highest activity of hydroxyl radical scavenging. Our results in the track with those reported by Md. Serajul and his colleagues in protein hydrolystes of Grass turtle [42]. On the other hand,

the IC_{50} of protein hydrolysates obtained at DH 19.21% was the best recorded value with 11.63 mg/mL compared with IC_{50} values at DH 20.32%, 17.80% and 25.13% which showed 16.87, 11.97 and 15.24 mg/mL respectively with a slight significant difference ($P \leq 0.05$).

4. Conclusion

Fish protein hydrolysates were produced from bighead carp (Hypophthalmichthys nobilis) using ficin and flavourzyme proteases and two substrates (fresh and microwave-dried fillets). Under optimum conditions for two proteases, protein hydrolysates obtained from fresh substrate showed a significant variation in different characteristics compared with hydrolysates obtained using microwave-dried substrate. The results revealed that the nutritional and functional properties were significantly influenced by the degree of hydrolysis. The DHs recorded higher values in the hydrolysates derived from fresh substrate compared to the hydrolysates derived from microwave-dried substrate for ficin and flavourzyme proteases. Total amino acids showed higher values in protein hydrolysates produced from fresh substrate in compare with hydrolysates produced from microwave-dried substrate for ficin and flavourzyme respectively. The findings of the current work observed enhancement of solubility and emulsification properties for all protein hydrolysates and noticeably in hydrolysates obtained from fresh substrate. Eventually, the hydrolysates products showed excellent nutritional properties in term of using as protein additives in food application. Further studies about effect of hydrolysis time, using some techniques to improve the degree of hydrolysis along with functional properties and peptide fractions will be under consideration.

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Conflicts of Interest

The authors declare no conflict of interest.

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