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Effect of Enzyme Type on the Properties of Chinese Sturgeon (*Acipenser sinensis*) Protein Hydrolysates Produced by Enzymatic Hydrolysis Process

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Abstract

Protein hydrolysate is small fragments of peptides that contain many amino acids, and they can be prepared from fish meat and their by-products. The enzymatic hydrolysis process is the most effective method to recover the nutrients and bioactive peptides with preserving their nutritional value. In this study, papain and alcalase 2.4L enzymes have been employed to evaluate the preparation efficiency of protein hydrolysate from Chinese sturgeon by enzymatic hydrolysis process and its antioxidant properties. Papain was the more effective enzyme to obtain the highest degree of hydrolysis and yield, which were 20.62% and 16.77%, respectively. Increased degree of hydrolysis using papain led to an increase in the percentage of molecular weights (≤1 kDa), and total amino acids which were 98.26% and 97.82 g/100g protein, respectively. The solubility of the protein was significantly affected by enzyme type and pH, where the highest solubility was achieved by using the papain enzyme and pH 2, which was 97.39%. While alcalase 2.4L hydrolysate has achieved the highest antioxidant activities by DPPH and ABTS assays, which reached 81.42% and 87.75% at a hydrolysate concentration of 5 mg/mL, respectively. The findings indicate that papain and alcalase 2.4L enzymes can play a promising role in the production of fish protein hydrolysate with improved functional properties for potential food and pharmaceutical applications.

Keywords

Chinese Sturgeon, Papain, Alcalase 2.4L, Protein Hydrolysate, Antioxidant Properties

1. Introduction

Fish and its by-products are valuable nutritious foodstuffs and the protein nutritional properties are associated with their amino acids content in conjunction with the physiological employments of specific amino acids during digestion and absorption [1]. Food proteins are considered as a source of the essential amino acids, while they are also seen as biologically-active peptides, which are protein fragments that affect the life-affecting functions of the human organism [2]. Enzymatic hydrolysis process of fish proteins has been employed as a modern approach to take advantage of the biomass of unused fish, where the use of proteolytic enzymes is often an attractive procedure for improving functional and antioxidant properties of food proteins with preserving their nutritional values [3]. Protein solubility is an important functional characteristic, which affected by the substrates and enzymes used and the degree of hydrolysis (DH) [4]. Many proteolytic enzymes are most applied to hydrolyze the fish proteins to obtain fish protein hydrolysates [5]. Bioactive peptides are specific small protein fragments that act as sources of nitrogen and amino acids. These ingredients have numerous potential physiological functions within the human

body including antioxidant, antibacterial, antithrombotic and antihypertensive activities [6]. Recently, considerable many researches have focused on the release of bioactive peptides with desired functional properties from food proteins to be used as functional food ingredients aimed at health maintenance [4, 6-8]. Chinese sturgeon (*Acipeneser sinensis*) is one of the largest freshwater fish species in the world and the fastest-growing, where the adult fish reaches a length of 5 m and their weight ranges from 200 to 500 kg [9]. China is one of the largest countries contributing to the breeding of sturgeon, contributing more than 77% of the world's sturgeon meat production in 2017 [10]. Given the importance of the fish protein hydrolysate as a source of protein and the potential antioxidant activities, this study aims to compare the production and properties of the Chinese sturgeon protein hydrolysates obtained by using two different enzymes.

2. Material and Methods

2.1. Materials

A. sinensis samples were received from the Yangtze River by Hua Da Aquatic Products Science and Technology Industry Co., Ltd. Papain enzyme (Enzyme activity/1000 casein, pH 6.0, 40°C: ≥6000/(USP-U/mg)) was obtained from Ourchem Co., Ltd, Guangdong Province, China, and Alcalase 2.4L (2.4 AU-A/g) was obtained from Nanjing Chengna Chemical Co., Ltd. China. The enzymes used in the experiments were kept refrigerated at 4°C. All chemicals and reagents used were of high purity and analytical grade.

2.2. Preparation of Samples for Enzymatic Hydrolysis

The frozen samples were transported to the laboratory, cleaned and kept at −20°C. Before the enzymatic hydrolysis process, the samples were moved from the freezing stage to the cooling stage at 4°C for 12 h, and then minced to carry out the hydrolysis process.

2.3. Preparation of Sturgeon Protein Hydrolysates

To get the protein hydrolysates by using papain enzyme, the optimal conditions found by Noman et al. [9] were applied, and by using alcalase 2.4L according to Noman et al. [11]. The enzymatic hydrolysis processes were performed in jacketed 250 mL glass containers equipped with a stirrer (IKA C-MAG HS 4 S 25) attached to a circulating water bath (Shanghai Blue pard Yiheng Technical Co., Ltd, Shanghai, China) to adjust the temperature during hydrolysis. After the incubation period ends, the enzyme's activity was prevented by heating for 15 min at 95°C in a water bath [12]. The hydrolysates were cooled down to room temperature and centrifuged at 10,000 rpm and 4°C for 20 min. Finally, the supernatants were freeze-dried under vacuum at −50°C (SCIENTZ-10ND, Ningbo SCIENTZ Biotechnol Co., Ltd., Ningbo, China) to obtain the lyophilized protein hydrolysates, which were stored at −20°C until further analysis.

2.4. Protein Yield Recovery and Degree of Hydrolysis

The protein hydrolysate yield for each hydrolysate was calculated as the proportion of lyophilized protein hydrolysate weight to the raw material sample used according to the following formula:

$$\text{Yield (\%)} = [\text{FPH (g)}/\text{Raw material (g)}] \times 100 \quad (1)$$

The degree of hydrolysis was evaluated using formal titration according to the method described by Noman et al. [11]. One gram and a half from hydrolysate mixture were weighed, and the weight was made up to 50 g by using distilled water. The mixture pH was set to 7.0 using sodium hydroxide (0.1 N). Ten milliliters of formaldehyde solution 38% (v/v) were added to the mixture and left for 5 min at room temperature. The titration process was continued to pH 8.5 using 0.1 N standard sodium hydroxide solution. The total nitrogen in the raw sturgeon sample was estimated using the Kjeldahl method, and the free amino groups and DH were calculated as follows:

$$\text{Free amino groups (\%)} = [V \times C \times 0.014007/W] \times 100 \quad (2)$$

$$\text{DH (\%)} = [\text{Percentage of free amino groups}/\%\ \text{TN}] \times 100 \quad (3)$$

where: V is mL of sodium hydroxide used; C is sodium hydroxide concentration used for titration (0.1 N); W is the amount of sample used (g).

2.5. Determination of Amino acid Composition

The protein hydrolysate samples were prepared to determine the amino acids profile according to the method of Noman et al. [9]. Almost 100 mg of the lyophilized protein hydrolysates were digested in 6 M of HCl at 120°C for 22 hr. The mixture was cooled to room temperature and then 4.8 mL of NaOH (10N) were added. By using distilled water, the mixture volume was made up to 25 mL, filtered using two layers of filter paper No. 40, and centrifuged for 10 min at 10,000 rpm. Amino acids profile was analyzed according to AOAC method [13] using a reversed-phase high-performance liquid chromatography (RP-HPLC) (Agilent 1100 HPLC; Agilent Ltd., Palo Alto, CA, USA). A Zorbax, 80 A C-18 column (250×4.0 mm, 5 µm particle size; Agilent, USA) with UV detection at 338 nm at 40°C was used to inject 1 µL of the prepared sample. The mobile phase A was 7.35 mM/L of sodium acetate/triethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v) at pH 7.5, which was adjusted using acetic acid, while the mobile phase B was 7.35 mM/L of sodium acetate/methanol/ acetonitrile (1:2:2, v/v/v) at pH 7.2. The results of amino acids were processed and displayed as grams of amino acids/100 g of protein.

2.6. Determination of Peptide Molecular Weight Distribution

Protein hydrolysates were analyzed according to the

method of Foh et al. [14] with modification. Gel permeation chromatography using a high-performance liquid chromatography system (HPLC, Waters 1525, Milford, MA, USA) with a TSK gel 2000 SWXL (300×7.8 mm) column (Tosoh, Tokyo, Japan), and process of equilibrating was performed with acetonitrile: water (40:60, v/v) in the presence trifluoroacetic acid (0.1%). One hundred milligrams of protein hydrolysate were dissolved with 10 mL of deionized water as a mobile phase. The suspensions were subjected to ultrasound treatment for 5 min and centrifuged for 10 min at 10,000 rpm. The supernatant was filtered and applied in the column with elution at a flow rate of 0.5 mL/min. The monitoring process was at 220 nm and 30°C. A molecular weight calibration curve was prepared from average retention times according to the following standards: Gly-Gly-Gly (189 Da), Gly-Gly-Try-Arg (451 Da), bacitracin (1422 Da), and Cytochrome C (12384 Da).

2.7. Protein Solubility

The solubility of the protein hydrolysates was measured according to the method described by Noman et al. [11].

2.8. Antioxidant Activity Determination

2.8.1. DPPH Radical Scavenging Activity

The DPPH radical-scavenging activity of protein hydrolysates prepared by using papain and alcalase enzymes was estimated according to the method of Jemil et al. [15] with some modifications. Briefly, 2 mL from each protein concentration (1, 2, 3, 4 and 5 mg/mL) were mixed with 2 mL of 0.1 mM DPPH in 95% methanol. The solutions were vortexed and left for 30 min in dark at room temperature, and the reduction of DPPH radical was measured at 517 nm using a UV-1800PC spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China). To prepare the control sample, distilled water was used instead of the sample. DPPH scavenging activity was calculated by the following formula:

$$\text{Scavenging activity (\%)} = [A_C - A_S / A_C] \times 100 \quad (4)$$

Where A_C is the absorbance of control sample, and A_S is the absorbance of sample.

2.8.2. ABTS Radical Scavenging Activity

ABTS radical scavenging activities of sturgeon protein hydrolysates were determined by the method reported by Ovissipour et al. [16] with some modifications. Initially, 7.4 mM ABTS and 2.6 mM potassium persulfate solutions were prepared, and equal amounts of stock solutions were mixed to prepare the work solution and left to react in the dark for 16 hr at room temperature. The resulting solution was diluted by methanol (98%) to obtain an absorbance of 0.70 ± 0.02 units at 734 nm before measuring the absorbance of the samples. Secondly, 100 µL from each concentration (1, 2, 3, 4 and 5 mg/mL) were mixed with 3.5 mL of $ABTS^{\cdot+}$ working solution and the mixture was left for 20 min in the dark at room temperature. To prepare the blank sample, distilled water was utilized instead of the sample. The absorbance was then measured at 734 nm using a UV-1800PC spectrophotometer. The ABTS• scavenging activity of protein hydrolysates was calculated according to the following formula:

$$\text{ABTS (\%)} = [1 - (A_S/A_B)] \times 100 \quad (5)$$

where A_S is the absorbance of sample, and A_B is the absorbance of blank sample.

2.9. Statistical Analysis

The data were subjected to analysis of variance (ANOVA) test using statistical analysis software (version 6.4; CoStat, Monterey, CA, USA). Statistical significance was established at P values less than 0.05.

3. Results and discussion

3.1. Protein Recovery and Degree of Hydrolysis

Protein recovery by using papain and alcalase 2.4 L enzymes was 18.67% and 15.27%, respectively as shown in Figure 1. From this result, it is clear that the use of papain enzyme achieves a significant increase at $p<0.05$, where the yield is clearly associated to the DH [4, 11]. The difference in the obtained yield may be due to the nature of the enzyme interacts with the substrate.

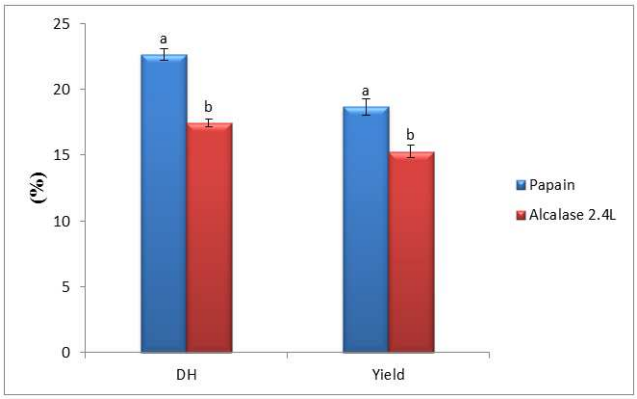


Figure 1. *Degree of hydrolysis and protein hydrolysate yield obtained by using papain and alcalase 2.4L enzymes under optimal conditions.*

The DH fundamentally used to monitor the reaction kinetics and describe the breakdown rate (hydrolysis) of food proteins [17], therefore, the DH can be defined as the proportion of the broken peptide bonds to the total numbers of bonds per unit weight in the substrate used in the hydrolysis process [18]. DH was significantly different when using the enzymes papain and alcalase as shown in Figure 1. From this result, it can note the DH was significantly higher by using papain, which was 22.64%, while it was 17.42% by using alcalase 2.4L under the optimal enzymatic hydrolysis conditions at an incubation period of 6 h. This difference may be due to several factors that affect the enzymatic hydrolysis process such as enzymes specificity [19]. On the other hand, Villamil et al. [20] reported that the temperature, enzyme

concentration, and pH affect the reaction rate, therefore, these factors will affect the DH in one way or another. Generally, increased DH indicates that more peptide bonds were broken through the enzymatic hydrolysis process [21].

3.2. Amino Acid Composition

The protein hydrolysate nutritional value depends on the amino acid content to achieve the needs of body functions, particularly the essential amino acid. Amino acids composition of sturgeon protein hydrolysates prepared using two enzymes was displayed in Table 1. The total amino acid was 97.82 and 89.44 (g/100 g protein) for papain and alcalase hydrolysates, respectively, where the hydrolysates showed significantly (p<0.05) different amino acids composition. The essential amino acids content of both hydrolysates was 49.62 and 43.41 (g/100 g protein), respectively. From the total amino acids content, the asparagine, glutamine, and alanine were found to be abundant, while the essential amino acids lysine, leucine, and arginine were more abundant in both hydrolysates. This essential amino acid content makes the sturgeon protein hydrolysate a potential source of addition in products with a poor protein content.

The significant differences between the amino acids content of papain and alcalase 2.4L hydrolysates might be due to the differences in the enzymatic cleavage of the substrate by these enzymes as evidenced by the DH, which was 22.64% for papain and 17.42% for alcalase as shown in the Figure 1. Comparing the amino acids content of Chinese sturgeon protein hydrolysates, we found that it differs from other protein sources, where the amino acids content of both protein hydrolysates in this study was lower than silver carp protein hydrolysate [22]. On the one hand, the amino acids content of Persian sturgeon protein hydrolysate was lower than that of the papain hydrolysate in this study, but it was more than that of the alcalase hydrolysate [12]. The differences in the amino acids content in different fish protein hydrolysates may be attributed to one or more of the following reasons: species, organs, age, location, feeding, and fishing season [9, 23].

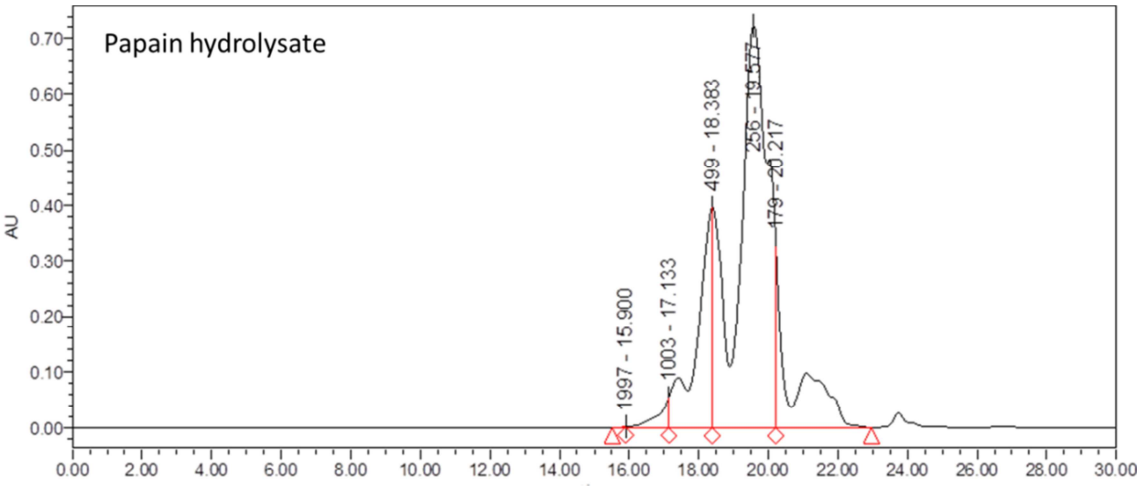
Table 1. *Amino acids composition of Chinese sturgeon protein hydrolysates (g/100 g protein) obtained under the optimal conditions by using papain and alcalase 2.4L enzymes (n=3, mean ± SD).*

Amino Acids	Papain hydrolysate	Alcalase 2.4L hydrolysate
Essential amino acid		
Histidine	2.97±0.07	2.13±0.11
Threonine	3.39±0.08	3.54±0.14
Arginine	6.46±0.09	5.74±0.13
Tyrosine	3.06±0.08	2.04±0.09
Valine	4.80±0.14	4.60±0.07
Methionine	2.96±0.07	2.03±0.08
Phenyl alanine	3.84±0.17	3.45±0.17
Isoleucine	4.38±0.16	4.21±0.14
Leucine	7.53±0.17	7.09±0.09
Lysine	10.23±0.21	8.58±0.12
Non –Essential amino acid		
Asparagine	11.14±0.17	9.67±0.21
Glutamine	18.23±0.21	16.90±0.19
Serine	3.69±0.11	3.22±0.11
Glycine	5.31±0.13	5.54±0.16
Alanine	5.80±0.12	6.30±0.08
Cystine-s	0.29±0.06	0.09±0.02
Proline	3.74±0.15	4.31±0.17
TAAs	97.82±1.07[a]	89.44±0.92[b]
TEAAs	49.62±0.62[a]	43.41±0.54[b]
TNEAAs	48.20±0.43[a]	46.03±0.48[b]
TEAAs/ TAAs%	50.73±0.31	48.54±0.28
TEAAs/ TNEAAs	1.03±0.06	0.94±0.11

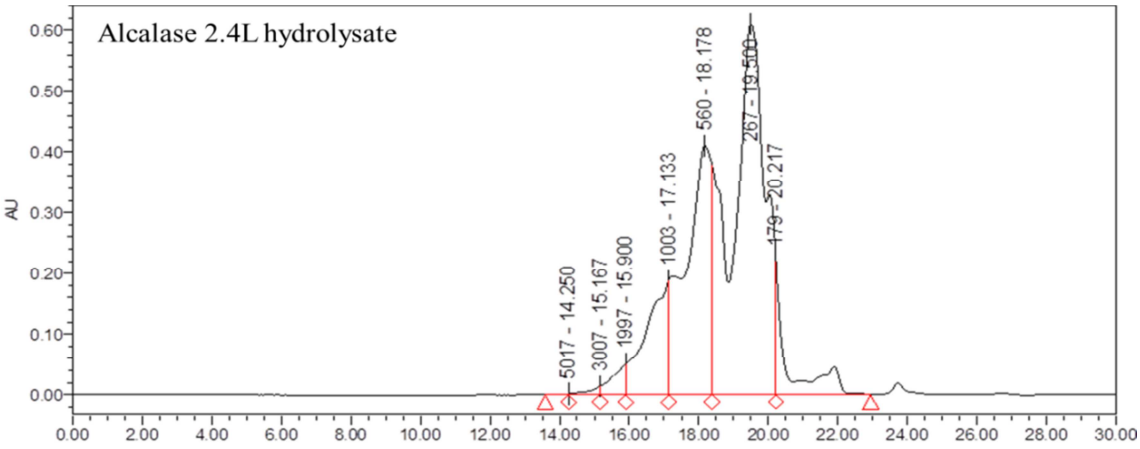
TAAs total amino acids, TEAAs total essential amino acids, TNEAAs total non–essential amino acids, TEAAs/ TAAs% percentage of essential amino acids to total amino acids, TEAAs/TNEAAs total essential amino acids to total non–essential amino acids.
*Different letters within the same row indicate significant difference at p< 0.05.

3.3. Molecular Weight Distribution

The molecular weight distribution of protein hydrolysates prepared from Chinese sturgeon with papain and alcalase 2.4L enzymes under the optimal conditions is displayed in Figure 2. The results showed that both protein hydrolysates contained a mixture of small peptides, especially the molecular weights <1 kDa, which were more than 98% for papain hydrolysate and more than 86% for alcalase hydrolysate. From this result, it is clear that the DH indicates the molecular weights obtained from the enzymatic hydrolysis process, and since the DH when using the papain enzyme was higher, the molecular weights were smaller. This result is consistent with the results of previous studies that found that the increase in the DH led to an increase in the percentage of small molecular weights [22, 24, 25]. Hou et al. [26] reported that protein hydrolysate with lower molecular weights have a higher nutritional availability.



	MW (KDa)					
	> 3	2-3	1-2	0.5-1	0.18-0.5	< 0.18
Content (%)	0.01	0.02	1.71	17.37	66.67	14.22



	MW (KDa)					
	> 3	2-3	1-2	0.5-1	0.18-0.5	< 0.18
Content (%)	0.45	1.79	10.94	27.27	53.41	6.14

Figure 2. *Molecular weight distribution profiles of Chinese sturgeon protein hydrolysates obtained by two enzymes under the optimal conditions.*

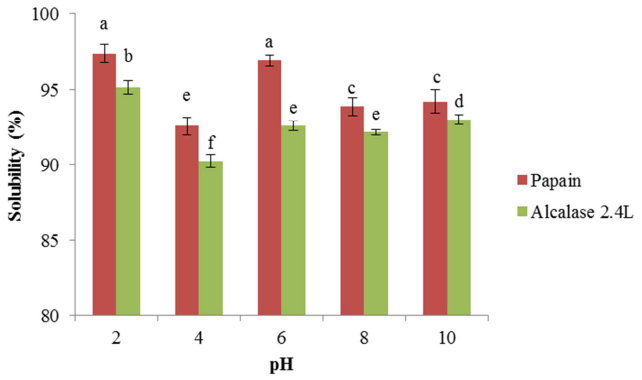


Figure 3. *Solubility of protein hydrolysate obtained using two different enzymes and at various pH. Data expressed as mean±SD of triplicate determinations. Different letters indicate significant differences at p< 0.05.*

3.4. Solubility of Protein Hydrolysate

Solubility plays an important role in controlling the functional properties of proteins and their use in various applications, such as emulsions and gels [27]. The solubility of protein hydrolysates prepared with different enzymes at pH values from 2 to 10 is shown in Figure 3. From the obtained results, it was found that both hydrolysates were highly soluble at different pH levels used, where the rate of melting exceeded 90%. Generally, papain hydrolysate was more soluble at different pH values because of the low molecular weights in papain hydrolysate [11].

The highest protein solubility was achieved at pH 2 with significant differences (p<0.05), while the lowest solubility was at pH 4 for both protein hydrolysates. The isoelectric points (IP) of proteins are between pH 4.5 and 5.5, therefore the net charge of the original proteins is reduced, and then more protein-protein interactions and less protein-water interactions occur [14]. Increased solubility may be attributed to the formation of carboxylic and amine groups from amino acids, which increase the hydrophilicity of the protein hydrolysate [28].

3.5. Antioxidant Activities of Hydrolysates

3.5.1. DPPH Radical Scavenging Activity

The scavenging capacity of both protein hydrolysates was measured against DPPH at 517 nm. The compounds that contribute to hydrogen (H^+) act to scavenge radicals and changing solution color from purple to yellow, which leads to a reduced absorption [29]. Figure 4A displays the DPPH radical-scavenging activity of sturgeon protein hydrolysates obtained by using papain and alcalase with different protein concentrations. Both protein hydrolysates showed that the radical scavenging activity significantly ($p< 0.05$) increased when the protein concentration was increased from 1 mg/mL to 5 mg/mL, however, the alcalase hydrolysate was more active at various protein concentrations, where the scavenging activity reached 78.45 and 81.42% at concentration of 5 mg/mL for papain and alcalase hydrolysates, respectively. This result is consistent with results of previous studies that found that increased protein concentration led to an increased scavenging activity [11, 29, 30]. The radical scavenging activity of proteins is generally associated with the amino acids sequence, amino acids composition, the molecular weights of peptides, and hydrophobic property resulting from different DH [31].

3.5.2. ABTS Radical Scavenging Activity

The antioxidant activity of sturgeon protein hydrolysates determined with the $ABTS^{+\bullet}$ scavenging method was displayed in Figure 4B. Alcalase hydrolysate was characterized by a higher activity as $ABTS^{+\bullet}$ scavengers than the papain hydrolysate at different protein concentrations (1-5 mg/mL), which was 80.63% of papain hydrolysate and 87.75% of alcalase hydrolysate at a protein concentration of 5 mg/mL. This difference in the antioxidant activity between the two studied samples may be due to the size of peptides resulting from the enzymatic hydrolysis process, where Sarmadi and Ismail [32] mentioned that the antioxidant activity of peptides with molecular masses of 500–1500 Da was higher than that of peptides with the molecular mass over 1500 Da and below 500 Da. Thus, it can be seen that more than 80% of the molecular weights of papain hydrolysate peptides were less than 500 Da, while it was in the alcalase hydrolysate less than 60%.

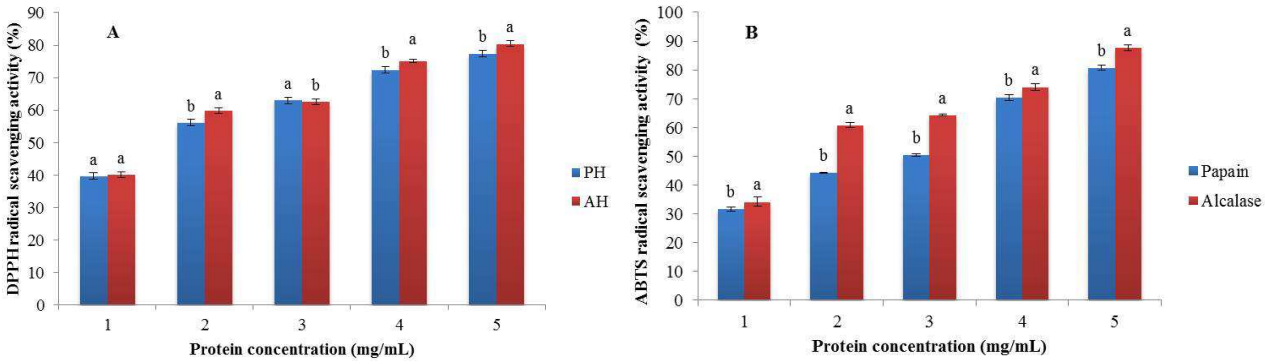


Figure 4. Antioxidant activities of protein hydrolysates obtained using two different enzymes, (A) DPPH radical-scavenging activity; (B) ABTS radical-scavenging activity. Data expressed as mean±SD of triplicate determinations. Different letters within the same protein concentration indicate significant differences at p < 0.05.

4. Conclusions

Chinese sturgeon protein hydrolysate was successfully prepared using Papain and Alcalase 2.4L as a biocatalyst. Both hydrolysates contained high levels of essential amino acids 49.62 and 43.41%, respectively. Furthermore, they showed improved solubility, as well as scavenging activity against DPPH and ABTS. The sturgeon protein hydrolysates could offer unique nutritional and physicochemical properties, hence an increase in its applications in the food industry. The results of this study could be helpful in the field of protein hydrolysis. Consequently, the efficiency of other enzymes should be studied to know their efficacy and their impact on other properties of the fish protein hydrolysate.

Conflict of Interest

There is no conflict of interest to declare.

Acknowledgements

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