

AN INVESTIGATION INTO COPPER-BINDING CAPACITY OF THE WHITE LEG SHRIMP HEAD (*Litopenaeus vannamei*) PROTEIN HYDROLYSATE

Tam Dinh Le Vo, Nhi Hoang Yen Bui, Truc Mai Xuan Le, Tam Le Minh Truong

Ho Chi Minh city University of Technology – Vietnam National University-Ho Chi Minh; vdlr@hcmut.edu.vn

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Abstract - The aim of this study is to obtain the copper-binding protein hydrolysate from the white leg shrimp head (WLSH) by-product using enzymatic hydrolysis. The outcome indicated that the copper-binding capacity (CBC) of the WLSH protein hydrolysate achieved the maximum value of 19.4 ± 0.5 mg Cu^{2+} /g protein with hydrolysis conditions including Flavourzyme preparation, pH 7.5, 50°C, the enzyme : substrate (E:S) ratio of 80 U/g protein and 5h of hydrolysis. Under the pH treatment in a range of 1-11 or heat treatment at 100°C for up to 180 min, its CBC remained over 80%. The water holding capacity (WHC) and the oil-holding capacity (OHC) of the protein hydrolysate were 4.1 ± 0.1 ml water/g protein hydrolysate powder and 4.5 ± 0.1 ml oil/g protein hydrolysate powder, respectively. The solution also encompassed up to 8 essential amino acids, accounting for 36.1% of the total amino acid content. The protein hydrolysate could serve not only as a copper chelating agent, preventing copper-deficient or superfluous relating diseases, but also as a texturizer and an amino acid supplement fortified in various types of food.

Key words - copper-binding activity; functional property; pH stability; thermal stability; protein hydrolysate; white leg shrimp head

1. Introduction

In our body, copper plays both enzymatic and non-enzymatic functions in diverse physiological processes such as neurotransmitters synthesis, enzymatic antioxidant system, angiogenesis, gas transport, and neurohormone homeostasis [1]. Copper deficiency is rare, however, copper transport and homeostasis corruption probably triggered fatal diseases such as Wilson disease and Menkes disease [1]. Besides food source, copper could be delivered via vitamin and mineral supplements for human beings, in form of cupric oxide which has lower bioavailability [2]. On the other hand, free copper ion in human body could generate reactive oxygen species which could lead to cardiovascular disease [3]. One of the most potential copper-chelating agents is marine peptide that may improve copper absorption as well as prevent copper-catalyzed oxidation reactions in human body [4]. As for food industry, copper is a catalyst for lipid oxidation reaction, having negative impacts on food quality attributes such as taste, texture, shelf life, appearance, and nutritional profile [5]. As a way to tackle these problems, the presence of chelator was reported from Osborn-Barnes and Akoh [5] to inhibit metal-catalyzed oxidation reaction. Some copper-binding peptides have been found to be potential chelators from aquatic life forms and by-products [6, 7].

Moreover, it was also reported that the application of enzymatic protein hydrolysate in the production of fish protein hydrolysate could improve the physicochemical properties of food proteins without losing their nutritional values [8]. Previous studies of fish protein hydrolysate have shown that, when added to food, they can contribute to water holding, emulsification and texture properties [8]. However, it is necessary that the protein hydrolysate

maintains its bioactivity and techno-functionality under the different conditions during food production as well as during gastrointestinal digestion. Hence, stability of bioactive protein hydrolysate at various environmental condition (pH, temperature, ...) must be obtained.

WLSH, a rich source of biomolecules, takes up to 50-70% of the whole shrimp [9]. Despite that, by-products are usually used for poultry and aqua feed [9]. Therefore, more researches on this by-product are required to increase its value. Besides, as far as we know, there have not been any publications about copper-binding protein hydrolysate from WLSH. This study was aim to (i) investigate the impacts of hydrolysis conditions (enzyme type, hydrolysis temperature, pH, E:S ratio and hydrolysis time) on the CBC of the WLSH protein hydrolysate; (ii) Determine its amino acid profile; (iii) Evaluate its pH and thermal stability; (iv) assess its functional properties.

2. Materials and Methods

2.1. Materials

2.1.1. WLSH

This study utilized WLSHs provided by a local shrimp processing manufactory in Long An province, Vietnam. They are shifted on ice to the Biochemical laboratory of Ho Chi Minh City University of Technology within 4 h, stored at -20°C in labelled polyethylene bags until used. Their moisture, protein, lipid and ash content (on dry weight basis) were $81.4 \pm 0.3\%$, $55.9 \pm 0.6\%$, $4.3 \pm 0.2\%$ and $23.1 \pm 0.2\%$, respectively, which were estimated based on the method of Association of Official Analytical Chemists [10].

2.1.2. Chemicals and enzyme preparations

Alcalase® 2.5L, Neutrase® 0.8L, Protamex® and Flavourzyme® 500MG with their optimal working conditions being presented in table 1 [7] were purchased from Novozymes (Denmark) and AB enzymes (Germany). Chemicals being of analytic grade were supplied by Sigma-Aldrich and Merck. Double-distilled water was used in this study.

Table 1. Optimal working pH and temperature of the proteases used in this study

Enzyme	Optimal pH	Optimal Temperature
Neutrase	8	50°C
Protamex	6.5	50°C
Flavourzyme	7	50°C
Alcalase	7.5	55°C

2.2. Methods

2.2.1. Preparation of WLSH protein hydrolysate

WLSH protein hydrolysates were prepared using method of Vo et al. [9] with slight modification. The

WLSH was dispersed in distilled water with the ratio of 1:4 (w/v) was heated at 90°C for 10 min to deactivate endogenous enzymes. Desired enzyme was added after pH value was controlled using 1M NaOH or HCl solution. After a required time of hydrolysis, the hydrolysates were heated for 10 min at 90°C to inactivate the enzymes, followed by centrifugation to obtain the supernatant. The method of Lowry et al. [11] was applied to determine the protein content of the protein hydrolysates. Freeze – dryer (Alpha 1–2/Ldplus, UK) was used to freeze-dry the collected supernatants and the powder was stored at –20°C until being used.

2.2.2. Effect of hydrolysis condition on the CBC of the protein hydrolysate

A single factor test in which one factor was changed on different levels while others being set was performed to examine the influences of five effective parameters including protease type, pH, temperature, E:S ratio and hydrolysis time on the CBC of the WLSH protein hydrolysate.

2.2.3. Determination of CBC

The method of Vo and Pham [6] was used to assess the CBC of WLSH protein hydrolysate. 1ml of protein hydrolysate was added to the mixture of 1ml of 2mM CuSO₄ solution, 1ml of 10% pyridine solution and 20μl of 0.1% pyrocatechol violet solution. The absorbance was recorded at 632 nm and CBC was calculated as follows:

$$CBC (\mu g \text{ Cu}^{2+} / g \text{ protein}) = \frac{A_c - A_s}{A_c} * \frac{m_{\text{Cu}^{2+}}}{m_{\text{protein}}} \quad (1)$$

Where, A_c: the absorbance of the blank; A_s: the absorbance of the sample; m_{Cu²⁺}: mass of Cu²⁺ (μg); m_{protein}: mass of protein in WLSH protein hydrolysate (g).

2.2.4. Analysis of amino acid composition

First, the WLSH protein hydrolysate was completely hydrolyzed using 6M HCl solution for 23 h at 110 ± 2°C. Subsequently, ion-exchange chromatography was performed to separate amino acids, which were then detected in forms of Ninhydrin derivatives. Quantities of free amino acids in sample was determined using standard solutions of amino acids by measuring their absorbance at 440 nm for Proline and 570 nm for the rest amino acids [10].

2.2.5. pH and thermal stability of copper-binding protein hydrolysate

The pH and thermal stability of copper-binding WLSH protein hydrolysate were obtained using the methods of Sripokar et al. [8]. 5ml of 40 mg protein/ml solution was prepared from the protein hydrolysate powder and distilled water. Prior to incubation at room temperature for 30 min, the sample solutions' pHs were adjusted to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 using 1 M HCl or 1 M NaOH solution for pH stability. After that, 1M phosphate buffer was used to adjust the pHs of samples to 7.0. Finally, distilled water was added until the volume of samples achieved 20 ml before determining their residual CBCs.

To determine thermal stability, 5ml of 40 mg protein/ml sample solution was heated at 100°C for 0, 15, 30, 45, 60, 90, 120, 150 and 180 min. Then, prior to having their residual CBCs tested, the treated samples were suddenly

cooled in iced water. The CBC of protein hydrolysate without pH or heat treatment was set at 100%.

2.2.6. Determination of OHC

OHC of the WLSH protein hydrolysate was assessed using the procedure published in the study of Vo et al. [12]. First, 10 ml of vegetable oil was added into 50mL centrifugal tube containing 0.5g of sample. Then the suspension was left at still for 30 min at temperature of 25±1°C (30s of agitation was performed every 10 min) before being centrifuged and recorded the volume of the supernatant. The volume of oil adhering to the tube wall was estimated by the same protocol in the absence of sample. This assay was conducted in triplicate and the OHC was depicted as the volume (mL) of oil absorbed by 1g of protein hydrolysate powder.

2.2.7. Determination of WHC

The centrifugation method presented by Vo et al. [12] was employed to determine WHC of the WLSH protein hydrolysate. First, 0.5g of sample was suspended in 20 mL of distilled water in centrifuge tube and then scattered with a vortex mixer for 30s. Then, the dispersion was left at room temperature for 6 h prior to being centrifuged, filtered with Whatman No. 1 filter paper, and recorded the volume of the filtrate. The difference between the initial volume of distilled water added to the protein sample and the volume of the filtrate was calculated, and the WHC was presented as mL of water absorbed per 1g of protein hydrolysate powder.

2.2.8. Statistical Analysis

Data were presented as averages ± standard deviations of triplicate experiments. Statgraphics Centurion 18 software was used to assess the estimation of variance (one-way ANOVA) and the signification (Tukey method) (p<0.05).

3. Results and discussion

3.1. Effect of hydrolysis condition on CBC of WLSH protein hydrolysate

3.1.1. Effect of enzyme type

It was found from this study that Flavourzyme showed the highest potential for generating the WLSH protein hydrolysate with the highest CBC of 6.2 ± 0.6 mg Cu²⁺/g protein (Figure 1a). As Flavourzyme preparation exhibits both exo- and endopeptidase activities, it was capable of releasing more bioactive peptides, displaying a broad substrate specificity [12]. Besides, Flavourzyme was reported from He et al. [13] to usually produce the protein hydrolysate possessing high amount of hydrophobic amino acids, which forms a hydrophobic barrier to restrain the access of water molecules and stabilize the peptide-copper ion complex. Hence, Flavourzyme was used in further experiments.

3.1.2. Effect of pH

As can be seen in Figure 1b, at pH 7.5, the CBC achieved the peak of 8.3±0.1 mg Cu²⁺/g protein. Kozłowski et al. [14] demonstrated that the environmental pH possibly alters charge distribution, ionization ability and conformation of enzyme and substrates, impacting enzyme catalytic activity and CBC of protein hydrolysate. The enzyme catalytic activity was lowered at other pH values

as the enzyme active site was progressively deformed and its function was also negatively affected. [12]. Therefore, pH 7.5 was set for further experiments.

3.1.3. Effect of temperature

Temperature affected not only the reaction rate of molecules, the activation energy of the catalytic reaction, and the thermal stability of enzyme and substrate [12], but also the bioactivity of the protein hydrolysate. In this study, CBC of the proteolysate peaked at 8.5 ± 0.5 mg Cu^{2+} /g protein at the temperature of 50°C, optimal temperature (Figure 1c). Under the effect of temperature, the conformation change of substrate and enzyme revealed the hydrophobic or electron-donating amino acid side chains inside the substrate [6], boosting the formation and stabilization of coordination bond between peptides and copper ions. At non-optimal temperature, due to the decrease in their movement or change in their configuration, the interaction between enzyme and substrate molecules was diminished, reducing the formation of copper-binding peptides. Therefore, hydrolysis temperature of 50°C was chosen for further study.

3.1.4. Effect of E:S ratio

The CBC of the WLSH protein hydrolysate was showed from figure 1d to be inversely proportional to the E:S ratio in the E:S range from 50 to 70 U/g protein. It is possibly deduced that the higher the E:S ratio is, the more the proteolysis rate elevates, possibly lessening the resonant structure of peptides in the protein hydrolysate, lowering CBC of the protein hydrolysate. To clarify the relationship between peptide resonant structure and its CBC, take the collaboration of Asn and adjacent Phe as an example. As such combination creates a protective barrier around Cu^{2+} , it can improve CBC by stabilizing Cu-N link of the peptide complex [14]. However, CBC of the WLSH exhibited the highest value of 15.9 ± 0.7 mg Cu^{2+} /g protein at the E:S ratio of 80 U/g protein (Figure 1d). This could be explained by the large amount of peptides obtained from protein hydrolysate exposed adequate copper-binding side chains and possessed appropriate size to reduce steric obstacle at the E:S ratio of 80 U/g protein. It was obvious from figure 4 that when boosting the E:S ratio from 80 to 90 U/g protein, there was a significant decrease in CBC. Vo and Pham [6] assumed that through early steps of hydrolysis, larger quantity of enzyme could degrade high potential copper-binding peptides forming, reducing CBC of protein hydrolysate. Hence, the E:S ratio of 80 U/g protein was used for further analyses.

3.1.5. Effect of hydrolysis time

CBC-hydrolysis time profile (Figure 1e) of WLSH protein hydrolysate is similar to its CBC-E:S ratio profile. When the hydrolysis time was prolonged from 2 to 4h, CBC of the protein hydrolysate reduced. The protein hydrolysate expressed the highest CBC of 19.4 ± 0.5 mg Cu^{2+} /g protein at the hydrolysis time of 5h. However, overlong hydrolysis (6h) lessened the CBC of the protein hydrolysate, which is probably due to the degradation of bioactive peptides generating at primary stage during extensive hydrolysis [7]. Additionally, CBC of the protein hydrolysate was also decreased by feedback inhibition

caused by hydrolysis products, lowering the enzyme catalyzing activity [7]. In this study, hydrolysis time of 5 h was chosen for further studies.

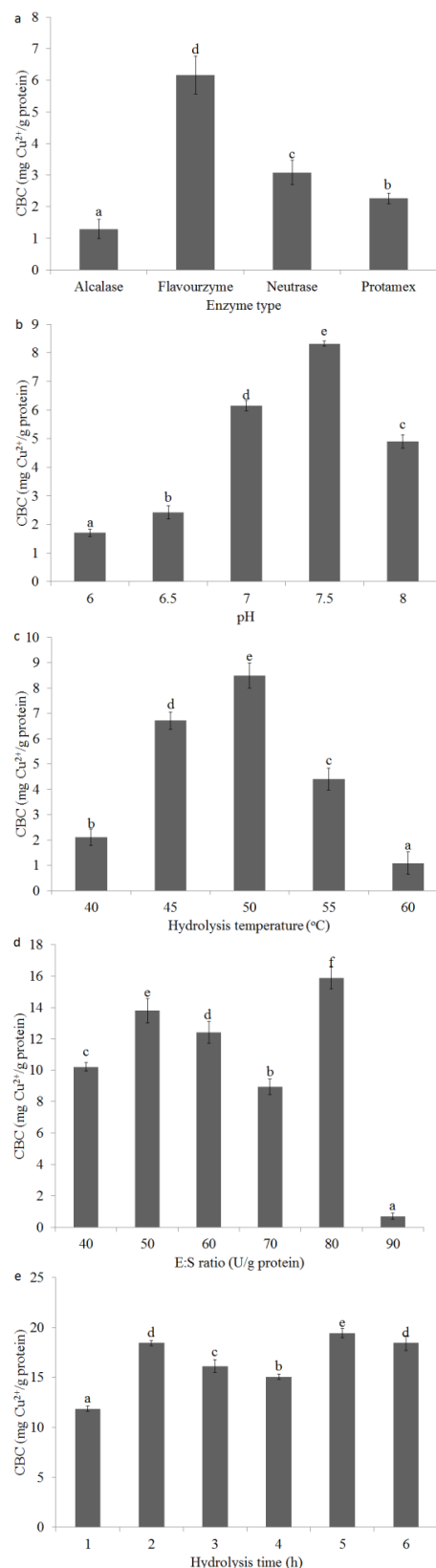


Figure 1. Effect of enzyme type (a), temperature (b), pH (c), E:S ratio (d) and hydrolysis time (e) on CBC of WLSH protein hydrolysate. Bars with different letters indicate significant differences ($p < 0.05$)

3.2. Amino acid composition of WLSH protein hydrolysate

Table 2 presented the amino acid profile of the WLSH protein hydrolysate. In terms of the nutritional value, with the exception of Trp, almost entire 9 essential amino acids could be supplied from the protein hydrolysate. The amino acid make-up plays an important role in bioactivity of the protein hydrolysate. In particular, Glu, known as binding-site for minerals of containing-peptides, took up the largest proportion in amino acid content of the protein hydrolysate, enhancing its CBC. Similarly, Asp stimulated the CBC of the protein hydrolysate by donating free electrons in O-carboxyl to Cu^{2+} empty orbitals [7]. Moreover, nitrogen atoms in -imidazole ring of His and/or in ϵ -amide of Lys are acted as electron donors that stimulates the CBC of the protein hydrolysate [6, 7]. With the presence of S-containing amino acids, e.g. Cys and Met, via free electrons of S atoms in their HS- and $\text{CH}_3\text{-S}$ -group, CBC of the protein hydrolysate is also improved [6]. Furthermore, hydroxyl group of Thr and Ser were also known as another chelating-site of copper-binding peptides [7]. Peptide- Cu^{2+} complex is stabilized by high content of aliphatic amino acids (Ala, Leu, Ile, Val and Phe) generating hydrophobic barrier, shielding one side of the complex plane from the strike of water molecules [6, 7].

Table 2. Amino acid profile of the WLSH protein hydrolysate

Essential amino acid	Content (mg/L)	Non-essential amino acid	Content (mg/L)
His	400	Arg	1100
Ile	700	Cys	1100
Leu	1400	Gly	1600
Lys	1800	Tyr	800
Met	500	Ala	1500
Phe	800	Asp	1700
Thr	900	Glu	2800
Val	1000	Ser	1000
Total	20800	Pro	1700

3.3. pH and thermal stability of copper-binding protein hydrolysate from WLSH

A wide range of pH value is displayed in food products, varying between 3 and 7 [15]. In human body, at different digestion stages, food encounters different pH including pH 1.5-6.5 in stomach and neutral pH (7.0-8.5) in duodenum, small and large intestine [8]. Physico-chemical changes of proteins could possibly be stimulated by pH adjustment comprising of denaturation, intermolecular interactions, solvability, which trigger crosslinking, lysine damage, non-specific cleavage of peptides and damaged amino acids, impacting on bioactivity of protein hydrolysate [16]. Therefore, pH stability of bioactive protein hydrolysate is a crucial characteristic for gastrointestinal stability and its application in food products. In this study, WLSH protein hydrolysate reached over 80% of CBC in pH range from 1 to 10, particularly achieved 100% at pH 7 (Figure 2a). However, at pH 11, there was a significant decrease in stability of CBC with relative activity of approximately 65%. This could be attributed to the degradation of bioactive peptides triggered

by alkaline hydrolysis [17]. Similarly, being consistent with our research, starry triggerfish muscle protein hydrolysate exhibited fine resistance against acidic and weak alkaline conditions [8]. It can probably be concluded that copper-binding protein hydrolysate from WLSH has a potential to be added to food products.

Due to thermal sensitiveness of protein, heat treatment is a popular unit operation in food manufacturing which can influence their functional characteristics [12]. Heat could alter conformational of protein/peptide as it changes the non-covalent interactions (ion linkage and Val der Waals force) and disulfide bonds existing in tertiary and/or quaternary structure, stimulating aggregation caused by hydrophobic interaction, altering CBC of the protein hydrolysate [18]. It could be observed from Figure 2b that WLSH protein hydrolysate kept over 80% of CBC after heating at 100°C for up to 180 min. It was possibly owned to the good balance between hydrophilic and hydrophobic interaction in the WLSH protein hydrolysate, preventing peptide aggregation [19]. By hindering the formation of the secondary structure and generating intermolecular hydrophobic bond, high content of Pro and Ile (Table 2) in the protein hydrolysate also contributed to its heat stability [20]. Similar observation has been published in study of Sripokar et al. [8]. Great thermal stability provides potential opportunity for application of WLSH protein hydrolysate in food products. However, in order to assure that the demanded physiological benefit of a food product is received, the loss of stability of bioactivity must also be assessed under severe treatment conditions, such as sterilization, grilling and frying.

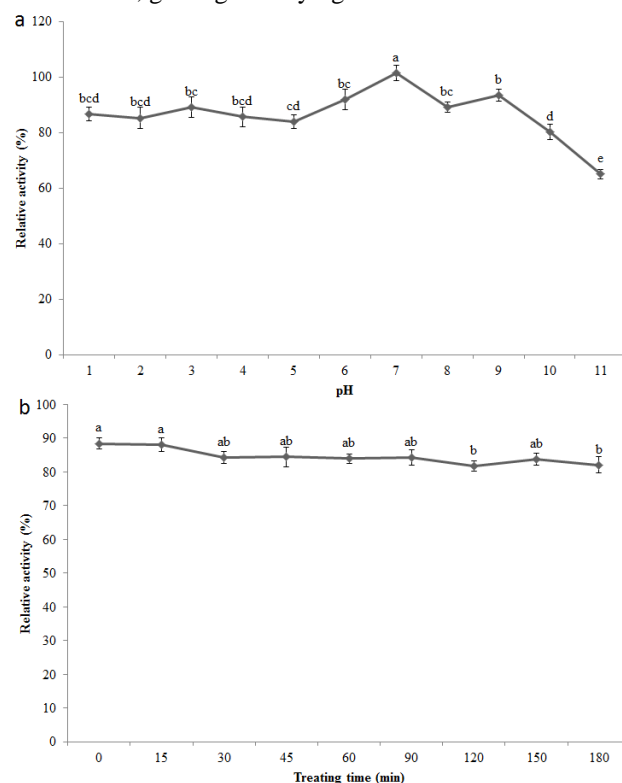


Figure 2. pH (a) and thermal (b) stability of copper-binding protein hydrolysate from WLSH. Different letters indicate significant differences ($p < 0.05$)

3.4. WHC and OHC of WLSH protein hydrolysate

OHC, a quantity of oil directly held by protein, is a crucial parameter that influences the savor of a food product. The oil-holding mechanism of protein hydrolysate is supposed to be the physical entrapment of oil. The greater the protein bulk density is, the greater the OHC is [12]. The protein hydrolysate powder displaying great OHC could be served for inhibiting phase separation and simultaneously enhancing the palatability and taste retention of some food products such as sausage, cake, mayonnaise, and other salad dressing [21]. In this test, the OHC of the WLSH protein hydrolysate obtained 4.5 ± 0.1 ml oil/g protein hydrolysate powder, 2.8 times higher than that of *Acetes* protein hydrolysate (DH 52.58%) [12]. This difference is a consequence of the dissimilarity in hydrophilic polar side chains of peptides in these protein hydrolysates [22]. Besides, OHC of protein hydrolysate was also affected by other factors such as degree of hydrolysis, the peptide surface hydrophobicity, and enzyme-substrate specificity [21]. Protein hydrolysates having high DH involve a large number of short-chain peptides with supreme hydrophilicity, diminishing the peptide-lipid interaction, hence reducing OHC [23]. Better hydrophobic peptides express strong OHC as they possibly form hydrophobic bonds to oil substances, rising protein-lipid complex stability [12].

WHC displays the capacity of absorbing water and preserving it versus gravitational attraction within a protein matrix. It has an impact on the texture and integrity of food products, for instance, frozen fish fillets or meat [24]. The WHC of the WLSH protein hydrolysate achieved 4.1 ± 0.1 ml water/g protein hydrolysate powder, which was 1.3 and 1.2 times greater than those of protein hydrolysates from tilapia protein [25] and Chinese sturgeon [22], in order. It was also revealed from Cumby et al. [23] that amino acid composition and peptide molecular weight were decisive factors for WHC of a protein hydrolysate. As lower-molecular-weight peptides are commonly more hydrophilic, short-chain peptides are more advantageous in holding water than larger-size peptides [23]. Furthermore, during enzymatic hydrolysis, the augment in proportion of polar groups including -COOH and -NH₂ groups shows a substantial influence on the quantity of adsorbed water [24]. As illustrated in table 2, three main amino acids in the WLSH protein hydrolysate, which were Glu, Asp and Lys, were interpreted to be able to bind 2-7 water molecules [26]. A conclusion could be drawn from the result that the WLSH protein hydrolysate could be considered as a moisture keeping agent for protein gel products or beef and fish muscle [24].

4. Conclusion

This study finds out a hydrolysis condition to obtain the WLSH protein hydrolysate possessing the highest CBC. This research also provides data on pH and thermal stability of CBC, functional characteristics and amino acid composition of the protein hydrolysate, benefiting for its application in food products. Further researches, however, are required to be conducted, on sensory properties and *in*

vivo activity of foods fortified with WLSH proteolysate powder.

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