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ORIGINAL PAPER

Identification of Copper-Binding Peptides and Investigation of Functional Properties of *Acetes japonicus* Proteolysate

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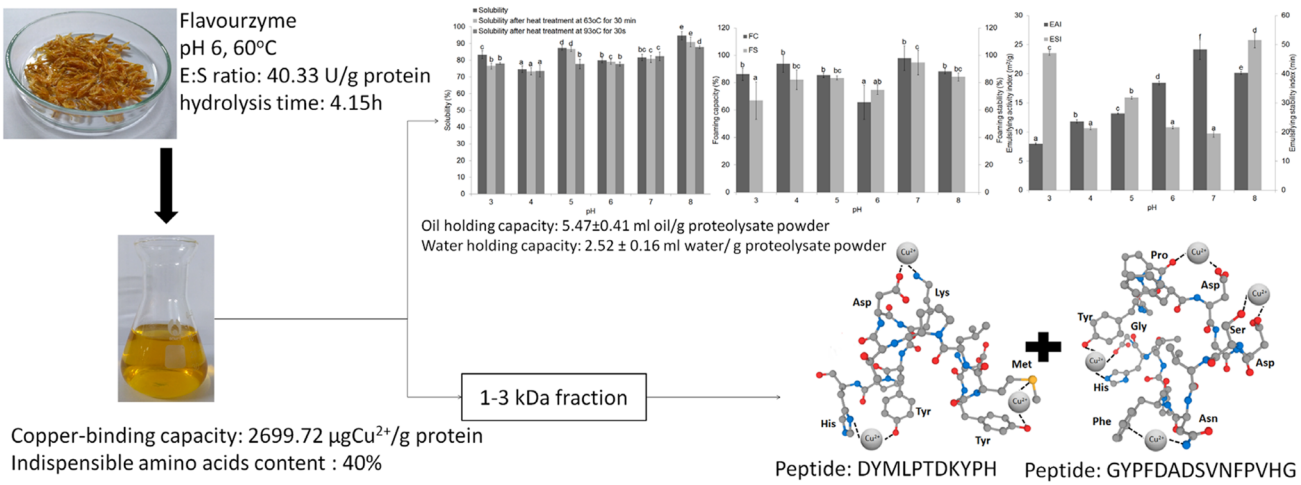
Abstract

In this research, two copper-binding peptides were identified in the proteolysate of *Acetes japonicus*. Firstly, the examination of the effects of hydrolysis conditions including enzyme type, pH, temperature, enzyme:substrate (E:S) ratio and hydrolysis time on the copper-binding capacity (CBC) was carried out. Secondly, to optimize the hydrolysis, response surface methodology (RSM) was applied via E:S ratio and hydrolysis time for maximizing the CBC of the proteolysate. After that, the amino acid composition and functional properties of the proteolysate was evaluated. Simultaneously, before being tested for CBC, four peptide fractions of 10–30 kDa, 3–10 kDa, 1–3 kDa, and < 1 kDa were recovered from the proteolysate using ultrafiltration. Finally, mass spectrometer / mass spectrometer (MS/MS) was used to determine molecular weight and amino acid sequence of bioactive peptide. The result showed that when using Flavourzyme, pH 6, 60 °C, E:S ratio of 40.33 U/g protein and hydrolysis time of 4.15 h, CBC reached its peak at 2699.72 μgCu^{2+}/g protein. Proteolysate, of which Leu was the major amino acid, the indispensible amino acids comprised of approximately 40% total amino acid content. The proteolysate also exerted great solubility, heat stability, foaming and emulsifying property, oil and water holding capacity (OHC and WHC, respectively). The 1–3 kDa fraction exhibited the highest CBC of 2754.73 ± 91.87 μg Cu^{2+}/g protein, from which two peptides (Asp-Tyr-Met-Leu-Pro-Thr-Asp-Lys-Tyr-Pro-His (1378.6 Da) and Gly-Tyr-Pro-Phe-Asp-Ala-Asp-Ser-Val-Asn-Phe-Pro-Val-His-Gly (1620.7 Da)) were detected. This is the first time that the copper affinity peptides were recovered from the small shrimp that could be utilized as a natural source for copper enhancement.

Graphic Abstract



Keywords *Acetes japonicus* · Copper-binding capacity · Copper-binding peptide · Enzymatic hydrolysis · Functional property

Extended author information available on the last page of the article

Statement of Novelty

The aims of this study are to evaluate copper-binding capacity and functional properties of *Acetes japonicus* proteolysate and to detect bioactive peptides from the proteolysate, suggesting a new way of utilizing the cheap protein source. Nowadays, however, there have been few publications on copper-binding activity of the proteolysate/peptide derived from aquatic life forms or by-products. Compared to previous studies, in the present work, three aspects of the proteolysate being evaluated include bioactivity, technological characteristics and nutritional composition. Also, the prediction of the de novo structures and interactions of identified peptides with cupric ion are also made in this paper.

Introduction

Copper, a cofactor for various enzymes and an essential trace mineral for human, contributes to various biochemical processes including cellular respiration, peptide amidation, neurotransmitter biosynthesis, pigment formation, connective tissue strength and central nervous system development [1]. Generally, copper can be absorbed from the stomach to the distal small intestine in mammals and humans [2]. However, only a small amount of dietary copper exists in soluble form in the stomach, so its absorption at the site is not considered to be nutritionally significant [2]. Also, the reduction of cupric to cuprous ions caused by ascorbic acid lowered the bioavailability of this element [2]. Although deficiencies are rare, genetically impaired functions of copper have caused fatal diseases in humans and animals. Wilson and Menkes diseases, in particular, result from mismanagement of copper transportation and homeostasis [3]. Diarrhea is a common ailment in young children suffering from a lack of zinc, magnesium or copper [3]. Besides, copper shortage affects human bone marrow hematopoiesis and nervous system [4]. Daily diet could provide copper for human body, but the presence of high amount of zinc, molybdenum and sulfur could suppress copper absorption, leading to inadequate intake of copper [3]. Wapnir [2] reported that the average true copper absorption from typical diets in developed societies is in the range from 30 to 40% of copper intake. Beside to food source, mineral supplements could be used to deliver copper for human beings, but they contains copper in forms of cupric oxide which has low bioavailability. Protein was reported to improve copper absorption and bioavailability by enhancing its solubility and intestinal bulk flow [2]. In contrast, copper excess was revealed to be risk for cardiovascular disease because of its catalytic activity for Haber Weiss reaction that generates reactive oxygen species [5, 6]. There was an evidence that the presence of a chelator could diminish free copper level in human body, benefiting in treating fibrotic, inflammatory, neural degeneration, autoimmune diseases and cancer [7]. In addition, unsaturated copper-binding sites on albumin and peptides in plasma prevent sudden release of copper ions in blood, inhibiting intravascular haemolysis [8].

Proteins are usually utilized in the chemical, pharmaceutical and food industries as emulsification, foaming, encapsulation and gelation agents. However, intact proteins are molecularly heavy and have intra-molecular bonds that obstruct them from exhibiting their functional properties [9]. Enzymatic hydrolysis was clearly demonstrated to be an efficient method to improve the functional properties of proteins while also remain their nutritional values [10]. Besides, marine protein hydrolysates not only possessed good solubility over a wide range of ionic strength and pH but also could tolerate strong heat without precipitating, as well as showing good technological features of food products including water holding, texture, gelling, whipping and emulsifying properties [11].

In Vietnam, several low economic valued aquatic life forms have been exploited inefficiently, of which *Acetes japonicus* is one. Although high protein content was found in the small shrimp, it is predominantly served as an ingredient in production of low price products such as shrimp paste and dry shrimp. The discovery of Cu-binding proteolysate was made in this study in order to maximize the exploitation of small shrimp in terms of nutritional value, functional features and/or bioactivity. Except for normal nutritional value, copper deficiency or overabundance relating diseases can be prevented and/or food texture or food taste can be improved as Cu-binding proteolysate/peptide being used as a natural Cu-carrier and/or a food additive, respectively. So far, there have been a limited number of publications on copper-binding proteolysate/peptide from the small shrimp.

The objectives of this study were to (i) evaluate the effects of hydrolysis conditions on the CBC of proteolysate and optimize the hydrolysis via E:S ratio and hydrolysis time for maximizing the CBC using RSM; (ii) analyze the amino acid composition of the proteolysate; (iii) investigate functional properties of the proteolysate; (iv) recover peptide fractions by using ultrafiltration centrifugal devices and test their CBC; (v) identify the amino acid sequences and molecular weights of copper affinity peptides.

Materials and Methods

Materials

Acetes japonicus

Acetes used in this investigation was bought from a company in Ninh Thuan province, Vietnam with the moisture of 12.3 ± 0.12%. Moisture content was determined using air oven method (100 °C for 5 h; AOAC number 950.46B), ash content was analysed using basic heating technique (550 °C for 5 h; AOAC number 920.15), and crude protein was examined by nitrogen combustion procedure (AOAC number 992.15) with conversion factor of N*6.25 [12]. The crude lipid content was tested using method of Folch et al. [13]. Chemical composition of *Acetes japonicus* that contained 72.8 ± 0.7% crude protein, 4.3 ± 0.2% crude lipid and 16.8 ± 0.2% ash (on dry weight basis) were determined.

Enzyme Preparations and Chemicals

Proteases including Alcalase® 2.5L (Serine endo-protease from *Bacillus licheniformis*, optimal pH of 7.5 and optimal temperature of 55 °C), Neutrase® 0.8L (Zinc metallo endo-protease from *Bacillus amyloliquefaciens*, optimal pH of 7 and optimal temperature of 55 °C), Protamex® (endo-protease from *Bacillus subtilis*, optimal pH of 6.5 and optimal temperature of 50 °C) and Flavourzyme® 500MG (mixture of endo- and exo-protease from *Aspergillus oryzae*, optimal pH of 7 and optimal temperature of 50 °C) and Corolase® 7089 (serine metallo endo-protease from *Bacillus subtilis*, optimal pH of 7 and optimal temperature of 55 °C) were obtained from Novozymes (Denmark) and AB enzymes (Germany). Chemicals were purchased from Sigma-Aldrich and Merck. All reagents were in analytic quality. Double-distilled water was used in tests.

Methods

Preparation of *Acetes japonicus* Hydrolysates

The preparation of hydrolysates was performed according to the procedure of Vo et al. [14] with slight modification. Water was added to the small shrimp with the *Acetes*:water ratio of 1:8 (w/v) and the mixture was then heated at 90 °C for 10 min to deactivate endogenous enzymes. Desired enzyme was added after controlling the pH value of the mixture using 1 M NaOH or HCl solution and this pH value was managed every 15 min during hydrolysis. After the required hydrolysis time, the reaction was disabled in order to deactivate the enzyme by heating the hydrolysate for 10 min at 90 °C. The supernatant was collected by centrifuging the hydrolysate. The method of Lowry et al. [15] and Nielsen et al. [16] were applied to determine the protein content and degree of hydrolysis (DH) of the proteolysate, respectively. The obtained supernatants were freeze-dried using freeze-dryer (Alpha 1–2/Ldplus, UK) and stored at -20 °C until being used.

Effects of Hydrolysis Condition on the CBC of *Acetes proteolysate*

A single factor test approach, which was carried out by having one factor altered with various levels while others being given, was applied to evaluate the impacts of five effective parameters including hydrolysis enzyme type, pH, temperature, E:S ratio and hydrolysis time on the CBC of the proteolysate.

Determination of CBC of Proteolysate

The CBC of proteolysate was determined using the method of Kong, Xiong [17]. After demineralization by macroporous resin (Amberlite IRC-748I sodium form, Acros), 1 mL of proteolysate was added to a mixture of 1 mL of 2 mM $CuSO_4$ solution, 1 mL of 10% pyridine solution and 20 µL of 0.1% pyrocatechol violet solution. The absorbance was measured at 632 nm and CBC was calculated using the following formula:

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

where A_c denotes the absorbance of the control (distilled water instead of sample); A_s is the absorbance of the sample; $m_{Cu^{2+}}$ is the initial weight of cupric ion, μg, $m_{protein}$ is the weight of protein of proteolysate, g.

Optimization of E:S Ratio and Hydrolysis Time for Maximizing the CBC of the *Acetes proteolysate*

A randomised, quadratic central composite circumscribe response surface design was used to optimize E:S ratio and hydrolysis time. CBC of the hydrolysate was the dependant variable. The Modde software (version 5.0) was used to generate experimental planning and to process data. Each factor in the design was investigated at five different levels ($-\sqrt{2}$, -1, 0, $+1$, $+\sqrt{2}$). The total number of experiments was 13 and the number of central experiments was five.

Amino Acid Composition Analysis

The proteolysate was hydrolyzed with 6 M HCl solution for 23 h at 110 ± 2 °C to estimate its amino acid content.

Next, the amino acids were separated using ion-exchange chromatography, followed by being derivatized with Ninhydrin and finally being detected. Standard solutions of amino acids were used to quantify free amino acids in samples by measuring their absorbance at 440 nm for Pro and 570 nm for other amino acids (AOAC 994.12 [12]).

Determination of Solubility

The method of Li et al. [18] was slightly modified to evaluate the solvability of the proteolysate. The mixtures of hydrolysate samples (100 mg) scattered in 10 mL of deionized water had their pH adjusted to 3, 4, 5, 6, 7 and 8 using 1 M HCl or NaOH solution. The mixture was then stirred at room temperature for 30 min prior to centrifugation. After dissolving the sample in 0.5 M NaOH solution, total protein content of sample was estimated using the method of Lowry et al. [15]. Protein solubility was calculated as follows:

$$Solubility\,(\%) = \frac{Protein\ content\ in\ supernatant * 100}{Total\ protein\ content\ in\ sample} \quad (2)$$

Heat Stability

The heat stability of the proteolysate was obtained following the procedure of Li et al. [18] with a minor variation. The pH of the solution consisting of 100 mg of hydrolysate dissipating in 10 mL of deionized water was adjusted in the range from 3 to 8 using 1 M HCl or 1 M NaOH solution. The solution was heated at 63 °C for 30 min or 93 °C for 30 s and then kept in ice-water for 10 min before being centrifuged. The heat stability of the proteolysate was expressed as its solubility after heat treatment.

Determination of Foaming Capacity (FC) and Foaming Stability (FS)

The method of Li et al. [18] with a minor variation was applied to evaluate the FC of the proteolysate. The FC was evaluated in the pH ranging from 3 to 8. The homogenization of proteolysate (40 mL, 10 mg.mL^{-1}) was done. The whipped specimen (40 mL, 10 mg.mL − 1) was then instantly moved into a 100 mL cylinder and the total volume was recorded after 30 s. The FC was determined by the following equation:

$$FC(\%) = \frac{A - B}{B} * 100 \quad (3)$$

where A indicates the volume of the proteolysate after being whipped (mL); B represents the volume of the proteolysate before being whipped (mL).

The whipped sample was left at 20 °C for 3 min and the volume of the whipped sample was then monitored. The FS was evaluated via the following equation:

$$FS(\%) = \frac{A_t - B}{B} * 100 \quad (4)$$

where A_t expresses the volume of the proteolysate after standing (mL); B depicts the volume of the proteolysate before being whipped (mL).

Determination of Emulsifying Activity Index (EAI) and Emulsifying Stability Index (ESI)

Emulsifying property of the proteolysate was determined using the method of Li et al. [18] with a minor alteration. 5 mL of vegetable oil was blended with 15 mL of 10 mg protein mL^{-1} hydrolysate to create a mixture, of which the pH was altered to 3, 4, 5, 6, 7 or 8 using 0.1 M NaOH or 0.1 M HCl solution. The homogenization was then carried out and 50 µL aliquot of the emulsion was taken from the bottom of the container at 0 min and 10 min after homogenization. Afterwards, the blend was added with 4.95 mL of 1 mg mL^{-1} sodium dodecyl sulfate solution and the absorbance was determined at 500 nm. The EAI and ESI were estimated by the equation:

$$EAI\left(m^2/g\right) = \frac{2 * 2.303 * A_0}{0.25 * protein\ weight(g)} \quad (5)$$

$$ESI(min) = \frac{A_0 * \Delta t}{\Delta A} * 100 \quad (6)$$

where $\Delta A = A_0 — A_{10}$; $\Delta t = 10$ min; A_0 and A_{10} are the absorbances of the samples taken at 0 min and 10 min after homogenization, respectively.

Determination of OHC

The procedure of Putra et al. [19] with slight adjustment was utilized to evaluate the OHC of the proteolysate. Firstly, 50 mL centrifugal tube containing 0.5 g of each sample mixed with 10 mL of vegetable oil was left for 30 min at temperature of 25 ± 1 °C (30 s of agitation was performed every 10 min). The mixture was then centrifuged and the volume of the supernatant was measured. The estimation of the amount of oil clinging to the wall of the tube was carried out with the same protocol without any sample. This assay was carried out in triplicate and the OHC was described as the volume (mL) of oil being absorbed by 1 g of proteolysate.

Determination of WHC

The WHC was determined by the centrifugation method presented by Putra et al. [19]. Firstly, 20 mL of water was used to rehydrate 0.5 g of sample in each centrifuge tube, which was then scattered with a vortex mixer for 30 s. Next, the dispersion was left at room temperature for 6 h prior to being centrifuged. The collected volume was accurately measured after the supernatant was filtered with Whatman No. 1 filter paper. The difference between the initial volume of distilled water added to the protein sample and the volume of the supernatant was evaluated, and the result was depicted as mL of water being absorbed per 1 g of protein sample.

Fractionation of Proteolysate

Four peptide fractions of 10–30 kDa, 3–10 kDa, 1–3 kDa, and < 1 kDa from the proteolysate were obtained by using ultrafiltration centrifugal devices of 30 kDa, 10 kDa, 3 kDa and 1 kDa (Thermo—Fisher Scientific, Pall, USA). Then the collected fractions were tested for their CBC.

Identification of Copper-Binding Peptide

Prior to being submitted to a mass spectrometer, dried samples were cleaned using C_{18} columns as manufacturer's instruction. In brief, 50 µg of each dried sample (from three biological replicates) were re-suspended in 100 µL of loading buffer C containing 0.1% trifluoroacetic acid (TFA) in 3% of acetonitrile (ACN), and then loaded onto a C_{18} MicroSpin™ column (The Nest Group, USA). Buffer A consisting of 0.1% formic acid (FA) in 3% ACN was used to wash the column twice, from which the peptides were eluted from the column with 100 µL of buffer B (0.1% FA in 97% ACN), and then dried in a vacuum concentrator (Eppendorf, USA). 3 µL of sample from cleaned peptides being dissolved in 20 µL of buffer A was withdrawn and submitted onto a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Germany) coupled with a nano uHPLC 3000 system (Dinonex, UK) and operated at a flow rate of 0.3 µL/min. Peptides were separated using a C_{18} column with a 105 min gradient of buffer B as follows: 3% for 5 min, then ramped up to 10% for 5 min, 50% for 75 min, 90% for 1 min, then kept at 90% for 4 min before being ramped back to 3% buffer B for 1 min then maintained at 3% for 14 min. The MS was operated in positive mode with resolutions of full MS and $ddMS^2$ set at 60,000 and 15,000 respectively. For full MS and $ddMS^2$, AGC targets were set at 3×10^6 and 5×10^4, respectively, while maximum IT times were set at 100 and 20 ms, respectively. A full mass scan ranging from 375 to 1500 m/z was applied for MS whilst the mass scan of 100–1500 m/z was applied for $ddMS^2$ and default charge state of ion was set at 2.

All raw data files from MS analysis were submitted to MaxQuant (V 1.5.3.8) for protein identification against *Acetes* database (consisting of 34 entries) downloaded in October 2019 from Uniprot (https://www.uniprot.org/). No specific enzyme mode was used for searching with max missed cleaves of two while minimum peptide length of four and maximum peptide mass of 1000 Da were set; tolerances of 20 and 4.5 ppm were applied for MS and MS/MS, respectively. For identification of both peptides and proteins, a false discovery rate (FDR) was used. Only peptides observed from three biological replicates (with score > 50) were used and their MS/MS annotations were manually examined to ensure that good coverage of b- and y- series ions was observed.

Statistical Analysis

Data were presented as means ± standard deviations of triplicate determinations. Analysis of variance (one-way ANOVA) was performed on the data, and the significance was determined using Tukey method ($p < 0.05$). These analyses were performed using the Statgraphics Centurion software.

Results and Discussion

Effect of Hydrolysis Conditions on CBC of *Acetes proteolysate*

Enzymatic hydrolysis is an environment-friendly method to produce a large and diversified range of products for human and animal consumption, which could improve bioactivities, functional and sensory properties of a proteolysate and at the same time maintain its nutritional values [20]. However, hydrolysis enzyme should be properly chosen and used under a suitable condition.

Effect of Protease Type

In this study, among four proteases, Flavourzyme was the best choice for obtaining the *Acetes* proteolysate with the highest CBC of 1708.26 ± 14.52 µg Cu^{2+}/g protein. Neutrase, Corolase, Protamex and Alcalase proteolysates showed the second, third, fourth and fifth strongest CBC, respectively (Fig. 1a). Both exopeptidases and endopeptidases are included in Flavourzyme preparation, which has a broad substrate specificity, releasing more bioactive peptides [21]. Besides, it was reported that Flavourzyme usually produces the proteolysate with high concentration of hydrophobic amino acids, which would prevent the access of water molecules and stabilize the peptide-metal

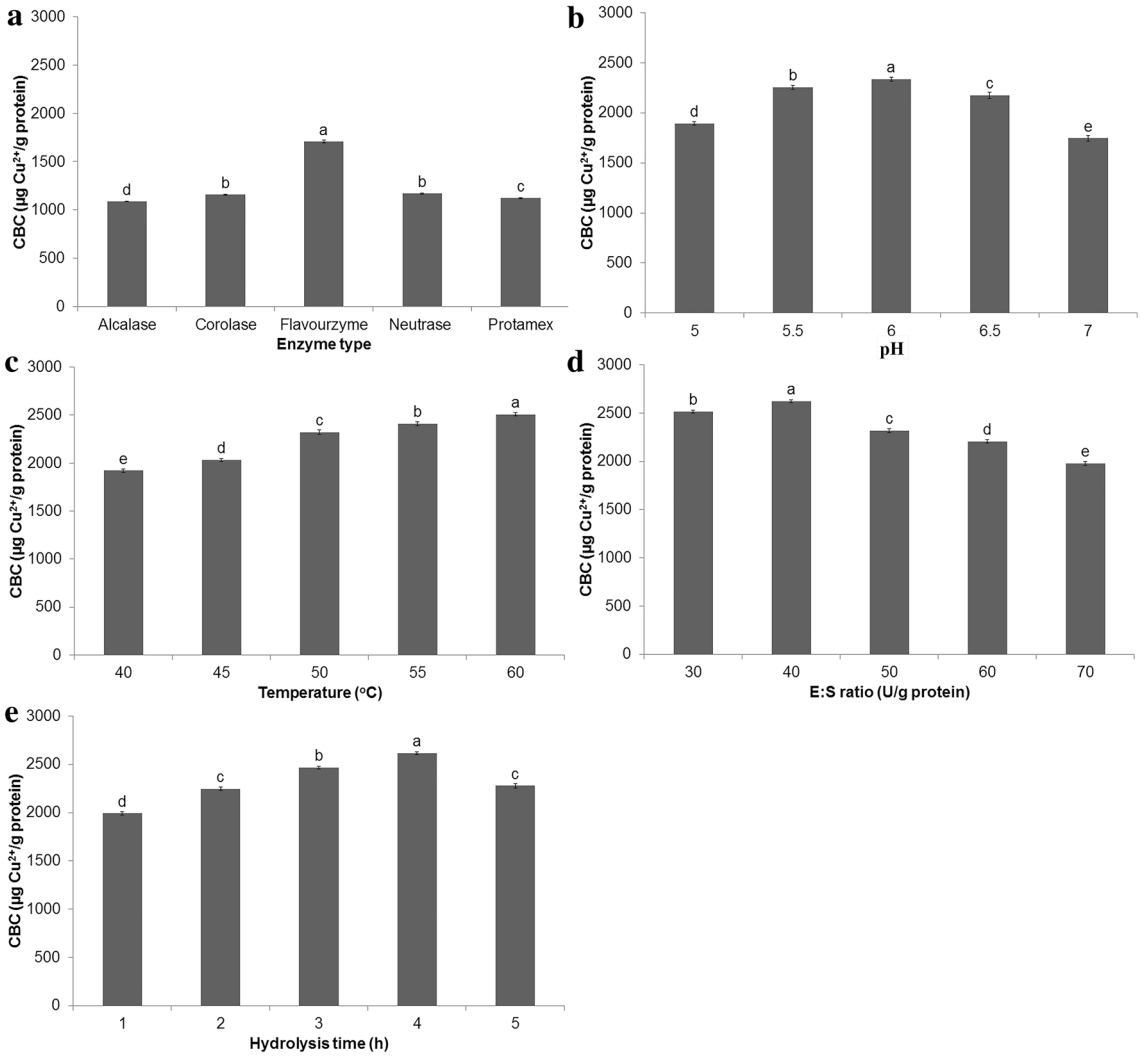


Fig. 1 Effect of enzyme type (**a**), temperature (**b**), pH (**c**), E:S ratio (**d**) and hydrolysis time (**e**) on CBC of the proteolysate. Bars with different letters indicate significant differences ($p < 0.05$)

ion complex [22, 23] by forming a hydrophobic fence. Thus, Flavourzyme was used as hydrolysis enzyme for further studies.

Effect of pH

As for the effect of pH value on CBC of proteolysate, the CBC achieve the highest value of 2337.38 ± 19.28 µg Cu^{2+}/g protein at pH 6, optimal pH (Fig. 1b). It can be clarified that the environmental pH, through changing the substrate and enzyme's charge distribution and conformation, affecting catalytic activity of enzyme and CBC of proteolysate, has a significant effect on their ionization ability [24]. At non-optimal pH, the amount of copper-binding peptides diminished due to low catalytic activity of enzyme. Hence, pH 6 was selected for further experiments.

Effect of Temperature

Concerning the effect of temperature on CBC of the proteolysate, CBC reached a peak of 2506.73 ± 18.71 µg Cu^{2+}/g protein at an optimal level of 60 °C (Fig. 1c). According to Arrhenius function, it could be stated that the enzyme-catalyzed reaction rate increases as elevating temperature [25], releasing high amount of bioactive peptides from intact protein, enhancing CBC of the proteolysate. Hence, hydrolysis temperature of 60 °C was chosen for further studies.

Effect of E:S Ratio

Figure 1d showed that the CBC of the *Acetes* proteolysate increased from 2519.90 ± 14.29 to 2623.31 ± 14.19 µgCu^{2+}/g protein as rising E:S ratio from 30 to 40 U/g protein. It can be elucidated that upon raising the E:S proportion, the

proteolysis rate elevates. As a result, a higher amount of small peptides is freed, improving bioactivity of the proteolysate. Nevertheless, when all substrates are converted to products as the hydrolysis speed is constant, the increase in E:S ratio would not enhance the CBC [26]. Moreover, Chen et al. [27] assumed that a greater amount of enzyme could damage copper-binding peptides created through early steps of hydrolysis, reducing bioactivity of proteolysate. So, the E:S ratio of 40 U/g protein was applied for further analyses.

Effect of Hydrolysis Time

As depicted at Fig. 1e, CBC reached a peak of 2617.04 ± 13.90 μgCu^{2+}/g protein at hydrolysis time of 4 h. Amino acid residues that express the capability of binding cupric ions [28], soaring the CBC of the proteolysate are generated from enzymatic hydrolysis. In contrast, feedback inhibition caused by hydrolysis products might lower the enzyme activity [29], decreasing CBC of the proteolysate. So, 4 h was selected as the hydrolysis time for additional tests.

Optimization of E:S Ratio and Hydrolysis Time for Maximizing CBC of the *Acetes proteolysate* Using RSM.

To set up an appropriate model, multiple regression analysis was performed on the experimental data and the final predictive function was achieved as follows:

$$Y = 2696.39 - 278.69X_1^2 - 111.01X_2^2 - 67.63X_1X_2 \quad (7)$$

where Y, X_1, X_2 are the CBC (μg Cu^{2+}/g protein), E:S ratio (U/g protein) and hydrolysis time (hour), respectively. The E:S ratio fluctuated from 30 to 50U/g protein and the hydrolysis time was varied from 3 to 5 h. The effect of each variable on the response was determined at 95% confidence level. Three terms of X_1^2, X_2^2 and X_1X_2 were estimated as significant effects whilst the linear coefficient of X_1 and X_2 were the insignificant factors. The regression model was significant ($p < 0.05$) with the coefficient of determination (R^2) of 0.976.

In order to determine optimal levels of the variables for the CBC, a three-dimensional surface plot (Fig. 2) was constructed according to the quadratic function (7). The optimal condition included the E/S ratio of 40.33 U/g protein and the hydrolysis time of 4.15 h with a predictive maximal response of 2699.72 μg Cu^{2+}/g protein, 3.16% higher than that of before optimization.

To verify the precision of the model, three independent replicates were carried out for measuring the CBC under the optimal condition. The average CBC was 2707.81 ± 8.7 μg Cu^{2+}/g protein, which was similar to the predicted value from quadratic function (7).

Amino Acid Composition

Table 1 showed the amino acid composition of the *Acetes* proteolysate. As for the nutritional value, the proteolysate could supply almost all 9 essential amino acids for human with the exception of Trp. The amino acid make-up is one of the most effective factors on bioactivity of the proteolysate. In particular, the CBC of the proteolysate was strengthened by high content of lipophilic amino acids including Ala, Leu, Ile, Val and Phe via generating hydrophobic fence, shielding one side of the complex plane from the attack of water molecules [23, 30, 31]. Besides, through donating free electrons in carboxyl oxygen atoms to cupric ion empty orbitals,

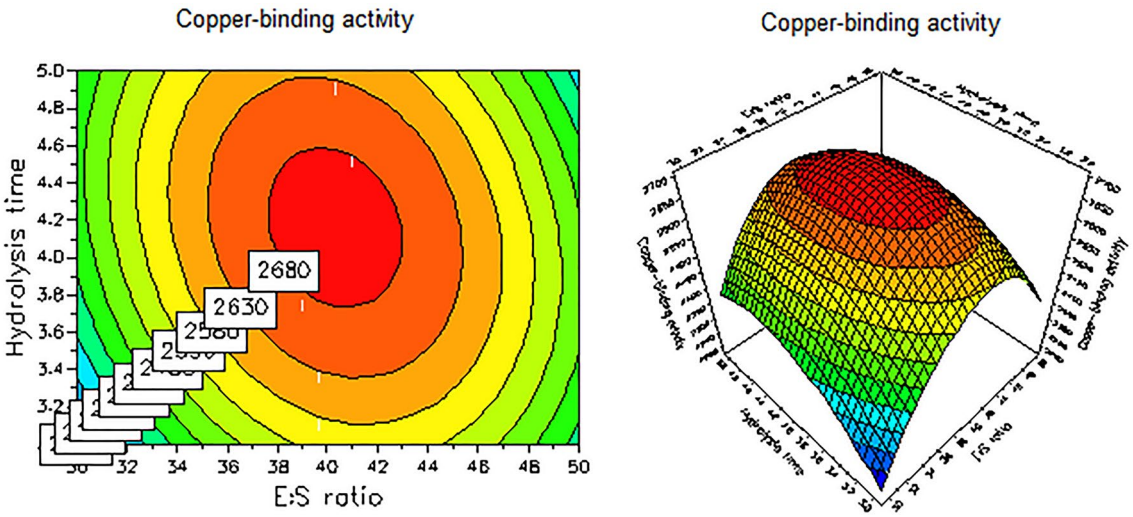


Fig. 2 Response surface chart for the CBC of the *Acetes* proteolysate

Table 1 Amino acid profile of the *Acetes* proteolysate

Essential amino acid	Content (mg/L)	Non-essential amino acid	Content (mg/L)
His	66	Arg	1479
Ile	529	Cys	168
Leu	1117	Gly	602
Lys	801	Tyr	487
Met	20	Ala	891
Phe	439	Asp	395
Thr	374	Glu	833
Val	677	Ser	436
Total	10,079	Pro	765

Glu and Asp affect the CBC of the proteolysate [32]. CBC of the proteolysate is also enhanced by the presence of sulfur-containing amino acids such as Cys and Met via free electrons of S atoms in their sulfhydryl and thioether group [32]. Moreover, N-imidazole of His and N-amide of Lys are considered as electron donors which supports the CBC of the proteolysate [33, 34].

Functional Properties of the Proteolysate

Solubility

Solvability is not only one of the most essential functional features of protein and proteolysate, but it also impacts on other functional properties consisting of emulsification and foaming capability [19]. By cleaving protein into smaller peptides with polar residues by enzymatic hydrolysis, they could form hydrogen bonds with water and enhance the solubility of a proteolysate [20]. Moreover, it was concluded by Moreover, Kristinsson, Rasco [35] that the solubility of a proteolysate was also enhanced by the balance between hydrophilic and hydrophobic force of peptides. Besides, the environmental pH also influences on the solvability of a proteolysate. It affects the charge of peptides, produces proteolysates with the lowest solubility at isoelectric point and the greatest solvability when the peptides charges maximally [35]. Great solvability is also important in other functional practices including emulsions, foams and gels, as solvable proteins assist homogeneous dispersibility of molecules in colloidal systems, improving the interfacial features [36]. As shown in Fig. 3a, *Acetes* proteolysate exerted great solubility with over 74% in the evaluated pH range and reached a peak of 94.63 ± 2.47% at pH 8, 1.2 and 3.2 times higher than those of Alcalase and Papain proteolysates from golden apple snail, respectively [19]. The size, the hydrophobic–hydrophilic balance and the charge of peptides formed through enzymatic hydrolysis account for the dissimilarity in the solvability [36]. The result suggests that the *Acetes* proteolysate might be used in food products to enhance their properties.

Heat Stability

Heat treatment is a general unit operation in food processing that can impact the functional features due to thermal sensitiveness of protein [18]. During heat handling, protein solubility is an efficient factor of the denaturation level of protein, assisting in controlling emulsification, foaming, extraction, and gelation processes [37]. Heat durability of proteolysate was described as its solubility after thermal processing at a fixed condition. In this study, the solvability of the proteolysate kept above 73% after both heat treatment modes (Fig. 3a), which was 1.1 times lower than that of grass carp proteolysate [18]. It was probable because protein aggregation during heat handling was caused by the poorer balance between hydrophilic and hydrophobic force [18]. In addition, variation in protein sources from dissimilar aquatic species affects that of heat stability of proteolysate. It was reported by Nurdiani et al. [38] that heat-stable proteins were detected in aquatic life form inhabiting in the water with high ambient temperature.

Foaming Property

The forming of foam of a proteolysate was affected by transportation, penetration and rearrangement of molecules at the air–water interface [39]. So as to express great foaming capacity (FC), protein molecules need to rapidly migrate to, unfold and reorganize at the air–water interface to reduce surface tension [40]. In addition, it was reported by Li et al. [18] that the lower the solvability of proteolysate was, the lower the migration speed of protein molecules to the air/water interface was, resulting in a lower FC. Besides, it was revealed by Naqash, Nazeer [41] that pH impacted on the FC of the proteolysate through the net charge of peptides in proteolysate. As demonstrated in Fig. 3b, the FC of the protein hydrolysate obtained the greatest value of 97.98 ± 9.08% at pH 7, which were higher than those of proteolysates from sole skin, squid skin and round scad [42]. These differences results from the great variation of longer chain peptides created through enzymatic hydrolysis, generating thicker and stronger films covering air bubbles [39].

To enhance the foaming stability (FS), protein molecules are required to create intermolecular polymers that embrace air bubbles, as intermolecular cohesiveness as well as elasticity of the protein polymers are essential for generation of stable foams [36]. The level of protein–protein interaction within the matrix that associates with the ionic repulsion of peptides determines the FS [41]. The proteolysate in this research exhibited the highest FS of 94.07 ± 9.02% at pH 7

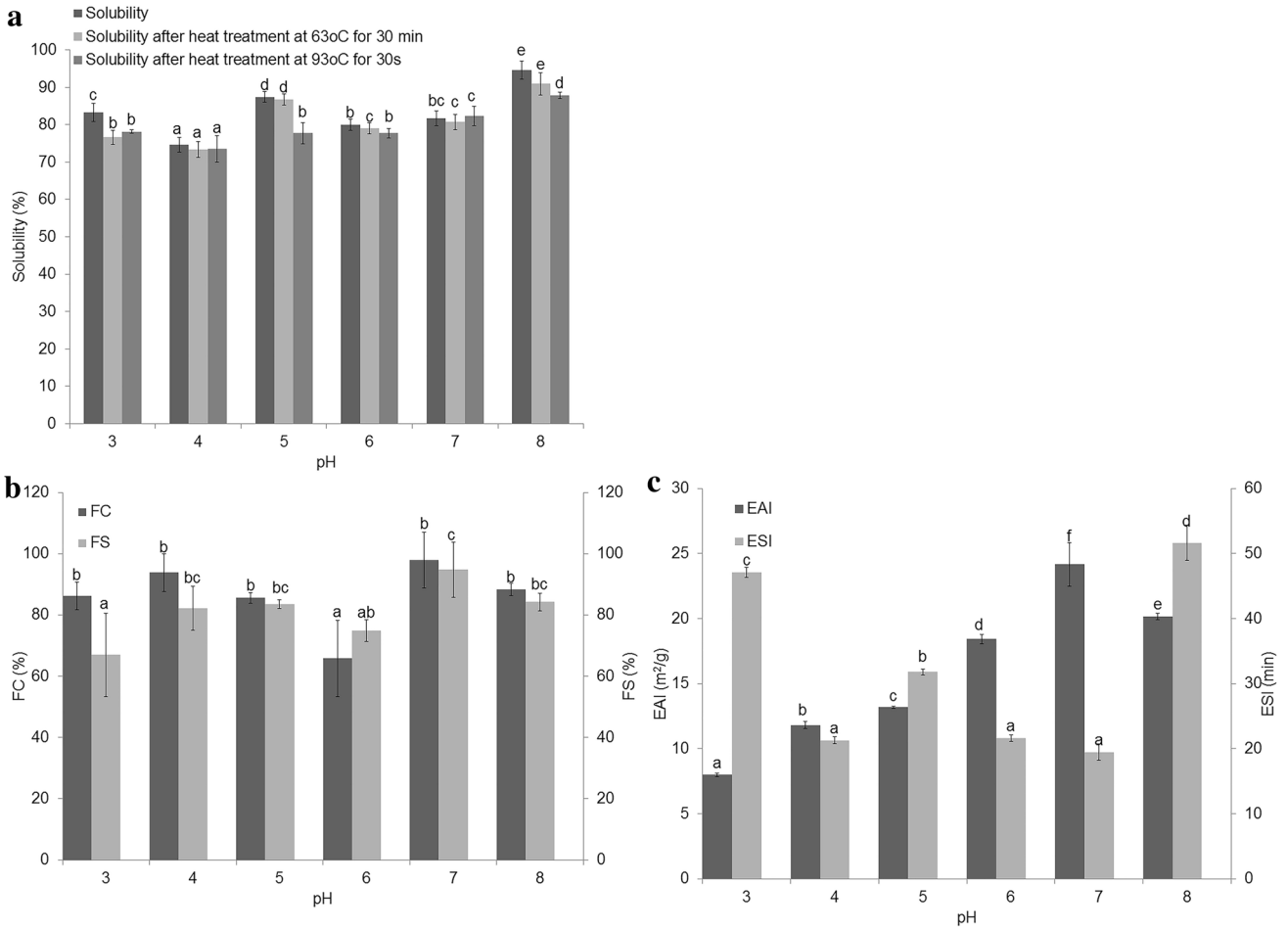


Fig. 3 Solubility and heat stability (**a**), foaming property (**b**) and emulsifying property (**c**) of *Acetes* proteolysate. The same color bars with different letters indicate significant differences ($p < 0.05$)

(Fig. 3b). Altogether, the *Acetes* proteolysate might be used for some products to improve their foaming capacity.

Emulsifying Property

The mechanism of emulsification of hydrolysate is the adsorption of peptides to the surface of freshly formed oil droplets through homogenization, forming a protective membrane to block their coalescence [20]. Protein solubility also affects emulsification via rapid migration to and adsorption at the oil–water interface of protein molecules [35]. As illustrated in Fig. 3c, at pH 8, the EAI and ESI of the proteolysate reached the peaks of 24.15 ± 1.66 m^2/g at pH 7 and 51.64 ± 2.71 min at pH 8, respectively. The ESI value was remarkably higher than that of round scad proteolysate [11]. Pacheco-Aguilar et al. [43] reported that great ESI was obtained at high pH as negatively charged peptides at alkaline pH results in the orientation of the peptides at the oil–water interface. Besides, it was also reported by Latorres et al. [44] that alkaline pH, by creating the repulsion of negative charges of peptides, benefits their better orientation at the oil–water interface, therefore, improving emulsifying feature of proteolysate. It was published that the emulsifying feature of proteolysate is directly involved in the surface property, molecular size and hydrophobicity of peptides [45]. Proteolysates with low DH often comprise larger peptides that assist their emulsifying feature via having good balance between hydrophilic and hydrophobic groups [35]. Moreover, Putra et al. [19] unveiled that the variation in emulsifying property of different hydrolysates results from their different amino acid compositions. Furthermore, the emulsifying stability is improved by the combination of greatly elastic protein layers being absorbed on the surfaces of oil droplets that were created by tertiary proteins and their steric effect via generating strong and thick films around oil droplets [39]. As a whole, the proteolysate in this study might be considered to apply in some food products to enhance their emulsion feature.

OHC and WHC

OHC, an amount of oil directly bound by protein, is an important factor that affects the taste of a product. The physical entrapment of oil is presumed to be the oil-holding mechanism of proteolysate, and the greater the bulk density of protein is, the higher the OHC is [35]. Besides, other factors impact on OHC of hydrolysate including degree of hydrolysis, the surface hydrophobicity of peptides, and enzyme–substrate specificity [45]. Proteolysates having high DH consist of a large amount of small peptides that is superior hydrophilicity, decreasing the interaction between peptide and lipid, reducing OHC [46]. Better hydrophobic peptides exhibit great OHC as they can form hydrophobic bonds to lipid, increasing durability of protein-lipid complex [37]. In this test, the OHC of the *Acetes* proteolysate obtained 5.47 ± 0.41 mL oil/g proteolysate powder, higher than those of grass carp proteolysate and Chinese sturgeon proteolysate 2.27 times and 2.32 times, respectively [10]. This difference results from the variation in hydrophilic polar side chains of peptides in these proteolysates [10]. The finding proposes that the proteolysate powder might be used for retarding phase separation as well as for enhancement of palatability and taste retention of some food products [45].

WHC describes the protein capacity of absorbing water and maintaining it against gravitational force within a protein matrix. It influences on texture and integrity of food products such as frozen fish fillets or meat [19]. The WHC of the *Acetes* proteolysate gained 2.52 ± 0.16 mL water/g proteolysate powder, which was 1.27 and 1.31 times higher than those of proteolysates from tilapia protein [47] Chinese sturgeon [10], respectively. Cumby et al. [46] revealed that amino acid profile and peptide size were important factors determining the WHC of a proteolysate. Lower-molecular-weight peptides are more effective in keeping water than larger-size peptides as smaller peptides are often more hydrophilic [46]. Furthermore, the rise in concentration of polar groups encompassing carboxyl and amino groups during enzymatic hydrolysis has substantial effect on the amount of adsorbed water [19]. The result reaches a conclusion that the *Acetes* proteolysate might be utilized as a moisture keeping agent for some food products.

Determine the Copper-Binding Capacity of Peptide Fractions

The copper-binding activity of the peptide fractions were presented in Fig. 4. The 1–3 kDa fraction had the highest copper-binding capacity (2754.73 ± 91.87 µg Cu^{2+}/ g protein) followed by the < 1 kDa, 10–30 kDa, 3–10 kDa fractions, and the lowest copper affinity belonged to the > 30 kDa fraction. The steric obstacle of larger peptides decreases their capacity to migrate to and chelate target metal ions, therefore results in low metal-chelating activity [39]. Intarasirisawat et al. [39] also published that small peptide was not capable of chelating metal ion. Hence, the peptide fraction of 1–3 kDa was used for peptide identification.

Identification of Copper-Binding Peptides

Two novel peptides (P1: DYMLPTDKYPH and P2: GYPFDADSVNFPVHG) were detected from the 1–3 kDa peptide fraction as shown in Table 2. All b- and y- series ions were also observed as illustrated in Fig. 5. Their positions in protein Sodium–potassium ATPase alpha-subunit and structures (drawn by chemSketch software) were demonstrated in

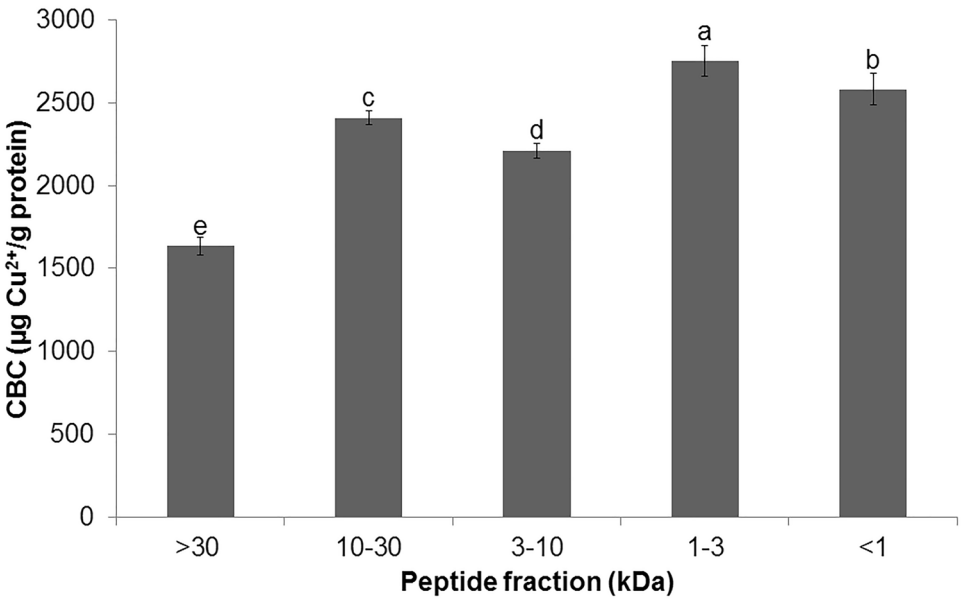


Fig. 4 CBC of peptide fractions. Bars with different letters indicate significant differences ($p < 0.05$)

Table 2 List of detected peptides found in *Acetes japonicus* proteolysate

Peptides	P1	P2
Mass (Da)	1378.6	1620.7
Protein/protein name	C7ADP5/ Sodium–potassium ATPase alpha-subunit (Fragment)	
Amino acid composition (%)[a]:		
Acidic	18.18	13.33
Basic	18.18	6.67
Neutral	27.27	40.00
Hydrophobic	36.36	40.00

[a]analyzed from https://www.biosyn.com/peptidepropertycalculator/peptidepropertycalculator.aspx

Figs. 6, 7, respectively. The de novo spatial structure of the detected peptides was depicted using PEP-FOLD 3 (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3). Details of 3-D model and interactions with Cu^{2+} of these peptides could be seen in Figs. 8, 9, 10.

From the sequences of two identified peptides in this study, His clearly contributed to CBC of the identified peptides probably due to the coordination between free electrons imidazole nitrogen atom and the empty orbital of cupric ion [34]. In addition, it was revealed by Matera-Witkiewicz et al. [48] that replacing Asp residue in a cooper-binding peptide sequence would remarkably reduce its copper affinity. Besides, Asn from the peptide P2, as a matter of fact, can stabilize Cu–N bond of the peptide complex and the combination of Asn and adjacent Phe forming a protective fence around copper ion, hence enhance the copper-binding capacity [23]. Gly from the sequence of peptide P2 possibly stabilize the peptide-Cu complex due to decrease strain in chelate ring owing to its flexibility [23]. Also, Pro_3 in the sequence of peptide P2 takes part in stabilization the Cu-peptide complex through increasing the propensity of a peptide chain to bend [23]. Moreover, the aromatic ring of Tyr_2 and Phe_4 in peptide P2 contributes to the stability of the peptide-Cu complex which bases on not only the ionic interaction but also the stacking between two rings [23]. Furthermore, Val contributes to the increase of CBC owing to its aliphatic side chain creating a hydrophobic barrier which hinders the attack on cooper ion of water molecule [30, 31]. Met from the P1 peptide is a residue increasing copper affinity of peptide via its thioether group [32].

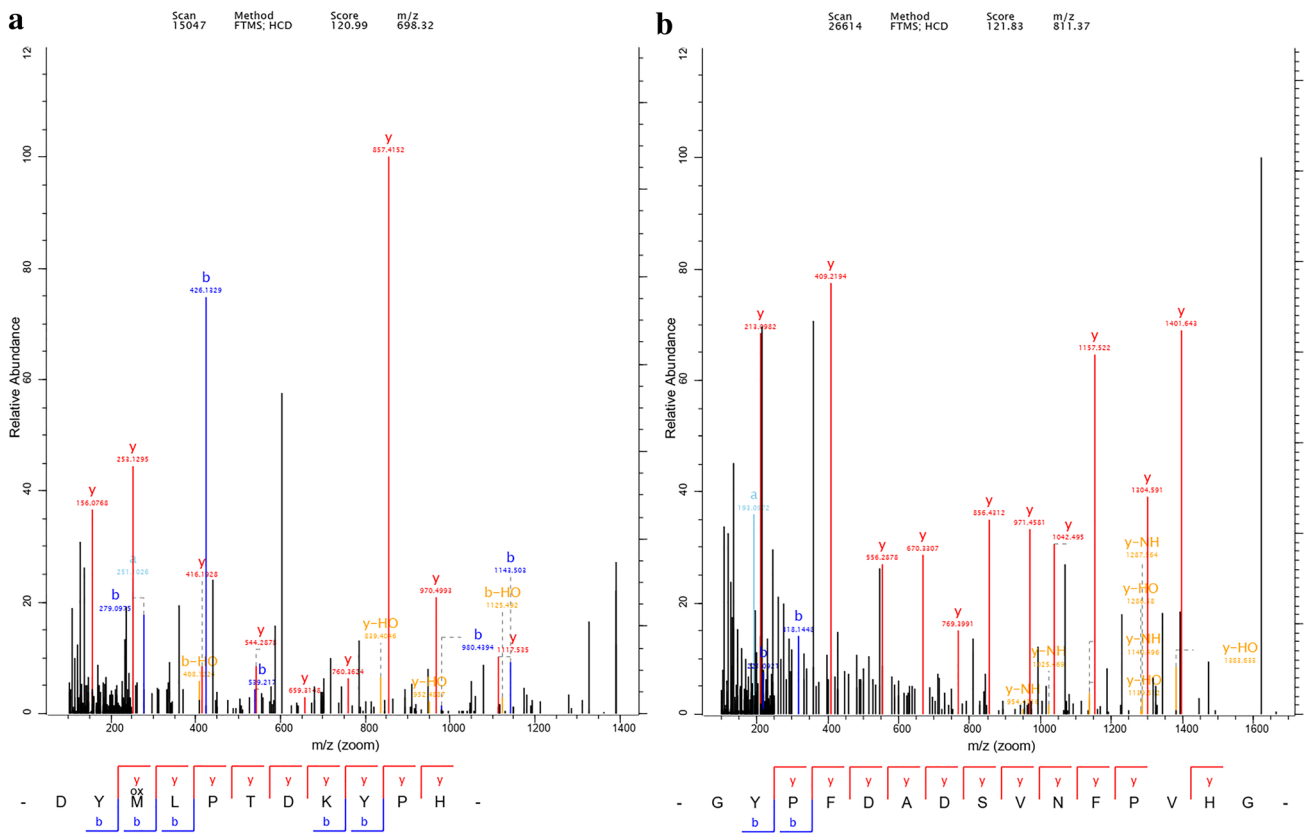


Fig. 5 MS/MS annotation of detected peptide of P1 (**a**) and P2 (**b**)

Fig. 6 Positions of peptide P1 and P2 in the Sodium–potassium ATPase alpha-subunit sequence

```
        10         20         30         40         50
TSEDQSGCQY DKTSQGWKAL SRIAALCNRA EFKVGQENTP ILKREVNGDA
        60         70         80         90        100
SEAALLKCVE LAIGDVKNWR ARNKKVCEIP FNSTNKYQVS IHETEDKNDP
       110        120        130        140        150
RYLVVMKGAP ERILERCSTI FINGEEKPLD EEMKEAFNNA YLELGGLGER
       160 P1     170        P2 180        190
VLGFCDYMLP TDKYPHGYPF DADSVNFPVH GLRFVGLMSM IDL
```

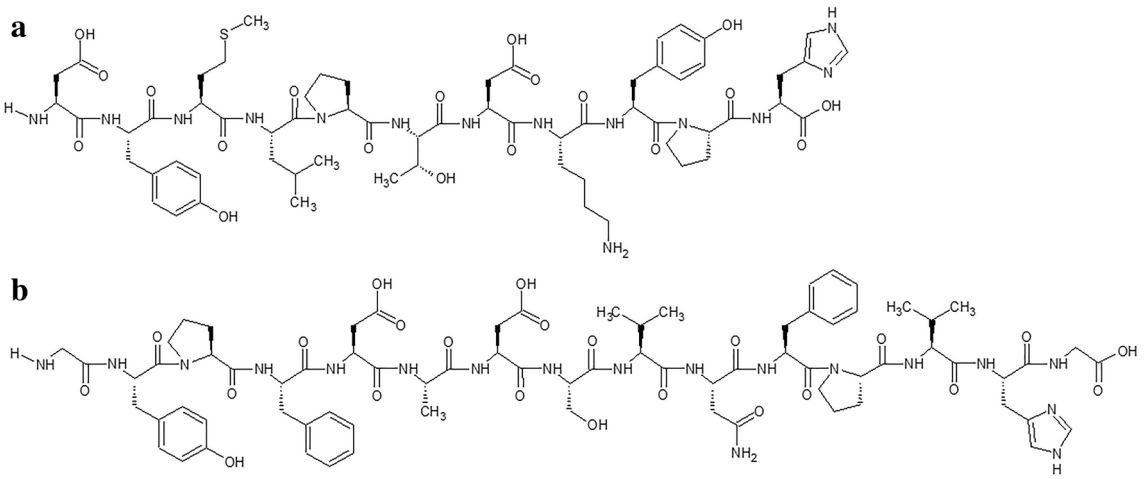


Fig. 7 The structure of peptide P1 (**a**) and P2 (**b**)

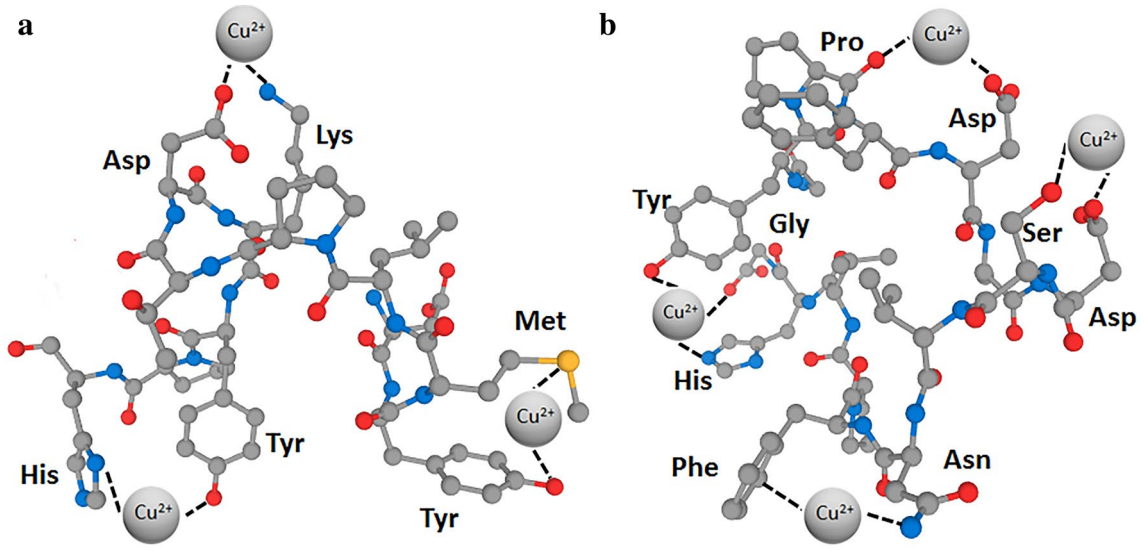


Fig. 8 The de novo 3-D structure of peptide P1 (**a**) and P2 (**b**) performed in balls and sticks, and uniform color in which grey, blue, red and yellow represent for C, N, O and S, respectively

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Fig. 9 Details of predicted 3-D model of the peptide P1

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ATOM     40  OD2 ASP     5      23.690  35.350  30.750  1.00  0.00           O
ATOM     41  C   ASP     5      28.130  34.500  31.050  1.00  0.00           C
ATOM     42  O   ASP     5      28.260  33.850  32.080  1.00  0.00           O
ATOM     43  N   ALA     6      29.110  35.220  30.510  1.00  0.00           N
ATOM     44  CA  ALA     6      30.560  35.010  30.770  1.00  0.00           C
ATOM     45  CB  ALA     6      31.350  35.510  29.560  1.00  0.00           C
ATOM     46  C   ALA     6      31.140  35.610  32.070  1.00  0.00           C
ATOM     47  O   ALA     6      32.340  35.580  32.310  1.00  0.00           O
ATOM     48  N   ASP     7      30.250  36.260  32.800  1.00  0.00           N
ATOM     49  CA  ASP     7      30.410  36.920  34.110  1.00  0.00           C
ATOM     50  CB  ASP     7      29.590  38.230  34.030  1.00  0.00           C
ATOM     51  CG  ASP     7      28.270  38.060  33.260  1.00  0.00           C
ATOM     52  OD1 ASP     7      27.300  37.560  33.880  1.00  0.00           O
ATOM     53  OD2 ASP     7      28.340  38.120  32.010  1.00  0.00           O
ATOM     54  C   ASP     7      29.910  36.030  35.270  1.00  0.00           C
ATOM     55  O   ASP     7      30.570  35.880  36.300  1.00  0.00           O
ATOM     56  N   SER     8      28.710  35.500  35.070  1.00  0.00           N
ATOM     57  CA  SER     8      28.060  34.470  35.920  1.00  0.00           C
ATOM     58  CB  SER     8      26.640  34.180  35.420  1.00  0.00           C
ATOM     59  OG  SER     8      25.800  35.330  35.570  1.00  0.00           O
ATOM     60  C   SER     8      28.810  33.130  36.030  1.00  0.00           C
ATOM     61  O   SER     8      28.680  32.430  37.030  1.00  0.00           O
ATOM     62  N   VAL     9      29.530  32.740  34.970  1.00  0.00           N
ATOM     63  CA  VAL     9      30.270  31.460  34.970  1.00  0.00           C
ATOM     64  CB  VAL     9      29.790  30.510  33.850  1.00  0.00           C
ATOM     65  CG1 VAL     9      28.360  30.040  34.110  1.00  0.00           C
ATOM     66  CG2 VAL     9      29.890  31.140  32.460  1.00  0.00           C
ATOM     67  C   VAL     9      31.810  31.550  34.980  1.00  0.00           C
ATOM     68  O   VAL     9      32.440  32.000  34.030  1.00  0.00           O
ATOM     69  N   ASN    10      32.370  30.820  35.940  1.00  0.00           N
ATOM     70  CA  ASN    10      33.840  30.590  36.120  1.00  0.00           C
ATOM     71  CB  ASN    10      34.090  29.710  37.350  1.00  0.00           C
ATOM     72  CG  ASN    10      33.640  30.330  38.680  1.00  0.00           C
ATOM     73  OD1 ASN    10      33.400  31.520  38.830  1.00  0.00           O
ATOM     74  ND2 ASN    10      33.480  29.530  39.690  1.00  0.00           N
ATOM     75  C   ASN    10      34.620  29.970  34.940  1.00  0.00           C
ATOM     76  O   ASN    10      35.850  29.910  34.970  1.00  0.00           O
ATOM     77  N   PHE    11      33.910  29.530  33.910  1.00  0.00           N
ATOM     78  CA  PHE    11      34.520  28.840  32.750  1.00  0.00           C
ATOM     79  CB  PHE    11      33.880  27.460  32.540  1.00  0.00           C
ATOM     80  CG  PHE    11      34.210  26.470  33.670  1.00  0.00           C
ATOM     81  CD1 PHE    11      33.300  26.290  34.710  1.00  0.00           C
ATOM     82  CD2 PHE    11      35.420  25.790  33.670  1.00  0.00           C
ATOM     83  CE1 PHE    11      33.600  25.420  35.750  1.00  0.00           C
ATOM     84  CE2 PHE    11      35.720  24.920  34.720  1.00  0.00           C
ATOM     85  CZ  PHE    11      34.820  24.740  35.760  1.00  0.00           C
ATOM     86  C   PHE    11      34.320  29.680  31.480  1.00  0.00           C
ATOM     87  O   PHE    11      33.190  30.110  31.220  1.00  0.00           O
ATOM     88  N   PRO    12      35.380  29.870  30.680  1.00  0.00           N
ATOM     89  CA  PRO    12      35.320  30.600  29.400  1.00  0.00           C
ATOM     90  CB  PRO    12      36.740  30.530  28.840  1.00  0.00           C
ATOM     91  CG  PRO    12      37.610  30.440  30.090  1.00  0.00           C
ATOM     92  CD  PRO    12      36.780  29.550  31.030  1.00  0.00           C
ATOM     93  C   PRO    12      34.330  29.930  28.450  1.00  0.00           C
ATOM     94  O   PRO    12      34.580  28.810  27.970  1.00  0.00           O
ATOM     95  N   VAL    13      33.140  30.520  28.350  1.00  0.00           N
ATOM     96  CA  VAL    13      32.060  30.070  27.440  1.00  0.00           C
ATOM     97  CB  VAL    13      30.700  30.690  27.780  1.00  0.00           C
ATOM     98  CG1 VAL    13      30.170  30.100  29.090  1.00  0.00           C
ATOM     99  CG2 VAL    13      30.730  32.220  27.860  1.00  0.00           C
ATOM    100  C   VAL    13      32.380  30.180  25.930  1.00  0.00           C
ATOM    101  O   VAL    13      31.740  30.870  25.140  1.00  0.00           O
ATOM    102  N   HIS    14      33.310  29.300  25.560  1.00  0.00           N
ATOM    103  CA  HIS    14      33.860  29.140  24.210  1.00  0.00           C
ATOM    104  CB  HIS    14      35.210  28.410  24.300  1.00  0.00           C
ATOM    105  CG  HIS    14      35.190  27.010  24.940  1.00  0.00           C
ATOM    106  ND1 HIS    14      35.270  26.750  26.250  1.00  0.00           N
ATOM    107  CD2 HIS    14      35.230  25.860  24.280  1.00  0.00           C
ATOM    108  CE1 HIS    14      35.350  25.430  26.400  1.00  0.00           C
ATOM    109  NE2 HIS    14      35.330  24.880  25.190  1.00  0.00           N
ATOM    110  C   HIS    14      32.900  28.520  23.180  1.00  0.00           C
ATOM    111  O   HIS    14      31.690  28.390  23.410  1.00  0.00           O
ATOM    112  N   GLY    15      33.390  28.580  21.950  1.00  0.00           N
ATOM    113  CA  GLY    15      32.770  27.930  20.790  1.00  0.00           C
ATOM    114  C   GLY    15      33.980  27.310  20.090  1.00  0.00           C
ATOM    115  O1  GLY    15      34.530  26.380  20.720  1.00  0.00           O
ATOM    116  O2  GLY    15      34.550  28.080  19.300  1.00  0.00           O
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Fig. 10 Details of predicted 3-D model of the peptide P2

Conclusion

Copper-binding peptide/proteolysate could be considered as a new potential product originated from *Acetes japonicus*, which is quite cheap and underestimated in terms of both utilization and economical evaluation. It was suggested that the *Acetes* proteolysate not only was an amino acid supplement, a taste and/or texture improving food additive but also a source of copper carrying protein/peptides, which could be further investigated for treatment of copper relating diseases. From the proteolysate, two novel copper affinity peptides were isolated with the amino acid sequences of DYMLPTDKYPH and GYPFDADSVNFPVHG, which could be promising alternative biocompounds for current copper supplements. For further applications, however, clinical experiments are required.

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