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Investigation of Calcium-Binding Capacity and Functional Properties of *Acetes Japonicus* Protein Hydrolysate

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Calcium-binding capacity (CaBC), amino acid profile and functional properties of proteolysate derived from *Acetes japonicus* were evaluated in this research. Firstly, effect of hydrolysis parameters including enzyme type, enzyme to substrate (E:S) ratio and hydrolysis time on the CaBC of the proteolysate was examined using single factor test method. Then, amino acid component and technological features involving solubility, heat stability, foaming and emulsifying property, oil holding capacity (OHC) and water holding capacity (WHC) of the protein hydrolysate were assessed. The result showed that the proteolysate exhibited the greatest CaBC of 261.61 ± 3.89 mg Ca^{2+}/g protein, 1.77 folds lower than that of casein phosphopeptide, under the hydrolysis condition encompassing Flavourzyme, pH 6.5, 50 °C, E:S ratio of 60 U/g protein and hydrolysis time of 5 hours. Solvability and heat stability of the proteolysate achieved over 75 % and over 70 % in the pH range from 5 to 8 The foaming capacity (FC), foaming stability (FS), emulsifying activity index (EAI) and emulsifying stability index (ESI) reached the peaks of 46.33 ± 0.57 %, 40.22 ± 0.38 %, 15.92 ± 0.07 m^2/g and 48.06 ± 1.59 min at pH 8. The OHC and WHC of the proteolysate accomplished 4.76 ± 0.41 ml oil/g proteolysate powder and 2.81 ± 0.08 ml water/g proteolysate powder. The proteolysate contained 8 out of 9 essential amino acids for human, comprising approximately 45 % of total amino acid content. On the whole, the proteolysate could be served for calcium enhancement which could protect human body from disorders caused by calcium shortage or as a functional protein preparation to improve food properties. The findings likewise increase the economic value for the small shrimp which is quite cheap in Vietnam.

1. Introduction

Of all the divalent metal ions, calcium is an essential mineral for human, which takes part in blood coagulation, neurotransmission, muscle contraction mitosis, and structural support of the skeleton (Jung et al., 2006). Calcium also affects several physiological functions such as cell proliferation, responses to hormones and neurotransmitters release (Huang et al., 2011). Rickets, osteoporosis, hypertension, obesity and kidney stone are known as consequences of calcium deficiency (Vo et al., 2018), which could be prevented by calcium-fortified products or calcium supplements in forms of inorganic calcium, organic calcium, or calcium-binding peptides. Inorganic calcium supplements, calcium carbonate or calcium chloride, may generate calcium phosphate precipitate during gastrointestinal digestion, lowering the absorption and bioavailability of calcium and trigger intestinal side effects such as flatulence and bloating (Chen et al., 2014). Organic calcium, calcium lactate and calcium gluconate, shows low therapeutic effects in clinical trial due to their low bioavailability and low proportion of calcium (Chen et al., 2014). Calcium-binding peptides could be a suitable alternative to enhance calcium bioavailability owing to the capability of remaining soluble form of calcium throughout changes in pH during digestion (Vo et al., 2018). The consumption of small peptides has many advantages which use little energy, increase migration velocity, and transporters being difficult to flood (Noman et al., 2018). Aquatic products and by-products have been considered to be a great protein source to produce calcium-binding peptides. These peptides were found in the proteolysate of tilapia scale (Chen et al., 2014), tilapia protein (Charoenphun et al., 2013) and shrimp by-product (Huang et al., 2011).
Acetes japonicus is one of the low economic valued aquatic species and has been exploited inefficiently. Although high protein content was found in the small shrimp, it is predominantly served as an ingredient in

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production of shrimp paste and dry shrimp which are low price products. To exploit the small shrimp more effectively in terms of nutritional value and/or bioactivity, Ca-binding proteolysate was discovered in the study. Ca-binding proteolysate/peptide can be used as a natural Ca-chelator besides its normal nutritional value to prevent Ca-deficiency relating diseases. So far, there have been few publications on Ca-chelating proteolysate/peptide from the small shrimp.

In this study, effects of hydrolysis condition on CaBC of *Acetes* proteolysate were investigated. Then, the amino acid profile of the proteolysate was analyzed. After that, functional features of the proteolysate consisting of solubility, heat stability, emulsifying and foaming property, water holding capacity (WHC) and oil holding capacity (OHC) were evaluated as well.

2. Materials and methods

2.1 Materials

Acetes japonicus was purchased from a local company in Vietnam with the moisture of approximately 12.3 ± 0.1 %. The compositions of the small shrimp comprising 72.8 ± 0.7 % crude protein, 4.3 ± 0.2 % crude lipid and 16.8 ± 0.2 % ash (on dry weight basis) were determined using the method of AOAC (2000).

Alcalase® 2.5L, Neutrase® 0.8L, Protamex®, Corolase® 7089 and Flavourzyme® 500MG were obtained from Novozymes (Kalundborg, Denmark) and AB enzymes (Darmstadt, Germany). Chemicals were acquired from Sigma-Aldrich (St. Louis, Missouri, United States) and Merck (Darmstadt, Germany). All reagents were in analytic quality. Double-distilled water was employed in tests.

2.2 Methods

The preparation of hydrolysates was performed according to the procedure of Vo et al. (2018) with a slight modification. First, the *Acetes* and water with the ratio of 1:8 (w/v) were mixed and then heated at 90 °C for 15 min to deactivate endogenous enzymes. Then enzyme preparation was added with the desired ratio after pH value of 6.5 was controlled employing 1 M NaOH or HCl solution. After the required hydrolysis time at 50 °C, the reaction was terminated by heating the hydrolysates for 15 min at 90 °C in order to deactivate the enzyme. The hydrolysates were then centrifuged to collect the supernatants. The obtained supernatants were freeze-dried and stored at -20 °C until used.

The effect of three factors including enzyme type (Alcalase® 2.5L, Neutrase® 0.8L, Protamex®, Corolase® 7089 and Flavourzyme® 500MG), E:S ratio (20, 30, 40, 50, 60 and 70 U/g protein) and hydrolysis time (2, 3, 4, 5 and 6 h) on the CaBC of the *Acetes* proteolysate were examined using the single factor test method which was performed by one factor varied with different levels while the others fixed.

Calcium–binding assay was carried out utilizing the approach of Jung et al. (2006). The solubility, heat stability, foaming and emulsifying property of the proteolysate were assessed in pH range from 3 to 8 using the method of Li et al. (2012). The procedure of Putra et al. (2018) was employed to evaluate the OHC and WHC of the *Acetes* proteolysate. The amino acid composition was analyzed by the method of AOAC (2000).

Data were presented as means ± standard variations of triplicate measurements. Analysis of variance (one-way ANOVA) was implemented on the data, and the significance was determined using Tukey method ($P < 0.05$). These assessments were run implementing the Statgraphics Centurion 18 application.

3. Results and discussion

3.1 Effect of hydrolysis condition on CaBC of the proteolysate

In this research, Flavourzyme proteolysate displayed the highest CaBC (209.7 ± 3.5 mg Ca^{2+}/g protein), followed by the Neutrase, Corolase, Alcalase and Protamex proteolysates (Figure 1a). Specificity of enzyme was known as a decisive factor of CaBC because enzymatic hydrolysis generated electron-donating peptides which were capable of coordinating to Ca^{2+} (Nourmohammadi et al., 2017). Among these proteases, Flavourzyme preparation is fungus-originated, containing both exopeptidases and endoproteases which has a wide range of substrate specificity, improving CaBC of the proteolysate (Jung et al., 2006). Cumby et al. (2008) published that Flavourzyme hydrolysis could preserve hydrophilic amino acid residues such as Asp, Lys and His, bringing up CaBC of proteolysate. Hence, Flavourzyme was used in subsequent experiments.

Figure 1b showed that the CaBC of the proteolysate was directly proportional to the E:S ratio in the E:S range of 20 - 60 U/g protein. It could be elucidated that when rising the E:S proportion, the proteolysis rate elevated, releasing small peptides, improving CaBC of the proteolysate. When the hydrolysis speed is constant, all substrates are converted to products, the increase in E:S ratio did not enhance the CaBC (Kang et al., 2018). Figure 1b showed a decrease in CaBC when augmenting the E:S ratio from 60 to 70 U/g protein. According to Chen et al. (2014), greater amount of enzyme could damage Ca-binding peptides formed during early steps of hydrolysis, lowering CaBC of proteolysate. So, the E:S ratio of 60 U/g protein was used for further analysis.

As depicted in Figure 1c, CaBC reached the peak of 261.61 ± 3.89 mg Ca^{2+}/g protein at the hydrolysis time of 5 h. Enzymatic hydrolysis created active amino acid residues expressing the capability of binding metal ions (Liu et al., 2010), increasing the CaBC of the proteolysate. As elongating the hydrolysis time, the deeper cleavage of the enzyme on generated peptides or the decrease in enzyme activity dropped the proteolysate's CaBC. Similar observation of the hydrolysis time-CaBC profile was found in the study of Charoenphun et al. (2013) for tilapia protein hydrolysate. Hence, 8 h was selected for additional tests.

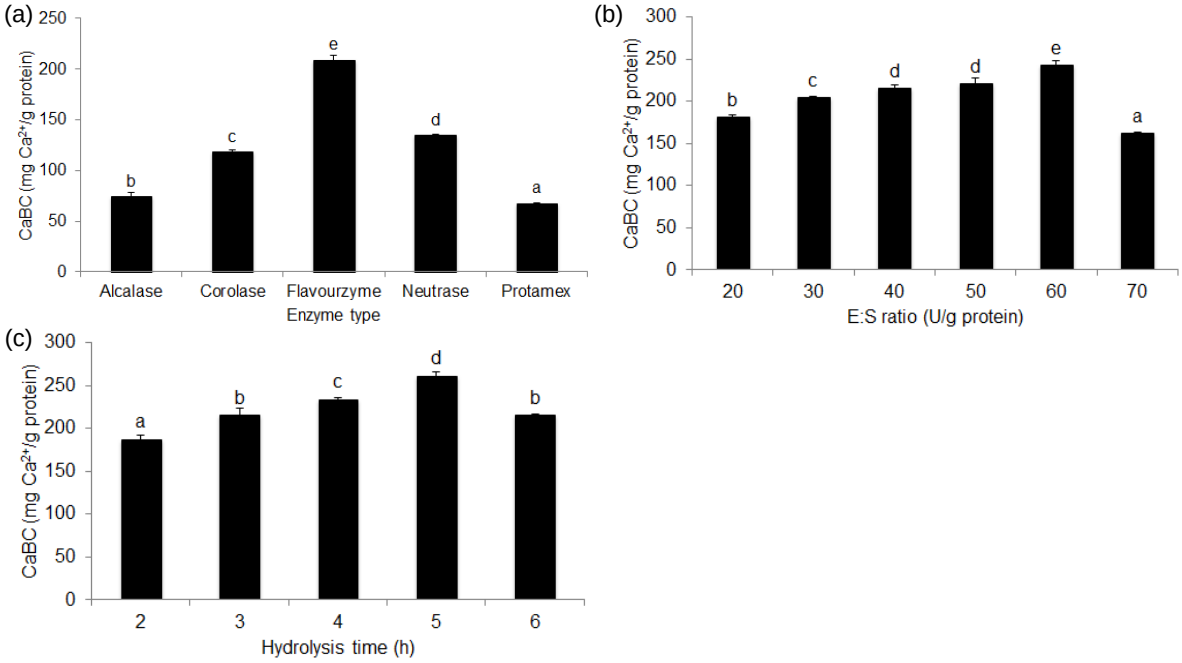

Figure 1: Effect of (a) enzyme type, (b) E:S ratio and (c) hydrolysis time on CaBC the proteolysate. Bars with different characters are significantly different (p<0.05).

3.2 Functional properties of the *Acetes* proteolysate

Solvability is not only one of the most essential functional features of protein and proteolysate, but it also impacts on other functional properties including emulsifying and foaming capacity (Putra et al., 2018). The environmental pH influences on the solvability of proteolysate via affecting the peptide charge, producing proteolysate exhibiting the lowest solubility at isoelectric point and the greatest solvability when being charged maximally (Li et al., 2013). As shown in Figure 2a, the *Acetes* proteolysate had solubility of over 75 % in the pH range from 5 to 8. The lowest solvability of the protelolyate was gained at pH 4 which may be approximate to the isoelectric point of the *Acetes* protein. The *Acetes* proteolysate owned higher solubility than that of proteolysate from golden apple snail (Putra et al., 2018). The size, the hydrophobic–hydrophilic balance and the charge of peptides formed through enzymatic hydrolysis account for the dissimilarity in the solvability (Ktari et al., 2012). These functional properties suggested that the *Acetes* proteolysate might be used for boosting features of some food products.

During heat handling, protein solvability is an efficient index of the denaturation level of protein, aiding in controlling emulsification, foaming, extraction, and gelation processes (Zayas, 1997). In this study, the solvability of the proteolysate was greater than 70 % after heat handling at 63 °C for 30 min in the pH range from 5 to 8 (Figure 2a). The heat durability of the proteolysate was quite lower than that of grass carp proteolysate (Li et al., 2012). It was probably because of the poorer balance between hydrophilic and hydrophobic force, causing protein aggregation during heat handling (Li et al., 2012). Besides, Nourmohammadi et al. (2017) unveiled that the increase in heat stability may be because of the hindrance of forming of the secondary structure by Pro, Ile and amino acids with identically charged side chains along with the creation of internal hydrophobic bonds in protein molecules.

Transportation, penetration and rearrangement of molecules at the air–water interface influence on the forming of foam (Intarasirisawat et al., 2012). So as to express great FC, protein molecules need to rapidly migrate to, unfold and rearrange at the air–water interface (Putra et al., 2018). Li et al. (2012) reported that the lower the solvability of proteolysate was, the slower the protein molecules migrated to the air/water interface,

as a result, lowering the FC. Besides, it was uncovered by Naqash and Nazeer (2013) that pH impacted on the FC of the proteolysate through the net charge of peptides. As demonstrated in Figure 2b, the FC of the proteolysate reached the maximum of 46.33 ± 0.57 % at pH 8 and the minimum of 37.00 ± 0.57 % at pH 4. These values were higher than those of proteolysates from sole skin, squid skin and round scad (Li et al., 2013). These variances were attributed to the amount variation of longer chain peptides formed via enzymatic hydrolysis which could generate a thicker and stronger film covering air bubbles (Intarasirisawat et al., 2012).

To boost the FS, protein molecules have to create intermolecular polymers that embrace air bubbles, thus, intermolecular cohesiveness as well as elasticity of the protein polymers are essential for generation of stable foams (Ktari et al., 2012). The level of protein-protein interaction within the matrix associating with ionic repulsion of peptides quantifies the FS (Naqash and Nazeer, 2013). The FS of the proteolysate exhibited over 35 % in the wide pH range from 3 to 8 (Figure 2b), which was higher than that of pink perch muscle proteolysate (Naqash and Nazeer, 2013). Taken together, the *Acetes* proteolysate might be applied for some products to enhance their foaming capacity.

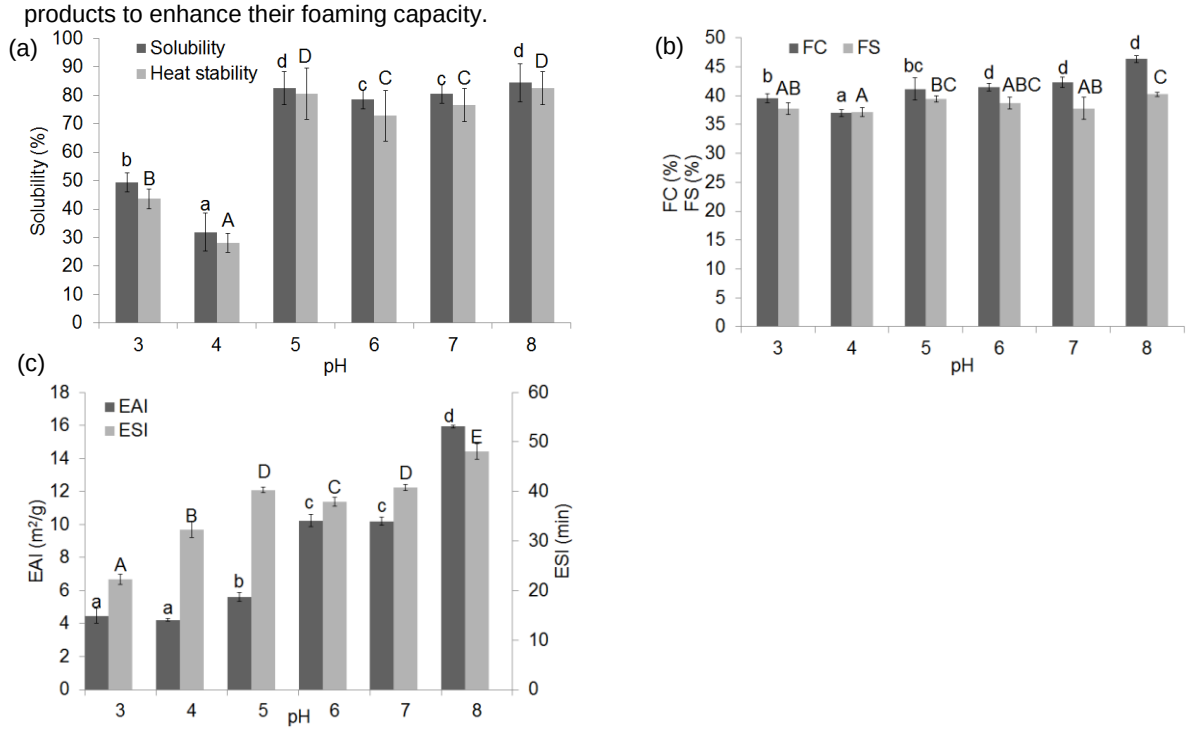

Figure 2: Solvability and thermal durability (a), foaming property (b) and emulsifying property (c) of the proteolysate. Bars with different characters are significantly different (p<0.05).

The adsorption of peptides to the surface of freshly formed oil droplets through homogenization, forming a protective membrane to block their coalescence is supposed to be the mechanism of emulsification of hydrolysate (Ktari et al., 2012). Protein solvability also had an effect on emulsification via rapid migration to and adsorption at the oil-water interface of protein molecules (Zayas, 1997). As illustrated in Figure 2c, at pH 8, the EAI and ESI of the proteolysate reached the peaks of 15.92 ± 0.07 m^2/g and 48.06 ± 1.59 min. These values were similar to those of round scad proteolysate (Thiansilakul et al., 2007). The research of Latorres et al. (2018) reported that alkaline pH created the repulsion of negative charges of peptides, benefiting their better orientation at the oil-water interface, therefore, improving emulsifying feature of proteolysate. Proteolysates possessing low DH often comprise more large peptides which assist to their emulsifying feature via having good balance between hydrophilic and hydrophobic groups (Li et al., 2012). Putra et al. (2018) unveiled that the variation in emulsifying property of different hydrolysates may be because of their different amino acid composition. The combination of greatly elastic protein layers being absorbed on the surfaces of oil droplets which were created by tertiary proteins and their steric effect improve the emulsifying stability via generating strong and thick films around oil droplets (Intarasirisawat et al., 2012). As a whole, the proteolysate in this study might be considered to apply in some food products to boost their emulsion feature.

OHC, the amount of oil directly bound by protein, is a critical parameter that affects the taste of the product. The physical entrapment of oil is assumed to be the oil-holding mechanism of proteolysate, and the greater the bulk density of protein is, the higher the OHC is (Zayas, 1997). Some other factors impact on OHC of

hydrolysate embracing degree of hydrolysis, the surface hydrophobicity of peptides, and enzyme-substrate specificity (Santos et al., 2011). High DH proteolysates consists of large amount of small peptides which is superior hydrophilicity, decreasing the interaction between peptide and lipid, reducing OHC (Cumby et al., 2008). Better hydrophobic peptides express high OHC because of their capacity of forming hydrophobic bonds to lipid, increasing durability of protein-lipid complex (Zayas, 1997). In this test, the OHC of the Flavourzyme proteolysate accomplished 4.76 ± 0.41 ml oil/g proteolysate powder, greater than that of the hydrolysates from Chinese sturgeon and grass carp (Noman et al., 2018). These differences were attributed to the variation in hydrophilic polar side chains of peptides in these proteolysates (Noman et al., 2018). The finding suggests that the proteolysate powder might be employed to retard phase separation as well as to improve palatability and taste retention of some food products (Santos et al., 2011).

WHC depicts the protein capacity of absorbing water and maintaining it against gravitational force within a protein matrix. It affects texture and integrity of food products such as frozen fish fillets or meat (Putra et al., 2018). The WHC of the *Acetes* proteolysate gained 2.80 ± 0.08 ml water/ g proteolysate powder, lower than that of proteolysates from golden apple snail (Putra et al., 2018). Cumby et al. (2008) uncovered that amino acid profile and peptide size were important parameters deciding the WHC of a proteolysate. Lower molecular weight peptides were more effectual in keeping water than larger size peptides since smaller peptides are often more hydrophilic (Cumby et al., 2008). The rise in amount of polar groups encompassing carboxyl and amino groups during enzymatic hydrolysis has considerable effect on the amount of adsorbed water (Putra et al., 2018). The result revealed the *Acetes* proteolysate might be used as a moisturizer for some food products.

3.3 Amino acid composition of the *Acetes* proteolysate

Table 1 presented the amino acid profile of the *Acetes* proteolysate, which has an effect on bioactivity and functional features of the proteolysate. Arg, the major amino acid in the proteolysate, could provide electron of N atom in its guanidino group for the formation of peptide-calcium bond. Besides, the hindrance the attack of water molecules of hydrophobic amino acids (Ile, Leu, Val, Phe, Ala and Pro), which comprised over 40 % total amino acid amount of the *Acetes* proteolysate, stabilized the peptide-calcium bond, strengthening the CaBC (Vo et al., 2018). Moreover, N-imidazole of His and N-amide of Lys as well as aromatic ring of Phe and Tyr were known as rich electron sources which supported the CaBC of the proteolysate (Vo et al., 2018). Likewise, the CaBC of the proteolysate was contributed by the existence of acidic amino acid such as Glu and Asp through donating their free electrons on O-carboxyl to calcium ions (Chen et al., 2014). Furthermore, Ser, Thr and Tyr played a vital function in the metal-binding activity of the proteolysate owing to their hydroxyl groups which served as coordinated combination sites for calcium ion (Jung et al., 2006). Furthermore, 8 out of 9 essential amino acids could be found in the *Acetes* proteolysate with the proportion of 45 % total amino acid content, indicating its potential to be used as an indispensable amino acid supply.

Table 1: Amino acid profile of the Acetes proteolysate

Essential amino acid	Content (mg/L)	Non-essential amino acid	Content (mg/L)
His	174	Arg	2,036
Ile	624	Cys	170
Leu	1,545	Gly	496
Lys	1,251	Tyr	506
Met	72	Ala	958
Phe	669	Asp	438
Thr	505	Glu	907
Val	752	Ser	809
Total	12,573	Pro	661

4. Conclusion

This study proposes an approach to utilize the small shrimp to obtain proteolysate having the CaBC and functional features. It may be used for human calcium absorption enhancement as a natural calcium binder, or to boost food functional properties. This original revelation points out that the proteolysate produces great worth for the low valued small shrimp with a new application trend. Nevertheless, further clinical studies on CaBC and organoleptic evaluation of proteolysate-fortified foods need extra investigation.

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