Influence of heat, proteases, and time on the hydrolysis of cricket protein

Siriprapa Jitmoleerat, Wasaporn Preteseille Chanput*

Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand

*Corresponding author. E-mail address: wasaporn.c@ku.th

Abstract

It has been increasing consumer interest in new protein sources, in which edible insects are one of the popular alternatives. Cricket is a protein-rich edible insect compared to others; therefore, it drives so much attention to protein hydrolysate production. This research aims to study the influence of processing factors on some parameters of cricket protein hydrolysate. The percentage of degree of hydrolysis (%DH) and molecular weight distribution of cricket protein were examined. The results showed that the %DH of cricket protein hydrolysate using Alcalase® was significantly higher than those of Neutrase® throughout the hydrolysis time up to 120 minutes. The %DH was increased in proportion to the hydrolysis time. Heat treatment at 80 ^{o}C for 30 minutes prior to enzymatic hydrolysis, did not show significant difference in terms of %DH, compared to the non-heat samples (except heat treated followed by Alcalase 90 minutes). The highest %DH of cricket protein obtained from Alcalase hydrolysis was observed at 120 minutes (73.88±2.71%). From the results of SDS-PAGE, most protein bands were absent after enzymatic hydrolysis at 60, 90, and 120 minutes. Most of the protein fragments were below 10-15 kDa.

Keywords: Alcalase, cricket, enzymatic hydrolysis, Neutrase, SDS-PAGE

Introduction

According to the Food and Agriculture Organization (FAO) of the United Nations, the global population will rise (United Nations, 2019). There may be a shortage of food, space for living, cultivating, and area to farm animals in the future. On top of that, there might be insufficient protein production capacity due to the huge amount of land required and greenhouse gas emissions. As a result, consumers have been increasingly turning to alternative proteins. Edible insects provide a nutritious alternative to animal protein for human consumption. It is a trendy nowadays due to its high protein content, fast reproductive rate, little natural resources required for rearing, and few lower greenhouse gas emissions than livestock (Kim et al., 2019). In Southeast Asia, insects have been consumed from the past up to nowadays. Insect consumption is currently popular and in high demand in the market. According to the report that represents the benefit of insects (high in protein, fiber, and vitamin B12, as well as being biologically active), the trading value of insects has increased by 20% per year over the last five years. The global edible insect business is currently worth 12,800 million baht (Van Huis et al., 2013).

House cricket (*Acheta domesticus*) is native to South-West Asia and has a protein content of 64.4–70.8% dry weight (Brogan et al., 2021). Although cricket is rich in protein, consumers hesitate to consume them due to their unappealing appearance. Protein hydrolysate serves several functions, including increasing solubility, water-holding capacity, emulsifying ability, and foam formation ability. Furthermore, protein hydrolysate is also beneficial to human health. It can control the operation of various systems in the human body and reduce the risk of developing certain diseases. It was discovered that insect protein hydrolysate possesses a wide range of biological activities, including antioxidant properties, anti-hypertensive properties, anti-diabetic properties, anti-inflammatory properties, and properties to reduce the allergenicity (Hall et al., 2017; Hall et al., 2018). Therefore, the aim of this study was to investigate the effect of heat treatment, types of proteases and hydrolysis time on cricket protein hydrolysate preparation using commercial enzymatic hydrolysis by determination of % degree of hydrolysis and molecular weight distribution prior to further study on, for instance, biological properties as well as reduction of allergenicity.

Materials and Methods

Materials

Unless specified, chemicals used were of reagent grade and obtained from suppliers: Sodium hydroxide, o-phthalaldehyde, 2-mercaptoethanol, brilliant blue G250, bromophenol blue, and ammonium persulphate were purchased from Sigma Aldrich (St. Louis, MO, USA). Hydrochloric acid 37% was obtained from QRëC™ (Quality Reagent Chemical, New Zealand). Sodium dodecyl sulphate, sodium tetraborate and tris (hydroxymethyl)methylamine were purchased from Ajax finechem Pty Ltd (New South Wales, Australia).

Methanol and acetic acid (glacial) were bought from MERCK™ (Darmstadt, Germany). Glycine and glycerol were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Whole house crickets (*Acheta domesticus*) were purchased from Global Bugs Asia (Bangkok, Thailand). Alcalase® (Protease from *Bacillus licheniformis*, ≥ 2.4 U/g) and Neutrase® (Protease from *Bacillus amyloliquefaciens*, ≥ 0.8 U/g) were used in the hydrolysis reaction and obtained from Sigma Aldrich (St. Louis, MO, USA).

Preparation of cricket protein hydrolysate using proteases

The frozen whole crickets were stored at 4 °C overnight to thaw. The following morning, samples were washed twice with tap water, then boiled in distilled water at a ratio of 1:2 (w/v) for 5 minutes. Then, drained water and blended with a blender in distilled water at a ratio of 1:2 (w/v) for 2 minutes until homogenous. The blended cricket was divided into 3 methods for protein hydrolysate preparation, (1) no heat and no enzyme addition; (2) no heat and enzymatic hydrolysis; and (3) heat at 80 °C for 30 minutes followed by enzymatic hydrolysis. Enzymatic hydrolysis was performed using 3% (E/S) at pH 8 for alcalase and pH 7 for neutrase. Then, the samples were incubated in a shaking water bath and subjected to constant shaking at 60 °C and 55 °C, respectively, for 15, 30, 60, and 90 minutes. Samples were boiled at 100 °C for 5 minutes to stop the reaction and centrifuged at 7,000 rpm at 4 °C for 15 minutes. The supernatant was then analyzed for %degree of hydrolysis and molecular weight distribution.

%Degree of hydrolysis (%DH)

According to the method of Alu'datt et al. (2012), 50 µL of clear protein hydrolysate was mixed with 2 mL of o-phthalaldehyde reagent, incubated for 2 minutes, and measured at wavelength 340 nm. The %DH was calculated using the following Equation 1:

$$\%DH = \frac{MW \times Abs\ (340nm)}{(\delta \times \varepsilon \times \rho)} \times 100 \qquad (1)$$

MW represents the mean of molecular weight of amino acids in crickets (136.65), δ is the dilution factor, ε is the constant (6000 m^{-1} cm^{-1}) and ρ is the soluble protein (mg/mL).

SDS-PAGE

The method of Hall et al. (2018) was used with a slight modification. A 20 µg of cricket protein hydrolysates with %DH greater than 50% were mixed with the loading dye in a ratio of 1:1 and heated to 95 °C for 5 minutes. Then, the protein marker and samples were loaded into gel slots using 12% separating gel and

4% stacking gel. The gel was run at 100 volts for 2 hours. After that, the gel was stained by soaking in the staining solution for 1 hour, then washed it off with the de-staining solution until the gel was transparently clear.

Statistical analysis

Data were analyzed using one-way ANOVA ($p \leq 0.05$) and differentiate the means by Duncan's multiple range test (DMRT). Value $p \leq 0.05$ was considered to be statistically significant. All statistical analyses were performed using the SPSS (Statistics Package for the Social Sciences) program version 22.

Result and Discussion

%Degree of hydrolysis (%DH)

Cricket protein hydrolysate is derived from enzymatic or chemical hydrolysis of cricket protein. Hydrolysis causes protein molecules to be cleaved into polypeptides or short-chain peptides, which can improve the protein's functional and biological properties (Hall et al., 2018). The digestion can be speculated using %degree of hydrolysis (%DH) which is an indicator of peptide bond cleavage and the breakdown of the structured proteins into smaller peptides. In this experiment, blended whole crickets were heated and hydrolysed by two different proteases at different time points.

Fig. 1 shows that %DH can be affected by the types of enzymes, hydrolysis time, and temperature. The control (no heat + no enzyme) at 15, 30, 60, 90, and 120 minutes of digesting time had the lowest %DH (less than 12%). Addition of alcalase and neutrase could significantly elevate %DH throughout the digesting time 15-120 minutes compared to the control ($p \leq 0.05$). Surprisingly, the samples treated by heat treatment prior to enzymatic hydrolysis (heat + enzyme) was not significantly different from the non-heated sample (no heat + enzyme), except alcalase digestion at 90 minutes. It can be explained that heating cricket powder at 80 ^{o}C might not be sufficient to loosen the complex cricket protein structure which is bonded with chitin, fat and phenolic compounds (Choi et al., 2017; Tan et al., 2021). Higher temperature assisted with pressure could be worth for the future trial. In both enzymes, particularly alcalase, when hydrolysis time was increased, the %DH was also significantly higher due to the enzymatic reaction. Obviously, alcalase could better cleave cricket protein than neutrase (Fig. 1) ($p \leq 0.05$). The %protein yield of alcalase and nutrase was observed maximumly 17% and 7% at 90 minutes digesting time, respectively. Even if both alcalase and neutrase are endopeptidases and have been widely used to produce protein hydrolysate (Aspevik et al., 2016), they have different principles of function. Alcalase is a serine protease that attacks the peptide bond through a serine residue at the active site. However, neutrase is a metalloprotease that randomly hydrolyses internal peptide bonds with zinc metal as a co-catalyst (Nielsen, 2010). Although the zinc content of house cricket has been reported to be 16-16.9

mg/100 g dry weight, this this may not be sufficient (Koseckova et al.,2022). Another reason could be that the enzyme unit of alcalase is 3 times higher than nutrase, which can contribute to more efficiently protein cleavage.

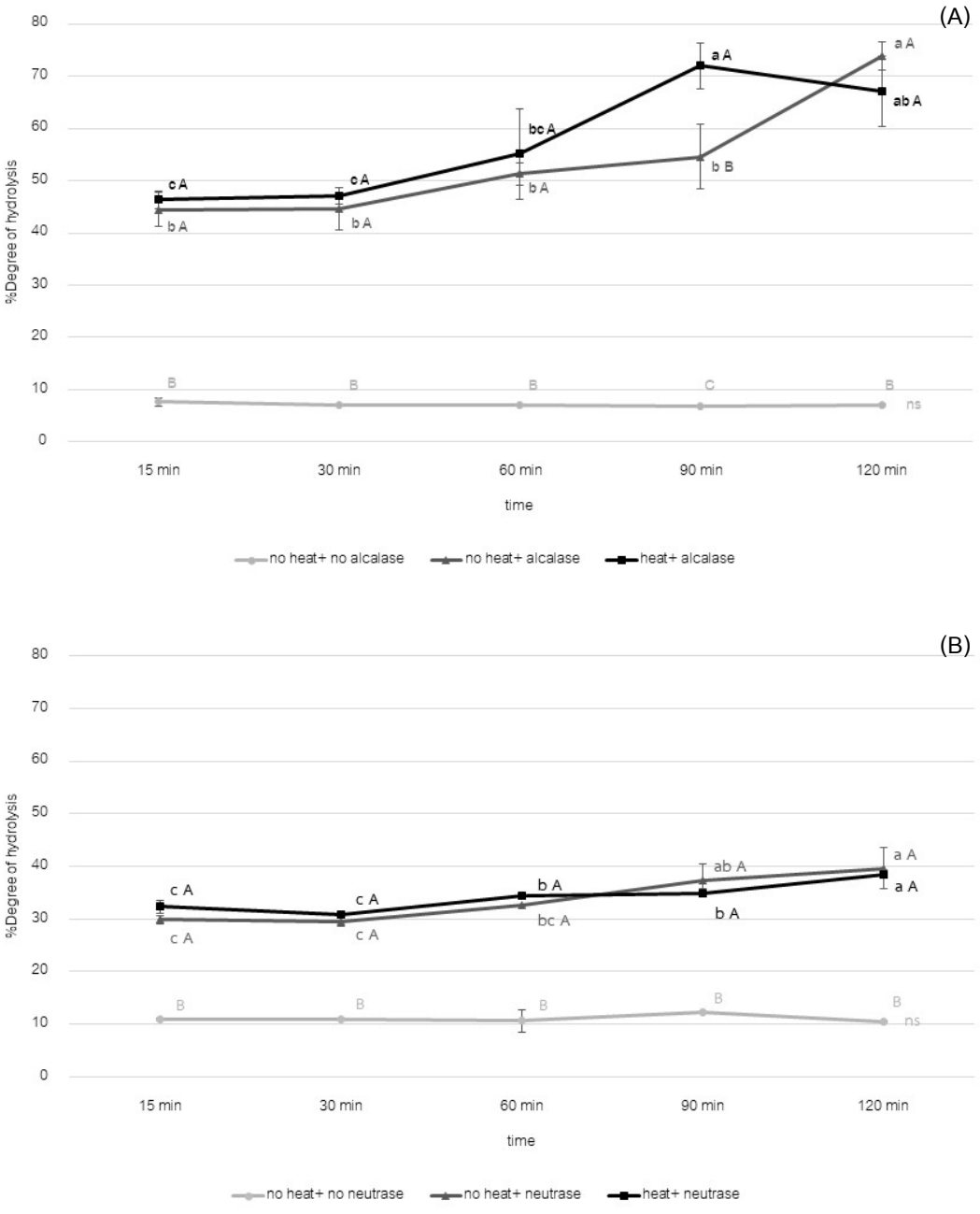

Fig. 1 Effect of heat treatment and enzymatic hydrolysis using (A) alcalase and (B) neutrase on %degree of hydrolysis (%DH). Values are means ± standard deviation of replications. The degree of hydrolysis measurement was performed using two independent x two technical replicates of each treatment (n = 4).

[A-E]Different superscripts in vertical values indicate significantly different ($p \leq 0.05$)

[a-b]Different superscripts in horizontal values indicate significantly different ($p \leq 0.05$)

As indicated by Hall et al. (2018) that protein hydrolysate with %DH > 50 showed great biological properties and lower allergenicity. Therefore, in this study, samples with %DH > 50 were selected to study protein molecular weight profile. These samples are heat + alcalase at 60, 90, and 120 minutes, no heat + alcalase at 60, 90, and 120 minutes. Among all selected samples, the highest %DH was observed at no heat+ alcalase for 120 minutes (73.88±2.71^{a}), heat + alcalase for 90 minutes (71.96±4.33^{ab}), and 120 minutes (67.18±6.77^{b}), respectively.

SDS-PAGE.

SDS-PAGE protein profile showed several bands ranging from 10 to 250 kDa in the control sample (no heat + no alcalase) at the digestion time of 60, 90, and 120 minutes (Fig. 2), indicating no protein digestion occurred. Most protein bands were absent in the samples after alcalase hydrolysis, in which mostly protein molecular weight below 10-15 kDa could be visible due to successful enzymatic hydrolysis. In accordance with Hall and Liceaga (2020), after cricket protein was hydrolysed with alcalase, the size of protein was observed below 6 kDa. The main allergenic protein in cricket is tropomyosin with the size of 37 kDa (Hall and Liceaga, 2021) The tropomyosin band is evidence visible in all control treatments; no heat+no enzyme at 60, 90, and 120 minutes of digesting time. Alcalase hydrolysis after 60 minutes could make the tropomyosin band became invisible, which can be implied the possible reduction of allergenicity. However, further allergenicity test, for example, ELISA or western blot should be further done to obtain the definite conclusion.

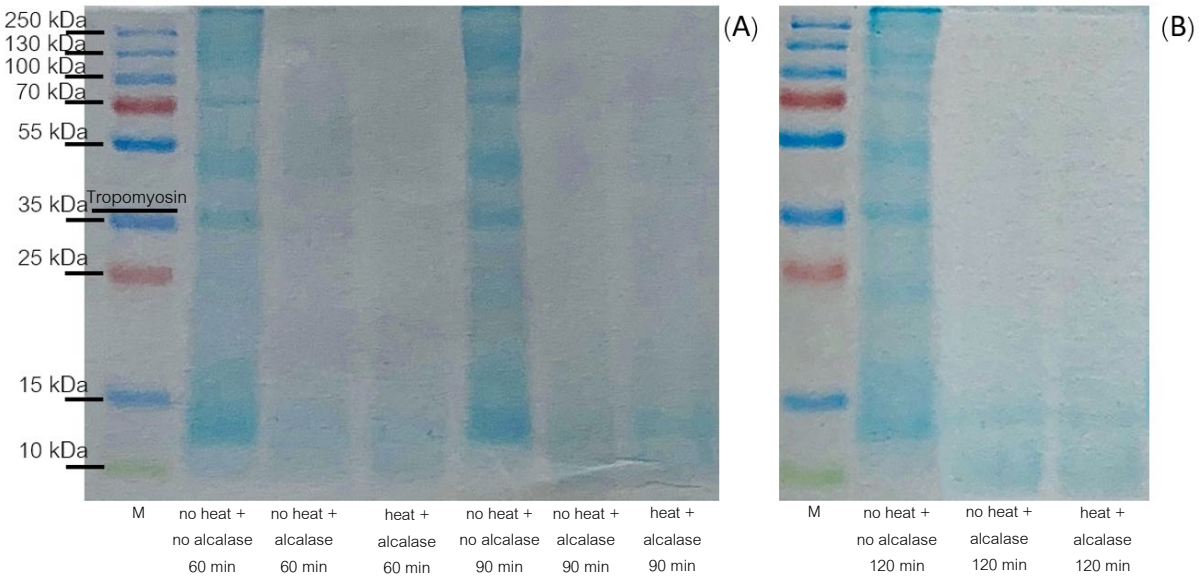

Fig. 2 Molecular weight distribution of cricket protein hydrolysate including controls (no heat + no alcalase) and alcalase treatments for (A) 60, 90, and (B) 120 minutes. (M; Molecular weight marker)

Conflict of Interest

The authors declare that there are no conflicts of interest.

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