Biological Science

Enzymatic Hydrolysis of Cricket (Gryllodes sigillatus) Protein: Influence of Alcalase and Neutrase Enzyme on Functional Properties of Recovered Protein

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Abstract

This study investigated the effects of enzyme concentration and hydrolysis time on improving the functional properties of cricket protein. The goal was to develop alternative sustainable protein from insects. Cricket protein was hydrolyzed using two proteolytic enzymes: Neutrase 0.8L and Alcalase 2.4L. We tested enzyme concentrations of 1.5 and 3.0%(w/w), and exposure times of 45 and 90 min. For both enzymes, the percentage yield increased as the enzyme concentration and hydrolysis time increased. The protein content of Neutrase-produced hydrolysate was higher (>80%) than that of Alcalase-produced hydrolysate (>70%). The highest degree of hydrolysis (DH) was 38.8% for Alcalase (at 3.0%, 90 min) and 70.2% for Neutrase (at 3.0%, 90 min). Both were higher than control (17.8% at 90 min). Hydrolysis by both enzymes improved the solubility of cricket protein hydrolysates over a range of pH values, exhibiting >56% soluble protein at pH 3 and >90% at pH 7 and 8. The hydrolysates had good foaming capacity (36.56% and 28.23%) and emulsification properties (44.03% and 44.47%). Emulsion stability and foaming stability were greatly high, exceeding 96% and 97.96%. These improvements in the functional properties of cricket protein hydrolysates give them potential uses in a range of food products.

Keyword: cricket; protein; hydrolysis; functional properties

1. Introduction

The Food and Agriculture Organization of the United Nations reports (FAO) estimates the world population to be 9 billion in 2050 (Huis et al., 2013). This will result in a growth in the demand for meals, with a rise of 70% by 2050. Protein consumption is increasing every year, and demand is expected to grow continuously, necessitating the search for alternative sources. New strategies for food production are needed. Solutions include reducing meat consumption and changing diets towards foods requiring less agricultural land. Insects are considered a viable source of protein because they are widely

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integrated into food cultures around the globe, have superior nutritional quality, and are more sustainable than traditional protein sources. Insects are sources of proteins, lipids, carbohydrates, certain vitamins, and minerals such as calcium, iron, or zinc. The energy content of insects is on average comparable to that of meat (on a fresh weight basis), except for pork, which has a particularly high-fat content (Sirimungkararat et al., 2008). Insects are also a good source of essential amino acids and polyunsaturated fatty acids (Rumpold and Schlüter, 2013). Currently, many commercial food products are enriched with protein from beans. However, insect protein is superior in terms of nutritional properties, containing all the essential amino acids. Insects are richer in protein content (40-61%) than legumes (23.5% protein), lentils (26.7%), or soybean (41.1%) (Blásquez et al., 2012). Studies show that, in typical Western societies, consumers find insects more appealing when used as an ingredient in foods with familiar flavors and textures (Tan et al., 2016). There is a higher readiness to eat processed insect products than whole insects (Hartmann et al., 2015). This suggests a potential strategy for food development, especially in regions that are unwilling to adopt novel eating habits due to a lack of sensory appeal.

Enzymatic hydrolysis is a processing method that is known to improve the functional properties of food proteins. Enzymatic hydrolysis converts protein into peptides of various sizes and free amino acids. It comprises of the nucleophilic attack of a water molecule on the covalent peptide bond between the amino and carboxyl groups of 2 adjacent amino acids, catalyzed by a peptidase (Wouters et al., 2016). The main effects of enzymatic hydrolysis on protein structure, which are a decrease of molecular mass of the proteins and an increase in both the number of ionizable groups and the accessibility of hydrophobic regions in the protein structure (Panyam and Kilara, 1996). These effects impact the functional properties of the resulting protein hydrolysates. These improve functional properties by enhancing solubility and associated surface properties related to the feasibility of the formation and stabilization of air-water (foams) and oil-water (emulsions) interfaces (Polanco-lugo et al., 2014), which are important properties in food product applications.

Many studies have shown that edible insects are a good source of protein, minerals, amino acids, and fatty acids. However, little consideration has been given to the functional properties, or the influence of hydrolytic enzymes on the functional properties of insect protein. This study investigated the improvement of the functional properties, including protein solubility, emulsifying, and foaming properties of cricket protein by using enzyme hydrolysis.

2. Methods

2.1 Materials

Raw frozen crickets (*Gryllodes sigillatus*) were purchased from Varin Cricket Farm (Lam Luk Ka, Pathum Thani, Thailand). Enzymes

used were Alcalase 2.4L (*Bacillus licheniformis*, □ 2.4 U/g) and Neutrase 0.8L (*Bacillus amyloliquefaciens,* □ 0.8 U/g) from Brenntag (Brenntag, Bangkok, Thailand). All other chemicals and reagents used in the experiments were of analytical grade and high purity.

Table 1 Degree of hydrolysis, crude protein content, and yield of cricket protein hydrolysates using Alcalase and Neutrase, compared with control.

Enzyme	Trials	E/S (%)	Time (min)	DH (%)	Crude protein (%)	Yield (%)
Alcalase	A1	1.5	45	37.8 ± 0.5^{d}	71.1 ±0.4^{d}	9.7 ± 0.1^{cd}
	A2	1.5	90	38.4 ± 0.3^{d}	72.1 ± 0.2^{d}	9.8 ± 0.1^{c}
	A3	3.0	45	26.6 ± 0.2^{e}	85.9 ± 0.7^{c}	12.1 ± 0.0^{a}
	A4	3.0	90	38.8 ± 0.3^{d}	70.3 ± 0.7^{de}	12.1 ± 0.0^{a}
Neutrase	N1	1.5	45	59.6 ± 0.9^{b}	95.4 ± 0.7^{b}	8.0 ± 0.1^{e}
	N2	1.5	90	58.7 ± 1.0^{b}	99.1 ± 0.8^{a}	8.3 ± 0.2^{e}
	N3	3.0	45	59.4 ± 0.3^{b}	98.2 ± 0.8^{ab}	9.3 ± 0.0^{d}
	N4	3.0	90	70.2 ± 0.4^{a}	80.9 ± 0.9^{d}	10.3 ± 0.0^{b}
Control	C1	n/a	45	8.3 ± 0.1^{f}	67.4 ± 1.7^{e}	2.9 ± 0.2^{f}
	C2		90	17.8 ± 0.0^{g}	59.8 ± 0.4^{f}	3.4 ± 0.1^{g}

2.2 Cricket protein hydrolysates (CPH) preparation

CPHs were prepared following the protein hydrolysis procedure of Hall *et al*. (2017) with modifications. Crickets (200 g) were thawed at 4°C overnight. The thawed crickets were rinsed once with distilled water at a 1:2 (w/v) ratio. The crickets were then mixed with distilled water at a ratio of 1:2 (w/v) and homogenized in a commercial blender for 2 min. The mixture (600 ml) was pasteurized in a water bath at 90°C for 15 min. The suspension was pre-incubated and adjusted to pH 7.0 or 8.0 at 50°C for optimal enzyme activity, using Neutrase 0.8L and Alcalase 2.4L (Aluko, 2018), respectively. The hydrolysis reaction was started by adding enzyme (Alcalase or Neutrase) at levels of 1.5% and 3.0% (w/w) for 45 and 90 min, corresponding to eight trials (Table 1). The enzymatic reaction was terminated by increasing the temperature to 90°C for 20 min. Next, the hydrolyzed cricket was left at 4 °C before centrifuging at 18,000 × g for 15 min (Tomy MX-305, Amuza, San Diego, CA, USA). The supernatant was separated from the pellet. The supernatant, mainly cricket protein hydrolysates (CPHA using Alcalase and CPHN using Neutrase), was collected, freeze-dried, and kept at -18°C until further use. The control sample consisted of an equal amount of cricket

subjected to the same processing with no enzyme added.

2. 3 Determination of degree of hydrolysis (DH)

The percentage degree of hydrolysis (DH), defined as the percentage of the total number of peptide bonds in a protein cleaved during hydrolysis, was determined using the formal titration method (Adler-Nissen, 1986). This is based on the reaction of amino acids with formaldehyde, liberating one H^+ ion from the amino group, which is potentiometrically titrated with a sodium hydroxide solution. For each trial, 2.5 ml of CPH was placed in a glass test tube, and the pH was adjusted to 8.5 using 0.01 N NaOH. After adding 1 ml of 35% formaldehyde, the solution was incubated for 1 min at room temperature and titrated with 0.01 N NaOH to pH 8.5. The volume of NaOH necessary to reach the endpoint was recorded. The degree of hydrolysis (DH) of the substrate was expressed as:

$$\square\square\ (\%) = \square \times \square_{\square} \times \frac{1}{\square} \times \frac{1}{\square_{\square}} \times \frac{1}{\square_{\square\square\square}} \times 100$$

Where B is the base used in mL, N_B is the normality of the base used, and $1/\alpha$ is the average degree of dissociation of the α-amino groups related with the pK of the amino groups at a particular pH and temperature. At 50°C and pH 7.0 or 8.0, the average pK values for the α-amino groups of peptides and protein are 2.27 and 1.13, respectively (Adler-Nissen, 1986), M_P is the mass of protein being hydrolyzed in g (%N × 6.25) and h_{tot} is the total number of peptide bonds in the protein substrate (8.64 meq/g cricket flour).

2.4 Crude protein content

The concentration of crude protein was measured using the Kjeldahl method (AOAC, 984.13) with a CPH nitrogen conversion factor of 6.25.

2.5 Yield

Total solids yield was measured as the ratio of protein hydrolysate produced to the mass of starting raw material.

2.6 Protein solubility

Protein solubility was determined using the method of Hall et al. (2017) and Chobert *et al*. (1988) with modifications. CPH (0.2 g) dispersions in 20 ml phosphate buffer of pH 3.0, 7.0, and 8.0 were magnetically stirred at room temperature for 30 min and centrifuged at 7,500 × g (4°C) for 15 min. After appropriate dilution, the protein contents of the supernatants were measured by the biuret method using bovine serum albumin as a standard calibration curve. Protein solubility was calculated as the percentage distribution of protein in the supernatant over the total crude protein content in the CPH.

2.7 Emulsifying properties

Emulsifying activity (EA) was determined using the method of Wu *et al*. (2009). 15 mL of 1% (w/v) CPH solution and 15 ml of vegetable oil were homogenized at 20,000 rpm for 1 min. The mixture was centrifuged at 3000 × g for 5 min and the volume of the individual layers was recorded. Emulsifying stability (ES) was

measured by heating the emulsion at 80°C for 30 min and centrifuging. EA and ES were evaluated as follows:

$$\text{EA}\ (\%) = \frac{V_e}{V} \times 100$$

$$\text{ES}\ (\%) = \frac{V_{30}}{V_e} \times 100$$

Where V is the total volume of tube contents, V_e is the volume of the emulsified layer, and V_{30} is the volume of the emulsified layer after heating.

2.8 Foaming properties

Foaming capacity (FC) was determined using the aeration method of Waniska and Kinsella (1979). CPH dispersions of 0.75 g in 25 mL of deionized water (final pH 6.8) were equilibrated with a stir bar for 10 min at room temperature. The mixture was aerated by a homogenizer at 16,000 rpm for 2 min. Foaming stability (FS) was calculated as the percentage of foam remaining after 30 min, as follows:

$$\text{FC}\ (\%) = \frac{\text{Volume after aeration} - \text{Volume before aeration}}{\text{Volume before aeration}} \times 100$$

$$\text{FS}\ (\%) = \frac{\text{Volume after 30 min}}{\text{Volume before aeration}} \times 100$$

2.9 Experimental design and statistical analysis

All trial conditions were replicated twice using a full factorial design. All analyses were conducted in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Tukey's pairwise comparison of means, with a level of significance of 0.05. Analysis of variance was performed on SPSS for Windows version 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1 Degree of hydrolysis (DH)

Controlled hydrolysis is critical to preserve the nutrients and improve the functional properties of the protein. DH is an indicator for the cleavage of peptide bonds and the breakdown of the structured proteins into smaller peptides (Purschke et al., 2018). Enzyme substrate (E/S) concentration, temperature, pH, and hydrolysis time affect the number of peptides released and the size and conformation of proteins. The amino acid sequence imparts different functional and bioactive properties to the hydrolysate product (Chalamaiah et al., 2012).

In the present study, the type of enzyme (Alcalase and Neutrase), E/S concentration and hydrolysis time (trials A1-N4) were varied to observe the effects on protein functionality. The results showed that DH increased significantly as the E/S concentration of both enzymes increased ($p < 0.05$) (Table 1). The percentage of DH was higher when using Neutrase than Alcalase, with ranges of 59.4% - 70.2% and 26.6% - 38.8%, respectively. Samples hydrolyzed with 3.0% E/S for 45 min (26.6% trial A3) and 1.5% E/S for 90 min (58.7% trial N2) had the lowest DH for both enzymes. When the highest concentration of enzyme (3.0% E/S)

was treated for 90 min, the maximum DH was 38.8% for Alcalase and 70.2% for Neutrase. Hydrolysis with Neutrase led to higher overall protein degradation due to Neutrase is a zinc metallo endo-protease that randomly hydrolyses internal peptide bonds and also facilitates the enzymatic synthesis of oligopeptides by the reverse proteolysis reaction with zinc metal as co-catalyst. Since Alcalase and Neutrase are endopeptidases, their hydrolysis processes may be expected to break peptide bonds from non-terminal amino acids randomly, facilitating further hydrolysis of proteins. Alkalase is an alkaline non-specific serine protease from Bacillus subtilis that initiates the nucleophilic attack on the peptide bond through a serine residue at the active site. Alcalase acts as an esterase, enabling it to catalyze stereoselective hydrolysis of some esters. Alcalase also efficiently hydrolyzes amino esters that include heterocyclic amino esters. In comparison, Neutrase is a neutral, zinc metallo endo-protease from Bacillus amyloliquefaciens that randomly hydrolyses internal peptide bonds and facilitates the enzymatic synthesis of oligopeptides by the reverse proteolysis reaction with zinc metal as co-catalyst. Neutrase contains a zinc ion at its active site that acts as a catalyst in the hydrolysis of the peptide bonds. Mwangi et al. (2016) showed zinc content ranging between 13.9 and 22.4 mg/100 g DM for four cricket species. As zinc is a cofactor of Neutrase, it activates the water molecule for the hydrolysis of the peptide bonds. That is probably why the activity of neutrase was higher than Alcalase. Thus, Neutrase's activity was influenced by the zinc content in cricket. As expected, the control had small DH values of 8.3% at 45 min and 17.8% at 90 min. This may reflect a small amount of protein denaturation and hydrolysis experienced by all samples during enzyme–inactivation treatment at 90°C (Chi et al., 2003).

3.2 Total crude protein content and yield

Table 1 shows the total crude protein content and yield of CPH trials with the two proteolysis enzymes. As expected, the total crude protein content of the control was lower than that of CPHA and CPHN, confirming previous studies (Zhang et al., 2012; Hall et al., 2017). The crude protein content of CPH was higher than that reported by Hall et al. (2017, 60-70%) using Alcalase. A higher crude protein content of CPH was observed in the Neutrase-hydrolyzed samples (80-99%) than the Alcalase-hydrolyzed samples (70-85%). Zhao et al. (2012) reported that the Alcalase hydrolyzed samples (14.51%) had a higher protein content than the Neutrase hydrolyzed samples (12.56%) for enzymatic hydrolysis of rice dreg protein.

The yield of a protein hydrolysate is an important criterion, as maximum recovery is desirable for the production of hydrolyzed food proteins (Zhao et al., 2012). The yield of CPH increased from 9.7% to 12.2% for CPHA, and from 8.0% to 10.3% for CPHN, as the E/S concentration and hydrolysis times were increased. Yields of cricket protein hydrolysates from Alcalase have been reported to be 5.2–11.7% (Hall et al., 2017). The highest yields for

both enzymes were observed at E/S 3% and 90 min exposure, reaching 12.2% (trial A4) for Alcalase and 10.3% (trial N4) for neutrase. Yields of CPH from Alcalase digestion were higher than that from Neutrase digestion. This is because there was higher solubilization of proteins by Alcalase than Neutrase. Alcalase is an alkaline protease and was more effective in dissolving cricket protein than the neutral neutrase. The control produced small yields (2.9-3.4%). This may reflect enzyme hydrolysis, which maximizes the yield.

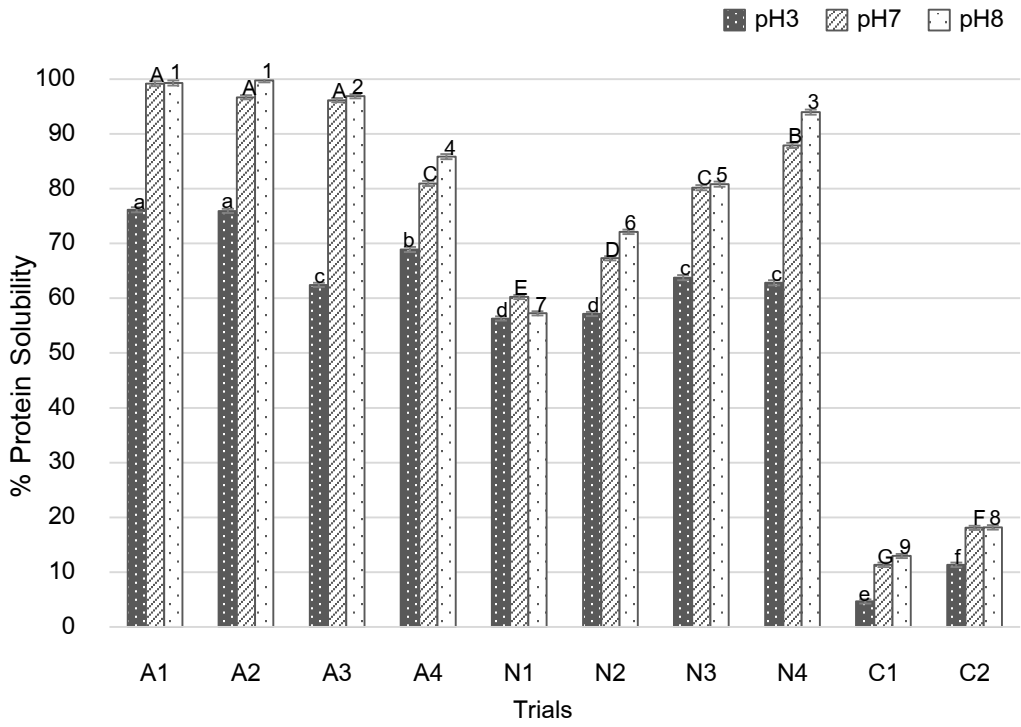

Figure 1 Protein solubility of CPH prepared using Alcalase and Neutrase at pH 3, 7, and 8. Values represent the mean and standard deviations from triplicates. Different letters indicate significant differences within pH ($p < 0.05$). Trials are reported in Table 1.

3.3 Protein solubility

Solubility is one of the most important functional properties of protein hydrolysates (Kristinsson and Rasco, 2000). Good solubility is required for many practical applications, especially for making emulsions, foams, and gels in designed food products. Solubility at pH 3, 7, and 8 is shown in Figure 1. Both hydrolysates were significantly ($p < 0.05$) more soluble than control over a wide pH range, with more than 60% solubility. The maximum solubility was observed in trials A1 (76.15%) and N3 (63.79%) at pH 3, trials A1 (99.19%) and trial N4 (87.93%) at pH 7, and trials A2

(99.8%) and N4 (94.01%) at pH 8. Solubility under all treatments was highest at pH 8 and lowest at pH 3. This is consistent with fundamental factors affecting the solubility of the protein, which is greater under alkaline than acid conditions. Our results may indicate that the isoelectric point of CPH is close to pH 3. The high solubility of protein hydrolysates was attributed to size reduction and the formation of polypeptide units, increasing repulsive interactions between peptides, and hydrogen bonding with water molecules (Zhao et al., 2012). Enzymatic hydrolysis potentially affected the molecular size and hydrophobicity, as well as polar and ionizable groups of protein hydrolysate (Chalamaiah et al., 2010). From these results, it was suggested that high solubility of CPH was due to the size reduction, an increase in the number of polar groups by enzymatic hydrolysis, formation of a smaller and more hydrophilic and more solvated polypeptide unit. The high solubility of CPH might also be due to the removal of insoluble fractions by centrifugation before freeze-drying (Chalamaiah et al., 2010). These increased the solubility of the hydrolyzed protein. These results demonstrate the potential for using CPH in many food formulations such as nutrition and sports beverages.

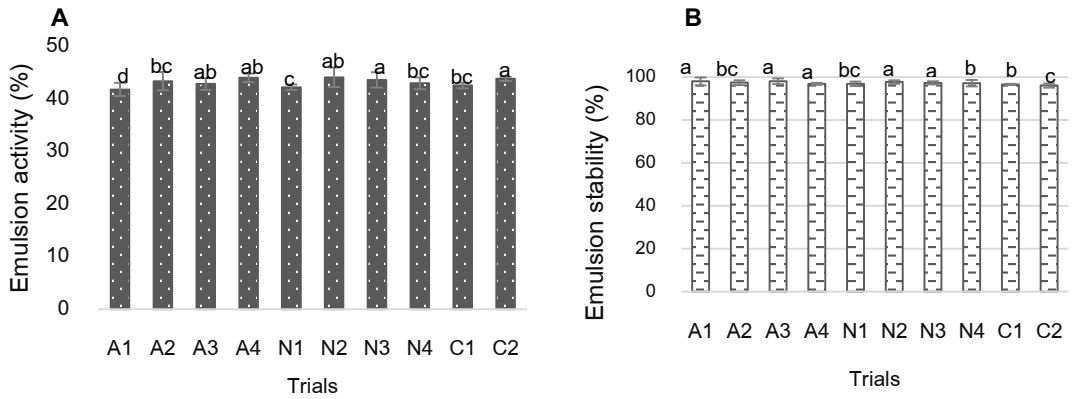

Figure 2 Emulsion activity (A) and emulsion stability (B) of CPH prepared using Alcalase and Neutrase. Different letters show significant differences ($p < 0.05$).

3.4 Emulsifying properties

Figure 2 shows the emulsifying activity (EA) and emulsifying stability (ES) of CPH. The ability of a protein to interact with oil or other protein molecules to form an interfacial layer depends on its flexibility and the accessibility of surface- exposed groups. It is crucial in hydrophobicity (Li-Chan and Nakai, 1991). As shown in Figure 2, the EA of CPH from both enzymes was similar (typically exceeding 40%). In particular, after heating at 80°C for 30 min, the ES of all treatments significantly increased (typically exceeding 95%). A previous study reported that control emulsions decreased to

near 0 m2/g while CPH emulsions remained relatively stable (Hall et al., 2017). Protein hydrolysates are surface-active materials and promote an oil-in-water emulsion because of their hydrophilic and hydrophobic groups and their charge (Chalamaiah et al., 2010). Generally, enzymatic hydrolysis of proteins can expose previously inaccessible amino acids in the parent protein. The release of these surface-stabilizing residues can increase hydrophobic interactions and thus, facilitate emulsion formation (Hall et al., 2017). It has been suggested that the protein hydrolyzed in the product, with a greater exposed net charge, form strong cohesive films that surround oil droplets, maintaining emulsion stability (Li-Chan and Nakai, 1991). The high emulsifying properties of CPH facilitate potential application in emulsion food formulations.

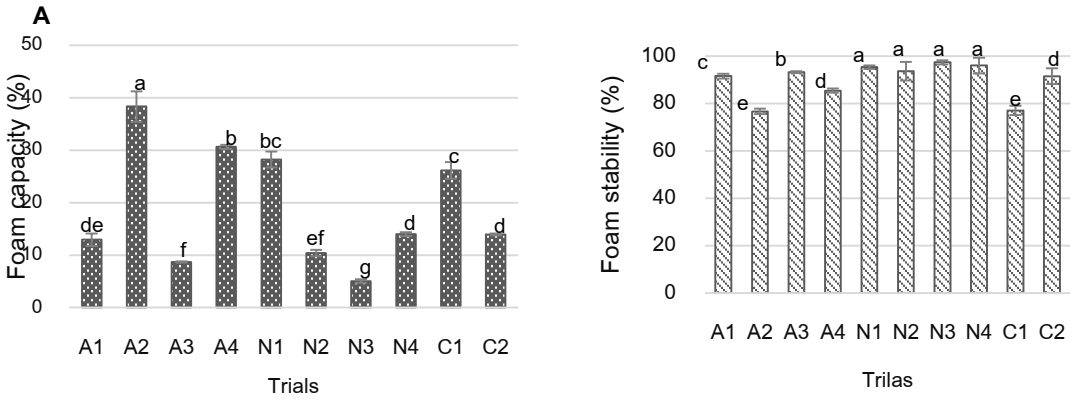

Figure 3 Foam capacity (A) and foam stability (B) of CPH prepared using Alcalase and Neutrase. Different letters show significant differences ($p < 0.05$).

3.5 Foaming properties

Foam formation is controlled by the transportation, penetration, and reorganization of molecules at the air-water interface. Figure 3 shows the foaming capacity (FC) and foaming stability (FS) of CPH for both enzymes. The highest FC was 36.56% in trial A2 and 28.23% in N1. The lowest FC when using Alcalase and neutrase was in trials A3 (8.7%) and N3 (5.05%) at 3.0% E/S and 45 min. The initial FC in all trials was lower than those reported by Hall et al. (2017). While hydrolysis may promote interfacial activity, explaining the increased emulsion capacity of hydrolysates, it also reduces the molecular weight of the polypeptides. The polypeptide size affects the strength of the interfacial layer and the formation of networks. Hydrolysis could then negatively influence FC (Hall et al., 2017). FS was high in all trials. The most substantial ability to stabilize foam was observed in trials A3 (93.29%) and N3 (97.42%). To exhibit good foaming properties, a protein must be capable of migrating rapidly to the air–water interface,

unfolding and rearranging at the interface (Halling, 1981). Sanchez and Patio (2005) described that an increase in protein concentration resulted in a higher diffusion rate. The higher foam stability of CPH may be due to high protein content.

4. Conclusion

Alcalase and neutrase enzymes were successfully used to improve the functionality of cricket protein hydrolysates. Compared with the un-hydrolyzed control, the hydrolysates had high emulsion activity, high emulsion stability, high foam stability, and moderate foaming capacity. The hydrolysates also exhibited good solubility, making them appropriate for use in foods with a wide range of pH values. Controlled enzymatic hydrolysis of cricket protein yields hydrolysates for use in a range of food formulations. The results suggested that these hydrolysates might serve as potential sources of protein.

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