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Characterization and bioactive protein hydrolysates from two-spotted cricket (*Gryllus bimaculatus* De Geer) and short-tail cricket (*Brachytrupes portentosus* Lichtenstein)

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ABSTRACT

The two-spotted cricket (*Gryllus bimaculatus*) and short-tail cricket (*Brachytrupes portentosus*) are economically significant edible insects in Thailand, boasting up to 60% protein content. This study investigates the effects of different types of proteases on the production of protein hydrolysates and biological activities from two cricket species, divided into 4 groups based on extraction and digestion methods as proteins extracted by heat at 100°C, proteins digested with the protease SD-AY 10, pepsin, and a combination of protease SD-AY 10 and pepsin. None of the 4 protein groups inhibited pathogenic *E. coli*, *Salmonella enteritidis*, and *Salmonella typhimurium*. The total antioxidant capacity (TAC) assessment showed that proteins from *G. bimaculatus* digested with SD-AY 10 had a significantly higher antioxidant level (5.5 nmol/µl), while proteins from *B. portentosus* digested with pepsin had a similarly high antioxidant level (5.59 nmol/µl), both significantly higher than other groups ($p<0.05$). Proteins from both crickets digested by enzymes were safe for RAW 246.7 macrophage cells at concentrations from 1.56 to 25% (v/v) and effectively inhibited nitric oxide production. Protein hydrolysates from *G. bimaculatus* and *B. portentosus* inhibited nitric oxide production at a concentration of 25% (v/v) equivalent to ß-glucan. Phagocytic activity was also observed in protein hydrolysates from both cricket species, stimulating RAW 246.7 cells at concentrations of 1.56-25% (v/v). However, protein hydrolysate from *B. portentosus*, digested with pepsin at a concentration of 1.56-12.5% (v/v) showed higher phagocytic activity values (152.52-163.86%) compared to ß-glucan (149.18%). The results showed that protein hydrolysates from two cricket species, digested by enzymes, exhibited antioxidant activity, inhibited nitric oxide production, are safe for cells, and hold potential as future supplements for human food and animal feed additives.

Keywords: Edible insects, Protein hydrolysates, Bioactive compound

INTRODUCTION

Population growth and global warming have significantly impacted food security. As protein sources may become insufficient to meet rising demand, prompting scientists to explore edible insects as an alternative protein source for humans and animals over the past decade. Edible insects have been investigated in various fields including breeding, food research, safety, storage, and packaging [1]. Thailand's high biodiversity provides up to 300 species of edible insects. The widespread consumption of insects began with the outbreak of the Patanga locust in 1978, which caused severe damage to agricultural crops, leading to a change from natural harvesting to insect farming practices [2]. Consequently, Thailand has witnessed a substantial expansion in its insect-related export market, encompassing a wide array of products such as fresh and frozen insects and processed forms such as fried, roasted, insect powder, protein shakes, and bars, with an estimated annual value of 28.57 million USD [3].

Edible insects are an emerging source of protein, and scientists are interested in using insect proteins to create functional foods and nutritional supplements such as protein extracts, protein hydrolysates, and peptides. These substances can exhibit biological activity including antioxidants, anti-diabetic agents, anti-hypertensive agents, anti-cancer agents, as well as pathogen inhibitors, immune stimulants, and growth promoters [4]. The production of protein hydrolysates and peptides is primarily through protease hydrolysis processes such as alcalase, pepsin, papain, bromelain, trypsin, chymotrypsin, neutral protease, and proteinase-k [5-8]. Enzymes utilized in the process of protein digestion to produce protein hydrolysate may yield different biologically active substances depending on the protein source, despite being the same type of enzyme. Zielinska et al. [9] studied the antioxidant and anti-inflammatory effects of using enzymes in the gastrointestinal tract to digest proteins from three species of edible insects: *Gryllodes sigillatus*, *Tenebrio molitor*, and

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Schistocerca gregaria. Different types of enzymes, when digesting the same protein, can produce varying antioxidant activities. This variation results from differences in protein size and the number of positive and negative charges in the structure, which affect the biological activity [10], which is important for applications in the food, cosmetics, health products, and animal dietary supplement industries.

The two-spotted cricket (*Gryllus bimaculatus*) and short-tail cricket (*Brachytrupes portentosus*) are economically important insects cultivated for their high nutritional value. Protein, fat, and carbohydrate percentages of *G. bimaculatus* were 57.02%-70.2%, 13.90%-33.14%, and 13.90%, respectively with *B. portentosus* 48.69%-59%, 20.60%-26.15%, and 5.15%, respectively [8, 11, 12]. Hall et al. [13] studied insect protein hydrolysates from whole crickets (*Gryllodes sigillatus*) using alcalase for enzymatic hydrolysis to enhance functional properties. The hydrolysates, composed of smaller peptides and amino acids, showed improved solubility, emulsifying, and foaming abilities. These hydrolysates are widely applied in food products for their nutritional and functional benefits, contributing to better texture, stability, and overall product quality. This research used protease enzymes to produce protein hydrolysates and investigated the biological activities of these two species. The knowledge gained will promote the production of high-value insect products for use as health enhancers, cosmetics, and animal health supplements.

MATERIALS AND METHODS

Extraction of soluble proteins from crickets

The extraction process of the two species of crickets, *G. bimaculatus* and *B. portentosus* involved weighing 50 g of fresh cricket, adding 400 ml of distilled water, blending thoroughly and then packing the mixture in a 500 ml glass bottle before sterilization in an autoclave at 100°C for 30 min. After sterilization, the precipitate was separated by centrifugation at a speed of 5000 rpm for 15 min, and the supernatant was stored at -20°C.

Protein hydrolysate production using enzymes

Three samples of cricket protein hydrolysates were used.

1. Protein hydrolysates were obtained from the action of the enzyme SD-NY10 (Amano, Japan) at a concentration of 300 units/100 ml of soluble protein extract. The water-soluble protein extract were adjusted to pH 7 and incubated in a temperature-controlled bath at 50°C for 8 h. After incubation, the enzyme reaction was stopped by boiling in water for 10 min and the samples were stored at -20°C.

2. 100 ml of the extracted soluble protein were adjusted to pH 3 using 0.5 N hydrochloric acid. The enzyme pepsin at a concentration of 300 units/100 ml of soluble protein extract. (Sigma-Aldrich, Germany) was then added and the mixture was incubated in a water bath shaker at 37°C for 2 h. The enzyme reaction was stopped by boiling the mixture in water for 10 min.

3. Protein hydrolysate samples digested with the enzyme SD-NY10 were then digested with the pepsin enzyme, as described above, and stored at -20°C.

Protein analysis

Protein content was measured using the Bradford assay with ready-made Bradford reagent (Bio-Rad, USA) diluted with distilled water at a ratio of 1:4. A 0.5 ml sample was taken to determine the protein amount, and 5 ml of the diluted Bradford reagent was added. The mixture was thoroughly mixed, and the absorbance was measured at a wavelength of 595 nm. The experiment was repeated three times and compared with a standard curve of bovine serum albumin at concentrations of 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mg/ml (Sigma-Aldrich, Germany).

Analysis of protein molecular weights by SDS-PAGE

Protein samples from the crickets and standard protein were transferred on precast acrylamide gels with continuous concentrations of 4-12% (Invitrogen, USA) using electricity of 170 volts for 45 min. Protein bands were fixed on the gel plates with the Fixing solution for 1 h, stained with Coomassie brilliant blue for 2 h, and then the stain was washed off with the Destain solution overnight. The molecular weight of the sample protein was compared with the standard band displayed on the gel. The procedure for staining the gel was adapted from Green and Sambrook [14].

Pathogenic bacterial inhibition by the agar well diffusion method

The experiment used three bacterial strains: *Escherichia coli* TISTR 117, *Salmonella typhimurium* TISTR 292, and *Salmonella enteritidis* TISTR 2519. The bacteria were cultured in nutrient broth and incubated at 37°C for 18-24 h. The bacterial concentrations were then adjusted to $1x10^8$ CFU/ml using 0.85% sodium chloride and compared with the McFarland Standard 0.5. The bacteria were spread on nutrient agar using the 3-way streaking method. Wells were created with a No. 3 cork borer, and 100 µl of the substances from both species of crickets were added into each well. Each plate was replicated three times and incubated at 37°C for 18-24 h. The inhibitory effect was examined by measuring the diameter in millimeters of the clear zones formed.

Antioxidant activity

The total antioxidant capacity was assessed using a total antioxidant capacity assay kit (TAC) from Sigma-Aldrich, Germany, and compared to the standard Trolox measurements at a wavelength of 570 nm using a microplate reader (Multiskan Go,

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Thermo Scientific, Finland). A critical evaluation criterion was ensuring that the absorbance value of the cricket protein sample did not surpass the standard curve. If this occurred, then sample dilution was necessary.

Cytotoxicity testing

A RAW 264.7 cell culture (Mouse macrophage cell line ATCC ® number TIB-71™), ATCC, Virginia, USA was used.

1. RAW 264.7 cell cultures were prepared in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic in a 75 cm² surface area cell culture flask and incubated at 37°C with 5% CO_2 until they reached a density of 80%. The cells were then dissociated into a single cell suspension and counted to a concentration of $1x10^5$ cells/ml. Subsequently, the cells were cultured in a 96-well culture plate and incubated at 37°C with 5% CO_2.

2. The six test samples were prepared as protein hydrolysates from the two species of cricket. These test samples were filtered through a 0.22-micron filter and then diluted in cell culture media to concentrations of 50, 25, 12.5, 6.25, 3.13, and 1.56% (v/v). The positive control solution was supplemented with 10% DMSO in the culture medium, while the negative control solution was used DMEM. Each experimental group was replicated three times, and cells were incubated for 24 h. After incubation, cell viability was assessed through succinate dehydrogenase enzyme activity in living cells according to the protocol in ISO 109935-5. 100 ml of MTT solution (1 mg/ml) were then added and incubated at 37°C for 2 h. After incubation, the MTT solution was removed, and 100 µL of DMSO were added to the cells. The formation of a purple substance was observed and measured, with light absorbance value at a wavelength of 570 nm (ABS_{570}).

3. The cell survival percentage was calculated and compared between the test sample and control groups as follows:

$$\text{Cell viability \%} = \frac{ABS_{570} \text{ of test sample}}{ABS_{570} \text{ of the control group}} x100$$

Substances with a cell viability value below 70% were considered cytotoxic.

Stimulating nitric oxide production in the macrophage cell line

1. The cell line RAW 246.7 was cultured using the same method as in the cytotoxicity test but with a cell concentration of $2x10^5$ cells/ml. The same procedure for testing and protein hydrolysate extraction from cricket samples was used in the cytotoxicity study. The positive control for this test was a lipopolysaccharide solution (L4931) from *Escherichia coli* of Sigma-Aldrich, Germany at a concentration of 1 µg/ml in the culture medium, while the negative control was a diclofenac solution at a concentration of 40 µg/ml in the cell culture medium. Each group was replicated three times.

2. Nitric oxide quantification

The RAW 264.7 cell line was cultured in a cell culture medium with the test sample, positive, and negative controls for 24 h. Then, the obtained cell culture medium was analyzed to measure the quantity of nitric oxide using a Griess reagent kit (Thermo Fisher Scientific, USA). The quantity of nitrite produced by the cells was assessed by the spectrophotometric analysis of light absorption at a wavelength of 570 nm and compared with a nitrite standard. Nitric oxide quantification was calculated as follows:

$$\text{Nitric oxide (µmol)} = \frac{ABS_{570} \text{ of test sample}}{\text{the slope value on the standard graph}}$$

The method was adapted from Zhang et al. [15].

Phagocytic activity analysis of macrophage cells

The RAW 264.7 cell culture method and test substances were the same as in the cytotoxicity test, with ß-glucan at a concentration of 100 µg/ml in the culture medium used as a positive control. The negative control group consisted of cells cultured in Dulbecco's modified Eagle's medium (DMEM). All experimental groups were replicated three times. After incubation for 24 h, the cell culture medium was removed, and 100 µl of neutral red solution (at a concentration of 0.075% in PBS) was added to each well and incubated for 3 h at 37°C. After this incubation, the neutral red solution was removed, the cells were washed once with PBS pH 7.4, and Destain was added for 1 h according to the method of Zhang et al. [15]. The absorbance was then measured at a wavelength of 540 nm to calculate the phagocytic activity as:

Phagocytic activity = (ABS_{540} of test sample – ABS_{540} of the negative control unit) x 100

RESULTS AND DISCUSSION

Characterization of protein hydrolysates derived from two species of cricket

Water-soluble proteins from *G. bimaculatus* and *B. portentosus* were extracted using heat at 100°C for 30 min in an autoclave. The resulting water-soluble protein yields were 0.84 and 0.92 mg/ml, respectively. Subsequent enzymatic digestion of the water-soluble proteins produced three types of peptide hydrolysates: protein hydrolysates from digestion with the enzyme SD-AY 10, SD-AY 10 with pepsin, and pepsin. When different enzymes were used to digest the water-soluble proteins, the amounts of proteins digested varied. Peptides digested with SD-AY 10+pepsin from both cricket species had the highest

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protein digestion values of 96-98%, followed by peptides digested with pepsin at 83-84%. The lowest digestion was observed in peptides digested using the SD-AY 10 enzyme (Table 1).

Table 1 Soluble protein hydrolysates (%) from two cricket species digested with pepsin and SD-AY 10.

Cricket species	Soluble protein hydrolysates (%)		
	SD-AY 10	Pepsin	SD-AY 10+Pepsin
G. bimaculatus	37	84	96
B. portentosus	72	83	98

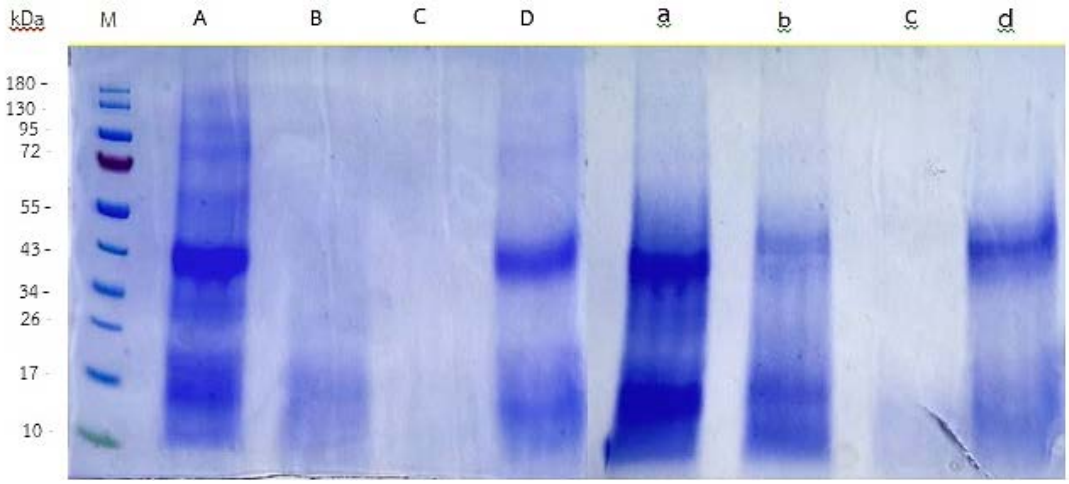

Figure 1 SDS-PAGE patterns of *B. portentosus* and *G. bimaculatus* proteins. A-D=proteins from *B. portentosus* and a-d protein from *G. bimaculatus*: A, a = protein extracted with heat 100°C, B b = protein hydrolysate by SD-AY 10; C c = protein hydrolysate by SD-AY 10+pepsin and D d = protein hydrolysate by pepsin.

The protein hydrolysate obtained from *B. portentosus* digested with SD-AY 10 had a higher level of digested protein compared to *G. bimaculatus* (Table 1). Different amino acid compositions in the protein structure impacted the specific binding site of the enzyme SD-AY 10 for the amino acid serine [16], leading to limited enzymatic activity. This observation aligned with Leni et al. [17]. They utilized the enzyme bromelain for protein digestion from larvae of *Alphitobius diaperinus* and *Hermetia illucens* and recorded distinct protein digestibility values that differed by 13.3% and 23.1%, respectively.

Analysis of protein hydrolysate solutions derived from both cricket species via SDS-PAGE at an acrylamide gel concentration of 4-12% revealed water-soluble proteins extracted at 100°C from *B. portentosus* with molecular weights of 10, 17, 30, 43, 60, 72, and 95 kDa. The proteins digested with SD-AY 10 exhibited bands in the molecular weight range of 17 kDa, while the proteins digested with SD-AY 10+pepsin resulted in no protein bands on the gel. Proteins digested with pepsin displayed bands at molecular weights of 43 and 15 kDa. Proteins extracted with heat from *G. bimaculatus* showed bands at 10, 17, and 43 kDa, while proteins digested with the enzyme SN-AY 10 demonstrated a lighter band at 43 kDa and more intense bands between 17 and 10 kDa. Proteins digested with SD-AY 10+pepsin and pepsin exhibited analogous outcomes to those from *B. portentosus* (Figure 1). Proteins digested with SD-AY 10+pepsin showed 96-98% protein digestion (Table 1) with no protein bands on the gel, suggesting protein digestion to a size of less than 10 kDa. These findings align with previous research utilizing endo-proteases such as papain, bromelain, pepsin, and protease enzymes from microorganisms, which digested proteins extracted from water-based edible insect sources, yielding protein sizes ranging from 10 to 35 kDa [8, 18-20].

Biological activity

1. Effective inhibition of pathogenic microorganisms

The protein samples derived from *G. bimaculatus* and *B. portentosus* were used to test the inhibition of pathogens *E. coli*, *S. enteritidis*, and *S. typhimurium* included heat-extracted soluble protein, and protein hydrolysates obtained through digestion with SD-AY 10, SD-AY 10 combined with pepsin, and pepsin using the agar well diffusion method. Results indicated that the protein samples from both species of crickets were unable to inhibit all three species of pathogens, consistent with results reported by Flores et al. [20]. They extracted proteins from the edible insects *Tenebrio molitor* and *Ulomoides dermestoides* digested with protease from *Aspergillus oryzae*, yielding peptides with molecular weights ranging from 45 kDa to less than 10 kDa, which were unable to inhibit *Salmonella* spp. Similarly, Sousa et al. [21] found that

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protein hydrolysates of the edible insect *Alphitolios cliperinus*, obtained from alcalase digestion, were unable to inhibit the growth of *E. coli* and *S. enteritidis*. These results highlighted that protein hydrolysates from the 150 insect species studied were ineffective in inhibiting pathogenic microorganisms.

2. Total antioxidant capacity

The total antioxidant capacity (TAC) assesses the resistance to free radicals generated within the body during the process of oxygen metabolism, which produces reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radical (HO^+), peroxyl radical (ROO^+), and superoxide radical (O_2^+) [22]. This test evaluated the water-soluble protein extracts and protein hydrolysates from two species of crickets for their antioxidant efficiency. Compared to the standard Trolox, *G. bimaculatus* protein hydrolysates digested with SD-AY 10 exhibited the highest antioxidant efficiency at 5.5 nmol/µl, significantly higher than the other protein solutions ($p<0.05$). Conversely, proteins extracted with heat at 100°C demonstrated the lowest efficiency. The heat-extracted protein exhibited lower antioxidant activity compared to the enzymatically hydrolyzed protein, particularly in the case of *G. bimaculatus*. According to the SDS-PAGE results, several large proteins were present, ranging from 43 to 130 kDa. The size of these proteins influences their antioxidant capacity, with smaller proteins demonstrating greater antioxidant activity. This observation aligns with findings that smaller proteins exhibit stronger antioxidant properties [23]. For *B. portentosus*, protein hydrolysates digested pepsin displayed efficient antioxidant activity, significantly higher than other protein solutions ($p<0.01$) with an efficiency value of 5.59 nm/µl (Figure 2). Consistent with numerous studies exploring protein digestion in edible insects using alkaline protease enzymes and digestive enzymes [20, 21, 24, 25].

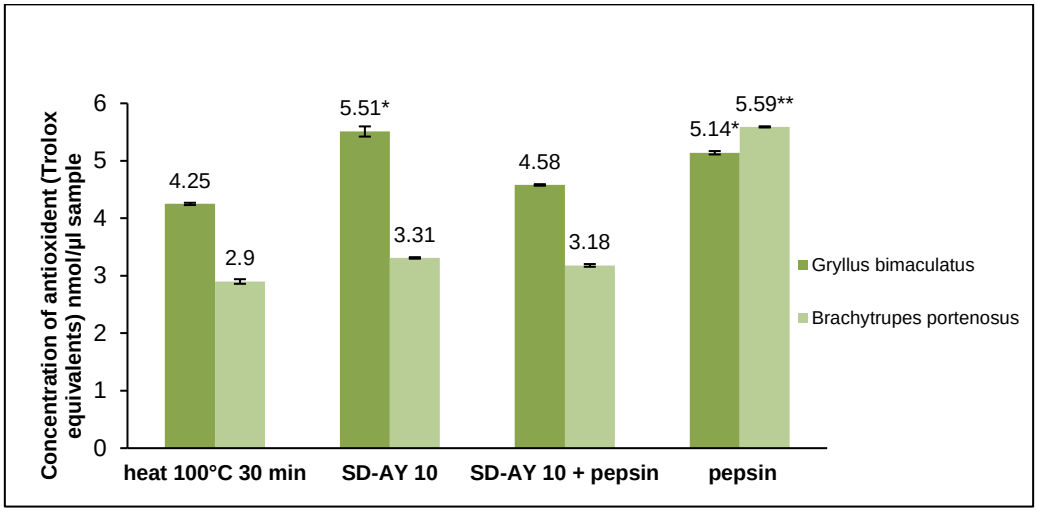

Figure 2 Effect of protein hydrolysates from *B. portentosus* and *G. bimaculatus* on total antioxidant capacity
*Significant difference ($p\leq0.05$), **significant difference ($p\leq0.01$).

The molecular weight of the peptides influenced the antioxidant properties. The experimental group digested with a single type of SD-NY10 of *G. bimaculatus*, with molecular weights ranging from 10 to 17 kDa, displayed better antioxidant efficiency than those utilizing SD-AY 10 with pepsin, which had a molecular size lower than 10 kDa, consistent with Jang et al. [26] and Flores et al. [20] within this molecular weight range. The antioxidant efficiency of peptides depends on various factors such as amino acid composition, hydrophilic-hydrophobic properties, polarity of the structure, enzyme types, and methods used to break down protein structures into peptides [4, 27].

3. Cytotoxicity

Peptide samples from the two cricket species were divided into three experimental groups: proteins digested with SD-AY 10, SD-AY 10+pepsin, and pepsin. Each experimental group utilized concentrations of protein solutions such as 1.56, 3.13, 6.25, 12.5, 25, and 50% (v/v) of RAW 264.7 cell culture medium. Results revealed that peptides within the concentration range of 1.56-25% (v/v) in all experimental groups exhibited cell safety, with over 70% cell viability, especially peptides from each cricket species. When comparing peptides derived from hydrolysis using three different enzyme treatments, those from *G. bimaculatus* hydrolyzed with SD-AY10 exhibited the highest cell viability ($p<0.01$). In contrast, proteins from *B. portentosus* digested with pepsin demonstrated superior safety, with cell survival rates ranging from 104% to 109% ($p<0.01$). However, cell toxicity was observed across all experimental groups at a 50% (v/v) sample concentration, indicating that higher concentrations of cricket protein hydrolysates resulted in increased cytotoxicity. These findings are consistent with those of Riolo et al. [28], who reported that elevated concentrations of protein hydrolysates from black soldier fly (*Hermetia illucens)* were harmful to fibroblast cells (L-929). (Table 2).

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Table 2 Effect of protein hydrolysates from *B. portentosus* and *G. bimaculatus* on the viability of the RAW 264.7 cell line.

Cell viability of RAW 264.7 (%)					
Type of protein hydrolysates treatment	**Sample concentration % (v/v)**	**SD-AY 10**	**SD-AY 10 + pepsin**	**pepsin**	**DMSO 10% (v/v)**
G. bimaculatus	1.56	90.52±0.97^{a}	79.69±0.26^{c}	86.45±2.72^{b}	17.11±0.09
	3.13	87.48±0.87^{a}	75.60±0.46^{c}	83.63±2.30^{b}	
	6.25	86.86±1.29^{a}	76.37±0.86^{c}	82.29±3.08^{b}	
	12.5	84.79±0.66^{a}	73.09±0.68^{b}	76.47±2.88^{b}	
	25	82.69±0.66^{a}	73.68±0.82^{b}	73.50±2.08^{b}	
	50	61.00±1.33^{a}	50.36±0.82^{c}	53.40±0.51^{b}	
B. portentosus	1.56	88.09±1.26^{b}	84.36±1.96^{c}	109.36±1.53^{a}	17.11±0.09
	3.13	87.08±0.72^{b}	86.80±1.03^{b}	106.68±0.69^{a}	
	6.25	89.11±0.45^{b}	86.19±1.14^{c}	108.37±1.45^{a}	
	12.5	88.21±1.69^{b}	85.89±0.46^{c}	109.67±0.60^{a}	
	25	85.27±1.50^{b}	79.48±1.06^{c}	104.67±1.42^{a}	
	50	33.80±1.23^{c}	47.06±1.45^{b}	63.34±1.40^{a}	

Note: ^{a,b,c}Means within a row with different letters are statistically significant ($p<0.01$)

Table 3 Effect of protein hydrolysates from *B. portentosus* and *G. bimaculatus* on nitric oxide production of the RAW 264.7 cell line.

Nitric oxide production of the RAW 264.7 cell line						
Type of protein hydrolysates treatment	**Sample concentration % (v/v)**	**SD-AY 10**	**SD-AY 10 + pepsin**	**pepsin**	**LPS (1 μg/ml)**	**Diclofenac (40 μg/ml)**
G. bimaculatus	1.56	35.48±0.27^{b}	33.72±0.56^{b}	86.45±2.72^{a}	34.02±0.54	15.51±1.50
	3.13	37.23±0.57^{b}	36.02±0.77^{b}	83.63±2.30^{a}		
	6.25	33.81±0.70^{b}	32.97±0.51^{b}	82.29±3.08^{a}		
	12.5	22.90±0.22^{b}	21.84±0.33^{b}	76.47±2.88^{a}		
	25	9.30±0.71^{a}	6.89±0.13^{b}	9.43±0.98^{a}		
	50	-	-	-		
B. portentosus	1.56	40.25±1.11^{a}	33.74±0.45^{b}	34.60±1.10^{b}	34.02±0.54	15.51±1.50
	3.13	39.56±0.46^{a}	33.30±0.56^{c}	34.75±0.86^{b}		
	6.25	34.34±0.91^{a}	30.64±0.27^{b}	30.47±0.60^{b}		
	12.5	22.26±1.00^{a}	19.73±0.29^{b}	21.01±0.57^{a}		
	25	7.66±0.36^{a}	6.73±0.94^{ab}	5.94±0.22^{b}		
	50	-	-	-		

Note: ^{a,b,c}Means within a row with different letters are statistically significant ($p<0.01$)

4. Effect of protein hydrolysates on the production of nitric oxide (NO)

In all the experimental groups, protein hydrolysates derived from the two cricket species, at a concentration of 25% (v/v), exhibited a higher inhibitory effect on nitric oxide production than diclofenac (anti-inflammatory substance) at 15.51 μmol. The amount of nitric oxide ranged from 5.94 to 9.43 derived from *G. bimaculatus* hydrolyzed using SD-AY10+pepsin exhibited significantly reduced nitric oxide (NO) levels ($p<0.01$). Similarly, peptides obtained from *B. portentosus* hydrolyzed with pepsin also showed the lowest NO levels ($p<0.01$). Notably, at a concentration of 50% (v/v), NO was undetectable (Table 3).

In most experimental groups, protein hydrolysate concentrations ranged from 1.56% to 12.5% (v/v) and demonstrated the ability to trigger nitric oxide production comparable to the positive control using LPS (34.02 μmol) as a stimulant, except for protein from *G. bimaculatus* digested with pepsin, which stimulated high levels of nitric oxide production ranging from 76.47 to 86.45 μmol. Nitric oxide is considered harmful to cells and classified as a pro-inflammatory substance. An excessive amount of nitric oxide stimulates immune cells to produce more substances that induce cell inflammation [29, 30]. The stimulation and inhibition of nitric oxide production by protein hydrolysates from both cricket species depended on various factors including the type of

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protein-digesting enzyme, insect species, protein molecular weight, and testing substance concentration. The reported of Yoon et al. [31] investigated the use of the enzyme Flavourzyme/alcalase and a combination of two enzymes in digesting proteins from the three insect species: *Tenebrio molitor*, *G. bimaculatus*, and *Bombyx mori*. The peptides tested had molecular weights ranging from less than 10 to 15 kDa. The protein concentration used in nitric oxide production was tested in RAW 624.7 cells at 0.1, 0.3, and 0.5 mg/ml. Protein hydrolysate from B. mori with both enzymatic digestions inhibited nitric oxide production better than the other two species, with a concentration of 0.5 mg/ml demonstrating the highest inhibitory effect.

5. Effect of peptide hydrolysates on phagocytic activity

Phagocytic activity measures the response of white blood cells to the invasion of foreign agents such as bacteria, viruses, or substances (polysaccharides, proteins, hydrolysates, and herbal extracts) [29]. White blood cells destroy invaders by releasing pro-inflammatory substances including reactive oxygen species, reactive nitrogen species, interleukin-1β, and tumor necrosis factor-α [32]. Thus, measuring phagocytic activity assesses the ability of white blood cells to destroy foreign substances when stimulated. Testing the protein hydrolysates from both cricket species revealed that at a concentration of 50% (v/v) in all experimental groups, the protein hydrolysates exhibited no phagocytic activity, which correlated with the production of nitric oxide and the cell survival rate. Notably, analysis of protein hydrolysates from *G. bimaculatus* revealed that proteins digested with pepsin at concentrations ranging from 1.56% to 12.5% (v/v) exhibited higher phagocytic activity than the positive control (149.18%), with activity values between 152.52% and 163.86%. Additionally, when comparing peptides hydrolyzed by the three enzymes, those treated with pepsin showed the highest phagocytic activity ($p<0.01$). Conversely, protein hydrolysates from *B. portentosus* at concentrations of 12.5% and 25% (v/v) showed phagocytic activity between 154.16% and 158%, exceeding the positive control (Table 4). Peptides hydrolyzed predominantly by pepsin had the highest phagocytic activity ($p<0.01$). These differences in macrophage activity and nitric oxide (NO) levels could be due to the distinct peptides generated by each enzymatic digestion.

Table 4 Effect of protein hydrolysates from *B. portentosus* and *G. bimaculatus* on phagocytic activity of the RAW 264.7 cell line.

Phagocytic activity of the RAW 264.7 cell line						
Type of protein hydrolysates treatment	**Sample concentration % (v/v)**	**SD-AY 10**	**SD-AY 10 + pepsin**	**pepsin**	**ß -glucan (100 μg/ml)**	**control**
G. bimaculatus	1.56	108.16±2.29^{c}	141.11±2.52^{b}	156.69±2.88^{a}	149.18±2.41	100
	3.13	138.58±5.44^{b}	139.19±1.36^{b}	159.01±0.75^{a}		
	6.25	128.75±4.57^{c}	141.59±0.66^{b}	163.86±2.90^{a}		
	12.5	137.21±3.19^{b}	138.34±0.38^{b}	152.52±4.91^{a}		
	25	145.97±1.39^{a}	131.43±0.81^{b}	142.76±5.53^{a}		
	50	-	-	-		
B. portentosus	1.56	128.9±71.48^{a}	115.05±1.65^{b}	131.19±2.26^{a}	149.18±2.41	100
	3.13	137.09±0.24^{a}	116.01±0.56^{c}	130.58±2.77^{b}		
	6.25	138.92±0.42^{a}	124.82±2.69^{b}	139.02±2.56^{a}		
	12.5	150.21±3.41^{a}	130.70±1.12^{b}	154.16±3.32^{a}		
	25	136.98±3.78^{b}	123.93±0.92^{c}	158.60±3.27^{a}		
	50	-	-	-		

Note: ^{a,b,c}Means within a row with different letters are statistically significant ($p<0.01$)

Results demonstrated that protein hydrolysates or peptides generated through protease digestion enhanced the phagocytic activity of macrophage cells. Various factors influenced cell activation including the molecular weight and concentration of the activating proteins, as well as their correlation with the cell survival rate [33-35].

CONCLUSIONS

The protein hydrolysates utilized in this study, derived from *G. bimaculatus* and *B. portentosus*, were digested with the enzymes SD-AY 10, pepsin, and a combination of SD-AY 10 and pepsin. Evaluation of biological activity revealed that *G. bimaculatus* protein digested with SD-AY 10 exhibited the highest antioxidant activity, while protein hydrolysates from *B. portentosus* hydrolyzed with pepsin had the highest antioxidant activity. Cytotoxicity assessment conducted on RAW 246.7 macrophages indicated that protein hydrolysates in all experimental groups were cell-safe at concentrations ranging from 1.56% to 25% (v/v). Protein hydrolysates from both cricket species inhibited nitric oxide production at a concentration of 25% (v/v), exerting anti-inflammatory effects. However,

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concentrations of 1.56% to 6.25% (v/v) stimulated the immune system and induced phagocytic activity. Proteins from *G. bimaculatus* digested with pepsin exhibited the highest phagocytic activity at concentrations ranging from 1.56% to 12.5% (v/v), while *B. portentosus* proteins digested with pepsin displayed the highest phagocytic activity at concentrations of 12.5% to 25% (v/v). Results suggested that protein hydrolysates from *G. bimaculatus* and *B. portentosus* showed significant biological activity and acted as antioxidants, anti-inflammatory agent, and immune stimulants. Further investigations should explore the development of other novel bioactive compounds derived from Thailand's biological resources. These compounds hold promises for applications as pharmaceutical, cosmetic, and health supplements for both humans and animals, thereby enhancing the value and economic potential of insects in the country.

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