

Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (*Gryllobates sigillatus*) protein

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ABSTRACT

Food-derived bioactive peptides have gained attention for their role in preventing chronic diseases. Edible insects are viable sources of bioactive peptides owing to their high protein content and sustainable production. In this study, whole crickets (*Gryllobates sigillatus*) were alcalase-hydrolyzed to a degree of hydrolysis (DH) ranging from 15 to 85%. Antioxidant activity, angiotensin converting enzyme (ACE), and dipeptidyl peptidase-4 (DPP-IV)-inhibition of the cricket protein hydrolysates (CPH) were evaluated before and after simulated gastrointestinal digestion (SGD). Antioxidant activity was similar among CPH, whereas ACE and DPP-IV inhibition was greater ($p < 0.05$) in CPH with 60–85% DH. Bioactivity improved after SGD. CPH allergenicity was evaluated using human shrimp-allergic sera. All sera positively reacted to tropomyosin in the unhydrolyzed cricket and CPH with 15–50% DH, whereas 60–85% DH showed no reactivity. In conclusion, CPH (60–85% DH) had the greatest bioactive potential and lowest reactivity to tropomyosin, compared with other CPH and the unhydrolyzed control.

1. Introduction

Scientific evidence confirms that food derived proteins and peptides can trigger physiological responses in the body that heavily influence protective effects on disease and their risk factors. Despite their potency, conventional drugs are less favored due to their high cost and side-effects (Li-Chan, 2015). Currently, there is a growing interest in the applications of food proteins and peptides in the form of functional foods or nutraceuticals as alternatives to conventional treatments. Peptides released from dietary proteins by enzymatic hydrolysis have demonstrated bioactivities including, antioxidant, antidiabetic, antihypertensive, antithrombotic, immunomodulating, osteoprotective, antimicrobial, anticarcinogenic and growth-promoting properties. To date, antioxidant, antihypertensive and antidiabetic peptides, from foods, are most intensively studied (Li-Chan, 2015).

Due to their high protein content, nutrient composition, and environmental factors, edible insects are currently being assessed as contributors to growing protein demands worldwide (Hall, Jones, O'Haire, & Liceaga, 2017). Rumpold and Schlüter (2013) compiled the protein contents for 236 different types edible insects. Crickets in particular, have approximately 61.32% protein, 13.41% fat, 9.55% fiber, and 3.85% ash (based on dry matter). In previous work, we established

that hydrolyzing whole crickets with a commercial enzyme yielded protein hydrolysates with improved (techno) functional properties that can be useful for food ingredient applications (Hall et al., 2017). Meanwhile, several edible insect species have been evaluated for bioactivity (Nongonierma & FitzGerald, 2017). For instance, Vercruysse and colleagues tested four different insect species for angiotensin I-converting enzyme (ACE) inhibiting capabilities (Vercruysse, Smagghe, Beckers, & Camp, 2009b). After digestion with gastrointestinal and mucosal enzymes, all insect protein hydrolysates could inhibit ACE activity, while the whole unhydrolyzed insects had the lowest inhibiting activity. Protein hydrolysates from cotton leafworm larvae (*Spodoptera littoralis*) (Vercruysse et al., 2009b), silkworm (*Bombyx mori*) (Wu, Jia, Yan, Du, & Gui, 2015), and modified peptides from silkworm pupa (*Bombyx mori*) (Tao et al., 2017) also demonstrated the ability to inhibit ACE *in vitro*. Similarly, *in vitro* antioxidant capabilities of peptides derived from insect protein hydrolysates have been reported. Cotton leafworm larvae (*Spodoptera littoralis*) (Vercruysse, Smagghe, Beckers, & Camp, 2009a), cricket (*Amphiacusta annulipes*), super worm (*Zophobas morio*) and locust (*Locusta migratoria*) (Zielińska, Karaś, & Jakubczyk, 2017) protein hydrolysates all demonstrated antioxidant activity comparable to that of other food protein hydrolysates such as fish, wheat germ, and flax seed protein (Chalamaiah, Dinesh

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Kumar, Hemalatha, & Jyothirmayi, 2012; Karamac, Kosinska-Cagnazzo, & Kulczyk, 2016; Zhu, Zhou, & Qian, 2006).

Zhang and associates (Zhang et al., 2016) evaluated the potential antidiabetic activity of silkworm peptides using a quantitative structure activity relationship approach to predict α -glucoside inhibitory peptides. The authors identified two potent peptides Ser-Gln-Ser-Pro-Ala (IC_{50} 20 μ m) and Gln-Pro-Gly-Arg (IC_{50} 65.8 μ m); the former having similar values as the positive control used in the study (Zhang et al., 2016). Although research is still in its early stage, knowledge already attained shows that edible insects can be a sustainable source of bioactive peptides for human health.

A major concern regarding insect consumption is possible allergenic responses. Research shows that some edible insect species contain tropomyosin proteins that cross-react with those of shrimp and potentially other crustaceans (Palmer, 2016). Although novel food allergens are not unique, it is essential to evaluate the potential risks to provide information to those likely affected. Tropomyosin, a major shrimp allergen, is a myofibrillar protein consisting of a coiled-coil dimer with molecular masses of 35–38 kDa (Pedrosa, Boyano-Martínez, García-Ara, & Quirce, 2015). Since dust mites and other arthropods such as crickets, have a 75–85% tropomyosin sequence identity (Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002), IgE cross-reactivity is greatly expected in edible insect species. Current observations regard tropomyosin as the major allergen responsible for cross-reactivity between crustaceans, mollusks, mites, and cockroaches (Pedrosa et al., 2015). Since house cricket (*Acheta domesticus*) contains reactive tropomyosin (Palmer, 2016), we suspect that the tropical banded crickets (*Gryllobates sigillatus*) used in this study and widely farmed in the U.S. for human consumption, will follow a similar trend.

Given the progression of insects as food, the objective of this study was to evaluate the effect of enzymatic hydrolysis on the bioactive properties of cricket protein hydrolysates before and after simulated gastrointestinal digestion. Modifications to tropomyosin and its reactivity to IgE from shellfish allergic patients after various alcalase hydrolysis conditions was also studied.

2. Materials

Unless specified, all chemicals utilized were reagent grade and obtained from suppliers: VWR International (Radnor, PA, USA), Sigma Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA). Human Dipeptidyl Peptidase IV (DPP-IV, ≥ 4500 units/ μ g protein) and substrate Gly-Pro p-nitroanilide hydrochloride, Angiotensin Converting Enzyme (ACE) from rabbit lung and substrate Hippuryl-L-Histidyl-L-Leucine (HHL) were all purchased from Sigma Aldrich (St. Louis, MO, USA). Whole, frozen, unpasteurized, adult (6 weeks old) tropical banded crickets (*Gryllobates sigillatus*) were purchased from Entomo Farms (Norwood, ON, Canada). Alcalase® (protease from *Bacillus licheniformis*, P2.4 U/g) was obtained from Sigma Aldrich (St. Louis, MO, USA). A total of 12 sera, including atopic and non-atopic controls, were kindly provided by the Food Allergy Research and Resource Program at the University of Nebraska-Lincoln. Serological data for all sera are summarized by (Palmer, 2016).

3. Methods

3.1. Preparation of cricket protein hydrolysates (CPH)

CPH preparation is described by (Hall et al., 2017). In short, thawed whole crickets were washed and homogenized using two volumes of water for 2 min. After pasteurization, the pH was adjusted to 8.0 using 5 M NaOH and incubated at 50 °C in a water bath. Alcalase was added at different concentrations and hydrolysis times for optimal enzyme activity (Table 1). Following hydrolysis, samples were heated again to inactivate the enzyme, cooled and centrifuged. Supernatant was collected, freeze-dried, and stored in polystyrene tubes at -20 °C until

Table 1

Sample code descriptions, hydrolysis conditions and degrees of hydrolysis (DH).

Sample code (based on DH) ⁺	E:S ¹ (%)	Time ¹ (min)	DH ^{1,2} (%)
Control ⁺	–	90	4.5 $\pm$ 0.5
15	0.25	10	15.2 $\pm$ 0.1
20	0.25	30	23.1 $\pm$ 0.8
30	0.5	10	31.3 $\pm$ 0.3
40	0.5	20	42.5 $\pm$ 0.6
50	3.0	20	51.8 $\pm$ 0.6
60	3.0	40	60.5 $\pm$ 0.6
70	3.0	60	72.8 $\pm$ 0.9
85	3.0	80	84.6 $\pm$ 1.2

¹ E:S (% w/w) is the concentration of alcalase used and time (min) is the hydrolysis time required to obtain different degrees of hydrolysis (%).

² Results are mean of triplicate analysis $\pm$ SD.

* Sample codes are theoretical values used to represent the actual %DH. For example, sample code 15 indicates DH = 15.2%.

⁺ Control samples contained no enzyme and similar heat treatments as CPH samples.

further use.

3.2. Degree of hydrolysis (DH)

DH was estimated using the trinitrobenzenesulfonic acid (TNBS) method as described in (Hall et al., 2017). DH was defined as the percent ratio of the number of peptide bonds cleaved (h) to the total number of peptide bonds per unit weight (h_{tot}), and expressed as:

$$\% \text{ Degree of hydrolysis (DH)} = \left[\frac{h}{h_{tot}} \right] \times 100$$

where h_{tot} of cricket protein was determined by fully hydrolyzing whole crickets with 12 N HCl for 24 h (1:1) at 90 °C and quantifying the total number of amino groups. The h_{tot} for tropical banded crickets used in this study was calculated to be 7.28 meq/g. The values of (h) were obtained by reference to a standard curve of Abs at 420 nm versus mg L⁻¹ amino nitrogen (using L-leucine).

3.3. Simulated gastrointestinal digestion (SGD)

In vitro digestion was performed as reported by You and others (You, Zhao, Regenstien, & Ren, 2010) with modifications. CPH (30 mg/ml protein) were dispersed in 1 M Tris buffer (pH 8.0) and allowed to equilibrate at room temperature for 30 min. Gastric digestion was simulated by adjusting the solution to pH 2 with HCl (6 M) then incubating with pepsin (4% w/w of protein) for 2 h at 37 °C. At the end of the gastric phase, pH was adjusted to 6.8 using 1 M NaHCO₃, then to pH 7 with 6 M NaOH. Bile salts (10 mg/ml) and pancreatin (4% w/w protein) were added and incubated for 2 h at 37 °C to simulate the intestinal digestion phase. Both phases were incubated in a water bath with continuous shaking. SGD was terminated by heating the digest at 90 °C for 10 min, cooling at room temperature and centrifuging (12,000 $\times$ g) (Avanti J-26S Centrifuge, Beckman-Coulter INC. CA, USA) for 15 min. Supernatants were collected, referred to as CPH-digests (CPHD), freeze dried, and stored at -20 °C until further use.

3.4. Antioxidant capacity

The antioxidant capacity of CPH before and after SGD was measured in the aqueous extracts. CPH and CPHD samples were suspended (1 mg/ml) in deionized water and vortexed for 1 min. All antioxidant assays were carried out in 96-well plates and measured using a Multiskan™ FC Microplate Photometer (Waltham, MA, USA).

3.4.1. 2,2'-azino-bis 3-ethylbenzthiazoline-6- sulphonic acid (ABTS)

ABTS assay was carried out using a slightly modified procedure

described in (Ketnawa & Liceaga, 2017). The ABTS stock solution (7.4 mM ABTS in 2.45 mM potassium persulfate) was first incubated in dark at 25 °C for 16 h. Stock solution was diluted with distilled water to prepare a working solution with an absorbance of 0.700 ± 0.02 at 734 nm. ABTS was prepared fresh for each assay. A 10 μ l aliquot of sample (1 mg/ml) was allowed to react with 294 μ l of the ABTS working solution for 10 min in dark conditions at 30 °C. Absorbance was read at 734 nm. Results are expressed in mM Trolox equivalent (TE)/mg sample.

3.4.2. 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The DPPH assay was done according to the method of (Nguyen, Jones, Kim, San Martin-Gonzalez, & Liceaga, 2017). Samples (100 μ l) were mixed with 99% EtOH (100 μ l) and 25 μ l of the DPPH (0.02% in EtOH) solution. After incubation for 30 min in dark conditions (RT), the absorbance was read at 510 nm. Results are expressed in mM TE/mg sample. Sample control, for background correction, was prepared by using 25 μ l EtOH instead of the DPPH solution.

3.4.3. Ferric ion reducing antioxidant power (FRAP)

The FRAP assay was done using the method from (Ketnawa & Liceaga, 2017) with modifications. Samples (200 μ l) were mixed with 200 μ l 1% potassium ferrocyanide (in 0.2 M phosphate buffer) and incubated at 50 °C for 20 min. After incubation, 800 μ l of 10% TCA was added then centrifuged at $10,000 \times g$ for 5 min. Into a 96-well plate, an aliquot of supernatant (100 μ l) added with 100 μ l of dd-water, followed by adding 20 μ l of 0.1% Ferric Chloride (in dd-water) to initiate reaction. The reaction was incubated for 10 min at RT in dark conditions, then absorbance read at 700 nm. Results are expressed in mM TE/mg sample.

3.4.4. Metal ion chelating (MIC)

The assay followed a modified version given in (Ketnawa & Liceaga, 2017). Briefly, in a 96-well plate CPH (300 μ l) was mixed with 5 μ l of 2 mM FeCl₂ and 10 μ l of 5 mM Ferrozine was added to initiate reaction. After incubation for 10 min at RT in dark conditions, absorbance was read at 522 nm. MIC was calculated using the following equation:

$$\% \text{ MIC Inhibition} = \left[\frac{\text{Abs}(\text{control}) - \text{Abs}(\text{sample})}{\text{Abs}(\text{control})} \right]$$

3.5. Dipeptidyl peptidase IV (DPP-IV) inhibitory activity

The effect of the hydrolyzed cricket protein samples on DPP-IV activity was determined following the method described by (Lacroix & Li-Chan, 2012) with some modifications. Samples were reconstituted in 100 mM Tris-HCl buffer (pH 8.0) to 1.25 mg/ml final assay concentration. In a 96 well plate, 25 μ l of sample solution was pre-incubated with 25 μ l of substrate Gly-Pro *p*-nitroanilide hydrochloride (6 mM) at 37 °C for 10 min. To initiate the reaction, 50 μ l of human DPP-IV (4.5 unit/ml) was added and incubated at 37 °C for 1 h. The reaction was terminated by adding 100 μ l of 1 M sodium acetate buffer, pH 4.0. The absorbance of liberated *p*-nitroanilide was measured at 405 nm using a Multiskan™ FC Microplate Photometer. Each test sample was analyzed in triplicate. Absorbance values were corrected with sample blanks in which DPP-IV was replaced with Tris-HCl buffer (100 mM, pH 8.0). The positive control (no inhibitor) used buffer in place of the sample. The negative control (no DPP-IV activity) used buffer instead of the DPP-IV solution. Percent DPP-IV inhibition was calculated as follows:

$$\text{DPP-IV inhibition (\%)} = 1 - \left[\frac{\text{Abs Sample} - \text{Abs Sample blank}}{\text{Abs positive control} - \text{Abs negative control}} \right]$$

3.6. Angiotensin converting enzyme (ACE) inhibitory activity

ACE-inhibitory activity was measured using the method described by (Martínez-Alvarez, Batista, Ramos, & Montero, 2016). The substrate Hippuryl-His-Leu (HHL) was hydrolyzed by ACE to hippuric acid (HA) and histidyl-leucine (HL). The relative amounts of liberated HA and HHL not cleaved were measured. Reactants were dissolved in 100 mM sodium phosphate buffer (pH 8.3) with 300 mM NaCl. CPH solutions (25 μ l) were combined with 25 μ l of substrate (HHL) and then incubated for 4 min at 37 °C. ACE was also incubated at the same time before being added. Then, 80 μ l of ACE (5 mU) was added to initiate the reaction and incubated at 37 °C in a water bath with constant stirring. Reaction was terminated with 50 μ l of 1 M HCl, and the solution was filtered using a 0.22 μ m nylon filter. The control reaction contained 25 μ l buffer in place of inhibitor (CPH). Finally, the amount product HA were quantified by high pressure liquid chromatography (HPLC) (Model 600E, Waters Corporation, Milford MA) on an analytical C18 column (YMC Pack ODS AM 12505-2546 wt, YMC America, Inc., Allentown, PA, USA). ACE inhibition was calculated as follows:

$$\text{ACE Inhibition (\%)} = \left(1 - \frac{A_{\text{inhibitor}}}{A_{\text{control}}} \right) \times 100$$

$A_{\text{inhibitor}}$ and A_{control} represent the relative areas (A) of the HA peaks with and without inhibitors. The half maximal inhibitory concentration (IC₅₀) was used to express the potency of the samples to inhibit ACE activity. The IC₅₀ value was expressed in mg of protein per ml. All IC₅₀ analyses were carried out at four different CPH concentrations (0.05, 0.5, 1, and 5 mg/ml). Each CPH sample was assayed in triplicate.

3.7. SDS-PAGE and immunoblotting

Samples were dissolved in zwitterionic-chaotropic buffer (2D-gel extraction buffer; 50 mM Tris-HCl, pH 8.8, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 M urea, 2 M thiourea, 2% w/v 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 67 mM Dithiothreitol (DTT) and shaken for 1 h at RT. Samples were prepared for SDS-PAGE by adding an aliquot to 2X Laemmli Sample Buffer (1mg/ml) and either used immediately or stored at –20 °C until used. Samples were loaded (10 μ l) onto 4–12% gradient gels (Bis Tris, NuPAGE, ThermoScientific, Waltham, MA) and ran with MES SDS running buffer (NuPAGE, ThermoScientific, Waltham, MA) at 200 V for 40 min. Gels were used for blotting or stained with Coomassie R-250 overnight with destaining overnight. Precision Plus Protein™ Dual Xtra Prestained Protein Standards (Biorad, Hercules, CA) were used as molecular weight standards.

Following SDS-PAGE, immunoblotting of CPH samples and unhydrolyzed control were performed as described by (Palmer, 2016). All sera samples were sourced by the Food Allergy Research and Resource Program at the University of Nebraska-Lincoln and used in a previous study where serological and ImmunoCAP® ISAC information is detailed (Palmer, 2016). Control sera (K and L) had no adverse reaction to shellfish and were subjected to ImmunoCAP® ISAC. Briefly, shrimp allergic sera or control sera (1:10 dilution) was used as the primary antibody with mouse anti-human IgE conjugated with horseradish peroxidase (1: 10,000; Southern Biotech, Birmingham, AL) as the secondary antibody. Immunoblots were visualized after development with a chemiluminescent substrate (Super Signal West Dura Extended Duration Substrate Kit; Thermo Scientific, Waltham, MA) according to manufacturer's instructions and using a Kodak Gel Logic 440 image station (Eastman Kodak, Rochester, NY) equipped with Kodak 1D v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT).

4. Results and discussion

4.1. Degrees of hydrolysis (DH)

It was established in a previous study that whole crickets (*G. si-gillatus*) can be hydrolyzed using alcalase (Hall et al., 2017). The h_{tot} used in the present study was 7.28 meq/g, whereas, (Hall et al., 2017) reported 8.64 meq/g for the same cricket species. Since h_{tot} depends on the number of total amino acids in the raw material, variations can exist from batch-to-batch, different sourced material, or processing treatments (i.e. whole cricket vs. cricket flour). Hence, it is imperative to determine h_{tot} for each substrate to accurately calculate DH.

In this work, various CPH were produced to evaluate the effects of hydrolysis (DH) on bioactivities and allergenicity. Predetermined enzyme-substrate (E/S) concentration and hydrolysis times were used to achieve CPH with a range of DH (15–85%) (Table 1). As expected, DH increased with higher E/S concentration and longer hydrolysis times. It was observed that for each E/S concentration, a 20-minute increase in hydrolysis time increased DH by approximately 10% (data not shown). Lower enzyme concentrations, 0.25% and 0.5%, were enough to achieve CPH ranging from 15 to 42% DH within 10 and 30 min. To achieve higher a DH (i.e. smaller peptides), E/S concentration was increased to 3% and hydrolyzed for 20, 40, 60 and 80 min, which produced CPH with 50–85% DH. After the pasteurization step, the control (no added enzyme) had a DH of 4.5%. This is a common observation as heat treatments can contribute to protein denaturation (Hall et al., 2017).

4.2. Antioxidant capacity

Antioxidant capacity of CPH before and after (CPHD) digestion with gastrointestinal enzymes (SGD) are shown in Table 2. Before SGD, antioxidant potential varied significantly amongst CPH. Hydrolysates showed lower ($p < 0.05$) ABTS scavenging values (except CPH with 85% DH) compared with the non-hydrolyzed cricket protein (control). CPH 15–30 (15–30% DH) had lower values than other CPH with no significant difference between them. However, the scavenging trend of DPPH radicals was opposite. CPH 15–40 had higher values while 50–85 and control showed the lowest (Table 2). The difference in scavenging patterns of ABTS and DPPH could be responsible for these observations. ABTS is more accessible to hydrophilic peptides, whereas hydrophobic peptides can readily interact with peroxy radicals such as DPPH (Chalamaiah et al., 2012). More substantially, scavenging activity of both ABTS and DPPH increased ($p < 0.05$) after SGD. All CPHD showed higher ABTS scavenging values. CPHD 20, 50, 60, 70, 85 and

control also showed higher DPPH scavenging after SGD. There was no difference between CPHD samples, suggesting that release of antioxidant rich peptides, after SGD, was similar in all CPH. Higher scavenging values can be a result of smaller molecular weight peptides (likely di- or tri-peptides), which have demonstrated to impart higher antioxidant potential. Contrasting results were observed in a study with cotton leaf worm (*Spodoptera littoralis*) hydrolysates, there were no differences in radical scavenging activity whether hydrolyzed with alcalase only or alcalase and gastrointestinal proteases (Vercruysse et al., 2009a).

The ability of CPH to act as a reducing component was measured by the FRAP assay (Table 2). Again, before SGD, only slight differences between the hydrolysates were observed. CPH 40–70 had the highest ($p < 0.05$) ferric ion reducing ability (897–991 $\mu\text{mol TE}$) and the non-digested protein (control) had negligible (near 0 $\mu\text{mol TE}$) activity. The reducing power of CPHD improved compared with CPH alone; FRAP values reached as high as 1439 $\mu\text{mol TE}$ (control). Strong reducing potential of protein hydrolysates has been attributed to the availability of hydrogen protons and electrons caused by cleavage of the peptide bond (Ketnawa & Liceaga, 2017). This explains the improvement in reducing potential of the control after SGD, as well as the improvement in all CPHD.

CPH also exhibited the ability to chelate metal ions (Fe^{2+}), with minor differences between samples (Table 2). CPH 50, 70 and 85 had the highest % MIC inhibition (53–71%), whereas CPH 15–40 and 60 had values ranging from 45 to 58% MIC inhibition. The control before SGD showed the lowest MIC inhibition (18.6%). The smaller peptides produced by SGD presented higher chelating properties in all samples, excluding CPH 50 which had no significant difference. Inhibition values reached as high as 83% (CPHD 85). Zielinska et al. also measured ABTS, DPPH, FRAP, and chelating activity of crickets (*G. sigillatus*), after various heat treatments. They found that each antioxidant activity (excluding FRAP) improved after heat treatments, compared with the raw, untreated crickets (Zielińska, Baraniak, & Karaś, 2017). Vercruysse and colleagues observed comparable trends with cotton leafworm (*S. littoralis*) protein hydrolysates. After hydrolysis with a combination of alcalase, gastrointestinal enzymes, or other proteases, antioxidant activity increased with only minor differences between the hydrolysates. Instead, results were attributed to other factors such as presence of certain amino acids and synergistic/antagonistic effects of mixed peptides (Vercruysse et al., 2009a). In a different study, Zielinska and their team (Zielińska et al., 2017) evaluated antioxidant potential of various insect proteins digested with gastrointestinal proteases, they found that the activity varied depending on the insect and not DH. In contrast, silkworm (*B. mori*) protein hydrolysates were prepared with different

Table 2
Antioxidant activities of cricket protein hydrolysates and their digests (CPHD) after SGD.

Sample Code ¹	ABTS ⁺ ($\mu\text{mol TE}/\text{mg sample}$)		DPPH ⁺ ($\mu\text{mol TE}/\text{mg sample}$)		FRAP ⁺ ($\mu\text{mol TE}/\text{mg sample}$)		MIC ⁺ (% MIC Inhibition)	
	CPH ²	CPHD ²	CPH ²	CPHD ²	CPH ²	CPHD ²	CPH ²	CPHD ²
15	381.1 $\pm$ 10.0 ^c	799.4 $\pm$ 8.2 ^{a*}	1458.5 $\pm$ 37.2 ^a	1590.8 $\pm$ 1.1 ^a	719.6 $\pm$ 5.0 ^g	1090.1 $\pm$ 22.4 ^{ab*}	45.3 $\pm$ 1.0 ^c	82.7 $\pm$ 2.9 ^{a*}
20	433.9 $\pm$ 3.8 ^d	772.0 $\pm$ 7.1 ^{a*}	1350.2 $\pm$ 13.9 ^b	1798.2 $\pm$ 5.3 ^{a*}	787.6 $\pm$ 5.6 ^f	1117.4 $\pm$ 18.6 ^{ab*}	48.4 $\pm$ 0.3 ^{cd}	81.8 $\pm$ 0.2 ^{ab*}
30	403.2 $\pm$ 8.1 ^c	721.4 $\pm$ 23.7 ^{ab*}	1490.5 $\pm$ 19.0 ^a	1575.9 $\pm$ 11.9 ^a	817.1 $\pm$ 6.7 ^e	1074.4 $\pm$ 4.1 ^{b*}	46.5 $\pm$ 3.4 ^{de}	78.2 $\pm$ 1.1 ^{ab*}
40	539.9 $\pm$ 9.9 ^{bc}	764.6 $\pm$ 27.1 ^{a*}	1433.0 $\pm$ 6.2 ^a	1622.9 $\pm$ 4.5 ^a	991.3 $\pm$ 5.5 ^a	1278.3 $\pm$ 18.8 ^{ab*}	58.5 $\pm$ 4.1 ^c	72.9 $\pm$ 0.6 ^{b*}
50	512.0 $\pm$ 4.2 ^c	775.1 $\pm$ 18.2 ^{a*}	1351.1 $\pm$ 6.0 ^b	1791.82 $\pm$ 7.9 ^{a*}	897.1 $\pm$ 8.6 ^d	1186.0 $\pm$ 16.9 ^{ab*}	71.8 $\pm$ 0.4 ^a	74.6 $\pm$ 1.0 ^a
60	663.3 $\pm$ 6.9 ^a	761.8 $\pm$ 32.3 ^{a*}	1144.9 $\pm$ 15.4 ^d	1740.7 $\pm$ 3.8 ^{a*}	929.3 $\pm$ 3.4 ^c	1241.3 $\pm$ 4.6 ^{ab*}	53.9 $\pm$ 0.2 ^{cd}	78.7 $\pm$ 0.9 ^{ab*}
70	550.7 $\pm$ 8.8 ^b	761.0 $\pm$ 21.5 ^{a*}	1224.7 $\pm$ 9.7 ^c	1926.3 $\pm$ 4.6 ^{a*}	964.8 $\pm$ 4.2 ^b	1208.7 $\pm$ 5.6 ^{ab*}	61.4 $\pm$ 2.0 ^{bc}	72.7 $\pm$ 6.0 ^{ab*}
85	516.8 $\pm$ 13.5 ^c	689.0 $\pm$ 13.8 ^{ab*}	872.4 $\pm$ 17.7 ^e	1628.2 $\pm$ 9.2 ^{a*}	906.6 $\pm$ 4.1 ^d	1137.4 $\pm$ 2.8 ^{ab*}	67.3 $\pm$ 0.3 ^{ab}	82.9 $\pm$ 2.6 ^{ab*}
Control	634.5 $\pm$ 7.1 ^a	584.2 $\pm$ 29.1 ^b	780.6 $\pm$ 19.3 ^f	1588.51 $\pm$ 6.7 ^{a*}	5.0 $\pm$ 0.0 ^{h*}	1439.7 $\pm$ 8.5 ^{a*}	18.6 $\pm$ 2.9 ^f	76.6 $\pm$ 0.6 ^{ab*}

¹ Sample codes descriptions are provided in Table 1.

² CPH represents cricket protein hydrolysates, hydrolyzed using alcalase excluding the control sample. CPHD represents the digests of each sample after simulated gastrointestinal digestion (SGD).

⁺ ABTS, DPPH, and FRAP results are expressed $\mu\text{mol Trolox equivalent per mg of sample}$ ($\mu\text{mol TE}/\text{mg}$). MIC results are expressed at % MIC inhibition. Values represent mean observations of triplicate analysis $\pm$ SD. Samples that do not share letters are significantly different ($p < 0.05$).

* Represent values that are significantly different before and after SGD.

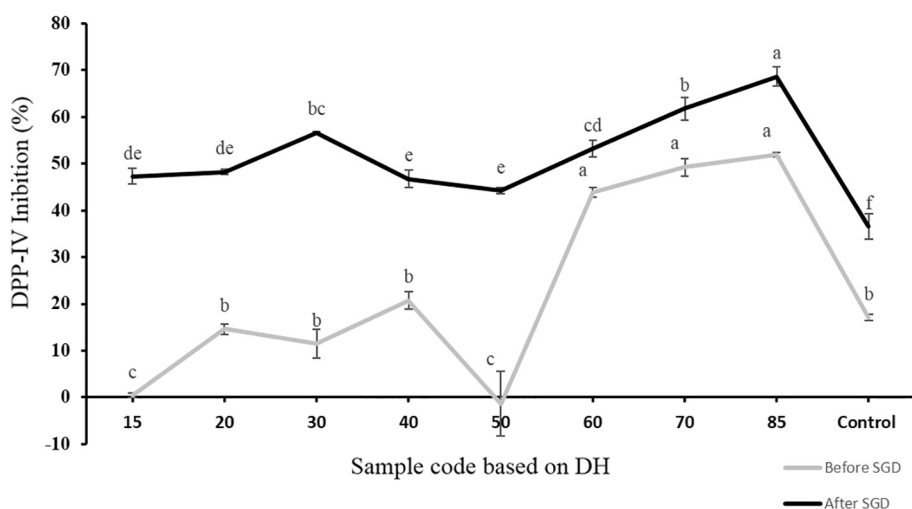


Fig. 1. Dipeptidyl peptidase IV (DPP-IV) Inhibition % of CPH and control before (black) and after the simulated gastrointestinal model (grey). Values represent mean observations of three replicates $\pm$ SD. Samples that do not share letters are significantly different ($p < 0.05$). There was significant difference ($p < 0.05$) between all CPH and CPHD.

proteases, including alcalase, and the results showed a correlation between DH and scavenging activity (Yang et al., 2013).

Overall, CPH exhibited high antioxidant activity that seemed independent of the DH, considering the minor differences between samples. As expected, the antioxidant capacity of CPH significantly increased ($p < 0.05$) after gastrointestinal digestion (Table 2), indicating that the digestive process *in vivo* may exert a beneficial effect on the release of bioactive compounds regardless of the antioxidant mechanism evaluated.

4.3. Dipeptidyl peptidase-4 inhibition (DPP-IV)

The results for the antidiabetic potential of CPH and CPHD at 1.25 mg/ml (final assay concentration) using human derived DPP-IV enzyme, are given in Fig. 1. All CPH, before SGD, had low DPP-IV inhibition ranging from 0 to 50%. After SGD, DPP-IV inhibition increased for all samples, with CPHD 60–85 exerting the highest ($p < 0.05$) inhibition between 62 and 69%. CPH composed of smaller molecular weight peptides (60–85% DH) showed the highest inhibitory activity both before and after SGD. In a recent study, Nongonierma, Lamoureux, and FitzGerald (2018) evaluated the DPP-IV inhibitory capacity of soluble protein hydrolysates from tropical banded cricket (*G. sigillatus*) powder; they also observed an improvement in activity following SGD of both a hydrolysate and non-hydrolyzed sample. This is not surprising since most peptides reported to have DPP-IV inhibitory activity are identified as di- or tri-peptides (Khaket, Redhu, Dhanda, & Singh, 2015; Lacroix & Li-Chan, 2012). A similar trend has been reported for other dietary proteins, such as salmon skin gelatin hydrolysates (Li-Chan, Hunag, Jao, Ho, & Hsu, 2012) and quinoa protein hydrolysates (Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015), where lower molecular weight peptides had higher inhibiting activity.

Kinetic studies reveal that DPP-IV catalytic activity is mostly determined by amino acid sequence and substrate structural characteristics (Lambeir, Durinx, Scharpé, & De Meester, 2003). Considering this, the ability of CPH to inhibit DPP-IV required successful binding to the active site and/or the enzyme-substrate complex. Thus, the inhibitory activity can be attributed to substrate-like features of CPH peptides that have interfered with the combination of DPP-IV and Gly-Pro-pNa (assay substrate). Results presented here also suggest that CPHD, after gastrointestinal digestion, may contain peptides structurally favorable to possess antidiabetic properties *in vivo*. Further research is required to justify this possibility.

Despite the lack of studies evaluating the antidiabetic properties of other insect-derived proteins, inhibition values presented in this work are comparable to other peptides in the literature. For example, hydrolysates from cuttlefish (*Sepia officinalis*) viscera showed up to 60%

inhibition at the end of *in vitro* gastrointestinal digestion (Cudennec et al., 2015). Similarly, silver carp (*Hypophthalmichthys molitrix*) hydrolysates had inhibition values near 55% after a 3-hour hydrolysis with alcalase (Zhang et al., 2016).

Porcine DPP-IV is most commonly used when assessing *in vitro* DPP-IV inhibitory activity of food-derived proteins (Khaket et al., 2015; Lacroix & Li-Chan, 2012; Li-Chan et al., 2012; Nongonierma et al., 2015). However, human and porcine DPP-IV are not identical (with only 88% overall sequence identity) which may cause variability among inhibition patterns (Lacroix & Li-Chan, 2015). In a comparison study, (Lacroix & Li-Chan, 2015) demonstrated that porcine DPP-IV was more susceptible to inhibition than the recombinant human enzyme. The authors also found differences in the mode of inhibition, attributing it to the inhibitor binding to less conserved regions of the porcine DPP-IV enzyme (Lacroix & Li-Chan, 2015). Despite lack of structural confirmation, porcine DPP-IV may have a different binding affinity, particularly to dietary proteins, which decreases the predictability or creates an overestimate of the inhibiting activity towards human DPP-IV. This suggests that CPH and CPHD can have stronger inhibiting effects towards porcine DPP-IV, compared with values presented in this work (Fig. 1). In this study, using human recombinant DPP-IV represents a relatively more accurate representation of the inhibition patterns of CPH and CPHD *in vivo*. The correlation between *in vitro* assays and *in vivo* results rely heavily on the accuracy of the *in vitro* techniques, as well as the biological origin of the enzymes. Thus, we demonstrated that CPH and CPHD peptides could inhibit human recombinant DPP-IV activity up to 52% before and 69% after SGD, depending on enzyme concentration and hydrolysis time.

4.4. Angiotensin converting enzyme (ACE) inhibitory activity

Inhibiting the angiotensin converting enzyme action is now one of the major options for treating hypertension (Li-Chan, 2015). Food-derived bioactive peptides are being assessed for potential to lower blood pressure by inhibiting ACE. Results are expressed as a percentage of product formed (HA) to relative substrate (HHL) used. In this study, IC_{50} values were determined for CPH and CPHD samples that inhibited ACE $> 70\%$. Inhibition percentages and IC_{50} values are shown in Table 3.

Overall, ACE inhibition increased with DH. This observation was consistent in each CPH sample, except CPH 50 which had the lowest values amongst all samples tested. CPH 40 and 60–85 all inhibited ACE activity $> 80\%$ and $> 90\%$, respectively, at the concentration tested (5 mg/ml). To evaluate the effect of gastrointestinal proteases, ACE inhibition of CPHD 40 and 60–85 were also measured. Compared to CPH-60-85 before SGD, ACE inhibition decreased slightly in the CPHD

Table 3
Angiotensin I Converting Enzyme Inhibition (%).

Sample Code ¹ (based on DH)	ACE Inhibition ² (%)	IC ₅₀ ³ (mg/mL)
Control	60.1 ± 1.3 ^d	–
15	45.3 ± 3.6 ^f	–
20	51.6 ± 1.8 ^e	–
30	67.4 ± 0.1 ^c	–
40	82.4 ± 0.2 ^{b*}	0.096 ± 0.007 ^{a*}
50	37.1 ± 2.8 ^g	–
60	95.3 ± 0.0 ^{a*}	0.040 ± 0.002 ^{b*}
70	96.4 ± 0.1 ^{a*}	0.015 ± 0.001 ^{c*}
85	95.2 ± 0.1 ^{a*}	0.033 ± 0.002 ^{b*}
CPHD-40	90.0 ± 0.4 ^{b*}	0.089 ± 0.008 ^{a*}
CPHD-60	92.8 ± 0.4 ^{a*}	0.066 ± 0.001 ^{b*}
CPHD-70	92.6 ± 0.1 ^{a*}	0.062 ± 0.000 ^{b*}
CPHD-85	92.8 ± 0.4 ^{a*}	0.063 ± 0.004 ^{b*}

¹ Sample codes descriptions are provided in Table 1. CPHD represents the SGD digests of the sample.

² Angiotensin I converting enzyme (ACE) inhibition % at 5 mg/mL protein concentration.

³ The half maximal inhibitory concentration (IC₅₀) were determined for CPH that showed > 70% inhibition at 5 mg/mL and their respective CPHD. Values represent mean observations of triplicate analysis ± SD. Samples that do not share letters are significantly different ($p < 0.05$).

* Represent a significant ($p < 0.05$) difference between CPH and CPHD.

(Table 3). IC₅₀ values also increased (lower ACE inhibition) for CPHD 60–85. Conversely, CPH 40 had greater ACE inhibition (%) and lower IC₅₀ values, after SGD (CPHD-40). Results suggest that extensive hydrolysis (i.e. extensive peptide cleavage) can decrease ACE inhibition capacity. This is illustrated in CPHD-60–85, which required higher IC₅₀ concentrations than the alcalase hydrolyzed sample (CPH-60–85). Nonetheless, ACE was inhibited > 90% with CPH- and CPHD-60, 70 and 85.

There are some reports of ACE inhibiting potential of other hydrolyzed insect proteins. The trend observed in this study is in compliance with the data presented by (Vercruyse, Smagghe, Herregods, & Van Camp, 2005). In their work, the various insect protein hydrolysates prepared with gastrointestinal proteases inhibited *in vitro* ACE activity more than the alcalase hydrolysates and unhydrolyzed protein (Vercruyse et al., 2005). IC₅₀ values in our study were all lower than the values presented by Vercruyse and others (Vercruyse et al., 2005), meaning higher ACE inhibiting capabilities of the CPH and CPHD. For example, in their study, the lowest IC₅₀ value (0.588 mg/ml ± 1.134) was generated from silkworm (*bombyx mori*) protein hydrolysates produced from *in vitro* gastrointestinal proteolysis. Whereas in our study, IC₅₀ values ranged from 0.051 to 0.089 mg/ml by the CPHD (after gastrointestinal digestion). Lower IC₅₀ values from the various insect protein hydrolysates may be the result of excessive hydrolysis. The authors reported DH values as high as 90% for all insect protein hydrolysates (Vercruyse et al., 2005). Greater inhibiting capacity could also be attributed to differences in insect species or the fact that sequential digestion (alcalase followed by gastrointestinal proteases) was used in this work. However, in another study, alcalase hydrolysis followed by gastrointestinal proteolysis on water-soluble protein fractions of cotton leafworm (*S. littoralis*) did not increase the ACE inhibiting activity (Vercruyse et al., 2009a). ACE inhibiting values generated by CPH and CPHD are lower or similar in range with other ACE inhibiting food protein hydrolysates such as date seeds (Ambigaipalan, Al-Khalifa, & Shahidi, 2015), flounder fish muscle (Ko et al., 2016), and carp muscle tissue (Borawska, Darewicz, Vegarud, Iwaniak, & Minkiewicz, 2015).

4.5. Effects of enzyme hydrolysis on allergenicity

Palmer (Palmer, 2016), identified cross-reactive allergens

(primarily tropomyosin) in house crickets (*A. domesticus*) using IgE immunoblotting with shrimp allergic human sera. Western blots showed that patient IgE positively reacted with the tropomyosin band at 37 kDa (Palmer, 2016). With known reactivity to shrimp, control tropomyosin, and house cricket, the same patient serum was used to measure IgE reactivity in the tropical banded cricket (*G. sigillatus*) protein hydrolysates used in this study.

Western blots (Fig. 2A–L) show that all ten shrimp-allergic sera reacted positively to the control (unhydrolyzed cricket protein) showing a band at 37 kDa, corresponding to intact tropomyosin (Palmer, 2016) (Huang, Liu, Cai, Weng, Maleki, Su et al., 2010). At the same concentration, all sera also reacted to tropomyosin in CPH 10–50. Compared with the unhydrolyzed control, IgE binding was lower in CPH hydrolyzed 15–40% showing a much lighter tropomyosin band. Interestingly, CPH 50 (52% DH) reactivity was the same as the control with a strong band, particularly in sera shown in Fig. 2A, E, F and H–J. Conversely, CPH hydrolyzed 60–85% showed no tropomyosin bands at 37 kDa, possibly because reactivity was abolished at the higher DH. Three of the ten sera (Fig. 2A, C, and F) reacted to bands around and below 20 kDa in all samples, which could be proteolytic fragments of tropomyosin. The bands could also be an indication of sarcoplasmic calcium binding or myosin light chain proteins that have been identified as a shellfish allergen at 20 kDa (Ayuso et al., 2008). Moreover, the control (atopic and non-atopic) sera (Fig. 2K and L) did not react to any CPH sample or unhydrolyzed control.

From these findings, it is evident that IgE reactivity to tropomyosin was altered by alcalase hydrolysis. Depending on the extent of hydrolysis (% DH), reactivity decreased (CPH 10–40), showed no change (CPH 50), or was eliminated (CPH 60–85). The decreased antibody to antigen IgE binding in CPH 10–40 and 60–85 may be a result of proteolytic hydrolysis of the conformational tropomyosin epitopes (Mills, Madsen, Shewry, & Wichers, 2003). There is also a possibility that the smaller peptides in CPH 60–85 are less immobilized by the immunoblots. Whereas, enzymatic treatment under CPH 50 conditions (Table 1), may have exposed previously inaccessible tropomyosin epitopes, leading to an unchanged or even increased reactivity (Fig. 2A–J).

In literature, tropomyosin is usually reported as heat stable and resistant to gastrointestinal digestion (Palmer, 2016). However, similar results to this study have been reported by others who observed changes in tropomyosin IgE binding after treatment with gastrointestinal enzymes (Gámez et al., 2015; Huang et al., 2010; Mejrhit, Azdad, Chda, El Kabbou, Bousfiha, Bencheikh et al., 2017). For example, (Mejrhit et al., 2017) found that shrimp tropomyosin IgE binding was decreased after heat and pepsin treatments. Unfortunately, the effects of commercial enzymes, such as alcalase, on tropomyosin reactivity have been scarcely reported. In this study, we demonstrated that hydrolyzing cricket protein with 3% (w/w) alcalase to achieve 60–85 DH, changed the binding characteristics of IgE (from shrimp allergic human sera) to tropomyosin in crickets (*G. sigillatus*). Our results also indicate that the extent of hydrolysis (% DH) was influential in the tropomyosin reactivity. Further structural analysis should be conducted to evaluate the conformational changes that resulted in an increase or decrease of reactivity. More importantly, these observations emphasize a potential to obtain bioactive peptides from whole tropical banded crickets via enzymatic hydrolysis conditions used in this study, which would not result in an allergic reaction to shellfish allergic individuals.

5. Conclusion

This study demonstrates that cricket protein hydrolysates generated using alcalase could exert multiple *in vitro* bioactive properties. Antioxidant properties were independent of DH, whereas DPP-IV inhibiting values seemed higher in CPH with increased hydrolysis (higher %DH, smaller peptides). Simulated gastrointestinal digestion improved ($p < 0.05$) DPP-IV potential in all CPH. Alcalase hydrolysis alone was

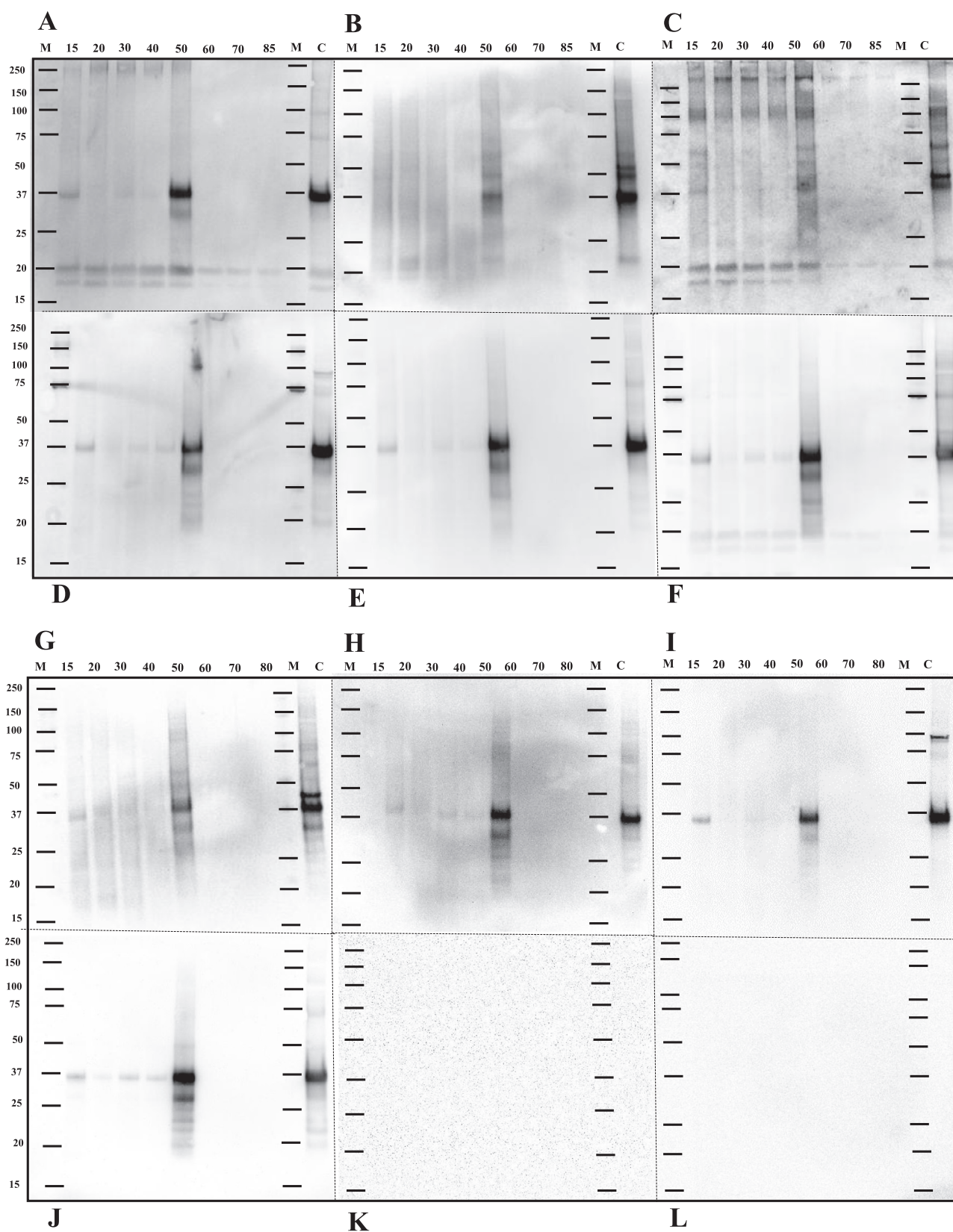


Fig. 2. (A–L). IgE and control immunoblots of cricket protein hydrolysates and unhydrolyzed cricket. A 10 µg of sample protein were separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane and subjected to immunoblotting with shrimp allergic sera (A–J), atopic control sera (K) and non-atopic control sera (L). Exposure times were 2 min each. Samples per lane are: molecular markers (M), numbers 15–85 represent sample codes for CPH 15–85 (descriptions provided in Table 1), and unhydrolyzed cricket (C). Both control sera (K and L) had no reactivity to any CPH sample.

successful at producing peptides that can readily inhibit *in vitro* ACE activity, especially those peptides with higher DH. Gastrointestinal digestion significantly increased their ACE inhibiting activity but the IC₅₀ values decreased slightly in CPHD 60–85.

Most of the CPH had lower tropomyosin IgE reactivity from shrimp allergic sera, compared with unhydrolyzed cricket. However, hydrolyzing cricket protein to 60–85% DH was necessary to completely remove the IgE reactivity to tropomyosin. Interestingly, CPH 50 (52% DH) showed similar reactivity to that of the unhydrolyzed control sample and thus requires further investigation.

These findings provide preliminary data to support the potential of using cricket protein hydrolysates as a natural source for bioactive peptides. Further research is needed to determine the bioavailability of CPH peptides. Additional research is also essential to isolate potent peptides for each bioactivity and to assess ACE and DPP-IV modes of inhibition, mechanisms, as well as their efficacy *in vivo*. In terms of allergenicity, the structural characteristics of tropomyosin in the CPH need to be investigated to elucidate the cause of increased/decreased IgE binding observed in this study. Nevertheless, cricket protein proved to be a suitable source of biologically active peptides to generate multifunctional hydrolysates that could be incorporated into functional foods or used as nutraceuticals as natural alternatives to anti-hypertensive and antidiabetic drugs or synthetic antioxidants.

Author contributions

This work was carried out in collaboration between all authors. Authors F. Hall, P. Johnson and A. Liceaga all contributed in the design of the study. F. Hall collected the data, wrote the first draft of the manuscript, carried out the literature searches. All authors read, revised and approved the final manuscript.

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Conflict of interest

Authors have declared that no competing interests exist. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

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