BIOACTIVE AND ALLERGENIC PROPERTIES OF EDIBLE CRICKET (*GRYLLODES SIGILLATUS*) PEPTIDES

By

Felicia G. Hall

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STATEMENT OF COMMITTEE APPROVAL

Dr. Andrea Liceaga, Chair
Department of Food Science, Purdue University

Dr. Kee-Hong Kim
Department of Food Science, Purdue University

Dr. Lavanya Reddivari
Department of Food Science, Purdue University

Dr. Philip Johnson
Department of Food Science and Technology, University of Nebraska-Lincoln.

Approved by:
Dr. Arun Bhunia

I dedicate this to my father, Ray, and grandmother, Andrea, who spoke success over me since birth. Although they were not alive to see it, I hope I made them both proud. This would also have not been possible without the unwavering support of my mother, Sharmaine, brother, Jordan, and husband, Snook, who have pushed, encouraged, and spoiled me throughout this entire journey, even when miles away.

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LIST OF ABBREVIATIONS

ACE 2: Angiotensin converting enzyme 2

ACE: Angiotensin I converting enzyme

Ang I: Angiotensin I

Ang II: Angiotensin II

CGMP: cyclic guanosine-monophosphate

CPH: cricket protein hydrolysates

CPHD: cricket protein hydrolysates digesta (after SGID)

CVDs: Cardiovascular diseases

ELISA: Enzyme-linked immunosorbent assay

eNOS: endothelial nitric oxide synthase

HUVEC: Human umbilical vein endothelial cells

ICAM-1 : Intercellular adhesion molecule 1

IL : interleukin

iNOS : inducible nitric oxide synthase

IκB : inhibitor kappaB

MCP-1: Monocyte chemotactic protein-1

MW: Microwave

NF-κB: Nuclear factor kappaB

NO: Nitric oxide

NOS: Nitric oxide synthase

RAAS: Renin angiotensin system

ROS: Reactive oxygen species

SGID: Simulated gastrointestinal digestion

SHR: Spontaneously hypertensive rats

TNF: Tumor necrosis factor

VCAM-1: Vascular adhesion molecule-1

ABSTRACT

Cardiovascular diseases (CVD) and their risk factors remain the leading cause of morbidity and mortality in North America. Food-derived bioactive peptides (BAP) have been shown to play a role in regulating physiological pathways of CVD risk factors including hypertension, diabetes, and chronic inflammation. Common sources of BAP include dairy and plant proteins. In addition to being an alternative protein source, it is now accepted that edible insect proteins can also confer health benefits beyond nutrition. However, as with any novel protein source, allergenicity remains a major concern surrounding edible insect consumption.

This dissertation aimed to: 1) Evaluate the bioactive potential of peptides from an edible cricket species and; 2) determine the effects of BAP production methods on immunoreactivity. First, peptide-rich extracts were generated from farmed food-grade crickets via enzymatic hydrolysis techniques with the commercial protease Alcalase™. To measure the *in vitro* bioavailability, cricket peptides were also subject to simulated gastrointestinal digestion (SGD). Peptides and their digests were tested for relevant bioactivities and active groups were further fractionated by chromatographic methods to identify the major peptides responsible for the bioactivity. When tested for *in vitro* antihypertensive and anti-glycemic properties, cricket peptides were found to inhibit the activities of angiotensin converting enzyme, dipeptidyl peptidase-4, α-amylase, and α-glucosidase. The anti-inflammatory potential was expounded using RAW-264.7 macrophages and human umbilical vein endothelial cells (HUVEC). Cricket peptides (after SGD) effectively lowered NF-κB, MCP-1, and IL-6 production in cells without affecting their viability. Proteomic analyses identified 18 sequences from the enriched cationic peptide fraction that showed the highest activity. Three novel peptides were identified via molecular docking, as potent ACE-inhibitors and binding was similar to that of the commercial drug captopril. Key binding characteristics included interaction with hydrophobic amino acids (Phe, Pro, Leu) near the C-terminal position and coordination with Zn (II) ions near the ACE active site.

Immunological reactivity was measured by IgE-binding from shrimp-allergenic patient sera to antigens present within cricket peptides. Our studies demonstrate that immunoreactivity was impacted by enzymatic hydrolysis, depending on the conditions and heating source used. Tropomyosin (a major shrimp allergen) was extracted from both untreated crickets and protein hydrolysates, and verified as the major reactive protein. Tropomyosin reactivity decreased (under

both partial and extensive hydrolysis) or retained (under conditions which prevented epitope cleavage). However, using microwave-assisted enzymatic hydrolysis-was effective at decreasing tropomyosin reactivity in all immunoassays tested (IgG and IgE). Proteomic and immunoinformatic analyses revealed prominent allergen binding regions of cricket tropomyosin available for cleavage during enzymatic hydrolysis. Conserved antigen regions showed greater homology with other crustacean species, but not with other well studied allergenic insect proteins (i.e., cockroach). Lastly, LC-MS/MS and FT-Raman spectrometry suggests that reactivity was affected due to distinct epitope cleavage within the protein instead of decreased antigen extractability/solubility.

The findings of this dissertation support that edible cricket proteins are a potential source of bioactive peptides for functional food or nutraceutical development. Additionally, using protein extraction methods such as microwave-assisted hydrolysis seems a promising tool for minimizing the immunoreactivity of the allergen present in this edible cricket species.

CHAPTER 1. GENERAL INTRODUCTION

Cardiovascular diseases (CVD) remain the leading cause of morbidity and mortality for men, women, and most racial and ethnic groups within North America (Sievenpiper & Lavie, 2018). CVD afflicts nearly 121.5 million adults (estimates for 2013-2016), of that, 29 million suffered from type 2 diabetes (T2D) and 13.34 million from hypertension. T2D and hypertension are regarded as major life-style related diseases and as such, changes in key health factors such as physical activity, regulating alcohol consumption, mitigating overweight/obesity, and a healthy diet intervention can reduce the risk of CVD and its risk factors (Association, 2017; Estruch et al., 2006). Both diseases are also common comorbidities. Patients with T2D are twice as likely to experience hypertension than those without (Sowers, Epstein, & Frohlich, 2001). Moreover, hypertensive individuals often experience insulin resistance and are at higher risks of developing T2D (Sowers et al., 2001). As such, T2D and hypertension are interlinked by similar risk factors such as obesity, endothelial dysfunction, vascular inflammation, arterial remodeling, and atherosclerosis (Petrie, Guzik, & Touyz, 2018; Sowers, 2013). Similar, these diseases share some cardiovascular complications such as oxidative stress, chronic inflammation, up-regulation of the renin-angiotensin-aldosterone system (RAAS) and activation of the immune system (Petrie et al., 2018).

Among other factors, onset and progression of diabetes, hypertension, and chronic inflammation can be heavily regulated by diet (Estruch et al., 2006; Mohamed, 2014). Several studies have demonstrated reduced systemic inflammation following a diet prescribed to be effective in patients with T2D and inflammation (Nowlin, Hammer, & D'Eramo Melkus, 2012). For example, implantation of the Dietary Approached to Stop Hypertension (DASH) diet, which is characterized by a high intake in fruits, vegetables, and whole grains, was also effective in reducing C-reactive protein (elevated levels linked T2D and inflammation) (Nowlin et al., 2012). Subsequent research studies have demonstrated that biologically active compounds in foods are largely responsible for the disease attenuating effects of the DASH diet (Erdmann, Cheung, & Schröder, 2008; Lutz, Fuentes, Ávila, Alarcón, & Palomo, 2019; Most, 2004; Rangel-Huerta, Pastor-Villaescusa, Aguilera, & Gil, 2015). Knowing this, many active compounds were/are characterized for their potential role towards CVD treatment and/or prevention. As such, there is

an increasing interest in exploring bioactive food components or functional foods as an alternative therapy for disease management.

1.1 Bioactive Food Peptides and Protein in Health

Years of scientific evidence have confirmed that food derived bioactive compounds can have beneficial effects on human health. Bioactive substances, in general, are defined as "food components that can affect biological processes or substrates and, hence, have an impact on body function or condition and ultimately human health" (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). To significantly impact biological processes, these dietary components must be consumed in large enough quantities. Therefore, the definition of a 'bioactive food substance' applies with two major considerations:

1. To classify as "bioactive", a dietary component should impart a measurable biological effect at a physiologically realistic level (Perez-Gregorio & Simal-Gandara, 2017; Guaadaoui et al., 2014)
2. The "bioactivity" should have the potential to affect human health in a positive way. This excludes biologically active effects such as toxicity, allergenicity, and mutagenicity (Guaadaoui et al., 2014).

Aside from plant derived bioactive components such as phenolic and phytochemical compounds, **food peptides** can also perform physiochemical roles that promote human health. The primary function of dietary proteins is to supply the body with appropriate amounts of indispensable amino acids and organic nitrogen to maintain adequate protein status in healthy individuals. In terms of proteins' nutritional significance, it is one of the major macronutrients used by the body for building repairing and maintaining tissues. An additional nutritive effect was discovered, in 1979, when peptides with opioid activity were isolated for the first time from partial proteolysis of milk proteins (Schlimme & Meisel, 1995). It was an instrumental discovery to know that bioactive peptides can provide nutritional as well as nonnutritive health benefits. Since then, peptides from dietary proteins have been studied for potential modulators of various regulatory processes in the body to benefit human health (Möller et al., 2008). Bioactive peptides vary in size and structure, but typically comprise of 2-20 amino acid residues, and in some cases, contain more than 20. Depending on residue sequence, dietary bioactive peptides can exhibit diverse activities for moderating human health, including; neurological (i.e. opioid peptides), cardiovascular (i.e.

antihypertensive peptides and anti-inflammatory), and digestive (i.e. immunomodulatory peptides) systems, as well as bone health (i.e. calcium-binding peptides) and weight management (i.e. anti-obesity and satiety-inducing peptides) **(Table 1).** It is often reported that bioactive peptides are multifunctional and can exert numerous health promoting physiological activities after digestion (Agyei, Potumarthi, & Danquah, 2015; Lammi, Aiello, Boschin, & Arnoldi, 2019). At present, milk proteins seem to be the greatest source of bioactive peptides and the most intensively studied; likely because of its role in our early-life stages, daily milk intake, and its products proved to be physiologically important to infants and adults (Burgess, 2014). Peptides with bioactivities, however, have been released from numerous food plant and animal sources.

In addition to traditional protein sources, underused protein rich foods such as marine by-products (i.e. fish and shellfish waste), meat industry by-products (i.e. deboning and trimming), industrial food waste (i.e. fruit, vegetable residues), edible insects (i.e. silk moth and crickets), as well as, grains, seeds, and legumes are viable alternatives to the human diet (Aguilar-Toalá, Deering, & Liceaga, 2020; Aguilar-Toalá & Liceaga, 2020; Hall, Johnson, & Liceaga, 2018; Harnedy & FitzGerald, 2012; Nongonierma & FitzGerald, 2017; Ryder, Bekhit, McConnell, & Carne, 2016; Urbizo-Reyes, San Martin-González, Garcia-Bravo, Vigil, & Liceaga, 2019). Due to their health promoting effects, proteins/peptides from these sources are used as ingredients or additives on the development of functional food products (Korhonen & Pihlanto, 2003; Li-Chan, 2015). As with any novel protein ingredient, allergenicity of bioactive peptide-containing functional foods should be considered since at-risk individuals can show allergic reactions after ingestion or even non-oral contact (Hartmann, Wal, Bernard, & Pentzien, 2007; Liu et al., 2020; Meisel, 2007).

1.2 References

Aguilar-Toalá, J., Deering, A., & Liceaga, A. (2020). New Insights into the Antimicrobial Properties of Hydrolysates and Peptide Fractions Derived from Chia Seed (Salvia hispanica L.). *Probiotics and antimicrobial proteins*.

Aguilar-Toalá, J., & Liceaga, A. (2020). Identification of chia seed (Salvia hispanica L.) peptides with enzyme inhibition activity towards skin-aging enzymes. *Amino Acids*, 1-11.

Agyei, D., Potumarthi, R., & Danquah, M. K. (2015). Food-derived multifunctional bioactive proteins and peptides: Sources and production. *Biotechnology of bioactive compounds. Sources and applications. Wiley, Oxford*, 483-506.

Association, A. D. (2017). 9. Cardiovascular disease and risk management. *Diabetes Care, 40*(Supplement 1), S75-S87.

Burgess, K. (2014). Milk and Dairy Products in Human Nutrition (2013), by E. Muehlhoff, A. Bennett and D. McMahon, Food and Agriculture Organisation of the United Nations (FAO), Rome. E-ISBN: 978-92-5-107864-8 (PDF). Available on web-site (publications-sales@ fao. org). *International Journal of Dairy Technology, 67*(2), 303-304.

Erdmann, K., Cheung, B. W., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry, 19*(10), 643-654.

Estruch, R., Martínez-González, M. A., Corella, D., Salas-Salvadó, J., Ruiz-Gutiérrez, V., Covas, M. I., . . . Vinyoles, E. (2006). Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Annals of internal medicine, 145*(1), 1-11.

Guaadaoui, A., Benaicha, S., Elmajdoub, N., Bellaoui, M., & Hamal, A. (2014). What is a bioactive compound? A combined definition for a preliminary consensus. *International Journal of Nutrition and Food Sciences*, *3*(3), 174-179.

Hall, F., Johnson, P. E., & Liceaga, A. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food chemistry, 262*, 39-47.

Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods, 4*(1), 6-24.

Hartmann, R., Wal, J.-M., Bernard, H., & Pentzien, A.-K. (2007). Cytotoxic and allergenic potential of bioactive proteins and peptides. *Current pharmaceutical design, 13*(9), 897-920.

Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides-opportunities for designing future foods. *Current pharmaceutical design, 9*(16), 1297-1308.

Lammi, C., Aiello, G., Boschin, G., & Arnoldi, A. (2019). Multifunctional peptides for the prevention of cardiovascular disease: A new concept in the area of bioactive food-derived peptides. *Journal of Functional Foods, 55*, 135-145.

Li-Chan, E. C. (2015). Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Current Opinion in Food Science, 1*, 28-37.

Liu, L., Li, S., Zheng, J., Bu, T., He, G., & Wu, J. (2020). Safety considerations on food protein-derived bioactive peptides. *Trends in food science & technology, 96*, 199-207.

Lutz, M., Fuentes, E., Ávila, F., Alarcón, M., & Palomo, I. (2019). Roles of phenolic compounds in the reduction of risk factors of cardiovascular diseases. *Molecules, 24*(2), 366.

Meisel, H. (2007). Editorial [Hot Topic: Food-Derived Bioactive Proteins and Peptides as Potential Components of Nutraceuticals (Executive Editor: H. Meisel)]. *Current pharmaceutical design, 13*(9), 873-874.

Mohamed, S. (2014). Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovasular disease. *Trends in food science & technology, 35*(2), 114-128.

Möller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: indication for health effects. *European journal of nutrition, 47*(4), 171-182.

Most, M. M. (2004). Estimated phytochemical content of the dietary approaches to stop hypertension (DASH) diet is higher than in the Control Study Diet. *Journal of the American Dietetic Association, 104*(11), 1725-1727.

Nongonierma, A. B., & FitzGerald, R. J. (2017). Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review. *Innovative Food Science & Emerging Technologies, 43*, 239-252.
Nowlin, S. Y., Hammer, M. J., & D'Eramo Melkus, G. (2012). Diet, inflammation, and glycemic control in type 2 diabetes: an integrative review of the literature. *Journal of nutrition and metabolism, 2012*.
Petrie, J. R., Guzik, T. J., & Touyz, R. M. (2018). Diabetes, hypertension, and cardiovascular disease: clinical insights and vascular mechanisms. *Canadian Journal of Cardiology, 34*(5), 575-584.
Perez-Gregorio, R., & Simal-Gandara, J. (2017). A critical review of bioactive food components, and of their functional mechanisms, biological effects and health outcomes. Current Pharmaceutical Design, 23(19), 2731-2741.
Rangel-Huerta, O. D., Pastor-Villaescusa, B., Aguilera, C. M., & Gil, A. (2015). A systematic review of the efficacy of bioactive compounds in cardiovascular disease: phenolic compounds. *Nutrients, 7*(7), 5177-5216.
Ryder, K., Bekhit, A. E.-D., McConnell, M., & Carne, A. (2016). Towards generation of bioactive peptides from meat industry waste proteins: Generation of peptides using commercial microbial proteases. *Food chemistry, 208*, 42-50.
Schlimme, E., & Meisel, H. (1995). Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Molecular Nutrition & Food Research, 39*(1), 1-20.
Sievenpiper, J. L., & Lavie, C. J. (2018). Dietary prevention of cardiovascular diseases. *Progress in cardiovascular diseases, 61*(1), 1-2.
Sowers, J. R. (2013). Diabetes mellitus and vascular disease. *Hypertension, 61*(5), 943-947.
Sowers, J. R., Epstein, M., & Frohlich, E. D. (2001). Diabetes, hypertension, and cardiovascular disease: an update. *Hypertension, 37*(4), 1053-1059.
Urbizo-Reyes, U., San Martin-González, M. F., Garcia-Bravo, J., Vigil, A. L. M., & Liceaga, A. M. (2019). Physicochemical characteristics of chia seed (Salvia hispanica) protein hydrolysates produced using ultrasonication followed by microwave-assisted hydrolysis. *Food Hydrocolloids, 97*, 105187.

CHAPTER 2. LITERATURE REVIEW AND RESEARCH OBJECTIVES

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Liceaga, A.M., & Hall, F., 2019. Nutritional, Functional and Bioactive Protein Hydrolysates. In: Melton, L., Shahidi, F., Varelis, P. (Eds.), Encyclopedia of Food Chemistry, vol. 3, pp. 456–464. Elsevier. ISBN: 9780128140260

The expectation of food as a venue for essential nutrients for life sustenance and growth has shifted towards the prospect of curing and/or preventing various forms of diseases (Tadesse & Emire, 2020). Moreover, lifestyle changes and socioeconomic trends, worldwide, demand

consumption and development of foods with increased health benefits (Betoret, Betoret, Vidal, & Fito, 2011). Producing bioactive peptides with multifunctional activities from food sources constitutes an attractive approach since they can be used to safely enhance the nutritional quality of food products. Bioactive peptides can be used as ingredients in functional foods, nutraceuticals, dietetic supplements, or as "natural" therapeutics, aimed at prevention and treatment of ailments as well as benefiting overall health (Mohamed, 2014) (**Table 2.2**). Over the last decade, interest in functional foods and nutraceuticals with bioactive peptides and protein hydrolysates has increased (Li-Chan, 2015). As the global market of bioactive peptides increases, functional food products continue to be developed (**Table 2.1**). Consequently, there has also been a surge of scientific research assessing the potentials of various food sources for bioactive peptides. Although they are usually much less potent than synthetic pharmaceutical drugs, dietary bioactive peptides are less likely to cause major side effects due to their natural ability to be metabolized and excreted. There are still, however, major challenges to commercialization including industrial scale production, inadequate proof of bioactivity, and low bioavailability and metabolic fate (Li-Chan, 2015). As research continues to develop more effective and efficient ways of production and utilization of food resources, research involving the discovery and potential of new food biologically active peptides (bio-peptides) is equally as important

Table 2.1. Commercial products available on the market with bioactive peptides/protein hydrolysates, their food source, health claims and manufacturer.

Product name	Health benefits	Food Product	Bioactive peptides/protein source	Manufacturer
Lactoprodan® Hydro 365, ALPHA, Whey Protein	Regulates blood sugar and improves synthesis of muscle glycogen stores	Whey protein hydrolysate (ingredient)	Whey and casein	Arla Food Ingredients
SureStart™ 917 and 948	Promotes digestive ease and hypoallergenic	Whey and milk protein Hydrolysate (ingredient)	Whey and milk	NZMP (Fonterra)
Beautycoll® (Peptan)	Promotes healthy ageing, joint and bone health, mitigates skin aging, supports connective tissues	Collagen peptides (ingredient)	Collagen	Beautycoll
ProMod Liquid Protein Fruit Punch (Pro-Stat)	Promotes ulcer healing	Collagen hydrolysate (beverage)	Collagen	Abbott
Capolac®	Improves calcium absorption	Casein phosphopeptides (ingredient)	Milk	Arla Food Ingredients
Prodiet F200/Lactium	Stress-relieving effects	Milk casein hydrolysate (capsules, tablets, powder, and drinks)	Milk	Ingredia
Bonito peptide, Vasotensin®	Helping to regulate the Angiotensin Converting Enzyme (ACE)	Bonito derived peptide (ingredient), Dietary supplements	Bonito fish	Nippon Supplement Inc, Metagenics

Seacure®	Promotes wound healing and supports immune system regulation	Protein dietary supplement	Pacific whiting fish	Proper Nutrition Inc.
Fortide	Improving feed efficiency, nutrient digestion and intestinal histology	Soybean bioactive peptide (ingredient)	Soybean	Chengdu Mytech Biotech Co. Ltd
Calpis	Angiotensin Converting Enzyme (ACE) inhibiting activity	Sour milk (beverage)	Milk	Calpis Co.
Valtyron	Angiotensin Converting Enzyme (ACE) inhibitory effect	Supplement in health drinks, vegetable juices and beverages	Sardine muscle	Senmi Ekisu Co.
PEPTIBAL®	Maintains healthy gut immune system reduces the inflammation	Protein hydrolysates in capsules	Shark	Virage Santé INC.,
Verisol	improves skin physiology	Protein powder	Porcine and bovine	Gelita
PeptAIde™	Help modulate inflammation	Brown rice derived peptides suitable for sports nutrition formats	Brown rice	BASF

Table 2.2. Types of Bioactive Peptides and Key Structural Features.

Activity	Structural elements	Remarks	Reference
ACE inhibitory	Pro or hydroxy-Pro as C-terminus	Usually resistant to degradation by digestive enzymes	(Matsufuji et al., 1994; Vermeirssen, Van Camp, & Verstraete, 2004)
	Pro, Lys or Arg as C-terminus	Preferred C-terminal residues with contribution to the ACE inhibitory potency	(Meisel, 1997)
	Tyr or Phe as C-terminus	Dipeptides with a C-terminal Tyr produced a higher antihypertensive effect compared to dipeptides with C-terminal Phe	(Suetsuna, 1998)
Antioxidant	High amounts of His and hydrophobic amino acids	Contribution to the antioxidant potency	(Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Peña-Ramos, Xiong, & Arteaga, 2004)
	Peptides with a Pro-His-His sequence	Peptides with a Pro-His-His sequence showed the greatest antioxidant activity among all tested peptides	(Chen et al., 1998)
Antithrombotic	Ile^{108}, Lys^{112} and Asp^{115}residues of casoplatelin	Important for the antithrombotic activity	(Fiat, Levy-Toledano, Caen, & Jollès, 1989)
	Sugar content	Positive correlation with antithrombotic activity	(Chabance et al., 1995)
Hypocholesterolemic	Low ratios of methionine–glycine and lysine–arginine in the dietary protein	Favors a hypocholesterolemic effect	(Kritchevsky, Tepper, Czarnecki, & Klurfeld, 1982; Morita et al., 1997; Wergedahl et al., 2004)

Activity	Structural elements	Remarks	Reference
	High amounts of hydrophobic amino acids	Hydrophobic peptides can bind bile acids and thereby enhance the fecal steroid excretion	(Iwami, Sakakibara, & Ibuki, 1986; Makino, Nakashima, Minami, Moriyama, & Takao, 1988)
Anti-obesity	Peptide length	Peptide length influences CCK-releasing activity (different among each dietary protein)	(Nishi, Hara, Hira, & Tomita, 2001)
	Multiple Arg residues	Necessary condition for CCK release through direct binding to intestinal cells	(Nishi, Hara, Asano, & Tomita, 2003)

2.1 Enzymatic hydrolysis to release bioactive peptides from food

In general, bioactive peptides are inactive within the amino acid sequence of its parent, native protein. Such peptides can be liberated by digestive enzyme proteolysis *in vivo* and are, thus, released during gastrointestinal passage. Consequently, their physiological effect is contingent on gastrointestinal (GI) functions which can differ per individual and diet. Variations in GI functions result in unpredictability of the peptide's regulatory effects. Liberation can also occur during food processing (e.g. ripening and fermentation), but again the resulting peptides and their bioactivity may vary (**Figure 2.1**). For that reason, research efforts have been aimed at utilizing controlled conditions to produce bioactive peptides. Microbial fermentation is also widely used, in many countries, to produce foods that contain bioactive peptides. For instance, milk protein fermentation with LAB has been a popular approach to develop nutritious functional foods with bioactive peptides (Hayes, Ross, Fitzgerald, & Stanton, 2007). Similarly, fermented soybean products (i.e., natto and tempeh) are known to contain antioxidant peptides (Wongputtisin, Khanongnuch, Pongpiachan, & Lumyong, 2007).

Nevertheless, enzymatic proteolysis has proved to be the most efficient and reliable method to generate peptides with specified bioactivities. Gastrointestinal or commercial proteases are utilized in these kinds of studies; either alone, in combination, or sequentially. Typically, the process begins with starting material that is either; (a) whole unmodified food (i.e. whole milk or plant); (b) food after processing pretreatment to improve hydrolysis (i.e. microwave and ultra-sonication); or (c) isolated/concentrated protein. Next is homogenization of the protein and water mixture. An appropriate protein to water ratio should be used to ensure sufficient water for enzyme mobility or not an excessive amount that may negatively affect the hydrolysis. Pasteurization of the solution may follow depending on the autolytic activity of the substrate (protein) used. Temperature and pH are adjusted to create an optimal environment for the enzyme to catalyze; this step is critical since peptide breakage depends on the enzyme's activity. The enzyme is added at a desired concentration, either in terms of weight per amount of substrate or regulated by enzyme activity. After the desired hydrolysis time/degree of hydrolysis, the enzyme is inactivated by increasing temperature or adjusting pH outside the optimal range. The hydrolysate slurry is cooled, separated (i.e. centrifugation, ultrafiltration, chromatography etc.) and stored (**Figure 2.1).** Before storage, usually an aliquot of the hydrolysate is used to assay the degrees of hydrolysis (DH). DH is used

as a descriptor of the hydrolysis, measuring the amount of peptide bonds broken compared to the total amount of peptides in the un-hydrolyzed protein (Alder-Nissen, 1986).

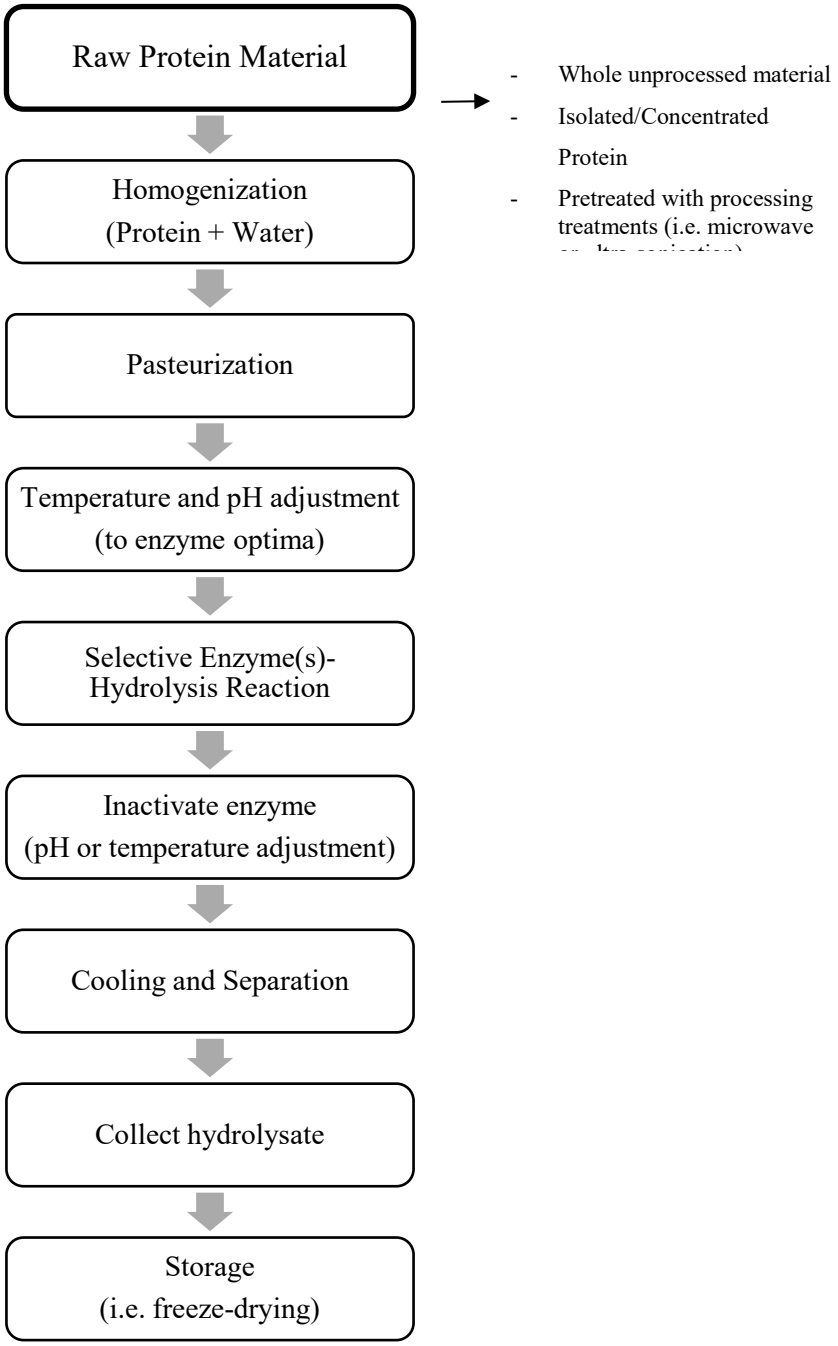

Figure 2.1. General Process of Enzymatic Hydrolysis. Adapted from (Hall and Liceaga, 2019).

2.2 Functions of Bioactive Peptides

Vascular diseases such as coronary artery disease, stroke, hypertension, diabetes and atherosclerosis are major health epidemics worldwide. In the United States alone, cardiovascular disease (CVD) is the leading cause of death (Association, 2017). Moreover, CVD ranks among the top five causes of death in less-developed countries. Consequently, improving diet and lifestyle choices are major strategies for reducing risks of vascular disease. Other vascular diseases risk reduction strategies are aimed at prevention and treatment of the causative factors such as high blood pressure, poor lipid profile or insulin resistance. Dietary bioactive peptides, as mentioned, can promote disease mitigating activities in the body such as antioxidant, antihypertensive and antidiabetic actions. **The focal point of this review centers on bioactivities relevant to CVD risk factors: antioxidant, antihypertension, anti-inflammatory, and antidiabetic properties which will be the major assays in this project.**

2.2.1 Antioxidant peptides

Highly reactive, oxygen containing molecules are a normal by-product of essential biochemical reactions, as those in the human body (Machlin & Bendich, 1987). If the reactive molecules contain one or more unpaired electrons, it is then characterized as a free radical. They can either donate or accept an electron from other molecules, behaving as oxidants or reductants. Oxygen containing free radicals present in many disease states include hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), peroxyl ($ROO^{\cdot}$) and lipid peroxyl ($LOO^{\cdot}$). Molecules such as; hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and lipid peroxide (LOOH) that are not free radicals, but can easily lead to free radical reactions in living organisms (Genestra, 2007). Due to their available electron, free radical molecules are highly unstable and can react with organic substances in the body such as lipids, proteins, and nucleic acids leading to cell damage and homeostatic disruption. Reactive oxygen free radicals are generated by various mechanisms, either endogenously (i) by-products of mitochondrial electron transport reactions; (ii) products of metal catalyzed reactions; (iii) produced by neutrophils and macrophages during inflammation; or by exogenous means (iv) UV light radiation and X-ray and gamma rays; (v) atmospheric pollutants, cigarette smoke, and industrial chemicals (Bagchi & Puri,

1998; Cadenas, 1989). Because free radicals are so unstable and have potential to damage cells and tissues, the body requires a critical balance of free radical generation and antioxidant defense mechanisms. An unfavorable balance that leads to oxidative damage is termed oxidative stress. Mounting evidence links oxidative stress to inflammatory conditions (arthritis, vasculitis, glomerulonephritis, lupus erythematous, adult respiratory diseases syndrome), diseases related to blood supply restriction (stroke and heart diseases), hypertension, diabetes, cancer, and neurological disorders Alzheimer's and Parkinson's) (Jay, Hitomi, & Griendling, 2006; Klaunig & Kamendulis, 2004; Lobo, Patil, Phatak, & Chandra, 2010).

Antioxidants are molecules capable of stabilizing or deactivating (via chemical interactions) free radicals, reactions promoted by oxygen or peroxides present within human cells or food materials (Gülcin, 2012). The human body has evolved with antioxidant systems that work synergistically to protect the cells and organ systems from free radical damage. Like free radicals, antioxidants can be obtained endogenously or exogenously. Endogenous antioxidants are critical to maintain optimal systematic health and well-being, however, under conditions that promote oxidative stress (e.g. excess alcohol consumption or mental stress) they may not be sufficient and exogenous antioxidants may be necessary to maintain optimal cellular functions. Exogenous sourced antioxidants refer to those obtained from the diet or dietary supplements. Consuming antioxidant rich foods has proven effective in promoting human health by increasing the antioxidant load in the body (Lobo et al., 2010). Antioxidant rich foods and food derived antioxidants such as vitamin C, A, & E, and phytochemicals (e.g. lycopene, green tea extracts, flavonoids, isoflavones, and polyphenols) has gained scientific attention over the years (Lobo et al., 2010).

More recently, bioactive peptides started being assessed for antioxidant potential from various food sources. Peptidic antioxidants are unique, compared to non-peptide antioxidants, in that they have the potential to act as multifunctional antioxidants that can be involved in several oxidative pathways. Peptide antioxidant capacity is attributed to complex interactions between their ability to inactivate reactive oxygen species (ROS), scavenge free radicals, chelate prooxidative transition metals, reduce hydroperoxides, and enzymatically eliminate specific oxidants**.** Food proteins/peptides also demonstrated ability to promote enzymatic antioxidant defense systems already present in the body (such superoxide dismutase and glutathione peroxidase). Moreover, antioxidant peptides from food are considered safe and healthy compounds

with low molecular weight and easy absorption (Sarmadi & Ismail, 2010). Structurally, most food derived antioxidant peptides contain 2-20 amino acids with a molecular weight less than 6 kDa (Sarmadi & Ismail, 2010). Exact structural characteristics vary significantly, depending on the type of peptide and antioxidant mechanism.

In vitro studies, using multiple chemical assays, are widely used as an indication of the potential of food-derived peptides to control various oxidative processes in the human body. These in vitro assays are classified as either methods based on hydrogen atom transfer (HAT) or methods based on electron transfer (ET). HAT assays involve a competitive scheme with antioxidant and substrate for thermally generated peroxyl radicals, whereas ET assays measure the peptide's capacity to reduce an oxidant. Other assays simply measure scavenging activity of biologically relevant oxidants (i.e. singlet oxygen, superoxide anion, peroxynitrite, and hydroxyl radical). Detailed discussions of the methodology and principles behind *in vitro* antioxidant capacity assays have been reviewed extensively (Decker, Warner, Richards, & Shahidi, 2005; D. Huang, Ou, & Prior, 2005; Magalhães, Segundo, Reis, & Lima, 2008; Moon & Shibamoto, 2009; Prior, Wu, & Schaich, 2005; Yoo, Kim, & Lee, 2007). **Table 2.3** compiles the most common *in vitro* antioxidant assays and the oxidative inhibiting mechanisms they represent in the human body. Although these methods are good for screening and assessing preliminary data there are draw-backs, mainly concerning lack of relevance to biological systems. Therefore, it is preferable to use at least two different chemicals assays, if necessary, and validate antioxidant capacity in more physiologically relevant cell based and whole organism systems (Foltz, van der Pijl, & Duchateau, 2010). There are a plethora of reports confirming the antioxidant capacity of food derived peptides and peptide-rich protein hydrolysates *in vivo, cell-based assays, and in vitro mechanisms* (**Table 2.5).** For example, (Manso et al., 2008) demonstrated that malondialdehyde (MDA-a reactive compound) levels decreased in the aortic tissue of spontaneously hypertensive rats after long term administration of egg white protein hydrolysates (0.5g/kg/day). Likewise, rapeseed protein hydrolysates (hydrolyzed with alcalase and flavourzyme) decreased MDA levels in rats 12.8% and 46.9% after 50 and 150 mg/kg/day intraperitoneal injections, respectively (Xue et al., 2009). Moreover, the rapeseed protein hydrolysates could also prevent hemolysis in rat red blood cells, inhibiting up to 40% at 0.5 mg/mL (Xue et al., 2009). Examples of from cell-based, *in vitro* and *in vivo* studies, in the last ten years, are summarized in **Table 2.5.**

Table 2.3. *In vitro* assays measuring antioxidant capacity and their mechanisms

assays involving hydrogen atom transfer reactions ROO• + AH → ROOH + A• ROO• + LH →ROOH + L•	ORAC (oxygen radical absorbance capacity)
	TRAP (total radical trapping antioxidant parameter)
	Crocin bleaching assay
	IOU (inhibited oxygen uptake)
	Inhibition of LDL oxidation
assays by electron-transfer reaction M(n) + e (from AH) → $AH^{\bullet+}$ + M (n − 1)	TEAC (Trolox equivalent antioxidant capacity)
	FRAP (Ferric ion reducing antioxidant power)
	DPPH (diphenyl-1-picrylhydrazyl)
	ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6 sulfonic acid))
	Copper (II) reduction capacity
Other common assays	TOSC (total oxidant scavenging capacity)
	inhibition of Briggs−Rauscher oscillation reaction
	chemiluminescence
	electrochemiluminescence

2.2.2 Antihypertensive peptides

High blood pressure or hypertension is currently the leading, but preventable, cause of premature death affecting more than 1/3 of the global population (K. T. Mills et al., 2016). As a major CVD risk factor, many strategies are aimed at treating and preventing high blood pressure. Hypertension is recognized as multifactorial, resulting from a combination of both environmental (i.e., stress, alcohol, smoking and dietary habits) and genetic factors. Although blood pressure is controlled by several interacting biochemical pathways, decades of research highlight that the renin-angiotensin-aldosterone system (RAAS) plays a vital role in regulating human electrolyte and blood pressure homeostasis. Inappropriate activation of the RAAS can modulate blood pressure through various processes, including changes in degrees of blood vessel restriction, increased activity of the sympathetic nervous system, and changes in renal salt and water homeostasis. Hence, the benefits of inhibiting RAAS activity make it an ideal target for treating

hypertension and has been well recognized (Crowley et al., 2006). At the cellular level, RAAS activity can be blocked by compounds (with specific structural characteristics) at various points with five distinct possible areas of inhibition **(Figure 2.2)**: (1) aldosterone inhibitors, (2) renin inhibitors, (3) angiotensin I converting enzyme (ACE) inhibitors, and (4) angiotensin receptor blockers. The angiotensin I converting enzyme catalyzes the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) and degrades the vasodilation compound bradykinin. Ang II itself raises blood pressure by several actions, mainly causing vasoconstriction and increasing aldosterone simulation and renal actions (**Figure 2.3**) (G.-H. Li, Le, Shi, & Shrestha, 2004). Hence, ACE inhibition is a major target and considered the most effective treatment resulting in overall antihypertensive effects.

Over the years, synthetic drugs have been designed to interact with and inhibit these regulatory points in the RAAS **(Table 2.4)**, to control and treat hypertension. There are a variety of antihypertensive drugs are available on the market aimed at targeting these points in the RAAS. Benazepril and Captopril and, for example, are popular synthetic compounds designed to inhibit ACE activity which is a major treatment method for hypertension. Synthetic inhibitors function by binding to ACE active sites, thereby 'blocking' or inhibiting the binding of angiotensin I to the target site. Consequently, substrate is not available for the ACE catalyzed conversion of angiotensin I to angiotensin II which causes hypertension. Although effective, synthetic antihypertensive drugs have potential negative impacts on human health (Lee, Hong, Jeon, Kim, & Byun, 2009). Emphasis on using safer and more effective medications to control high blood pressure has resulted in a strong demand for exploration of natural remedies to treat hypertension. Numerous food components have been used to lower blood pressure without any negative side effects (Vermeirssen, van der Bent, Van Camp, van Amerongen, & Verstraete, 2004). Bioactive peptides from food proteins have been shown effective at reducing hypertension and are safer than traditional drugs, which makes them a promising alternative when choosing between a synthetic drug and naturally-occurring bioactive food component. Bioactive peptide's ability to inhibit ACE and renin activity, just as synthetic compounds, are due to their structural features that allow sufficient binding to the active site and preventing the formation of the vaso-constricting angiotensin II (**Figure 2.4**). Analytical and chemometric experiments have revealed that ACE preferentially binds to peptides rich in hydrophobic amino acid residues (aromatic or branched-side chains) at the C- terminal; proline, tryptophan, phenylalanine, or tyrosine residues at these

positions are also speculated to strengthen the inhibitory activity of peptides, such as those on some synthetic ACE inhibitors (Murray & FitzGerald, 2007). According to model proposed by (Cushman, Cheung, Sabo, & Ondetti, 1982) and (Ondetti & Cushman, 1982), suitable C-terminal tripeptides can interact with the S_1, S_1' and S_2' at the ACE active site.

The ACE inhibitory or antihypertensive effects have been evaluated in many sources of dietary peptides *in vivo* and *in vitro* (**Table 2.5**). *In vivo*, spontaneously hypertensive rats (SHR) are used to evaluate overall antihypertensive capability. Generally, SHR are first fed extracts or food products containing a specified dose of the peptide, then diastolic and systolic blood pressure measured at specified time intervals. Studies using SHR are considered the most accurate experimental methods of evaluating antihypertensive peptides (Tavares, Sevilla, Montero, Carrón, & Malcata, 2012). This is likely because rats have similar vascular reactivity and renal functions to that of humans (Iwaniak, Minkiewicz, & Darewicz, 2014). *In vitro* techniques are still widely used to assess potential antihypertensive activity or as a selection tool. The most popular methods utilize spectrophotometric, fluorometric, colorimetric, radiochemical and chromatography techniques (FitzGerald, Murray, & Walsh, 2004; Murray & FitzGerald, 2007). ACE inhibitory effect is typically expressed as the percentage inhibition achieved by a specified peptide concentration or given as the $IC_{50,}$ defined as the peptide concentration required to lower the ACE activity to 50% of the initial value (Donkor, Henriksson, Vasiljevic, & Shah, 2005). While the inhibitory mode of ACE is evaluated by performing Lineweaver-Burk plots. Each *in vitro* method works by, essentially, monitoring the release of product from the substrate. The most popular synthetic substrates used are HHL (hippuryl-L-histidyl-L-leucine) and FAPGG (furanacryloyl-L-phenylalanyl-glycylglycine). In the former, ACE releases hippuric acid (HA) while the FAPGG the products are FAP (furanacryloyl-L-phenylalanine) and GG (glycylglycine). The products are then quantified using one of the mentioned techniques. For example, HA and FFP can be measured spectrophotometrically at 228 nm and 340 nm, respectively. If the reaction mixture contains an ACE inhibitory peptide the release of product (HA and FFP) is inhibited, which lowers the absorbance values (Iwaniak et al., 2014).

Table 2.4. Class of drugs designed to treat hypertension and their mechanism of action

Class	Mechanism of action
Aldosterone antagonists	Blocks the binding of aldosterone to principle cells of the renal collecting ducts
Renin Inhibitors	Interfere with the first rate limiting step in the synthesis of Ang I from angiotensinogen
ACE Inhibitors	Inhibits the direct conversion of Ang I into Ang II
Angiotensin receptor inhibitors	Blocks the binding of Ang I to AT1 receptors

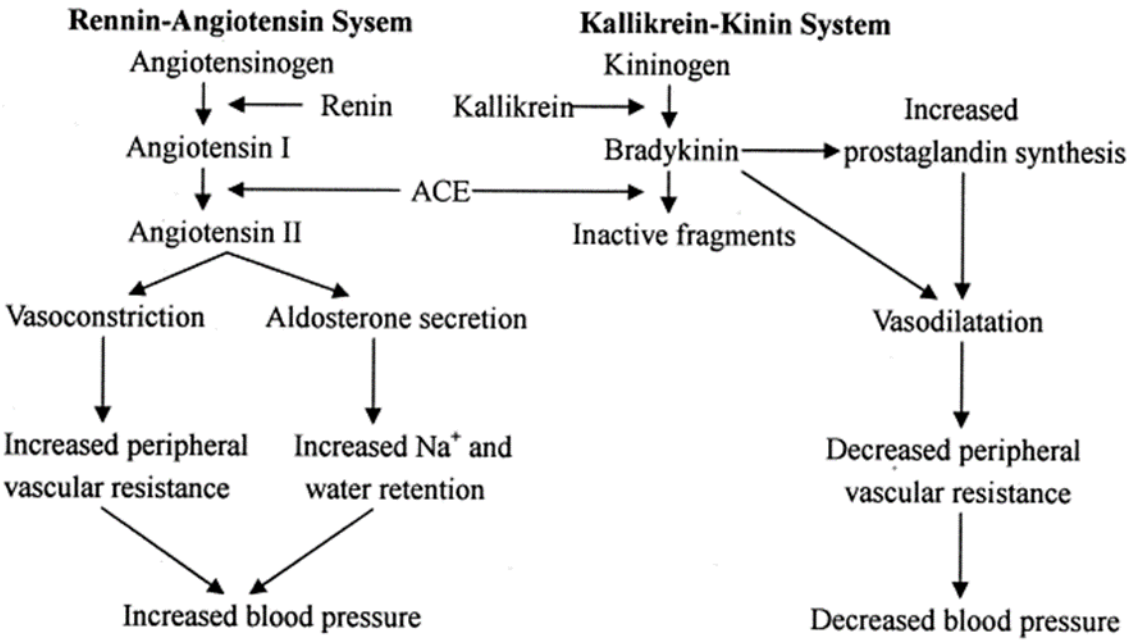

Figure 2.2. Role of ACE in blood pressure regulation. Obtained from (G.-H. Li et al., 2004)

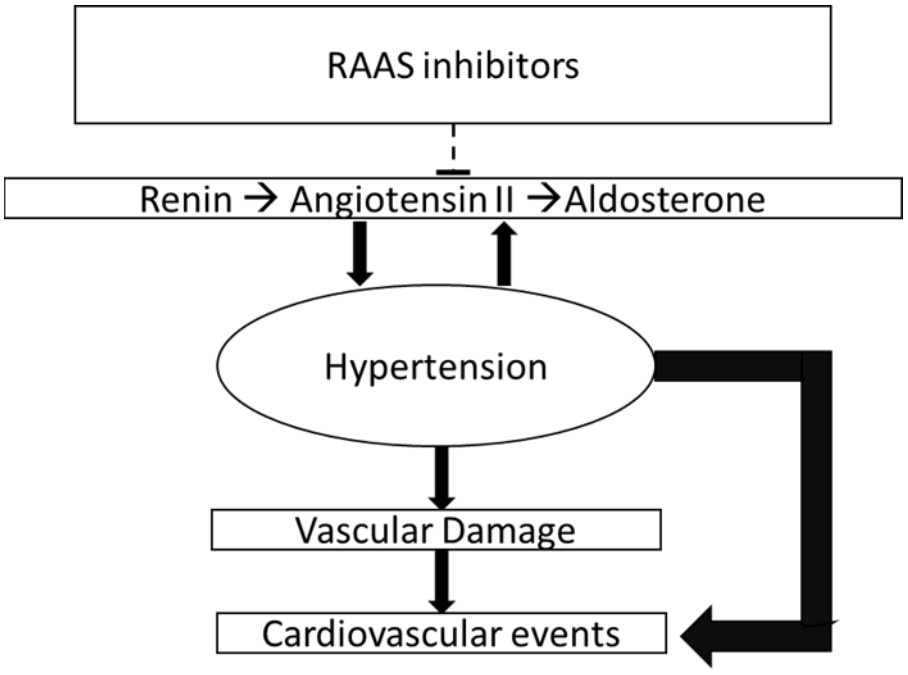

Figure 2.3. Overview of the Renin–angiotensin system and association with CVD

Table 2.5. Representative food derived antioxidant, antihypertensive, antidiabetic, and anti-inflammatory peptides/hydrolysates demonstrated *in vitro*, cell based, and some *in vivo* systems.

Protein Source	Preparation	Active state	Assay System	Bioactivity Observed	Reference
Tuna Frame	Alcalase, Neutrase, pepsin, papain, α-chymotrypsin and trypsin hydrolysis	Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro	Spontaneously hypertensive rats (SHR)	Decreased systolic blood pressure after oral administration	(Lee et al., 2010)
Oyster	Pepsin hydrolysis	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	Chemical assays SHR	In vitro ACE inhibitory activity antihypertensive activity orally administered to SHR	(Wang et al., 2008)
Amaranth seed	pronase, papain, trypsin, chymotrypsin and alcalase hydrolysis	Hydrolysates	SHR	Lowered overall blood pressure after intra-gastric administration	(Fritz et al., 2012)
Bovine Casein from whole milk	Pepsin hydrolysis of isoelectric casein	Hydrolysates (< 3 kDa)	SHR	Decreased arterial, systolic and diastolic blood pressure after oral administration	(Miguel et al., 2009)
Wheat gluten	Commercial product from Nisshin Pharma (Tokyo, Japan)	Hydrolysates pyroglutamyl leucine (pyroGlu-Leu)	*Caenorhabditis elegans* Hepatitis rats	suppressed the adverse effects caused by heat-stress or oxidative stress on nematodes hepatoprotective effects: decreased serum aspartate and alanine aminotransferases	(Zhang et al., 2013) (Sato et al., 2013)

Chicken collagen	Various dual combinations of 'proreases'	Hydrolysates	human dermal fibroblasts	Anti-inflammatory actions: oxidative stress, type I collagen synthesis and cellular proliferation	(Offengenden Chakrabarti & Wu, 2018)
Blue whiting (*Micromesistius poutassou*)	Alcalase 2.4L and Flavourzyme 500L	Hydrolysates	pancreatic BRIN-BD11 cells GLUTag cells. normal healthy mice	mediated insulin and glucagon-like peptide-1 increase in membrane potential, intracellular calcium and cyclic AMP concentration Glucose-lowering in normal healthy mice (4hr).	(Egerton et al., 2018)
Chicken	Pepsin hydrolysis	Hydrolysate	SHR	Reduced cellular superoxide. Increased plasma radical scavenging. Reduction in aorta MDA levels	(Manso et al., 2008)
Rapeseed	Alcalase/Flavourzyme hydrolysis of defatted and dehulled rapeseed	Hydrolysate	Wistar rat	Reduction in serum MDA levels	(Xue et al., 2009)
Douchi (Chinese fermented soybean)	Steamed soybeans fermented with *Aspergillus oryzae*	Peptides (released via fermentation	Wistar rat	Increased liver and kidney SOD and CAT superoxide dismutase and catalase activity. Reduction in liver and kidney MDA levels	(Wang et al., 2008)
Tilapia *(Oreochromis niloticus)*	Amano A2, Amano N, Flavourzyme, Neutrase, and Cryotin-F hydrolysis of tilapia protein isolates	Hydrolysates	Mononuclear cells from whole human blood chemiluminescent assays	Scavenged reactive oxygen species Ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC)	(Raghavan, & Kristinsson, Leeuwenburgh, 2008)

Croaker (Otolithes ruber) muscle		Lys-Thr-Phe-Cys-Gly-Arg-His	Wistar rat	Increased SOD, CAT, and GST activity	(Nazeer et al., 2012)
Pacific cod (Gadus macrocephalus) skin	Pepsin, trypsin, α-chymotrypsin hydrolysis of extracted gelatin	Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro	RAW264.7 mouse monocyte cells	Increased SOD, CAT, and GST activity. Reduced oxidation levels of cellular membrane lipids, protein, and DNA.	(Himaya et al., 2012)
Salmon byproduct from pectoral fin	Pepsin hydrolysis	Hydrolysate fraction (1–2 kDa)	Chang liver cells	Increased GSH levels. Inhibited lipid peroxidation	(Ahn, Je, & Cho, 2012)
Eggshell membrane	Alcalase and Protease S hydrolysis	Hydrolysate	Human intestinal epithelial Caco-2 cell In vitro chemical assays	Increased GSH levels. Inhibited formation of DCF and IL-8 secretion. scavenging activity against DPPH and hydroxyl radicals	(Shi et al., 2014)
Chia seed byproducts (after oil extraction)	Alcalase and flavourzume	Hydrolysate	*Saccharomyces cerevisiae*	increased survival when exposed to hydrogen peroxide	(Coelho et al., 2019)

2.2.3 Antidiabetic peptides

Diabetes mellitus (DM) is another major risk factor of CVD that affects a substantial portion of the population (382 million person). Prevalence of diabetes mellitus is increasing perpetually and expected to reach 592 million people by 2035 (Atlas, 2013). Incidences of DM are rising due to a variety of factors including; population growth, urbanization, increase in sedentary lifestyles and obesity (Whiting, Guariguata, Weil, & Shaw, 2011). DM is a chronic metabolic disorder, caused by defective insulin secretion and/ or action, and characterized by hyperglycemia (surplus of sugar in the blood stream). There are two main types of diabetes; insulin-dependent Type I (T1D) and non-insulin dependent Type II (T2D), of them T2D is more common and accounts for 90-95% of all cases. Diagnosis of T2D is not fully elucidated to one cause, but develops from a combination of reduced levels of glucagon-like peptide levels (GLP-1), hepatic glucose and β-cell dysfunction and defects in insulin resistance (Sena, Bento, Pereira, Marques, & Seiça, 2013).

For individuals with T2D, various treatment strategies are necessary, the first step is a change in diet, lifestyle, and regular physical exercise; antihyperglycemic medications are introduced when conventional therapies fail to keep blood glucose at goal levels (Asif, 2014). In general, diabetic drug treatments aims to control blood sugar levels before and after eating (Asif, 2014). A safe combination of agents, with different mechanisms of action, can be used to achieve this. Therapeutic treatments include inhibiting enzymes involved in blood glucose regulation and glucose-lowering agents. For example, dipeptidyl peptidase-IV and alpha-glucosidase play a significant role in the development of hyperglycemia in T2D (Kim & Egan, 2008). Accordingly, inhibition of theses enzymes is a major target for treatment and drug discovery.

Alpha-glucosidase (EC 3.2.1.20) is a key enzyme in carbohydrate synthesis and breakdown located in the brush border of the small intestine (Van der Laar et al., 2005). The resulting hydrolyzed dietary carbohydrate is a main source of glucose in the blood. Thus, inhibiting alpha-glucosidase activity delays carbohydrate digestion and glucose absorption in the blood. Consequently, postprandial glycaemia is reduced (Krentz & Sinclair, 2012). Dipeptidyl peptidase-IV (DPP-IV/CD26; EC.3.4.14.5) is a multifunctional transmembrane glycoprotein, involved in various biological processes (Fan et al., 2004). It is a serine type protease that contains N-terminal dipeptidase activity, which selectively removes dipeptides after Pro or Ala residues. DPP-IV is

present in almost all organs in the body and is involved in several physiological processes, namely incretin degradation which contributes to the deterioration in glucose homeostasis (Kim & Egan, 2008). Incretins GLP-1 and GIP are gut hormones released into plasma after the ingestion of food, increasing two- to three-fold in concentration. GLP-1 and GIP enhance meal-induced insulin secretion in a glucose-dependent manner, delay gastric emptying, and suppress pancreatic glucagon (Kim & Egan, 2008). In the presence of DPP-IV, GLP-1 and GIP are degraded as these gut-derived hormones are physiological substrates of DPP-IV. The development of the DPP-IV inhibitors has been a popular approach in the management of diabetes (Kim & Egan, 2008).

Like hypertension drug treatments, diabetic researchers have been focused on developing naturally sourced anti-hyperglycemic agents that are safe and free of side effects. Nutritional intervention has been established as the safest method for prevention and management of T2D, namely to inhibit intestinal DPP-IV to prevent incretin degradation (Asif, 2014). Food proteins and food protein hydrolysates have shown to impact several biomarkers of diabetes. Therefore, many studies have been carried out to identify food peptides with DPP-IV and alpha-glucosidase inhibitory activity. Among those proteins, milk derived peptides and amino acids can influence postprandial glycemic regulation and insulin secretion in both normal and type II diabetic individuals (Petersen et al., 2009). It was suggested that the antidiabetic properties of milk proteins are primarily attributed to the presence of bioactive peptides, released during GI digestion, that can influence the simulation of gut-derived hormones and possibly inhibit enzymes involved in glucose homeostasis. This anti-hyperglycemic effect may be a consequence of various pathways and mechanisms, the exact sequences responsible for these activities have not yet been completely identified (Ricci-Cabello, Olalla Herrera, & Artacho, 2012). Antidiabetic properties of bioactive peptides are still in the beginning stages, thus *in vitro* techniques are typically used. *In vitro* techniques typically involve enzymatic systems or cell lines to measure insulin secretion/resistance. Enzymatic inhibition assays simply measure the hydrolyzed substrate product (by DPP-IV or alpha-glucosidase) via colorimetric or chromatographic methods. To measure potential *in vivo,* diabetes can be induced in several animal species; most experiments are carried out on rodents (Fröde & Medeiros, 2008). The animal is then fed with the antidiabetic agent at specified dosages. After a feeding period, the blood is collected for various biochemical estimations including; reduction of blood glucose, serum insulin, cholesterol, triglycerides, glycosylated hemoglobin, protein and glycogen content in the liver (Fernandes, Lagishetty, Panda, & Naik, 2007) and others.

There are a few protein hydrolysate/peptides that were evaluated in models directly attenuate T2D conditions or indirectly by inhibiting pro-inflammatory molecules exasperate or that can promote hyperglycemia and/or hypertension (**Table 2.5**).

2.2.4 Interlink between hypertension, diabetes, and inflammation

Type 2 diabetes and hypertensive states can arise from multiple pathologies associated with cardiovascular diseases (CVD). Both diseases frequently coexist, especially when obese and insulin resistance states are present (**Figure 2.4**). Chronic hyperglycemia and insulin resistance can initiate mechanisms related to vascular diseases through oxidative stress, increased formation of advanced glycation end products (RAGE), endothelial dysfunction and promoting a low-grade inflammatory state. Hypertension can exasperate diabetes-associated vascular complications since its own pathology involved vascular dysfunction and proinflammatory conditions (**Figure 2.3**). Vascular/endothelial dysfunction, in particular, is characterized by decreased vasodilatation, proinflammatory conditions, and increased risk of blood clots (Rajendran et al., 2013). In these conditions, the proinflammatory state is characterized by an increased expression of adhesion molecules (i.e. ICAM-1 and VCAM-1), cytokines such as monocyte chemoattractant protein-1 (MCP-1), and inflammatory mediators such as TNF-α and IL-1β (Rajendran et al., 2013). Numerous clinical studies already support the link between metabolic dysfunctions (i.e. diabetes), hypertension, and inflammation. For example, patients with T2D showed increased levels of neutrophils and lymphocytes, which was correlated to insulin sensitivity mediated adipose tissue inflammation (Petrie, Guzik, & Touyz, 2018). Further, genetic studies have demonstrated protective effects and anti-inflammatory properties of antidiabetic strategies (i.e. fat loss, metformin, statin, pioglitazone, and insulin). Increased levels of inflammatory cytokines, TNF-α, IL-1β, and IL-12 can promote insulin release in the pancreatic islets and promote insulin sensitivity.

In this context, controlling comorbidities, targeting vascular health, and an anti-inflammatory state are viable strategies to reduce complications of diabetes and hypertension. Bioactive peptides from food proteins provide anti-inflammatory effects through multiple pathologies including via antihypertensive (i.e. RAAS regulation), antioxidant (i.e. ROS inhibition), and antidiabetic (i.e. insulin regulation) pathways as described previously. Recently, more direct anti-inflammatory activities of bioactive peptides have been observed, mainly via attenuation of leukocyte-endothelial interactions (**Table 2.5)**. (Chakrabarti et al., 2014; Guha &

Majumder, 2019). For example, egg and milk bioactive peptides (liberated through proteolysis) are reported to be effective in attenuating expression of cytokine-induced inflammatory proteins in endothelial cells (Guha & Majumder, 2019; Majumder, Chakrabarti, Davidge, & Wu, 2013; Majumder, Mine, & Wu, 2016). These effects often coexist with antioxidant and ACE-inhibitory properties, enhancing their multifunctional benefits (W. Huang et al., 2012). Whey protein hydrolysates, primarily, have demonstrated these multi-capabilities *in vivo* (animal models)(Chatterton, Nguyen, Bering, & Sangild, 2013).

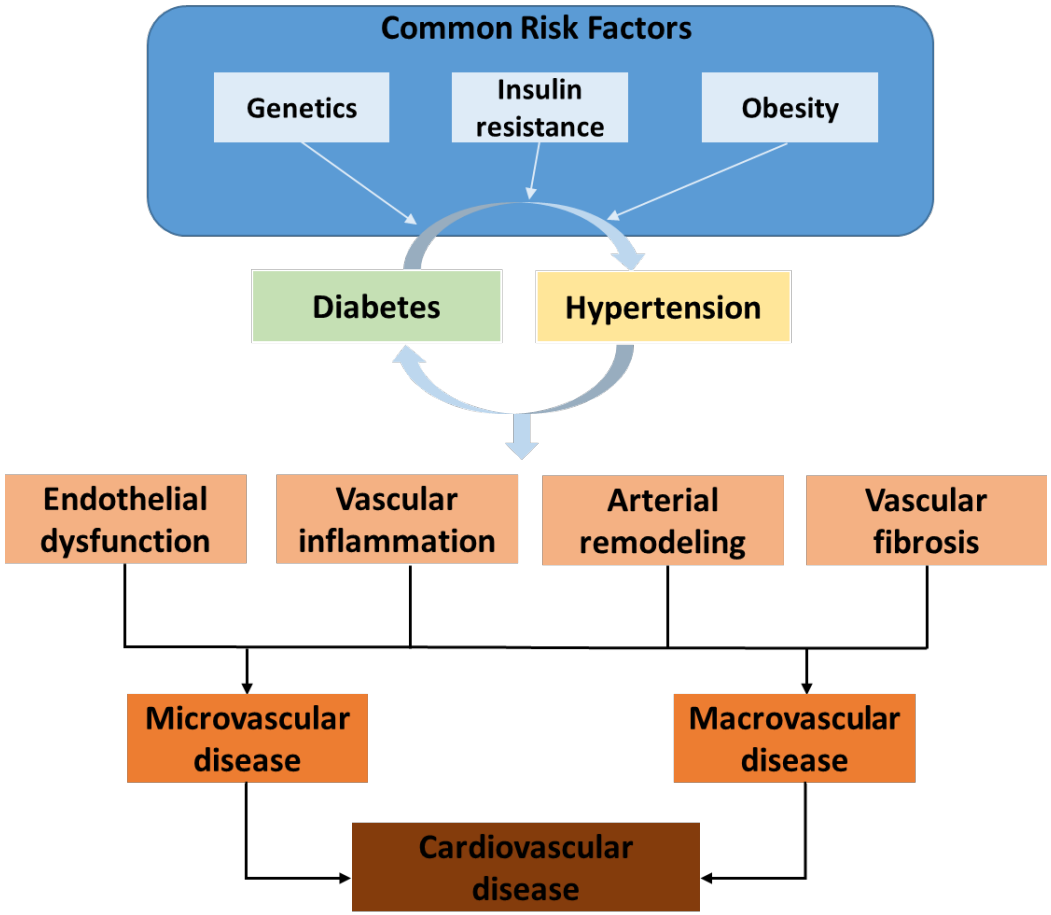

Figure 2.4. Vascular processes whereby diabetes and hypertension predispose to cardiovascular disease. Common risk factors can promote diabetes and hypertension, which are associated with vascular inflammation, endothelial dysfunction, and structural remodeling that sequentially lead to macrovascular and microvascular disease. Vascular damage and endothelial dysfunction are amplified when diabetes and hypertension coexist. Image adapted from (Petrie, Guzik, & Touyz, 2018).

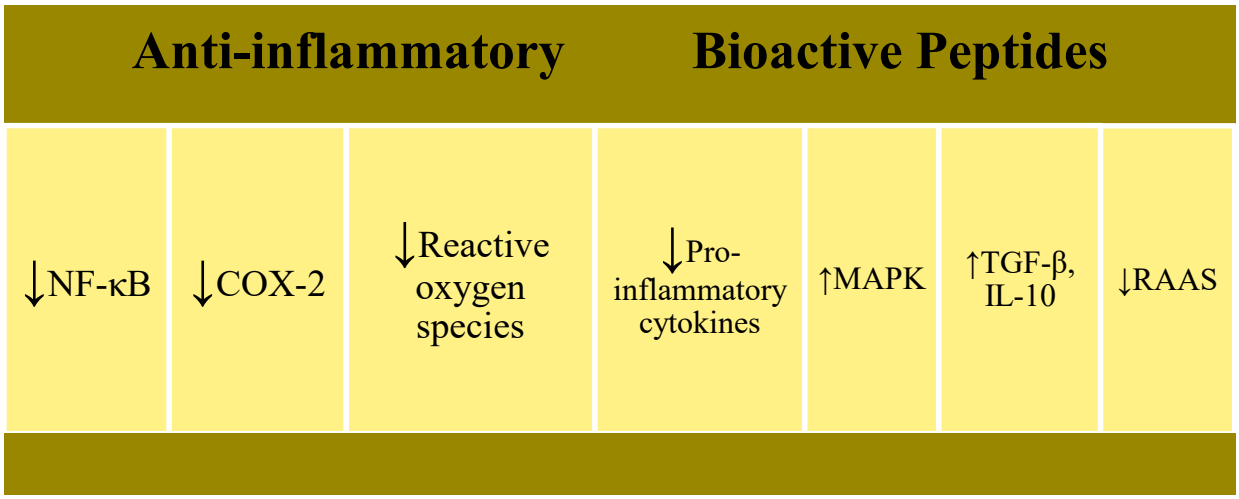

Figure 2.5. The potential mechanisms of action of anti-inflammatory bioactive peptides and peptide-rich protein hydrolysates. MAPK: mitogen activated protein kinase; NF-κB: nuclear factor-κB; COX-2: cyclo-oxygenase-2; TGF-beta: transforming growth factor-beta; IL-10: interleukin-10; RAS: renin-angiotensin system; ROS: reactive oxygen species. Image and description obtained from (Chakrabarti, Jahandideh, & Wu, 2014)

2.3 Bioactive Peptides from Underutilized Protein Resources: Agricultural By-Products, Invasive Fish Species, and Edible Insects

Due to the global population growth and dietary shifts towards high protein consumption, there is an increasing demand for more sustainable protein sources. Subsequently, investigations revealed that these protein-rich materials were great sources of bioactive peptides to positively influence human health (Harnedy, & FitzGerald, 2012; Lafarga, & Hayes, 2014; Nongonierma, & FitzGerald, 2017). Our lab, particularly, elucidated the bioactive potential of peptide from various underused protein sources such as fish by products and invasive species, underutilized legumes and seeds such as chia, hemp, and canary seeds (Urbizo-Reyes et al., 2019; Ketnawa, Wickramathilaka, & Liceaga, 2018; Suwal et al., 2018**)**. For example, peptides from chia seeds, liberated with sequential enzymatic hydrolysis, exhibited inhibiting activity towards enzymes (collagenase, hyaluronidase, tyrosinase and elastase) responsible for aging and cosmetic skin ailments (Aguilar-Toalá & Liceaga, 2020). In another study Ketnawa et al. (2017) demonstrated the antioxidant potential of fish frame protein hydrolysates. Even further, the authors documented how the extraction method used (microwave-assisted enzymatic hydrolysis) decreased reactivity of fish allergen parvalbumin. In vivo studies confirm these observations. Protein hydrolysates from anchovy (*Engraulis encrasicolus)* waste not only exhibited protection against LPS-induced

inflammation in macrophage cells (RAW 264.7) via COX-2, NF-kB; but also down-regulated expression of proinflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6) in ApoE knockout mice (Giannetto et al., 2020). Likewise, chia seed protein hydrolysates increase survival of yeast (*Saccharomyces cerevisiae*) after exposure to hydrogen peroxide demonstrating its antioxidative protective effects (Coelho et al., 2019). These studies, along with others alike, provide evidence to support their use as nutraceuticals or ingredients in functional foods to promote human health.

Protein from edible insects are also being evaluated as an alternative to animal protein for human food (Hall, Jones, O'Haire, & Liceaga, 2017; Spiegel, Noordam, & Fels-Klerx, 2013; Van Huis, 2003). Insects are well recognized as nutritious foods providing a significant source of proteins (including essential amino acids), carbohydrates, fats, some minerals and vitamins, as well as caloric value (Roos, N., 2018). Likewise, insect rearing requires much less food and breeding space relative to livestock production, along with a smaller climate impact in terms of greenhouse gases (GHG) and ammonia emissions. With this knowledge, the Food and Agriculture Organization of the United Nations (FAO) launched documentation and programs supporting edible insect consumption as a major future prospect for food and feed security (Van Huis, 2013). Sensory evaluations quickly discovered that due to western cultural taboos, acceptance was more likely if edible insects were prepared with familiar flavors and textures (Luna et al., 2020; Mishyna, Chen, & Benjamin, 2020). Physicochemical analyses determined that incorporating insects, in their whole form, into food products resulted in unstable and poor functionality (Hall et al., 2017; Luna et al., 2020; Kim et al., 2016). Thus, extracting the nutritional components (e.g. chitin, fats, and proteins) to use as food ingredients is suggested as a means to improve consumer acceptance and encourage modern entomophagy (Sosa & Fogliano, 2017). The identification of a wide range dietary bioactive peptides in other underutilized protein sources have led to the exploration of edible proteins as a source of biologically active peptides (Aguilar-Toalá, Deering, & Liceaga, 2020; Aguilar-Toalá & Liceaga, 2020; Ketnawa & Liceaga, 2017; Nongonierma & FitzGerald, 2017; Urbizo-Reyes, San Martin-González, Garcia-Bravo, Vigil, & Liceaga, 2019).

Numerous bioactive components are believed to be present in insects such as chitin, polyphenols, enzymes, and peptides/proteins since they possess their own proactive and modulating pathologies (Mlcek, Borkovcova, Rop, & Bednarova, 2014). Research on insect-derived bioactive peptides is relatively new but still demonstrates their promising potential. Vercrussye and others (2005) were the first to describe the bioactive potential of silk moth

(*Bombyx mori*) peptides by evaluating the *in vitro* ACE inhibitory activity of the hydrolyzed insect protein. Since then, numerous studies evaluating and generating insect-derived bioactive peptides, via enzymatic hydrolysis, have been reported. A comprehensive article detailing insect bioactive peptides has also been recently reported (Nongonierma & FitzGerald, 2017). Silk fibroin from *Bombyx mori* has been the most extensively studied and is reported to have antioxidant, antihypertensive, antidiabetic, and hypocholesterolemic properties *in vivo* (Nongonierma & FitzGerald, 2017).

Tropical banded crickets (*Gryllodes sigillatus*) were utilized in this study due to their relevance in North America. Among edible insect species, crickets are the most farmed within North America. There are currently more than 30-startup companies specializing in cricket farming, flours, and food products. With a protein content around 70% (dry weight) thorough analysis of its protein, as a source of bioactive peptides, is merited. Recently, (Nongonierma, Lamoureux, & FitzGerald, 2018) reported antidiabetic, DPP-IV inhibitory activity, of enzymatically hydrolyzed cricket (*G. sigillatus*) flour; as well as the effects of gastrointestinal digestion (SGID). They found that inhibition was highest after digestion with GI enzymes and in hydrolysates with smaller molecular weight peptides (Nongonierma & FitzGerald, 2017), a common feature in food-derived bioactive peptides. Tropical banded cricket protein hydrolysates has also exhibited *in vitro* antioxidant and anti-inflammatory activities (Zielińska, Baraniak, & Karaś, 2017). The same group also demonstrated antioxidant, ACE, lipase and α-glucosidase inhibiting and anti-inflammatory properties of cricket (*G. sigillatus*), mealworm (*Tenebrio molitor*), and locust (*Schistocerca gragaria*) protein hydrolysates and their identified peptides (Zielińska, Baraniak, & Karaś, 2018; Zielinska, Karas, Baroniak, & Jakubczyk, 2020).

Given their relative novelty, in vitro studies on edible insect peptides are crucial because rapid assessment of extraction techniques (i.e. enzymatic hydrolysis), treatments (i.e. sonication), and their compounds, (i.e. peptides, chitin, phenolics) can be studied simultaneously to determine their impact on bioactivity before confirming in vivo viability. In previous years, edible insects were not considered a potential food source and thus their micro-and macro molecule composition were not characterized in-depth. For example, the full proteome of edible insect species such as crickets are yet to be determined. Hence, characterization, safety analyses, and in vitro determinations are still very necessary to support the evidence towards edible insects as a therapeutic alternative. Although there are only a few reports cricket protein bioactive peptides,

interest in the topic is high and increasingly gaining interest along with other insect species **(Table 2.6)**.

Table 2.6. Reported bioactive properties of some edible insect protein and peptides

Insect common name	Species	Enzyme(s)	Bioactivity	Reference
Silkworm, Bumblebee, Locust, Leaf worm	*B. mori* *B. terrestris* *S. gregaria* *S. littoralis*	SGID (pepsin, trypsin and chymotrypsin), Alcalase or thermolysin.	ACE inhibition	(Vercruysse et al., 2005)
	S. littoralis	SGID (pepsin, trypsin and chymotrypsin)	ACE inhibition	(Vercruysse, Smagghe, Matsui, & Van Camp, 2008)
	S. littoralis	SGID (pepsin, trypsin and chymotrypsin), Alcalase or thermolysin.	ACE inhibition and antioxidant (DPPH scavenging and FRAP)	(Vercruysse, Smagghe, Beckers, & Van Camp, 2009)
Silkworm	*B. mori* pupae defatted dry powder	Acid protease	ACE inhibition	(W. Wang et al., 2011)
	B.mori larvae	SGID (pepsin and trypsin)	ACE inhibition and antioxidant (DPPH scavenging, Fe^{2+}chelating and Fe^{2+}reducing activities)	(Q.-Y. Wu, Jia, Tan, Xu, & Gui, 2011)
	B. mori chrysalises	Alcalase 2.4 L, Flavourzyme, Protamex, papain, trypsin and pepsin	Antioxidant (DPPH scavenging)	(Yang et al., 2013)
Beetle	*T. molitor* larvae flour	Alcalase	ACE inhibition	(Dai, Ma, Luo, & Yin, 2013)
Silkworm	*B. mori* pupae	Alkaline protease	ACE inhibition	(X. Li et al., 2014)
	B. mori pupae	Acid protease from *A. usamii*	ACE inhibition	(W. Wang, Wang, & Zhang, 2014)

	B. mori pupae powder	Alcalase	ACE inhibition	(Jia, Wu, Yan, & Gui, 2015)
	B. mori pupae powder	SGID (pepsin, trypsin and chymotrypsin)	ACE inhibition	(Q. Wu, Jia, Yan, Du, & Gui, 2015)
	B. mori pupae	neutral proteases	ACE inhibition	(Tao et al., 2017)
	B. mori pupae	Alcalase	ACE inhibition and antioxidant (DPPH scavenging, Fe^{2+}chelating and total reducing activities)	(Zhou, Ren, Yu, Jia, & Gui, 2017)
Cockroach, Locust, Beetle, Superworm	*B. dubia* *G. portentosa* *L. migratoria* *A. annulipes* *Z. morio*	Direct hydrolysis by SGID (α-amylase, pepsin and pancreatin)	Antioxidant (ABTS and DPPH scavenging, Fe^{2+}chelating, Fe^{2+}reducing an and Cu^{2+}chelating activities), ACE, lipase, and α-glucosidase, anti-inflammatory (LOX and COX-2 inhibition)	(Zielińska et al., 2017, 2018; Zielinska et al., 2020)

2.4 Safety concerns: allergenicity of insect protein

Potential allergens and toxins are major safety concerns surrounding insect consumption and represent additional challenges toward consumer acceptance. Insect-induced allergic reactions are typically associated with external interactions caused by components of defensive or poisonous secretions from bites and stings. However, some edible insect species may be toxic to humans or induce allergic responses after consumption. Although not as common, official reports of adverse reactions have been mostly associated with consumption of silkworm pupae (Liu, Tian, & Chen, 2001), teak caterpillar cocoons *(Hyblaea puera)* (Lukiwati, 2010) and grasshoppers (Srivastava, Babu, & Pandey, 2009; Vetter, 1995). Currently, de Gier *et al.* (2018) provides the most recent compilation of case studies describing allergic reactions following insect consumption. Their work details thirty individual reports of allergic reactions following consumption include beetles, moth larvae, caterpillars, locusts, grasshoppers, cicadas, bees, and color additive carmine derived from cochineal extract (*Dactylopius coccus*) ~~var~~. The authors document clinical symptoms that ranged from mild localized reactions to severe systemic reactions such as anaphylactic shock (de Gier & Verhoeckx, 2018). Similar to other food proteins, insects can induce IgE mediated allergic reactions in sensitive individuals (Ribeiro, Cunha, Sousa-Pinto, & Fonseca, 2018). Generally, allergic reactions (post consumption) occur when the immune system overreacts to harmless food substances (allergens) that trigger the production of antibodies (IgE) as an immune defense to "protect" the body from infections and diseases (Galli, Tsai, & Piliponsky, 2008). Major food allergies include, milk, egg, peanut, tree nuts, shellfish, fish, wheat, and soy. However, cross-reactivity is possible across different species (i.e. crustaceans and insects)(Aalberse, Akkerdaas, & Van Ree, 2001). Multiple comprehensive reviews are available that describe aspects of food allergies, epidemiology, pathogenesis, diagnosis and treatment (Ebo & Stevens, 2001; Nurmatov et al., 2017; Sicherer & Sampson, 2018)

Although clinical reports and characterization of edible insect allergies are scarce, numerous *in vitro* analyses reveal the identity of antigens and IgE binding proteins from various species that may correlate to an allergic reaction after ingestion. A growing body of literature illustrate two potential allergenic risks with edible insects following ingestion. Major groups of invertebrates such as insects (mites, cockroaches, and crickets), crustaceans (shrimps, prawns, crabs, and lobsters), and mollusks (squids, oysters, octopus, and mussels) are investigated due to their capacity to trigger food or contact allergic reactions in susceptible individuals. Invertebrate pan-allergens proteins belong to protein families involved in various functional (i.e. muscle contraction proteins, enzymes, storage

proteins) and structure activities (i.e. actin, myosin, tubulins). Due to their high degree of sequential and structural conservation, cross-allergenic properties are often reported between the pan-allergens of this group. Consequently, a number of IgE binding cross-reactive allergens were identified in various edible insect species including crickets, mealworms, super worms, and locusts (Abdelmoteleb et al., 2018; Broekman et al., 2015; F. Hall, Johnson, & Liceaga, 2018; Leni et al., 2020; Palmer, 2016; Reese, Ayuso, & Lehrer, 1999; Yuan et al., 2017). Molecular characterization of these cross-reactive allergens are limited, however, amino acid sequence comparisons of insect antigens and that of shrimp reveal homologies of up to 80%, which may correlate to an allergic reaction post-consumption (Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002; Reese et al., 1999; Wong, Huang, & Lee, 2016). The major shrimp allergen, tropomyosin, is speculated to be a cross-sensitizing allergen in some edible insects, including crickets, due to reported immunological relationships between crustaceans, cockroaches and dust mites (Ayuso et al., 2002; Wong et al., 2016). Tropomyosin is a highly conserved protein that can exist in different isoforms, found in all species of vertebrate and invertebrate's muscle and non-muscle cells. Evidence also suggests that shrimp can cross react with other arthropods and insects, including grasshopper, fruit flies, mealworms, crickets, and grasshoppers (Broekman et al., 2015; Leni et al., 2020; Reese et al., 1999; Van Broekhoven, Bastiaan-Net, de Jong, & Wichers, 2016). It is also suspected that epitopes on tropomyosin are conserved among invertebrates including insects (Leung et al., 1996). For this reason, it is suggested that persons with crustacean allergies avoid consuming insects and insect-containing foods.

Hence, a major concern regarding insect-derived bioactive peptides, in functional foods, is a potential allergic response for at-risk consumers. Although novel food allergens are not unique, it is essential to evaluate the potential risks to provide information to those likely affected and as knowledge for the progression of scientific investigations. Research confirms that some edible cricket species contain tropomyosin proteins that cross-react with those of shrimp and potentially other crustaceans (Abdelmoteleb et al., 2018; Palmer, 2016). Since dust mites and other arthropods such as crickets, have a 75-85% tropomyosin sequence identity (Ayuso et al., 2002), IgE cross-reactivity is greatly expected in edible insect species. Palmer and their team (Palmer, 2016), for the first time, 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). Since house cricket (*Acheta domesticus)* contains reactive tropomyosin (Palmer, 2016), we suspect that the tropical banded

crickets (*Gryllodes sigillatus)* used in this study and widely farmed in the U.S. for human consumption, possess a similar reactivity profile that should be investigated.

2.4.1 Impact of processing and proteolysis on allergenicity

Food proteins involved in sensitization or clinical allergic reactions are susceptible to physicochemical changes during food processing and incorporation into complex food structures. Heat treatment applied during food processing can alter the immunochemical reactivity of food antigens by modifying the three-dimensional structure of proteins. Nevertheless, protein secondary and tertiary structures can be altered as a result of thermal and non-thermal processing, as well as, interfacial adsorptions such as air-water (foams), and oil-water (emulsion) systems. Depending on the IgE-binding epitopes, processing can result in protein unfolding and aggregation, including chemical modifications such as glycation (E. C. Mills & Mackie, 2008; Tian, Rao, Zhang, Tao, & Xue, 2018; Zhao, Huo, Qian, Ren, & Lu, 2017). Such physicochemical changes will, by nature, modify the way in which food proteins are broken down during digestion, absorption, and presented to the immune system. Consequently, it is now understood that protein allergenicity can be inherently altered by food processing. In their review, Wegrzyn and Fiocchi detail the effects of high temperature and food matrices on the most commonly studied food allergens (Nowak-Wegrzyn & Fiocchi, 2009). Shrimp allergens are reported to readily undergo the Maillard reaction (glycation) upon boiling which results in the formation of neo-epitopes that can retain and, in some cases, increase allergenic responses. In other foods however, glycation as a result of high temperature may result in decreased allergenicity in cases such as peanuts and cherry allergens. Given the high amino acid sequence identity (60-85%) among arthropod allergens, it is suspected that edible insect allergens, either primary or cross-reactive, would behave similarly to well-studied shellfish and crustacean antigenic proteins post-processing.

Edible insect preparations are often consumed after certain degree of heat and processing (such as roasting or pasteurizing). Understanding the allergenic properties of insects/insect-containing foods as effected by these processing techniques is critical for shrimp allergic, as well as, potential newly sensitized individuals. Numerous investigations already describe the effects of processing and heat on shrimp/shell-fish allergens. In general, several allergenic arthropod proteins are considered heat-stable and form dimers or oligomers enhancing antibody reactivity. There are also accounts of reactive fragments of crustacean allergens in processing foods (Kamath, Rahman,

Komoda, & Lopata, 2013; Lopata, O'hehir, & Lehrer, 2010; Rahaman, Vasiljevic, & Ramchandran, 2016). Given their novelty, there are limited accounts on the effects of processing on edible insect allergens. However, some species are documented having altered allergenic profiles after processing compared to the unprocessed (raw) insect. For example, thermal processing on edible locusts (*Patanga succincta*) exhibited different IgE-binding proteins using pooled sera from prawn-allergic patient sera (n=16) (Phiriyangkul, Srinroch, Srisomsap, Chokchaichamnankit, & Punyarit, 2015). Enolase, HEX1B, and arginine kinase were identified as raw locust allergens, whereas fried locust only contained HEX1B and enolase as allergens. In the same study, two other allergenic proteins were undetected in raw locusts as opposed to 'cooked': pyruvate kinase and GAPDH (Phiriyangkul et al., 2015). Broekhoven *et al.* (2016) observed diminished, but not eliminated, allergenic response of tropomyosin IgE cross-reactivity in three edible mealworm species after heat processing and *in vitro* digestion. In a similar study, the authors also observed differences in IgE-binding between untreated and heated yellow mealworm extracts (Broekman et al., 2015). However, the authors attribute this to decreased protein solubility (observed in the western blotting) since the skin prick analyses showed similar reactivity between the heat-treated and unprocessed mealworm extracts (Broekman et al., 2015).

Fermentation and enzymatic hydrolysis are the only treatments that have so far demonstrated efficacy in lowering allergenicity in foods, depending on the protein (Besler, Steinhart, & Paschke, 2001). Both techniques are often used to produce hypoallergenic milk protein hydrolysates for functional food development (Abd El-Salam & El-Shibiny, 2019). Shrimp tropomyosin tends to show a decrease in human IgE binding under pepsin hydrolysis, and is considered a possible method to reduce sensitivity (Mejrhit et al., 2017). Likewise, cross-reactivity and allergenicity of skin prick tests (n= 5 crustacean allergic patients) to migratory locusts (*locusta migratoria*) decreased? after hydrolysis with different enzymes and heat treatments. The patients reacted to most or all of the non-hydrolyzed sample with no reactions to the enzymatically and heat-treated sample extracts (Pali-Schöll et al., 2019). Edible insects and their associated products will likely always require warning labels for at-risk allergic consumers. However, preliminary studies to-date demonstrate the potential of hypoallergenic insect protein extracts or bioactive peptides. Further, risk assessment of novel foods should include understanding how protein extraction methods such as enzymatic hydrolysis impact existing immune reactivity. In general, factors that alter the allergenicity of food proteins are not well-

known. In addition to cooking, thermal, and non-thermal processing techniques, the structure of the food matrix (i.e. processed snacks or food fortification) may also affect allergen stability and release which would impact elicitation of reactions after consumption.

2.5 Research objectives and project aims

Despite the growing interest in edible insects, the treatment/prevention of diseases with their protein/peptides has not yet been validated with enough scientific criteria to support their use as functional ingredients. More research is, therefore, needed to shed light on the mechanisms of action and potential of these bioactive compounds to be employed as new therapeutic drugs. Consequently, it is also necessary to evaluate their allergenic properties as insect proteins are known to cross-react with crustacean allergens.

Therefore, the main objectives of this project are to **(1) assess the efficacy, *in vitro*, of bioactive peptides from edible crickets; and (2) evaluate allergenic properties of cricket peptides and the effects of proteolysis conditions on allergenicity.**

To achieve this, the following specific aims were established;

- **Aim 1**: Determine conditions required to liberate bioactive peptides from cricket protein and measure their potential, *in vitro,* antioxidant, antihypertensive, and antidiabetic activity

- **Aim 2**: Assess the impact of the Alcalase™ proteolysis treatments on cricket allergenic cross-reactivity

- **Aim 4**: Examine the effects of microwave heating, during proteolysis, on peptide bioactivity and antigen immunoreactivity

- **Aim 5**: Characterize tropomyosin immunoreactivity comparing the use of conventional and microwave heating during proteolysis

- **Aim 6**: Identify and characterize peptides responsible for the bioactivity observed in cricket protein hydrolysates (CPH)

- **Aim 7**: Evaluate the potential of CPH with antidiabetic and antihypertensive properties to attenuate cellular inflammatory conditions. Also, determine cellular permeability of cricket peptides.

2.6 References

Aalberse, R. C., Akkerdaas, J., & Van Ree, R. (2001). Cross-reactivity of IgE antibodies to allergens. *Allergy, 56*(6), 478-490.

Abd El-Salam, M. H., & El-Shibiny, S. (2019). Reduction of milk protein antigenicity by enzymatic hydrolysis and fermentation. A review. *Food Reviews International*, 1-20.

Abdelmoteleb, M., Palmer, L. K., Pavlovikj, N., Marsh, J. T., Johnson, P. E., & Goodman, R. E. (2018). Bioinformatics and Proteomics Evaluations to Consider IgE Binding Assays for Potential Cross-Reactivity Between House-Cricket (Aceta domesticus) Used in Food, Crustaceans and Cockroaches. *Journal of Allergy and Clinical Immunology, 141*(2), AB263.

Aguilar-Toalá, J., Deering, A., & Liceaga, A. (2020). New Insights into the Antimicrobial Properties of Hydrolysates and Peptide Fractions Derived from Chia Seed (Salvia hispanica L.). *Probiotics and antimicrobial proteins*.

Aguilar-Toalá, J., & Liceaga, A. (2020). Identification of chia seed (Salvia hispanica L.) peptides with enzyme inhibition activity towards skin-aging enzymes. *Amino Acids*, 1-11.

Ahn, C.-B., Je, J.-Y., & Cho, Y.-S. (2012). Antioxidant and anti-inflammatory peptide fraction from salmon byproduct protein hydrolysates by peptic hydrolysis. *Food Research International, 49*(1), 92-98.

Alder-Nissen, J. (1986). *Enzymic Hydrolysis of Food Proteins*: Elsevier Applied Science Publishers.

Asif, M. (2014). The prevention and control the type-2 diabetes by changing lifestyle and dietary pattern. *Journal of education and health promotion, 3*.

Association, A. D. (2017). 9. Cardiovascular disease and risk management. *Diabetes Care, 40*(Supplement 1), S75-S87.

Atlas, I. D. (2013). 6th edn. *International Diabetes Federation.–Brussels, Belgium*, 160.

Ayuso, R., Reese, G., Leong-Kee, S., Plante, M., & Lehrer, S. B. (2002). Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosins. *International archives of allergy and immunology, 129*(1), 38-48.

Bagchi, K., & Puri, S. (1998). Free radicals and antioxidants in health and disease: a review.

Besler, M., Steinhart, H., & Paschke, A. (2001). Stability of food allergens and allergenicity of processed foods. *Journal of Chromatography B: Biomedical Sciences and Applications, 756*(1-2), 207-228.

Betoret, E., Betoret, N., Vidal, D., & Fito, P. (2011). Functional foods development: Trends and technologies. *Trends in food science & technology, 22*(9), 498-508.

Broekman, H., Knulst, A., den Hartog Jager, S., Monteleone, F., Gaspari, M., De Jong, G., . . . Verhoeckx, K. (2015). Effect of thermal processing on mealworm allergenicity. *Molecular nutrition & food research, 59*(9), 1855-1864.

Cadenas, E. (1989). Biochemistry of oxygen toxicity. *Annual review of biochemistry, 58*(1), 79-110.

Chabance, B., Jollès, P., Izquierdo, C., Mazoyer, E., Francoual, C., Drouet, L., & Fiat, A.-M. (1995). Characterization of an antithrombotic peptide from α-casein in newborn plasma after milk ingestion. *British Journal of Nutrition, 73*(4), 583-590.

Chakrabarti, S., Jahandideh, F., & Wu, J. (2014). Food-derived bioactive peptides on inflammation and oxidative stress. *BioMed research international, 2014*.

Chalamaiah, M., Ulug, S. K., Hong, H., & Wu, J. (2019). Regulatory requirements of bioactive peptides (protein hydrolysates) from food proteins. *Journal of Functional Foods, 58*, 123-129.

Chatterton, D. E., Nguyen, D. N., Bering, S. B., & Sangild, P. T. (2013). Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *The international journal of biochemistry & cell biology, 45*(8), 1730-1747.

Chen, H.-M., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of agricultural and food chemistry, 46*(1), 49-53.

Coelho, M. S., de Araujo Aquino, S., Latorres, J. M., & de las Mercedes Salas-Mellado, M. (2019). In vitro and in vivo antioxidant capacity of chia protein hydrolysates and peptides. *Food hydrocolloids*, 91, 19-25.

Crowley, S. D., Gurley, S. B., Herrera, M. J., Ruiz, P., Griffiths, R., Kumar, A. P., . . . Coffman, T. M. (2006). Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proceedings of the National Academy of Sciences, 103*(47), 17985-17990.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1982). Development and design of specific inhibitors of angiotensin-converting enzyme. *The American journal of cardiology, 49*(6), 1390-1394.

Dai, C., Ma, H., Luo, L., & Yin, X. (2013). Angiotensin I-converting enzyme (ACE) inhibitory peptide derived from Tenebrio molitor (L.) larva protein hydrolysate. *European Food Research and Technology, 236*(4), 681-689.

de Gier, S., & Verhoeckx, K. (2018). Insect (food) allergy and allergens. *Molecular immunology, 100*, 82-106.

Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring antioxidant effectiveness in food. *Journal of agricultural and food chemistry, 53*(10), 4303-4310.

Donkor, O., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2005). Probiotic Strains as Starter Cultures Improve Angiotensin-converting Enzyme Inhibitory Activity in Soy Yogurt. *Journal of Food Science, 70*(8).

Ebo, D., & Stevens, W. (2001). IgE-mediated food allergy–extensive review of the literature. *Acta Clinica Belgica, 56*(4), 234-247.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). Characterization of protein hydrolysates from blue whiting (Micromesistius poutassou) and their application in beverage fortification. *Food chemistry*, *245*, 698-706.

Fan, H., Yan, S., Stehling, S., Marguet, D., Schuppan, D., & Reutter, W. (2004). Dipeptidyl peptidase IV/CD26 in T cell activation, cytokine secretion and immunoglobulin production. *Dipeptidyl Aminopeptidases in Health and Disease*, 165-174.

Fernandes, N. P., Lagishetty, C. V., Panda, V. S., & Naik, S. R. (2007). An experimental evaluation of the antidiabetic and antilipidemic properties of a standardized Momordica charantia fruit extract. *BMC complementary and alternative medicine, 7*(1), 29.

Fiat, A.-M., Levy-Toledano, S., Caen, J. P., & Jollès, P. (1989). Biologically active peptides of casein and lactotransferrin implicated in platelet function. *Journal of Dairy Research, 56*(3), 351-355.

FitzGerald, R. J., Murray, B. A., & Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *The Journal of Nutrition, 134*(4), 980S-988S.

Foltz, M., van der Pijl, P. C., & Duchateau, G. S. (2010). Current in vitro testing of bioactive peptides is not valuable. *The Journal of nutrition, 140*(1), 117-118.

Fröde, T., & Medeiros, Y. (2008). Animal models to test drugs with potential antidiabetic activity. *Journal of Ethnopharmacology, 115*(2), 173-183.

Galli, S. J., Tsai, M., & Piliponsky, A. M. (2008). The development of allergic inflammation. *Nature, 454*(7203), 445-454.

Genestra, M. (2007). Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cellular signalling, 19*(9), 1807-1819.

Giannetto, A., Esposito, E., Lanza, M., Oliva, S., Riolo, K., Di Pietro, S., Abbate, J.M., Briguglio, G., Cassata, G., Cicero, L. and Macrì, F. (2020). Protein Hydrolysates from Anchovy (*Engraulis encrasicolus*) Waste: In Vitro and In Vivo Biological Activities. *Marine Drugs*, 18(2), 86.

Guha, S., & Majumder, K. (2019). Structural-features of food-derived bioactive peptides with anti-inflammatory activity: A brief review. *Journal of food biochemistry, 43*(1), e12531.

Hall, F.G., and Liceaga, A. M. (2019). Nutritional, Functional and Bioactive Protein Hydrolysates. In Melton, L., Shahidi, F., and Varelis, P. (Eds.), *Encyclopedia of Food Chemistry*, 3, 456-464. Elsevier. doi.org/10.1016/B978-0-08-100596-5.21776-9

Hall, F., Johnson, P. E., & Liceaga, A. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food chemistry, 262*, 39-47.

Hall, F. G., Jones, O. G., O'Haire, M. E., & Liceaga, A. M. (2017). Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. *Food chemistry, 224*, 414-422.

Hayes, M., Ross, R. P., Fitzgerald, G. F., & Stanton, C. (2007). Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part I: overview. *Biotechnology Journal, 2*(4), 426-434.

Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of functional foods*, 4(1), 6-24.

Himaya, S., Ngo, D.-H., Ryu, B., & Kim, S.-K. (2012). An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. *Food Chemistry, 132*(4), 1872-1882.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of agricultural and food chemistry, 53*(6), 1841-1856.

Huang, W., Shen, S., Nimalaratne, C., Li, S., Majumder, K., & Wu, J. (2012). Effects of addition of egg ovotransferrin-derived peptides on the oxygen radical absorbance capacity of different teas. *Food chemistry, 135*(3), 1600-1607.

Iwami, K., Sakakibara, K., & Ibuki, F. (1986). Involvement of post-digestion 'hydrophobic'peptides in plasma cholesterol-lowering effect of dietary plant proteins. *Agricultural and biological chemistry, 50*(5), 1217-1222.

Iwaniak, A., Minkiewicz, P., & Darewicz, M. (2014). Food-originating ACE inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. *Comprehensive Reviews in Food Science and Food Safety, 13*(2), 114-134.

Jay, D., Hitomi, H., & Griendling, K. K. (2006). Oxidative stress and diabetic cardiovascular complications. *Free Radical Biology and Medicine, 40*(2), 183-192.

Jia, J., Wu, Q., Yan, H., & Gui, Z. (2015). Purification and molecular docking study of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide from alcalase hydrolysate of ultrasonic-pretreated silkworm pupa (Bombyx mori) protein. *Process Biochemistry, 50*(5), 876-883.

Ketnawa, S., & Liceaga, A. M. (2017). Effect of microwave treatments on antioxidant activity and antigenicity of fish frame protein hydrolysates. *Food and Bioprocess Technology, 10*(3), 582-591.

Ketnawa, S., Wickramathilaka, M., & Liceaga, A. M. (2018). Changes on antioxidant activity of microwave-treated protein hydrolysates after simulated gastrointestinal digestion: Purification and identification. *Food Chemistry*, 254, 36-46.

Kim, W., & Egan, J. M. (2008). The role of incretins in glucose homeostasis and diabetes treatment. *Pharmacological reviews, 60*(4), 470-512.

Klaunig, J. E., & Kamendulis, L. M. (2004). The role of oxidative stress in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol., 44*, 239-267.

Krentz, A. J., & Sinclair, A. J. (2012). The Evolution of Glucose-Lowering Drugs for Type 2 Diabetes *Nutritional and Therapeutic Interventions for Diabetes and Metabolic Syndrome* (pp. 459-474): Elsevier.

Kritchevsky, D., Tepper, S. A., Czarnecki, S. K., & Klurfeld, D. M. (1982). Atherogenicity of animal and vegetable protein: influence of the lysine to arginine ratio. *Atherosclerosis, 41*(2-3), 429-431.

Lee, J. K., Hong, S., Jeon, J.-K., Kim, S.-K., & Byun, H.-G. (2009). Purification and characterization of angiotensin I converting enzyme inhibitory peptides from the rotifer, Brachionus rotundiformis. *Bioresource Technology, 100*(21), 5255-5259.

Leni, G., Tedeschi, T., Faccini, A., Pratesi, F., Folli, C., Puxeddu, I., . . . Depraetere, S. (2020). Shotgun proteomics, in-silico evaluation and immunoblotting assays for allergenicity assessment of lesser mealworm, black soldier fly and their protein hydrolysates. *Scientific Reports, 10*(1), 1-10.

Leung, P. S., Chow, W. K., Duffey, S., Kwan, H. S., Gershwin, M. E., & Chu, K. H. (1996). IgE reactivity against a cross-reactive allergen in crustacea and mollusca: evidence for tropomyosin as the common allergen. *Journal of Allergy and Clinical Immunology, 98*(5), 954-961.

Li-Chan, E. C. (2015). Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Current Opinion in Food Science, 1*, 28-37.

Li, G.-H., Le, G.-W., Shi, Y.-H., & Shrestha, S. (2004). Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition research, 24*(7), 469-486.

Li, X., Li, Y., Huang, X., Zheng, J., Zhang, F., & Kan, J. (2014). Identification and characterization of a novel angiotensin I-converting enzyme inhibitory peptide (ACEIP) from silkworm pupa. *Food Science and Biotechnology, 23*(4), 1017-1023.

Liu, X., Tian, J., & Chen, D. (2001). An allergic shock case that resulted from consuming silkworm pupae. *Chinese Recuperative Medicine, 10*(2), 80.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy reviews, 4*(8), 118.

Lukiwati, D. R. (2010). Teak caterpillars and other edible insects in Java. *Forest insects as food: humans bite back*, 99.

Luna, G. C., Martin-Gonzalez, F. S., Mauer, L. J., & Liceaga, A. M. (2020). Cricket (Acheta domesticus) protein hydrolysates' impact on the physicochemical, structural and sensory properties of tortillas and tortilla chips. *Journal of Insects as Food and Feed*, 1-12.

Machlin, L. J., & Bendich, A. (1987). Free radical tissue damage: protective role of antioxidant nutrients. *The FASEB Journal, 1*(6), 441-445.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica chimica acta, 613*(1), 1-19.

Majumder, K., Chakrabarti, S., Davidge, S. T., & Wu, J. (2013). Structure and activity study of egg protein ovotransferrin derived peptides (IRW and IQW) on endothelial inflammatory response and oxidative stress. *Journal of agricultural and food chemistry, 61*(9), 2120-2129.

Majumder, K., Mine, Y., & Wu, J. (2016). The potential of food protein-derived anti-inflammatory peptides against various chronic inflammatory diseases. *Journal of the Science of Food and Agriculture, 96*(7), 2303-2311.

Makino, S., Nakashima, H., Minami, K., Moriyama, R., & Takao, S. (1988). Bile acid-binding protein from soybean seed: isolation, partial characterization and insulin-stimulating activity. *Agricultural and biological chemistry, 52*(3), 803-809.

Manso, M. A., Miguel, M., Even, J., Hernández, R., Aleixandre, A., & López-Fandiño, R. (2008). Effect of the long-term intake of an egg white hydrolysate on the oxidative status and blood lipid profile of spontaneously hypertensive rats. *Food Chemistry, 109*(2), 361-367.

Matsufuji, H., Matsui, T., Seki, E., Osajima, K., Nakashima, M., & Osajima, Y. (1994). Angiotensin I-converting enzyme inhibitory peptides in an alkaline protease hydrolyzate derived from sardine muscle. *Bioscience, biotechnology, and biochemistry, 58*(12), 2244-2245.

Meisel, H. (1997). Biochemical properties of regulatory peptides derived from mil proteins. *Peptide Science, 43*(2), 119-128.

Mejrhit, N., Azdad, O., Chda, A., El Kabbaoui, M., Bousfiha, A., Bencheikh, R., . . . Aarab, L. (2017). Evaluation of the sensitivity of Moroccans to shrimp tropomyosin and effect of heating and enzymatic treatments. *Food and Agricultural Immunology, 28*(6), 969-980.

Mills, E. C., & Mackie, A. R. (2008). The impact of processing on allergenicity of food. *Current opinion in allergy and clinical immunology, 8*(3), 249-253.

Mills, K. T., Bundy, J. D., Kelly, T. N., Reed, J. E., Kearney, P. M., Reynolds, K., . . . He, J. (2016). Global disparities of hypertension prevalence and control. *Circulation, 134*(6), 441-450.

Mishyna, M., Chen, J., & Benjamin, O. (2020). Sensory attributes of edible insects and insect-based foods–Future outlooks for enhancing consumer appeal. *Trends in Food Science & Technology*, 95, 141-148.

Mlcek, J., Borkovcova, M., Rop, O., & Bednarova, M. (2014). Biologically active substances of edible insects and their use in agriculture, veterinary and human medicine. *Journal of Central European Agriculture, 15*(4), 0-0.

Mohamed, S. (2014). Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovasular disease. *Trends in food science & technology, 35*(2), 114-128.

Moon, J.-K., & Shibamoto, T. (2009). Antioxidant assays for plant and food components. *Journal of agricultural and food chemistry, 57*(5), 1655-1666.

Morita, T., Oh-hashi, A., Takei, K., Ikai, M., Kasaoka, S., & Kiriyama, S. (1997). Cholesterol-lowering effects of soybean, potato and rice proteins depend on their low methionine contents in rats fed a cholesterol-free purified diet. *The Journal of nutrition, 127*(3), 470-477.

Murray, B., & FitzGerald, R. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. *Current pharmaceutical design, 13*(8), 773-791.

Nazeer, R., Kumar, N. S., & Ganesh, R. J. (2012). In vitro and in vivo studies on the antioxidant activity of fish peptide isolated from the croaker (Otolithes ruber) muscle protein hydrolysate. *Peptides, 35*(2), 261-268.

Nishi, T., Hara, H., Asano, K., & Tomita, F. (2003). The soybean β-conglycinin β 51–63 fragment suppresses appetite by stimulating cholecystokinin release in rats. *The Journal of nutrition, 133*(8), 2537-2542.

Nishi, T., Hara, H., Hira, T., & Tomita, F. (2001). Dietary protein peptic hydrolysates stimulate cholecystokinin release via direct sensing by rat intestinal mucosal cells. *Experimental Biology and Medicine, 226*(11), 1031-1036.

Nongonierma, A. B., & FitzGerald, R. J. (2017). Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review. *Innovative Food Science & Emerging Technologies*.

Nongonierma, A. B., Lamoureux, C., & FitzGerald, R. J. (2018). Generation of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides during the enzymatic hydrolysis of tropical banded cricket (Gryllodes sigillatus) proteins. *Food & function*.

owak-Wegrzyn, A., & Fiocchi, A. (2009). Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. *Current opinion in allergy and clinical immunology, 9*(3), 234-237.

Nurmatov, U., Dhami, S., Arasi, S., Pajno, G. B., Fernandez-Rivas, M., Muraro, A., . . . Beyer, K. (2017). Allergen immunotherapy for IgE-mediated food allergy: a systematic review and meta-analysis. *Allergy, 72*(8), 1133-1147.

Offengenden, M., Chakrabarti, S., & Wu, J. (2018). Chicken collagen hydrolysates differentially mediate anti-inflammatory activity and type I collagen synthesis on human dermal fibroblasts. *Food Science and Human Wellness*, *7*(2), 138-147.

Ondetti, M., & Cushman, D. (1982). Enzymes of the renin-angiotensin system and their inhibitors. *Annual review of biochemistry, 51*(1), 283-308.

Pali-Schöll, I., Meinlschmidt, P., Larenas-Linnemann, D., Purschke, B., Hofstetter, G., Rodríguez-Monroy, F. A., . . . Jäger, H. (2019). Edible insects: Cross-recognition of IgE from crustacean-and house dust mite allergic patients, and reduction of allergenicity by food processing. *World Allergy Organization Journal, 12*(1), 100006.

Palmer, L. (2016). Edible Insects as a Source of Food Allergens.

Peña-Ramos, E. A., Xiong, Y. L., & Arteaga, G. E. (2004). Fractionation and characterisation for antioxidant activity of hydrolysed whey protein. *Journal of the Science of Food and Agriculture, 84*(14), 1908-1918.

Petersen, B. L., Ward, L. S., Bastian, E. D., Jenkins, A. L., Campbell, J., & Vuksan, V. (2009). A whey protein supplement decreases post-prandial glycemia. *Nutrition Journal, 8*(1), 47.

Petrie, J. R., Guzik, T. J., & Touyz, R. M. (2018). Diabetes, hypertension, and cardiovascular disease: clinical insights and vascular mechanisms. *Canadian Journal of Cardiology, 34*(5), 575-584.

Phiriyangkul, P., Srinroch, C., Srisomsap, C., Chokchaichamnankit, D., & Punyarit, P. (2015). Effect of food thermal processing on allergenicity proteins in Bombay locust (Patanga succincta). *International Journal of Food Engineering, 1*(1), 23-28.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of agricultural and food chemistry, 53*(10), 4290-4302.

Raghavan, S., Kristinsson, H. G., & Leeuwenburgh, C. (2008). Radical scavenging and reducing ability of tilapia (Oreochromis niloticus) protein hydrolysates. *Journal of agricultural and food chemistry, 56*(21), 10359-10367.

Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G., & Nishigaki, I. (2013). The vascular endothelium and human diseases. *International journal of biological sciences, 9*(10), 1057.

Reese, G., Ayuso, R., & Lehrer, S. B. (1999). Tropomyosin: An invertebrate pan–allergen. *International Archives of Allergy and Immunology, 119*(4), 247-258.

Ribeiro, J. C., Cunha, L. M., Sousa-Pinto, B., & Fonseca, J. (2018). Allergic risks of consuming edible insects: A systematic review. *Molecular nutrition & food research, 62*(1), 1700030.

Ricci-Cabello, I., Olalla Herrera, M., & Artacho, R. (2012). Possible role of milk-derived bioactive peptides in the treatment and prevention of metabolic syndrome. *Nutrition reviews, 70*(4), 241-255.

Roos, N. (2018). Insects and human nutrition. *In Edible insects in sustainable food systems* (pp. 83-91). Springer, Cham.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides, 31*(10), 1949-1956.

Sato, K., Egashira, Y., Ono, S., Mochizuki, S., Shimmura, Y., Suzuki, Y., ... & Nakamura, Y. (2013). Identification of a hepatoprotective peptide in wheat gluten hydrolysate against D-galactosamine-induced acute hepatitis in rats. *Journal of agricultural and food chemistry*, *61*(26), 6304-6310.

Sena, C. M., Bento, C. F., Pereira, P., Marques, F., & Seiça, R. (2013). Diabetes mellitus: new challenges and innovative therapies *New Strategies to Advance Pre/Diabetes Care: Integrative Approach by PPPM* (pp. 29-87): Springer.

Shi, Y., Kovacs-Nolan, J., Jiang, B., Tsao, R., & Mine, Y. (2014). Antioxidant activity of enzymatic hydrolysates from eggshell membrane proteins and its protective capacity in human intestinal epithelial Caco-2 cells. *Journal of Functional Foods, 10*, 35-45.

Sicherer, S. H., & Sampson, H. A. (2018). Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *Journal of Allergy and Clinical Immunology, 141*(1), 41-58.

Sosa, D. A. T., & Fogliano, V. (2017). Potential of Insect-Derived Ingredients for Food Applications *Insect Physiology and Ecology*: InTech.

Spiegel, v. d. M., Noordam, M., & Fels-Klerx, H. (2013). Safety of novel protein sources (insects, microalgae, seaweed, duckweed, and rapeseed) and legislative aspects for their application in food and feed production. *Comprehensive Reviews in Food Science and Food Safety, 12*(6), 662-678.

Srivastava, S., Babu, N., & Pandey, H. (2009). Traditional insect bioprospecting-As human food and medicine. *Indian Journal of Traditional Knowledge, 8*(4), 485-494.

Suetsuna, K. (1998). Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from Allium sativum L (garlic). *The Journal of Nutritional Biochemistry, 9*(7), 415-419.

Tadesse, S. A., & Emire, S. A. (2020). Production and processing of antioxidant bioactive peptides: A driving force for the functional food market. *Heliyon, 6*(8), e04765.

Tao, M., Wang, C., Liao, D., Liu, H., Zhao, Z., & Zhao, Z. (2017). Purification, modification and inhibition mechanism of angiotensin I-converting enzyme inhibitory peptide from silkworm pupa (Bombyx mori) protein hydrolysate. *Process Biochemistry, 54*, 172-179.

Tavares, T., Sevilla, M. Á., Montero, M. J., Carrón, R., & Malcata, F. X. (2012). Acute effect of whey peptides upon blood pressure of hypertensive rats, and relationship with their angiotensin-converting enzyme inhibitory activity. *Molecular nutrition & food research, 56*(2), 316-324.

Tian, Y., Rao, H., Zhang, K., Tao, S., & Xue, W. T. (2018). Effects of different thermal processing methods on the structure and allergenicity of peanut allergen Ara h 1. *Food science & nutrition, 6*(6), 1706-1714.

Urbizo-Reyes, U., San Martin-González, M. F., Garcia-Bravo, J., Vigil, A. L. M., & Liceaga, A. M. (2019). Physicochemical characteristics of chia seed (Salvia hispanica) protein hydrolysates produced using ultrasonication followed by microwave-assisted hydrolysis. *Food Hydrocolloids, 97*, 105187.

Van Broekhoven, S., Bastiaan-Net, S., de Jong, N. W., & Wichers, H. J. (2016). Influence of processing and in vitro digestion on the allergic cross-reactivity of three mealworm species. *Food chemistry, 196*, 1075-1083.

Van de Laar, F. A., Lucassen, P. L., Akkermans, R. P., Van de Lisdonk, E. H., Rutten, G. E., & Van Weel, C. (2005). Alpha-glucosidase inhibitors for type 2 diabetes mellitus. *Cochrane database of systematic reviews*, (2).

Van Huis, A. (2003). Insects as food in sub-Saharan Africa. *International Journal of Tropical Insect Science, 23*(03), 163-185.

Van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G., & Vantomme, P. (2013). *Edible insects: future prospects for food and feed security* (No. 171). Food and Agriculture Organization of the United Nations.

Vercruysse, L., Smagghe, G., Beckers, T., & Van Camp, J. (2009). Antioxidative and ACE inhibitory activities in enzymatic hydrolysates of the cotton leafworm, Spodoptera littoralis. *Food chemistry, 114*(1), 38-43.

Vercruysse, L., Smagghe, G., Herregods, G., & Van Camp, J. (2005). ACE inhibitory activity in enzymatic hydrolysates of insect protein. *Journal of agricultural and food chemistry, 53*(13), 5207-5211.

Vercruysse, L., Smagghe, G., Matsui, T., & Van Camp, J. (2008). Purification and identification of an angiotensin I converting enzyme (ACE) inhibitory peptide from the gastrointestinal hydrolysate of the cotton leafworm, Spodoptera littoralis. *Process biochemistry, 43*(8), 900-904.

Vermeirssen, V., Van Camp, J., & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92*(3), 357-366.

Vermeirssen, V., van der Bent, A., Van Camp, J., van Amerongen, A., & Verstraete, W. (2004). A quantitative in silico analysis calculates the angiotensin I converting enzyme (ACE) inhibitory activity in pea and whey protein digests. *Biochimie, 86*(3), 231-239.

Vetter, R. (1995). A case of ingestant allergy from eating a grasshopper. *The Food Insects Newsletter, 8*(5).

Wang, D., Wang, L.-j., Zhu, F.-x., Zhu, J.-y., Chen, X. D., Zou, L., & Saito, M. (2008). In vitro and in vivo studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food). *Food Chemistry, 107*(4), 1421-1428.

Wang, W., Wang, N., & Zhang, Y. (2014). Antihypertensive Properties on Spontaneously Hypertensive Rats of Peptide Hydrolysates from Silkworm Pupae Protein. *Food and Nutrition Sciences, 5*(13), 1202.

Wang, W., Wang, N., Zhou, Y., Zhang, Y., Xu, L., Xu, J., . . . He, G. (2011). Isolation of a novel peptide from silkworm pupae protein components and interaction characteristics to angiotensin I-converting enzyme. *European Food Research and Technology, 232*(1), 29-38.

Wergedahl, H., Liaset, B., Gudbrandsen, O. A., Lied, E., Espe, M., Muna, Z., . . . Berge, R. K. (2004). Fish protein hydrolysate reduces plasma total cholesterol, increases the proportion of HDL cholesterol, and lowers acyl-CoA: cholesterol acyltransferase activity in liver of Zucker rats. *The Journal of nutrition, 134*(6), 1320-1327.

Whiting, D. R., Guariguata, L., Weil, C., & Shaw, J. (2011). IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes research and clinical practice, 94*(3), 311-321.

Wong, L., Huang, C. H., & Lee, B. W. (2016). Shellfish and house dust mite allergies: is the link tropomyosin? *Allergy, asthma & immunology research, 8*(2), 101-106.

Wongputtisin, P., Khanongnuch, C., Pongpiachan, P., & Lumyong, S. (2007). Antioxidant activity improvement of soybean meal by microbial fermentation.

Wu, Q.-Y., Jia, J.-Q., Tan, G.-X., Xu, J.-L., & Gui, Z.-Z. (2011). Physicochemical properties of silkworm larvae protein isolate and gastrointestinal hydrolysate bioactivities. *African Journal of Biotechnology, 10*(32), 6145-6153.

Wu, Q., Jia, J., Yan, H., Du, J., & Gui, Z. (2015). A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (Bombyx mori) protein: biochemical characterization and molecular docking study. *Peptides, 68*, 17-24.

Xue, Z., Yu, W., Liu, Z., Wu, M., Kou, X., & Wang, J. (2009). Preparation and antioxidative properties of a rapeseed (Brassica napus) protein hydrolysate and three peptide fractions. *Journal of agricultural and food chemistry, 57*(12), 5287-5293.

Yang, R., Zhao, X., Kuang, Z., Ye, M., Luo, G., Xiao, G., . . . Xiong, Z. (2013). Optimization of antioxidant peptide production in the hydrolysis of silkworm (Bombyx mori L.) pupa protein using response surface methodology. *J Food Agric Environ, 11*, 952-956.

Yoo, K. M., Kim, D., & Lee, C. Y. (2007). Evaluation of different methods of antioxidant measurement. *Food Science and Biotechnology, 16*(2), 177.

Yuan, F., Lv, L., Li, Z., Mi, N., Chen, H., & Lin, H. (2017). Effect of transglutaminase-catalyzed glycosylation on the allergenicity and conformational structure of shrimp (Metapenaeus ensis) tropomyosin. *Food chemistry, 219*, 215-222.

Zhang, W., Lv, T., Li, M., Wu, Q., Yang, L., Liu, H., ... & Wang, D. (2013). Beneficial effects of wheat gluten hydrolysate to extend lifespan and induce stress resistance in nematode Caenorhabditis elegans. *PLoS One*, *8*(9), e74553.

Zhao, R.-J., Huo, C.-Y., Qian, Y., Ren, D.-F., & Lu, J. (2017). Ultra-high-pressure processing improves proteolysis and release of bioactive peptides with activation activities on alcohol metabolic enzymes in vitro from mushroom foot protein. *Food chemistry, 231*, 25-32.

Zhou, Z.-F., Ren, Z.-X., Yu, H.-Y., Jia, J.-Q., & Gui, Z.-Z. (2017). Effects of different modification techniques on molecular structure and bioactivity of Bombyx mori pupa protein. *Journal of Asia-Pacific Entomology, 20*(1), 35-41.

Zielińska, E., Baraniak, B., & Karaś, M. (2017). Antioxidant and anti-inflammatory activities of hydrolysates and peptide fractions obtained by enzymatic hydrolysis of selected heat-treated edible insects. *Nutrients, 9*(9), 970.

Zielińska, E., Baraniak, B., & Karaś, M. (2018). Identification of antioxidant and anti-inflammatory peptides obtained by simulated gastrointestinal digestion of three edible insects species (Gryllodes sigillatus, Tenebrio molitor, Schistocerca gragaria). *International journal of food science & technology, 53*(11), 2542-2551.

Zielinska, E., Karas, M., Baroniak, B., & Jakubczyk, A. (2020). Evaluation of ACE, α-glucosidase, and lipase inhibitory activities of peptides obtained by in vitro digestion of selected species of edible insects. *European Food Research and Technology*.

CHAPTER 3. EFFECT OF ENZYMATIC HYDROLYSIS ON BIOACTIVE PROPERTIES AND ALLERGENICITY OF CRICKET (*GRYLLODES SIGILLATUS*) PROTEIN

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Hall, F., Johnson, P. E., & Liceaga, A.M. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (*Gryllodes sigillatus*) protein. *Food Chemistry*, *262*, 39-47. DOI: https://doi.org/10.1016/j.foodchem.2018.04.058

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 (*Gryllodes 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.

3.1 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, immunomodulation, osteoprotective, antimicrobial, anti-carcinogenic 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) complied 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 et al., 2012, Karamac et al., 2016, Zhu et al., 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 (IC50 20 μm) and Gln-Pro-Gly-Arg (IC50 65.8 μm); 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 (*Gryllodes 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.

3.2 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/µg 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 (Gryllodes 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).

3.3 Methods

3.3.1 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 further use.

Table 3.1. 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 ± 0.5
15	0.25	10	15.2 ± 0.1
20	0.25	30	23.1 ± 0.8
30	0.5	10	31.3 ± 0.3
40	0.5	20	42.5 ± 0.6
50	3.0	20	51.8 ± 0.6
60	3.0	40	60.5 ± 0.6
70	3.0	60	72.8 ± 0.9
85	3.0	80	84.6 ± 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 ± 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.

3.3.2 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 (htot), and expressed as:

$$\% \text{ Degree of hydrolysis (DH)} = [\frac{h}{htot}] \times 100$$

where htot 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 htot 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).

3.3.3 Simulated gastrointestinal digestion (SGD)

In vitro digestion was performed as reported by You and others (You, Zhao, Regenstein, & 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×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.

3.3.4 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).

3.3.5 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 µl aliquot of sample (1 mg/mL) was allowed to react with 294 µl 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.

3.3.6 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 µl) were mixed with 99% EtOH (100 µl) and 25 µl 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 µl EtOH instead of the DPPH solution.

3.3.7 Ferric ion reducing antioxidant power (FRAP)

The FRAP assay was done using the method from (Ketnawa & Liceaga, 2017) with modifications. Samples (200 µl) were mixed with 200 µl 1% potassium ferrocyanide (in 0.2 M phosphate buffer) and incubated at 50 °C for 20 min. After incubation, 800 µl of 10% TCA was added then centrifuged at 10,000×g for 5 min. Into a 96-well plate, an aliquot of supernatant (100 µl) added with 100 µl of dd-water, followed by adding 20 µl 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.

3.3.8 Metal ion chelating (MIC)

The assay followed a modified version given in (Ketnawa & Liceaga, 2017). Briefly, in a 96- well plate CPH (300 µl) was mixed with 5 µl of 2 mM FeCl2 and10 µl 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:

$$\% \text{ MIC Inhibition} = \left[\frac{Abs\ (control) - Abs\ (sample)}{Abs\ (control)}\right] * 100$$

3.3.9 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 µl of sample solution was pre-incubated with 25 µl of substrate Gly-Pro p-nitroanilide hydrochloride (6 mM) at 37 °C for 10 min. To initiate the reaction, 50 µl 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 µl 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:

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

3.3.10 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 µl) were combined with 25 µl 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 µl 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 µl of 1 M HCl, and the solution was filtered using a 0.22 µm nylon filter. The control reaction contained 25 µl 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:

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

$A_{inhibitor}$ 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.

3.3.11 SDS-PAGE and immunoblotting

Samples were dissolved in zwitterionic-chaotrophic 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-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 67 mM Dithiothreitol (DTT) and shaken for 1 hr at RT. Samples were prepared for SDS-PAGE by adding an aliquot to 2X Laemmeli Sample Buffer (1mg/mL) and either used immediately or stored at −20 °C until used. Samples were loaded (10 µl) 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 de-staining overnight. Precision Plus Protein™ Dual Xtra Pre-stained Protein Standards (Bio-Rad, 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).

3.4 Results and Discussion

3.4.1 Degrees of hydrolysis (DH)

It was established in a previous study that whole crickets (*G. sigillatus*) can be hydrolyzed using alcalase (Hall et al., 2017). The htot used in the present study was 7.28 meq/q, whereas, (Hall et al., 2017) reported 8.64 meq/g for the same cricket species. Since htot 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 htot 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 3.1**). 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).

3.4.2 Antioxidant capacity

Antioxidant capacity of CPH before and after (CPHD) digestion with gastrointestinal enzymes (SGD) are shown in **Table 3.2**. 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 peroxyl 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 SGID. There was no difference between CPHD samples, suggesting that release of antioxidant rich peptides, after SGID, 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).

Table 3.2. Antioxidant activities of cricket protein hydrolysates and their digests (CPHD) after simulated gastrointestinal digestion (SGID)

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

[+]ABTS, DPPH, and FRAP results are expressed μmol Trolox equivalent per mg of sample (μmol TE/mg). MIC results are expressed at % MIC inhibition. Values represent mean observations of three replicates ± SD. Samples that do not share letters are significantly different ($p < 0.05$). "*" represent values that are significantly different before and after SGID

The ability of CPH to act as a reducing component was measured by the FRAP assay (**Table 3.2**). 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 μmol TE) and the non-digested protein (control) had negligible (near 0 μmol TE) activity. The reducing power of CPHD improved compared with CPH alone; FRAP values reached as high as 1439 μmol 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 SGID, 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 leaf worm (*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 and found that the activity varied depending on the insect and not DH. In contrast, silkworm (*B. mori*) protein hydrolysates were prepared with different 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.

3.4.3 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 **Figure 3.1**. All CPH, before SGID, 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 et al., 2015, Lacroix and 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.

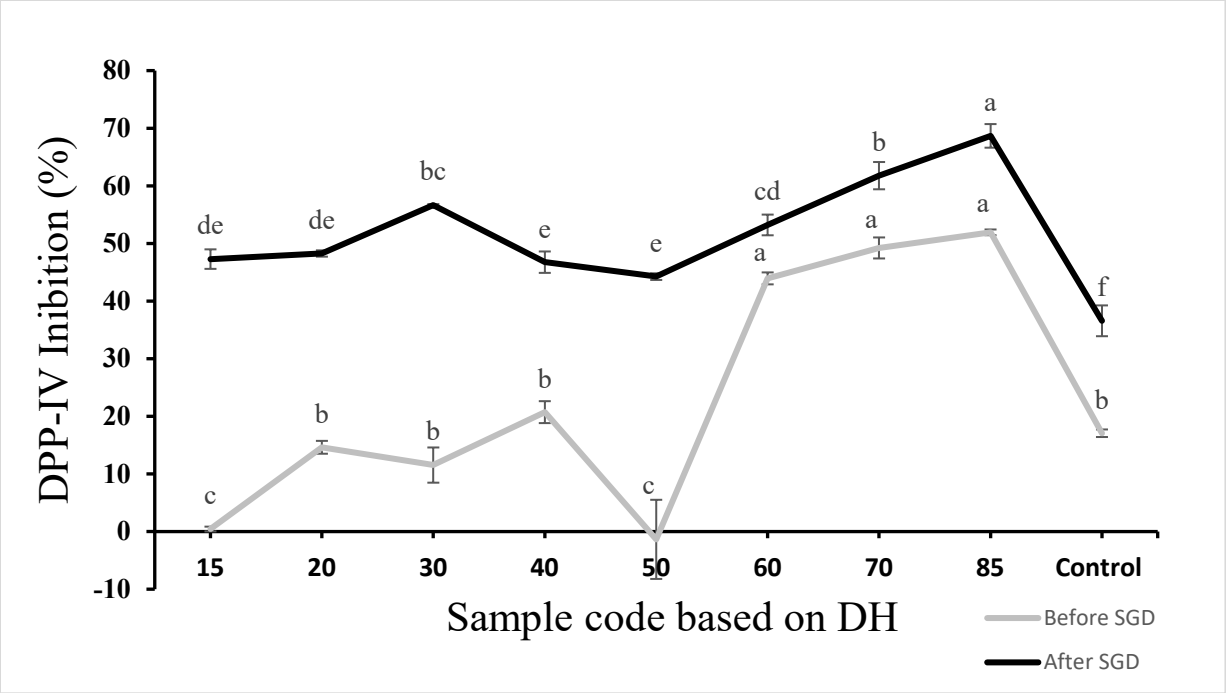


Figure 3.1 Dipeptidyl peptidase IV (DPP-IV) Inhibition % of CPH and control before (black) and after the simulated gastrointestinal model (grey). Values represent mean observations of three replicates ± SD. Samples that do not share letters are significantly different ($p < 0.05$). There was significant difference ($p < 0.05$) between all CPH and CPHD.

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 and 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. 3.1**). 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.

3.4.4 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 3.3.**

Table 3.3 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	$82.4 \pm 0.2^{b*}$	$0.096 \pm 0.007^{a*}$
50	37.1 ± 2.8^{g}	–
60	$95.3 \pm 0.0^{a*}$	$0.040 \pm 0.002^{b*}$
70	$96.4 \pm 0.1^{a*}$	$0.015 \pm 0.001^{c*}$
85	$95.2 \pm 0.1^{a*}$	$0.033 \pm 0.002^{b*}$
CPHD-40	$90.0 \pm 0.4^{b*}$	$0.089 \pm 0.008^{a*}$
CPHD-60	$92.8 \pm 0.4^{a*}$	$0.066 \pm 0.001^{b*}$
CPHD-70	$92.6 \pm 0.1^{a*}$	$0.062 \pm 0.000^{b*}$
CPHD-85	$92.8\ 0.4^{a*}$	$0.063 \pm 0.004^{b*}$

[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 (IC_{50}) 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.

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). 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 follows the data presented by (Vercruysse, 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 (Vercruysse et al., 2005). IC50 values in our study were all lower than the values presented by Vercruysse and others (Vercruysse 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 (Vercruysse 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 leaf worm (*S. littoralis*) did not increase the ACE inhibiting activity (Vercruysse 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).

3.4.5 Effects of enzyme hydrolysis on allergenicity

Palmer and others (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. 3.2. A–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 (Huang, et al., 2010; Palmer, 2016). 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 **Figure 3.2. A, 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. 3.2. A, 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. 3.2K and L**) did not react to any CPH sample or unhydrolyzed control.

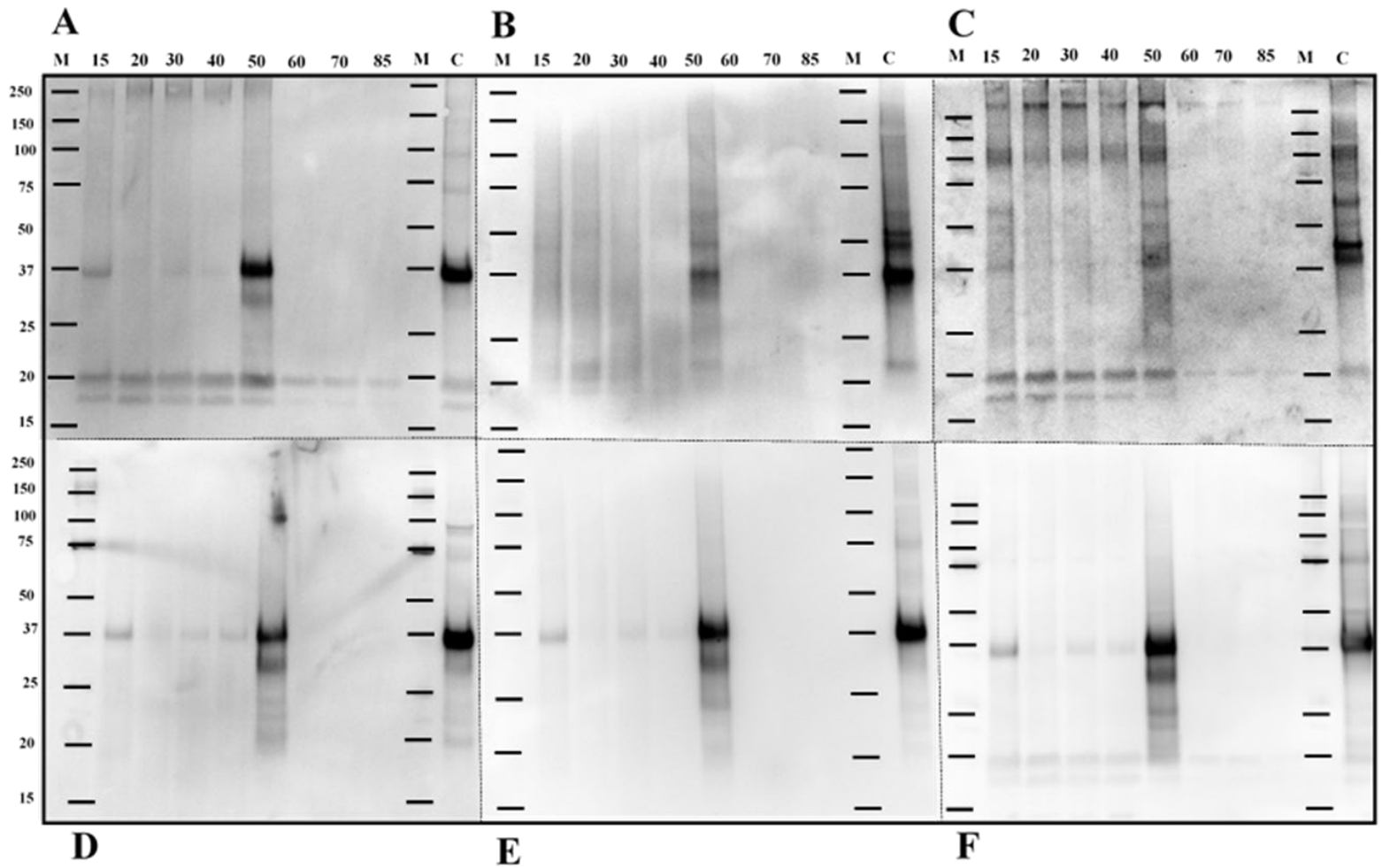
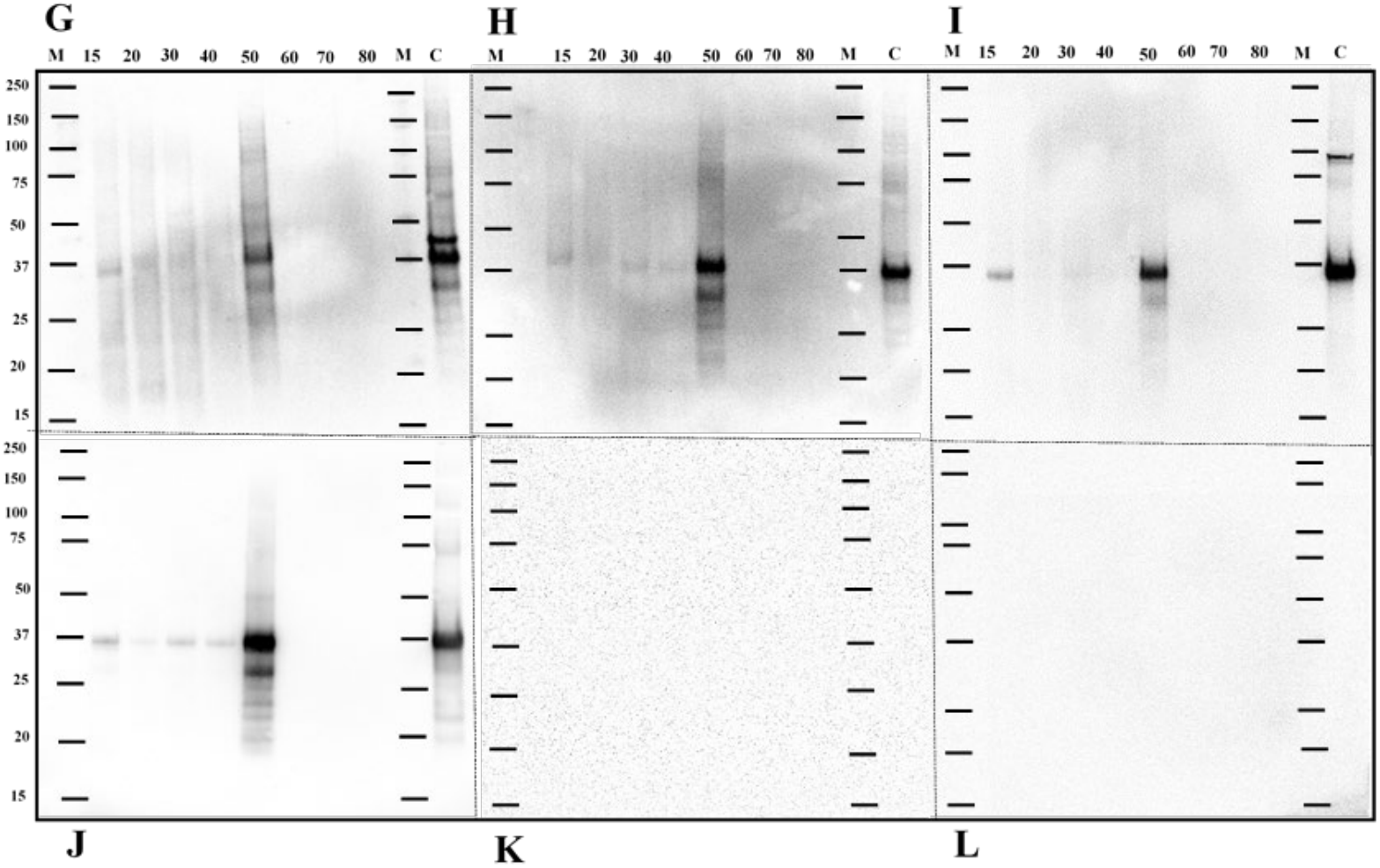


Figure 3.2. (A–L). IgE and control immunoblots of cricket protein hydrolysates and unhydrolyzed cricket. A 10 µg of sample protein were separated by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane and subjected to immunoblotting with shrimp allergic sera (A–J).

Figure 3.2 continued. 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



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 3.1**), may have exposed previously inaccessible tropomyosin epitopes, leading to an unchanged or even increased reactivity (Fig. 3.2A–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 Kabbaoui, 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.

3.5 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 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 antihypertensive and antidiabetic drugs or synthetic antioxidants.

3.6 References

Ambigaipalan, P., Al-Khalifa, A. S., & Shahidi, F. (2015). Antioxidant and angiotensin, I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using Alcalase, Flavourzyme and Thermolysin. Journal of Functional Foods, 18, 1125-1137.

Ayuso, R., Grishina, G., Bardina, L., Carrillo, T., Blanco, C., Ibáñez, M. D., Sampson, H. A., & Beyer, K. (2008). Myosin light chain is a novel shrimp allergen, Lit v 3. Journal of Allergy and Clinical Immunology, 122(4), 795-802.

Ayuso, R., Reese, G., Leong-Kee, S., Plante, M., & Lehrer, S. B. (2002). Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosins. International Archives of Allergy and Immunology, 129(1), 38-48.

Borawska, J., Darewicz, M., Vegarud, G. E., Iwaniak, A., & Minkiewicz, P. (2015). Ex vivo digestion of carp muscle tissue - ACE inhibitory and antioxidant activities of the obtained hydrolysates. Food & function, 6, 211-218.

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. In Food chemistry, vol. 135 (pp. 3020-3038).

Cudennec, B., Balti, R., Ravallec, R., Caron, J., Bougatef, A., Dhulster, P., & Nedjar, N. (2015). In vitro evidence for gut hormone stimulation release and dipeptidyl-peptidase IV inhibitory activity of protein hydrolysate obtained from cuttlefish (Sepia officinalis) viscera. Food Research International, 78, 238-245.

Gámez, C., Zafra, M. P., Sanz, V., Mazzeo, C., Ibáñez, M. D., Sastre, J., & del Pozo, V. (2015). Simulated gastrointestinal digestion reduces the allergic reactivity of shrimp extract proteins and tropomyosin. Food chemistry, 173, 475-481.

Hall, F. G., Jones, O. G., O'Haire, M. E., & Liceaga, A. M. (2017). Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. Food Chemistry, 224, 414-422.

Huang, Y.-Y., Liu, G.-M., Cai, Q.-F., Weng, W.-Y., Maleki, S. J., Su, W.-J., & Cao, M.-J. (2010). Stability of major allergen tropomyosin and other food proteins of mud crab (Scylla serrata) by in vitro gastrointestinal digestion. Food and chemical toxicology, 48(5), 1196-1201.

Karamac, M., Kosinska-Cagnazzo, A., & Kulczyk, A. (2016). Use of different proteases to obtain flaxseed protein hydrolysates with antioxidant activity. International Journal of Molecular Sciences, 17.

Ketnawa, S., & Liceaga, A. M. (2017). Effect of Microwave Treatments on Antioxidant Activity and Antigenicity of Fish Frame Protein Hydrolysates. Food and Bioprocess Technology, 10(3), 582-591.

Khaket, T. P., Redhu, D., Dhanda, S., & Singh, J. (2015). In Silico Evaluation of Potential DPP-III Inhibitor Precursors from Dietary Proteins. International Journal of Food Properties, 18, 499-507.

Ko, J. Y., Kang, N., Lee, J. H., Kim, J. S., Kim, W. S., Park, S. J., Kim, Y. T., & Jeon, Y. J. (2016). Angiotensin I-converting enzyme inhibitory peptides from an enzymatic hydrolysate of flounder fish (Paralichthys olivaceus) muscle as a potent anti-hypertensive agent. Process Biochemistry, 51, 535-541.

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012). Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an in silico approach. Journal of Functional Foods, 4, 403-422.

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2015). Comparison of the susceptibility of porcine and human dipeptidyl-peptidase IV to inhibition by protein-derived peptides. Peptides, 69, 19-25.

Lambeir, A.-M., Durinx, C., Scharpé, S., & De Meester, I. (2003). Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Critical reviews in clinical laboratory sciences, 40, 209-294.

Li-Chan, E. C. (2015). Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. Current Opinion in Food Science, 1, 28-37.

Li-Chan, E. C. Y., Hunag, S. L., Jao, C. L., Ho, K. P., & Hsu, K. C. (2012). Peptides derived from Atlantic salmon skin gelatin as dipeptidyl-peptidase IV inhibitors. Journal of Agricultural and Food Chemistry, 60, 973-978.

Martínez-Alvarez, O., Batista, I., Ramos, C., & Montero, P. (2016). Enhancement of ACE and prolyl oligopeptidase inhibitory potency of protein hydrolysates from sardine and tuna by-products by simulated gastrointestinal digestion. Food & function, 7(4), 2066-2073.

Mejrhit, N., Azdad, O., Chda, A., El Kabbaoui, M., Bousfiha, A., Bencheikh, R., Tazi, A., & Aarab, L. (2017). Evaluation of the sensitivity of Moroccans to shrimp tropomyosin and effect of heating and enzymatic treatments. Food and Agricultural Immunology, 1-12.

Mills, E., Madsen, C., Shewry, P., & Wichers, H. (2003). Food allergens of plant origin—their molecular and evolutionary relationships. Trends in Food Science & Technology, 14(4), 145-156.

Nguyen, E., Jones, O., Kim, Y. H. B., San Martin-Gonzalez, F., & Liceaga, A. M. (2017). Impact of microwave-assisted enzymatic hydrolysis on functional and antioxidant properties of rainbow trout Oncorhynchus mykiss by-products. Fisheries Science, 83(2), 317-331.

Nongonierma, A. B., & FitzGerald, R. J. (2017). Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review. Innovative Food Science & Emerging Technologies.

Nongonierma, A. B., Lamoureux, C., & FitzGerald, R. J. (2018). Generation of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides during the enzymatic hydrolysis of tropical banded cricket (Gryllodes sigillatus) proteins. Food & function.

Nongonierma, A. B., Le Maux, S., Dubrulle, C., Barre, C., & FitzGerald, R. J. (2015). Quinoa (Chenopodium quinoa Willd.) protein hydrolysates with in vitro dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant properties. Journal of cereal science, 65, 112-118.

Palmer, L. (2016). Edible Insects as a Source of Food Allergens.

Pedrosa, M., Boyano-Martínez, T., García-Ara, C., & Quirce, S. (2015). Shellfish allergy: a comprehensive review. Clinical reviews in allergy & immunology, 49(2), 203-216.

Rumpold, B. A., & Schlüter, O. K. (2013). Nutritional composition and safety aspects of edible insects. Molecular nutrition & food research, 57(5), 802-823.

Tao, M., Wang, C., Liao, D., Liu, H., Zhao, Z., & Zhao, Z. (2017). Purification, modification and inhibition mechanism of angiotensin I-converting enzyme inhibitory peptide from silkworm pupa (Bombyx mori) protein hydrolysate. Process Biochemistry, 54, 172-179.

Vercruysse, L., Smagghe, G., Beckers, T., & Camp, J. V. (2009a). Antioxidative and ACE inhibitory activities in enzymatic hydrolysates of the cotton leafworm, Spodoptera littoralis. Food Chemistry, 114, 38-43.

Vercruysse, L., Smagghe, G., Beckers, T., & Camp, J. V. (2009b). Antioxidative and ACE inhibitory activities in enzymatic hydrolysates of the cotton leafworm, Spodoptera littoralis. Food chemistry, 114(1), 38-43.

Vercruysse, L., Smagghe, G., Herregods, G., & Van Camp, J. (2005). ACE inhibitory activity in enzymatic hydrolysates of insect protein. Journal of Agricultural and Food Chemistry, 53, 5207-5211.

Wu, Q., Jia, J., Yan, H., Du, J., & Gui, Z. (2015). A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (Bombyx mori) protein: Biochemical characterization and molecular docking study. Peptides, 68, 17-24.

Yang, R., Zhao, X., Kuang, Z., Ye, M., Luo, G., Xiao, G., Liao, S., Li, L., & Xiong, Z. (2013). Optimization of antioxidant peptide production in the hydrolysis of silkworm (Bombyx mori L.) pupa protein using response surface methodology. Journal of Food, Agriculture and Environment, 11, 952-956.

You, L., Zhao, M., Regenstein, J. M., & Ren, J. (2010). Changes in the antioxidant activity of loach (Misgurnus anguillicaudatus) protein hydrolysates during a simulated gastrointestinal digestion. Food chemistry, 120(3), 810-816.

Zhang, Y., Chen, R., Chen, X., Zeng, Z., Ma, H., & Chen, S. (2016). Dipeptidyl Peptidase IV-Inhibitory Peptides Derived from Silver Carp (Hypophthalmichthys molitrix Val.) Proteins. Journal of Agricultural and Food Chemistry, 64, 831-839.

Zhang, Y., Wang, N., Wang, W., Wang, J., Zhu, Z., & Li, X. (2016). Molecular mechanisms of novel peptides from silkworm pupae that inhibit α-glucosidase. Peptides, 76, 45-50.

Zhu, K., Zhou, H., & Qian, H. (2006). Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. Process Biochemistry, 41(6), 1296-1302.

Zielińska, E., Baraniak, B., & Karaś, M. (2017). Antioxidant and Anti-Inflammatory Activities of Hydrolysates and Peptide Fractions Obtained by Enzymatic Hydrolysis of Selected Heat-Treated Edible Insects. Nutrients, 9(9), 970.

Zielińska, E., Karaś, M., & Jakubczyk, A. (2017). Antioxidant activity of predigested protein obtained from a range of farmed edible insects. International Journal of Food Science & Technology, 52(2), 306-312.

CHAPTER 4. EFFECT OF MICROWAVE-ASSISTED ENZYMATIC HYDROLYSIS OF CRICKET (*GRYLLODES SIGILLATUS*) PROTEIN ON ACE AND DPP-IV INHIBITION AND TROPOMYOSIN-IgG BINDING[2]

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Abstract

Techniques, such as microwave-assisted enzymatic hydrolysis, provide options to generate insect-bioactive peptides. However, allergenicity of these novel bioactive peptides remains a concern. Cricket protein was enzymatically hydrolyzed using conventional heating or microwave radiation. Protein hydrolysates were evaluated for DPP-IV and ACE inhibition activity, and tropomyosin-IgG reactivity (a major cricket allergen). ACE and DPP-IV inhibition were highest in the microwave-hydrolyzed protein (IC50 = 0.096 mg/ml and 0.27 mg/mL, respectively). All samples displayed tropomyosin-IgG reactivity; however, the lowest binding was obtained with the microwave-hydrolyzed protein. Raman spectroscopy revealed conformational changes, particularly in the Amide I and S-S regions, which may correlate to the observations in the immunochemical reactivity. In conclusion, microwave-assisted enzymatic hydrolysis can be a useful method for generating bioactive peptides from insect proteins and lowering their immunoreactivity, validating the potential of these treatments to generate bioactive, hypoallergenic peptides for food and pharmaceutical applications.

4.1 Introduction

With a broadening adoption of insects as human food, there is now a growing body of research supporting therapeutic potential of various insect species (Nongonierma and FitzGerald, 2017, van Huis, 2018). Proteolysis is the primary method used to liberate peptides from native insect proteins with various bioactivities (Dai et al., 2013, Hall et al., 2018, Lee et al., 2017, Nongonierma and FitzGerald, 2017, Nongonierma et al., 2018, Zielińska et al., 2018). Yet, enzymatic hydrolysis and other processing methods may alter the allergenic properties of these

insect-derived peptides and hydrolysate mixtures. Immunological investigations confirm the presence of cross-reactive allergens in some insect species that are susceptible to trigger allergic reactions in shellfish allergic individuals (Abdelmoteleb et al., 2019, Broekman et al., 2015, Van Broekhoven et al., 2016). Among them, tropomyosin is identified as a major insect pan-allergen that is reportedly resistant to heat and proteolysis (Ozawa et al., 2018). This implies that peptides released during processes such as enzymatic hydrolysis may retain or contain new immune reactivity.

Using controlled enzymatic hydrolysis in a previous study, tropical banded cricket protein hydrolysates demonstrated high angiotensin converting enzyme (ACE) and dipeptidyl peptidase-IV (DPP-IV) inhibition activity before and after simulated gastrointestinal digestion. However, these peptides, in particular the one with half of its peptide bonds hydrolyzed (i.e., 50% DH), still displayed tropomyosin IgE binding to shrimp-allergic patient sera. We hypothesized that as proteolysis reached 50%, the hydrolysate mixture contained peptides with residual allergenicity and tropomyosin binding capacity (Hall et al., 2018). Other contributing factors such protein folding or aggregation induced during conventional (convection heating) enzymatic hydrolysis could preserve epitope regions. Conformational changes during heating would also likely influence protease cut-sites that prevent cleavage of existing epitopes or expose new antigenic epitopes. Thus, modifications that occur during conventional (water bath) hydrolysis would ultimately influence allergenic (i.e. IgE-binding) characteristics.

The use of microwave radiation combined with enzymatic hydrolysis to decrease allergenicity and improve peptide bioactivity has been previously documented (Izquierdo et al., 2008, Ketnawa and Liceaga, 2017). These effects are postulated to result from a combination of thermal and non-thermal interactions with the organic material that are not usually accessible during convection heating (de la Hoz, Diaz-Ortiz, & Moreno, 2005). Although more research is needed to explain the influence of the electric field on chemical transformation, several studies already demonstrate the use of microwave irradiation to decrease hydrolysis time by increasing the reaction rate (Urbizo-Reyes, San Martin-González, Garcia-Bravo, Malo, & Liceaga, 2019), and increase overall product yield (Reddy, Huang, Chen, Chang, & Ho, 2013).

As we generate novel bioactive peptides from emerging protein sources, such as insects, it is important that we also contemplate the potential immune reactivity or the production of novel antigens within these bioactives. Therefore, the aim of this study was to apply microwave-assisted

enzymatic hydrolysis to generate protein hydrolysates from whole crickets and evaluate the effect on the peptides' immune reactivity.

4.2 Materials and Methods

4.2.1 Materials

Reagent grade chemicals were used for each method, unless specified. Supplies include: Sigma Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA). Human Dipeptidyl Peptidase IV (DPP-IV, ≥ 4500 units/µg protein) and substrate Gly-Pro p-nitroanilide hydrochloride, Human recombinant Angiotensin Converting Enzyme (ACE, 10 U/mg protein) 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 (Gryllodes sigillatus) were purchased from EntomoFarms (Norwood, ON, Canada). Alcalase® (protease from *Bacillus licheniformis*, ≥ 2.4 U/g) was obtained from Sigma Aldrich (St. Louis, MO, USA).

4.2.2 Preparation of cricket protein hydrolysates (CPH)

Conventional enzymatic hydrolysis

Conventional enzymatic hydrolysis is described in detail elsewhere (Hall, Jones, O'Haire, & Liceaga, 2017); modifications are stated. After homogenizing (2 min) whole crickets in 2 volumes of double distilled-water, slurries were pasteurized in a water bath (90 °C) for 15 min, then pH (8.0) and temperature (55 °C) adjusted to enzyme optima. Alcalase (7.54 U) was used to achieve approximately a 50% degree of hydrolysis in order to replicate tropomyosin reactivity observed in a previous study (Hall et al., 2018).

Microwave-assisted enzymatic hydrolysis

The microwave unit with an Xpress™ vessel carousel (MarsExpress™ CEM Co., Matthews, NC, USA) was programmed to heat solutions to 55 °C and remain constant with varying power (600 Watts; 80% power maximum). Vessel temperatures were maintained using an infrared sensor that adjusted power to avoid temperature fluctuations. After pasteurizing whole cricket slurries, as

described in the previous step, Alcalase was added (Table 1) and solutions distributed (40 mL) into individual microwave vessels capped with fixed pressure release valves with constant stirring. Following hydrolysis, Alcalase was heat inactivated (90 °C) in a water bath (15 min) for both methods, samples were cooled to room temperature and centrifuged. Supernatants were collected, lyophilized, and stored in polystyrene tubes at − 20 °C until further use. Table 1 details the parameters used for both conventional and microwave-assisted enzymatic hydrolysis.

Degree of hydrolysis (%DH)

%DH was determined using the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979) with modifications (Hall et al., 2017). %DH is defined as the percent ratio of peptide bonds cleaved (h) to the total number of peptide bonds per unit weight (htot), and expressed as:

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 whole tropical banded crickets (*G. sigillatus*) 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 (420 nm) versus mg/mL amino nitrogen (using L-leucine).

4.2.3 SDS-PAGE

Sample preparation followed previously a described method (Hall et al., 2018). After dissolving in 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-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 67 mM Dithiothreitol (DTT)), samples were added 1:1 to 2X Laemmeli Sample Buffer (1 mg/mL final concentration) and used immediately or stored at −20 °C. Samples were then loaded (10 µL) onto 4–12% gradient gels (Bis Tris, NuPAGE, ThermoScientific, Waltham, MA) with MES SDS running buffer at 200 V for 40 min. Gels were used for blotting or stained with silver stain according to the manufacturer's instructions (Pierce™ Silver Stain Kit, ThermoScientific, Waltham, MA). Novex™ Sharp Pre-stained Protein Standard (Biorad, Hercules, CA) were used as molecular weight standards.

4.2.4 In vitro bioactive properties (DPP-IV & ACE inhibition)

Dipeptidyl peptidase-IV (DPP-IV) inhibition

The effect of the microwave irradiation on DPP-IV inhibition activity was determined following the method proposed by Lacroix and Li-Chan (2015) with modifications (Hall et al., 2018). Samples were dissolved in 100 mM Tris-HCl buffer (pH 8.0) to 1.25 mg/mL (final assay concentration). In a 96 well plate, 25 µL of sample solution was pre-incubated with 25 µL of substrate Gly-Pro p-nitroanilide hydrochloride (6 mM) at 37 °C for 10 min. Fifty microliters of human recombinant DPP-IV (4.5 U) was added, reaction incubated at 37 °C for 1 hr, then terminated with 100 µL 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 (insert company, city, State). Absorbance values were corrected with sample blanks in which DPP-IV was replaced with Tris-HCl buffer (100 mM, pH 8.0). Positive controls (no inhibitor) included buffer instead of sample and the negative control (no DPP-IV) used buffer in place of DPP-IV solution. Percent DPP-IV inhibition was calculated as follows:

Angiotensin converting enzyme (ACE) inhibition

ACE-inhibition activity was measured with a described protocol (Martínez-Alvarez, Batista, Ramos, & Montero, 2016) with modifications (Hall et al., 2018) using human recombinant ACE. Samples, substrate, and ACE were reconstituted in 100 mM sodium phosphate buffer (pH 8.3) with 300 mM NaCl. Sample solutions (10 µL) were combined with 25 µL of substrate (HHL) then incubated for 4 min at 37 °C. ACE. Then, 80 µL of ACE (50 mU) was added to initiate the reaction and incubated at 37 °C in a water bath with constant shaking for 2 hr. ACE was also incubated at the same time before being added. Reaction was terminated with 50 µL of 1 M HCl, and solution filtered using a 0.22 µm nylon filter. The control reaction contained 25 µL buffer in place of inhibitor (CPH). During the reaction, Hippuryl-His-Leu (HHL) was hydrolyzed by ACE to hippuric acid (HA) and histidyl-leucine (HL). The relative amounts of product HA and unreacted HHL 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). Percent ACE inhibition was calculated as follows:

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

4.2.5 Mode of inhibition

Various substrate, HHL (1.25, 2.5, 5.0 and 10.0 mM) and Gly-Pro p-nitroanilide hydrochloride (4, 6, 8 and 12 mM), concentrations were incubated in the presence/absence of inhibitor, 2.5 and 5 mg/L (ACE) and 5 and 10 mg/L (DPP-IV). Inhibition % were measured as described above. Initial reaction velocities were determined from the formation of product over time. Inhibition type was determined according to Lineweaver-Burk graphs of reciprocal product concentration, Hippuric acid (HA) and p-nitroanilide (PN), absorbance values versus the reciprocal of substrate concentrations. K_m and V_{max} values at different sample concentrations were also determined according to the Lineweaver-Burk plots.

4.2.6 Tropomyosin-IgG sandwich ELISA

The following solutions were prepared using analytical grade chemicals: 50 mM carbonate/bicarbonate buffer, pH 9.6; phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T); 1% bovine serum albumin; 1 mM ABTS in 70 mM citrate phosphate buffer, pH 4.2. Monoclonal antibody 1A6 (mAb), mouse IgG1, polyclonal antibody (pAb), rabbit anti-shrimp tropomyosin, and standard natural shrimp tropomyosin were packaged as a kit purchased from Indoor Biotechnologies (Charlottesville, VA, USA). For the sandwich ELISA, 100 µL of mAb (diluted 1:1000 in carbonate-bicarbonate buffer) was used to coat single wells of polystyrene microtiter plates (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 4 °C, overnight. The plate was washed with PBS-T 3x for 5 min, then free binding sites of wells were blocked with 1% BSA for 1 hr at RT. After washing with PBS-T (3 × 5 min), diluted samples and standard (50–0.1 ng/mL in PBS-T) was added and incubated for another 1 hr at room temperature. Rabbit anti shrimp pAb (1:1000 in PBS-T) was added after another wash step and incubated for 1 hr at room temp. The plate was washed again with PBS-T and goat anti-rabbit horseradish peroxidase

conjugated antibody (diluted 1:1000 in 1% BSA, PBS-T) was incubated in wells for another hour at room temperature. After the final wash step with PBS-T, substrate solution (1 mM ABTS) was added and the enzymatic color reaction was performed in the dark for 15 min at room temperature. Finally, absorbance was measured at 405 nm in a UV/VIS Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA)

4.2.7 FT-Raman spectroscopy

Spectra were obtained using a Bruker MultiRAM system (Bremen, Germany) equipped with a 1064 nm laser source and a liquid nitrogen cooled Ge detector. Spectral data were collected over the range 400–1800 cm−1 at a resolution of 4 cm−1 under fixed measurement parameters (laser power: 300 mW, average of two measurements, 500 scans). The OPUS software package provided by Bruker Optics (Billerica, MA, USA) was used for spectral acquisition, instrument control and preliminary file manipulation. Each sample measurement was repeated twice, and the measurement with the largest deviation with respect to the average was eliminated. Spectra were taken of the aqueous solutions in Tris-HCl buffer (50 mM, pH 7.5) at 10 mg/mL protein. A cricket myofibrillar extract was used reference for the secondary structure of untreated muscle proteins compared to the heated-no enzyme, heated, and hydrolyzed protein samples. The myofibrillar extract was obtained using the sequential salt-buffer extraction method described by Ruttanapornvareesakul et al. (2005).

4.2.8 Total amino acid analysis.

The total amino acid composition of all samples was analyzed by UPLC Amino acid Analysis Solution using the AccQ·Tag Ultra Derivatization kit with UV detection (Water Corporations, Milford, MA, USA) by the Danforth Center's Proteomics and Mass Spectrometry Facility (St Louis, Missouri, USA) as previously described (Suwal, Ketnawa, Liceaga, & Huang, 2018). Samples were pre-oxidized using performic acid and subject to acid hydrolysis with 0.5% phenol/6 M HCl in a vapor-phase hydrolysis vessel under vacuum. Cysteine (Cys), Methionine (Met) and Tryptophan (Trp) are destroyed by the acid hydrolysis. The pre-oxidation step using performic acid prior to the standard acid hydrolysis yields stable forms of Cysteic acid (Cya) and methionine

sulfoxide (MetS), which can therefore be measured. The quantity (moles) of amino acids in each peptide fraction was calculated using a series of standard solution run before the samples.

4.2.9 Statistical analysis

All analyses were conducted in triplicate, unless indicated otherwise, then analyzed using Minitab 16® statistical software (State College, PA, USA). Statistical analyses of observed differences among means consisted of analysis of variance (ANOVA), using a General Linear Model (GLM); followed by Tukey's pairwise comparison of means, with a level of significance defined at 5%

Table 4.1. Sample code descriptions, hydrolysis parameters, degrees of hydrolysis, DPP-IV and ACE IC_{50} values

Samples*	E:S[1] (%)	Time[1] (min)	Heating method	Degree of hydrolysis[1, 2] (%DH)	DPP-IV (IC_{50} mg/mL)	ACE (IC_{50} mg/mL)
WB-C	n.a.	20	Water bath	5.2 ± 0.7^{a}	4.05 ± 0.68^{a}	2.50 ± 0.35^{a}
MW-C	n.a.	10	Microwave	10.5 ± 0.9^{b}	0.31 ± 0.03^{b}	1.43 ± 0.17^{b}
WB-CPH	3.0	20	Water bath	56.2 ± 1.0^{c}	0.65 ± 0.04^{c}	0.20 ± 0.05^{c}
MW-CPH	3.0	10	Microwave	48.1 ± 3.2^{c}	0.27 ± 0.04^{b}	0.096 ± 0.01^{d}

*Sample codes descriptions are as follows: WB-C= conventionally heated control with no enzyme; MW-C= microwave heated control with no enzyme; WB-C= cricket protein enzymatically hydrolyzed using conventional heating; and MW-CPH= cricket protein enzymatically hydrolyzed with microwave heating.

[1]E: S (%) 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 determinations ± SD.

[3]The half-maximal inhibitory concentration (IC_{50}) were determined for sample with ≥ 70% inhibition at 5 mg/mL (ACE) and 20 mg/mL (DPP-IV). Samples that do not share the same letters are significantly different (p <0.05).

4.3 Results and Discussion

4.3.1 Characterization of hydrolysates

Hydrolysis conditions and degree of hydrolysis varied per treatment as detailed in **Table 4.1**. Water bath heated (WB-C) and microwave-heated (MW-C) controls were incubated without added enzyme and achieved hydrolysis estimated to 5.2% and 10% DH, respectively. Protein cleavage, in these control samples was expected due to protein denaturation occurring during heating (e.g., pasteurization at 90 °C and incubation at 55 °C) (Hall et al., 2017). Both enzyme-treated samples (WB-CPH and MW-CPH) neared 50% DH, as intended (**Table 4.1**). Similar to control samples, protein cleavage/reaction occurred much quicker under microwave heating (10 min) compared to conventional hydrolysis (20 min). Likewise, microwave accelerated reactions are frequently observed. Most studies attribute these effects to expedited conformational changes and protein bond re-orientation, which allows faster cleavage by the enzyme and increased hydrolysis efficiency (Izquierdo et al., 2008, Nguyen et al., 2017, Peachey et al., 2008, Zhong et al., 2005).

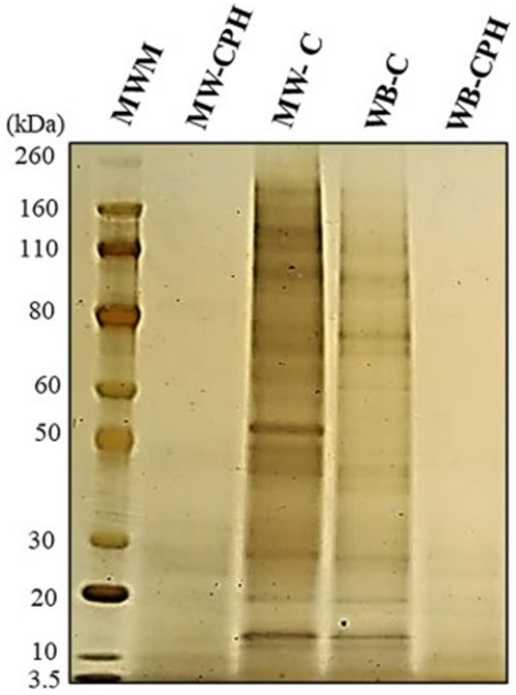

Figure 4.1. Comparison between SDS-PAGE protein profiles of MW-CPH (lane 2), MW-C (lane 3), WB-C (lane 4), and WB-CPH (lane 5). Sample descriptions are detailed in Table 1. MWM = Molecular Weight Marker.

Table 4.2. Total amino acid composition (mg/g) in microwave and conventional treated samples

Amino Acid	WB-CON	MW-CON	MW-CPH	WB-CPH
Asp + Asn	45.581	48.239	71.952	54.237
Glu + Gln	65.502	100.548	132.689	97.085
Ser	17.626	19.83	26.803	21.747
Gly	13.0.42	23.449	27.62	20.736
His	1.876	4.274	4.861	3.002
Thr	13.125	15.259	20.752	0
Ala	16.266	30.312	38.688	14.775
Pro	17.101	20.739	25.827	19.092
Arg	16.266	25.009	27.847	23.037
Tyr	0.275	0.858	0.241	0.152
Val	18.832	18.694	25.523	19.747
Met	7.218	7.038	11.448	8.649
Cys	0.124	0.174	0.172	0.147
Ile	11.356	12.881	16.513	13.222
Leu	21.187	26.456	32.494	26.226
Phe	3.732	12.716	11.485	9.34
Lys	16.873	23.228	30.76	23.458
Total	**272.94**	**389.704**	**505.675**	**354.652**

Table 4.3. Summary of the total amino acid composition (mg/g) in samples.

Group of amino acids[a]	WB-CON	MW-CON	MW-CPH	WB-CPH
HAA	95.967	129.694	162.219	111.203
AAA	4.007	13.574	11.726	9.492
PCAA	35.015	52.511	63.468	49.497
NCAA	111.083	148.787	204.641	151.322
EAA	94.199	120.546	153.836	103.644

[a] Hydrophobic amino acids (HAA): alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine; positively charged amino acids (PCAA): arginine, histidine, lysine; negatively charged amino acids (NCAA): aspartic, glutamic, threonine, serine; Branched chain amino acids (BCAA): leucine, isoleucine, valine aromatic amino acids (AAA): phenylalanine, tryptophan and tyrosine; essential amino acids (EAA): histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

4.3.2 ACE and DPP-IV inhibition

ACE and DPP-IV inhibition are commonly assayed in vitro, to measure potential treatment of hypertension and diabetes, respectively (Nongonierma & FitzGerald, 2017). IC_{50} values for DPP–IV and ACE inhibition activity of the cricket protein hydrolysates derived from conventional, water bath (WB-CPH) and microwave-assisted hydrolysis (MW-CPH) are given in Table 1; while Lineweaver-Burk plots illustrate the mode of inhibition (**Figure 4.2**). Water bath (conventional) heated samples without enzyme (WB-C) showed the lowest inhibiting activity for both activities assayed (**Table 4.1**). In contrast, the control sample exposed to microwave heating (MW-C) significantly improved IC50 values for DPP-IV (0.31 mg/mL) and ACE (1.43 mg/mL). When the proteolytic enzyme was added (MW-CPH), the IC50 values for DPP-IV and ACE improved to 0.27 and 0.096 mg/mL, respectively. Greater inhibition activity was also observed in the water bath heated samples with enzyme (WB-CPH), compared to their control (**Table 4.1**). WB-CPH and MW-CPH demonstrate the efficacy of enzymatic hydrolysis, regardless of heating type, to release bioactive peptides from whole crickets.

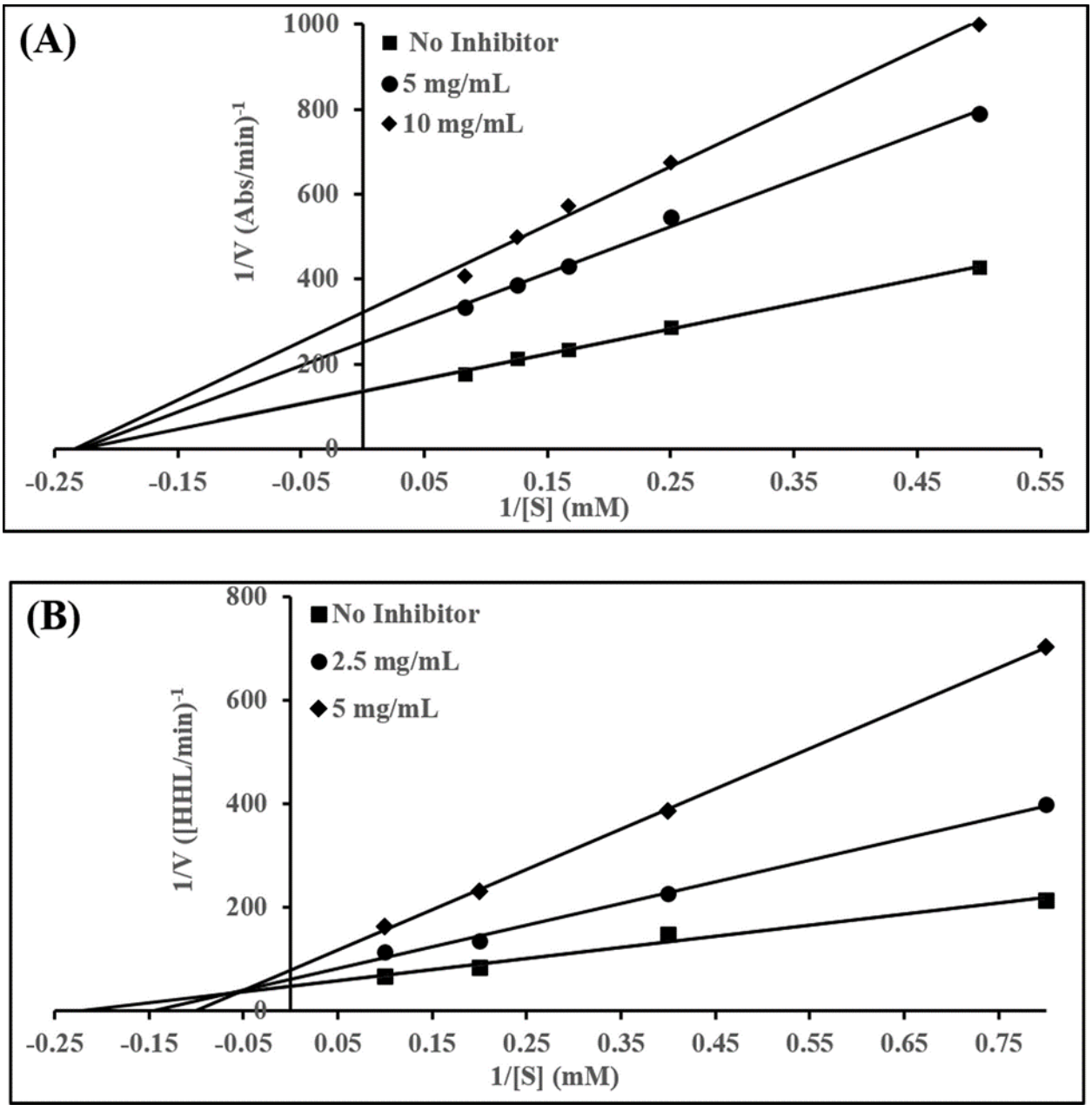

Figure 4.2. The Lineweaver-Burk plot of the inhibitory effects on (A) DPP-IV and (B) ACE activities of MW-CPH. Activities were determined in the absence and presence of different concentrations of the sample. Sample descriptions are detailed in Table 1. Values are represented as the mean ± SD, n = 3.

Although both enzyme-treated samples had a similar extent of hydrolysis (~50% DH), greater inhibition activity was observed when proteolysis occurred under microwave heating (MW-CPH). Microwave-assisted enzymatic hydrolysis has proven to be superior at releasing bioactive peptides from whole crickets. It is reported that microwave energy can increase the rate of protein unfolding, enhancing proteolysis to deliver peptide fragments that may not have been otherwise accessible to the proteolytic enzyme during conventional hydrolysis (Charoensiddhi et al., 2015, Ketnawa and Liceaga, 2017, Nguyen et al., 2017). To the best of our knowledge, this is the first report utilizing microwave-assisted hydrolysis to generate insect protein hydrolysates with promising DPP-IV and ACE inhibiting activity (in vitro).

Nevertheless, ACE (0.096–2.50 mg/mL) and DPP-IV (0.270–4.054 mg/mL) IC_{50} values in this work are similar to other protein hydrolysates reported in literature. For example, Dai and others (2013) observed ACE inhibiting activity from mealworm larvae (*Tenebrio molitor*) protein hydrolysates (IC_{50} 0.39 mg/mL) which improved after separating lower molecular weight peptide fractions (0.23 mg/mL); the authors also demonstrated lower systolic blood pressure in spontaneously hypertensive rats (Dai et al., 2013). Lower IC_{50} values were reported in our previous work using cricket protein hydrolysates (0.015 – 0.096 mg/mL) after simulated gastrointestinal digestion, affirming the influence of peptide size on ACE-inhibiting potential (Hall et al., 2018). Regarding DPP-IV inhibition, Nongonierma and others (2018) documented IC50 values ranging from 0.40 to 1.01 mg/mL from cricket-flour protein hydrolysates. Similarly, hydrolyzed mealworm protein concentrates and isolate displayed IC_{50} values of 0.63 mg/mL and 0.60 mg/mL, respectively (Lacroix, Dávalos Terán, Fogliano, & Wichers, 2019). In contrast with these other findings, human recombinant varieties of both enzymes were used in this study and may have a lower binding affinity and selectivity compared to rabbit (ACE) or porcine (DPP-IV) varieties (Bull et al., 1985, Lacroix and Li-Chan, 2015). Use of the former would represent, a relatively, more accurate estimation of inhibitory capabilities in vivo.

To clarify the ACE and DPP-IV inhibition pattern of MW-CPH, both enzymes were measured with varying concentrations of substrate with and without inhibitor. MW-CPH displayed a non-competitive inhibiting pattern towards DPP-IV (**Figure 4.20 A**). The Lineweaver-Burk plots exhibited decreased V_{max} and unchanged K_m, suggesting that peptides released during microwave-assisted hydrolysis interacted with a non-active site position on DPP-IV to produce a dead-end complex. Most food-derived DPP-IV inhibitors are competitive in nature, interacting directly with

the active site of the enzyme (Nongonierma and FitzGerald, 2013, Patil et al., 2015, Zhang et al., 2016). Plots of ACE inhibiting activity (**Figure 4.2 B**) described a mixed-type pattern characterized by apparent K_m and V_{max} changes at different concentrations (Shi, Chen, Wang, Song, & Qiu, 2005). This behavior suggests that, when interacting with ACE, MW-CPH peptides could bind to both the free enzyme and enzyme-substrate complex, limiting its overall activity. Most food protein hydrolysates are also reported as competitive inhibitors of ACE with some accounts of mixed mode, indicating interaction with the active site or other areas to prevent substrate binding (Ahn et al., 2012, Girgih et al., 2011, Udenigwe et al., 2009). Overall, these results suggest that cricket protein hydrolysates produced using microwave-assisted hydrolysis were able to form enzyme–substrate–inhibitor or enzyme–inhibitor complexes to decrease activity of both enzymes. Although in vivo efficacy of MW-CPH requires confirmation, microwave heating combined with enzymatic hydrolysis proves a suitable approach for releasing bioactive peptides form insect protein.

4.3.3 Tropomyosin-IgG reactivity

Tropomyosin-IgG reactivity was quantified to assess whether hydrolyzing cricket protein under microwave irradiation can alter antigen binding. Hydrolysates prepared conventionally with enzyme (WB-CPH), elicited an IgG response similar to the control sample derived from microwave heating with no enzyme (MW-C) (Figure 4.3). Conversely, protein hydrolysates obtained using microwave-assisted hydrolysis (MW-CPH) showed the lowest ($p < 0.05$) antigenic reactivity of all treatments, with < 10 ng/mL of quantifiable tropomyosin. Both WB-CPH and MW-CPH were hydrolyzed to near 50% DH with the same enzyme concentration (3% w/w, Alcalase), and yet microwave-hydrolyzed samples elicited the lowest IgG reactivity.

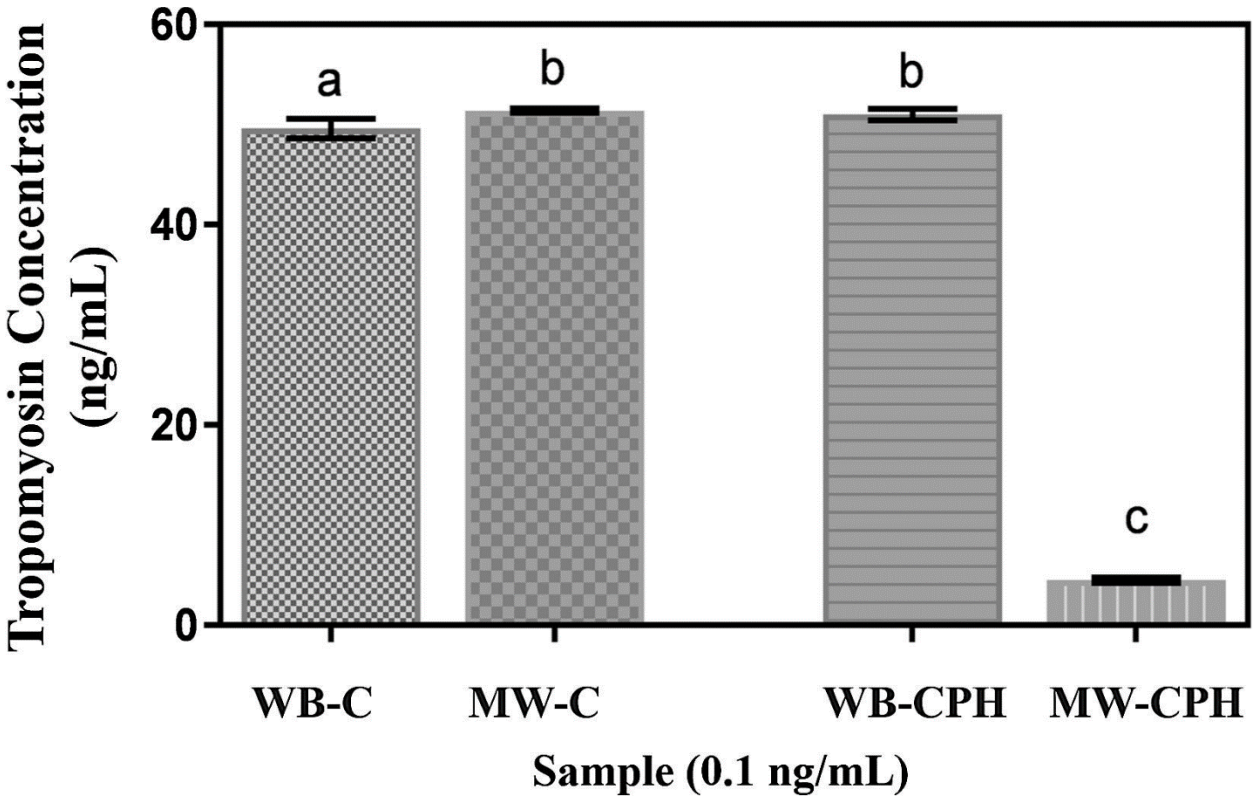

Figure 4.3. Comparison of conventional and microwave treated samples tropomyosin-IgG reactivity. Tropomyosin equivalents were calculated using the standard curve for shrimp tropomyosin. All values are represented as the mean ± SD, n = 6. Means with different letters above the bars are significantly different ($p > 0.05$).

Similar to the release of bioactive peptides, expatiated protein unfolding likely increased enzyme cleavage accessibility to active epitope regions, thus lowering immunoreactivity. The different binding patterns, comparing microwave and conventional hydrolysis, illustrate the influence of the heating type on protein/peptide allergenicity (Verhoeckx et al., 2015). In the MW-C sample, it is possible that epitopes were more exposed but not cleaved since no enzyme was added, amplifying binding at the antigenic site. In the event of conventional proteolysis (WB-CPH), residual peptides with unchanged or higher antibody binding affinity were generated. Overall, the most effective decrease in IgG-binding occurred using a combination of microwave heating and enzymatic hydrolysis (MW-CPH). Tropomyosin, a major insect allergen, is considered heat resistant due to its α-helical, coiled-coil dimeric structure. The helical secondary structure of tropomyosin collapses upon heating at temperatures above 80 °C, yet the native conformation may reform upon cooling and consequently its immunoreactivity as well (Ozawa, Watabe, & Ochiai,

2011). Nonetheless, studies investigating the effects of processing on *in vitro* immunological activity typically report decreased antibody detection after extensive enzymatic treatments, high temperatures, or other novel processing technologies such as high-pressure treatments. Similar to findings in this work, decreased tropomyosin reactivity is often observed following a combination of processing methods (e.g., proteolysis combined with high-pressure heating). For instance, tropomyosin-IgG binding affinity decreased after high heat treatment (90 °C) followed by pepsin hydrolysis, but exhibited the lowest reactivity when treatments were combined (Mejrhit et al., 2017). Likewise, IgE-binding and skin prick tests using house dust mites and crustacean allergic patient sera, decreased in migratory locust (*Locusta migratoria*) extracts that were both thermally and enzymatically treated (Pali-Schöll et al., 2019).

It is important to note that processing methods that alter epitopes to decrease reactivity can also create new binding regions that have potential to induce new sensitization and allergic responses (Davis et al., 2001, Verhoeckx et al., 2015). Phiriyangkul, Srinroch, Srisomsap, Chokchaichamnankit, and Punyarit (2015) identified more cross-reactive proteins to shrimp-allergic patient sera in raw, unprocessed Bombay locust (*Patanga Succincta*) compared to the fried insect. In another study, altered cross-reactivity was also observed after various processing and simulated gastro-intestinal digestion (SGID) of three edible mealworm species (Van Broekhoven et al., 2016). Several proteins were degraded during SGID, whereas, tropomyosin epitopes appeared stable against dehydration, boiling, and digestion, but not frying (Van Broekhoven et al., 2016). Changes in protein solubility, influenced by processing methods, may also be a consequential factor when considering an increase/decrease in detected allergenicity. In this case, allergen solubility in the appropriate assay buffer would be the main determinant on in vitro immunoreactivity. Researchers concluded that heat processing did not increase IgE-binding capacity, but was instead attributed to improved protein solubility (Broekman et al., 2015). Samples assayed in our study were extracted with chaotropic buffer prior to IgG analysis. Sample protein solubility was ranked as follows MW-CPH > WB-CPH > MW-C > WB-C (data not shown), suggesting that in this case, solubility may not be a major determinant in the antigen-antibody binding. ELISA IgG-binding quantification revealed structural changes induced by proteolysis combined with microwave irradiation, which altered tropomyosin's antibody-binding capacity. Given that changes in IgG-binding does not necessarily translate to altered allergenic potential, future work will be focused on IgE reactivity.

4.3.4 Raman spectroscopy

Figure 4.4 shows a comparison between conventional and microwave-assisted hydrolysis along with untreated myofibrillar (MF) protein extract from whole crickets, which includes tropomyosin proteins. These spectra provide evidence of the key constituents, showing component-specific peaks that allow for qualitative analysis. For example, the Amide I (1600–1700 cm−1) region provides secondary structural information about proteins that involves C-double bond-O stretching, C -single bond-N stretching, and N-single bond-H in plane bending of backbone peptide groups (Alix, Pedanou, & Berjot, 1988). In the amide I region (**Figure 4.4A**) the cricket myofibrillar extract shows several peaks between 1601 cm−1 and 1659 cm−1 indicating a predominantly poly-proline helical structure (McColl, Blanch, Hecht, Kallenbach, & Barron, 2004). Poly-proline helices (PPII) are considered a secondary-structure class with major conformational elements commonly observed in myofibrillar proteins (i.e. dominant among insect proteins) (Cubellis et al., 2005, Ma et al., 2001, Yi et al., 2016). PPII is also a significant local structure present among unfolded peptides (Kentsis, Mezei, Gindin, & Osman, 2004). When the cricket protein was treated using conventional or microwave-assisted hydrolysis, the peaks became more defined into a band at 1659 cm−1, indicative of a predominance in α-helical structure. In addition to intensity differences, the distribution in this region remained mostly unchanged among all four treatments. The conventionally heated control (WB-C) showed the highest intensity, while MW-CPH showed the lowest band intensity. Further, absence of a band at 1680 cm−1 and 1235–1240 cm−1 suggests absence of β-sheet structure and amide III conformation, respectively.

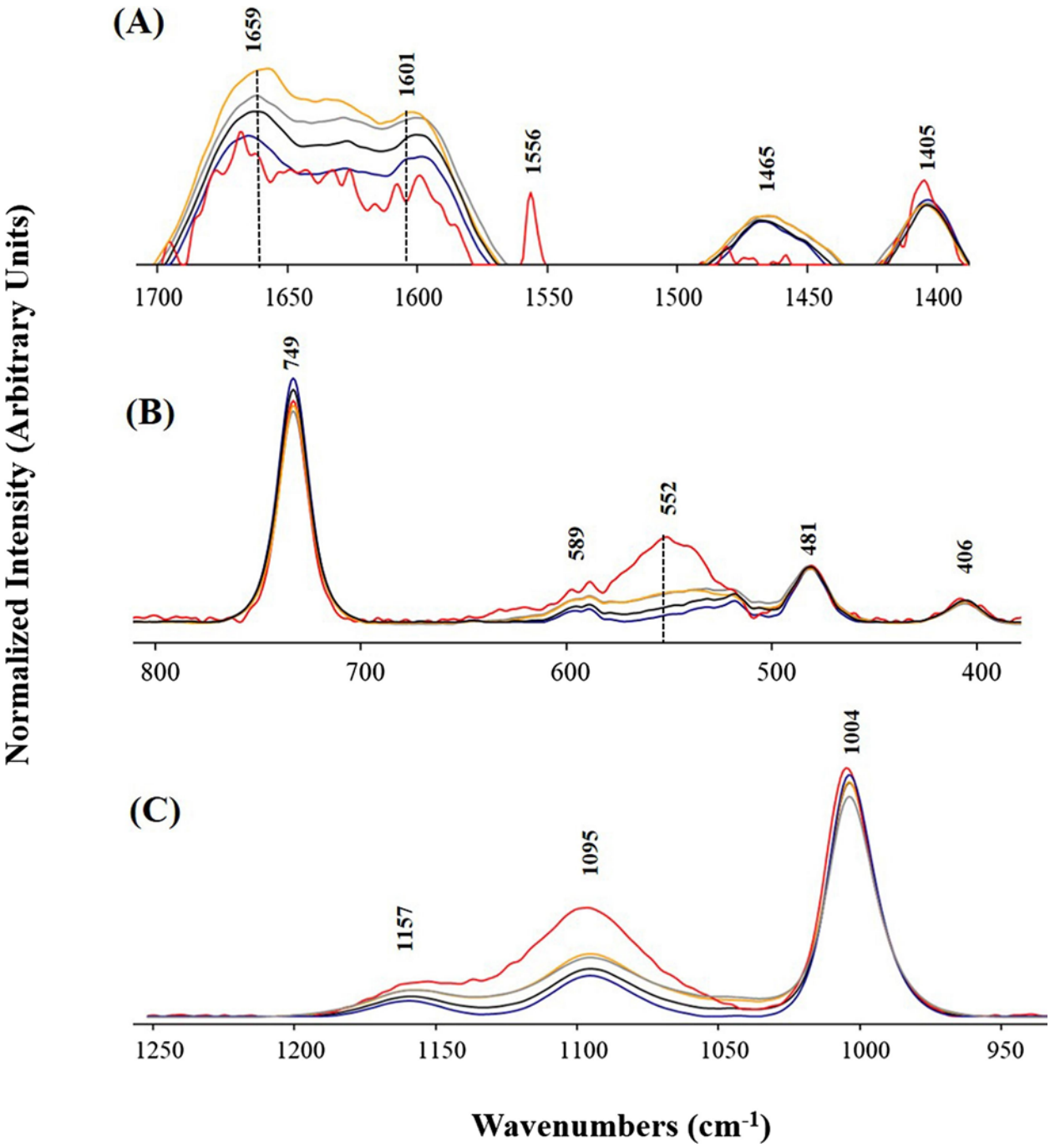

Figure 4.4. (A–C) Effects of proteolysis and microwave treatment on Raman Spectra (400–1700 cm−1) of cricket protein. Color codes are as follows: Myofibrillar protein extract (red), Control (yellow), Microwave Control (grey), Conventionally hydrolyzed CPH (black), and MW-assisted hydrolyzed CPH (blue). Samples were measured in Tris-HCl buffer, pH 7.0 with a concentration of 10 mg/mL protein. Spectra show normalized intensities and are overlapped using the OPUS spectroscopy software. Vertical dashed lines were drawn to highlight the change of characteristic Raman bands along with the change in treatment.

The myofibrillar extract elicited a small band near 1556 cm−1 (**Fig. 4.4 A**) which is reported to be directly associated with the C2 = C3 vibration mode on the indole ring of tryptophan residues. The absence of this peak in the other samples suggests changes in the microenvironment of tryptophan induced by heat and/or enzyme treatments (Rygula et al., 2013). There were also major peak intensity changes at 1095 cm^{-1} corresponding to C-single bond-N bonding that may correlate to peptide bond cleavage (**Fig. 4.4 B**). This is supported by the myofibrillar extract having the highest peak intensity, followed by controls (WB-C and MW-C), and the hydrolyzed cricket protein (WB-CPH and MW–CPH) being the least intense. Peptide cleavage may also be responsible for intensity changes in bands associated with CH_2 and CH_3 bending vibrations (1405 cm^{-1}).

Investigations confirm that gauche-gauche-gauche, gauche-gauche-trans, and trans-gauche-trans conformations of S-single bond-S linkages between cysteine typically result in Raman bands near 510, 525, 540 and 580 cm^{-1}, respectively (Kurouski et al., 2012). The presence of S-single bond-S bonds is observed by the presence of bands at 552 cm^{-1} with a small shoulder at 589 cm^{-1} (Fig. 4C) in the myofibrillar extract. Notably, the maximum intensity of this band shifted to lower frequencies (552 cm^{-1}) and intensities in the other samples, which may indicate loss of disulfide bridges and protein unfolding/denaturing that would significantly affect conformation. As found with other protein allergens, reduction of disulfide bonds increased digestibility and lower allergenicity (Buchanan et al., 1997). Similar changes were detected in heated treated myofibrillar proteins, where unfolding and conformational changes are exemplified by spectra changes in the S-S region avid (Bouraoui et al., 1997, David et al., 2008). Xiang-Liang and others (2011) observed a decrease in the normalized intensity of S-single bond-S Raman bands (500–600 cm^{-1}) as temperature increased from 30 to 70 °C; bands disappeared near higher temperatures and reappeared upon cooling. The authors attribute these changes to heat-induced modifications to pork myofibrillar proteins during gelation (Xu, Han, Fei, & Zhou, 2011). In our study, Raman spectra revealed peptide unfolding/denaturation primarily in MW-CPH. These results corroborate with earlier results that microwave radiation induced protein unfolding, providing distinct cleavage regions during proteolysis, and may be responsible for the decrease in tropomyosin-IgG binding. Treatments of pure tropomyosin would provide a clearer evaluation of structural changes and effects on antibody binding. However, the intent of the current study was to assess the effects of

processing on whole hydrolysate mixtures, as this would resemble the type of cricket protein that consumers will ingest.

4.4 Conclusion

The present work demonstrates the efficiency of microwave irradiation coupled with enzymatic hydrolysis at generating bioactive peptides from insect protein. Results demonstrated the superiority of microwave-assisted proteolysis to expedite the hydrolysis reaction and obtain peptides with improved DPP-IV and ACE inhibition capacity. However, careful consideration needs to be taken when potential allergens or the development of new antigens are generated during production of bioactive peptides. Based on the tropomyosin-IgG results, microwave-assisted proteolysis could be a potential tool for decreasing allergenicity of bioactive peptides. However, further confirmation is needed using IgE reactivity to provide a more accurate assessment of potential allergenicity after consumption, while also revealing the presence of other allergens besides tropomyosin. Bioavailability models and in vivo assays would serve to confirm the antihypertensive and antidiabetic properties observed. Future investigations should also consider insect bioactive peptides within a food matrix and the effect on antigenicity and/or allergenicity.

4.5 References

Abdelmoteleb, M., Palmer, L. K., Marsh, J., Johnson, P., & Goodman, R. E. (2019). Bioinformatics and Proteomics Evaluations of Potential IgE Cross-Reactive Proteins in Novel Edible Insects and Shrimp for Food Safety. *Journal of Allergy and Clinical Immunology, 143*(2), AB237.

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*(6), 1256-1262.

Ahn, C.-B., Jeon, Y.-J., Kim, Y.-T., & Je, J.-Y. (2012). Angiotensin I converI copy ting enzyme (ACE) inhibitory peptides from salmon byproduct protein hydrolysate by Alcalase hydrolysis. *Process Biochemistry, 47*(12), 2240-2245

.

Alix, A., Pedanou, G., & Berjot, M. (1988). Fast determination of the quantitative secondary structure of proteins by using some parameters of the Raman amide I band. *Journal of Molecular Structure, 174*, 159-164.

Angiolillo, L., Del Nobile, M. A., & Conte, A. (2015). The extraction of bioactive compounds from food residues using microwaves. *Current Opinion in Food Science, 5*, 93-98.

Bouraoui, M., Nakai, S., & Li-Chan, E. (1997). In situ investigation of protein structure in Pacific whiting surimi and gels using Raman spectroscopy. *Food Research International, 30*(1), 65-72.

Broekman, H., Knulst, A., den Hartog Jager, S., Monteleone, F., Gaspari, M., De Jong, G., . . . Verhoeckx, K. (2015). Effect of thermal processing on mealworm allergenicity. *Molecular nutrition & food research, 59*(9), 1855-1864.

Buchanan, B., Adamidi, C., Lozano, R., Yee, B., Momma, M., Kobrehel, K., et al. (1997). Thioredoxin-linked mitigation of allergic responses to wheat. *Proceedings of the National Academy of Sciences, 94*(10), 5372-5377.

Bull, H., Thornberry, N. A., & Cordes, E. H. (1985). Purification of angiotensin-converting enzyme from rabbit lung and human plasma by affinity chromatography. *Journal of Biological Chemistry, 260*(5), 2963-2972.

Charoensiddhi, S., Franco, C., Su, P., & Zhang, W. (2015). Improved antioxidant activities of brown seaweed Ecklonia radiata extracts prepared by microwave-assisted enzymatic extraction. *Journal of Applied Phycology, 27*(5), 2049-2058.

Cubellis, M. V., Caillez, F., Blundell, T. L., & Lovell, S. C. (2005). Properties of polyproline II, a secondary structure element implicated in protein–protein interactions. *Proteins: Structure, Function, and Bioinformatics*, *58*(4), 880-892.

Dai, C., Ma, H., Luo, L., & Yin, X. (2013). Angiotensin I-converting enzyme (ACE) inhibitory peptide derived from Tenebrio molitor (L.) larva protein hydrolysate. *European Food Research and Technology, 236*(4), 681-689.

David, C., Foley, S., Mavon, C., & Enescu, M. (2008). Reductive unfolding of serum albumins uncovered by Raman spectroscopy. *Biopolymers: Original Research on Biomolecules, 89*(7), 623-634.

Davis, P. J., Smales, C. M., & James, D. C. (2001). How can thermal processing modify the antigenicity of proteins? *Allergy, 56*, 56-60.

de la Hoz, A., Diaz-Ortiz, A., & Moreno, A. (2005). Microwaves in organic synthesis. Thermal and non-thermal microwave effects. *Chemical Society Reviews, 34*(2), 164-178.

Girgih, A. T., Udenigwe, C. C., Li, H., Adebiyi, A. P., & Aluko, R. E. (2011). Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (Cannabis sativa L.) protein hydrolysates. *Journal of the American Oil Chemists' Society, 88*(11), 1767-1774.

Hall, F., Johnson, P. E., & Liceaga, A. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food chemistry*, *262*, 39-47.

Hall, F. G., Jones, O. G., O'Haire, M. E., & Liceaga, A. M. (2017). Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. *Food chemistry*, *224*, 414-422.

Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: production, bioavailability and incorporation into foods. *Advances in colloid and interface science, 165*(1), 23-35.

Izquierdo, F. J., Peñas, E., Baeza, M. L., & Gomez, R. (2008). Effects of combined microwave and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins. *International Dairy Journal, 18*(9), 918-922.

Kentsis, A., Mezei, M., Gindin, T., & Osman, R. (2004). Unfolded state of polyalanine is a segmented polyproline II helix. *PROTEINS: Structure, Function, and Bioinformatics, 55*(3), 493-501.

Ketnawa, S., & Liceaga, A. M. (2017). Effect of Microwave Treatments on Antioxidant Activity and Antigenicity of Fish Frame Protein Hydrolysates. *Food and Bioprocess Technology, 10*, 582-591.

Kurouski, D., Washington, J., Ozbil, M., Prabhakar, R., Shekhtman, A., & Lednev, I. K. (2012). Disulfide bridges remain intact while native insulin converts into amyloid fibrils. *PloS one, 7*(6), e36989.

Lacroix, I. M., Dávalos Terán, I., Fogliano, V., & Wichers, H. J. (2019). Investigation into the potential of commercially available lesser mealworm (A. diaperinus) protein to serve as sources of peptides with DPP-IV inhibitory activity. *International Journal of Food Science & Technology*, *54*(3), 696-704.

Lacroix, I. M., & Li-Chan, E. C. (2015). Comparison of the susceptibility of porcine and human dipeptidyl-peptidase IV to inhibition by protein-derived peptides. *Peptides, 69*, 19-25.

Lee, W., Kim, M.-A., Park, I., Hwang, J. S., Na, M., & Bae, J.-S. (2017). Novel direct factor Xa inhibitory compounds from Tenebrio molitor with anti-platelet aggregation activity. *Food and chemical toxicology, 109*, 19-27.

Ma, K., Kan, L.-s., & Wang, K. (2001). Polyproline II helix is a key structural motif of the elastic PEVK segment of titin. *Biochemistry, 40*(12), 3427-3438.

Martínez-Alvarez, O., Batista, I., Ramos, C., & Montero, P. (2016). Enhancement of ACE and prolyl oligopeptidase inhibitory potency of protein hydrolysates from sardine and tuna by-products by simulated gastrointestinal digestion. *Food & function, 7*(4), 2066–2073. doi:10.1039/c5fo01603g.

McColl, I. H., Blanch, E. W., Hecht, L., Kallenbach, N. R., & Barron, L. D. (2004). Vibrational Raman optical activity characterization of poly (L-proline) II helix in alanine oligopeptides. *Journal of the American Chemical Society, 126*(16), 5076-5077.

Mejrhit, N., Azdad, O., Chda, A., El Kabbaoui, M., Bousfiha, A., Bencheikh, R., . . . Aarab, L. (2017). Evaluation of the sensitivity of Moroccans to shrimp tropomyosin and effect of heating and enzymatic treatments. *Food and Agricultural Immunology, 28*(6), 969-980.

Nguyen, E., Jones, O., Kim, Y. H. B., San Martin-Gonzalez, F., & Liceaga, A. M. (2017). Impact of microwave-assisted enzymatic hydrolysis on functional and antioxidant properties of rainbow trout Oncorhynchus mykiss by-products. *Fisheries science, 83*, 317-331.

Nongonierma, A. B., & FitzGerald, R. J. (2013). Dipeptidyl peptidase IV inhibitory properties of a whey protein hydrolysate: Influence of fractionation, stability to simulated gastrointestinal digestion and food–drug interaction. *International Dairy Journal, 32*(1), 33-39.

Nongonierma, A. B., & FitzGerald, R. J. (2017). Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review. *Innovative Food Science & Emerging Technologies, 43*, 239-252.

Nongonierma, A. B., Lamoureux, C., & FitzGerald, R. J. (2018). Generation of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides during the enzymatic hydrolysis of tropical banded cricket (Gryllodes sigillatus) proteins. *Food & function*.

Ozawa, H., Umezawa, K., Takano, M., Ishizaki, S., Watabe, S., & Ochiai, Y. (2018). Structural and dynamical characteristics of tropomyosin epitopes as the major allergens in shrimp. *Biochemical and biophysical research communications, 498*(1), 119-124.

Ozawa, H., Watabe, S., & Ochiai, Y. (2011). Thermodynamic characterization of muscle tropomyosins from marine invertebrates. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 160*(2-3), 64-71.

Pali-Schöll, I., Meinlschmidt, P., Larenas-Linnemann, D., Purschke, B., Hofstetter, G., Rodríguez-Monroy, F. A., et al. (2019). Edible insects: Cross-recognition of IgE from crustacean-and house dust mite allergic patients, and reduction of allergenicity by food processing. *WAO Journal*.

Patil, P., Mandal, S., Tomar, S. K., & Anand, S. (2015). Food protein-derived bioactive peptides in management of type 2 diabetes. *European journal of nutrition, 54*(6), 863-880.

Peachey, E., McCarthy, N., & Goenaga-Infante, H. (2008). Acceleration of enzymatic hydrolysis of protein-bound selenium by focused microwave energy. *Journal of Analytical Atomic Spectrometry, 23*(4), 487-492.

Phiriyangkul, P., Srinroch, C., Srisomsap, C., Chokchaichamnankit, D., & Punyarit, P. (2015). Effect of food thermal processing on allergenicity proteins in Bombay locust (Patanga succincta). *International Journal of Food Engineering, 1*(1), 23-28.

Reddy, P. M., Huang, Y.-S., Chen, C.-T., Chang, P.-C., & Ho, Y.-P. (2013). Evaluating the potential nonthermal microwave effects of microwave-assisted proteolytic reactions. *Journal of proteomics, 80*, 160-170.

Ruttanapornvareesakul, Y., Ikeda, M., Hara, K., Osako, K., Kongpun, O., & Nozaki, Y. (2005). Effect of shrimp head protein hydrolysates on the state of water and denaturation of fish myofibrils during dehydration. *Fisheries science, 71*(1), 220-228.

Rygula, A., Majzner, K., Marzec, K. M., Kaczor, A., Pilarczyk, M., & Baranska, M. (2013). Raman spectroscopy of proteins: a review. *Journal of Raman Spectroscopy, 44*(8), 1061-1076.

Shi, Y., Chen, Q.-X., Wang, Q., Song, K.-K., & Qiu, L. (2005). Inhibitory effects of cinnamic acid and its derivatives on the diphenolase activity of mushroom (Agaricus bisporus) tyrosinase. *Food chemistry, 92*(4), 707-712.

Suwal, S., Ketnawa, S., Liceaga, A. M., & Huang, J. Y. (2018). Electro-membrane fractionation of antioxidant peptides from protein hydrolysates of rainbow trout (Oncorhynchus mykiss) byproducts. *Innovative Food Science & Emerging Technologies*, *45*, 122-131.

Udenigwe, C. C., Lin, Y.-S., Hou, W.-C., & Aluko, R. E. (2009). Kinetics of the inhibition of renin and angiotensin Ifverting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods, 1*(2), 199-207.

Urbizo-Reyes, U., San Martin-González, M. F., Garcia-Bravo, J., Malo, A. L., & Liceaga, A. M. (2019). Physicochemical characteristics of chia seed (Salvia hispanica) protein hydrolysates produced using ultrasonication followed by microwave-assisted hydrolysis. Food Hydrocolloids, 105187.

Van Broekhoven, S., Bastiaan-Net, S., de Jong, N. W., & Wichers, H. J. (2016). Influence of processing and in vitro digestion on the allergic cross-reactivity of three mealworm species. *Food Chemistry, 196*, 1075-1083.

van Huis, A. (2018). Insects as Human Food *Ethnozoology* (pp. 195-213): Elsevier.

Verhoeckx, K. C., Vissers, Y. M., Baumert, J. L., Faludi, R., Feys, M., Flanagan, S., et al. (2015). Food processing and allergenicity. *Food and chemical toxicology, 80*, 223-240.

Xu, X.-L., Han, M.-Y., Fei, Y., & Zhou, G.-H. (2011). Raman spectroscopic study of heat-induced gelation of pork myofibrillar proteins and its relationship with textural characteristic. *Meat science, 87*(3), 159-164.

Yi, L., Van Boekel, M. A., Boeren, S., & Lakemond, C. M. (2016). Protein identification and in vitro digestion of fractions from Tenebrio molitor. *European Food Research and Technology, 242*(8), 1285-1297.

Zhang, Y., Chen, R., Chen, X., Zeng, Z., Ma, H., & Chen, S. (2016). Dipeptidyl Peptidase IV-Inhibitory Peptides Derived from Silver Carp (Hypophthalmichthys molitrix Val.) Proteins. *Journal of Agricultural and Food Chemistry, 64*, 831-839. doi:10.1021/acs.jafc.5b05429

Zhong, H., Marcus, S. L., & Li, L. (2005). Microwave-assisted acid hydrolysis of proteins combined with liquid chromatography MALDI MS/MS for protein identification. *Journal of the American Society for Mass Spectrometry, 16*(4), 471-481.

Zielińska, E., Baraniak, B., & Karaś, M. (2018). Identification of antioxidant and anti-inflammatory peptides obtained by simulated gastrointestinal digestion of three edible insects species (Gryllodes sigillatus, Tenebrio molitor, Schistocerca gragaria). *International Journal of Food Science & Technology, 53*(11), 2542-2551.

CHAPTER 5. CHARACTERIZING CHANGES IN EDIBLE CRICKET ALLERGENICITY AFTER PROTEOLYSIS AND HEAT TREATMENTS

This chapter is under review in Journal of Insects for Food and Feed as:
Hall, F., & Liceaga, A.M. (2020). Effect of novel processing methods on edible cricket (*Gryllodes sigillatus*) allergenicity: tropomyosin isolation and proteomic characterization.

Abstract

Edible insects continue to be a promising source of alternative protein for food and feed; as such, allergenicity and toxicity remain an important safety concern. Currently, traditional and innovative processing methods are being utilized to improve sensory qualities and improve consumer acceptance. Therefore, there is increasing interest to understand the impact of these processes on their allergenic reactivity. A major cricket pan-allergen, tropomyosin, is a viable model to study the influence of processing on the immunoreactivity of edible insects. This study examines IgE reactivity of edible cricket (*Gryllodes sigillatus*) tropomyosin following heat and/or enzymatic treatment. Tropomyosin was extracted from raw, heated, and protease-treated cricket protein, respectively, using selective precipitation and characterized by electrophoresis, mass spectrometry, and FT-Raman. Immunoreactivity of treated cricket protein and tropomyosin extracts were analyzed by immunoblotting and ELISA. Immunoinformatics revealed homology with other arthropod tropomyosins and potential epitope regions. Both immunoblotting and ELISA demonstrated that tropomyosin reactivity remained stable after convection and microwave heating when the protease was not added. In contrast, IgE (anti-shrimp) binding to tropomyosin extracted from protease-treated cricket protein partially decreased under convection heating (WB-CPH), while protease treatments under microwave heating (MW-CPH) significantly decreased IgE binding, showing almost no reactivity. Additionally, proteomic analysis revealed fewer abundance of intact tropomyosin in MW-CPH, compared with WB-CPH, suggesting decreased amounts of intact epitope regions. Building on our observations, the microwave-assisted protease treatment could be an effective method for generating hypoallergenic cricket protein ingredients used to formulate insect-based food products.

5.1 Introduction

Environmental and sustainability efforts continue to promote edible insects as a source of alternative protein (Salter, 2019). Similar to any novel protein, food allergens are a major safety concern surrounding edible insects. Several investigations have identified potential antigens and IgE binding proteins in various insect species, which may correlate to an allergic reaction after consumption (Feng et al., 2018; Ribeiro et al., 2018). Consequently, a growing body of literature agrees that a high degree of cross-reactivity exists between homologous proteins found in crustaceans and other arthropods (Leoni et al., 2019; Pali-Schöll et al., 2019; Volpicella et al., 2019). In crickets, tropomyosin, arginine kinase, and glyceraldehyde 3-phosphate dehydrogenase have been identified as highly allergenic (Hossny et al., 2019). However, in 2019, Kamemura et al. (2019) identified tropomyosin as the main reactive allergen in field crickets against shrimp-specific IgE. Likewise, tropomyosin was recognized as the major reactive allergen in unprocessed cricket species such as house (*Acheta domesticus)* (Abdelmoteleb et al., 2018) and tropical banded crickets (*Gryllodes sigillatus*) (Hall et al., 2018a).

Currently, insect-based foods are customized for western palates by developing new food products via traditional (e.g. roasting, defatting) insect flours and novel (e.g. enzymatic proteolysis, sonication) processing techniques that allow for insects to be used as protein-rich ingredients in food formulation (Gravel and Doyen, 2019; Hall et al., 2017; Melgar-Lalanne et al., 2019). Following processing, allergenicity can remain the same, increase, or decrease, depending on the processing method and conditions used. Most studies report IgE binding capacity retained after thermal treatments such as baking or frying (Broekman et al., 2015; Jeong et al., 2016; Phiriyangkul et al., 2015; Van Broekhoven et al., 2016), while others observed altered reactivity after extensive heating (e.g. boiling), protease treatment, or newer technologies such as high-pressure processing, microwave heating, and food irradiation (Hall et al., 2018a; Leni et al., 2020; Mills and Mackie, 2008; Phiriyangkul et al., 2015). In previous studies, we observed the impact of enzymatic proteolysis on tropomyosin reactivity in crickets (*G. sigillatus*); our results showed that IgE binding decreased with increased proteolysis, although reactivity remained intact under some conditions (Hall et al., 2018a; Hall and Liceaga, 2020). Other scientists also reported depleted IgE binding activity in black soldier fly (*Hermetia illucens*) and lesser mealworm (*Alphitobius diaperinus*) after protease treatment (Leni et al., 2020). Broekman and their team (Broekman et al., 2015) explained these contradicting observations by demonstrating, *in vitro*, that

mealworm IgE-binding antigen changes were mainly due to altered protein solubility. However, there is strong evidence of altered food protein allergenicity, *in vivo*, after processing (de Gier and Verhoeckx, 2018; De Martinis et al., 2020). To further explain this phenomenon, investigators have focused on assessing the effects of processing on purified/isolated antigens. For instance, thermal-treated oyster tropomyosin (Cra g 1) showed higher IgE reactivity than the raw form as a result of protein denaturation and polymer formation (Fang et al., 2018). Similar analyses have also correlated protein structural changes to altered reactivity of individual allergens such as egg white ovalbumin heated under electrostatic conditions or peanut Ara h 1 after roasting processes (Claude et al., 2017; Tian et al., 2018). These studies elucidate structure-specific modifications that may occur under processing to help explain the change or lack thereof in allergenicity.

In this context, the aim of this study was to evaluate the influence of two heat treatments and enzymatic proteolysis, two novel processing methods used to prepare food grade insect protein, on tropomyosin reactivity. To achieve this, we characterized the IgE binding behavior of isolated tropomyosin extracted from tropical banded crickets (*G. sigillatus*) after processing conditions that included enzymatic proteolysis, convection, and microwave heating. To mimic realistic processing conditions, tropomyosin was extracted from the treated cricket rather than processing the purified antigen. Since insects will likely be consumed predominantly in its processed state, we should continue to consider the impact of various protein-processing technologies on known allergens.

5.2 Materials and methods

5.2.1 Materials

Frozen, adult (>6-weeks) food-grade tropical banded crickets (*G. Sigillatus*) were purchased from 3 Cricketeers (Hopkins, MN) and stored at -20°C until analyses. All chemicals were reagent grade and purchased from either Sigma Aldrich-Millipore or Thermo Fisher Scientific, unless otherwise stated. Rabbit anti-shrimp tropomyosin, Mab 1A6, and shrimp tropomyosin standard were sourced together from Indoor Biotechnologies (Charlottesville, VA).

Patient Sera

Study protocols were approved by the institutional review board (IRB) of Purdue University, West Lafayette, IN. Human serum samples were kindly donated by The Jaffe Food Allergy Institute at the Icahn School of Medicine (Food Allergy Research and Education Initiative) containing no identifiable information. Sera (#1-4) were from individuals with a shrimp allergy positive for shrimp-specific IgE by ImmunoCAP (> 5 S-IgE) and history of immediate-type reaction to shrimp. A pool was prepared of the four sera and used in the indirect ELISA as described. The cockroach allergic sera (#5) was purchased from Discovery Life Science's (Huntsville, AL) clinical research remnant sample bank.

5.2.2 Tropomyosin extraction from crickets, verification and identification

Tropomyosin was extracted according to Huang & Ochiai. (2005) with modifications. Frozen, edible crickets were homogenized with 25 mM Tris-HCl buffer with 0.1 M KCl, pH 8.0 (Buffer 1). After centrifugation (10,000 × g for 15 min), the supernatant was stored and pellet re-suspended again in Buffer 1. Washing, homogenization with Buffer 1, and centrifugation was repeated three times to remove sarcoplasmic proteins. Washings from buffer 1 were pooled and designated as sarcoplasmic extracts (SE). The final precipitate was then washed another three times with four volumes of absolute acetone. After the final wash, residues were placed on a filter paper and dried overnight at 4ºC. The dried powder was re-suspended in 5-fold (w/v) of Buffer 2 (1 M KCl, 0.5 mM DTT, 25 mM Tris-HCl, pH 8.0, 0.1 mM $CaCl_2$), and stirred at 4°C overnight. Aliquots of buffer 2 represented myofibrillar extracts (ME). The solution was centrifuged (15,000 ×g for 15 min), then subjected to isoelectric precipitation at pH 4.5 with 1 N HCl. After another centrifugation, the pellet was re-suspended in 1 M KCl/0.5 mM DTT and isoelectric precipitation was repeated once more. The precipitated protein was dissolved in 50 mM Tris buffer; pH adjusted to 7.6 with 1 N NaOH, then clarified by centrifugation (designated as the extract after isoelectric precipitation: IP). Supernatant was then fractionated with $(NH_4)_2SO_4$ to 40–60% (v/v) saturation, stirred at 4°C for 4 h, and centrifuged (15,000 × g, 15 min). The precipitate was reconstituted and dialyzed with a Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's protocol. Extracts of the final stage were designated as IP-AS. Efficiency of the extraction conditions was assessed using frozen crickets and frozen shrimp (as positive control) and aliquot removed at each stage of the extraction process as follows: **SE**= sarcoplasmic proteins extracted with buffer 1, **ME**= myofibrillar proteins extracted with buffer 2, **IP**= Isoelectric

precipitate at pH 4.6 of myofibrillar extract**,** and **IP-AS**= extract after ammonium sulfate fractionation of the isoelectric precipitate, also designates the final extraction step.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used as verification of the tropomyosin extraction process above. Tropomyosin samples were prepared for electrophoresis as previously described (Hall, Johnson, & Liceaga, 2018a). Briefly, after dialysis, protein concentration was measured with the bicinchoninic acid (BCA) assay using BSA standards (ThermoScientific, Walthman, MA) then dissolved in chaotropic buffer and aliquoted to 2X Laemmeli Sample Buffer (1 mg/mL). Samples were loaded (10 µL) 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 Brilliant Blue R-250 (Bio-Rad, Hercules, California) overnight followed by de-staining with R-250 de-staining solution overnight (Bio-Rad, Hercules, California). Novex Sharp Pre-Stained protein standards (Thermo Fisher, Waltham, MA) were used as molecular weight standards.

In-gel digestion and LC-MS/MS identification

Electrophoresis was performed as described above. Bands visualized in the ~37 kDa area during the tropomyosin extraction were excised and processed at the Purdue Proteomics core facility in the Bindley Biosciences Center for in-gel extraction and LS-MS/MS analysis following standard and previously described procedures (Jiménez, Huang, Qiu, & Burlingame, 1998; Mittal, Aryal, Camarillo, Ferreira, & Sundararajan, 2019; Sooreshjani, Gursoy, Aryal, & Sintim, 2018). Briefly, bands of interest were cut into cubes for in-gel digestion and washed with 50:50 (v/v) acetonitrile/25 mM ammonium bicarbonate. After drying, samples were reduced with 10 mM dithiothreitol for and cysteine residues alkylated with 55 mM iodoacetamide. Samples were digested with sequence grade Lys-C/Trypsin (Promega Corporation, Madison, WI) in a Barocycler NEP2320 (Pressure Biosciences, Inc.) at 50°C under 20,000 psi. Following digestion, peptides were extracted by sonication using 60% acetonitrile/5% trifluoroacetic acid. Supernatants were dried and re-suspended for LC-MS/MS analysis.

Reverse-phase LC-ESI-MS/MS was used to analyze samples on the UltiMate 3000 RSLCnano coupled to the Q Exactive High Field Hybrid Quadrupole Orbitrap MS and a Nano-spray Flex ion source (Thermo Fisher Scientific). Peptides were loaded onto a trap column (300

μm ID × 5 mm, 5 μm 100 Å PepMap C18 silica), then separated on a reverse phase column (50 cm × 75 μm ID, 3 μm 100 Å PepMap C18 medium, Thermo Fisher Scientific). A data dependent top 20 method acquisition method was used with MS scan range of 400–1,600 m/z, resolution of 120,000 at 200 m/z, spray voltage of 2, AGC target of 3×10^6 and a maximum injection time of 100 milliseconds. MS/MS scans were acquired at a resolution of 15,000 at m/z 200 with an ion-target value of 1×10^5 and a maximum injection of 20 milliseconds was used.

MaxQuant software (v. 1.6.0.16)17-19 with its built-in Andromeda search engine was used for analysis of the LC-MS/MS data. The MS/MS spectra were searched against the Polyneoptera database from Uniprot (downloaded on April 16, 2019), for protein identification and relative quantification. Peptides were considered identified if the Mascot score was over 95% confidence limit. Label-free quantification intensities were used to calculate relative protein abundance. Normalized spectral counting quantification-exponentially modified protein abundance index (emPAI) were derived from Mascot search results and used for comparison of the relative abundance of proteins (Ishihama et al., 2005).

5.2.3 Tropomyosin extraction from cricket protein treatments

To investigate the effect of enzymatic proteolysis and heat treatments on tropomyosin immunoreactivity, first, cricket protein hydrolysates (CPH) were prepared following a previously described protocol (Hall & Liceaga, 2019). Briefly, CPH were prepared by either convection heating proteolysis in a water bath (WB-CPH) or by using microwave-assisted proteolysis (MW-CPH). In both cases, proteolysis was done with Alcalase (*Bacillus licheniformis* ≥2.4 U/g) at pH 8.0 and 55°C. Following proteolysis, CPH were pasteurized in a water bath at 90°C for 15 min, centrifuged (10,000 × g), and the supernatant collected. Control treatments consisted of cricket protein heated in a water bath (WB-Con) or microwave (MW-Con) with no enzyme added. CPH and control samples were lyophilized and stored frozen (-20°C) until use. Tropomyosin samples were extracted from these cricket protein treatments as described above, stored at -80°C, and used within 48 h.

5.2.4 Immunoreactivity of cricket protein treatments and their tropomyosin extracts

The effect of heating and enzymatic proteolysis treatments on immunoreactivity of cricket protein was evaluated. Reactivity was assessed on the treated samples (WB-Con, MW-Con, WB-CPH, and MW-CPH) and their respective extracted tropomyosin to ensure that the observed immunoreactivity was not influenced by the extraction conditions.

Immunoblotting using IgG and IgE

Cricket protein treatments and tropomyosin extracts were electrophoresed by SDS-PAGE as already described (Hall et al., 2018b). Following SDS-PAGE, protein bands were transferred to a PVDF membrane (30 V, 1 h). After washing with PBS-T, membranes were blocked using 5% BSA (w/v) for 2 h and incubated with pooled sera (1:10 dilution) overnight. Goat anti-human IgE conjugated with horseradish peroxidase (1:4,000; 1 h; ThermoFisher, Waltham,MA) was used as the secondary antibody. Immunoblots were visualized after development with a Thermo Scientific TMB-Blotting solution. Blots were washed between each step for 10 min four times with PBS-T (Phosphate-Buffered Saline, 0.1% Tween 20), unless otherwise stated.

Enzyme-linked immunosorbent assay (ELISA)

Binding to anti-tropomyosin IgG was quantified by sandwich ELISA using Monoclonal antibody 1A6 (mAb), tropomyosin standard and rabbit anti-shrimp tropomyosin packaged as a kit by Indoor Biotechnologies (Charlottesville, VA) (Hall & Liceaga, 2019). Washings occurred after each incubation with PBS-T 3x for 5 min. mAb (100 µL; diluted 1:1000 in carbonate-bicarbonate buffer) was used to coat single wells of polystyrene microtiter plates and incubated at 4 °C, overnight. Samples and standard (50-0.1 ng/mL in PBS-T) were incubated for 1 h at room temperature. Rabbit anti-shrimp tropomyosin pAb (1:1000 in PBS-T) was added after another wash step and incubated for 1 h at room temperature. The plate was washed again with PBS-T and goat anti-rabbit horseradish peroxidase conjugated antibody (diluted 1:1000 in 1% BSA, PBS-T) was incubated in wells for the final hour at room temperature.

IgE binding capacity was measured by indirect ELISA. Wells of a 96-well plate were coated with samples (100 µL, 0.1 µg/mL) in coating buffer (0.05M $NaHCO_3$, pH 9.6) overnight at 4°C. The wells were washed three times with PBS-T between each step. Following blocking with 5% BSA (w/v) in PBS-T for 2 h, plates were incubated with the serum pool (1:10, 100 µL) for 4 h

at 37°C. Then, 100 µL goat anti-human IgE-peroxidase (diluted to 1:5000 in PBS) was added to each well, and incubated at room temperature for 1 h. After the final wash step TMB-ELISA substrate solution (Thermo Fisher, Waltham, MA) was used for color development and absorbance read at 450 nm for both assays. All analyses were conducted in triplicate, unless indicated otherwise. Statistical analyses of observed differences among means consisted of analysis of variance (ANOVA), GraphPad Prism version 7 for Windows, La Jolla California USA; followed by Tukey's pairwise comparison of means, with a level of significance defined at 5%.

5.2.5 FT-Raman spectroscopy

Raman spectra was collected from tropomyosin derived from treated cricket protein samples with pure non-treated cricket tropomyosin used as comparison as described in (Hall & Liceaga, 2019). Spectra were obtained using a FT-Raman (Bruker MultiRAM system, Bremen, Germany) equipped with a 1064 nm laser source and a liquid nitrogen cooled Ge detector. Spectral data were collected over the range 400–1800 cm^{-1} at a resolution of 4 cm^{-1} under fixed measurement parameters (laser power: 300 mW, average of two measurements, 500 scans). The OPUS software package provided by Bruker Optics (Billerica, MA, USA) was used for spectral acquisition, instrument control and preliminary file manipulation. Each sample measurement was repeated twice, and the measurement with the largest deviation with respect to the average was eliminated. Spectra were taken of the aqueous tropomyosin extractions at 5 mg/mL protein.

5.2.6 Relative quantification of tropomyosin extracted from cricket protein treatments

Proteomic analysis was used to compare tropomyosin protein and peptides abundance extracted from WB-CPH and MW-CPH. Sample preparation, mass spectrometry analysis, bioinformatics and data evaluation were performed in collaboration with the Proteomics Core Facility at the Indiana University School of Medicine (IUSM), Indianapolis, IN. Methods described in brief below were adaptations from literature reports published elsewhere (Mosley, Florens, Wen, & Washburn, 2009; Mosley et al., 2011; Smith-Kinnaman et al., 2014) and vendor provided protocols. Tropomyosin extracts, from WB-CPH and MW-CPH, were re-suspended in 8 M Urea quantified by Bradford assay. Proteins were reduced with 5 mM TCEP at room temperature for 30 min, then alkylated with 10 mM chloroacetamide for 30 min in the dark at room temperature. Samples were

diluted to 1 M Urea with 50 mM Tris pH 8.5, and digestions were carried out using Trypsin/Lys-C Mass spec grade protease mix (Promega) at a 1:100 protease to substrate ratio, overnight at 37°C, reaction quenched then samples desalted.

Samples were analyzed using a trap column (2 µm particle size, 50 µm diameter) EasySpray (801A) column on an UltiMate 3000 HPLC and Q-Exactive Plus (Thermo Fisher, Waltham, MA) mass spectrometer. A data dependent top 20 method acquisition method was used with MS scan range of 350-1600 m/z, resolution of 70,000, AGC target 3e6, maximum IT of 50 ms. MS2 settings of fixed first mass 100 m/z, normalized collision energy of 36, isolation window of 1.5 m/z, resolution of 35,000, target AGC of 1e5, and maximum IT of 250 milliseconds. For dd acquisition a minimum AGC of 2e3 and charge exclusion of 1, and ≥7 were used.

Data analysis, including de novo and database searches were performed using PEAKS software (Bioinformatics Solutions), with Q-Exactive Plus parameters and search database of all TrEMBL/Swissprot *Gryllidae* proteins and common contaminants.

5.2.7 Allergenic potential of cricket tropomyosin and epitope predication

The allergic potential and cross-reactivity of cricket tropomyosin in general was verified using Allermatch (http://www.allermatch.org) according to the current recommendations of the FAO/WHO Expert Consultation. Comparison in Allermatch was based on UniProt and WHO–IUIS database. An 80 amino acid sliding window alignment was performed with a 35% cut-off percentage (Fiers et al., 2004). Results are given as percent sequence identity, which indicates the extent to which two sequences have the same residues at the same positions in an alignment. Allermatch further calculates the Expect value (E), which describes the number of hits one can expect to see by chance when searching a database of a particular size. An E-value closer to zero indicates a match is more significant. The sequence alignment was carried out by comparing the amino acid sequence of tropomyosin belonging to A0A4P8D324_ACHDO= house cricket and A0A2P1ANK0_9ORTH= field cricket, respectively. Additional tools, AlgPred (https://webs.iiitd.edu.in/raghava/algpred), ABCPred (http://www.imtech.res.in/ragh/abcpred/), and Bepipred (http://tools.iedb.org/bcell) were used to predict the location of linear epitopes.

5.3 Results and Discussion

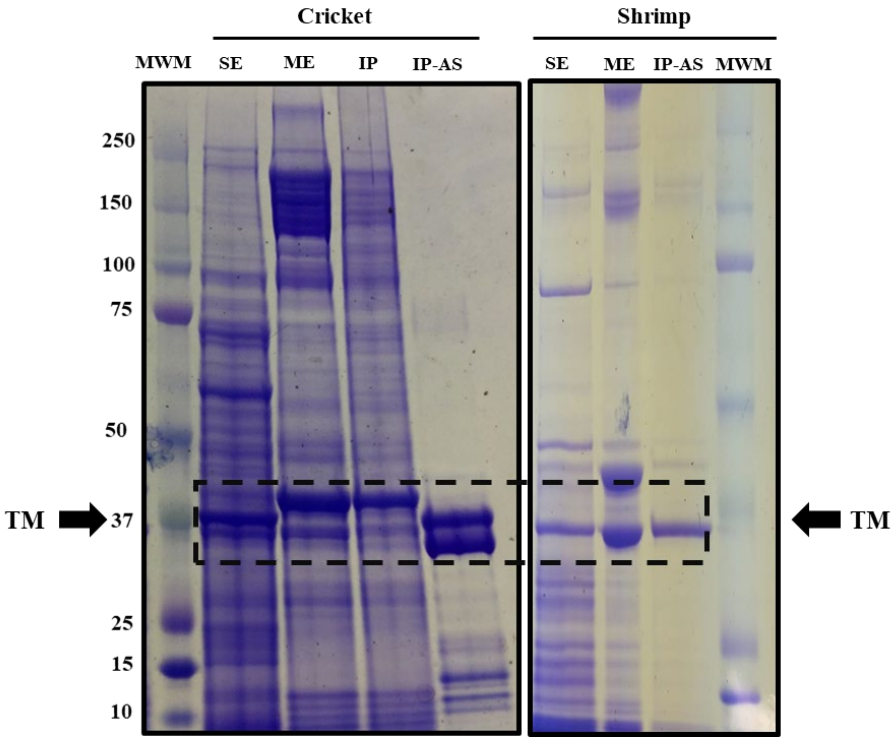

Figure 5.1. SDS-PAGE comparing extraction stages of tropomyosin from edible tropical banded crickets and store-bought shrimp
Lane names correspond to: MWM, molecular weight marker; SE, sarcoplasmic proteins extract using Buffer 1 (25 mM Tris-HCl buffer with 0.1 M KCl, pH 8.0); ME, myofibrillar proteins extracted with Buffer 2 (1 M KCl, 0.5 mM DTT, 25 mM Tris-HCl, pH 8.0, 0.1 mM CaCl2); IP, isoelectric precipitate of myofibrillar extract (pH 4.6); IP-AS, ammonium sulphate 60-70% fractions of IP. All samples were dialyzed before analysis. Whole frozen shrimp was used as a reference. Arrows indicate excised bands of interest.

5.4 Tropomyosin extraction and identification from edible crickets

Tropomyosin were first extracted from edible, frozen crickets and store-bought shrimp, as a reference, then quantified to confirm the efficacy of the extraction conditions. The major reactive bands were excised from each stage of the extraction process. Comparing the extraction stages, some expected differences are apparent in their protein profile (**Figure 5.1**). The sarcoplasmic extract (SE, lane 2), myofibrillar extract (ME, lane 3) and isoelectric precipitate (IP, lane 4) show a wide range of bands, with a consistent band observed near 37 kDa. The electrophoretogram also confirms that several proteins were removed after washing with the salt-containing buffer and isoelectric precipitation, confirming the water-soluble nature of sarcoplasmic proteins. A prominent double band near 37 kDa was evident after the isoelectric precipitate was further

clarified with ammonium sulfate fractionation (IP-AS). Bands at similar molecular weight (~37 kDa) have been established as the major reactive protein, tropomyosin, in crickets (Hall et al., 2018b; Kamemura et al., 2019) and other insects (Pali-Schöll et al., 2019). Hence, tropomyosin bands at ~37 kDa were targeted for extraction and characterization in the present study.

During tropomyosin quantification, peptide spectra matched mostly to tropomyosin proteins in isoelectric and ammonium sulphate precipitates from cricket (Cricket IP-AS) and shrimp (Shrimp IP-AS). Bands excised from the myofibrillar extract (ME) and isoelectric precipitates (IP) also matched to tropomyosin, as expected, but with a larger number of peptides matched to other structural proteins such as paramyosin and myosin heavy chain (Table 1). Interestingly, shrimp tropomyosin (SH-TR) showed higher matches to termite (*Cryptotermes secundus*) and cockroach (*Blattella germanica*) tropomyosin. Whereas, cricket tropomyosin (CR-TR) peptides had no matches to cockroach tropomyosin but did show higher matches with field cricket (*Teleogryllus emma*) tropomyosin isoforms (**Table 5.1**). Nonetheless, the main goal of this analysis was to identify reactive bands near 37 kDa and to validate the proposed tropomyosin extraction method and does not serve as absolute quantification. The abundance of non-tropomyosin proteins decreased with the subsequent extraction steps, confirming the efficacy to isolate tropomyosin from cricket protein treatments to further assess their immunoreactivity.

Table 5.1. Proteins identified in excised bands using LC-MS/MS and Polyneoptera database

Protein			Spectral Count			
Accession	**Name**	**Species**	**Cricket ME[a]**	**Cricket IP[b]**	**Cricket IP-AS[c]**	**Shrimp IP-AS[d]**
A0A2P0XJ16	Putative Per a allergen*	Periplaneta americana	671	354	44	
A0A2J7RDH4	Paramyosin, short form	Cryptoterms secundus	241	133	22	
A0A2P8ZN67	Paramyosin (Fragment)	Blattella germanica	286	142	16	
A0A109ZYM7	Arginine kinase (Fragment)	Aglaothorax diminutiva	142	114	105	
A0A109ZYQ8	Arginine kinase (Fragment)	Aglaothorax gurneyi	136	110	97	
A0A109ZYT6	Arginine kinase (Fragment)	Aglaothorax ovata gigantea	110	86	89	
A0A385MDB2	Calcium-transporting ATPase	Teleogryllus emma	120	94	114	
A0A1P8BJZ4	Myosin heavy chain isoform A	Locusta migratoria	120			
A0A2P9A976	Myosin Heavy Chain (Fragment)	Blattella germanica	101			
A0A067R416	Actin, clone 403	Zootermopsis nevadensis	66	89	103	18
A0A067QL86	Actin, muscle	Zootermopsis nevadensis	80	104		
A0A2P1ANK0	Tropomyosin isoform 2*	Teleogryllus emma	26	222	337	42
A0A2P1ANK6	Tropomyosin isoform 1*	Teleogryllus emma		210	349	943
Q9UB83	Tropomyosin*	Periplaneta americana				797
A0A067QXJ4	Tropomyosin*	Zootermopsis nevadensis		116	172	
A0A2J7PK46	Tropomyosin*	Cryptotermes secundus		127	219	
A0A2J7PK53	Tropomyosin*	Cryptotermes secundus				530
Q9NG56	Tropomyosin*	Blattella germanica				901

*Allergens belonging to the family of Tropomyosin proteins.

[a] ME: Extract from crickets with buffer 2 after removal of sarcoplasmic proteins in buffer 1

[b] IP: Isoelectric precipitate at pH 4.6 of myofibrillar extracts

[c] Cricket IP-AS: Extract from cricket after isoelectric precipitation and ammonium sulfate fractionation.

[d] Shrimp IP-AS: Extract from shrimp after isoelectric precipitation and ammonium sulfate fractionation

5.5 Immunoreactivity of cricket protein treatments and their tropomyosin extracts

Immunoblots and ELISA were used to evaluate allergenicity in treated cricket protein and their extracted tropomyosin (**Figures 5.2 and 5.3**) following heat and proteolysis treatments. Figure 2A displays anti-shrimp tropomyosin IgG activity of unfractionated protein treated samples. Both control samples (lanes 1 and 2), which were thermally treated with no enzymes (WB-Con and MW-Con), had similar reactivity with predominant bands near 37 kDa, and above 60 kDa. There were slight variations seen among the binding pattern, such as observing more higher molecular weight bands in MW-Con (lane 2) and at 37 kDa is slightly higher in WB-Con (lane 1). These differences are likely attributed to the effects of heating type on the reactive proteins/protein fragments. Microwave heating is known to impart more structural change in proteins compared with convection heating (Ketnawa & Liceaga, 2017). Considering cricket protein specifically, previous studies show that spontaneous hydrolysis (no added enzyme) occurred more under microwave heating (Hall & Liceaga, 2020). The enzymatic treated protein under convection, water bath, heating (WB-CPH), retained some reactive response around 35 kDa, while there was no apparent IgG reactivity in the sample enzymatically treated under microwave heating (MW-CPH). When the same treated cricket protein samples were probed with IgE (**Figure 5.2 B**), reactivity remained in the thermally treated controls (lane 1-2) and WB-CPH (lane 3), but was not visible in MW-CPH. Bands near 70 kDa were not as apparent compared with the IgG binding. When tropomyosin was extracted from each cricket protein treatment, the IgE response was similar (**Figure 5.2 C and D**) to that observed before tropomyosin extraction (**Figure 5.2 B**). Interestingly, no reactive bands in regions belonging to tropomyosin (32-37 kDa) were observed when probing samples against cockroach-allergic patient sera (**Figure 5.2 E**). Reactive bands were seen in WB-Con (**Figure 5.2** E; lane 1) and Mw-Con (**Figure 5.2** E; lane 2) differing in binding patterns. Band intensity were much less in WB-CPH (lane 3) and no reactivity in MW-CPH (lane 4). MW-CPH also displayed no reactivity for some anti-shrimp sera (**Figure 5.2** A, B and D; lane 4) and a decreased reactivity in others (**Figure 5.2** C; lane 4).

The overall trend demonstrates decreased IgE and IgG reactivity in the sample treated with enzymatic proteolysis and microwave heating (MW-CPH), including its extracted tropomyosin. Both ELISA assays corroborate this pattern. MW-CPH had significantly lower ($p < 0.05$) IgE and IgG reactivity, compared with the other treatments (**Figure 5.3** A and B) whether on the protein hydrolysate or its extracted tropomyosin. In contrast, there was no significant difference ($p > 0.05$)

in quantifiable tropomyosin between WB-Con, MW-Con and WB-CPH, for either protein hydrolysate samples or their extracted tropomyosin (**Figure 5.3** A & B). However, WB-CPH exhibited slightly decreased ($p < 0.05$) IgE response compared with shrimp tropomyosin, WB-Con, and MW-Con.

Comparing immunoreactivity between samples (**Figure 5.2**), bands above 60 kDa may correspond to cross-linked tropomyosin fragments caused by extensive heating. Similar binding patterns were observed in purified tropomyosin after enzymatic and thermal treatment. For example, a prominent 70 kDa band was detected after glycosylating purified tropomyosin from shrimp (*Metapenaeus ensis*) catalyzed with transglutaminase (Yuan et al., 2017). The authors also demonstrated decreased IgE reactivity at 35 kDa when glycosylated tropomyosin (>65 kDa) bands were present. Glycosylated tropomyosin (>65 kDa) with weaker immune responses in IgE, but not IgG, is reported in various studies (Fang et al., 2018; Nakamura et al., 2006; Wang, Ni, Wang, Li, & Fu, 2019). In some cases, protease digestibility was lowered following glycosylation indicating structural changes and consequently associated with reduced IgE-binding (Nakamura et al., 2006). Although others observed suppressed immunoreactivity in glycated tropomyosin, glycosylation might explain the retained response observed in the water bath heated (WB-CPH) as opposed to microwave treated (MW-CPH) samples. Further, we know from previous studies that this only occurs under specific conditions (convection heating in a water bath at 55ºC for 20 min), whereas, tropomyosin intensity weakened with the progress of proteolysis (Hall et al., 2018b). Considering the composition of whole crickets, it is possible that the conditions used to produce WB-CPH were sufficient to induce glycosylation and limit cleavage by the enzyme used to produce the CPH. However, this theory does not fully explain whether glycosylation occurs during heating alone or the role of proteolysis in decreasing IgE reactivity. Binding to other allergens (not tropomyosin) is also possible given the polyclonal nature of antibodies present in the sera. However, given our collective analyses (monoclonal IgG anti-tropomyosin binding, comparison before and after extraction, and MS identification) along with reported literature (Abdelmoteleb, Palmer, Marsh, Johnson, & Goodman, 2019; H. C. Broekman et al., 2017; Kamemura et al., 2019; Mejrhit et al., 2017; Wang et al., 2019; Yuan et al., 2017), we believe that the immunoreactivity demonstrated in our study is likely due to tropomyosin and/or its fragments. Nevertheless, the current results demonstrate that the overall IgE reactivity was suppressed after treating crickets with microwave heating and enzymatic proteolysis.

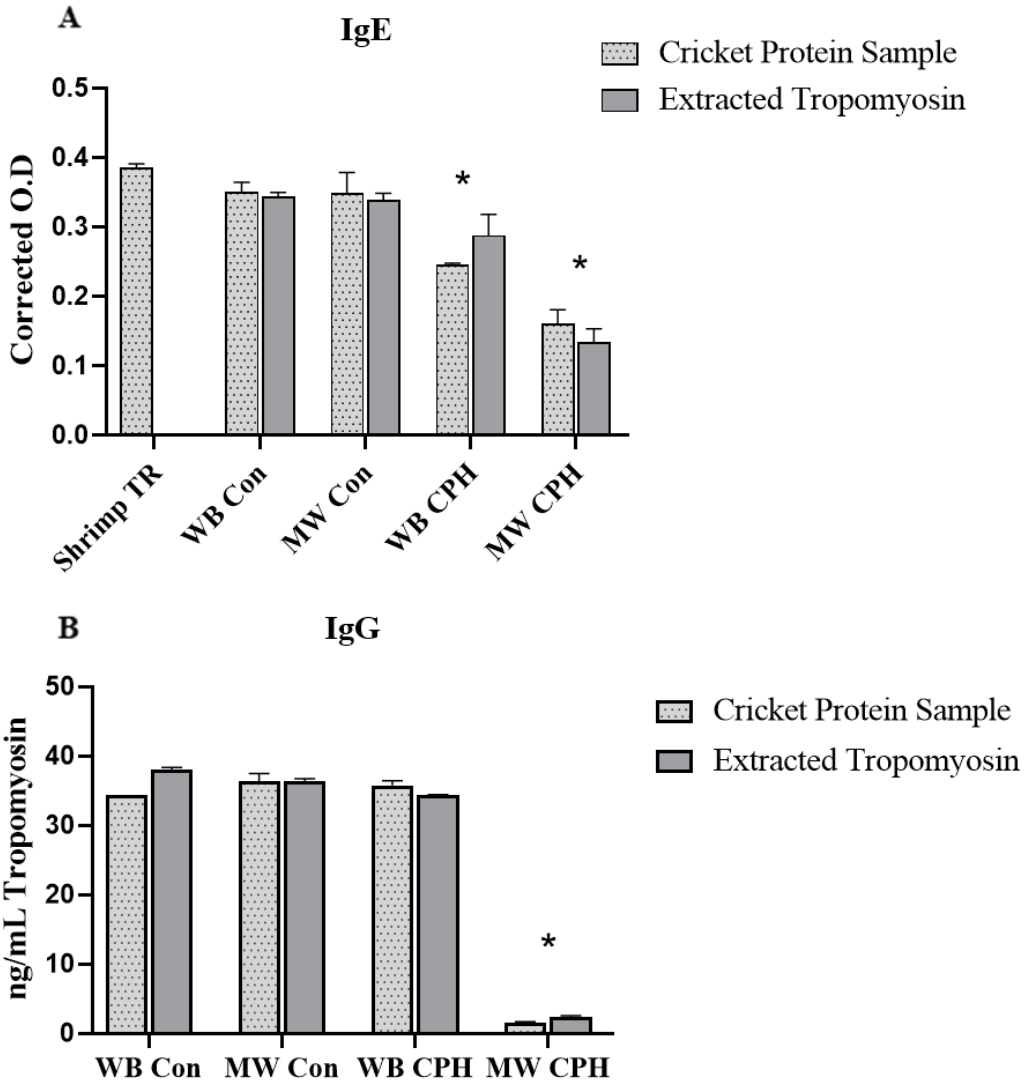

Figure 5.2. IgE indirect (A) and IgG sandwich (B) ELISA of treated cricket protein and their tropomyosin extracts.
Results are means of at least triplicate. Samples denoted with '*' indicate significant difference ($p<0.05$) from other samples tested. Purified shrimp tropomyosin was used as a positive reference for the IgE ELISA and used to create the standard curve for IgG ELISA quantification. 'Cricket protein samples' indicates the state before any tropomyosin extraction steps.
Sample descriptions are as follows: WB-Con= crickets heated in a water bath and no enzyme; WB-Con= crickets heated in a microwave and no enzyme; WB-CPH= cricket protein hydrolysates produced under water bath heating; MW-CPH= cricket protein hydrolysates produced under microwave heating.

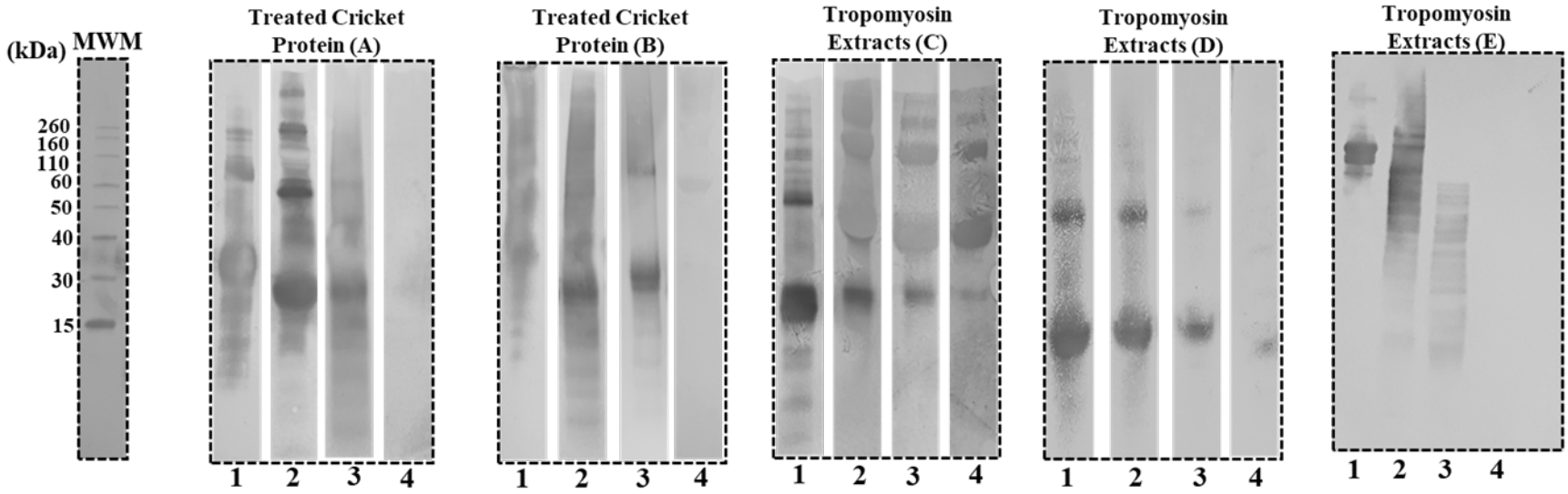

Figure 5.3 Immunoblot analysis of treated cricket protein and their tropomyosin extracts.

Immunoblots containing treated cricket protein samples were incubated with rabbit anti-tropomyosin IgG (A) or shrimp allergic patient sera # 1 (B). Immunoblots containing the tropomyosin extracts from the treated cricket protein samples were incubated with shrimp allergic patient sera #1 and 2 (C and D), or cockroach allergic patient sera (E).
Lane number corresponds to samples as follows: 1: WB-Con= crickets heated in a water bath and no enzyme; 2: WB-Con= crickets heated in a microwave and no enzyme; 3: WB-CPH= cricket protein hydrolysates produced under water bath heating; 4: MW-CPH= cricket protein hydrolysates produced under microwave heating. Unedited immunoblots are available in supplementary material (Fig S1.)

5.6 FT-Raman spectroscopy of treated cricket protein

Figure 4 compares spectra of untreated purified cricket tropomyosin to those from controls, heated but no enzyme added (**Figure 5.4 A**) and heated with enzyme proteolysis (**Figure 5.4** B). Specific regions such as, amide I (1600-1700 cm^{-1}), disulfide and SH bonds (500-659 cm^{-1}) were used to characterize changes in tropomyosin structure post-treatments (Hall & Liceaga, 2019). Other regions (~756, 1099, and 1450 cm^{-1}) are indicative of protein conformation and the local environment.

Proteins with a high α-helical content, illicit an amide I band centered near 1640–1660 cm^{-1}. A higher content of random coil structure (i.e., poly-proline helices) are ascribed to proteins with an amide I band at 1640-1646 cm^{-1} (Hall & Liceaga, 2019). In the present study, the maximum intensity of the 1641 cm^{-1} band in the untreated tropomyosin can be attributed to a high poly-proline α-helix content (**Figure 5.4**). After heating the cricket tropomyosin in a water bath (WB-Con), this peak intensifies and is slightly sharper than untreated tropomyosin. Microwave heating (MW-Con) induced a decreased amide I peak, appearing more like a broad band. Both enzymatic treatments (WB-CPH and MW-CPH) showed a similar trend with an increase in the amide I band width, indicating a loss of coiled structure. As the amide I band is sensitive to the changes of the hydrogen bonding involving the peptide linkages, changes in this region are expected given the nature of the treatments (Li-Chan, Nakai, & Hirotsuka, 1994). Enzymatic proteolysis and microwave heating are prominent with protein unfolding and cleavage (Gohi, Du, Zeng, & Cao, 2019). The increased peak intensity observed in WB-CPH, however, suggests a higher content of secondary structure that can be associated with protein cross-linking occurring during convection heating in a water bath (Sun et al., 2011). Wu et al. (Wu et al., 2019) observed similar amide I peak changes in myofibrillar proteins. As temperature increased, secondary structure content shift from strongly α-helical to β-sheet content, which they associated with protein network formation and gelation (Wu et al., 2019). The C–C stretching vibrations in 890–1100 cm^{-1} also reveal information about the secondary structure, with an intensity decrease at 1099 cm^{-1} after microwave and enzyme treatment. Gradual loss of α-helical structures leads to broadening and weakening of intensity of this band with an increase in disordered structures (Ikeda, 2003). The changes in the spectrum profile agreed with this theory and suggests that α-helices decreased to some extent in WB-Con and even more so in MW-Con, WB-CPH, and MW-CPH.

Bands located at 500–530 and 535–550 cm^{-1} are assigned to S–S bonds in the conformation of gauche–gauche-trans and trans-gauche-trans, respectively (Hall & Liceaga, 2019; Li-Chan et al., 1994). When untreated, the relative intensity of the 550 cm^{-1} was highest and only decreased slightly in WB-Con. Intensity decreased further in the microwave-heated control (MW-Con) and both hydrolysates. No major changes in frequency, and only intensity, indicate that these were unlikely conformational changes and mainly due to disulfide bond cleavage. CH_2 bending vibrations (1450-1470 cm^{-1}) elutes to specific involvement of the CH_2 bands assigned to the H-C-H methylene asymmetric bending or deformation (Howell & Li-Chan, 1996). WB-Con was the only sample to elicit a strong band at 1460 cm^{-1}, with a much smaller peak in the untreated tropomyosin (1473 cm^{-1}). Previous studies reported a higher peak intensity in this region for whey protein treated with high pressure compared with heated whey protein. The peak increase was attributed to marked changes in the environment around aliphatic residues or hydrocarbon changes after high pressure treatment due to increased surfaced activity and hydrophobicity (Ngarize, Adams, & Howell, 2005).

Based on these groups of peaks, Raman spectra allowed for monitoring of specific groups in tropomyosin after heated-only and enzymatic treatments. Dissociation of secondary structure and disulfide bonds clearly demonstrate unfolding (MW-Con) and peptide-bond cleavage (WB-CPH and MW-CPH). There were also indications of protein cross-linking/aggregation in WB-Con, compared with the treated samples. These observations may also suggest formation of glycosylated products in WB-Con. Similarly, glycated soy protein showed similar peak patterns demonstrating that glycation had the most effect of the secondary structure (Bu, Zhang, & Chen, 2015); further, antigenicity of the glycated soy products decreased with increasing glycosylation. In our study, there are not obvious differences between peak patterns in MW-CPH compared with WB-CPH, although they both differ from the untreated tropomyosin. Hence, specific structural modifications are unlikely to account for the differences observed, whereas epitope cleavage seems more responsible for IgE-binding variations instead of structural modifications.

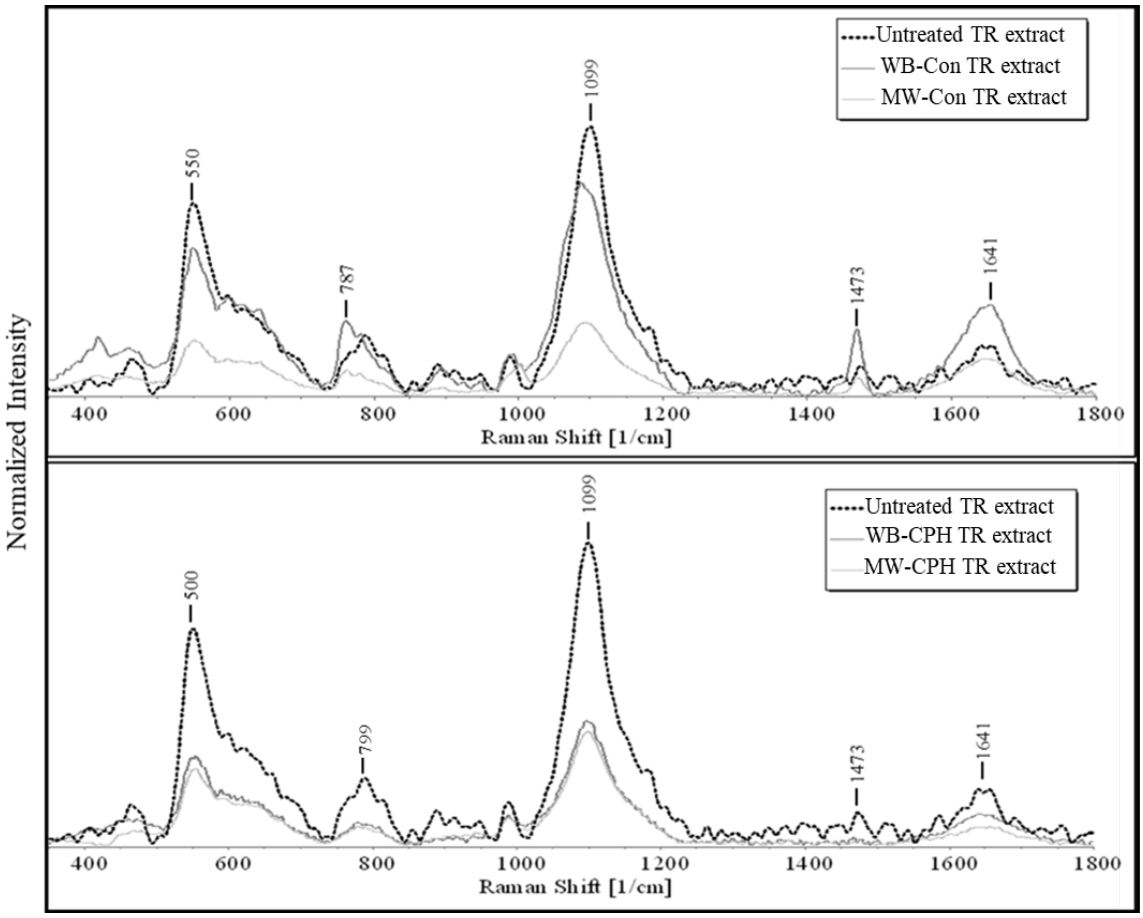

Figure 4. FT-Raman spectroscopy comparing thermally treated tropomyosin extracts (no enzyme) (A) and enzyme treated tropomyosin extracts (B) against untreated tropomyosin extracts

Samples codes are as follows: Untreated TR extract= extracted tropomyosin from untreated frozen crickets, WB-Con TR extract= extracted tropomyosin from convection heated crickets in a water bath with no enzyme, MW-Con TR extract= extracted tropomyosin from crickets heated in a microwave with no enzyme, WB-CPH TR extract= extracted tropomyosin from cricket protein hydrolysates produced under convection heating, MW-CPH TR extract= extracted tropomyosin from cricket protein hydrolysates produced under microwave heating.

5.7 Proteomic analysis comparing tropomyosin extracted from WB-CPH and MW-CPH

The relative quantification of extracted tropomyosin is illustrated in **Figure 5.5** A, and helps determine the extent to which the heat source used during proteolysis impacted tropomyosin detection by mass spectrometry. For both WB-CPH and MW-CPH, peptides from tropomyosin presented the highest average area, clearly representing the most abundant protein, amongst a few other structural proteins (Supp. Mat. Table S5.1). Peptides were almost evenly matched to tropomyosin from two cricket species, field (*Telegryllus emma*) and house crickets (*Acheta domesticus*). Between samples, WB-CPH had relatively more matched peptides to each species than MW-CPH. The initial presumption is that there was simply a higher amount of detectable tropomyosin due to limited extractability or modified peptides. Both cases are commonly observed amongst processed food samples and proteomic analysis of their allergens. In roasted peanuts, for instance, extractability of Ara h allergens is reduced and consequently their peptide abundance detected by mass spec (Tian et al., 2018). Van Broehekhovan et al. (2016) also demonstrated reduced extractability of edible mealworm allergens after various thermal treatments. To combat this, they suggest using detergents and chaotropic agents during extraction to accurately reflect IgE-binding characteristics and protein/peptide abundance. However, extraction conditions in the present study, were effective for microwave (MW-Con) and water bath heated (WB-Con) controls as well as water bath heated protein hydrolysates (MW-CPH). Hence, contrasting changes in allergenicity and lower peptide abundance observed in MW-CPH are probably more so attributed to specific protein cleavage during microwave-assisted enzymatic proteolysis.

In the likely event that processing with microwave heating and enzymatic proteolysis impacted measured allergenicity and LC-MS/MS detection, it would be valuable to establish a link between the tryptic peptide products of both enzyme-treated samples. Potential markers of processing might be the presence of peptides specific to either treatment and/or the relative abundance of shared peptides. The number of tryptic peptides produced from extracted tropomyosin are displayed in **Figure 5.5 B** and their relative abundance available in supplementary material (Table S5.2). A list of de novo only peptides, which takes into non-tryptic products, are provided in Table S5.3. As expected, the number of peptides is relatively large because some degree of proteolytic fragmentation already occurred during the proteolysis treatment. A total of 395 peptides were detected in MW-CPH and 421 in WB-CPH. Only a fraction of peptides (116) were detected in both samples with similar peptide scores. Sequence tags ADKINEDVQELTK

and KVQLVEEDLERSEER, are also predicted epitope regions, can be highlighted as an example of a peptide detected in both processed samples in a similar fashion (Table S5.2). On the other hand, some peptides were unique to only MW-CPH or their abundances were higher than water bath treated hydrolysates (WB-CPH). Tropomyosin peptides LLAEDADGKSDEVSRK and LAMVEADLER, for instance, were identified in both samples with a higher abundance present in WB-CPH.

Comparing tropomyosin tryptic peptides from the cricket protein treatments can provide a comprehensive analysis to differentiate changes caused by enzymatic proteolysis and the heating type (water bath versus microwave). We have identified a series of peptides corresponding to tropomyosin that were found to be specific for either WB-CPH or MW-CPH, and their detection might therefore be indicative of difference in trypsin/alcalase cleavage sites, availability, and protein structure changes induced by the heating treatment used.

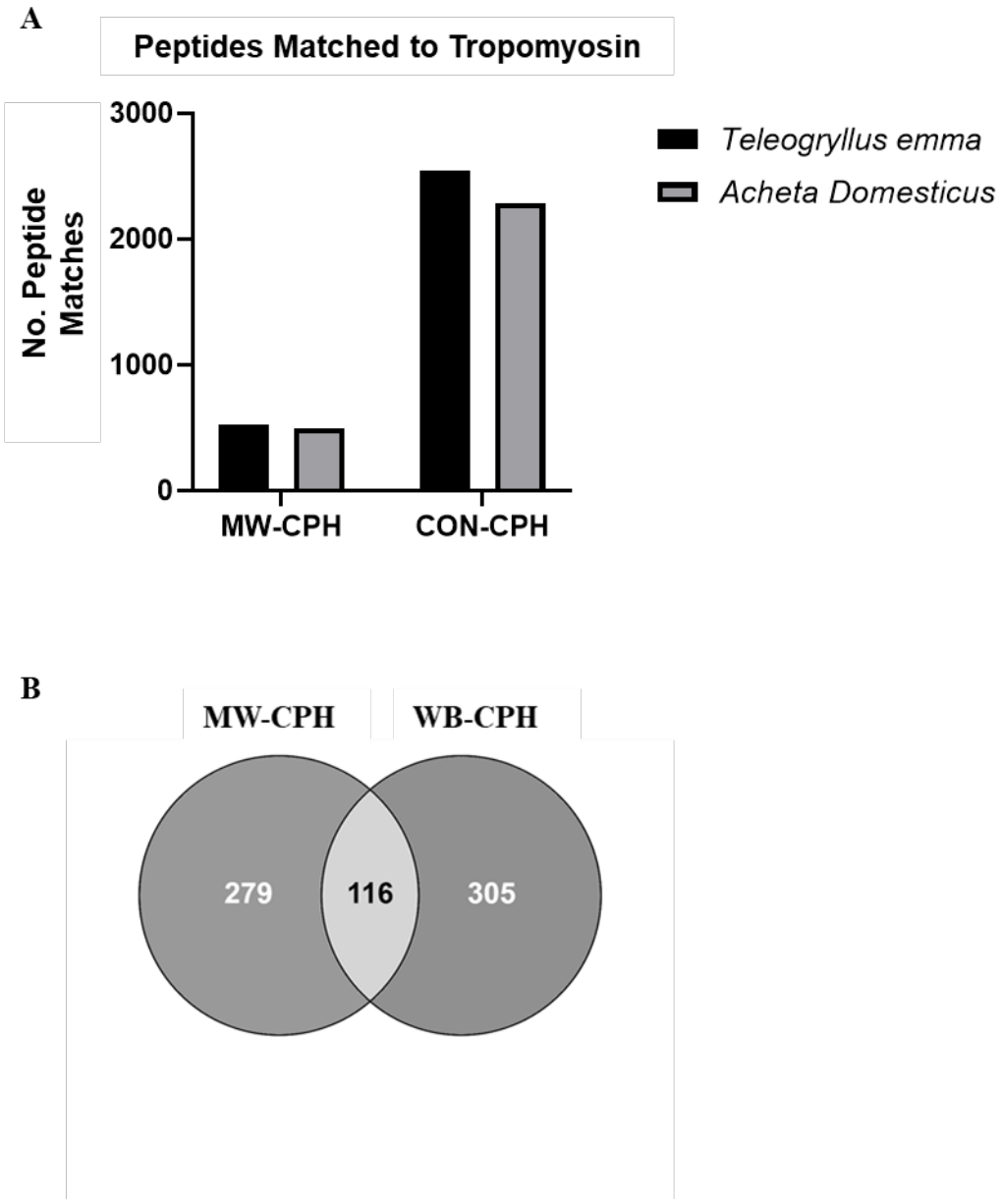

Figure 5.4. Abundance of tropomyosin extracted from WB-CPH and MW-CPH (A) and their identified peptide distribution (B)

```
A0A4P8D324_ACHDO    MDAIKKKMQAMKLEKDNAMDKADTCEGQAKDANNKADKINEDVQELTKKLAQVENDLITT
A0A2P1ANK0_9ORTH    MDAIKKKMQAMKLEKDNAMDKADTCEGQAKDANNKADKINEDVQELTKKLAQVENDLITT

A0A4P8D324_ACHDO    KANLEQANKDLEDKEKALQAAESEMAALNRKVQLVEEDLERSEERAATAATKLQEASEAA
A0A2P1ANK0_9ORTH    KANLEQANKDLEDKEKALQAAESEMAALNRKVQLVEEDLERSEERAATAATKLHEASEAA

A0A4P8D324_ACHDO    DEAQRMCKVLENRSQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFVEDELEVAE
A0A2P1ANK0_9ORTH    DEAQRMCKVLENRSQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFVEDELEVAE

A0A4P8D324_ACHDO    DRVKSGDSKIMELEEELKVVGNSLK----------------------------------
A0A2P1ANK0_9ORTH    DRVKSGDSKIMELEEELKVVGNSLKSLEVSEEKANQRVEEYKRQIKTLSVKLKEAEARAE

A0A4P8D324_ACHDO    -------------------------------------------
A0A2P1ANK0_9ORTH    YAEKTVKKLQKEVDRLEDNLFSDKEKYKSITDDLDSTFAELTGY
```

Figure 5.5. Sequence alignment of tropomyosin isoforms from *Telegryllus emma* (A0A2P1ANK0_9ORTH) and *Acheta domesticus* (A0A4P8D324_ACHDO)

5.8 Immunoinformatics of cricket tropomyosin

Tropomyosin sequences of field cricket [*Teleogryllus Emma* (A0A2P1ANK0)] and house cricket [*Acheta domesticus* (A0A4P8D324)] were acquired in UniProt **(Figure 5.6**) and used to search against other known allergens in Allermatch. Their sequence alignment (**Figure 5.6**) demonstrates the high shared homology between the two cricket species. Linear epitopes were predicted by ABCpred (S. Saha & G. P. S. Raghava, 2006), IEDB (Vita et al., 2019), and Algpred (S. Saha & G. Raghava, 2006). The prediction results of, shrimp- by each tool are combined in **Table 2**. The results of allergenicity assessment of by Allermatch using the full-length, 80-aa sliding window are shown in Table 2. Significance is assumed when the expected score is below 1.0 or a >50% identity match (www.allermatch.org). Allergens from various species of shrimp, crab, and insects showed >60% identity to cricket tropomyosin. The top two matches were tropomyosin from the silverfish (*Lepisma saccharina)* and northern shrimp (*Pandalus borealis*). There were also matches to other insect allergens such as mite (*Dermatophagoides farina*) paramyosin (Der f 11), American cockroach (*Periplaneta americana*) tropomyosin (Per a 7), and German cockroach (*Blattella germanica*) tropomyosin (Bla g 7). Interestingly, strong tropomyosin reactivity against cockroach allergic sera was not observed in this study despite them having significant homology (>65%; Table 2). Others have observed that cricket proteins' identities were higher to cockroach than to crustacean proteins, yet serum IgE also suggested difference in binding

(Abdelmoteleb et al., 2018). Future analysis with a larger sample size of anti-cockroach sera would better assess whether crickets are more cross-reactive to shrimp than cockroach tropomyosin. The combined results of the three immunoinformatic tools predicted a total of 31 peptides as potential linear epitopes within cricket tropomyosin allergen (Table 3). Of the unique peptide predictions, there was overlap in sequences by the different tools used. For example, the peptides RSQQDEERMDQ (IEDB), RSQQDEERMDQLTNQ (Algpred), and NRSQQDEERMDQLTNQ (ABCpred) all shared the 'RSQQDEERM' sequence. From the predicted epitope regions, the full peptide ADKINEDVQELTKK was identified in both water bath (WB-CPH) and microwave (MW-CPH) treated hydrolysates, with a higher abundance in WB-CPH (Supp. Mat. Table S2). Portions of other predicted epitope regions (VQLVEEDLER, VQLVEEDLERSEER, and ANLEQANKDLEDKEK) were also identified in both cricket protein treatments. These potentially active regions remained intact after alcalase and trypsin digestion, regardless of the heating treatment used. However, the relative abundance was higher in the WB-CPH for the indicated regions, which may suggest that more epitope regions remained intact after enzymatic proteolysis and water bath heated treatment. Whereas, microwave heating is known to increase the rate of protein unfolding, enhancing epitope region exposure to the protease which may have not been otherwise accessible during water bath heating (El Mecherfi et al., 2011; Ketnawa & Liceaga, 2017). Confirmatory assays involving IgE-binding assays with a sequenced version of these epitope regions would further confirm their involvement in the cricket tropomyosin cross-reactivity observed in this study.

Table 5.2. Cricket tropomyosin predicted sequence homology with reported allergens

Allergen	Sequence Link in SwissProt/NCBI	Full Alignment[a]	
		E-val	%ID
Lep s 1	CAC84590	1.7e-050	81.50%
Pan b 1.0101	CBY17558	4.8e-049	78.50%
Pen m 1	AAX37288	2.2e-040	67.30%
Pen a 1	11893851	1.2e-039	65.40%
Hom a 1.0102	AAC48288	9.5e-042	69.30%
Lit v 1.0101	EU410072	1.1e-040	67.30%
Hom a 1.0101	O44119	3.3e-041	67.80%
Per a 7.0102	AAD19606	2.9e-041	68.30%
Bla g 7.0101	AAF72534	4.5e-041	68.30%
Der f 10.0101	BAA04557	3.7e-041	67.80%
Chi k 10	CAA09938	7.4e-042	68.80%
Tyr p 10.0101	AAT40866	9.8e-038	65.60%
Blo t 10.0101	ABU97466	1.4e-041	68.30%
Met e 1	Q25456	3.6e-039	66.80%
Pan s 1	O61379	4.7e-041	67.30%
Lep d 10	Q9NFZ4	5.3e-038	66.20%
Der p 10	O18416	6.5e-042	68.30%
Cha f 1	Q9N2R3	2.1e-040	67.30%
Asc l 3.0101	ACN32322	3.5e-042	68.80%
Ani s 3	Q9NAS5	5.8e-042	69.30%
Hel as 1	CAB38044	3.7e-041	67.80%
Hal d 1	AAG08987	1e-039	65.40%
Mim n 1	AAG08989	6e-041	67.80%
Per v 1	AAG08988	2e-041	68.30%
Cra g 1	AAK96889	1.1e-040	67.30%
Der p 11	AAO73464	3.7e-041	67.80%
Blo t 11	AAM83103	3.5e-042	69.30%
Der f 11.0101	AAK39511	4.2e-041	68.30%

5.9 Conclusion

Allergenicity of foods depends on the contribution of several components that may react differently to processing treatments. The conditions of the heat treatment (e.g. source, intensity, length, and temperature) may also impact differently the structure of allergenic proteins, their interactions with other constituents within the matrix, and finally their immunoreactivity. Our aim was to evaluate the influence of heat treatment (convection or microwave) and enzymatic

proteolysis on tropomyosin antigen reactivity. Our results suggest that the decrease in allergenicity observed in microwave and enzymatically treated protein (MW-CPH) was associated with structural modifications of the protein, mainly with a loss of native structure and active tropomyosin epitope regions. More importantly, new allergenic peptide fragments were not formed during enzymatic proteolysis with microwave heating (MW-CPH). Protein folding or cross-linking reactions during convection heating in a water bath likely masked epitope region, which resulted in retained tropomyosin reactivity. These observations correlate with a higher abundance of tropomyosin observed in the water bath hydrolysate (WB-CPH) compared with the microwaved (MW-CPH) treatment.

Microwave-assisted protease treatment could be an effective method for lowering cricket tropomyosin immunoreactivity when formulating insect-based food products or developing bioactive peptides for therapeutic applications. Although our aim was not to decrease cross-reactivity of cricket protein, our results underline the impact of different heat treatments on tropomyosin binding. Further work is required to confirm the role, if any, of proteolysis treatments on tropomyosin solubility and subsequent IgE response. This information will support continued allergenic risk assessment of edible insects as more novel techniques are used to extract protein components of these emerging protein sources.

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5.11 References

Abdelmoteleb, M., Palmer, L. K., Marsh, J., Johnson, P., & Goodman, R. E. (2019). Bioinformatics and Proteomics Evaluations of Potential IgE Cross-Reactive Proteins in Novel Edible Insects and Shrimp for Food Safety. *Journal of Allergy and Clinical Immunology, 143*(2), AB237.

Abdelmoteleb, M., Palmer, L. K., Pavlovikj, N., Marsh, J. T., Johnson, P. E., & Goodman, R. E. (2018). Bioinformatics and Proteomics Evaluations to Consider IgE Binding Assays for Potential Cross-Reactivity Between House-Cricket (Aceta domesticus) Used in Food, Crustaceans and Cockroaches. *Journal of Allergy and Clinical Immunology, 141*(2), AB263.

Broekman, H., Knulst, A., den Hartog Jager, S., Monteleone, F., Gaspari, M., De Jong, G., . . . Verhoeckx, K. (2015). Effect of thermal processing on mealworm allergenicity. *Molecular nutrition & food research, 59*(9), 1855-1864.

Broekman, H. C., Knulst, A. C., de Jong, G., Gaspari, M., den Hartog Jager, C. F., Houben, G. F., & Verhoeckx, K. C. (2017). Is mealworm or shrimp allergy indicative for food allergy to insects? *Molecular nutrition & food research, 61*(9), 1601061.

Bu, G., Zhang, N., & Chen, F. (2015). The influence of glycosylation on the antigenicity, allergenicity, and structural properties of 11S-lactose conjugates. *Food Research International, 76*, 511-517.

Claude, M., Bouchaud, G. g., Lupi, R., Castan, L., Tranquet, O., Denery-Papini, S., . . . Brossard, C. (2017). How proteins aggregate can reduce allergenicity: comparison of ovalbumins heated under opposite electrostatic conditions. *Journal of agricultural and food chemistry, 65*(18), 3693-3701.

de Gier, S., & Verhoeckx, K. (2018). Insect (food) allergy and allergens. *Molecular immunology, 100*, 82-106.

De Martinis, M., Sirufo, M. M., Suppa, M., & Ginaldi, L. (2020). New perspectives in food allergy. *International journal of molecular sciences, 21*(4), 1474.

El Mecherfi, K. E., Saidi, D., Kheroua, O., Boudraa, G., Touhami, M., Rouaud, O., . . . Haertlé, T. (2011). Combined microwave and enzymatic treatments for β-lactoglobulin and bovine whey proteins and their effect on the IgE immunoreactivity. *European Food Research and Technology, 233*(5), 859-867. doi:10.1007/s00217-011-1581-y

Fang, L., Li, G., Gu, R., Cai, M., & Lu, J. (2018). Influence of thermal treatment on the characteristics of major oyster allergen Cra g 1 (tropomyosin). *Journal of the Science of Food and Agriculture, 98*(14), 5322-5328.

Feng, Y., Chen, X. M., Zhao, M., He, Z., Sun, L., Wang, C. Y., & Ding, W. F. (2018). Edible insects in China: Utilization and prospects. *Insect Science, 25*(2), 184-198.

Fiers, M. W., Kleter, G. A., Nijland, H., Peijnenburg, A. A., Nap, J. P., & Van Ham, R. C. (2004). Allermatch™, a webtool for the prediction of potential allergenicity according to current FAO/WHO Codex alimentarius guidelines. *BMC bioinformatics, 5*(1), 133.

Gohi, B. F. C. A., Du, J., Zeng, H.-Y., & Cao, X.-j. (2019). Microwave Pretreatment and Enzymolysis Optimization of the Lotus Seed Protein. *Bioengineering, 6*(2), 28.

Gravel, A., & Doyen, A. (2019). The use of edible insect proteins in food: Challenges and issues related to their functional properties. *Innovative Food Science & Emerging Technologies*, 102272.

Hall, F., Johnson, P. E., & Liceaga, A. (2018a). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food chemistry, 262*, 39-47.

Hall, F., Johnson, P. E., & Liceaga, A. (2018b). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food Chemistry*.

Hall, F., & Liceaga, A. (2019). Effect of microwave-assisted enzymatic hydrolysis of cricket (Gryllodes sigillatus) protein on ACE and DPP-IV inhibition and tropomyosin-IgG binding. *Journal of Functional Foods*, 103634.

Hall, F., & Liceaga, A. (2020). Effect of microwave-assisted enzymatic hydrolysis of cricket (Gryllodes sigillatus) protein on ACE and DPP-IV inhibition and tropomyosin-IgG binding. *Journal of Functional Foods, 64*, 103634.

Hall, F. G., Jones, O. G., O'Haire, M. E., & Liceaga, A. M. (2017). Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. *Food chemistry, 224*, 414-422.

Hossny, E., Ebisawa, M., El-Gamal, Y., Arasi, S., Dahdah, L., El-Owaidy, R., . . . Martinez, S. (2019). Challenges of managing food allergy in the developing world. *World Allergy Organization Journal, 12*(11), 100089.

Howell, N., & Li-Chan, E. (1996). Elucidation of interactions of lysozyme with whey proteins by Raman spectroscopy. *International journal of food science & technology, 31*(5), 439-451.

Huang, M.-C., & Ochiai, Y. (2005). Fish fast skeletal muscle tropomyosins show species-specific thermal stability. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 141*(4), 461-471.

Ikeda, S. (2003). Heat-induced gelation of whey proteins observed by rheology, atomic force microscopy, and Raman scattering spectroscopy. *Food Hydrocolloids, 17*(4), 399-406.

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., & Mann, M. (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Molecular & Cellular Proteomics, 4*(9), 1265-1272.

Jeong, K. Y., Son, M., Lee, J. Y., Park, K. H., Lee, J.-H., & Park, J.-W. (2016). Allergenic characterization of 27-kDa glycoprotein, a novel heat stable allergen, from the pupa of silkworm, Bombyx mori. *Journal of Korean medical science, 31*(1), 18-24.

Jiménez, C., Huang, L., Qiu, Y., & Burlingame, A. (1998). In-gel digestion of proteins for MALDI-MS fingerprint mapping. *Current protocols in protein science, 14*(1), 16.14. 11-16.14. 15.

Kamemura, N., Sugimoto, M., Tamehiro, N., Adachi, R., Tomonari, S., Watanabe, T., & Mito, T. (2019). Cross-allergenicity of crustacean and the edible insect Gryllus bimaculatus in patients with shrimp allergy. *Molecular immunology, 106*, 127-134.

Ketnawa, S., & Liceaga, A. M. (2017). Effect of microwave treatments on antioxidant activity and antigenicity of fish frame protein hydrolysates. *Food and Bioprocess Technology, 10*(3), 582-591.

Leni, G., Tedeschi, T., Faccini, A., Pratesi, F., Folli, C., Puxeddu, I., . . . Depraetere, S. (2020). Shotgun proteomics, in-silico evaluation and immunoblotting assays for allergenicity assessment of lesser mealworm, black soldier fly and their protein hydrolysates. *Scientific Reports, 10*(1), 1-10.

Leoni, C., Volpicella, M., Dileo, M. C., Gattulli, B. A., & Ceci, L. R. (2019). Chitinases as food allergens. *Molecules, 24*(11), 2087.

Li-Chan, E., Nakai, S., & Hirotsuka, M. (1994). Protein Structure-Function Relationships in Foods. Yada, RY; Jackman, RL; Smith, JL, Eds; Blackie Academic & Professional: Chapman & Hall Inc.: London, England.

Mejrhit, N., Azdad, O., Chda, A., El Kabbaoui, M., Bousfiha, A., Bencheikh, R., . . . Aarab, L. (2017). Evaluation of the sensitivity of Moroccans to shrimp tropomyosin and effect of heating and enzymatic treatments. *Food and Agricultural Immunology, 28*(6), 969-980.

Melgar-Lalanne, G., Hernández-Álvarez, A. J., & Salinas-Castro, A. (2019). Edible insects processing: Traditional and innovative technologies. *Comprehensive Reviews in Food Science and Food Safety, 18*(4), 1166-1191.

Mills, E. C., & Mackie, A. R. (2008). The impact of processing on allergenicity of food. *Current opinion in allergy and clinical immunology, 8*(3), 249-253.

Mittal, L., Aryal, U. K., Camarillo, I. G., Ferreira, R. M., & Sundararajan, R. (2019). Quantitative proteomic analysis of enhanced cellular effects of electrochemotherapy with Cisplatin in triple-negative breast cancer cells. *Scientific Reports, 9*(1), 1-16.

Mosley, A. L., Florens, L., Wen, Z., & Washburn, M. P. (2009). A label free quantitative proteomic analysis of the Saccharomyces cerevisiae nucleus. *Journal of proteomics, 72*(1), 110-120.

Mosley, A. L., Sardiu, M. E., Pattenden, S. G., Workman, J. L., Florens, L., & Washburn, M. P. (2011). Highly reproducible label free quantitative proteomic analysis of RNA polymerase complexes. *Molecular & Cellular Proteomics, 10*(2).

Nakamura, A., Sasaki, F., Watanabe, K., Ojima, T., Ahn, D.-H., & Saeki, H. (2006). Changes in allergenicity and digestibility of squid tropomyosin during the Maillard reaction with ribose. *Journal of agricultural and food chemistry, 54*(25), 9529-9534.

Ngarize, S., Adams, A., & Howell, N. (2005). A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures. *Food Hydrocolloids, 19*(6), 984-996.

Pali-Schöll, I., Meinlschmidt, P., Larenas-Linnemann, D., Purschke, B., Hofstetter, G., Rodríguez-Monroy, F. A., . . . Jäger, H. (2019). Edible insects: Cross-recognition of IgE from crustacean-and house dust mite allergic patients, and reduction of allergenicity by food processing. *WAO Journal*.

Phiriyangkul, P., Srinroch, C., Srisomsap, C., Chokchaichamnankit, D., & Punyarit, P. (2015). Effect of food thermal processing on allergenicity proteins in Bombay locust (Patanga succincta). *International Journal of Food Engineering, 1*(1), 23-28.

Ribeiro, J. C., Cunha, L. M., Sousa-Pinto, B., & Fonseca, J. (2018). Allergic risks of consuming edible insects: A systematic review. *Molecular nutrition & food research, 62*(1), 1700030.

Saha, S., & Raghava, G. (2006). AlgPred: prediction of allergenic proteins and mapping of IgE epitopes. *Nucleic acids research, 34*(suppl_2), W202-W209.

Saha, S., & Raghava, G. P. S. (2006). Prediction of continuous B-cell epitopes in an antigen using recurrent neural network. *Proteins: Structure, Function, and Bioinformatics, 65*(1), 40-48.

Salter, A. M. (2019). Insect Protein: A Sustainable and Healthy Alternative to Animal Protein? *The Journal of Nutrition, 149*(4), 545-546.

Smith-Kinnaman, W. R., Berna, M. J., Hunter, G. O., True, J. D., Hsu, P., Cabello, G. I., . . . Mosley, A. L. (2014). The interactome of the atypical phosphatase Rtr1 in Saccharomyces cerevisiae. *Molecular bioSystems, 10*(7), 1730-1741.

Sooreshjani, M. A., Gursoy, U. K., Aryal, U. K., & Sintim, H. O. (2018). Proteomic analysis of RAW macrophages treated with cGAMP or c-di-GMP reveals differentially activated cellular pathways. *RSC advances, 8*(64), 36840-36851.

Sun, W., Zhao, Q., Zhao, M., Yang, B., Cui, C., & Ren, J. (2011). Structural evaluation of myofibrillar proteins during processing of Cantonese sausage by Raman spectroscopy. *Journal of agricultural and food chemistry, 59*(20), 11070-11077.

Tian, Y., Rao, H., Zhang, K., Tao, S., & Xue, W. T. (2018). Effects of different thermal processing methods on the structure and allergenicity of peanut allergen Ara h 1. *Food science & nutrition, 6*(6), 1706-1714.

Van Broekhoven, S., Bastiaan-Net, S., de Jong, N. W., & Wichers, H. J. (2016). Influence of processing and in vitro digestion on the allergic cross-reactivity of three mealworm species. *Food chemistry, 196*, 1075-1083.

Vita, R., Mahajan, S., Overton, J. A., Dhanda, S. K., Martini, S., Cantrell, J. R., . . . Peters, B. (2019). The immune epitope database (IEDB): 2018 update. *Nucleic acids research, 47*(D1), D339-D343.

Volpicella, M., Leoni, C., Dileo, M. C., & Ceci, L. R. (2019). Progress in the Analysis of Food Allergens through Molecular Biology Approaches. *Cells, 8*(9), 1073.

Wang, Y., Ni, S., Wang, C., Li, X., & Fu, L. (2019). Cross-linking of shrimp tropomyosin catalyzed by transglutaminase and tyrosinase produces hypoallergens for potential immunotherapy. *Food & function, 10*(3), 1609-1618.

Wu, M., Cao, Y., Lei, S., Liu, Y., Wang, J., Hu, J., . . . Yu, H. (2019). Protein structure and sulfhydryl group changes affected by protein gel properties: process of thermal-induced gel formation of myofibrillar protein. *International Journal of Food Properties, 22*(1), 1834-1847.

Yuan, F., Lv, L., Li, Z., Mi, N., Chen, H., & Lin, H. (2017). Effect of transglutaminase-catalyzed glycosylation on the allergenicity and conformational structure of shrimp (Metapenaeus ensis) tropomyosin. *Food chemistry, 219*, 215-2

5.12 Appendix

Supplementary material

Figure S5.1: Unedited immunoblots against anti-tropomyosin IgG, shrimp (S1-S3) and cockroach allergic patient sera (S5)

Figure S5.2: Full length SDS-PAGE of tropomyosin extraction from cricket and shrimp

Table S5.1: Protein matches identified in the tropomyosin extracts from WB-CPH and MW CPH. Characterization parameters include protein abundance (area), sequence coverage, and unique peptide matches. Database searches were performed using PEAKS software, and of all TrEMBL/Swissprot Gryllidae proteins and common contaminants.

Table S5.2: Tryptic Peptide sequences identified in tropomyosin extracts from WB-CPH and MW-CPH. Characterization parameters include peptide abundance (area), and accession number of parent protein. Database searches were performed using PEAKS software, and of all TrEMBL/Swissprot Gryllidae proteins and common contaminants.

Table S5.3: De Novo Peptide sequences identified in tropomyosin extracts from WB-CPH (A) and MW-CPH (B). Parameters include: peptide sequence, average local confidence (ALC %), peptide length, peptide abundance (area), peptide mass and accession number of parent protein when available.

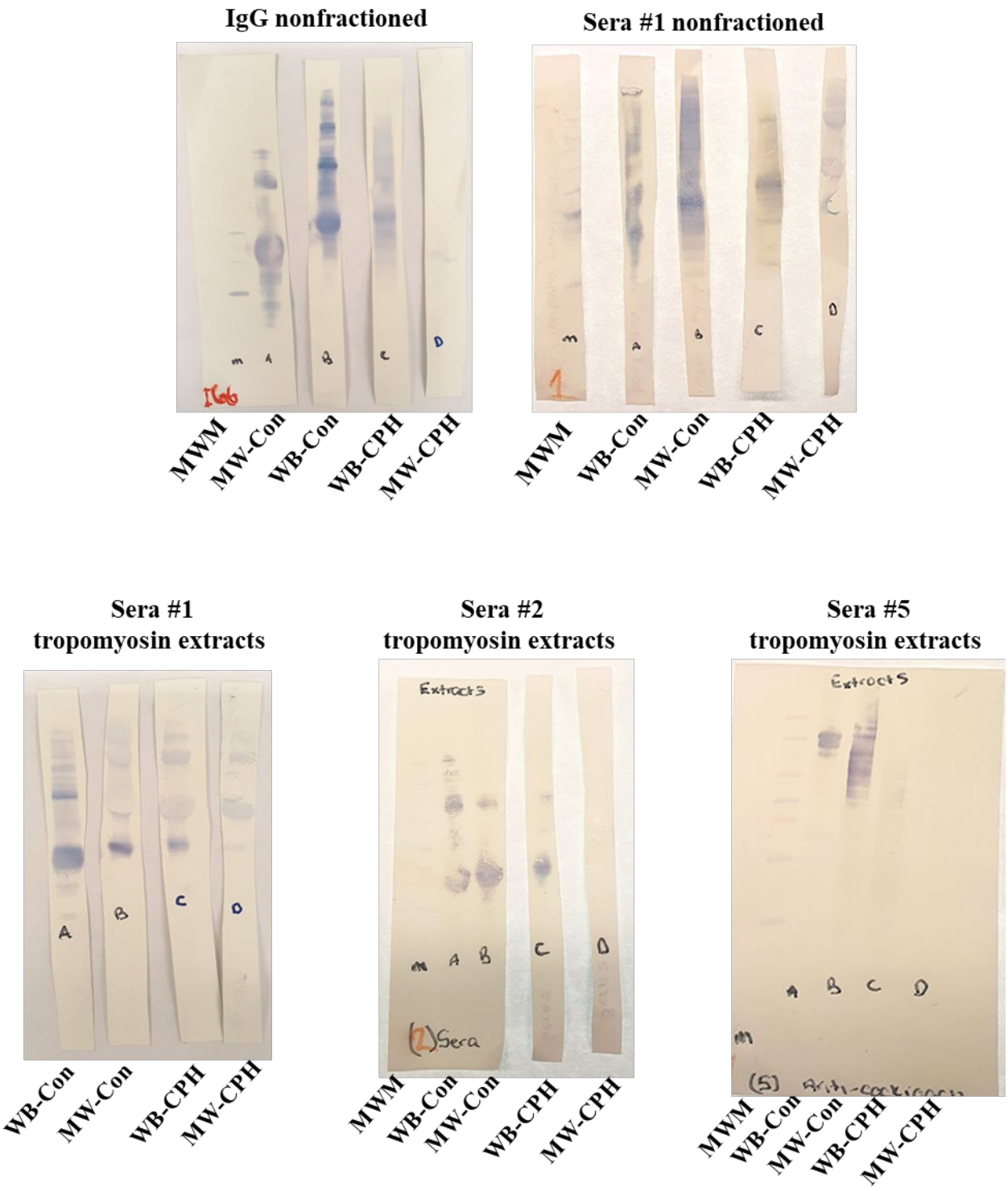

Figure S5.1: Unedited immunoblots against anti-tropomyosin IgG, shrimp (S1-S3) and cockroach allergic patient sera (S5)

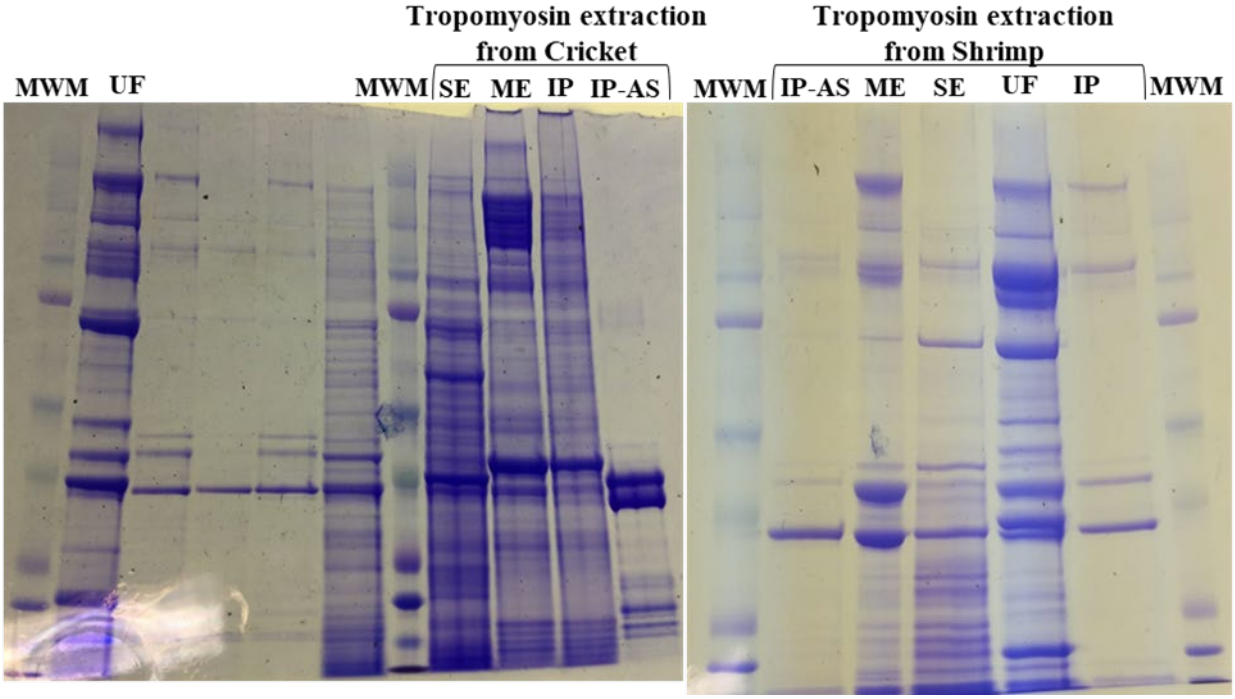

Figure S5.2: Full length SDS-PAGE of the tropomyosin extraction process from cricket and shrimp. Lane names correspond to: MWM, molecular weight markers; SE, sarcoplasmic proteins extracted with Buffer 1 (25 mM Tris-HCl buffer with 0.1 M KCl, pH 8.0); ME, myofibrillar proteins extracted with Buffer 2 (1 M KCl, 0.5 mM DTT, 25 mM Tris-HCl, pH 8.0, 0.1 mM CaCl2); IP, isoelectric precipitate of myofibrillar extract (pH 4.6); IP-AS, ammonium sulphate 60-70% fractions of IP; UF: cricket or shrimp protein before any extraction steps.

Table S5.1. Protein Matches									
Accession	**Coverage (%)**	**#Peptides**	**#Unique**	**TR_MW-CPH Area**	**TR_WB-CPH Area**	**MW-CPH Area (top-3 peptides)**	**WB-CPH Area (top-3 peptides)**	**Avg. Mass**	**Description**
A0A2P1ANK6	64	27	7	4.01E+08	2.10E+09	3.58E+08	1.94E+09	32784	Tropomyosin isoform 1 OS=Teleogryllus emma OX=62746 GN=Tm PE=2 SV=1
A0A4P8D324	77	25	1	7.09E+07	1.52E+09	7.09E+07	1.52E+09	23015	Tropomyosin 1 (Fragment) OS=Acheta domesticus OX=6997 PE=2 SV=1
A0A2P1ANK7	11	5	5	1.92E+08	2.42E+06	1.77E+08	1.99E+06	46709	Troponin T OS=Teleogryllus emma OX=62746 GN=TnT PE=2 SV=1
F2ZBU0	17	8	8	4.20E+08	2.94E+07	2.15E+08	1.05E+07	41822	Actin OS=Gryllus bimaculatus OX=6999 GN=Act PE=2 SV=1
A0A2P1ANK3	24	4	2	2.06E+07	1.47E+08	2.06E+07	1.47E+08	17281	Troponin C OS=Teleogryllus emma OX=62746 GN=TnC PE=2 SV=1

A0A385MDB2	2	2	2	6.68E+06	3.79E+05	6.68E+06	3.79E+05	109615	Calcium-transporting ATPase OS=Teleogryllus emma OX=62746 PE=2 SV=1
X2D1W0	19	2	2	7.65E+07	2.83E+05	7.65E+07	2.83E+05	14506	Apolipophorin-III (Fragment) OS=Gryllus bimaculatus OX=6999 PE=2 SV=1
D0EX54	10	2	2	3.85E+06	1.16E+06	3.85E+06	1.16E+06	24380	Ferritin OS=Teleogryllus emma OX=62746 PE=2 SV=1
C0SSX5	12	1	1	2.55E+06	1.64E+05	2.55E+06	1.64E+05	12858	14-3-3zeta (Fragment) OS=Gryllus bimaculatus OX=6999 GN=14-3-3z PE=2 SV=1
Q86R38	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	13949	Histone H3 (Fragment) OS=Stenopelmatus fuscus OX=202428 PE=3 SV=1
A0A0R7GPT7	7	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12137	Histone H3 (Fragment) OS=Phaneroptera falcata OX=396409 GN=H3 PE=3 SV=1
Q49SL8	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	13265	Histone H3 (Fragment) OS=Gryllus assimilis OX=128156 GN=H3 PE=3 SV=1

Q49SL3	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	13265	Histone H3 (Fragment) OS=Camptonotus carolinensis OX=114793 GN=H3 PE=3 SV=1
A0A0R7GPS3	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12324	Histone H3 (Fragment) OS=Tettigonia viridissima OX=62794 GN=H3 PE=3 SV=1
A0A0U2JM59	7	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	10795	Histone H3 (Fragment) OS=Gryllus bimaculatus OX=6999 GN=H3 PE=3 SV=1
T2BBX1	7	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	11430	Histone H3 (Fragment) OS=Anabrus simplex OX=316456 PE=3 SV=1
A0A144R6S6	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12325	Histone H3 (Fragment) OS=Cardiodactylus tankara OX=1108857 GN=H3 PE=3 SV=1
A0A144R6T7	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Lebinthus lifouensis OX=323479 GN=H3 PE=3 SV=1

D1LUF6	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Acheta domesticus OX=6997 GN=H3 PE=3 SV=1
A0A144R7L6	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Lebinthus sp. JD-2016 OX=1811910 GN=H3 PE=3 SV=1
A0A144R6P0	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Cardiodactylus enkraussi OX=1108854 GN=H3 PE=3 SV=1
Q1WH87	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Acris crepitans OX=317300 PE=3 SV=1
A0A0U2ZND9	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Cardiodactylus novaeguineae OX=1108856 GN=H3 PE=3 SV=1
A0A144R6Q5	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Cardiodactylus guttulus OX=1108855 GN=H3 PE=3 SV=1

W0TZ80	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Phaneroptera falcata OX=396409 GN=h3 PE=3 SV=1
A0A144R6X5	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Lebinthus villemantae OX=1108860 GN=H3 PE=3 SV=1
A0A0U3BH51	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Nisitrus vittatus OX=323487 GN=H3 PE=3 SV=1
A0A144R712	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Pixibinthus sonicus OX=1807130 GN=H3 PE=3 SV=1
A0A144R6W2	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Lebinthus santoensis OX=1108859 GN=H3 PE=3 SV=1
A0A144R6V1	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12294	Histone H3 (Fragment) OS=Lebinthus nattawa OX=1108858 GN=H3 PE=3 SV=1

A0A1P8L0N0	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12465	Histone H3 (Fragment) OS=Roeseliana roeselii OX=494379 GN=H3 PE=3 SV=1
A0A1P8L0K2	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12465	Histone H3 (Fragment) OS=Tettigonia viridissima OX=62794 GN=H3 PE=3 SV=1
T2BAI8	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12566	Histone H3 (Fragment) OS=Pholidoptera griseoaptera OX=302092 PE=3 SV=1
T2BCF5	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12566	Histone H3 (Fragment) OS=Phaneroptera falcata OX=396409 PE=3 SV=1
T2BC13	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12566	Histone H3 (Fragment) OS=Troglophilus neglectus OX=552514 PE=3 SV=1
T2BCE6	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12566	Histone H3 (Fragment) OS=Camptonotus carolinensis OX=114793 PE=3 SV=1
T2BBW6	6	1	1	3.34E+06	9.59E+05	3.34E+06	9.59E+05	12566	Histone H3 (Fragment) OS=Stenopelmatus fuscus OX=202428 PE=3 SV=1

Q49M29	24	4	4	1.00E+08	3.99E+07	7.26E+07	2.41E+07	22594	Myosin light chain OS=Gryllotalpa orientalis OX=213494 GN=MLC PE=2 SV=1
A0A4V1DVH3	76	21	4	6.19E+08	1.91E+09	4.87E+08	1.18E+09	23040	Tropomyosin 2 (Fragment) OS=Acheta domesticus OX=6997 PE=2 SV=1
A0A385MCP5	28	5	3	3.62E+07	6.91E+07	3.18E+07	6.67E+07	17378	Troponin C OS=Teleogryllus emma OX=62746 PE=2 SV=1

Table S5.2. Tryptic peptides identified in WB-CPH and MW-CPH

Peptide	Area MW-CPH	Area WB-CPH	MW-CPH	WB-CPH	Accession
LAEASQAADESER	2.10E+08	1.24E+09	2.10E+08	1.24E+09	A0A2P1ANK6:A0A4V1DVH3
SLEVSEEK	1.75E+08	1.62E+09	1.75E+08	1.62E+09	A0A2P1ANK6
ALLC(+57.02)EQQAR	1.84E+08	8.98E+08	1.84E+08	8.98E+08	A0A2P1ANK6:A0A4V1DVH3
SLADEER	1.01E+08	6.41E+08	1.01E+08	6.41E+08	A0A2P1ANK6:A0A4V1DVH3
MDQLTNQLK	7.29E+07	1.79E+09	7.29E+07	1.79E+09	A0A4P8D324
INEDVQELTK	4.81E+07	1.10E+09	4.81E+07	1.10E+09	A0A4P8D324
LQEASEAADEAQR	7.09E+07	1.52E+09	7.09E+07	1.52E+09	A0A4P8D324
LAQVENDLITTK	9.19E+07	1.39E+09	9.19E+07	1.39E+09	A0A4P8D324
VQLVEEDLER	9.73E+07	1.91E+09	9.73E+07	1.91E+09	A0A4P8D324

FLAEEADK	4.49E+07	3.00E+08	4.49E+07	3.00E+08	A0A4V1DVH3
ANLEQANK	1.32E+07	3.27E+08	1.32E+07	3.27E+08	A0A4P8D324
LATATAK	3.56E+07	1.55E+08	3.56E+07	1.55E+08	A0A2P1ANK6:A0A4V1DVH3
FLAEEADKK	1.32E+08	1.06E+09	1.32E+08	1.06E+09	A0A4V1DVH3
LLAEDADGKSDEVSR	2.59E+07	3.86E+08	2.59E+07	3.86E+08	A0A4P8D324
LEKDNAMDR	1.87E+07	4.31E+07	1.87E+07	4.31E+07	A0A2P1ANK6:A0A4V1DVH3
NVDDANEEALK	1.97E+07	1.40E+07	1.97E+07	1.40E+07	
EEEYKQQIK	1.61E+07	1.35E+08	1.61E+07	1.35E+08	A0A2P1ANK6
AEKAEEEAR	1.75E+05	5.11E+05	1.75E+05	5.11E+05	A0A2P1ANK6:A0A4V1DVH3
IQLLEEDLER	3.30E+08	2.01E+09	3.30E+08	2.01E+09	A0A2P1ANK6:A0A4V1DVH3
GFMTPER	3.65E+06	3.64E+06	3.65E+06	3.64E+06	
DLEDKEK	7.12E+06	2.46E+08	7.12E+06	2.46E+08	A0A4P8D324
IIAPPER	4.47E+07	1.01E+07	4.47E+07	1.01E+07	F2ZBU0
VYLC(+57.02)EGQK	1.15E+07	5.03E+06	1.15E+07	5.03E+06	
VVGNNLK	7.62E+07	5.36E+08	7.62E+07	5.36E+08	A0A2P1ANK6
LLAEDADGK	4.38E+07	7.98E+08	4.38E+07	7.98E+08	A0A4P8D324
SLYTGGR	8.64E+05	4.97E+05	8.64E+05	4.97E+05	
DSYVGDEAQSK	5.64E+07	3.73E+06	5.64E+07	3.73E+06	F2ZBU0
LEKDNAMDK	2.88E+06	8.97E+07	2.88E+06	8.97E+07	A0A4P8D324
SGSISTDM(+15.99)VGEILR	5.78E+06	1.02E+07	5.78E+06	1.02E+07	A0A2P1ANK3
GFIDTQK	1.50E+07	7.39E+06	1.50E+07	7.39E+06	Q49M29
AAEELKK	2.29E+06	1.39E+06	2.29E+06	1.39E+06	

SLGDLGEYM(+15.99)FDK	2.06E+06	1.04E+06	2.06E+06	1.04E+06	D0EX54
AFDAFDR	1.09E+07	6.05E+07	1.09E+07	6.05E+07	A0A2P1ANK3:A0A385MCP5
GYSFTTTAER	8.02E+07	1.54E+06	8.02E+07	1.54E+06	F2ZBU0
IIAPPERK	5.30E+06	5.08E+05	5.30E+06	5.08E+05	F2ZBU0
ALQTAEGEIAALNR	3.55E+08	1.12E+08	3.55E+08	1.12E+08	A0A4V1DVH3
DNAMDKADTC(+57.02)EGQAK	1.51E+06	5.45E+07	1.51E+06	5.45E+07	A0A4P8D324
DLTDYLMK	6.06E+07	7.09E+06	6.06E+07	7.09E+06	F2ZBU0
SYELPDGQVITIGNER	7.39E+07	1.91E+06	7.39E+07	1.91E+06	F2ZBU0
VVGDNLK	9.61E+06	2.20E+07	9.61E+06	2.20E+07	
MDSTEPPYSEAR	1.50E+06	9.95E+05	1.50E+06	9.95E+05	
SLEVSEEKANQR	1.13E+06	7.83E+06	1.13E+06	7.83E+06	A0A2P1ANK6
MDALENQLK	2.99E+08	2.04E+09	2.99E+08	2.04E+09	A0A2P1ANK6:A0A4V1DVH3
LKEAEAR	4.99E+07	4.20E+08	4.99E+07	4.20E+08	A0A2P1ANK6
KRLEEASK	1.72E+06	4.68E+04	1.72E+06	4.68E+04	
ATELWEAIVK	9.00E+07	1.28E+06	9.00E+07	1.28E+06	A0A2P1ANK7
AGFAGDDAPR	6.24E+07	3.82E+06	6.24E+07	3.82E+06	F2ZBU0
RLEEAER	1.12E+07	3.28E+05	1.12E+07	3.28E+05	A0A2P1ANK7
IQTIENELDQTQEQLMQVNAK	1.11E+08	1.36E+08	1.11E+08	1.36E+08	A0A2P1ANK6:A0A4V1DVH3
KYDEVAR	1.45E+07	1.12E+08	1.45E+07	1.12E+08	A0A2P1ANK6:A0A4V1DVH3
KGFMTPER	3.37E+06	9.72E+05	3.37E+06	9.72E+05	
QQFPDGAQAADK	3.45E+07	1.81E+05	3.45E+07	1.81E+05	X2D1W0
LEKDNAM(+15.99)DR	5.22E+06	1.58E+06	5.22E+06	1.58E+06	A0A2P1ANK6:A0A4V1DVH3

VIVITGDNK	1.83E+06	1.68E+05	1.83E+06	1.68E+05	A0A385MDB2
KAFDAFDR	4.89E+06	2.02E+07	4.89E+06	2.02E+07	A0A2P1ANK3:A0A385MCP5
DLTDYLM(+15.99)K	3.41E+07	5.80E+05	3.41E+07	5.80E+05	F2ZBU0
TLSDYNIQK	4.74E+06	2.49E+06	4.74E+06	2.49E+06	
KGC(+57.02)ISTEMVK	2.20E+05	2.45E+05	2.20E+05	2.45E+05	A0A385MCP5
DSYVGDEAQSKR	2.41E+06	1.90E+05	2.41E+06	1.90E+05	F2ZBU0
M(+15.99)SGGSDDDDVVINAFK	1.74E+07	1.18E+06	1.74E+07	1.18E+06	Q49M29
LAFVEDELEVAEDR	7.35E+07	1.28E+09	7.35E+07	1.28E+09	A0A4P8D324
MSGGSDDDDVVINAFK	2.43E+07	1.34E+07	2.43E+07	1.34E+07	Q49M29
IM(+15.99)ELEEELK	4.86E+07	2.17E+08	4.86E+07	2.17E+08	A0A4P8D324
AVTETGVELSNEER	2.55E+06	1.64E+05	2.55E+06	1.64E+05	C0SSX5
EYIAEWR	5.06E+07	2.18E+05	5.06E+07	2.18E+05	A0A2P1ANK7
VQLVEEDLERSEER	3.79E+07	4.86E+08	3.79E+07	4.86E+08	A0A4P8D324
LEDELVIEKEK	1.62E+06	6.39E+06	1.62E+06	6.39E+06	A0A2P1ANK6
ATFDSLGR	3.33E+07	3.30E+06	3.33E+07	3.30E+06	Q49M29
MFFESANEER	1.79E+06	1.18E+05	1.79E+06	1.18E+05	D0EX54
KAAEELKK	4.96E+05	1.08E+05	4.96E+05	1.08E+05	
SKEEDELKR	3.74E+06	9.64E+04	3.74E+06	9.64E+04	A0A2P1ANK7
ALQAAESEMAALNR	2.65E+07	2.86E+08	2.65E+07	2.86E+08	A0A4P8D324
IQTIENELDQTQEQLMQVNAK	1.69E+08	6.73E+08	1.69E+08	6.73E+08	A0A2P1ANK6:A0A4V1DVH3
EIAQDFK	3.34E+06	9.59E+05	3.34E+06	9.59E+05	Q86R38:A0A0R7GPT7
FSADEVDDAYEQMVIDDK	7.51E+06	1.41E+07	7.51E+06	1.41E+07	Q49M29

LAMVEADLER	1.78E+08	1.57E+09	1.78E+08	1.57E+09	A0A2P1ANK6:A0A4V1DVH3
KIQTIENELDQTQEQLMQVNAK	7.21E+07	2.13E+08	7.21E+07	2.13E+08	A0A2P1ANK6:A0A4V1DVH3
SQQDEERMDQLTNQLK	6.69E+06	7.99E+07	6.69E+06	7.99E+07	A0A4P8D324
FLIEEDAEAM(+15.99)QQELR	1.48E+07	1.37E+08	1.48E+07	1.37E+08	A0A2P1ANK3
AAQLSGDAQTAVR	4.19E+07	1.01E+05	4.19E+07	1.01E+05	X2D1W0
INEDVQELTKK	9.27E+06	2.18E+08	9.27E+06	2.18E+08	A0A4P8D324
FSADEVDDAYEQM(+15.99)VIDDK	2.41E+06	5.41E+05	2.41E+06	5.41E+05	Q49M29
IQLLEEDLERSEER	1.21E+08	4.14E+08	1.21E+08	4.14E+08	A0A2P1ANK6:A0A4V1DVH3
ADKINEDVQELTK	5.04E+07	1.21E+09	5.04E+07	1.21E+09	A0A4P8D324
DFEISDLNSQVNDLR	1.40E+07	5.29E+06	1.40E+07	5.29E+06	
MLEIPVDDATLK	1.28E+07	3.52E+07	1.28E+07	3.52E+07	A0A385MCP5
ANLEQANKDLEDKEK	3.08E+07	7.06E+08	3.08E+07	7.06E+08	A0A4P8D324
LEDELVIEK	1.69E+07	1.07E+08	1.69E+07	1.07E+08	A0A2P1ANK6
M(+15.99)LEIPVDDATLK	4.34E+06	2.40E+06	4.34E+06	2.40E+06	A0A385MCP5
SQQDEERM(+15.99)DQLTNQLK	1.77E+07	3.52E+07	1.77E+07	3.52E+07	A0A4P8D324
SLEVSEEKANQREEEYK	1.57E+06	6.11E+06	1.57E+06	6.11E+06	A0A2P1ANK6
RDFEISDLNSQVNDLR	2.45E+07	7.05E+06	2.45E+07	7.05E+06	
LAMVEADLERAEER	5.14E+07	1.55E+08	5.14E+07	1.55E+08	A0A2P1ANK6:A0A4V1DVH3
LAM(+15.99)VEADLERAEER	4.71E+07	2.38E+07	4.71E+07	2.38E+07	A0A2P1ANK6:A0A4V1DVH3
ALQAAESEM(+15.99)AALNR	7.04E+06	5.45E+06	7.04E+06	5.45E+06	A0A4P8D324
SSALDEQLK	3.61E+07	4.97E+05	3.61E+07	4.97E+05	A0A2P1ANK7
FLIEEDSEALEK	1.88E+07	3.13E+07	1.88E+07	3.13E+07	A0A385MCP5

WDLEHEVR	3.59E+07	2.60E+07	3.59E+07	2.60E+07	
KVQLVEEDLER	2.40E+07	6.64E+08	2.40E+07	6.64E+08	A0A4P8D324
GLSPDQVK	4.85E+06	2.12E+05	4.85E+06	2.12E+05	A0A385MDB2
EVDRLEDELVIEKEK	2.36E+07	4.04E+07	2.36E+07	4.04E+07	A0A2P1ANK6
KIQTIENELDQTQEQLMQVNAK	4.47E+07	4.41E+07	4.47E+07	4.41E+07	A0A2P1ANK6:A0A4V1DVH3
FLAEEADKKYDEVAR	8.77E+07	4.31E+08	8.77E+07	4.31E+08	A0A4V1DVH3
KLAM(+15.99)VEADLER	2.72E+07	3.88E+07	2.72E+07	3.88E+07	A0A2P1ANK6:A0A4V1DVH3
M(+15.99)DALENQLK	4.10E+07	7.18E+07	4.10E+07	7.18E+07	A0A2P1ANK6:A0A4V1DVH3
KLAQVENDLITTK	8.82E+06	1.13E+08	8.82E+06	1.13E+08	A0A4P8D324
KLAMVEADLER	8.57E+07	7.48E+08	8.57E+07	7.48E+08	A0A2P1ANK6:A0A4V1DVH3
RIQLLEEDLER	3.66E+07	1.79E+08	3.66E+07	1.79E+08	A0A2P1ANK6:A0A4V1DVH3
LAFVEDELEVAEDRVK	1.22E+07	1.89E+08	1.22E+07	1.89E+08	A0A4P8D324
IMELEEELK	4.71E+07	1.69E+09	4.71E+07	1.69E+09	A0A4P8D324
M(+15.99)DQLTNQLK	5.40E+06	5.50E+07	5.40E+06	5.50E+07	A0A4P8D324
ADKINEDVQELTKK	3.46E+06	8.71E+07	3.46E+06	8.71E+07	A0A4P8D324
IVELEEELR	4.31E+08	2.46E+09	4.31E+08	2.46E+09	A0A2P1ANK6:A0A4V1DVH3
LAM(+15.99)VEADLER	1.36E+08	2.22E+08	1.36E+08	2.22E+08	A0A2P1ANK6:A0A4V1DVH3
KVQLVEEDLERSEER	2.37E+06	4.57E+07	2.37E+06	4.57E+07	A0A4P8D324
EVDRLEDELVIEK	2.66E+08	1.27E+09	2.66E+08	1.27E+09	A0A2P1ANK6
KLAFVEDELEVAEDR	1.37E+06	2.78E+07	1.37E+06	2.78E+07	A0A4P8D324

Table S5.3 (B). De novo peptide sequences identified in tropomyosin extracts from WB-CPH					
Peptide	ALC (%)	length	Area	Mass	Accession Number
KAHEDYEAK	99	9	1.13E+09	1089.509	
KLC (+57.02)DDLHDAK	99	10	1.13E+09	1213.5762	
RLVEK	98	5	1.08E+09	643.4017	
RLYDK	98	5	8.79E+08	693.3809	
RLLEER	98	6	8.79E+08	814.4661	A0A2P1ANL5
RLEDDER	98	7	6.86E+08	931.4359	
RLENER	98	6	5.48E+08	815.4249	
KLLAEDADGK	98	10	5.32E+08	1058.5608	A0A2P1ANK0:A0A4P8D324
RLEAER	97	6	5.32E+08	772.4191	
RVESLEK	97	7	5.32E+08	859.4763	
RDTELTK	97	7	5.32E+08	861.4556	
HSDLDELLNEAK	97	12	5.32E+08	1382.6677	
RLDENER	97	7	4.21E+08	930.4518	
HWVR	97	4	3.40E+08	596.3183	
KDELK	97	5	2.96E+08	631.3541	
HLVLR	97	5	2.96E+08	636.4071	
RLDNDAEAK	97	9	2.26E+08	1030.5043	
RQLER	97	5	2.26E+08	700.398	
KVVEAETK	97	8	2.26E+08	902.5073	
KQQLK	97	5	2.26E+08	643.4017	
RLYDR	97	5	2.17E+08	721.3871	
RELVR	96	5	2.17E+08	671.4078	
LTHELDK	96	7	2.17E+08	854.4498	
RLEEVEGQR	96	9	2.17E+08	1114.573	
RLEDDER	96	7	2.17E+08	931.4359	
RLEDDER	96	7	1.68E+08	931.4359	
HYDEVQR	96	7	1.68E+08	945.4304	

REAELSK	96	7	1.58E+08	831.445	
KTFDNDGK	95	8	1.58E+08	923.4348	
LHEEQER	95	7	1.58E+08	939.441	
RLEDDERR	95	8	1.58E+08	1087.5369	
QVDHLGNEK	95	9	1.54E+08	1038.5094	
KLEEQR	95	6	1.38E+08	801.4344	
RLEVER	95	6	1.34E+08	800.4504	
HWVR	95	4	1.29E+08	596.3183	
RVLSK	95	5	1.27E+08	601.3911	
RDTELTK	94	7	1.21E+08	861.4556	
RQELDRR	94	7	1.21E+08	971.5261	
HYDEVQR	94	7	1.21E+08	945.4304	
THELDK	94	6	1.10E+08	741.3657	
RLDLQR	93	6	1.01E+08	799.4664	
LEDDERR	93	7	9.94E+07	931.4359	
RYQQQLK	93	7	9.78E+07	962.5297	A0A2P1ANK6
KDEK	93	4	9.54E+07	518.27	
RELLR	93	5	9.07E+07	685.4235	
KAEEEALR	92	8	9.07E+07	944.4927	A0A2P1ANK6:A0A4V1DVH3
TKQDDDVYR	92	9	9.07E+07	1138.5254	
KEAEAR	92	6	8.11E+07	702.366	A0A2P1ANK0:A0A2P1ANK6
RREEMAR	91	7	7.47E+07	946.4766	
K(+42.01)LLR	91	4	7.38E+07	570.3853	
KLLR	91	4	6.14E+07	528.3748	
RLLDEQNAR	91	9	5.68E+07	1113.5891	
KNQLELELDEEK	91	12	5.68E+07	1486.7515	
YDEVQR	91	6	5.39E+07	808.3715	
RLHELK	90	6	5.26E+07	794.4763	
SDDLLK	90	6	4.13E+07	689.3596	
S(+42.01)EELR	90	5	3.69E+07	674.3235	

VHLAK	90	5	3.53E+07	566.354	
HAETLK	90	6	3.48E+07	697.3759	
TNMEDDR	90	7	3.21E+07	879.3392	
YVFR	90	4	3.21E+07	583.3118	
C(+57.02)LSSSVR	90	7	3.21E+07	807.3909	
QTELQR	90	6	3.14E+07	773.4031	
RLEDDERR	89	8	3.01E+07	1087.5369	
KALETQLK	89	8	2.98E+07	929.5546	
LADEER	89	6	2.80E+07	731.345	A0A2P1ANK6:A0A4V1DVH3
LLEER	89	5	2.80E+07	658.3649	
S(+42.01)LVHEK	89	6	2.74E+07	753.4021	
EADLERAEER	89	10	2.34E+07	1216.5684	A0A2P1ANK6:A0A4V1DVH3
RLMDDNEVLK	89	10	2.34E+07	1231.623	
C(+57.02)NDLTTK	89	7	2.23E+07	850.3854	
TYLGGK	89	6	2.23E+07	637.3435	
RELLK	89	5	2.23E+07	657.4173	
KLC(+57.02)DDLHDAK	89	10	2.23E+07	1213.5762	
TLSVK	89	5	2.23E+07	546.3377	
HTVELK	89	6	2.07E+07	725.4072	
KNQLELELDEEK	88	12	1.88E+07	1486.7515	
LADEER	88	6	1.88E+07	731.345	A0A2P1ANK6:A0A4V1DVH3
ELELELR	88	7	1.88E+07	900.4916	
HTVELLHEEQER	88	12	1.75E+07	1518.7427	
HTVELR	88	6	1.75E+07	753.4133	
QEELR	88	5	1.75E+07	673.3395	
YEEVAR	88	6	1.73E+07	765.3657	
LDALK	88	5	1.69E+07	558.3377	
LHEPR	88	5	1.65E+07	650.35	
ELER	88	4	1.59E+07	545.2809	
HADVLAR	88	7	1.55E+07	780.4242	

SVLR	88	4	1.55E+07	473.2962	
HPHPEDC(+57.02)R	87	8	1.55E+07	1046.4353	
VVEAETK	87	7	1.54E+07	774.4123	
S(+42.01)LEEELK	87	7	1.43E+07	888.444	A0A2P1ANK0:A0A4P8D324
TNMEDDR	87	7	1.40E+07	879.3392	
RLEHLK	87	6	1.34E+07	794.4763	
K(+42.01)DVLR	87	5	1.28E+07	671.3966	
QLGLAQR	87	7	1.28E+07	784.4555	
RLLENR	87	6	1.27E+07	799.4664	
RLENEER	87	7	1.27E+07	944.4675	
RLEDELQGLR	87	10	1.24E+07	1227.657	A0A2P1ANK6
TGEALLR	87	7	1.24E+07	758.4286	
EEEYK	87	5	1.24E+07	696.2966	
TLSVK	87	5	1.24E+07	546.3377	
HLEQENSTLR	87	10	1.23E+07	1225.605	
QGRLEQANK	87	9	1.23E+07	1042.552	A0A2P1ANK0:A0A4P8D324
C(+57.02)DFTR	87	5	1.08E+07	697.2853	
KLAMVER	87	7	1.03E+07	845.4793	A0A2P1ANK6:A0A4V1DVH3
KVNNLEK	87	7	9.94E+06	843.4814	
KEAEAR	87	6	9.94E+06	702.366	A0A2P1ANK0:A0A2P1ANK6
WDVPEK	86	6	9.73E+06	772.3755	
YDEVAR	86	6	9.59E+06	751.35	A0A2P1ANK6:A0A4V1DVH3
RLEDDER	86	7	9.29E+06	931.4359	
LMEHPVDDDLLK	86	12	8.95E+06	1423.7017	
HAYDEVQR	86	8	8.62E+06	1016.4675	
FAER	86	4	8.28E+06	521.2598	
RVLSK	86	5	8.24E+06	601.3911	
KMQAMK	86	6	8.08E+06	735.3771	A0A2P1ANK0:A0A4P8D324
HLTELSK	86	7	8.04E+06	826.4548	
S(+42.01)ADLER	86	6	8.01E+06	731.345	

DLDRLEDELGLNK	86	13	7.84E+06	1528.7732	A0A2P1ANK6
AEFAER	86	6	7.84E+06	721.3395	A0A2P1ANK6
LLEER	86	5	7.79E+06	658.3649	
NEFAER	86	6	7.79E+06	764.3453	
REAELSK	86	7	7.49E+06	831.445	
HETAEGVR	86	8	7.44E+06	897.4304	
S(+42.01)LALDHANK	86	9	6.98E+06	1009.5192	
KNDTYDKEK	85	9	6.98E+06	1139.5459	
VHWVR	85	5	6.92E+06	695.3867	
LQEQEEHLEALVVK	85	14	6.79E+06	1663.8779	
TREVVSEEK	85	9	6.33E+06	1075.551	
ESGELPR	85	7	6.22E+06	786.3871	
TLDLR	85	5	6.01E+06	616.3544	
HEAER	85	5	5.79E+06	640.2928	
QDLLK	85	5	5.74E+06	615.3591	
AEFAER	85	6	5.60E+06	721.3395	A0A2P1ANK6
LLDELK	85	6	5.29E+06	729.4272	
KSLEVEVK	85	8	4.76E+06	930.5386	
KGQNLEYTK	85	9	4.63E+06	1079.561	
QLTER	85	5	4.60E+06	645.3445	
YLHR	85	4	4.40E+06	587.3179	
NLSVR	85	5	4.28E+06	587.3391	
LELR	85	4	4.23E+06	529.3224	
KYTAR	85	5	4.08E+06	637.3547	
S(+42.01)MLEEELK	84	8	3.94E+06	1019.4845	A0A2P1ANK0:A0A4P8D324
RLENEEER	84	8	3.93E+06	1073.51	
S(+42.01)LLR	84	4	3.86E+06	529.3224	
TTLEATLHQVENK	84	13	3.82E+06	1482.7678	
LTDSATR	84	7	3.75E+06	762.3871	
VEEYRK	84	6	3.56E+06	822.4235	

TTLEAK	84	6	3.53E+06	661.3646	
S(+42.01)EYKR	84	5	3.52E+06	723.3552	
KLQK	84	4	3.28E+06	515.3431	
ELELELR	84	7	3.28E+06	900.4916	
SDEVSR	84	6	3.22E+06	691.3137	A0A2P1ANK0:A0A4P8D324
S(+42.01)AEAR	84	5	3.20E+06	574.2711	
LELR	84	4	2.83E+06	529.3224	
GHDLHHPR	84	8	2.62E+06	967.4736	
TVDLPK	84	6	2.55E+06	671.3854	
YDEVAR	84	6	2.34E+06	751.35	A0A2P1ANK6:A0A4V1DVH3
ELELELR	84	7	2.32E+06	900.4916	
YELLR	83	5	2.32E+06	692.3857	
THSLLD	83	6	2.09E+06	684.3442	
RLEDELVHEK	83	10	2.03E+06	1266.6567	A0A2P1ANK6
LADLLK	83	6	1.84E+06	671.4218	
S(+42.01)EYKR	83	5	1.82E+06	723.3552	
ELVHEK	83	6	1.81E+06	753.4021	
S(+42.01)LLEELR	83	7	1.70E+06	900.4916	
KQVEDLR	83	7	1.68E+06	886.4872	
TGAHS(+79.97)R	83	6	1.68E+06	707.2752	
LLDLEK	83	6	1.67E+06	729.4272	
QLREEAEELVR	83	11	1.58E+06	1370.7153	
S(+42.01)HPVSDDLLK	83	10	1.52E+06	1151.5823	A0A2P1ANK3
TLHQLD	83	6	1.51E+06	725.3708	
TLSVK	83	5	1.43E+06	546.3377	
KLC(+57.02)DDLHD	83	8	1.42E+06	1014.444	
HAAWC(+57.02)R	83	6	1.42E+06	799.3548	
TGGAAEALAD	83	10	1.38E+06	874.4032	
EERLSFK	83	7	1.25E+06	907.4763	
S(+42.01)AEEAR	83	6	1.17E+06	703.3137	

S(+42.01)VELELDEEK	83	10	1.15E+06	1231.582	
S(+42.01)LQREAEELVR	83	11	1.15E+06	1370.7153	
A(+42.01)DDLPPEQK	82	9	1.10E+06	1053.4978	A0A2P1ANK3:A0A385MCP5
A(+42.01)DDLPPEQK	82	9	1.03E+06	1053.4978	A0A2P1ANK3:A0A385MCP5
GVTHELDK	82	8	1.02E+06	897.4556	
KFLC(+57.02)D	82	5	1.02E+06	681.3156	
T(+42.01)EALDRGDELGLNK	82	14	9.88E+05	1571.7791	
RLHELELELR	82	10	9.77E+05	1306.7356	
KLDELK	82	6	9.74E+05	744.4381	
EDLRDLDELVHEK	82	13	9.33E+05	1609.7947	
TGYYVEK	82	7	9.07E+05	858.4123	
NQLSDLTR	82	8	8.98E+05	945.488	
FSC(+57.02)EK	82	5	8.92E+05	669.2792	
WDVPEK	82	6	8.10E+05	772.3755	
VHWVR	82	5	8.02E+05	695.3867	
LLDLEK	82	6	7.93E+05	729.4272	
VEDLK	82	5	7.91E+05	602.3275	
ELELELR	82	7	7.71E+05	900.4916	
S(+42.01)EYKR	82	5	7.26E+05	723.3552	
RLHELELELR	82	10	7.12E+05	1306.7356	
HQELC(+57.02)PK	82	7	7.03E+05	910.433	
S(+42.01)VELELDEEK	82	10	6.97E+05	1231.582	
HYDEVQR	82	7	6.80E+05	945.4304	
VEEVEDRLR	82	9	6.76E+05	1143.5884	
S(+42.01)VELELDEEK	82	10	6.58E+05	1231.582	
P(+42.01)ADRLED	82	7	6.47E+05	856.3926	
RLHELELELR	82	10	6.17E+05	1306.7356	
LYDR	82	4	5.90E+05	565.286	
LLDLEK	82	6	5.58E+05	729.4272	
TLSVK	81	5	5.40E+05	546.3377	

LEELR	81	5	5.28E+05	658.3649	
A(+42.01)DDLPPEQK	81	9	5.18E+05	1053.4978	A0A2P1ANK3:A0A385MCP5
GHDLHHPR	81	8	5.18E+05	967.4736	
S(+42.01)DLVHEK	81	7	4.99E+05	868.429	
NALLNK	81	6	4.97E+05	671.3966	
VEEYRK	81	6	4.80E+05	822.4235	
KALQAAESEMVR	81	12	4.72E+05	1331.6868	A0A2P1ANK0:A0A4P8D324
HTVELLHEEQER	81	12	4.43E+05	1518.7427	
EVERVEDELVHEK	81	13	4.19E+05	1609.7947	
A(+42.01)DDLPPEQK	81	9	4.02E+05	1053.4978	A0A2P1ANK3:A0A385MCP5
YEFR	81	4	3.80E+05	613.2859	
RLEEDHER	81	8	3.76E+05	1082.5105	
TARLEDSYK	81	9	3.22E+05	1081.5403	
MQAMK	81	5	3.12E+05	607.2822	
LLDELK	81	6	3.09E+05	729.4272	
S(+42.01)VEDEEAWLR	81	10	2.93E+05	1274.5779	
KVLENR	81	6	2.91E+05	757.4446	A0A2P1ANK0:A0A4P8D324
KDHTLEDNLFNDK	81	13	2.72E+05	1587.7529	A0A2P1ANK0
AAEELK	81	6	2.65E+05	659.3489	A0A2P1ANL5
TYLK	81	4	2.30E+05	523.3006	
EVEDRLR	81	7	2.28E+05	915.4774	
NSYESVLR	81	8	2.27E+05	966.4771	
TLSVK	81	5	2.24E+05	546.3377	
TLS(+79.97)FEK	81	6	2.23E+05	803.3466	
RLHELELELR	81	10	2.23E+05	1306.7356	
S(+42.01)LEELK	81	6	2.05E+05	759.4014	
LVLR	81	4	1.91E+05	499.3482	
LDELR	81	5	1.75E+05	644.3493	
LYKEAEDGK	81	9	1.63E+05	1051.5186	
HFDKDR	81	6	1.63E+05	816.3878	

HSDLDELLNEAK	81	12	1.63E+05	1382.6677	
SWTR	81	4	1.59E+05	548.2707	
LEEELR	80	6	1.56E+05	787.4075	A0A2P1ANK6:A0A4V1DVH3
NSYESVLR	80	8	1.44E+05	966.4771	
EDELVHEK	80	8	1.03E+05	997.4716	
Q(+42.01)LSREAEELVR	80	11	9.76E+04	1370.7153	
LHEPR	80	5	9.75E+04	650.35	
V(+42.01)TDVRELLDEEK	80	12	6.95E+04	1486.7515	
REQLDRR	80	7		971.5261	
KLLAEEADK	80	9		1015.5549	A0A2P1ANK6:A0A4V1DVH3
LLENR	80	5		643.3653	
RLVEKDEELDALR	80	13		1584.8472	
S(+42.01)VELELDEEK	80	10		1231.582	
KLLGDDLD	80	8		887.46	A0A2P1ANK6
LKDELK	80	6		744.4381	
QVSLGGEDVQNK	80	12		1272.6309	
NQELTETR	80	8		989.4778	
LADELR	80	6		715.3864	
FNDKEK	80	6		779.3813	
EFEC(+57.02)DK	80	6		826.3167	
LLRPR	80	5		653.4337	
FDAFDR	80	6		769.3395	A0A2P1ANK3:A0A385MCP5
LADEER	80	6		731.345	A0A2P1ANK6:A0A4V1DVH3
RRLEDDER	80	8		1087.5369	
S(+42.01)AAAAAEAEAAR	80	12		1129.5364	A0A0P0QKK0

CHAPTER 6. IDENTIFICATION AND CHARACTERIZATION OF EDIBLE CRICKET PEPTIDES ON HYPERTENSIVE AND GLYCEMIC IN VITRO INHIBITION AND TEIR INFLAMMATORY EFFECT ON RAW 264.7 MACROPHAGE CELLS

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Abstract

Recent studies continue to demonstrate the potential of edible insects as a protein base to obtain bioactive peptides applicable for functional food development. This study aimed at identifying antihypertensive, anti-glycemic and anti-inflammatory peptides derived from the *in vitro* gastrointestinal digests of cricket protein hydrolysates. After sequential fractionation, the protein digest subfraction containing the lowest molecular weight (<0.5 kDa), hydrophobic (C18) and cationic peptides (IEX) was found responsible for the most bioactivity. The cationic peptide fraction significantly reduced ($p<0.05$) α-amylase, α-glucosidase, and angiotensin converting enzyme (ACE) activity in vitro, as well as inhibited the expression of NF-κB in RAW 264.7 macrophage cells. A total of 28 peptides were identified with mass spectrometry (LC-MS/MS) and de novo sequencing from the potent fraction. Three novel peptides YKPRP, PHGAP, and VGPPQ were chosen for molecular docking studies. PHGAP and VGPPQ exhibited a higher degree of non-covalent interactions with the enzyme active site residues and binding energies comparable to captopril. Results from this study demonstrate the bioactive potential of edible cricket peptides, especially as ACE inhibitors.

6.1 Introduction

Cardiovascular diseases (CVD) are currently the number one cause of death globally, estimating about 18 million lives per year. Diets high in saturated fats and sugar are a well-established risk factor in coronary heart disease since these pro-inflammatory diets are linked to other significant risk factors such as obesity, hypertension, and diabetes (Estruch et al., 2006). Hence, anti-inflammatory diets are recommended to prevent, treat, and manage CVD and its

related risk factors (Estruch et al., 2006). While fruits and vegetables are undoubtedly associated with preventing CVD risk factors, food proteins and peptides with biological activities have also been identified, suggesting their potential use as nutraceuticals and functional food ingredients for health promotion (Udenigwe & Aluko, 2012).

In recent years, consumption of edible insects has increased in western cultures as a response to demand for alternative, more sustainable and eco-friendly resources. The application of insect protein in food formulation has been widely reported in literature (Çabuk & Yılmaz, 2020; Hall, Jones, O'Haire, & Liceaga, 2017; Luna, Martin-Gonzalez, Mauer, & Liceaga, 2020) However, interest is emerging on the potential of protein derived from insects as sources of bioactive agents. Studies are continuously demonstrating various biologically active properties such as antidiabetic, antihypertensive, antioxidant, and anti-inflammatory properties (Hall, Johnson, & Liceaga, 2018; Vercruysse, Smagghe, Matsui, & Van Camp, 2008; Zielińska, Baraniak, & Karaś, 2017). Typically, the insect-derived protein components are in the form of extracts (i.e. water extracts, protein hydrolysates, or buffer extracts) and in other cases isolated/purified peptide or peptide mixtures. Various cricket protein preparations have exhibited multiple bioactive properties *in vitro*, yet enzymatic hydrolysis stands as one of the more effective methods used to liberate active peptides (Nongonierma & FitzGerald, 2017). We speculate that the active polypeptide fractions of insects are more readily available after proteolysis, as commonly seen with other protein derived biopeptides (Korhonen & Pihlanto, 2003, 2006). The protein hydrolysate is typically free of the insect body (i.e., exoskeleton) that contains high amounts of proteins but are complexed with the chitin polymerized matrix. For example, Vercruysse et al. (Vercruysse, Smagghe, Beckers, & Van Camp, 2009) reported that enzymatic hydrolysis was necessary to release bioactive peptides with ACE inhibitory activity from cotton leafworm *(Spodoptera littoralis*). Gastrointestinal digestion of the cotton leafworm protein hydrolysates, using pepsin, trypsin and chymotrypsin, improved the IC_{50} values significantly from 73 mg/mL to 0.7 mg/mL, representing high activity. The potency of the ACE inhibitory peptides (Ala-Val-Phe) from the protein hydrolysate was confirmed to have antihypertensive activity *in vivo* (Vercruysse et al., 2010).

In our own work, we confirmed that chitin was successfully removed when developing tropical banded cricket protein hydrolysates. In addition, these hydrolysates demonstrated better *in vitro* antioxidant, ACE and DPP-IV inhibiting activity, compared with the non-hydrolyzed cricket protein; this activity increased further after simulated gastrointestinal digestion (Hall et al.,

2018). Increased hydrolysis time showed the best bioactivity overall, owing to smaller molecular weight peptides. The present study aims to identify and characterize the bioactive peptides in cricket protein responsible for the ACE and DPP-IV inhibiting activity, while we also evaluate their anti-inflammatory activity on RAW 264.7 macrophage cells. Finally, we used *in silico* analysis to help elucidate the structural interactions between these bioactive peptides and ACE.

6.2 Materials and Methods

6.2.1 Materials

Unless specified, all chemical reagents were of analytical grade and obtained from suppliers: MilleporeSigma (St. Louis, MO, USA) and ThermoFisher Scientific (Waltham, MA, USA). Alcalase® (*Bacillus licheniformis*, 2.4 U/g), The Renin Inhibitor Screening Assay Kit, Human Dipeptidyl Peptidase IV (DPP-IV, ≥4500 units/µg protein) and substrate Gly-Pro p-nitroanilide hydrochloride, Angiotensin Converting Enzyme (ACE) from rabbit lung and substrate Hippuryl-L-Histidyl-L-Leucine (HHL), α-Amylase (Type VI-B, ≥5 units/mg) and Rat intestinal acetone powder (α-Glucosidase), were all purchased from MilleporeSigma (St. Louis, MO, USA). Whole, frozen, unpasteurized, adult (6 weeks old) tropical banded crickets (*Gryllodes sigillatus*) were purchased from Entomo Farms (Norwood, ON, Canada). Raw 264.7 macrophage cells were obtained from Novus Biologicals (Littleton, CO) whereas, Renilla Luciferase Activity Bioluminescence Detection Kit was purchased from Promega Corporation (Madison, WI, USA).

6.2.2 Cricket protein hydrolysate preparation

Cricket protein hydrolysates (CPH) and its digests were produced as previously reported (Hall et al., 2018). Briefly, crickets were hydrolyzed with alcalase (3.0% E/S) under controlled convection heating for 80 min at 55ºC followed by heating 15 min at 95ºC to terminate proteolysis. After centrifugation, supernatants (30 mg/mL) were subject to a simulated gastrointestinal digestion (SGID) as reported by You et al (You, Zhao, Regenstein, & Ren, 2010) with modifications (Ketnawa, Wickramathilaka, & Liceaga, 2018). SGID was initiated with the gastric phase with pepsin (4% w/w of protein) then intestinal digestion phase with bile salts (10 mg/mL) and pancreatin (4% w/w protein). Both phases were incubated at the enzyme pH optima in a water bath

with continuous shaking. Supernatants after filtration were collected, referred to as CPH-digests (CPHD), freeze dried, and stored at −20 °C until further use.

6.2.3 Molecular weight distribution of CPH and CPHD

The molecular weight distribution of CPH before and after *in vitro* digestion (CPHD) were evaluated following a described protocol (Ketnawa et al., 2018). Peptides were separated with Superdex™ Peptides 10/300 GL column (GE Healthcare Bio-Science AB, Uppsala, Sweden) on a Waters e2695 HPLC system equipped with a PDA detector (Waters Co., Milford, MA, USA). The fractionation ranged from 1000-7000 kDa. Briefly, samples (5 mg/mL) were filtered (0.45 μm) before injection (50 μL) at a flow rate of 0.5 mL/min with 100 mM sodium phosphate buffer (pH 7.0). Molecular weight standards were used for molecular weight calculation and ran with the same conditions, including bovine serum albumin (66,400 Da), aprotinin (6511 Da), vitamin B12 (1355 Da), cytidine (243 Da), dl-dithiotheritol (154 Da), and glycine (75 Da). Plots of retention time for molecular weight standards were used to construct the calibration curve, from which molecular distributions were computed. The logarithm of molecular weight (lg MW) and the retention time (RT) were in a linear relationship and the formula was calculated as $Rt = -0.2094$ (lg MW) + 1.2747 ($R2 = 0.9867$, $P < 0.05$).

6.2.4 CPHD peptide fractionation

Size exclusion chromatography (SEC)

The gastrointestinal digest was re-dissolved in 10 mg/mL elution buffer (20 mM sodium phosphate buffer with 300 mM NaCl, pH 7.3) and filtered (0.45 μm) before applied to a gel filtration Superdex® Peptide 10/300 GL (30 cm × 10 mm, 13 μm, GE Healthcare Life Sciences, Chicago, IL, USA). Elution was carried at room temperature on a Waters 2690 HPLC system (Waters Corporation, Milford, USA) equipped with an automatic sample injector and 2998 UV Photodiode array (PDA) detector at a flow rate of 0.5 mL/min. The absorbance of each fraction was measured at a wavelength of 214 nm. After collection, fractions were concentrated, reconstituted in assay buffers, then bioactivities measured as described above.

Reverse-phased high-performance liquid chromatography (RP-HPLC)

The fraction with the strongest overall *in vitro* bioactivity after SEC separation was further purified using preparative PH-HPLC on a XBridge^{TM} BEH130 pre C18 column (10 µm, 10 x 150 mm, Waters Inc., Milford, MA, USA), at a flow rate of 1 mL/min. Eluent A: Water/0.1% TFA; eluent B: Acetonitrile/01% TFA. After 50µL of sample (10 mg/mL) was injected into the C18 column, concentrations of eluent B were increasing as: 0–40 min, 0–100% (v/v); 40–50 min, 100% (v/v). Eluents were monitored at 218 nm, fractions collected (F1 to F6), concentrated and bioactivities measured as described above.

Ion exchange chromatography (IEX)

The most potent fraction (F6) from RP-HPLC was loaded onto a HiTrap SP HP cation exchange chromatography column (34 µm; GE Healthcare Life Sciences) with a flow rate of 1 mL/min. The sample was sequentially eluted with Citrate Buffer (pH 4.3) with 0–1 M NaCl and both anion and cationic fractions collected. Fractions were desalted and concentrated using Pierce™ C18 Tips (100 µL) following the manufactures' protocol (ThermoFisher Scientific, Walthman, MA, USA). The most potent fraction for the *in vitro* bioactivities, described above, was assessed for protein sequencing.

6.2.5 Anti-glycemic, anti-hypertensive, and anti-inflammatory assays

Dipeptidyl peptidase-IV (DPP-IV) inhibition

DPP-IV inhibiting activity was determined following the method described by (Lacroix & Li-Chan, 2012) with some modifications (F. Hall et al., 2018). Samples were reconstituted in 100 mM Tris-HCl buffer (pH 8.0). In a 96-well plate, 25 µL of sample solution was pre-incubated with 25 µL of substrate Gly-Pro p-nitroanilide hydrochloride (6 mM) at 37°C for 10 min. To initiate the reaction, 50 µL of human DPP-IV (4.5 unit/ml) was added and incubated at 37°C for 1 h. The absorbance of liberated p-nitroanilide was measured at 405 nm. Ile-Pro-Ile, DPP-IV inhibitor, was used as a reference following the same procedure. Positive control used buffer in place of inhibitor while the negative control used buffer in place of DPP-IV.

Alpha-amylase inhibition

The inhibition α-amylase activity was assayed using soluble starch as substrate according to the modified procedure by Awosika and Aluko (Awosika & Aluko, 2019). Samples were dissolved in 1 mL of 20 mM sodium phosphate buffer (pH 6.9) containing 6 mM NaCl. Briefly, 100 µL aliquot of each sample and 100 µL of α-amylase solution (1mg/mL) were added to test tubes and allowed to incubate for 10 min at 25°C. After incubation, 100 µL of a 1 g/100 mL starch solution (dissolved in the above buffer) was added and incubated at 25°C for another 10 min. The reaction was terminated by adding 200 µL of dinitrosalicylic acid (DNSA) color reagent (96 mM DNSA, 2 M sodium potassium tartrate tetrahydrate and 2 M NaOH) followed by incubation in a boiling water bath at 100°C for 5 min. Reactions were diluted with double distilled water (3 mL) then enzyme activity was quantified by measuring the maltose equivalents released from starch at 540 nm. A blank reading (buffer in place of enzyme) was subtracted from each absorbance. A pharmacological α-amylase inhibitor (acarbose) was assayed using the same protocol and used as a positive control.

Alpha-glucosidase inhibition

Inhibition of α-glucosidase was assayed according to previously described methods (Shobana, Sreerama, & Malleshi, 2009) with modifications (Awosika & Aluko, 2019). The enzyme solution was prepared by homogenizing rat intestinal acetone powder (300 mg) in 9 mL of 0.9% (w/v) NaCl solution, centrifuged and filtered. The reaction mixture consisted of substrate p-nitrophenyl-α-D-glucopyranoside (25 µL, 10 mM), PBS (pH 6.9, 25 µL, 0.1 M) and sample (50 µL) pre-incubated at 37°C for 10 min in a 96-well plate. Reaction was initiated by adding rat intestinal α-glucosidase solution (50 µL; 1.0 U/mL) and incubated at 37°C for 30 min. The absorbance of p-nitrophenyl release was measured at 405 nm. Acarbose was assayed with the same procedure and used as a positive control. Positive control used buffer in place of inhibitor while the negative control used buffer in place of α-glucosidase.

Angiotensin converting enzyme II inhibition

ACE-inhibitory activity was measured using the method described by (Martínez-Alvarez, Batista, Ramos, & Montero, 2016) with modifications described (Hall et al., 2018). 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. Samples (25 µL) were pre-incubated with 25 µL of substrate (HHL) for 4 min at 37 °C then 80 µL of ACE (5 mU) was added incubated at 37 °C for 120 min. The reaction was terminated with 50 µL of 1 M HCl then solution filtered with 0.22 µm nylon filters. The positive control reaction contained 25 µL buffer in place of inhibitor (CPH). The amount product HA were quantified by HPLC (Model 2690, Waters Corporation, Milford MA) on an analytical C18 column XBridge^{TM} BEH130 C18 column.

RAW 264.7-NF-κ-B cell culture and cell viability

The NF-κB luciferase reporter cell line (Novus Biologicals) was cultured in DMEM supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 3 µg/mL puromycin. All cell lines were maintained at 37°C with 5% CO_2. Cell viability was estimated by the MTT assay as described previously (P. Kumar, Nagarajan, & Uchil, 2018). Cells (3×10^4/well) were seeded in 96-well plates and treated with various sample concentrations (in phosphate buffered saline (PBS). After incubation for 24 hours, a 20-µL aliquot of MTT solution (5 mg/mL in PBS) was added to each well and incubated for a further 4 hours at 37°C. The purple formazan was diluted with 150 µL dimethyl sulfoxide. The absorbance was measured at 490 nm using a microplate reader. The percentage of viable cells was calculated with respect to the cells treated with phosphate-buffered saline (PBS).

Luciferase Assay

The NF-κB Luciferase reporter RAW 264.7 cell line (Novus Biologicals), which expresses an optimized Renilla luciferase reporter gene (RenSP) under the transcriptional control of an NF-κB response element, was used. The cells were seeded (2×10^5 cells/well) into 24-well plates for 16 hr, pretreated with the sample for 1 hr and then infected with Lipopolysaccharides (LPS; 500 ng; E. coli Serotype R515, Re, TLR grade) for 6 hr. Media from each well were aspirated, and then 100 µL PBS was added to each well. Luciferase assays were performed using the Promega Renilla Luciferase detection assay kit following the manufactures' protocol. Luminescence was measured as the relative luminescence units (RLU) using Spectramax (Spectramax, Molecular Devices). Control cells that were treated with media alone.

6.2.6 Identification of cationic peptides and molecular docking

Sample preparation, mass spectrometry analysis, bioinformatics and data evaluation were performed in collaboration with the Proteomics Core Facility at the Indiana University School of. Methods are described in literature reports published elsewhere (Mosley, Florens, Wen, & Washburn, 2009) and vendor provided protocols. Samples were analyzed using a 5 cm trap column and 15 cm (2 µm particle size, 50 µm diameter) EasySpray (801A) column on an UltiMate 3000 HPLC and Q-Exactive Plus (Thermo) mass spectrometer. Data analysis, including de novo were performed using PEAKS software (Bioinformatics Solutions), with Q-Exactive. From the generated peptides, only peptide with ALC > 80% were chosen to proceed to the following step. The peptide list was searched against the BIOPEP-UWM Database of Bioactive Peptides (Minkiewicz, Iwaniak, & Darewicz, 2019). Antihypertensive (AHT) prediction and amphilicity scores were generated by AHTPin (R. Kumar et al., 2015) in silico prediction algorithm of antihypertensive peptides (**Supp. Table. S2**). Molecular docking was performed using Accelrys Discovery Studio software and AutoDock Vina as previously described (Amaya, Cabrera, Matallana, Arevalo, & Guevara-Pulido, 2020). Among all identified peptides, all were predicted as antihypertensive peptides and were docked against ACE, along with the inhibitor drug captopril. For molecular docking, the crystal structure of human ACE bound with Lisinopril (PDB: 108A) was used. Before docking, Lisinopril and water molecules removed, polar hydrogen atoms added in the presence of cofactors (zinc and chloride ions). A binding site with a radius of 15 A and coordinates x: 41.58, y: 37.374, and z: 43.47 was created. All generated docking modes were evaluated according to affinity energy values.

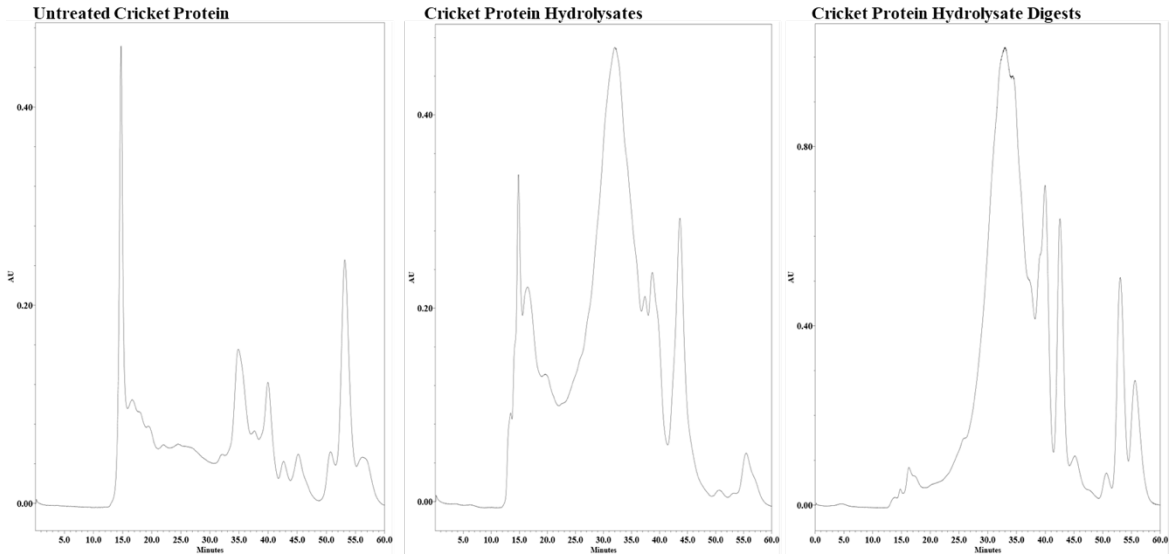
6.2.7 Statistical Analysis

All data are presented as mean ± SD from 4 to 8 independent experiments. Data analysis were performed by one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test using the PRISM 6 statistical software (Graph Pad Software, San Diego, CA). $P < 0.05$ was considered to be significant.

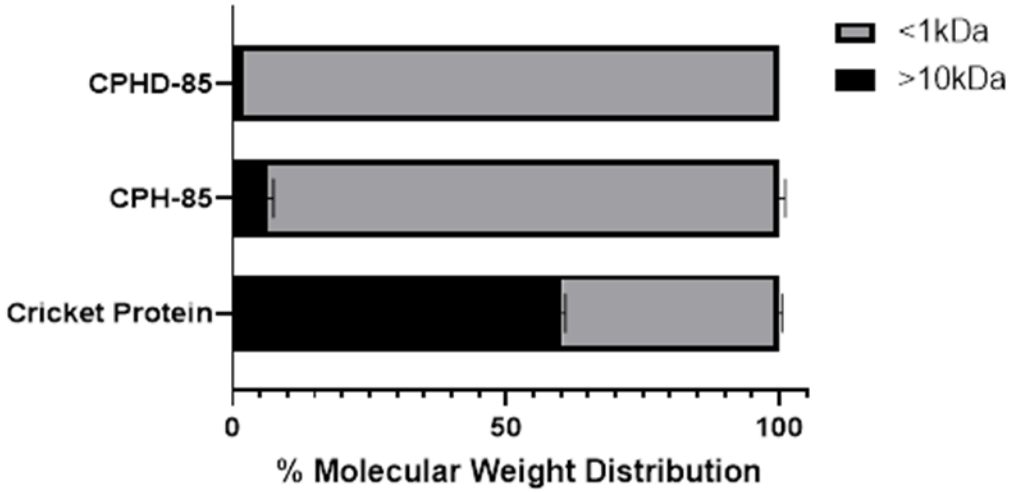
6.3 Results

6.3.1 Molecular weight distribution of CPH and CPHD

After controlled proteolysis with the commercial enzyme alcalase, the subsequent cricket protein hydrolysate (CPH) was subjected to *in vitro* simulated gastrointestinal digestion (SGID). Proteolysis with aclalase, followed by both pepsin and pancreatin generated a complex mixture of peptide containing digests (CPHD). Before enzyme treatment, more than 50% of the native cricket proteins were above 10 kDa (**Figure 6.1 B**). The alcalase treatment (3.0% E: S; 80 min) resulted in CPH consisting mostly of peptides below 1 kDa (**Figure 6.1 A**). After SGID, the composition of peptides compared with native proteins increased slightly; the digesta (CPHD) now contained more than 90% peptides below 1 kDa. The size exclusion chromatograms (**Figure 6.1 B**) visually illustrate the changes in molecular weight distribution of the untreated cricket protein, cricket protein hydrolysates (CPH) and its digesta (CPHD) after SGID. The untreated cricket protein chromatogram exhibited more peaks at earlier retention times (10-25 min) compared with the hydrolysate mixtures. Both CPH and CPHD chromatograms are absent of peaks seen with the cricket protein and instead a larger cluster of peaks at a later retention time (25-55 min) indicating substantial protein hydrolysis and generation of smaller peptides. The peptide molecular weight distribution of CPH and CPHD were mostly similar, indicating some resistance to digestion by the gastrointestinal proteases used in the SGID. Our previous work highlights the multifunctional bioactive properties of CPH and the improvement or retention of antihypertensive and antidiabetic activity after SGID.



(a)



(b)

Figure 6.1. Molecular weight distribution of cricket protein after enzymatic hydrolysis and in vitro digestion.

(a) Gel filtration peptide profile of non-hydrolyzed cricket protein, cricket protein hydrolyzed with alcalase, and CPH after digestion with gastrointestinal enzymes.

(b) Percentage of peptides above 10 kDa and below 1 kDa. Cricket protein = non-hydrolyzed crickets; CPH =cricket protein hydrolysates; CPHD= simulated gastrointestinal digests of CPH. Molecular weights were calculated from a standard curve with peptides of known mass. Error bars represent standard deviation on triplicate determinations.

6.3.2 CPHD Peptide fractionation

In our preliminary observations, the simulated gastrointestinal digests (CPHD) demonstrated inhibition capacity to enzymes involved in disease pathways such as hypertension, type-2 diabetes and inflammation (**Supp. Mat. Fig. S6.1**). Hence, the CPHD was separated by sequential chromatography to isolate and characterize the peptide or group of peptides responsible for the observed bioactivity. First, peptides were distinguished by size on a Superdex® Peptide 10/300 column (**Supp. Mat. Fig S6.2 A**) Four peak fractions collected (S1-S4), lyophilized, and then probed against DPP-IV, ACE, and α-glucosidase for screening **(Table 6.1).**

Taken together, fraction S4 illustrated superior inhibition activity against all enzymes (**Table 6.1**). Next, fraction S4 was separated by reversed phased chromatography (C18) resulting in six fractions F1 to F6. Of the C18 fractions, F6 showed the better inhibiting activity and being the fraction with the most hydrophobic peptides present within the CPHD **(Table 6.1).** Next, fraction F-VI was concentrated for a final separation via ion-exchange, which resulted in two peaks belonging to either anionic or cationic peptides (**Supp. Mat. Fig S6.2 C**).

Table 6.1. % Inhibition of ACE, DPP-IV, and α-glucosidase by CPHD peptide fractions

	Fraction	ACE (%)	DPP-IV (%)	α-Glucosidase (%)
Size exclusion	**S1**	32.3 ±0.31a	19.3 ±2.52a	62.2 ±1.09a
	S2	28.8 ±0.02b	16.98 ±0.30a	25.9 ±2.54b
	S3	47.5 ±0.87c	20.6 ±0.61a	83.6 ±1.14c
	S4	73.9 ±1.12d	68.0 ±2.52b	89.5 ±0.91d
Reversed-phased	**F1**	25.4 ±0.6e	5.1 ±0.01c	9.4 ±0.36e
	F2	29.4 ±0.04g	15.3 ±2.70d	11.6 ±0.04f
	F3	67.2 ±0.12h	18.4 ±0.59a	8.4 ±0.47g
	F4	32.2 ±0.19a	17.1 ±1.61a	7.3 ±1.51h
	F5	47.4 ±0.5c	21.0 ±1.96a	22.3 ±2.79i
	F6	93.4 ±0.08i	26.98 ±1.63e	48.2 ±5.79j
Positive inhibitor	**Captopril**	98.0 ±0.01j	n.a	n.a
	Ile-Leu-Pro	n.a	88.9 ±6.05f	n.a
	Acarbose	n.a	n.a	70.9 ±0.43k

[1] Results are reported as the percentage of inhibiting activity and is a mean of least triplicate (n=3) with standard deviation. S1-S4 are fractions collected from the gel filtration column. F1-F6 are fractions collected from the reversed phase C18 column. Captopril, peptide Ile-Leu-Pro, and Acarbose are commercially available therapeutic options to inhibit angiotensin converting enzyme II, Dipeptidyl peptidase-4, and α-glucosidase, respectively. n.a. =not applicable. Lowercase letters indicate significant differences between fractions within assays ($p < 0.05$).

6.3.3 Cationic peptide fractions show multifunctional bioactivity

To assess the extent of its multifunctional ability, the cationic fraction was tested against ACE, DPP-IV, α-amylase and α-glucosidase (**Table 6.2**). The DPP-IV inhibiting capacity was greater than 30% for all concentrations tested, never more than 50%, yet still exceeded the activity of the anionic fraction. Superior inhibiting activity was also observed for α-amylase, α-glucosidase, and ACE with IC_{50} values of 18.5, 13.9, and 1.9 μg/mL, respectively. In contrast, the anionic fraction IC_{50} values were 5082.7 μg/mL (α-amylase), 76.62 μg/mL (α-glucosidase), and 509 μg/mL (ACE), indicating that the most potent peptides were present in the cationic fraction (**Table 6.2**). The data also illustrates that bioactive efficacy increased as fractionation progressed. Peptide fractions displayed enhanced inhibiting ability after separated into charged groups (anion and

cation) compared with peptide fractions after SEC and C18 separation. This was true for all enzymes tested except DPP-IV.

Table 6.2. IC_{50} values for α-amylase, α-glucosidase, and ACE inhibition by IEX fractions

IEX fraction	α- Amylase (mg/mL)	α-Glucosidase (µg/mL)	ACE (µg/mL)
Cationic Peptides	18.537	13.902	1.922
Anionic Peptides	5082.75	76.623	509.0

IC_{50}: The peptide concentration required to inhibit 50% of enzyme activity

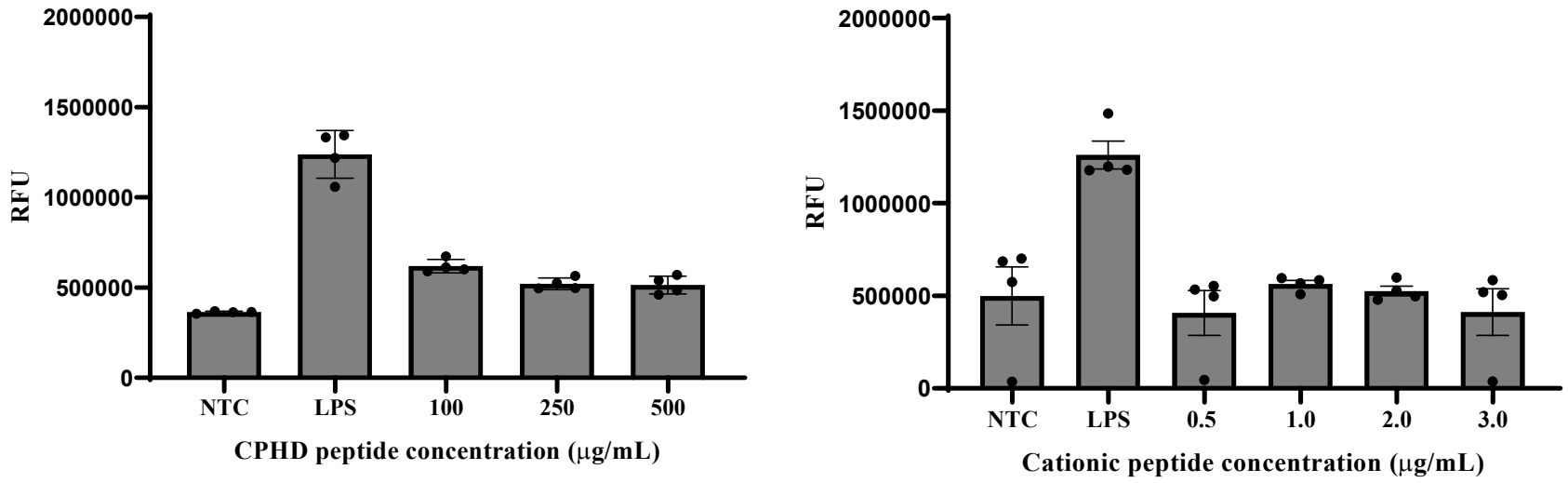
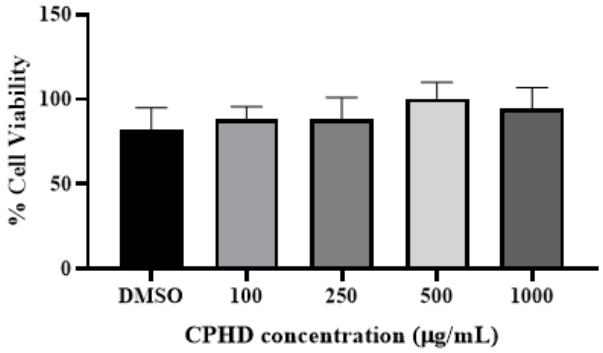


Figure 6.2. Peptides from (a) CPHD and its (b) Cationic Peptide fraction Inhibits LPS-Induced Inflammation in RAW 264.7 Cells.

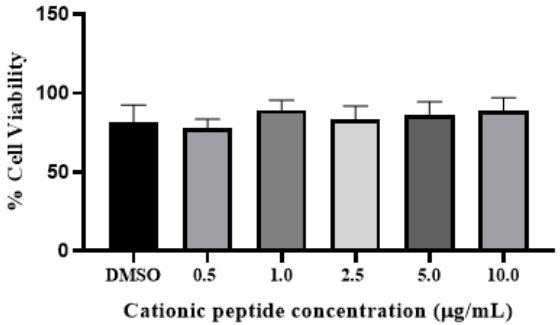
Different lowercase letters indicate significant differences between treatments ($p < 0.05$). Samples were analyzed in quadruplicate (n=4). Cells were stimulated for 6 hr with 500 ng of lipopolysaccharide (LPS) and/or pre-treated with CPHD (a), cationic fractions, or PBS (NTC= non treated control). The data are expressed as Renilla luciferase activity in relative light units (RLU). Data represent the mean ± SD of quadruplicate samples, representative of four independent experiments.

6.3.4 NF-κB expression in macrophage (RAW 264.7) cells

Treating RAW264.7 macrophage cells with LPS resulted in a significant increase ($p<0.05$) in inflammatory induction compared to that in the non-treated control. Treatment of RAW264.7 macrophage cells with the cationic fraction at various concentrations resulted in a non-concentration dependent inhibition of LPS-induced activation (**Figure 6.2**). There were significant differences ($p<0.05$) between the positive control (LPS only) and those dosed with CPHD and LPS. However, this inhibiting activity did not prove to be dose-dependent showing minor variation after treated with 0.5, 1, 2, or 3 μg/mL of the cationic peptide fraction. Comparing the inflammatory response between CPHD and its cationic fractions, CPHD also inhibited NF-κB (**Figure 6.2a**) in the presence of LPS activation. The inhibitory trend remained but requiring a much higher concentration of CPHD (100-500 μg/mL) than cationic peptides (0.5-3 μg/mL). Notably, the NF-κB defect observed by CPHD and cationic fractions were not due to cytotoxic effects (**Figure 6.3**). Damage to macrophages does not appear to be significant up to 10 μg/mL of cationic peptides (**Figure 6.3a**) and 1 mg/mL of the hydrolysate digesta (CPHD) after 12-hour exposure (**Figure 6.3b**).



(a)



(b)

Figure 6.3.Percentage of Macrophage Cell Viability after exposure to **(a)** CPHD and its **(b)** Cationic peptide fraction

CPHD= cricket protein hydrolysates after simulated digestion with gastrointestinal enzymes. Cells were incubated with CPHD (100-1000µg/mL), cationic peptide fraction (0.5-10 µg/mL), or DMSO (0.25%) for 12 hours to evaluate cytotoxicity by MTT assay with peptides. There was no significant ($p > 0.05$) reduction in viability compared with DMSO-treated cells. Data represent the mean ± SD representative of eight (n=8) independent experiments

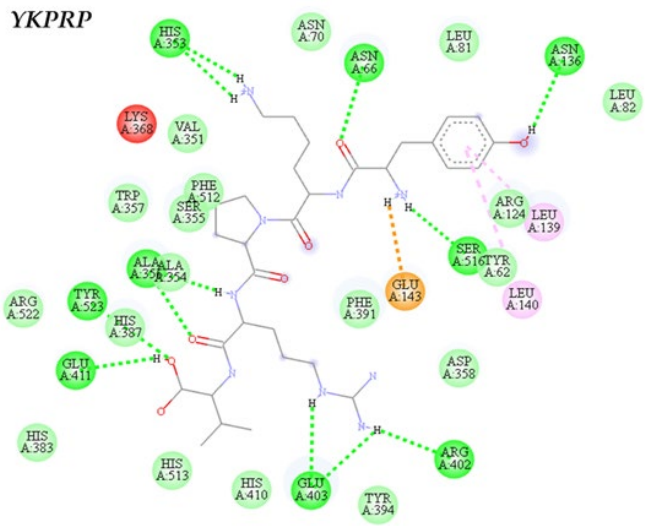
6.3.5 Peptide identification and molecular docking against ACE

Twenty-eight peptide sequences were identified within from the cationic peptide fraction between 384.52 and 659.84 Da and 4-6 amino acids (Supp. Mat. Table S6.1). A search against the Biopep database revealed that most of the peptides contained known ACE-inhibiting tri-peptides within their sequence. Other bioactive properties were also identified, including antioxidant, DPP-IV inhibition, and immunomodulation. Since all peptides were predicated to possess antihypertensive (AHT) capabilities (R. Kumar et al., 2015), they were used in molecular docking studies against human ACE and compared to known ACE inhibitor captopril. Of the best poses of peptides, three peptides YKPRP, PHGAP, VGPPQ had the lowest binding energy when bound to ACE and were mainly stabilized by hydrogen bonds and van der Waal interactions with ACE residues (Supp. Mat. Table S2). Peptide YKPRP (-39.75 kJ/mol) had a lower binding energy compared with PHGAP (-36.82 kJ/mol), VGPPQ (-36.87.26 kJ/mol), and captopril (-24.26 kJ/mol). The ACE molecule includes three main active sites; S1 with three residues Ala354, Glu384, and Tyr523; S2 comprising of Gln281, His353, Lys511, His513; and an S1' pocket including residue Glu162 (Qian et al., 2019). YKPRP formed multiple H-bond (**Figure 6.4a**), some of which being interactions with S1 (Tyr523, and Ala354) and S2' (His513) active site residues. Although not as many hydrogen bond and hydrophobic interactions, PHGAP mainly interacted with Tyr523, Ala354 residues of S1 and Tyr520, His353, His513, Gln281 of S2' and Phe457 of the hydrophobic pocket (**Figure 6.4b**). Likewise, VGPPQ showed a high degree of interactions with S1 (Ala354, Glu 384, Tyr523) and S2 (His352, His513) residues, most of which are van de Waals in nature (Supp. Mat. Table 2). The docking results also showed interactions between peptides PHGAP and VGPPQ and Zn2+ at the enzyme active site with distances less than 3.0A distance to the carbonyl oxygen of the peptide. These interactions are similar to that observed of captopril having all interactions with ACE S1 and S2', residues of the hydrophobic pocket (Phe 457, Phe527) and Zn2+ coordination on the sulfhydryl (**Figure 6.4d**).

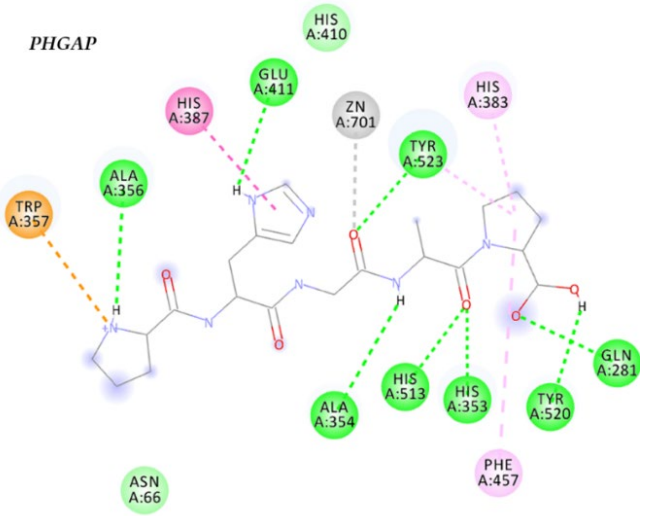
Table 6.3. Predicted binding energies, ZN (II) coordination, and ACE residues that interact with docked ligands.

			Interaction with ACE active site residues							
Ligand	**Affinity Energy (kJ/mol)**	**Zn (II)coordination**	**Glu384**	**Tyr523**	**Ala354**	**Gln281**	**Tyr520**	**Lys511**	**His513**	**His353**
Captopril	-24.26	sulfhydryl group of captopril	+	+	+	+	+	+	+	+
YKPRP	-39.75	None	-	+	+	-	-	-	+	+
PHGAP	-36.82	carboxylic acid group of glycine	-	+	+	+	+	-	+	+
VGPPQ	-36.87	carboxylic acid group of lysine	+	+	+	-	-	-	+	+

*Interactions are denoted with a "+" indicating that there was interaction between the ACE residue and ligand or "- "indicating no interaction.


YKPRP
HIS A:353
ASN A:70
ASN A:66
LEU A:81
ASN A:436
LEU A:82
LYS A:368
VAL A:351
PHE A:512
TRP A:357
SER A:355
ARG A:124
LEU A:139
SER A:516
TYR A:62
ALA A:354
GLU A:143
LEU A:140
ARG A:522
TYR A:523
HIS A:387
PHE A:391
GLU A:411
ASP A:358
HIS A:383
HIS A:513
HIS A:410
GLU A:403
TYR A:394
ARG A:402

(a)


PHGAP
HIS A:410
GLU A:411
HIS A:387
ZN A:701
HIS A:383
TYR A:523
ALA A:356
TRP A:357
GLN A:281
HIS A:513
HIS A:353
ALA A:354
TYR A:520
PHE A:457
ASN A:66

(b)

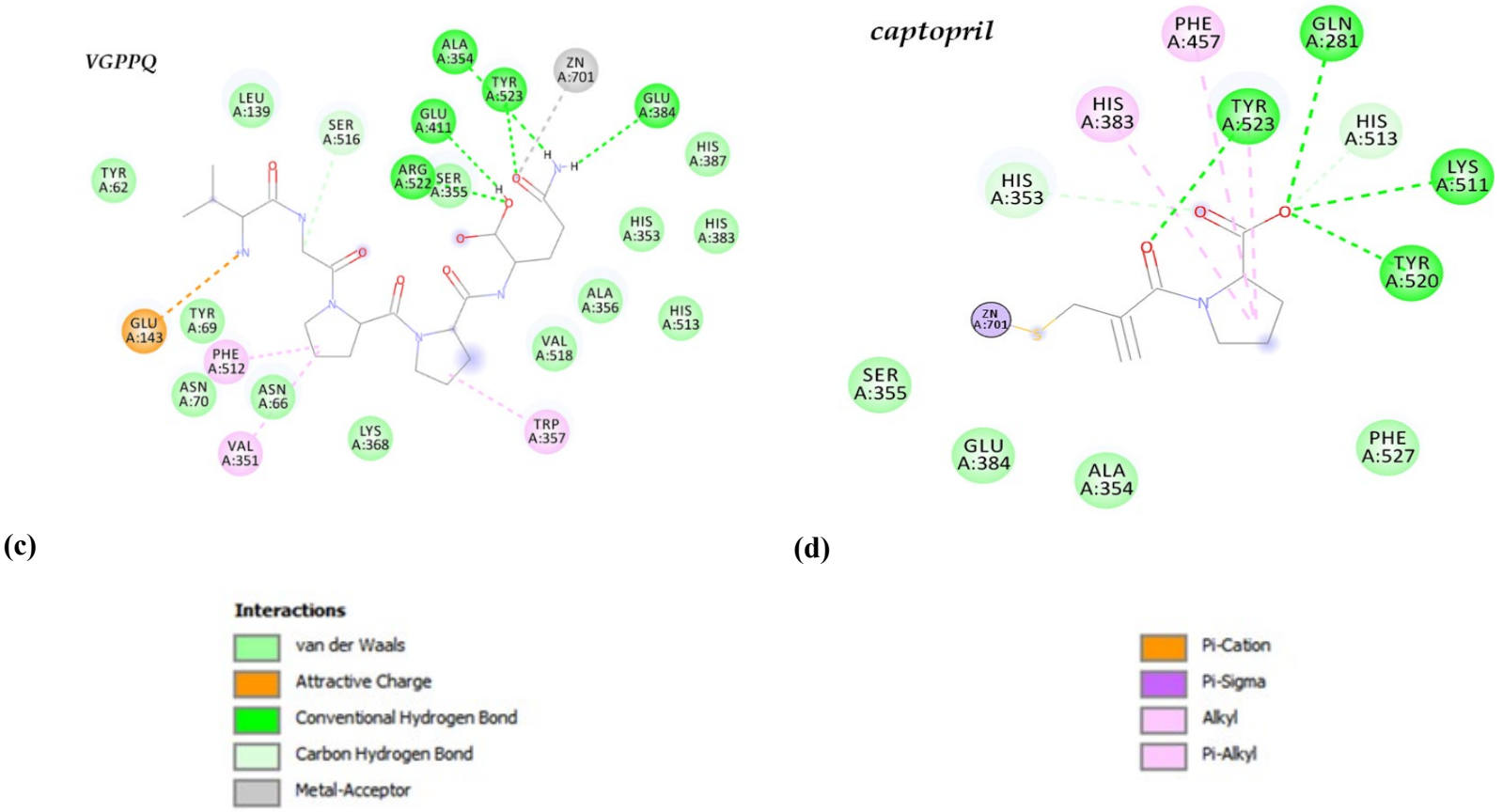


Figure 6.4. 2D model of predicted binding mode of **(a)** YKPRP, **(b)** PHGAP, **(c)** VGPPQ, and **(d)** captopril to the angiotensin-converting enzyme (ACE). Images were obtained with Discovery Studios Visualizer Software and the best scored docking pose is shown

6.4 Discussion

Cardiovascular disease is the major cause of morbidity and mortality in chronic glycaemia and hypertension (Petrie, Guzik, & Touyz, 2018; Sowers, Epstein, & Frohlich, 2001). As such, diabetes and hypertension are closely interlinked by similar risk factors, such as endothelial dysfunction, oxidative stress, arterial atherosclerosis, obesity and inflammation processes (Petrie et al., 2018). Diabetic and hypertensive pathways also overlap some cardiovascular complications related primarily to microvascular and macrovascular disease (Petrie et al., 2018). Common mechanisms, such as upregulation of the renin-angiotensin-aldosterone system, oxidative stress, inflammation, and activation of the immune system likely contribute to the close relationship between diabetes and hypertension. Nutritional interventions have been consistently proposed as a part of a comprehensive strategy to lower the incidence and severity of cardiovascular diseases and its risk factors (Estruch et al., 2006). In addition to the immense benefits of phenolic and non-peptic compounds, bioactive peptides are considered a viable treatment and prevention alternative owing to their inhibitory nature of various disease-related enzymes (Udenigwe & Aluko, 2012). Edible insects are undoubtedly an underexploited source of protein and bioactive peptides. Literature evidence shows that the most therapeutically effective insect preparations are in the form of peptides derived from protein hydrolysates, which have shown inhibitory effects towards inflammation, hypertension, diabetes and microbial growth (Nongonierma & FitzGerald, 2017).

In our previous work, we evaluated the potential antioxidant, ACE, and DPP-IV inhibiting capabilities of various cricket protein hydrolysates (CPH) using the commercial enzyme alcalase. CPH prepared with the highest enzyme concentration (3.0%) and incubated for 80 min showed the best bioactivity among other hydrolysate preparations. The observed activity remained or improved after SGID, suggesting further liberation of active peptides or stability towards gastrointestinal enzymes (Hall et al., 2018). This study highlighted conditions necessary to produce cricket protein hydrolysates that may be biologically active. However, the peptide/peptide group responsible has not yet been characterized. Hence, the main objective of this study was to isolate and characterize peptide groups present within cricket protein hydrolysates primarily responsible for its potential bioactivity. In general, active enzymatic hydrolysates contain peptides with a wide range of molecular weights, amino acid sequences, hydrophobicity and hydrophilicity, charge, and bioactive efficacies; therefore, this work focused on assay-directed fractionation. The SGID

digests of the protein hydrolysates were chosen as the starting material for fractionation, since peptides likely exhibit their properties after digestion and absorbance into the blood system.
Following the data described in **Figure 6.1 and Table 6.1**, the CPHD were first separated by molecular weight size. There were four major groups of peptides (Fraction S1-S4) with distinguished molecular weights and retention time. Fraction S4 was identified as having the best inhibiting activity towards ACE, DPP-IV and α-glucosidase, and was further separated using RP-HPLC. From the chromatographic profile at 214 nm (**Supp. Mat. Fig. 6.2a**) fraction S4 was still a complex mixture of peptides, which was then separated according to the hydrophobicity of the peptides (F1-F6). ACE and α-glucosidase inhibition assays revealed that fraction F6 had better inhibition activity compared with the other fractions. After two stages of fractionation, S6 and F4 likely contained hydrophobic peptides below 10-20 amino acids similar to known biologically relevant peptides (Udenigwe & Aluko, 2012). These peptides do not have any specific consensus on amino acid sequences that are responsible for their biological activity, but most of them maintain certain common features, such as containing a positive charge and relatively hydrophobic and amphipathic structure (Hoskin & Ramamoorthy, 2008). This trend corresponds well with that described by Vercruysse et al. (Vercruysse et al., 2008), where peptide fractions from cotton leaf worm (*S. littoralis*) were separated by SEC and RP-HPLC. Fractionation revealed an active peptide with an aromatic, hydrophobic amino acid at the carboxyl terminus, namely phenylalanine, and consisted of a hydrophobic amino acid at the amino terminus, alanine, which showed antihypertensive activity *in vitro* and *in vivo*. Fraction F6 was separated into two charged groups, anionic and cationic peptides, which confirmed that the most potent peptides were cationic in nature. The data conferred the cationic pool of peptides with superior inhibiting activity towards ACE, α-amylase, and α-glucosidase; DPP-IV inhibiting activity, however, decreased after the various stages of fractionation.

Cellular inflammation and dysfunction play a crucial role in several disorders, including cardiovascular disease risk factors such as hypertensive and hyperglycemic responses. Nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) is a central regulator of pro-inflammatory mediators and is an essential component linking chronic inflammation and CVD. Thus, regulating NF-κB is considered a potential therapeutic target for CVD risk factors. Since monocytogenes-induced NF-κB pathways is the most dominant host response in macrophage cells, we examined the ability of cricket peptides to inhibit NF-κB in the RAW 264.7-NF-κB-luciferase

reporter cell line. Our results demonstrate that the cation peptide fractions downregulated NF-κB expression in the RAW 267.4 NF-κB-reporter cell line. This reporter contains a firefly luciferase gene under the control of multimerized NF-κB responsive pathways, which are commonly used to assess the potency of anti-inflammatory molecules and peptides. During inflammatory scenarios, NF-κB positively modulates expression levels of several chemokines (e.g. MCP-1), proangiogenic factors, for example, vascular endothelial growth factor (VEGF), and cellular inhibitors of apoptosis (Lazennec & Richmond, 2010). In this study, NF-κB expression was stimulated by the bacterial cell wall component lipopolysaccharide (LPS) which is known to induce an inflammatory cascade by activating the TLR4-NFκB signaling pathway. Cells pre-treated with the cationic fraction demonstrated lower levels of NF-κB compared with LPS alone, verifying that this fraction contained some of the more potent peptides present within CPHD. (**Figure 6.2**). Notably, this anti-inflammatory activity was maintained and required a much lower concentration of the cationic peptides (1-10 µg/mL vs 100-500 µg/mL; **Figure 6.2**). Most food-derived peptides anti-inflammatory activities primarily by inhibiting signaling components of either nuclear factor-kappa light-chain-enhancer of activated B cells (NF-κB) or mitogen-activated protein kinase (MAPK) pathways, the two major pathways involved in chronic inflammation following uncontrolled signal activation (Guha & Majumder, 2019). Similar to the findings of this study, venom from wasp (*Nasonia vitripennis*) dose-dependently inhibited NF-κB signaling in NF-κB-reporter fibrosarcoma cells (Danneels et al., 2014). More than half of NF- κB was suppressed with 6.4 and 12.5 µg/mL of wasp venom. In addition to the NF-κB inhibitory action, the wasp venom also inhibited IL-6 (a proinflammatory cytokine) expression in LPS-activated macrophages, as well as, expression of two NF-κB target genes, IκBα and A20 (Danneels et al., 2014). In another study, peptide fractions from cricket (*G. sigilattus*) protein hydrolysates also demonstrated anti-inflammatory properties (Zielińska, Baraniak, & Karaś, 2018). The study detailed the efficacy of SEC isolated peptide fractions to inhibit lipoxygenase and cyclooxygenase-2 activity with IC_{50} values of 0.13 µg/mL and 0.26 µg/mL, respectively. The authors also noted that better anti-inflammatory activity was overserved for the peptide fractions compared with the hydrolysates (Zielińska et al., 2018).

Cationic bioactive peptides, particularly, have shown to be useful therapeutic agents against human diseases when exhibiting high bioactive potency and no cytotoxic activity against mammalian cells. The de novo sequencing revealed that the cationic peptides fractioned from

CPHD consisted of mainly hypotensive peptides (**Supp. Mat. Table S6.2**) which may have played a role in the superior ACE inhibiting activity overserved by this fraction (**Table 6.3**). Additionally, most of the identified sequences contain tri-peptides with reported activity, including antioxidant, antimicrobial, anti-glycemic, and immunomodulatory properties (Minkiewicz et al., 2019). This would explain the multifunctional ability of the cationic peptide fraction overserved in this study. Among the peptides identified, the molecular docking results revealed that peptides YKPRP, PHGAP, and VGPPQ had binding properties similar to those of commercial ACE inhibiting drug captopril (**Table 6.4**). All three peptides had binding energies greater than captopril, but similar to that of Lisinopril (-39.96 kJ/mol) (Girgih, He, & Aluko, 2014). The ability of peptides to form multiple hydrogen bonds and hydrophobic interactions is a key characteristic of peptide-induced inhibition by means of stabilizing the non-catalytic enzyme-peptide complex structure (He, Aluko, & Ju, 2014). All three peptides interacted with ACE active sites mainly via hydrogen bonding and van der Waals interactions. However, peptides PHGAP and VGPPQ showed more interaction with S1 and S2 active site residues. In addition to hydrogen and hydrophobic bonds, interaction between Zn2+ ion and peptides play a significant role in ACE inhibition (He et al., 2014). Both peptides PHGAP and VGPPQ also exhibited interaction with ZN2+ atoms binding to ACE in an analogous fashion to that observed of captopril (**Figure 6.4**), indicating that they would be potent ACE inhibitors. Even so, YKPR is reported as a known neuropeptide, analogue of the natural immunomodulatory tetra peptide tufsin (TKPR) and may be more. Similar peptides are reported as neuromodulators isolated from various insect species and other crustaceans such as lobster and crabs (Howard et al., 2010). For example, penta-peptide RYLPT (proctolin) is the first insect neuropeptide sequenced and chemically characterized. However, a study demonstrated that proctolin, although able to bind, were resistant to ACE hydrolysis, in vivo (Siviter et al., 2002). Peptides, like proctolin, that have a prolyl residue near the C-terminal are known to be resistant to hydrolysis by mammalian ACE due to the structural constraints imposed by the presence of imino acids (Siviter et al., 2002). This would explain why YKPR, in this study, demonstrated a lower binding affinity than other peptides, yet less interaction with active site residues. Whereas, peptides PHGAP and VGPPQ contains tripeptides GAP and GPP, respectively. Both of which are present in many ACE-inhibiting, antioxidant, DPP-IV-inhibiting and antimicrobial food-derived peptide sequences (**Supp. Mat. Table S6.1**) (Minkiewicz et al., 2019). Potent ACE inhibitors include hydrophobic amino acid residues near the C-terminal. Proline is reported as a desirable reside, but

also aliphatic peptides such lysine is responsible for the ACE-inhibitory capacity. Zielińska (Zielinska, Karas, Baroniak, & Jakubczyk, 2020) isolated ACE inhibitory peptides from cricket (*G.sigillatus*) protein hydrolysates. They identified sequence IIAPPER, LAPSTIK, KAPEEHPV, KVEGDLK which show no homology to peptides identified in this study. These differences are attributed to conditions used to liberate bioactive peptides and peptide isolation techniques, also demonstrated in their study. To the best of our knowledge, these bioactive peptide sequences derived from cricket protein hydrolysates have yet to be reported. Based on the results obtained in this study, analyzing the molecular mechanism involved between these identified peptide sequences on hypertensive, glycemic, and inflammatory responses as well as their in vivo bioactive effects will be the focus of future studies. The in vivo bioactive effects of cricket protein hydrolysates await to be confirmed. Considering the variety of activity displayed in this study, enhanced potency post-fractionation, and affinity of novel peptides towards ACE (a central regulator of the renin–angiotensin aldosterone system and known target for treating hypertension) highlights that cricket-derived peptides could play a significant role in the pathogenesis involved in cardiovascular dysfunction.

6.5 Conclusions

In this study, we selected cricket protein hydrolysates as a starting material to characterize and identify potent bioactive peptides upon simulated gastrointestinal digestion. After separation by size and hydrophobicity, fractions were screened for enzyme inhibitory capacity, then finally separated by charge. Subsequently, we examined the antihypertensive, antidiabetic activity, and anti-inflammatory activity of the final fraction and identified the peptide sequences using LC-MS/MS.

Taken together, cricket protein hydrolysates contain potent peptides with potential ACE, α-glucosidase, and α-amylase inhibiting capacity. Additionally, CPHD and its cationic fractions inhibited LPD-induced NFκB activity, but other molecular events such as peptide-TLR4 interaction remain unknown. Despite the effect of the protein hydrolysates and cationic peptide fractions on the NF-κB expression in macrophage cells, it should be noted that we did not observe a clear relationship between sample concentration and their biological effect. Notably, the sequences of ACE-inhibitory peptides were identified as three pentapeptides YKPRP, PHGAP, VGPPQ. Consumption of edible cricket peptides, alone or in the form of functional

foods may contribute to positive effects to conditions associated with inflammation and hypertension. Nevertheless, these results are a contribution to future research, which should be undertaken to verify the findings using in vivo models and clinical trials. Moreover, these results can be the basis for further research on other species of edible insects.

6.6 References

Amaya, J. A. G., Cabrera, D. Z., Matallana, A. M., Arevalo, K. G., & Guevara-Pulido, J. (2020). IN-SILICO design of new enalapril analogs (ACE inhibitors) using QSAR and molecular docking models. *Informatics in Medicine Unlocked*, 100336.

Awosika, T. O., & Aluko, R. E. (2019). Inhibition of the in vitro activities of α-amylase, α-glucosidase and pancreatic lipase by yellow field pea (Pisum sativum L.) protein hydrolysates. *International journal of food science & technology, 54*(6), 2021-2034.

Çabuk, B., & Yılmaz, B. (2020). Fortification of traditional egg pasta (erişte) with edible insects: nutritional quality, cooking properties and sensory characteristics evaluation. *Journal of Food Science and Technology*, 1-8.

Danneels, E. L., Gerlo, S., Heyninck, K., Van Craenenbroeck, K., De Bosscher, K., Haegeman, G., & de Graaf, D. C. (2014). How the venom from the ectoparasitoid Wasp Nasonia vitripennis exhibits anti-inflammatory properties on mammalian cell lines. *PloS one, 9*(5), e96825.

Estruch, R., Martínez-González, M. A., Corella, D., Salas-Salvadó, J., Ruiz-Gutiérrez, V., Covas, M. I., . . . Vinyoles, E. (2006). Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Annals of internal medicine, 145*(1), 1-11.

Girgih, A. T., He, R., & Aluko, R. E. (2014). Kinetics and molecular docking studies of the inhibitions of angiotensin converting enzyme and renin activities by hemp seed (Cannabis sativa L.) peptides. *Journal of agricultural and food chemistry, 62*(18), 4135-4144.

Guha, S., & Majumder, K. (2019). Structural-features of food-derived bioactive peptides with anti-inflammatory activity: A brief review. *Journal of food biochemistry, 43*(1), e12531.

Hall, F., Johnson, P. E., & Liceaga, A. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food chemistry, 262*, 39-47.

Hall, F. G., Jones, O. G., O'Haire, M. E., & Liceaga, A. M. (2017). Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. *Food chemistry, 224*, 414-422.

He, R., Aluko, R. E., & Ju, X.-R. (2014). Evaluating molecular mechanism of hypotensive peptides interactions with renin and angiotensin converting enzyme. *PloS one, 9*(3), e91051.

Hoskin, D. W., & Ramamoorthy, A. (2008). Studies on anticancer activities of antimicrobial peptides. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1778*(2), 357-375.

Howard, J. A., Yufit, D. S., Chetina, O. V., Teat, S. J., Capelli, S. C., & Pattison, P. (2010). Crystal structure of the insect neuropeptide proctolin. *Organic & Biomolecular Chemistry, 8*(22), 5110-5112.

Ketnawa, S., Wickramathilaka, M., & Liceaga, A. M. (2018). Changes on antioxidant activity of microwave-treated protein hydrolysates after simulated gastrointestinal digestion: Purification and identification. *Food chemistry, 254*, 36-46.

Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides-opportunities for designing future foods. *Current pharmaceutical design, 9*(16), 1297-1308.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International dairy journal, 16*(9), 945-960.

Kumar, P., Nagarajan, A., & Uchil, P. D. (2018). Analysis of cell viability by the MTT assay. *Cold Spring Harbor Protocols, 2018*(6), pdb. prot095505.

Kumar, R., Chaudhary, K., Chauhan, J. S., Nagpal, G., Kumar, R., Sharma, M., & Raghava, G. P. (2015). An in silico platform for predicting, screening and designing of antihypertensive peptides. *Scientific Reports, 5*, 12512.

Lacroix, I. M., & Li-Chan, E. C. (2012). Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates. *International Dairy Journal, 25*(2), 97-102.

Lazennec, G., & Richmond, A. (2010). Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends in molecular medicine, 16*(3), 133-144.

Luna, G. C., Martin-Gonzalez, F. S., Mauer, L., & Liceaga, A. (2020). Cricket (Acheta domesticus) protein hydrolysates' impact on the physicochemical, structural and sensory properties of tortillas and tortilla chips. *Journal of Insects as Food and Feed*, 1-12.

Martínez-Alvarez, O., Batista, I., Ramos, C., & Montero, P. (2016). Enhancement of ACE and prolyl oligopeptidase inhibitory potency of protein hydrolysates from sardine and tuna by-products by simulated gastrointestinal digestion. *Food & function, 7*(4), 2066–2073. doi:10.1039/c5fo01603g.

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM database of bioactive peptides: Current opportunities. *International journal of molecular sciences, 20*(23), 5978.

Mosley, A. L., Florens, L., Wen, Z., & Washburn, M. P. (2009). A label free quantitative proteomic analysis of the Saccharomyces cerevisiae nucleus. *Journal of proteomics, 72*(1), 110-120.

Nongonierma, A. B., & FitzGerald, R. J. (2017). Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review. *Innovative Food Science & Emerging Technologies, 43*, 239-252.

Petrie, J. R., Guzik, T. J., & Touyz, R. M. (2018). Diabetes, hypertension, and cardiovascular disease: clinical insights and vascular mechanisms. *Canadian Journal of Cardiology, 34*(5), 575-584.

Qian, B., Tian, C., Huo, J., Ding, Z., Xu, R., Zhu, J., . . . Villarreal, O. D. (2019). Design and evaluation of four novel tripeptides as potent angiotensin converting enzyme (ACE) inhibitors with anti-hypertension activity. *Peptides, 122*, 170171.

Shobana, S., Sreerama, Y., & Malleshi, N. (2009). Composition and enzyme inhibitory properties of finger millet (Eleusine coracana L.) seed coat phenolics: Mode of inhibition of α-glucosidase and pancreatic amylase. *Food chemistry, 115*(4), 1268-1273.

Siviter, R. J., Nachman, R. J., Dani, M. P., Keen, J. N., Shirras, A. D., & Isaac, R. E. (2002). Peptidyl dipeptidases (Ance and Acer) of Drosophila melanogaster: major differences in the substrate specificity of two homologs of human angiotensin I-converting enzyme. *Peptides, 23*(11), 2025-2034.

Sowers, J. R., Epstein, M., & Frohlich, E. D. (2001). Diabetes, hypertension, and cardiovascular disease: an update. *Hypertension, 37*(4), 1053-1059.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: production, processing, and potential health benefits. *Journal of food science, 77*(1), R11-R24.

Vercruysse, L., Smagghe, G., Beckers, T., & Van Camp, J. (2009). Antioxidative and ACE inhibitory activities in enzymatic hydrolysates of the cotton leafworm, Spodoptera littoralis. *Food chemistry, 114*(1), 38-43.

Vercruysse, L., Smagghe, G., Matsui, T., & Van Camp, J. (2008). Purification and identification of an angiotensin I converting enzyme (ACE) inhibitory peptide from the gastrointestinal hydrolysate of the cotton leafworm, Spodoptera littoralis. *Process Biochemistry, 43*(8), 900-904.

Vercruysse, L., Van Camp, J., Morel, N., Rougé, P., Herregods, G., & Smagghe, G. (2010). Ala-Val-Phe and Val-Phe: ACE inhibitory peptides derived from insect protein with antihypertensive activity in spontaneously hypertensive rats. *Peptides, 31*(3), 482-488.

You, L., Zhao, M., Regenstein, J. M., & Ren, J. (2010). Changes in the antioxidant activity of loach (Misgurnus anguillicaudatus) protein hydrolysates during a simulated gastrointestinal digestion. *Food chemistry, 120*(3), 810-816.

Zielińska, E., Baraniak, B., & Karaś, M. (2017). Antioxidant and Anti-Inflammatory Activities of Hydrolysates and Peptide Fractions Obtained by Enzymatic Hydrolysis of Selected Heat-Treated Edible Insects. *Nutrients, 9*(9), 970.

Zielińska, E., Baraniak, B., & Karaś, M. (2018). Identification of antioxidant and anti-inflammatory peptides obtained by simulated gastrointestinal digestion of three edible insects species (Gryllodes sigillatus, Tenebrio molitor, Schistocerca gragaria). *International Journal of Food Science & Technology, 53*(11), 2542-2551.

Zielinska, E., Karas, M., Baroniak, B., & Jakubczyk, A. (2020). Evaluation of ACE, α-glucosidase, and lipase inhibitory activities of peptides obtained by in vitro digestion of selected species of edible insects. *European Food Research and Technology*.

6.7 Appendix

Supplementary material

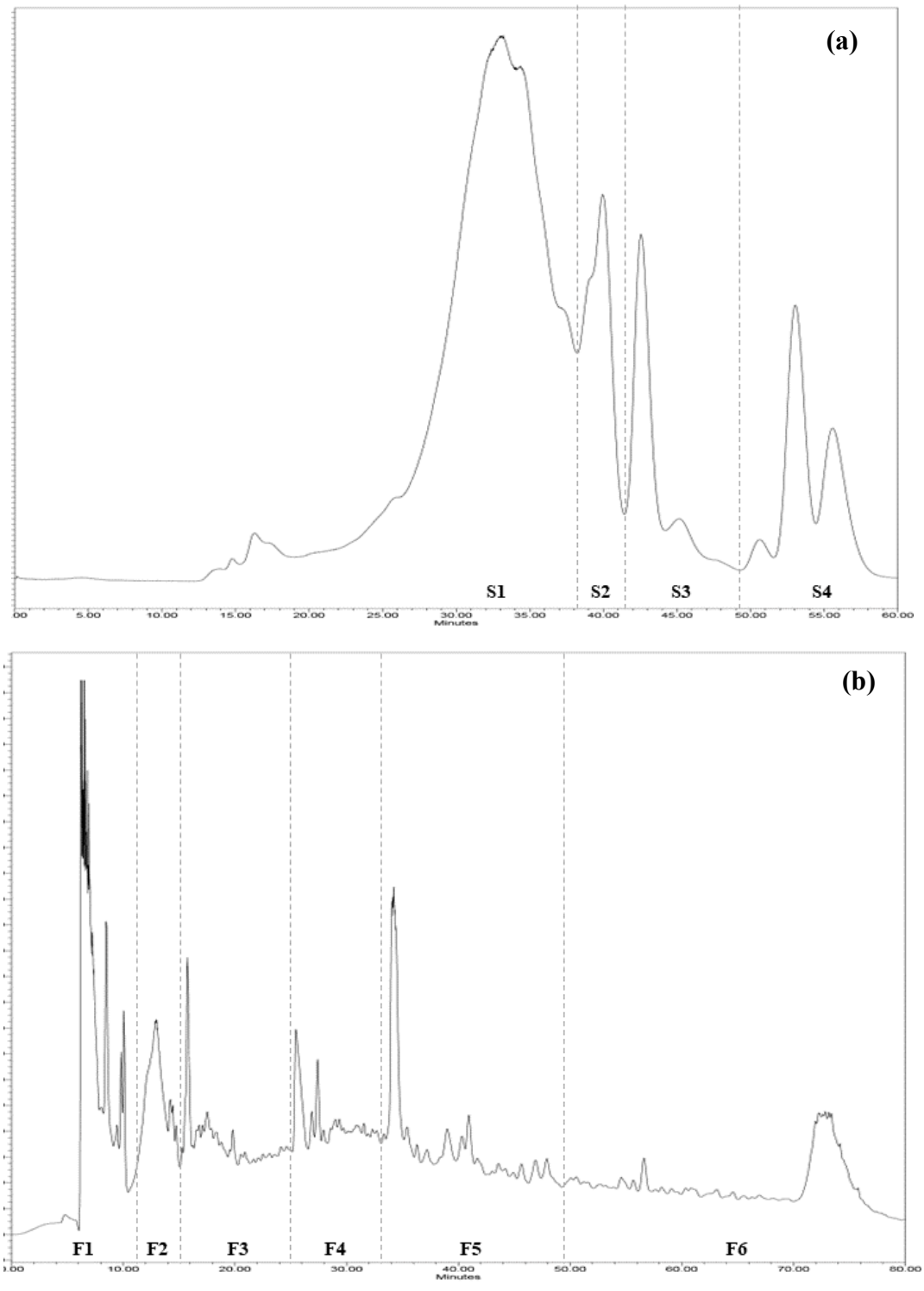
Figure S6.1: Representative elution profiles illustrating fractions collected from gel filtration (SEC) **(a)**, reverse-phased (C18) **(b),** and semi-preparative cation exchange (IEX) **(c)** columns. Chromatography was performed sequentially as follow: CPHD was separated by gel filtration; fraction S4 collected and separated by reverse-phase chromatography; and fraction F6 collected and separated by IEX. Elution profiled monitored at 280nm.

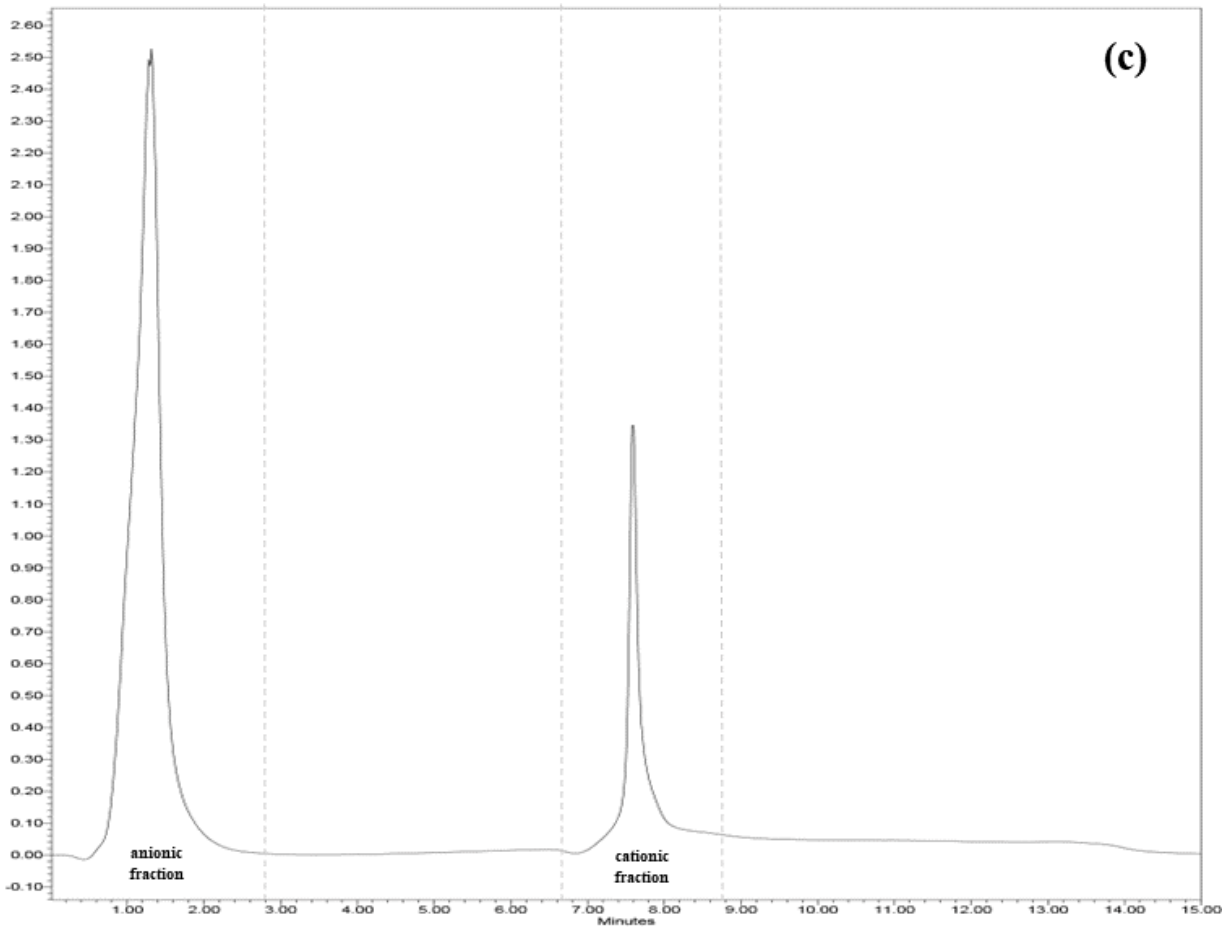
Figure S6.2. 2D and 3D image of molecular docking interaction, including interaction summary table by Discovery Studio software, between ligands **(a)** YKPRP, **(b)** PHGAP, **(c)** VGPPQ, **(d)** captopril and ACE (PDB: 108A)

Table S6.1: De novo peptide list, BioPEP database matches, and antihypertensive peptide prediction. Characterization parameters include de novo score, average local confidence (%) peptide abundance (area). Database searches were performed using PEAKS software. Antihypertensive peptide predication includes SVM score, prediction (AHT or non-AHT), and amphiphilic score. Peptides searched against the BioPEP database reports the known peptide and bioactivity reported.

Table S6.2: All residues interacting with ACE (PDB 108A) for each peptides YKPRP, PHGAP, VGPPQ and known ACE inhibitor captopril.

Table S6.3. Predicted binding affinity (kJ/mol) for peptides best docked pose against ACE


(a)
S1
S2
S3
S4
Minutes
(b)
F1
F2
F3
F4
F5
F6
Minutes

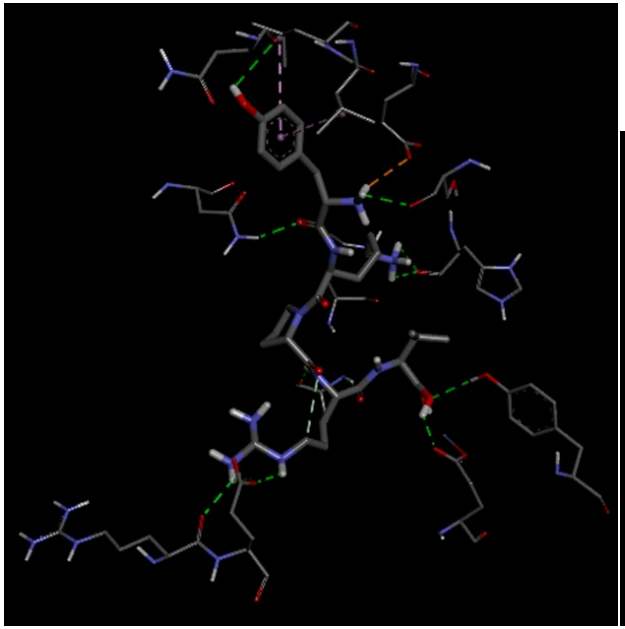

(c)
anionic fraction
cationic fraction
Minutes

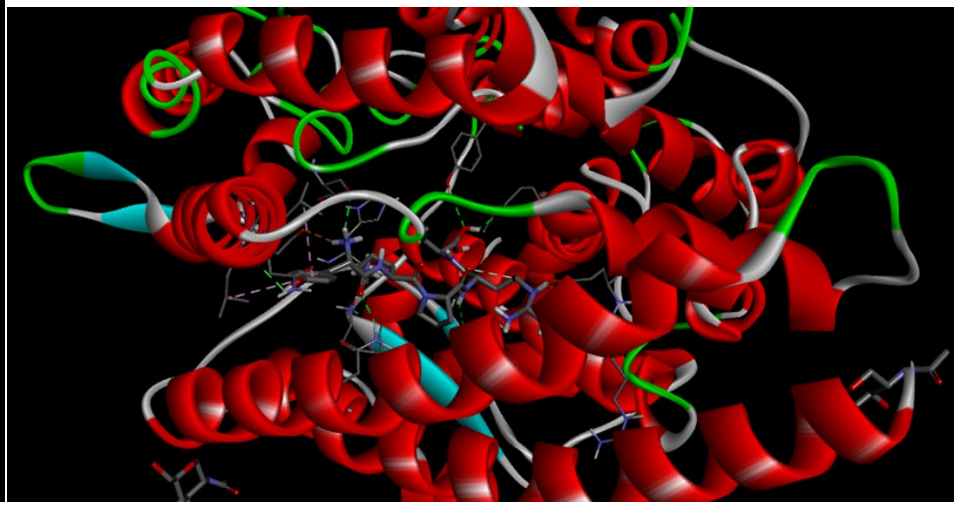
Peptide Sequence	De Novo Score	ALC (%)	Area	SVM Score	Prediction		Amphiphilicity Score	Mol wt.	Matched Peptide in BioPEP Database	Activity Reported
LPPK	85	85	1.76E+06	2.25	AHT		0.92	453.62	PPK	ACE inhibitor
GPPK	81	81	3.91E+06	2.05	AHT		0.92	397.52	GPP, PPK	ACE inhibitor
YKPRP	95	95	1.58E+06	2.02	AHT		2.24	659.84	exact match	immunomodulation
VLPQ	85	85	1.70E+07	1.59	AHT		0.31	455.6	VLP	ACE inhibitor
VKLAPG	83	83	2.01E+07	1.54	AHT		0.61	583.8	VKL, APG, LAP	DPPIV and ACE inhibitor
LLPQ	85	85	5.00E+06	1.43	AHT		0.31	469.62	LLP	ACE inhibitor
LQPL	89	89	4.38E+06	1.43	AHT		0.31	469.62	LQP	ACE and DPP-IV inhibitor
PHGAP	80	80	9.73E+05	1.35	AHT		0.29	477.58	PHG	not specified
VGPPQ	92	92	1.00E+07	1.33	AHT		0.25	496.63	GPP	ACE inhibition, antioxidant
FVPL	87	87	4.10E+06	1.29	AHT		0	474.64	FVP, VPL	ACE and DPPIV inhibitor
VHLP	87	87	1.69E+06	1.27	AHT		0.36	464.61	exact match	PEP inhibitor

PLVR	85	85	3.66E+06	1.27	AHT		0.61	483.65	LVR	ACE inhibitor
LVRP	85	85	3.16E+07	1.27	AHT		0.61	483.65	VRP, LVR	ACE inhibitor
VHLP	87	87	1.69E+06	1.27	AHT		0.36	464.61	exact match	PEP inhibitor
FVPH	82	82	1.07E+07	1.23	AHT		0.36	498.63	FVPH, FVP	antioxidant and ACE inhibitor
LVPK	83	83	2.54E+07	1.15	AHT		0.92	455.64	VPK	ACE
LLPR	98	98	2.25E+07	1.14	AHT		0.61	497.67	LLP	ACE
LPLR	95	95	2.46E+07	1.14	AHT		0.61	497.67	LPL	DPPIV inhibitor
LRPL	96	96	6.88E+07	1.14	AHT		0.61	497.67	LRP	ACE inhibitor
LLPF	80	80	1.14E+07	1.12	AHT		0	488.66	LLPF, LLP	antioxidant and ACE inhibitor
VGPL	84	84	2.72E+07	1.1	AHT		0	384.52	GPL	ACE inhibitor

Table S6.2. All residues interacting with ACE (PDB 108A) for each peptides YKPRP, PHGAP, VGPPQ and known ACE inhibitor captopril.

Interaction Ty (a)			:AP	VGPPQ
H-Bond	Gln281	Asn66	Glu411	Ala354
	Tyr523	Asn136	Ala356	Glu384
	Lys511	His353 (2)	Tyr523	Arg522
	Tyr520	Ala354	Gln281	Ser516
	His353	Ala356	Tyr520	Glu411
	His513	Glu411	His353	
		Glu403 (2)	His513	
		Arg402	Ala354	
		Ser516		
		Tyr523		
Vdw	Ser355	Asn70	His410	Tyr69
	Glu348	Leu81	Asn66	His353
	Ala354	Leu80		His383
	Phe527	Arg124		His513
		Tyr62		Val518
		Phe391		Ala356
		Asp358		Lys368
		Tyr394		Arg124
		His410		Tyr62
		His513		Leu139
		His383		Asn70
		Arg522		Asn66
		His387		His387
Other	Phe457	Leu139	Trp357	Phe512
	His383	Leu140	His383	Val351
		Glu143	Phe457	Trp357





Name	Color	Distance	Bond Category	Type	From	From	To	To
N:UNK1:H - A:GLU143:OE1	Yellow	2.99931	Hydrogen Bond; Electrostatic	Salt Bridge; Attractive Charge	N:UNK1:H	H-Donor; Positive	A:GLU143:OE1	H-Acceptor; Negative
N: UNK1: H - A: ALA356: O	Green	2.45711	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A: ALA356: O	H-Acceptor
N:UNK1:H - A:GLU411:OE2	Green	2.08941	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:GLU411:OE2	H-Acceptor
N: UNK1: H - A: ARG402: O	Green	2.37175	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A: ARG402: O	H-Acceptor

N:UNK1:H - A:GLU403:OE1	Green	2.2475	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:GLU403:OE1	H-Acceptor
N:UNK1:H - A:GLU403:OE1	Green	2.29977	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:GLU403:OE1	H-Acceptor
N: UNK1: H - A: SER516: O	Green	2.46192	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:SER516:O	H-Acceptor
N: UNK1: H - A: ASN136: O	Green	2.96341	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:ASN136:O	H-Acceptor
N: UNK1: H - A: HIS353: O	Green	2.66588	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:HIS353:O	H-Acceptor
N: UNK1: H - A: HIS353: O	Green	2.60396	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:HIS353:O	H-Acceptor
A: ASN66:HD21 - N: UNK1: O	Green	2.27171	Hydrogen Bond	Conventional Hydrogen Bond	A:ASN66:HD21	H-Donor	N:UNK1: O	H-Acceptor
A: ALA356: HN - N: UNK1: O	Green	2.05664	Hydrogen Bond	Conventional Hydrogen Bond	A:ALA356:HN	H-Donor	N:UNK1: O	H-Acceptor
A: TYR523: HH - N: UNK1: O	Green	2.56552	Hydrogen Bond	Conventional Hydrogen Bond	A:TYR523:HH	H-Donor	N:UNK1: O	H-Acceptor
N: UNK1: C - N: UNK1: O	Blue	3.43906	Hydrogen Bond	Carbon Hydrogen Bond	N:UNK1:C	H-Donor	N:UNK1: O	H-Acceptor
N:UNK1 - A:LEU139	Purple	4.69064	Hydrophobic	Pi-Alkyl	N:UNK1	Pi-Orbitals	A:LEU139	Alkyl
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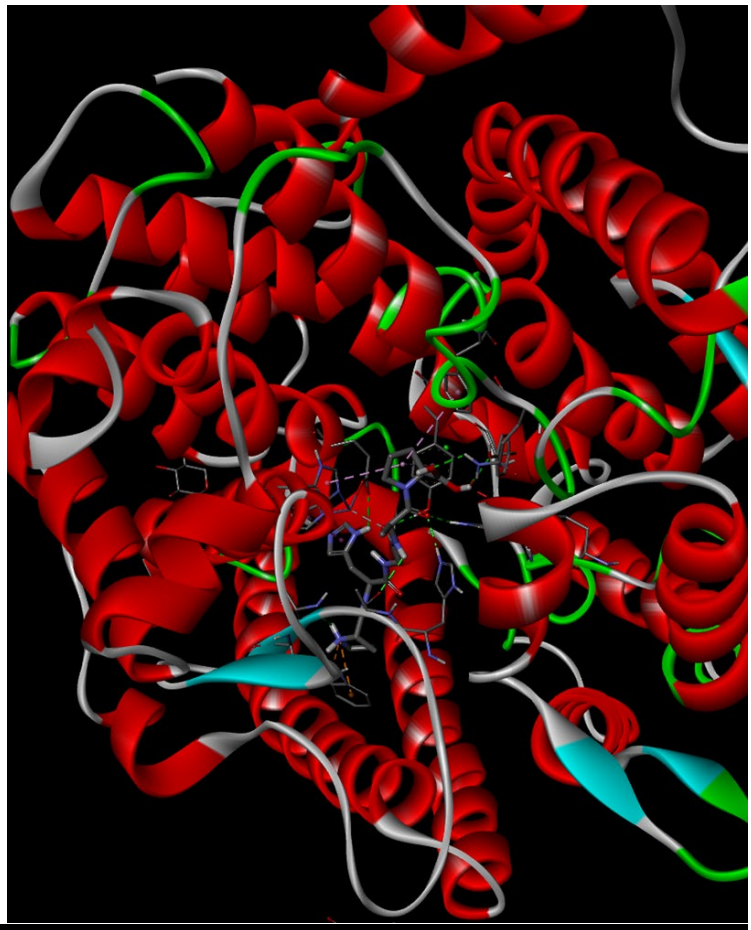

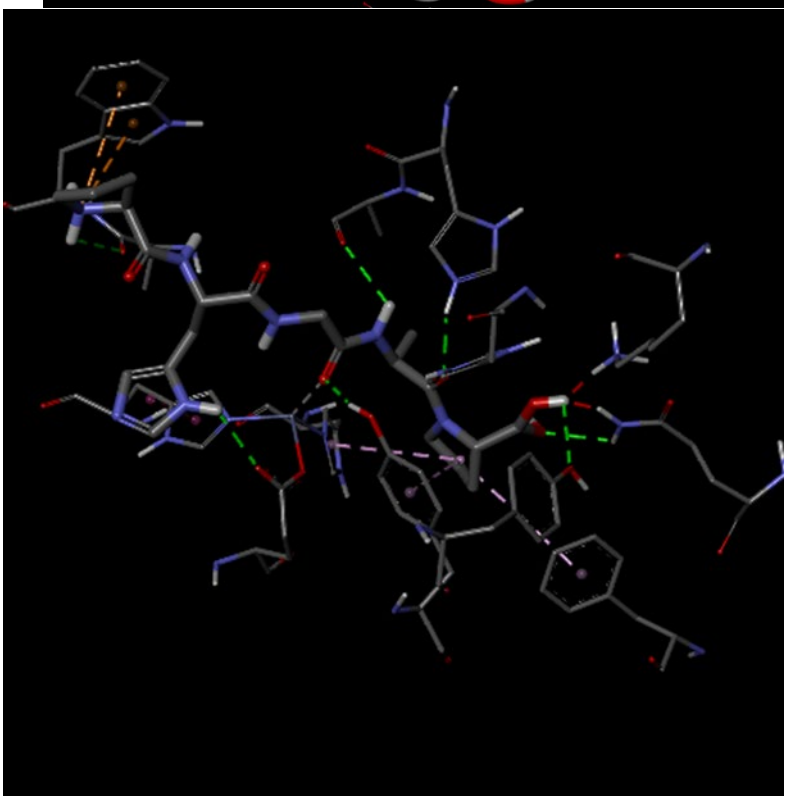

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A: ARG522:HH12 - N: UNK1: O	Green	2.04377	Hydrogen Bond	Conventional Hydrogen Bond	A:ARG522:HH12	H-Donor	N: UNK1: O	H-Acceptor
A: TYR523: HH - N: UNK1: O	Green	2.06106	Hydrogen Bond	Conventional Hydrogen Bond	A:TYR523:HH	H-Donor	N: UNK1: O	H-Acceptor
N: UNK1: H - N: UNK1: O	Green	2.58093	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	N: UNK1: O	H-Acceptor
N:UNK1:H - A:GLU411:OE2	Green	1.98992	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:GLU411:OE2	H-Acceptor
N: UNK1: H - A: ALA354: O	Green	2.49815	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A: ALA354: O	H-Acceptor
N:UNK1:H - A:GLU384:OE2	Green	2.5431	Hydrogen Bond	Conventional Hydrogen Bond	N:UNK1:H	H-Donor	A:GLU384:OE2	H-Acceptor
N: UNK1: C - A: SER516: O	Green	3.58085	Hydrogen Bond	Carbon Hydrogen Bond	N:UNK1:C	H-Donor	A: SER516: O	H-Acceptor
A: ZN701: Zn - N: UNK1: O	Grey	2.61485	Other	Metal-Acceptor	A:ZN701:Zn	Metal	N: UNK1: O	H-Acceptor
A:VAL351 - N:UNK1	Purple	4.99333	Hydrophobic	Alkyl	A:VAL351	Alkyl	N:UNK1	Alkyl
A:TRP357 - N:UNK1	Purple	4.42583	Hydrophobic	Pi-Alkyl	A:TRP357	Pi-Orbitals	N:UNK1	Alkyl
A:TRP357 - N:UNK1	Purple	4.46087	Hydrophobic	Pi-Alkyl	A:TRP357	Pi-Orbitals	N:UNK1	Alkyl
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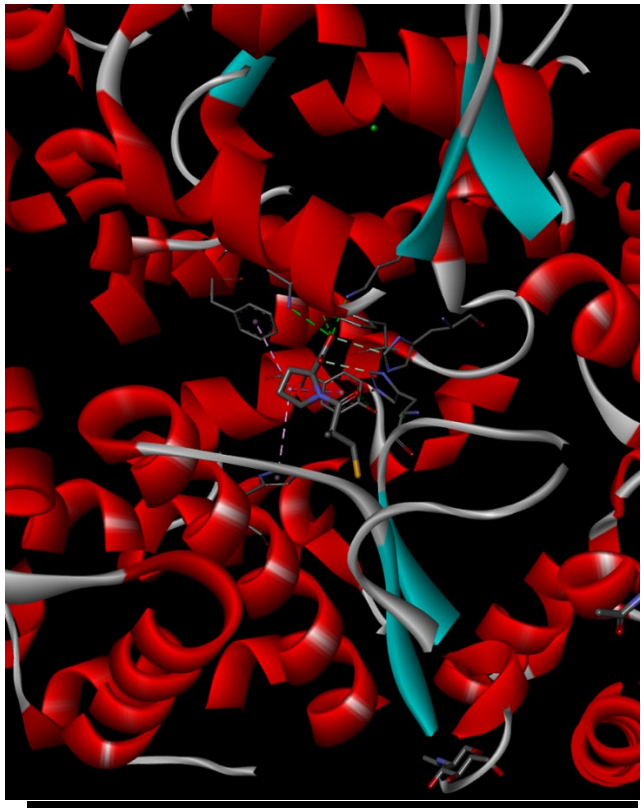
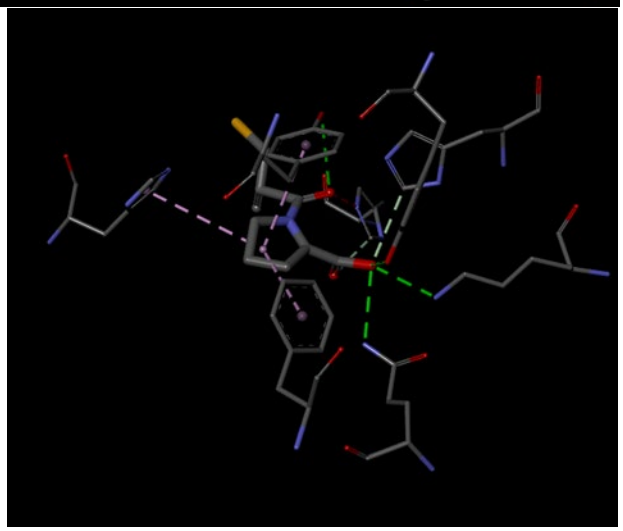
(d)





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A:GLN281:NE2 - A:MCO702:O2	Green	3.07382	Hydrogen Bond	Conventional Hydrogen Bond	A:GLN281:NE2	H-Donor	A:MCO702:O2	H-Acceptor
A: LYS368: NZ - A: HIS353: O	Green	2.7148	Hydrogen Bond	Conventional Hydrogen Bond	A:LYS368:NZ	H-Donor	A: HIS353: O	H-Acceptor
A: HIS383: N - A: VAL379: O	Green	2.93387	Hydrogen Bond	Conventional Hydrogen Bond	A:HIS383:N	H-Donor	A: VAL379: O	H-Acceptor
A: HIS383: N - A: VAL380: O	Green	2.98927	Hydrogen Bond	Conventional Hydrogen Bond	A:HIS383:N	H-Donor	A: VAL380: O	H-Acceptor
A:HIS383:ND1 - A:ASP415:OD1	Green	2.58083	Hydrogen Bond	Conventional Hydrogen Bond	A:HIS383:ND1	H-Donor	A:ASP415:OD1	H-Acceptor
A: GLY386: N - A: HIS383: O	Green	2.99023	Hydrogen Bond	Conventional Hydrogen Bond	A:GLY386:N	H-Donor	A: HIS383: O	H-Acceptor
A: PHE457: N - A: ASP453: O	Green	3.31829	Hydrogen Bond	Conventional Hydrogen Bond	A:PHE457:N	H-Donor	A: ASP453: O	H-Acceptor
A: PHE460: N - A: PHE457: O	Green	3.19714	Hydrogen Bond	Conventional Hydrogen Bond	A:PHE460:N	H-Donor	A: PHE457: O	H-Acceptor
A: SER461: N - A: PHE457: O	Green	2.786	Hydrogen Bond	Conventional Hydrogen Bond	A:SER461:N	H-Donor	A: PHE457: O	H-Acceptor
A: ARG468:NH1 - A: HIS513: O	Green	2.95001	Hydrogen Bond	Conventional Hydrogen Bond	A:ARG468:NH1	H-Donor	A: HIS513: O	H-Acceptor

A: LYS511: N - A: PRO508: O	Green	2.95861	Hydrogen Bond	Conventional Hydrogen Bond	A:LYS511:N	H-Donor	A: PRO508: O	H-Acceptor
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A:LYS511:NZ - A:MCO702:O2	Green	2.7296	Hydrogen Bond	Conventional Hydrogen Bond	A:LYS511:NZ	H-Donor	A:MCO702:O2	H-Acceptor
A: ILE514: N - A: LYS511: O	Green	3.122	Hydrogen Bond	Conventional Hydrogen Bond	A:ILE514:N	H-Donor	A: LYS511: O	H-Acceptor
A: SER517: N - A: HIS513: O	Green	2.86945	Hydrogen Bond	Conventional Hydrogen Bond	A:SER517:N	H-Donor	A: HIS513: O	H-Acceptor
A: VAL518: N - A: HIS513: O	Green	2.92662	Hydrogen Bond	Conventional Hydrogen Bond	A:VAL518:N	H-Donor	A: HIS513: O	H-Acceptor
A:TYR520:N - A:ASP465:OD2	Green	2.87542	Hydrogen Bond	Conventional Hydrogen Bond	A:TYR520:N	H-Donor	A:ASP465:OD2	H-Acceptor
A:TYR520:OH - A:MCO702:O2	Green	2.65723	Hydrogen Bond	Conventional Hydrogen Bond	A:TYR520:OH	H-Donor	A:MCO702:O2	H-Acceptor
A: TYR523: N - A: TYR520: O	Green	2.91276	Hydrogen Bond	Conventional Hydrogen Bond	A:TYR523:N	H-Donor	A: TYR520: O	H-Acceptor
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A: PHE527: N - A: TYR523: O	Green	3.01104	Hydrogen Bond	Conventional Hydrogen Bond	A:PHE527:N	H-Donor	A: TYR523: O	H-Acceptor

A: HIS353:CD2 - A: ALA354: O	Green	3.56757	Hydrogen Bond	Carbon Hydrogen Bond	A:HIS353:CD2	H-Donor	A: ALA354: O	H-Acceptor
A:HIS353:CE1 - A:MCO702:O3	Green	3.41253	Hydrogen Bond	Carbon Hydrogen Bond	A:HIS353:CE1	H-Donor	A:MCO702:O3	H-Acceptor
A:HIS383:CD2 - A:GLU384:OE2	Green	3.46531	Hydrogen Bond	Carbon Hydrogen Bond	A:HIS383:CD2	H-Donor	A:GLU384:OE2	H-Acceptor
A:HIS513:CE1 - A:HIS353:NE2	Green	3.56803	Hydrogen Bond	Carbon Hydrogen Bond	A:HIS513:CE1	H-Donor	A:HIS353:NE2	H-Acceptor
A:HIS513:CE1 - A:MCO702:O2	Green	3.30321	Hydrogen Bond	Carbon Hydrogen Bond	A:HIS513:CE1	H-Donor	A:MCO702:O2	H-Acceptor
A: PRO515: CD - A: LYS511: O	Green	2.99064	Hydrogen Bond	Carbon Hydrogen Bond	A:PRO515:CD	H-Donor	A: LYS511: O	H-Acceptor
A:LYS511:NZ - A:TRP279	Orange	4.56592	Electrostatic	Pi-Cation	A:LYS511:NZ	Positive	A:TRP279	Pi-Orbitals
A:LYS511:NZ - A:TRP279	Orange	4.25136	Electrostatic	Pi-Cation	A:LYS511:NZ	Positive	A:TRP279	Pi-Orbitals
A:SER461:CB - A:PHE457	Purple	3.7949	Hydrophobic	Pi-Sigma	A:SER461:CB	C-H	A:PHE457	Pi-Orbitals
A:HIS353 - A:PHE512	Pink	3.97506	Hydrophobic	Pi-Pi Stacked	A:HIS353	Pi-Orbitals	A:PHE512	Pi-Orbitals
A:HIS513 - A:PHE512	Pink	5.2462	Hydrophobic	Pi-Pi T-shaped	A:HIS513	Pi-Orbitals	A:PHE512	Pi-Orbitals
A:HIS513 - A:TYR520	Pink	4.56546	Hydrophobic	Pi-Pi T-shaped	A:HIS513	Pi-Orbitals	A:TYR520	Pi-Orbitals
A:TYR520 - A:TYR523	Pink	4.5206	Hydrophobic	Pi-Pi T-shaped	A:TYR520	Pi-Orbitals	A:TYR523	Pi-Orbitals
A:TRP279 - A:LYS511	Pink	4.09861	Hydrophobic	Pi-Alkyl	A:TRP279	Pi-Orbitals	A:LYS511	Alkyl
A:TRP279 - A:LYS511	Pink	5.17605	Hydrophobic	Pi-Alkyl	A:TRP279	Pi-Orbitals	A:LYS511	Alkyl

A:HIS383 A:MCO702	-	Pink	5.2964	Hydrophobic	Pi-Alkyl	A:HIS383	Pi-Orbitals	A:MCO702	Alkyl
A:PHE457 A:MCO702	-	Pink	5.35083	Hydrophobic	Pi-Alkyl	A:PHE457	Pi-Orbitals	A:MCO702	Alkyl
A:HIS513 A:VAL518	-	Pink	4.96896	Hydrophobic	Pi-Alkyl	A:HIS513	Pi-Orbitals	A:VAL518	Alkyl
A:TYR520 A:VAL464	-	Pink	4.20731	Hydrophobic	Pi-Alkyl	A:TYR520	Pi-Orbitals	A:VAL464	Alkyl
A:TYR523 A:MCO702	-	Pink	4.3695	Hydrophobic	Pi-Alkyl	A:TYR523	Pi-Orbitals	A:MCO702	Alkyl

CHAPTER 7. CRICKET PROTEIN HYDROLYSATES: INTESTINAL PERMEABILITY AND CELLULAR INFLAMMATORY RESPONSE

This chapter is under review:
Hall, F., & Liceaga, A.M. (2020). Cellular anti-inflammatory, antioxidant, and permeability of cricket peptides.

Abstract

The anti-inflammatory activity of cricket protein hydrolysate digesta (CPHD) obtained by hydrolysis with alcalase and stimulated gastrointestinal digestion was ascertained. This study is a continuation with sample CPHD-85 with strong in vitro antioxidant, DPP-IV and ACE inhibitory activity. For this purpose, CPHD was investigated for their anti-inflammatory capacity using an endothelial cell line (HUVEC) and an intestinal epithelial line (Caco-2). Effects CPHD on and secretion of monocyte chemoattractant protein 1, chemokine IL-8 (IL-8) and IL-6 and intercellular adhesion molecule-1 (ICAM-1 and VCAM) and COX-2 were evaluated stimulated inflamed cells. Upon TNF-α treatment, levels of MCP-1 and IL-8 were significantly reduced at 100 and 500 µg/mL. Pre-treatment with CPHD did not significantly decrease inflammation markers, IL-6 and COX-2 in epithelial cells when compared to IL-1β stimulated cells. The transport study revealed that some CPHD peptides (≥5%) are permeable with retained ACE and DPP-IV inhibitory activity. This suggests that metabolites produced in the course of intestinal absorption may also provide a protective effect against cellular dysfunction. Overall, these in vitro screening results, permeability and endothelial bioactivity, show the potential of cricket protein hydrolysates to be used as a functional food with anti-inflammatory properties.

7.1 Introduction

There is a growing interest in naturally sourced agents for the prevention and/or treatment of risk factors related to cardio vascular diseases such as chronic inflammation, hypertension and diabetes. In this context, several food-derived peptides with impact on cardiovascular health through their antihypertensive, antioxidant, anti-inflammatory, antidiabetic and even cholesterol attenuating effects have been produced by proteolysis of underutilized food and by-product

materials. Insect protein valuable resources for production of protein hydrolysates containing bioactive properties (Nongonierma & FitzGerald, 2017; Udenigwe & Aluko, 2012). Cricket protein hydrolysates (CPHD) digested with alcalase and gastrointestinal enzymes presented antioxidant, ACE and DPP-IV-inhibitory activity making them promising application as a functional food additive (Hall, Johnson, & Liceaga, 2018). However, their bioactivity and permeability within a cellular model has yet to be investigated. To date, insect protein hydrolysates and/or protein extracts have demonstrated modulatory effects against inflammatory reactions, through inhibition of the production of pro-inflammatory markers. For example, glycosaminoglycan (GAG) derived from cricket (Gryllus bimaculatus) reduced pro-inflammatory markers vascular endothelial growth factor (VEGF) in human umbilical vein endothelial cells (HUVEC), interleukin-6, prostaglandin E2-stimulated lipopolysaccharide in RAW 264.7 cells, and tumor necrosis factor (TNF)-α production in splenocytes (Ahn, Han, Hwang, Yun, & Lee, 2014) . Also, Hwang and their team reported protective effects from the same cricket species (Gryllus bimaculatus) protein extracts against acute alcoholic liver damage in mice. The authors associate such effects with suppression of ROS-mediated oxidative stress in the liver and small intestine (Hwang et al., 2019). Besides the existing efforts to describe the bioactive potential of insect-derived peptides, most in vitro studies do not consider their cellular activity and intestinal barrier permeation. Caco-2 epithelial and human umbilical vein endothelial cells have been extensively used to study the effects of bioactive ingredients in both normal and inflammatory conditions. In this regard, the aim of the present work was to evaluate the potential of CPHD to modulate the inflammatory response in epithelial cells and endothelial cells. The secretion markers (MCP-1, IL-6, IL-8, VCAM-1, and ICAM-1) were evaluated under inflammatory conditions induced. Additionally, the potential permeability of CPHD peptides were assessed.

7.2 Materials and methods

7.2.1 Chemicals

TNF-α purchased from Abcam, USA (ab9642), was used to induce inflammation in human umbilical vascular endothelial cells (HUVECs). The primary antibodies used for western blot and immunofluorescence experiments were as follows: anti-VCAM-1 (Abcam, ab134047, Lot # GR257919-53), anti-ICAM-1 (Santa Cruz, sc-8439, Lot #L1517), anti-E-selectin (Santa Cruz, sc-

137054, Lot #G2718), anti-β-actin (Abcam, ab6276, Lot #GR3270852-5). The secondary antibodies used for western blot were IRDye 800CW goat anti-rabbit IgG (#925-32211, Lot #C80718-11) and IRDye 680RD goat anti-mouse IgG (#925-68070, Lot #C80619-01) from LI-COR Biosciences, while for immunofluorescence were Alexa Fluor 488 (ab150113, Lot #GR3235892-2 and ab150077, Lot #GR3225145-2) and Alexa Fluor 594 (ab150116, Lot #GR3248932-1, and ab150080, Lot #GR3232361-2).

7.2.2 Endothelial monoculture

Human umbilical vein endothelial cells (HUVECs) were used in collaboration with Dr. Kaustav Majumder's FoodBioPepLAB at in the Department of Food Science and the University of Nebraska Lincoln following lab standardized protocol. First, the umbilical veins were flushed with buffer to remove blood clots and then cells were isolated with a type 1 collagenase-containing buffer. The cells were grown and maintained in a humidified environment at 36C with 5%CO2 in M199 buffer with phenol red supplemented with 20% FBS and L-Glutamine (Invitrogen, ThermoFisher), penicillin-streptomycin (Life Technologies) and 1% endothelial cell growth supplement. HUVEC cells within the passages 2–5 were used in this study.

7.2.3 Epithelial monoculture

Caco-2 (ATCC HTB-37) cells were grown in Eagle's minimum essential medium (EMEM; ATCC 30-2003) supplemented with 20% FBS and 1% penicillin–streptomycin (Gibco) at 37 °C and 5% CO2 in a humidified environment. Cells were grown for 6 days until they were confluent (80%) and then sub-cultured and used for further experiments. The cells used for absorption study were then grown for 21 days until they differentiated, and the cell medium was changed every other day. Caco-2 cells within the passages 22–25 were used in this study.

7.2.4 Caco-2 cell-permeability assay

The transepithelial transport of CPHD peptides across Caco-2 cell monolayers was conducted at the Plants for Human Health Institute at North Carolina State University in the lab of Dr. Mario Ferruzzi. Protocols used followed previously described methods standardized by this lab (Moser et al., 2016). DMEM (without FBS and phenol red) was used as a transport medium in the

permeability study. CPHD was reconstituted with the same DMEM to attain a final concentration of 0.5 mg/ml. Fully differentiated cell monolayers were gently washed twice with the transport medium in both the apical and basolateral compartments. After washing, cells were equilibrated with the transport medium for 1 h at 37 °C in 5% CO2 in a humidified incubator. After measuring TEER values, 150 µL of the sample (0.5 mg/ml in transport medium) were added to the apical compartment, and 700 µL of medium were added to basolateral compartment. The cell culture plates were incubated for a total of 4 hr in the humidified incubator. Permeates in the basolateral compartment were collected at desired time intervals (0, 0.5, 1, 2, and 4 hr) and replaced with transport media. The permeability of transport was expressed as the % (w/w) of peptides transported from the apical to basolateral layer. The collected basolateral solutions of the CPHD and were lyophilized and the determination of antioxidant, DPP-IV, and ACE inhibitory activity. Peptide concentrations were determined by using the BCA protein Assay kit (Thermo Fisher Scientific, Burlington, ON, Canada). Chromatograms of the samples before and after transport were analyzed using an Acquity BEH C18 column on a reverse-phase HP-liquid chromatography (RP-HPLC) system. Samples (50 µL) were eluted with the gradient dd-H2O and ACN with 0.1% TFA at 0.5 mL/min as follows: 1% B (0–5 min) and 1–50% B (5–30 min); absorbance was monitored at 220-280 nm.

7.3 Determination of anti-inflammatory activity

7.3.1 Adhesion molecule expression (ICAM-1 and VCAM-1) in HUVECs

Adhesion molecule expression in HUVEC cells were detected as described by Majumder et al. (Majumder, Chakrabarti, Davidge, & Wu, 2013). Cells grown in 24-well plates were pre-treated with the cricket protein hydrolysate digesta (CPHD) at three concentrations (10, 100, and 500 µg/ml) for 2 hr. Following pre-incubation, cells were treated with TNF (5ng/mL) for another 6 hr. Expression of adhesion molecules, ICAM-1 and VCAM-1, were determined using western blotting techniques and respective antibodies. After pre-treatment with CPHD, cells were stimulated for 6 hr with TNF. The cells were lysed with boiling hot buffer (2x Laemmli buffer with 0.2% Triton-X-100 and DTT). Samples were ran on a 4-20% SDS-PAGE (Bio-Rad), transferred to a nitrocellulose membrane and bands of interest were detected by specific antibodies. The membranes were blocked with Odyssey blocking buffer in TBS for 1 hr then incubated overnight

at 4 °C with primary antibodies at their recommended dilutions. The next day, the membranes were washed 3x with TBST and incubated with the secondary antibodies IRDye 800CW goat anti-rabbit and/or IRDye 680RD goat anti-mouse IgG, depending on the host of the primary antibody used, at a concentration of 1:10,000 at RT for 1 hr. After a final wash, the proteins were detected under fluorescent green and red channels in an Odyssey CLx imaging system (Li-Cor Biosciences). The protein expression was quantified using Image Studio software from Li-Cor, and the expression was normalized using β-actin as the loading control. Bands for ICAM-1 (mouse monoclonal antibody; Santa Cruz Biotechnologies) and VCAM-1 (rabbit polyclonal antibodies; Santa Cruz Biotechnologies) were normalized with Actin (rabbit polyclonal antibody; Abcam, Cambridge, MA). The protein bands were detected by a Li-cor Odyssey Bio-Imager and analyzed by densitometry. Cell lysates from none treated-control cells (NTC) were loaded on every gel and all data expressed as fold change over the untreated control.

7.3.2 Western blot analysis of cyclooxygenase-2 (COX-2) protein expression in Caco-2

Expression level of COX-2 was detected by western blot analysis, as previously described (Majumder, Chakrabarti, Davidge, & Wu, 2013; (Majumder et al., 2013). After stimulation pre-treatment with CPHD (12 hrs) and IL-1β stimulation (24 hrs), the culture medium was removed, and the whole cell lysate was prepared using boiling hot Laemmli's buffer containing 0.2% Triton X-100 and 50 μM dithiothreitol (DTT) as a reducing agent. The cell lysates were analyzed as described above. Subsequently, the membrane was incubated with rabbit polyclonal anti-COX-2 (1:2000 dilution) and anti-actin antibody (1:5000 dilution) (Abcam, Cambridge, MA, USA) as a loading control (Majumder et al., 2013). The protein bindings were detected using the fluorochrome-conjugated anti-rabbit secondary antibodies (1:10000 dilution) (Licor Biosciences, Lincoln, NB, USA). Protein bands were detected by a Licor Odyssey Bio-Imager and densitometry was performed using the Licor Image Studio Lite western blot analysis software (Licor Biosciences, Lincoln, NB, USA). COX-2 bands were normalized the levels of actin. The results were expressed as fold change over the positive control (IL-1β treatment alone).

7.3.3 Enzyme-Linked Immunosorbent Assay

Cell-free supernatant media from the HUVEC and Caco-2 inflammation studies were used to quantify the levels of monocyte chemoattractant protein-1 (MCP-1), IL-6, and IL-8 through ELISA. ELISAs were performed using specific kits for human CCL-2/MCP-1 (Thermo Scientific, #88-7399-88), IL-6 (#88-7066-880), and IL-8 (#88-8086-88) by following the manufacturer's guidelines. 96-well high-affinity protein-binding plates (Corning 9018) were coated with 100 µL of the capture antibody (1:250, v/v) diluted in 1× phosphate-buffered saline (PBS) and incubated overnight at 4 °C. Plates were then washed five times with wash buffer (1× PBS, 0.05% Tween) and blocked with 200 µL of 1× ELISA diluent for 1 h at room temperature on an orbital shaker. Plates were again washed five times, followed by the addition of 100 µL of standards and samples and overnight incubation at 4 °C. The following day, plates were washed five times and incubated with 100 µL of the detection antibody (1:250) diluted in 1× ELISA diluent for 1 h at room temperature. After washing, plates were incubated with 100 µL of the horse-radish peroxidase enzyme (1:250, v/v) diluted in 1× diluent for 30 min at room temperature. Finally, after a final wash, plates were incubated with 100 µL of tetramethylbenzidine substrate solution in the dark for 15 min at room temperature. The reaction was stopped with the addition of 50 µL of 1 M H2SO4, and the absorbance was measured at 450 nm. The concentrations of the cytokines and chemokine were quantified from the standard curves.

7.3.4 Statistical Analysis

All data presented were mean values ± SEM of four to eight independent experiments. Statistical analyses were performed using one-way or two-way analysis of variance. Differences with p values < 0.05 considered significant. Graphs and analysis for this study were done using GraphPad PRISM 6 statistical software (Graph Pad Software, San Diego, CA).

7.4 Results

7.4.1 Epithelial and Endothelial inflammatory response

Upon TNF-α treatment, levels of MCP-1, IL-8, ICAM-1 and VCAM-1 were significantly increased compared with the none-treated control (NTC). (Figures 6.1-6.3). HUVEC monolayers were pretreated with 10,100, or 500 µg/mL of CPHD did not excerpt any significant effect of TNF-

induced increased expression of ICAM-1 (Figure 6.2). Similarly, TNF stimulation significantly increased VCAM expression in endothelial cells. However, pretreatment with CPHD did not reduce the TNF-stimulated expression of VCAM at any concentration. A time-course study would reveal if longer incubation times are necessary to observe significant effects, as demonstrated in other food-derived bioactive peptides (Kleiveland, 2015). Although CPHD did not exert any effects on intercellular adhesion molecule expression, there were significant changes in the secretion of monocyte chemoattractant protein 1 (MCP-1) and chemokine IL-8 (IL-8) when HUVEC cells were stimulated with TNF-α. Both IL-8 and MCP-1 were significantly increased in cells stimulated with CPHD compared to NTC ($p<0.05$), but not in cells pretreated with CPHD. IL-8 expression remained that same after stimulation when pretreated with10 ug/mL of CPHD ($p>0.05$; Figure 6.3). Whereas higher concentrations (100 and 500 ug/mL) did inhibit some expression IL-8. Similar effects were observed with MCP-1 levels; however, greater inhibitory effects were seen when HUVEC cells were pretreated with 100 ug/mL CPHD (Figure 6.3). For both molecules, there were minimal dose-dependent activity observed between 100 and 500 ug/mL. However, the epithelial cellular response to CPHD observed in this study is the result of a 2-hour pre-treatment before TNF-stimulation. The anti-inflammatory effects may be time dependent and should be optimized.

To further analyze the anti-inflammatory effect of CPHD, the expression of COX-2 and IL-6 in Caco-2 epithelial cells induced to a pro-inflammatory state with IL-1β. As revealed in Figure 6.2a-b, stimulation of Caco-2 cells with IL-1β increased both COX-2 and IL-6 expression. Interestingly, pre-treatment with CPHD did not any significant effects on epithelial inflammatory response after a 12-hour incubation.

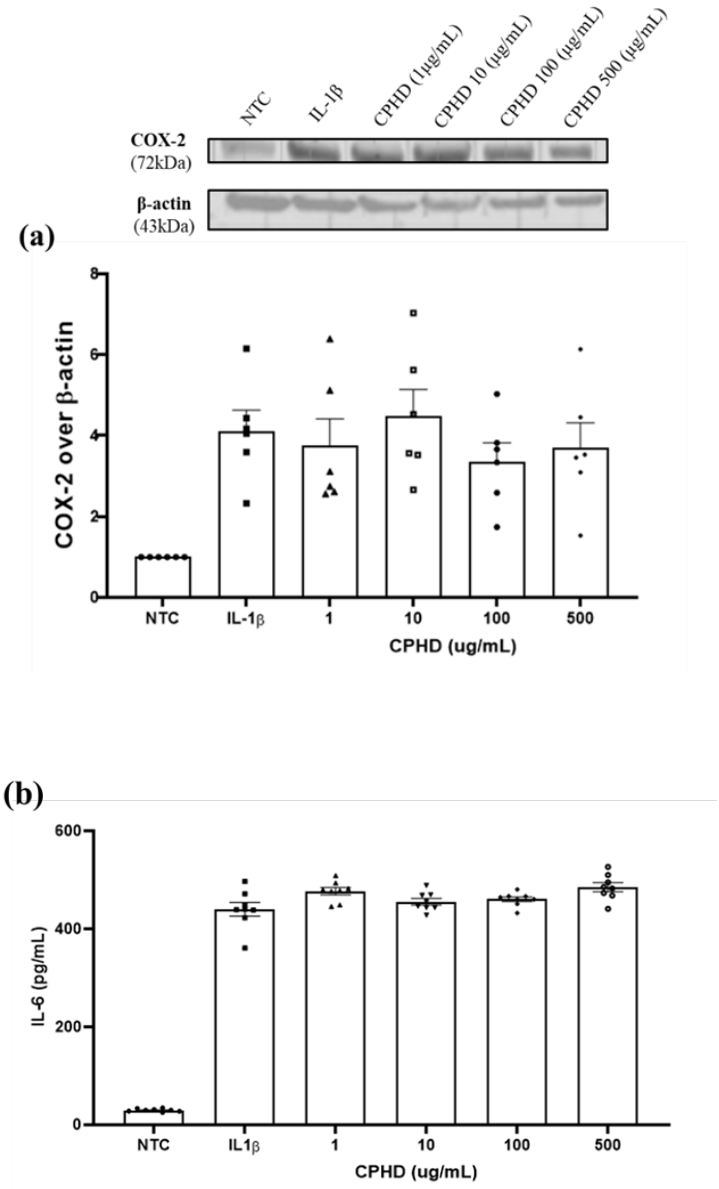

Figure 7.1. The expression levels of (a) COX-2 and (b) IL-6 measured in Caco-2 cell lysates (COX-2) or media (IL-6) obtained after 12 hr of pretreatment with CPHD (1, 10, 100, or 500 μg/mL, as indicated). IL-1β (5 ng/mL) was used as a positive inflammatory stimulator. Data for COX-2 levels are expressed as the fold change of protein expression for calculated by optical densitometry with respect to β-actin. IL-6 concentration was determined by ELISA analysis of cell media. All data is expressed as mean values ± SEM of 8 independent experiments.

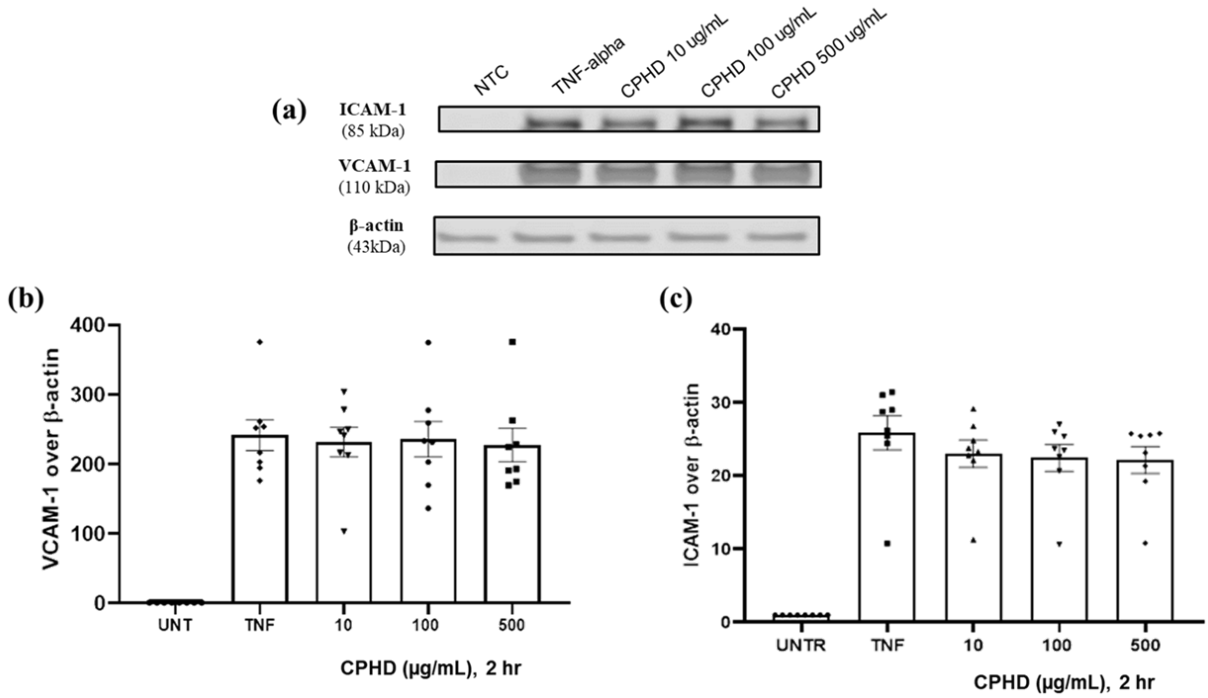

Figure 7.2. The expression levels of VCAM-1 and ICAM-1 measured by western blot in HUVEC cell lysates obtained after 2 hr of pretreatment with CPHD (10, 100, or 500 µg/mL, as indicated). TNF-α (5 ng/mL) was used as a positive inflammatory stimulator. (a) Representative blots for VCAM-1, ICAM-1 and β-actin bands. Data is expressed as the fold change of protein expression for (b) VCAM-1 and (c) ICAM-1 calculated by optical densitometry with respect to β-actin and expressed as mean values ± SEM of 8 independent experiments.

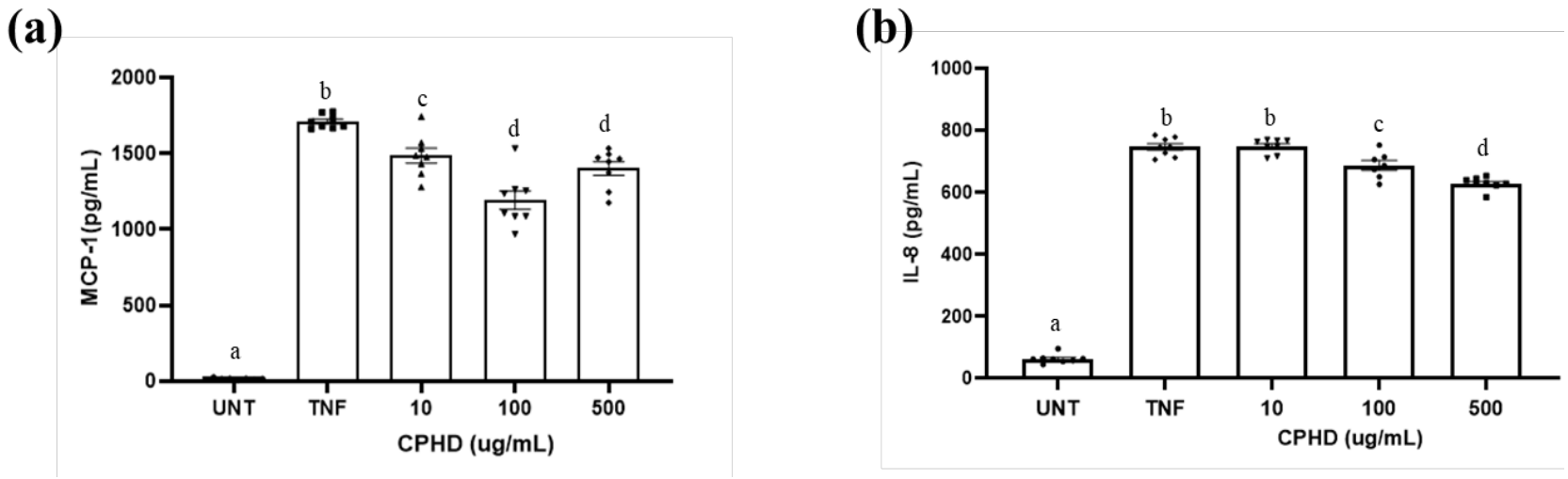

Figure 7.3. Cytokine concentrations of (a) MCP-1 and (b) IL-8 quantified by ELISA in HUVEC cell media obtained after 2 hr of pretreatment with CPHD (10, 100, or 500 μg/mL, as indicated). TNF-α (5 ng/mL) was used as a positive inflammatory stimulator. Data is expressed as the concentration (pg/mL) of mean values ± SEM of 8 independent experiments.

7.4.2 Effects of CPHD on Caco-2 cell viability

The cytotoxicity of Caco-2 cells was evaluated after a 12 hr exposure to different concentrations (0.5-5 mg/mL) of CPHD. The results suggest that concentrations higher than 1 mg/mL increases toxicity for the cells (viability < 80%) compared to DMSO. Based on these results, 0.5 mg/mL of CPHD was used for the transport study. Caco-2 incubation with 0.5 mg/mL CPHD did not cause any significant decrease in cell viability (Figure 6.4) and in vitro bioactivity was previously demonstrated at this concentration (see CHAPTER 3).

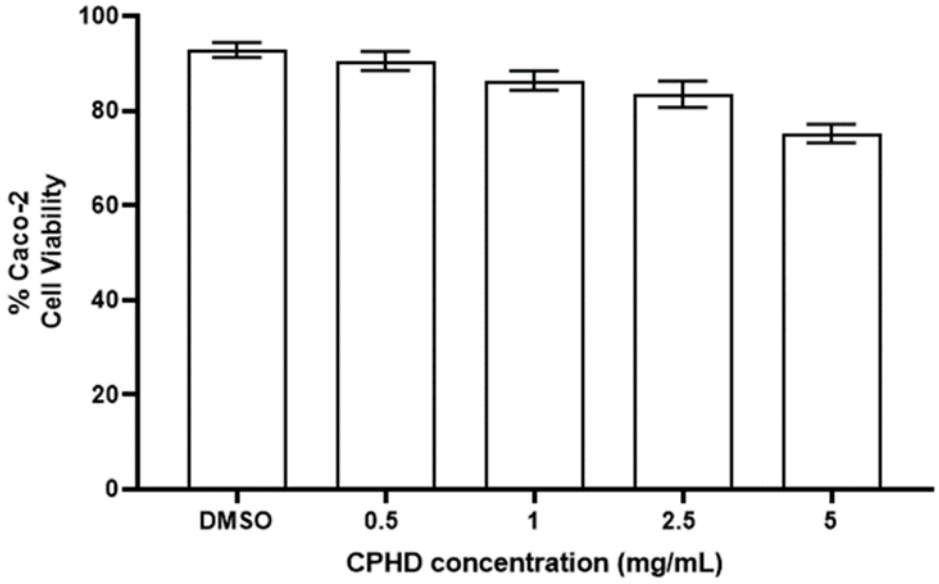

Figure 7.4. Effects of different concentrations of CPHD (0.5-5 mg/mL) on Caco-2 cell viability. Data is expressed at % cell viability means ± SEM of eight independent experiments.

7.4.3 CPHD transport and bioactivity of permeate

An aliquot of the basolateral material was collected at various time periods (0, 0.5, 1, 2, and 4 hrs) after adding CPHD (0.5 mg/mL) to the apical layer. Permeability % was calculated as the percentage of peptides that transported from the apical to basolateral Caco-2 monolayer. Figure 6.5a displays the % permeability of peptides measured at the desired time intervals. After 0.5 hrs, <1% peptides from CPHD were transported across the monolayer. The number of permeable peptides increased significantly ($p < 0.01$) after 1, 2, and 4 hrs with a maximum of 6.3% permeability. After concentrating the collected permeates, their ACE and DPP-IV inhibitory and ABST antioxidant activity were assessed for each time point. As demonstrated in Figure 6.5., there was no activity at 0 hr confirming that CPHD peptides did not immediately diffuse from the apical to basolateral layer. After 0.5 hrs, however, bioactivity began to be measurable. The activity (DPP-IV, ACE, and ABST continued to increase ($p < 0.05$) after 1 and 2 hours suggesting that CPHD peptides were transporting across the monolayer over time. At the final collection, after 4 hrs, ACE and DPP-IV inhibiting capacity were even greater compared to permeate collected after 2hr (Figure 6.5 b). Antioxidant activity, however, did not significantly improve from 2 to 4-hour incubation.

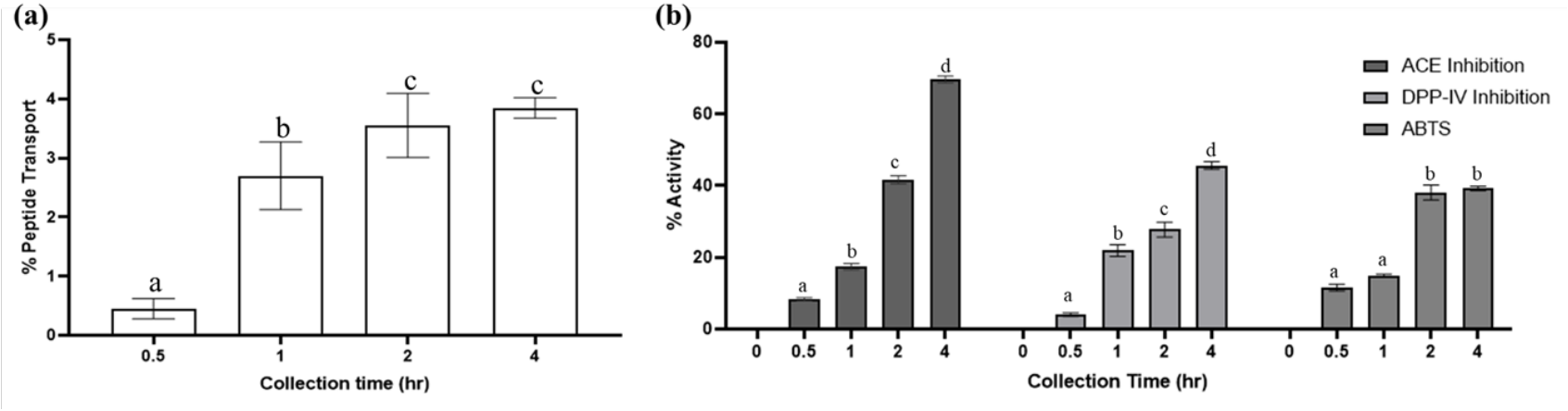

Figure 7.5. % Peptide Transport (a) and ACE, DPP-IV inhibitory and ABTS activity % (b) of Caco-2 permeates collected at 0.5, 1, 2, and 4 hours. Aliquots of the basolateral layer were removed at the desired time points (0-4 hours). The apical layers were also collected after 4 hours and analyzed. Results are means of four (n=6) independent experiments. Different letters represent a significant difference ($p < 0.05$) between time periods.

7.5 Discussion

The aim of this study was to investigate CPHD prepared by hydrolysis from an edible cricket species on inflammation associated with endothelial and epithelial dysfunction, an intestinal transport model served to evaluate the bioactivity of permeable peptides present within CPHD. This CPHD was previously characterized detailing conditions responsible for the antioxidant, DPPIV and ACE-inhibitory bioactivities observed (Hall et al., 2018). CPHD was desalted using solid phase extraction which is the common strategy to desalt and concentrate protein and peptide samples. This technique not only removes the excess of salts, but also increases the relative abundance of low MW peptides within the hydrolysate, which might be responsible for the bioactive properties. CPHD is a complex mixture of different compounds (mainly peptides, phenolic compounds, nucleotides, vitamins) that can all contribute to the biological activities, therefore desalting and concentration of CPHD was a crucial step. Moreover, the removal of the salt was necessary to maintain ideal cell conditions.

Secretion of endothelial markers under inflammatory and non-inflammatory conditions were assessed in in epithelial and endothelial cells. Upon TNF-α-induced endothelial inflammation, CPHD pre-treatment significantly ($p < 0.05$) suppressed the MCP-1 and IL-8 levels in an independent of dose. Treatment with 100 and 500 ug/mL CPHD prompted the largest decrease in the secretion of these inflammation markers. Considering that these effects were observed after a 2-hour pre-treatment, longer incubation periods may result in a great inhibition of inflammatory markers. Other food-derived bioactive peptides displayed maximum effects after 10 to 20-hour pre-treatments. Egg peptide IRW and IQW, for instance, exhibited minimal changes after 2, 4, and 6-hour pre-treatments but improved effects after 10 and 20 hours (Majumder et al., 2013). This was not the case for IL-1β induced epithelial inflammation. Elevated levels of cyclooxygenase-2 (COX-2) and IL-8 expression are primarily associated with chronic intestinal inflammation. CPHD did not exert major significant changes after a 20-hour pre-incubation before stimulation. This may indicate that while the transport study demonstrated permeability of CPHD peptide through the Caco-2 monolayer, they may not have any therapeutic benefits towards the inflammatory cascade present during chronic inflammatory conditions such as inflammatory bowel disease (IBD) and Crohn's disease (CD) (Majumder, Mine, & Wu, 2016).

In terms of cellular transport and potential bioavailability, the transport study demonstrated that some peptides within CPHD could successfully cross the Caco-2 monolayer and retain its

bioactivity. After 4 hours, permeates exhibited antioxidant, ACE, and DPP-IV inhibitory capacity; the activity was significantly greater than that observed at 0-2 hours (Figure xx). However, antioxidant capacity of permeates were much less than those previously seen with the entire CPH or CPHD. The permeability values, observed in this study, were considerate to represent moderate permeability (Deferme, Annaert, & Augustijns, 2008). Although studies regarding insect bioactive peptides are increasing, there are currently no known reports of their permeability or bio accessibility. The values seen in this study are, however, better or comparable to other food-derived hydrolysates and peptides. For example, protein hydrolysates from spent hens (by-product of the egg industry) exhibit 3.87 % (expressed as the % of peptides before and after transport, permeability after a 4hr incubation with Caco-2 monolayers (Fan, Yu, Liao, & Wu, 2020). Similar results were also reported for zein protein hydrolysates in vitro digests which showed ≤ 1.5 % permeable peptides after 4 hrs (Liang, Chalamaiah, Ren, Ma, & Wu, 2018). Bioactive peptides typically have an absorption rate less than 1%, depending on peptide length and interaction with intestinal cell transporters (Xu, Hong, Wu, & Yan, 2019). A relatively higher permeability % by CPHD is likely due to a greater proportion of low molecular weight peptides resulting from the multistep digestion with aclalase, then pepsin, and pancreatin. This assumption was later supported by the existence of more than half of peptides in CPHD being < 1 kDa (Figure 7.1). The transport process enhanced ACE and DPP-IV inhibition, indicating that further degradation during transport may have occurred (Lacroix et al, 2017). Peptidases in the Caco-2 monolayer which may contribute to the formation of new peptides during the transport over time. However, it should be noted that Caco-2 monolayers has lower carrier expression, secreting cells, and lower expression of uptake transporters compared to human intestinal tissue (Deferme et al., 2008).

Nevertheless, future studies should aim to characterize the composition of CPHD permeate after Caco-2 cellular transport model, in terms of intact (or new) peptides which may impact differentially the inflammatory markers production and the endothelial inflammatory gene expression. Additionally, given the complexity and diversity of the inflammatory response, further evaluation using different cellular and animal models is needed to validate the anti-inflammatory properties. Overall, the findings obtained in this work indicate that CPHD can potentially operate through an inhibitory regulation of the inflammatory compounds in endothelial cells.

7.6 Conclusion

The findings of this study demonstrated that in addition to the antioxidant and ACE and DPP-IV-inhibitory activities reported in previous work, cricket protein hydrolysates prepared with the commercial enzyme alcalase exhibited anti-inflammatory activity in an endothelial cell model. Further, a transport study illustrated that some peptides with retained bioactivity from CPHD successfully permeated across the Caco-2 monolayer over a period of 4 hours. Additionally, results suggested that CPHD may be able to attenuate expression levels of IL-8 and MCP-1 under pro-inflammatory conditions, although there were no significant effects on adhesion molecules ICAM-1 and VCAM-1 all while sustaining cellular viability. There were also no significant outcomes towards epithelial inflammatory responses in IL-6 and COX-2 expression. To the best of our knowledge, this is the first report of cellular anti-inflammatory activity exhibited by a cricket protein hydrolysate. These preliminary observations indicate that CPHD hydrolysate could be used as a therapeutic agent towards treatment of prevention of inflammatory-related diseases. However, further studies are needed to fully explore optimize test conditions (i.e. Concentration and incubation time) and anti-inflammatory effects in vivo.

7.7 References

Ahn, M. Y., Han, J. W., Hwang, J. S., Yun, E. Y., & Lee, B. M. (2014). Anti-inflammatory effect of glycosaminoglycan derived from Gryllus bimaculatus (a type of cricket, insect) on adjuvant-treated chronic arthritis rat model. Journal of Toxicology and Environmental Health, Part A, 77(22-24), 1332-1345.

Deferme, S., Annaert, P., & Augustijns, P. (2008). In vitro screening models to assess intestinal drug absorption and metabolism Drug absorption studies (pp. 182-215): Springer.

Fan, H., Yu, W., Liao, W., & Wu, J. (2020). Spent Hen Protein Hydrolysate with Good Gastrointestinal Stability and Permeability in Caco-2 Cells Shows Antihypertensive Activity in SHR. Foods, 9(10), 1384.

Hall, F., Johnson, P. E., & Liceaga, A. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. Food chemistry, 262, 39-47.

Hwang, B. B., Chang, M. H., Lee, J. H., Heo, W., Kim, J. K., Pan, J. H., . . . Kim, J. H. (2019). The edible insect Gryllus bimaculatus protects against gut-derived inflammatory responses and liver damage in mice after acute alcohol exposure. Nutrients, 11(4), 857.

Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., ... & Wichers, H. (2015). The impact of food bioactives on health: in vitro and ex vivo models (p. 338). Springer Nature.

Lacroix, I. M., Chen, X. M., Kitts, D. D., & Li-Chan, E. C. (2017). Investigation into the bioavailability of milk protein-derived peptides with dipeptidyl-peptidase IV inhibitory activity using Caco-2 cell monolayers. Food & function, 8(2), 701-709.

Liang, Q., Chalamaiah, M., Ren, X., Ma, H., & Wu, J. (2018). Identification of new anti-inflammatory peptides from zein hydrolysate after simulated gastrointestinal digestion and transport in caco-2 cells. Journal of agricultural and food chemistry, 66(5), 1114-1120.

Majumder, K., Chakrabarti, S., Davidge, S. T., & Wu, J. (2013). Structure and activity study of egg protein ovotransferrin derived peptides (IRW and IQW) on endothelial inflammatory response and oxidative stress. Journal of agricultural and food chemistry, 61(9), 2120-2129.

Majumder, K., Mine, Y., & Wu, J. (2016). The potential of food protein-derived anti-inflammatory peptides against various chronic inflammatory diseases. Journal of the Science of Food and Agriculture, 96(7), 2303-2311.

Moser, S., Lim, J., Chegeni, M., Wightman, J. D., Hamaker, B. R., & Ferruzzi, M. G. (2016). Concord and Niagara grape juice and their phenolics modify intestinal glucose transport in a coupled in vitro digestion/caco-2 human intestinal model. Nutrients, 8(7), 414.

Nongonierma, A. B., & FitzGerald, R. J. (2017). Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review. Innovative Food Science & Emerging Technologies, 43, 239-252.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: production, processing, and potential health benefits. Journal of food science, 77(1), R11-R24.

Xu, Q., Hong, H., Wu, J., & Yan, X. (2019). Bioavailability of bioactive peptides derived from food proteins across the intestinal epithelial membrane: A review. Trends in food science & technology, 86, 399-411.

CHAPTER 8. CONCLUSION AND FUTURE DIRECTIONS

8.1 Overall Conclusions

Cardiovascular diseases (CVD) and its risk factors are currently major causes of mortality and morbidity (Mensah et al., 2019). Hypertension and Type-2 Diabetes are well-defined risk factors that contribute to the development of CVD (Mensah et al., 2019). Treatment and prevention of these diseases are associated with major life-style changes such as diet and exercise. Under extreme conditions, pharmaceutical drugs are employed to control hypertensive and hyperglycemic states. However, commercially available drugs used to treat these diseases are very costly and linked with adverse side effects. Thus, bioactive food compounds and functional foods are gaining recognition as a complement therapy for prevention and management of diseases over the past few decade (Alkhatib et al., 2017; Asgary et al., 2018). Food-derived bioactive peptides have been shown to regulate physiological functions and can exert beneficial health properties. Thus, bioactive peptides are considered a lead compound ingredient to develop nutraceuticals and functional foods (Chakrabart et al., 2018). Various food-derived peptides have demonstrated mitigating effects towards oxidative, hypertensive, inflammatory, and glycemic disease pathways (Lorenzo et al., 2018; Lammi et al., 2018).

The main objective of the present research was to evaluate the efficacy, *in vitro*, of edible cricket peptides in physiologically relevant systems related to cardiovascular disease progression. This work also contributed to understanding the effect of the peptide production process on known cross-reactivity between insect protein and crustacean allergens, namely tropomyosin. Ultimately, results of these works will help to promote development of insect-containing foods by improving knowledge and understand of their bioactive and allergenic potential

The key findings of this project are outlined below:

1. The efficacy of bioactive peptides is dependent on the extraction conditions used to liberate active peptides within the parent protein. The aim of studies 1, 4, and 5 were to generate bioactive peptides from an edible cricket species. Cricket proteins were successfully hydrolyzed using the commercial enzyme alcalalse to yield peptide mixtures with biologically relevant properties. The peptides products with the lowest molecular weight peptides (CPH)

demonstrated *in vitro* antioxidant activity as well as the ability to inhibit the angiotensin converting enzyme, dipeptidyl peptidase-4 activity, alpha-amylase, and alpha-glucosidase. Pre-treatment with these cricket peptides inhibited MCP-1 and IL-8 levels in endothelial cells stimulated with TNF-α, while there were no changes in ICAM-1/VCAM-1 regulation under inflammatory conditions. Even further, NF-κB levels were lower in LPS-induced macrophage cells when cricket peptides were present. CPH peptides after a gastrointestinal digestion demonstrated transport ability ≥5% across Caco-2 monolayers with retained ACE and DPP-IV activity. After sequential fractionation, hydrophobic and cationic peptides below >0.6 kDa were identified as those responsible for the bioactivity observed within the complex mixture. The final fraction was verified by reducing ($p<0.05$) α-amylase, α-glucosidase, and angiotensin converting enzyme (ACE) activity *in vitro*, as well as inhibited the expression of NF-κB in RAW 264.7 macrophage cells. Proteomics affirmed the sequence of 28 peptides within the final fraction and three, YKPRP, PHGAP, and VGPPQ were chosen for molecular docking studies with the angiotensin converting enzyme. PHGAP and VGPPQ exhibited a higher degree of non-covalent interactions with the enzyme active site residues and binding energies comparable to captopril. Results from these studies demonstrate the bioactive potential of edible cricket peptides, especially as ACE inhibitors.

2.Evidence has confirmed that cricket protein can cross-react with common crustacean allergens. Enzymatic hydrolysis and microwave treatments are being considered as approaches to produce hypoallergenic peptides for food applications. In studies 2 and 3, we observed positive tropomyosin reactivity from cricket protein to IgE from shrimp-allergic patients. Allergenic responses were altered after enzymatically hydrolyzing the protein to varying degrees of hydrolysis (DH). Tropomyosin-IgE binding decreased as DH values increased with almost no response in cricket protein >60% hydrolyzed. Unexpectedly, tropomyosin-IgE reactivity was higher in samples with 50% DH than those with 10-40% or >50% DH. To examine this further, cricket proteins were hydrolyzed using microwave processing to obtain 50% DH. Microwave-treated samples exhibited a decreased IgG-tropomyosin response (4.6 ng/mL) compared to non-microwave treated hydrolysates (51.4 ng/mL). Further, Raman spectroscopy was used to elucidate tropomyosin structural changes induced by different treatment conditions. Pure tropomyosin, tropomyosin extracted from shrimp and whole crickets, all showed distinct peaks at 1647 nm (amide I), 1097 nm (C-H stretching), 782 nm (associated with the microenvironment

of Trp), and 555 nm (S-S trans-gauche-trans region). Shift in Raman bands, decreased peak intensity or absence of these bands were observed in the microwave-treated cricket protein, correlating to the decreased IgG reactivity. Isolating tropomyosin rich extracts from both microwave and conventionally produced hydrolysates revealed that differences in IgE binding, between these two samples, are likely due to distinct proteolytic peptide fragments present within the hydrolysate mixture instead of decreased solubility and extractability of the antigen. Proteomics and immunoinformatics revealed prominent epitope regions of tropical banded crickets (*Gryolles sigillatus*) available for cleavage during hydrolysis. We hypothesize that enzymatic hydrolysis combined with microwave heating cleaved linear epitopes which translated to lower IgE binding. These results demonstrate that common processing techniques can alter the allergenic properties of cricket proteins and should continue to be investigated.

8.2 Recommend Work

Future studies targeting functional food development using cricket peptides should include:

1. While the present study demonstrates potential antihypertensive, anti-inflammatory, and anti-glycemic properties of cricket protein hydrolysates, *in vitro*, further research is undoubtedly required to verify these findings and elucidated the mechanisms involved.

a. Studies involving *in vivo* systems, such as small rodents and *Caenorhabditis elegans*, are required to determine the preventive effects, if any, of these peptides. In particular, the antihypertensive capacity of the hydrolysates should be determined with mechanisms elucidated as to whether activity is primarily toward regulatory enzymes (ACE, renin, etc) or indirectly via inflammatory cascades.

b. It would also be valuable to study the bioavailability and bio-accessibility of the cricket peptides both of the raw peptides and within a food matrix.

2. Product development studies should determine appropriate food matrices (i.e. baked product, beverage, or ingredient powder) should be used in which cricket protein hydrolysates can be supplemented. It is critical to determine how cricket peptides can interact within food matrices.

a. Descriptive sensory evaluation would be useful to determine, not only consumer acceptance of a cricket-containing food, but also if the extensive proteolysis used generated any bitter peptides that would negatively impact its overall feasibility.

3. Finally, a quantitative analysis of the cricket allergens of the hydrolysates alone and within a food matrix would help support of the overall risk assessment of cricket peptides as a food ingredient.
    a. For analytical methods such as IgE-binding and proteomics, finding the optimal buffer and digestion conditions to detect cricket-allergen markers would contribute to detection and diagnostic analyses.

Findings of these studies will continue to support research of edible cricket peptides, their therapeutic actions and promote acceptance and commercialization of insect-containing foods.

8.3 References

Mensah, G. A., Roth, G. A., & Fuster, V. (2019). The global burden of cardiovascular diseases and risk factors: 2020 and beyond.

Alkhatib, A., Tsang, C., Tiss, A., Bahorun, T., Arefanian, H., Barake, R., ... & Tuomilehto, J. (2017). Functional foods and lifestyle approaches for diabetes prevention and management. Nutrients, 9(12), 1310.

Asgary, S., Rastqar, A., & Keshvari, M. (2018). Functional food and cardiovascular disease prevention and treatment: A review. Journal of the American College of Nutrition, 37(5), 429-455.

Chakrabarti, S., Guha, S., & Majumder, K. (2018). Food-derived bioactive peptides in human health: Challenges and opportunities. Nutrients, 10(11), 1738.

Lorenzo, J. M., Munekata, P. E., Gomez, B., Barba, F. J., Mora, L., Perez-Santaescolastica, C., & Toldra, F. (2018). Bioactive peptides as natural antioxidants in food products–A review. Trends in food science & technology, 79, 136-147.

Lammi, C., Aiello, G., Boschin, G., & Arnoldi, A. (2019). Multifunctional peptides for the prevention of cardiovascular disease: A new concept in the area of bioactive food-derived peptides. Journal of Functional Foods, 55, 135-145.