CRICKET (*ACHETA DOMESTICUS*) PROTEIN HYDROLYSATES: FUNCTIONAL PROPERTIES AND APPLICATION IN A FOOD MATRIX

by

Gabriela Calzada Luna

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STATEMENT OF COMMITTEE APPROVAL

Dr. Andrea Liceaga, Chair
Department of Food Science
Dr. Lisa Mauer
Department of Food Science
Dr. Fernanda San-Martin
Department of Food Science

Approved by:

Dr. Arun K. Bhunia
Head of the Graduate Program

To my parents. Without their unconditional love and support I would not be here today. Thank you.

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ABSTRACT

The farming of insects has been shown to require less land, feed, and water compared to traditional livestock maintenance, while proven to be a source of high-quality protein. The aversion of the Western culture towards edible insects is the major hurdle into their incorporation in the market, unveiling the challenge of integrating them into an existing familiar product. However, studies have shown that merely pulverizing insects into edible "flours" possesses difficulties on itself such as low solubility; severely altering the structural and sensory characteristics of food products upon their addition. Alternatively, scientists have turned to chemical protein isolation techniques to create insect flours with improved functionality. Furthermore, enzymatic proteolysis has shown to aid in extracting the protein bound to insoluble chitin and enhancing techno-functional properties. While this promising technique may open a range of possibilities, no research has been done regarding the incorporation of insect hydrolysates into a food matrix. The purpose of this work was to explore the production of insect hydrolysates with improved techno-functional properties and their impact in the physicochemical, structural, and sensory characteristics on a chosen model matrix: corn tortillas. Crickets (*Acheta domesticus*) were chosen due to their current relevance in the Western market.

Hydrolysates were produced with low (5%), medium (8%), and high (15%) degrees of hydrolysis (DH) either with Alcalase (AL) or Flavourzyme (FL). Alcalase cricket protein hydrolysates (CPH) resulted in higher fat content, which was suspected of possessing surface-activity. Overall, AL peptides displayed significantly ($p < 0.05$) higher emulsion and foam capacity and stability, suggesting stronger amphiphilic activity. On the other hand, FL peptides were more soluble and had a lower mean molecular weight, demonstrated by their lower glass transition temperatures.

Both of these developments may be explained by Alcalase endopeptidase activity and Flavourzyme primarily exopeptidase activity. Treatments resulted in AL-peptides with large and medium size molecular weights that included hydrophobic terminal ends, while FL peptides were smaller and likely contained free amino acids. The difference in molecular weights were seen upon their addition in the raw corn masa, where AL-CPH increased elastic and viscous behavior compared to control, whereas the smaller FL-CPH lowered them due to the plasticizing capability of hydrophilic small peptides. The ability of FL-CPH to interact with corn macromolecules was observed upon thermal treatment, resulting in FL-tortillas with superior strength and extensibility compared to AL-tortillas. In fact, AL-tortillas fragility was seen by the rollability test, showing a complete disintegration of the tortilla structure. Raman spectroscopy further showed the heat-induced intermolecular interactions of FL-peptides with the corn macromolecules. Raman bands at 1049 cm^{-1} in FL-tortillas allude to protein-starch complexes and the *gauche-gauche* region confirmed the presence of disulfide bridges in FL-tortillas, both of these developments were absent in AL-tortillas. Lastly, the formulation of corn chips with these CPH proved to be globally accepted by a population with diverse neophobia levels, confirming theories that consumers are willing to eat insects in an “invisible” format. Flavor and aroma profiles of the chips, quantified by a descriptive analysis study, revealed no commonalities between the two sets of chips. AL-chips were characterized as having corn, shrimp, and roasted peanut notes, while FL-chips were characterized as having tomato, ketchup, and French fry notes. Overall, enzymatic proteolysis was shown to generate cricket peptides with different characteristics, both able to be utilize as a functional ingredient for palatable food products.

CHAPTER 1: INTRODUCTION

The unequivocal rise in population and the effects of climate change on current agricultural systems stresses the importance of investing in alternative proteins. Challenges in this new era must be combated by educating populations in developing countries about the environmental risks associated with traditional animal agriculture, especially as their purchasing power rises (FAO, 2014; Ingram, Ericksen, & Liverman, 2012). The livestock agricultural sector is the main contributor of green-house-gas emissions and exploitation of fertile land, directly reducing biodiversity (Ingram et al., 2012). Creating alternative proteins that are both nutritious and environmentally friendly is not only important in the future growth of generations, but essential in the preservation of our ecosystems. Examples of alternative protein sources include plants, cultured meat and insects. Farming of insects has shown to be more environmentally friendly than traditional livestock practices in terms of water and land requirements, and lower green-house gas emissions; however, entomophagy is not yet a popular practice in today's Western cultures (Van Huis et al., 2013). Food scientists face a challenge of transforming people's opinion regarding insects from disgust to palatable food options (Gmuer, Nuessli Guth, Hartmann, & Siegrist, 2016). Several entomophagy-driven companies have decided to embrace the challenge by creating insect snacks, some containing whole insects and some containing insect flours (powders) (Van Huis et al., 2013). Experts state that the latter option would benefit in diminishing the Westernized psychological strains associated with insect consumption, although much uncertainty exists for food neophobic consumers, those with a negative disposition of trying new foods (Gmuer et al., 2016). These insect flours contain insoluble chitin from the insects' exoskeleton, which limits their techno-functional properties, especially restricting the functionality of the protein attached to the chitin (Hall, Jones, O'Haire, & Liceaga, 2017). In order to expand the use of these flours, scientists

have begun to isolate the insects' proteins to improve functional parameters, observing an increase in solubility (Zielińska, Karaś, & Baraniak, 2018). Beyond that, protein hydrolysis has shown to improve functional properties even more than using pure chemical isolation means. Parameters include high protein yields, high solubility at the proteins' isoelectric point and significant improvement in emulsion and foam capacity, making protein hydrolysis an attractive procedure to obtain novel ingredients (Hall et al., 2017). Much work has been done on protein hydrolysis from plant and milk sources that illustrate how limited disruption of proteins is enough to improve their functionality (Adler-Nissen & Olsen, 1979; Chobert, Bertrand-Harb, & Nicolas, 1988). While we know about the theoretical potential for enhanced protein functionality, there has been no work done in the application of these insect hydrolysates in a food matrix.

This work is further explores how enzymatic hydrolysis can enhance functional properties of insect proteins, especially at low degrees of hydrolysis. Furthermore, the application of the resulting hydrolysates in a chosen model matrix, corn tortillas and tortilla chips, was done to study the hydrolysates' impact on the physicochemical and structural features. Lastly, their sensory attributes and consumer acceptability were evaluated to determine their potential applicability as food ingredients in a market with a wide range of neophobic attitudes. The overall objective is to demonstrate the applicability and acceptability of cricket protein hydrolysates as ingredients with future applications in food formulation.

1.1: Hypotheses & Objectives

1. Improved techno-functional properties of whole cricket flour can be obtained via controlled enzymatic proteolysis with limited degrees of hydrolysis (DH), but their characteristics will differ depending on enzyme used.

Objectives:

   a. Obtain cricket protein hydrolysates (CPH) with a range of DH levels using two different enzymes.
      i. Determine appropriate treatments to obtain DH levels between 5 – 20%
      ii. Utilize Alcalase (endo peptidase) and Flavourzyme (exo and endo peptidase).
      iii. Characterize CPH by proximate composition, molecular weight distribution and glass transition temperatures.
   b. Show improvement of functionality by comparing CPH with cricket flour (CF)
      i. The following techno-functional parameters will be analyzed: emulsion capacity & stability, foaming capacity & stability, protein solubility, and water hydration capacity.

2. The incorporation of CPH in a corn tortilla will result in a novel product with higher protein content and altered physicochemical and structural characteristics depending on the enzymatic treatments.

Objectives:

   a. Formulate a corn tortilla with added CPH.
      i. Hydration tests will be carried out to find appropriate water to solid ratios for maximum hydration.
      ii. CPH-tortillas and control tortilla will be characterized: proximate composition, total amino acid analysis, color, and water activity.
   b. Compare the physicochemical and structural changes between enzymatic treatments.

i. Rheological and texture analysis of CPH and control corn dough and cooked tortillas will be analyzed.
ii. FT-Raman of CPH corn tortillas and CPH powders will be analyzed.

3. The incorporation of CPH into a corn chip will result in a product with unique sensory characteristics, depending on the enzyme used, and will be accepted by consumers.

Objectives:

a. Formulate a corn chip with added CPH.
    i. CPH-chips will be characterized: proximate composition and color.
b. Show acceptability of CPH-chips by a diverse population.
    i. Classify the consumers based on Food Neophobia levels.
    ii. Conduct consumer acceptability test on CPH-tortilla chips and correlate results with consumers' classification.
    iii. Conduct preference test to compare enzymatic treatment.

4. Different enzymatic treatments will produce CPH with specific flavor and aroma attributes.

Objectives:

a. Quantify the flavor and aroma profiles of CPH-chips formulated with two distinct enzymatic treatments.
    i. Formulate tortilla chips with CPH obtained via Alcalase and Flavourzyme treatments.
    ii. Conduct a descriptive analysis study to obtain flavor and aroma profiles.

1.2: References

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CHAPTER 2: REVIEW OF LITERATURE

2.1: Food Security

Food security is defined as "when all people, at all times, have physical and economic access to sufficient, safe, and nutritious food to meet their dietary needs and food preferences for an active and healthy life" (FAO, 1996). Given this definition, food security is unequivocally tied to environmental factors that affect the "physical" and "economic" access to food. The rise on the worlds' population is estimated to reach 9 billion by 2050 (FAO, 2014), meaning today's natural resources need to be enough to grant "sufficient, safe, and nutritious" food to all people, if we aim to claim food security status. Increasing physical access to foods directly relates to the amount of food production per unit area (Ingram, Ericksen, & Liverman, 2012). The amount of food yield in any given agricultural space will depend on various factors, including; price incentives, access to agrarian technology, investment in physical infrastructure, irrigation systems and a favorable climate (Ingram et al., 2012). The latter is, however, what holds most of the weight to allow all other factors a fair chance to close the gap between potential and actual yields of food production. For example, the Irish potato (*Solanum tuberosum*), one of the major crops in Zimbabwe, was identified to have a 77% yield gap due to water and nutrient limiting factors (Svubure, Struik, Haverkort, & Steyn, 2015). In Egypt, the sudden rise in population and the water scarcity problems have created significant consumption and production gaps in cereal, sugar, oil, legume and forage crops. Noteworthy changes to their economy considering Egypt was self-sufficient in almost every food commodity, except wheat, during the 1960s (Ouda et al., 2017). Several regions in Sub-Sahara Africa have anywhere from a 39 – 133 % agricultural yield gap that could be closed given appropriate technical efficiencies. In addition, closing these gaps would reduce the total amount

of green-house gas emissions emitted by 25 – 58% (Henderson et al., 2016). These food production gaps threaten the integrity of our current food system.

Progress on food security over the last two decades has been slow. Proportionally the amount of undernourished people worldwide dropped from 20% (1990-92) to about 16% (2004-06), however the total amount of "chronically hungry" people has been kept fairly constant (Ingram et al., 2012). Undernourished is hereof referred to as a permanent state of not consuming adequate amounts of macro and micro-nutrients to live a healthy lifestyle, while chronical hunger refers to multiple repeating periods of undernourishment. In addition, approximately 1 billion people are estimated to suffer from protein malnutrition (Wu et al., 2014).

Overall, food security problems are caused by a lack of fertile land and the vulnerability of crops, livestock, and fisheries to climate change (land degradation, water shortages, death of coral reefs, etc.) (Ingram et al., 2012). The Millennium Ecosystem Assessment argues that over the last 50 years humans have altered climate more than in any other period in history, causing the degradation of productive land, and fisheries, directly impacting food and water security (Ingram et al., 2012). In 2007, the Intergovernmental Panel on Climate Change (IPC) reported that human activity such as deforestation and fossil-fuel use were the primary causes for the unequivocal rise of temperature in the earth's atmosphere (Ingram et al., 2012), which have a direct impact on the rise of ocean levels and climate change. Unfortunately, the global food system has been both, a primary driving factor in the rise of greenhouse gas emissions, and has been affected by climate change as water shortages threaten food production (Ingram et al., 2012). The global food system is utterly linked to the various factors contributing to climate change, several examples showing critical boundaries

to avoid unacceptable climate change taken from Ingram et al. (2012) are shown in Table 1. In fact, agriculture is accountable for 25% of all carbon dioxide gas emissions, 50% of all methane gas emissions (particularly from livestock and rice) and 75% of all nitrogen dioxide (from fertilizers). In December 2009, several international organization such as World Bank, the Consultative Group on International Agricultural Research (CGIAR), the Food and Agricultural Organization (FAO), and the US Department of Agriculture (USDA) listed the increase in food production without the rise on greenhouse gas emissions as a major critical challenge to ensure food security in a world undergoing climate change (Ingram et al., 2012).

Table 1: Planetary Boundaries. Adapted from Ingram et. al (2012)

Process	**Boundary**	**Examples of food system/security links**
Biodiversity loss	Extinction rate no more than ten species per million per year	Agriculture one major cause of biodiversity loss. Agricultural genetic base needs diversity. Biodiversity enhances resilience of rural poor.
Nitrogen cycle	No more than 35MT per year removed for human use	Agriculture, transport and manufacturing use/produce nitrogen (fertilizer). Fisheries degrade by nitrogen runoff
Phosphorus cycle	No more than 11MT to oceans each year	Agriculture major source of phosphorous pollution. Fisheries damaged by phosphorous loading
Ocean acidification	No more than 2.75 global mean saturation of aragonite in sea water	Agricultural CO_2 emissions contribute to acidification. Fisheries degrade by acidification
Global freshwater use	No more than 4000km^{3}per year consumption by human activity	Agriculture and food processing consumer water. Food production depends on adequate water and food processing and consumption requires good water quality
Change in land use	No more than 15 percent of global land cover converted to cropland	Agriculture drives land-use conversion/ Forests provide environmental services that include food.

MT = million tones

In addition to the unsustainability of today's food system, people's dietary patterns are changing to include more meat and dairy products (Ingram et al., 2012), burdening even further the environmental footprint the livestock industry already has. The consumption of meat in Asia has increased significantly in the last decade, while North and South America has seen moderate increase in demand (Ingram et al., 2012). The economic rise of the middle class in China and India have sharply increased consumption of meat. Experts estimate a double demand in livestock by the year 2050 (465 million tons) compared to the numbers reported in the year 2000 (FAO, 2006). This change in meat consumption is more likely tied to increase in urbanization and higher incomes, and while it may represent an advancement in the economic status of these populations it will provoke dreadful consequences to the environment. It is estimated that the livestock industry occupies about 30% of non-polar land (3.9 billion hectares) while being one of the primordial polluting entities; emitting 37% of the Earths' methane gas emissions, adding 9000 kg of solid waste per year (in USA alone) and releasing drug-resisting pathogens onto soil and coastal zones (Aarnink, Keen, Metz, Speelman, & Verstegen, 1995; FAO, 2006; Goodland, 2013; Losey & Vaughan, 2006). In fact, 78% of agrarian land and 33% of crops are used exclusively for the nourishment of livestock (Steinfeld, Gerber, Wassenaar, Castel, & de Haan, 2006). It is clear that these numbers confirm the need to develop a more sustainable agriculture system, one that takes into account the rise in population and looks beyond conventional food sources.

While demand of meat and meat products has increased around the world, cereals still pose as the most important source of calories, especially for those countries living in poverty. In fact, two thirds of the calories consumed world-wide are provided by wheat, rice, and maize, while the poorest populations survive mainly with these staple crops, deprived of sufficient proteins,

vitamins and minerals (Cassman, 1999; WHO, 2005). Unfortunately, none of those 3 staple crops are a complete source of protein (one that contains all essential amino acids), suggesting that people living in poverty are lacking adequate protein intake (Ingram et al., 2012). In a world were undernourished populations are lacking access to protein, yet the primary protein sources are those that contaminate the most, the use of alternative protein sources is key in building a sustainable and healthy future. Goodland (2013) proposes the replacement of at least 25% of livestock products with alternative proteins as a possible solution that would reduce 4% or more of agricultural greenhouse gas emissions and subsequently allow the reforestation of agrarian land. The consumption of alternative protein sources will alleviate environmental stress caused by the livestock industry, yet it is a matter of creating these alternatives and pleasing consumers preferences to create long-term changes. There are various types of protein alternatives currently in the market and still undergoing developing stages. Among them are proteins from algae, cultured meat, modified vegetable proteins, and insects.

2.2: Corn Tortillas

Corn (maize) tortillas are a staple food in Latin America, but especially in Mexico. Mexico is the biggest consumer of corn tortillas per capita in the world, with an approximate consumption of 240 g of tortillas per day, and up to 400 g per day in rural areas (Amaya-Guerra, Alanis-Guzman, & Saldívar, 2004). Corn is not only consumed in the traditional tortilla form, but it is also transformed into corn patties and corn cakes depending on the region. Overall in Mexico, corn provides 45% of daily calories and 39% of daily protein intake. In rural areas, corn contributes 70% of total calories, and 50% of daily protein intake (Amaya-Guerra et al., 2004). Unfortunately, corn is not a good source of protein. The overall protein quantity of corn is approximately 9% on a dry basis and the protein efficiency ratio (PER) is very low (1.5) (Valle & Pérez-Villaseñor,

1974) compared to 2.50 for beef (Hopkins, Steinke, & Kolar, 1976). Furthermore, corn protein is not a complete source of amino acids, having lysine and tryptophan as limiting indispensable amino acids (Landry & Moureaux, 1982). When families from low socio-economic levels in Mexico face food shortages their primary food staple is corn tortillas, thus risking protein deficiency health concerns (Valle & Pérez-Villaseñor, 1974).

In addition to the localized importance that corn, and corn tortillas have in Mexico, the global market for tortillas has recently been valued at $12,324 million USD in 2018 by Future Market Insights. In Mexico, 94% of the tortillas are manufactured with corn, but wheat flour tortillas have a strong presence in the global market too. A general increase in demand for Mexican cuisine in North and Latin America is one major factor that has led to an increase in market growth (Future Market Insights, 2018).

The high consumption of corn tortillas and their low nutritional value makes this staple product a perfect model to fortify. Studies in the past have experimented fortifying corn tortillas with other protein sources. For instance, a study evaluated the addition of soybean presscake (a by-product in the production of soy bean oil) to corn tortillas and found that adding up to 40% resulted in products with acceptable rollability properties and high consumer acceptability scores (R.A. Anderson, 1969). Acceptability scores included overall flavor, appearance, and texture. Another study focused on fortifying corn tortillas with purified lysine and tryptophan, and compared them with tortillas fortified with the same two amino acids plus a protein concentrate from *Phaseolus lunatus* beans (Lecuona-Villanueva, Betancur-Ancona, Chel-Guerrero, & Castellanos-Ruelas, 2012). In both cases, the fortified corn tortillas showed no significant changes in rollability, tensile

strength, and elasticity. Fortification was made to supply sufficient amounts of the two limiting amino acids; however, there was a decrease in lysine bio-availability after tortilla processing was reported. Researchers speculate that the heat treatment that tortillas were subjected to induced a crosslinking chemical reaction with asparagine and glutamine, decreasing lysine availability in the *in vitro* digestibility study (Lecuona-Villanueva et al., 2012). Sensory acceptability testing in the same study showed that all fortified corn tortillas in average scored lower than the control, but the difference was not significant, and scores never surpassed a benchmark representing a *dislike* towards the product. In another study, the use of mealworm (*Tenebrio molitor*) whole insect flour was used to supplement corn tortillas (7%) and results showed an increase in protein and fat content by 2 and 1 %, respectively (Aguilar-Miranda, López, Escamilla-Santana, & Barba de la Rosa, 2002). Flour in this context is referred to as a grounded powder of the starting material. Researchers also reported acceptable texture and flavor of the corn tortillas by a small group of panelists.

2.2.1: Structural Integrity

Corn tortillas are made by an ancient process called nixtamalization. It involves cooking corn kernels in a lime ($Ca(OH)_2$) solution (0.5 – 2%) at around 80°C for about 50 min. and leaving them to soak for more than 8 hours. There are several physicochemical changes occurring within the kernels at this time that contribute to the structural formation of nixtamalized corn products. First, the high alkaline environment and excess water solubilizes the pericarp, which facilitates the absorption of water and ionic exchange to the endosperm. The endosperm of the corn kernel will hold about 98% of the starch. Because of the high temperature and excess water during nixtamalization, starch bodies around the periphery of the endosperm start to go through a process of gelatinization. This process involves absorption of water and swelling of the starch granules (Serna-Saldivar, Gomez, & Rooney, 1990). When granules reach their gelatinization temperatures

there is a loss of crystallinity, and birefringence. About 20% of starch granules within the endosperm are gelatinized due to the nixtamalization process. Secondly, the high alkalinity causes a rupture of disulfide bonds within ϒ- zein bodies, known to hold the external spherical structure of corn protein bodies, causing internal α- and β- zein bodies to be released. These are overall more hydrophobic, resulting in a loss of total solubility of zein (Ortega Martinez, Villegas, & Vasal, 1986). These zein proteins are then susceptible to deamination; a process where the ammonia group of glutamine and asparagine is removed, resulting in glutamic and aspartic acid. These negatively charged residues are prone to form protein complexes with calcium ions, strengthening the matrix and developing unique viscoelastic properties (Miklus, 1999). Santos et al. (2014) demonstrated the formation of calcium bridges within protein bodies by comparing the properties of a lime treated masa with a non-lime treated masa. In the study proteins were extracted and their SDS-PAGE profiles under reducing and non-reducing conditions were compared. The control (not lime-treated) showed stabilization of bands via disulfide bridges in a non-reducing environment, and absence of complexes under reducing conditions. Meanwhile, the lime treated samples exhibited the presence of complexes in the reducing environment, due to calcium-protein interactions. Furthermore, rheological studies on the nixtamalized corn dough (masa) revealed significantly higher elastic and viscous modulus compared to the non-lime treated control, showing the importance of protein-starch interactions via calcium bridges, as opposed to purely a gelatinized starch matrix, in the formation of its viscoelastic properties (Guzmán, Flores, Escobedo, Guerrero, & Feria, 2009). In order to successfully create corn tortillas fortified with foreign proteins, inter-molecular interactions between foreign amino acid residues, corn proteins and starch need to take place. The extent of calcium bridge formation between these components will determine the viscoelastic changes on the matrix. Note that the addition of any foreign protein

in corn masa will subsequently dilute the amount of starch in this starch-based matrix, thus limiting the structural capacity gelatinized starch has on the system.

2.2.2: Food Safety & Quality

For a product to be successful among consumers, it must remain at an adequate quality parameter throughout the time stated on its label. Shelf stability refers to limiting microbial growth below safety limits, chemically preserving quality of macro nutrients, and maintaining palatability as well as nutritional claims stated on the label (de W Blackburn, 2006). There is a myriad of factors that may affect the quality of a product during shelf life, but the first concern is limiting pathogenic microbial growth. A product's composition plays a key role in this parameter. Intrinsic factors are variables within a product that may be manipulated to limit microbial growth. These include; pH, water activity (a_w), and redox potential (Eh). Water activity is defined as the vapor pressure of water in a food relative to the vapor pressure of pure water (Fellows, 2009), a common lower threshold in the food industry is targeting a a_w of 0.86 to limit the growth of *Staphylococcus aureus*, one of the most salt-resistant food-borne pathogens (Tilkens, King, Glass, & Sindelar, 2015).

The physicochemical environment of tortillas is prone to mold and yeast growth, thus leading to the use of preservative blends specifically for this purpose. Mixes of different preservatives have been shown to increase their effectiveness while reducing optima concentration and leading to better flavor perception (Rolow, 2002). Almost all preservatives are in the form of organic acids, having greater efficacy when found in their undissociated form (Rolow, 2002). Mixes of calcium or sodium propionate, potassium sorbate, methyl or propyl paraben and sodium benzoate have been shown to effectively limit mold, yeast and bacteria growth in tortillas. The pKa (4.2- 5.0) of these organic acids is close to the nixtamalized corn tortilla pH (5.5), however decreasing the pH of the corn masa would increase their preservation efficacy and has been shown to increase shelf

life of corn tortillas from 8 to 20 days. Controversially, the flavor of lower pH corn tortillas present sour notes and discoloration (Rolow, 2002).

2.3: Entomophagy

Entomophagy has been practiced by homo sapiens even before learning how to hunt or cultivate agricultural products (Ramos-Elorduy, 2009). This ancient practice is still prevalent in several countries around the globe and holds valuable aspects of cultural identity that are appreciated among international presence (Ramos-Elorduy, 2009). In fact, 113 countries claim to have had entomophagy as a cultural tradition (MacEvilly, 2000). Some cultures even gave certain species spiritual attributes, for example consuming wasps was thought of bringing prosperity, protection and abundance (Ramos-Elorduy, 2009). Some insects were given such high value that humans treasured them from a religious stance, as is the case of the sacred beetle *Ateuches sacer* in Egypt, or the "sacred jumil" *Edessa cordifera* in Mexico, which was believed to have communication with God by the ancient Tlapanecos (Ramos-Elorduy, 2009). However, the consumption of insects across all indigenous groups world-wide goes beyond spiritual believes. It comes down to the wide availability since insects can be found in every ecosystem and can be consumed at all stages of development depending on the species; eggs, larvae, pupae, and adults (Ramos-Elorduy, 2009). Today, in Thailand, wasps, caterpillars, crickets and locusts are sold as expensive delicacies in high-end restaurants (mostly for tourists) while also being commonly sold in the local market (Abbasi & Abbasi, 2016). Japan considers rice-field grasshoppers (*inago*) and canned wasps as luxury food items, and ant larvae (*escamoles*) are sold at 25 USD per plate (~200 g) in Mexico (Abbasi & Abbasi, 2016). Social and economic movements in response to entomophagy are on the rise. Today's mass media has increasingly mentioned insects as alternative sources of protein to consider due to its lower environmental impact and high nutritional value (Ramos-Elorduy, 2009).

Schools have promoted insect eating days to commemorate its cultural value and familiarize the next generation on this ancient practice (Ramos-Elorduy, 2009). The Food Industry is slowly catching up as several entomophagy-focused start-ups have shown appearance in the last decade (Ramos-Elorduy, 2009; Wu et al., 2014; J. Yi, Zhu, McClements, & Decker, 2014). However, the eating of insects is still regarded with disgust among Western society (Gmuer, Nuessli Guth, Hartmann, & Siegrist, 2016), making the emotional barrier to adopt this practice one of the main challenges. Data suggests that incorporating insects as part of an ingredient could be a potential gateway for Westerners to overcome negative emotional barriers towards entomophagy. Westerners in this document refers to Europeans and non-aboriginal Americans, Canadians, non-aboriginal Australians, and New Zealanders. Consumer's risk analysis of eating a new food product is greatly affected by the setting in which they will experience this new item (Baker, Shin, & Kim, 2016). Greater psychological risk is perceived when a consumer is faced with a new product in a retail setting compared to a restaurant setting (Baker et al., 2016). Marketing insect-containing products in a retail setting by showing a full image of the insect significantly reduced a consumers' willingness to buy this item (Baker et al., 2016), elucidating the frail acceptance line a modern consumer has towards insect goods. Additionally, it's been demonstrated that consumers showed a more positive emotional response to food products that incorporated crickets in the form of a flour than those that incorporated crickets in its intact form (Gmuer et al., 2016). A flour refers to a grounded or a completely pulverized form of crickets. For the industry to effectively introduce insects into the market it is imperative to consider the best business strategy, taking into consideration product formulation and psychological assessments by the buyer.

In a world where consumers are able to readily demand information, comes a generation known as Millennials that have become aware of environmental issues and demand ethically sourced food products (Crowder, Shoulders, & Rucker, 2014). In fact, millennials' sensory perceptions of foods are recognized to be influenced by extrinsic cues as is the case with alternatively produced (AP) food products (organic, grass-fed, antibiotic free, etc.); normally attributed with higher degrees of environmental consciousness (Crowder et al., 2014). As this generation gains purchasing power it is important to consider the various stimuli that can be associated with the eating of insects such as; environmentally impact, nutritional properties, and an embracement of ancient practices.

2.3.1: Sustainability Aspects of Entomophagy

The Food and Agricultural Organization (FAO) of the United Nations has been one of the main promoters of entomophagy due to its lower environmental impact and the high economic opportunity that insect farming represents (Van Huis et al., 2013). FAO sponsors insect farmers around the world and educates locals on the feasibility and practicality of insect farming (Van Huis et al., 2013) in order to stimulate this educational movement. One of the main economic advantages with insect farming is their high food conversion efficiency, also referred to as efficiency conversion of ingested foods (ECI%), when compared to traditional livestock. ECI can be defined as the weight gained by an animal per pound of food intake (Nakagaki & Defoliart, 1991), making it a good quantifiable measure of a farms' productivity. The ECI of house crickets (*Acheta domesticus*) is about 6 times higher than that in cattle, 3 times higher than in pigs, and 5 times higher than in sheep (Nakagaki & Defoliart, 1991). Moreover, as previously mentioned, the accumulation of green-house gases (GHGs) in the atmosphere is the main cause of climate change directly attributed to human activity (Maharjan & Joshi, 2013), and traditional livestock is the single main contributor of GHGs of global agriculture (Goodland & Anhang, 2009). On the other

hand, insect farming only emits 1% of GHGs compared to the farming of ruminant animals and significantly lower to that of pigs (Oonincx et al., 2010). Emissions of ammonia, which causes water acidification and eutrophication, were much lower, in some cases even negligible, in insect farming when compared to traditional livestock (Oonincx et al., 2010). Insects can also multiply significantly faster while occupying smaller spaces. For instance, the house cricket is able to lay 1200 to 1500 eggs within 4 weeks, in comparison the breeding ratio for beef is 4:1 for every animal marketed (Capinera, 2004). In addition, shortages of land worldwide have forced animal agriculture to take over other natural reserves, making it a direct reducer of global biodiversity (Goodland & Anhang, 2009), while it is apparent that insect farming would occupy only a fraction of the land traditional animal agriculture uses. In fact, in countries where locals are accustomed to the consumption of crickets, mini-farms are set up in backyards using inexpensive materials (Abbasi & Abbasi, 2016). In contrasts, raising cattle requires voluptuous commercial equipment and significantly more space. Comparing the farming of mealworms with more traditional protein sources, Dennis G. A. B. (2012) states that in order to produce 1kg of mealworms only 3.6 m^2 is needed as "land use", compared to 9.3 m^2 for chickens (2.58 times), 11 m^2 for pork (3.03 times) and 39.6 m^2 (11 times) for beef.

2.3.2: Nutritional Properties of Insects

Nutritional properties of multiple insects have been analyzed and results reveal that most edible insects are nutritionally sufficient, and some even superior, compared to other forms of protein. The nutritional profiles of adult crickets, mealworms and locusts were analyzed by Zielińska, Baraniak, Karaś, Rybczyńska, and Jakubczyk (2015) and revealed a total protein percentage of 70%, 52.3%, and 76%, respectively. More importantly, a satisfactory amount of all essential amino acids (benchmarked by (WHO2007), measured as mg/g protein) was found in all three species.

Essential or indispensable amino acids are those the human body needs to consume through the diet since the body is unable to synthesize them, or synthesize enough quantities, to make proteins and other nitrogen containing compounds (Janice L Thompson, 2011). The eight essential amino acids for adults are; histidine, isoleucine, lysine, leucine, methionine, phenylalanine, threonine, and valine. In addition, tryptophan is also essential for infant growth (Janice L Thompson, 2011). The *Anaphe ventana* moth powder, regularly consumed by rural Nigerians, showed higher protein content than lamb and pork, and presence of essential minerals (Ashiru, 1989). Although consumption is decreasing, the intake of this larvae alleviates protein malnutrition in Nigeria. Furthermore, the nutritional quality of insect protein is enhanced by its high digestibility ratio when their exoskeleton is removed. Finke (2004) observed higher digestible protein of insects compared to vegetable sources, and when compared to soy protein, crickets (*Acheta domesticus*) were a superior amino acid source when fed to rats (Finke, DeFoliart, & Benevenga, 1989). Protein digestibility was measured on mealworm *T. molitor* (without exoskeleton) on an in vitro study, showing that the soluble fractions had 85% protein digestibility index, while protein digestibility of the non-soluble fractions was around 45% (Yi, Van Boekel, Boeren, & Lakemond, 2016). These numbers are comparable to protein digestibility values of beef, pork, turkey and salmon, which have been estimated to be 89%, 90%, 78%, and 85%, respectively (Kinyuru, Kenji, Njoroge, & Ayieko, 2010). A study done on *Henicus whellani* (cricket species endemic to Zimbabwe) showed an average protein content of 53% by weight, 10% ash content, and confirmed the presence of flavonoids (Musundire, Zvidzai, Chidewe, Samende, & Manditsera, 2014). Flavonoids are polyphenols naturally occurring in fruits and vegetables that have been linked with “antibacterial, antiviral, anti-inflammatory, anti-allergenic, and vasodilatory properties” (Duarte et al., 1993; Hanasaki, Ogawa, & Fukui, 1994; Middleton, 1994). The presence of these beneficial polyphenols

is more likely a direct effect of the crickets' diet. This aspect is important to consider if mass production of crickets is to be carried out in the future. High ash content is directly related to high amounts of essential minerals such as calcium, phosphorous, potassium, and magnesium (Womeni et al., 2012). Zielińska et al. (2015) also demonstrated the presence of essential fatty acids in all three insect samples (crickets, mealworms and locusts). The human body required regular dietary consumption of two particular poly-unsaturated fatty acids, linoleic acid (omega-6) and alpha linolenic acid (omega-3), for the synthesis of essential biological compounds important for optimal health (Janice L Thompson, 2011). Intake of omega-3 fatty acids is also directly related to a decrease in heart disease development by "reducing inflammation and blood triglycerides" as well as "increasing high-density lipoproteins" (Janice L Thompson, 2011). In general, insects' fatty acid profiles are comparable to those of fish and poultry, with some insects containing even higher amounts of poly-unsaturated fatty acids (PUFA) (Zielińska et al., 2015). Currently, Americans eat sufficient amounts of fats, but mostly in the form of saturated fatty acids (Janice L Thompson, 2011). This class of lipids, along with trans fatty acids, have been linked to higher rates of coronary diseases (Janice L Thompson, 2011). The biggest source of saturated fatty acids comes from consumption of red meat such as beef and pork, as well as dairy products (Janice L Thompson, 2011). This information suggests that the incorporation of insect consumption as part of the American diet would contribute to the consumption of healthier lipids, and the additional benefits associated with phytochemical compounds (flavonoids) while providing sufficient amounts of essential amino acids. Furthermore, at a global scale the 850 million undernourished people in the world (Ingram et al., 2012), would obtain superior nutritional quality with respect to lipids with an entomophagy diet, than with a traditional meat-based diet.

2.3.3: Insect Protein

The incorporation of insects into the Western diet will depend on the success of the food industry in creating food products that are appealing and palatable to the Western consumer. As mentioned earlier, the incorporation of insects in a "flour" form may alleviate psychological strains by associating insect-containing foods with more familiar foods. The extraction of protein from insects is largely of interest due to its role in satisfying protein demands and creating sustainable protein alternatives. Unfortunately, the study of insect proteins has been largely understudied in the past, however its importance as a relevant food ingredient has emphasized the importance of this research area. The techno-functional properties were the first to be studied, outlining the limitations of whole insect flours as food ingredients. Solubility profiles of various whole insect flours have been shown to have an isoelectric point (pI) around pH 3- 5, while all of them showed poor solubility (< 30%) across a wide pH range, including flours from grasshoppers (*Schistocerca gregaria*), the large African cricket, and *Locusta migratoria* (Adebowale, Adebowale, & Oguntokun, 2005; Mishyna, Martinez, Chen, & Benjamin, 2019; Purschke, Meinlschmidt, Horn, Rieder, & Jäger, 2018). In addition, large African cricket flour and *L. migratoria* flours also showed poor emulsion and foaming properties, limiting the possible food vehicles in which these flours could be used. The poor functionality of these whole insect flours is most likely due to the presence of chitinous fibers within them (Abbasi & Abbasi, 2016; Hall, Jones, O'Haire, & Liceaga, 2017). Chitin is an insoluble fiber (poly-N-acetyl glucosamine chains) that creates the bulk of the structurally stiffened insect cuticle, along with proteins and phenolic compounds (Andersen, Peter, & Roepstorff, 1996). The functionality of the insect cuticle will determine the type of molecular structural arrangement needed for specific mechanical properties. The stiffness of the exoskeleton originates from the procuticle, which through the process of schlerotization, pro-cuticular proteins

undergo modifications to have cross-links with catecholamine derivatives via oxidative couplings (Andersen, Hojrup, & Roepstorff, 1995). In addition, cuticular proteins are known to possess β-sheets as part of their secondary structure with a conserved sequence motif, known as the "R&R consensus sequence" suspected of stabilizing chitin-protein interactions (Rebers & Riddiford, 1988). This motif is rich in aromatic amino acid residues, likely stabilizing the interaction with chitin via planar base stacking with saccharides or by facilitating the access of aromatic residues to participate in schlerotization (Willis, Papandreou, Iconomido & Stavros, 2012).

The isolation of proteins via alkaline extractions has been shown to improve techno-functional properties in *L. migratoria* powders, obtaining 100% solubility at alkaline pH and significant improvement under acidic conditions (Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016). Similar protein isolation techniques were used to obtain high protein powders from *Tenebrio molitor*, *G. sigillatus*, and *Schitocerca gregaria*, and their functionalities were compared to their whole flour counterpart. Results showed protein isolates had significantly higher water and oil holding capacity than the flours, while solubility profiles surpassed 90% in alkaline mediums for all protein isolates (Zielińska, Karaś, & Baraniak, 2018). Another protein isolation technique combining alkaline protein isolation with enzymatic proteolysis has also shown to improve techno-functional properties of proteins from crickets (*G. sigillatus*) compared to its un-hydrolyzed control. The reported improvements include higher solubility across a pH (3 – 10) range, higher emulsifying capacity and stability, and higher foaming capacity (Hall et al., 2017).

While *in vitro* assays have shown the limited functionality of whole insect flours, studies on the functional and sensory changes in food matrices with whole insect flours further show the need to

restructure this novel ingredient. The baking of bread made with whole cricket (*Acheta domesticus*) flour to replace 10 or 30% of wheat flour resulted in breads with significantly higher firmness (g) level and darker color with increasing amount of cricket flour, attributes normally disliked by buyers. More importantly, sensory evaluation scores were below 5 for overall liking in a 9-point hedonic scale (1= dislike extremely, 5 = neither like nor dislike, 9 = like extremely) for breads with 10%, and scores below 3 for breads with 30% cricket (Osimani et al., 2018). Similarly, mealworm burgers had overall poor acceptability by consumers who had never tried insects but knew of insects as food, and among those who did not know of insects as food ingredients (Tan, Fischer, van Trijp, & Stieger, 2016). Food products that have been rated as acceptable by panelists normally have low amounts of insects. For instance, the baking of bread with 5 and 10% mealworm powders were rated as acceptable by a small panel of judges and had similar leavening percentage and slightly lower firmness compared to the control. The difference in sensorial evaluation between the bread formulated with crickets and mealworms denotes the importance of food appropriateness, an important factor in consumer acceptability of any novel product (Tan et al., 2016).

2.4: Protein Hydrolysates

Hydrolysis of proteins refers to the rupture of the peptide bond between two amino acids, this can be done via enzymatic reaction, heat treatments or alkali/acidic reactions (Pasupuleti & Demain, 2010). The products obtained upon protein hydrolysis are called protein hydrolysates. Degree of hydrolysis (DH) is a measure to quantify the amount of peptide bonds cleaved relative to the total amount of peptide bonds in a substance (Pasupuleti & Demain, 2010). Protein hydrolysates, also known as peptones or peptides, are commercially used in a variety of industries for various uses including: manufacture of vaccines, industrial scale fermentation processes, manufacture of

bioactive compounds and optimization of functional ingredients in the food industry (Pasupuleti & Demain, 2010). For example, during WWII limited protein hydrolysis was applied to soy protein isolates to mimic egg whites in baked goods in order to alleviate the severe shortage (Adler-Niseen, 1986). This long history of protease usage has spurred research into the further investigation of this methodology. Chemical approaches to protein hydrolysis involve subjecting the substrate to extreme acidic or alkaline conditions for long periods of time, thus resulting in the destruction of nutritional components (such as tryptophan) and chemically affecting amino acid side chains (Tavano, 2013). Alternatively, enzymatic hydrolysis can be done under mild conditions in a highly specific manner (Tavano, 2013). Enzymatic hydrolysis is widely used in an industrial scale because it can be performed under mild operational conditions, the amount of enzyme needed is small to process large batches and there is no destruction of amino acids (Pasupuleti & Demain, 2010). A myriad of proteases is currently used for different commercial purposes. They are classified based on the nature of origin (bacterial, fungal, etc.), catalytic site (endo- vs exo-peptidase), and catalytic triad (aspartic protease, serine, etc.) (Whitehurst & Van Oort, 2009).

Enzyme's specificity for peptide cleavage will create different hydrolysates with unique amino acid profiles and molecular weight distributions (Tavano, 2013). For example, a study on hydrolysis of whey protein utilizing different enzymes but constant DH (4%) proved to have different molecular weight distributions (Whitehurst & Van Oort, 2009). In other words, peptides obtained from the same substrate with the same rupturing degree will contain different peptide sizes depending on the enzyme used. The amino acid sequence and molecular weight distribution in specific sets of peptides will ultimately affect peptides' functional properties. For this reason, it is important to compare protease activity across a variety of enzymes applied to the same protein substrate. Alcalase is a single-chain serine endo-protease produced by *Bacillus lecheniformis,* also

known as Subtilisin Carlsberg (Walsh, 2002). An endo-protease is a type of enzyme that cleaves internal peptide bonds, as opposed to an exo-peptidase which catalyzes the breakage of bonds closer to the amino or carboxyl end of the polypeptide chain (Walsh, 2002). Alcalases' single domain catalytic triad consists of serine 221, aspartate 32, and histidine 64 (Walsh, 2002). Alkaline conditions (8-10 pH) and temperatures ranging 50-65˚C are optimum for Alcalase catalytic activity (Walsh, 2002). Alcalase is an enzyme widely used in the food industry for its' high efficient and potent activity (Whitehurst & Van Oort, 2009). In addition, Alcalase has been known to effectively separate chitin from the rest of the polypeptide unit from shrimp (Synowiecki & Al-Khateeb, 2000), resulting in a chitin-free product with a higher degree of functionality. A study also demonstrated increased functionality of cricket hydrolysates compared to the intact cricket protein when utilizing Alcalase (Hall., Jones, O'Haire, & Liceaga, 2017), however it is essential to compare functionality of peptides cleaved with different enzymes due to the protease's high degree of specificity. Flavourzyme is an enzyme sourced from the fungi *Aspergillus oryzae* and is especially attractive because as an endo- and an exo- protease it will tend to not only decrease the molecular size of the protein, but it will also cleave hydrophobic terminal amino acids. A polypeptide without its hydrophobic tail will exhibit lower levels of bitterness (Whitehurst & Van Oort, 2009), an important advantage for the formulation of food ingredients. The food industry already utilizes proteases from *Aspergillus oryzae* in baking applications. Low additions of this protease will cause limited breakage of the gluten lattice, thus decreasing a dough's resistance to stretching and subsequently facilitating the retention of CO_2 gas (Walsh, 2002). Because Alcalase and Flavourzyme are well known in the food industry, they are attractive enzymes to produce and compare functionalities of respective insect protein hydrolysates.

2.5: Proteins: Functional Properties

In order for the food industry to consider insects as reliable sources of protein, the study of the functional properties relevant to product formulation is important. The term "functional properties" or "techno-functional properties" in this document refers to the capacity of an ingredient to form stable emulsions, foams, to solubilize in an aqueous medium, and to form stable gels. Because food matrices are highly complex systems, improvement of functional parameters will facilitate the adoption of this new ingredient for future food formulations. Current start-up companies are already utilizing insects in their formulation with a similar end goal: to encourage the consumption of more sustainable sources of food. Some examples include protein bars by Chapul™ (Goodland & Anhang, 2009), tortilla-cricket chips by Chirps™ (Lockeretz, 2007) and even pasta by Bugsolutely™ (Van Huis et al., 2013). These companies are mainly incorporating crickets in the form of a powder or "flour", conscious of the consumers reluctance to see whole insects in their food. However, the use of cricket flour is limited due to a lack of functional properties (emulsification, foamability, solubility, and gelling), thus limiting the maximum concentration of insect substance allowable within a product. Most of the companies have focused on utilizing crickets, as opposed to other insects, due to their relative approachable stance with Westerners. Furthermore, we have decided to choose crickets because of its relevance in the Western market today.

2.5.1: Emulsification

Emulsifiers are of extreme importance in the food industry due to their role in stabilizing complex food matrices. The importance of producing food grade emulsifiers started in the second half of the twentieth century, when the food industry was challenged to produce and distribute masses of shelf stable food products (Hasenhuettl, 2008). Today, approximately 500,000 metric tons of

emulsifiers are produced worldwide and the United States alone sales 225-275 million USD of emulsifiers. The Food and Drug Administration (FDA) is the official regulator of food ingredients, in the United States, giving them the right to dictate limits on the use of emulsifiers in any food product. The FDA assigns different status to food ingredients depending on their level of risk, generally recognized as safe (GRAS) status being the one with the least amount of restrictions (Hasenhuettl, 2008). Emulsifiers are compounds with amphiphilic properties, able to interact with polar and non-polar mediums and create miscibility within the two. In other words, an emulsifier is able to disperse oil droplets in water mediums, or water droplets in a lipid medium, depending on the chemical nature of the emulsifier (Hasenhuettl, 2008). Emulsifiers can be derived from various sources, protein-derived are one of the most common due to their wide availability and GRAS status. However, proteins naturally enclose their hydrophobic groups in the interior of the structure, exposing only hydrophilic side chains to interact with an aqueous environment (Tavano, 2013). A common solution to this structural problem is protein hydrolysis. The rupturing of peptide bonds can expose ionizable groups and hydrophobic side chains (Tavano, 2013), increasing their amphiphilic properties. In addition, hydrolysis decreases the protein's molecular weight (Tavano, 2013) providing a more flexible structure to surround hydrophobic molecules. Studies show that a limited amount of DH significantly improves emulsifying properties, however large DH values (15% or more) could negatively affect functionality (Diniz, 1997). Slightly disrupting polypeptide chains increases their surface area, promoting their surface-active sites to better bind with aqueous and nonpolar phases (Diniz, 1997). Light disruptions on polypeptide chains affecting tertiary structure and not secondary structure provide greater flexibility (Panyam, 1996), this can enable a protein to act as an emulsifier more effectively while maintaining its integrity in surface activity. There are three parameters used to evaluate the emulsifying properties of food ingredients;

emulsion activity, capacity, and stability (Panyam, 1996). Emulsion activity is an indication of "the maximum interfacial area per unit weight of protein" between oil and protein in a stable solution (Panyam, 1996). Emulsion capacity quantifies amount of hydrophobic molecule dispersed per unit weight of protein under specific conditions, while stability is an indicator of a protein's ability to sustain the emulsion over time under constant temperature and gravitational field (Panyam, 1996).

In cases were high DH values exhibit higher emulsifying capacity a tendency of a weak emulsion stability is very common. This can be attributed to the inability of the smaller peptide chains to maintain surface activity. In fact, a study compared peptides' emulsifying capacity of soy protein cleaved by Alcalase and Neutrase, and found that a 5% DH and a 2-6% DH showed the best performance for Alcalase and Neutrase, respectively (Adler-Nissen & Olsen, 1979), while a DH beyond 5% decreased emulsifying capacity. Mietsch, Fehér, and Halasz (1989) also compared the emulsifying capacity of soy and casein peptides treated with the same two enzymes (Alcalase and Neutrase), concluding significant improvement in emulsion capacity but no improvement in emulsion stability. The degree of improvement was not equal among samples, soy proteins showed superior improvement changes when treated under the same conditions compared to casein. Another study compared emulsifying activity of casein and whey peptides hydrolyzed with trypsin, and found increased functionality up to a 10% DH (Chobert, Bertrand-Harb, & Nicolas, 1988). However, emulsion stability of these peptides was lower than the un-hydrolyzed control.

Emulsions with low solubility make quantification of instability difficult to measure consistently. The turbidimetric method derived from Pearce and Kinsella (1978) work has been used throughout

literature for its objectivity and consistency. This method relies on the Mie theory of light scattering that states there is a simple relationship between "the turbidity of the emulsified solution and the interfacial area". The turbidity of an emulsion can be quantified using a spectrophotomer, where turbidity is proportional to absorbance:

$$T = \frac{2.303A}{l}$$

Where T = turbidity, A= absorbance, and l= length of the cuvette.

Subsequently, an increase in turbidity would have an increase in absorbance, which would be directly related to an increase in interfacial water – to – oil area, such that:

$$\text{Interfacial area} = 2T$$

2.5.2: Foaming

Foaming is the phenomenon in which air or gas particles are dispersed in a liquid medium, naturally increasing in volume. A foam may be stabilized by an amphiphilic particle, such as an emulsifier. The characteristic dual surface activity needed to stabilize an emulsion is also needed to stabilize a foam. The nonpolar end interacts with air particles, while the polar end interacts with the aqueous medium. The nature of this mechanism makes foams sensitive to oil, because the presence of lipid substance inherently competes with air particles to interact with the lipophilic ends of the emulsifier. Foam capacity is the term used to characterize the degree of volume growth achievable by an emulsifier, while foam stability indicates the time needed to rupture the foam matrix (Panyam, 1996). A protein's ability to stabilize a foam can also be enhanced through limited protease activity due to the higher interfacial surface area of active sites and an increase in polypeptides' flexibility from tertiary structure disruption (Panyam, 1996). Foam capacity of cricket protein hydrolysates by Alcalase significantly increased compared to control when

hydrolyzed at 0.5% enzyme concentration with a DH of 26% (Hall. et al., 2017). The nature of the substrate as well as the cutting sites of the enzyme will give peptides different foam stability and capacity indexes, thus different enzymes will result in peptides with different functionalities. For instance, a study compared the DH values of soy proteins cleaved by Alcalase vs Neutrase. They found that the peptides with a treatment that achieved a 3-4% DH showed a 12-fold improvement in foam expansion when treated with Alcalase, compared to Neutrase which only showed a 4-fold improvement (Whitehurst & Van Oort, 2009). In that same study, a DH beyond 5% lowered foam and emulsion capacity. Lower DH are generally recognized to have better foaming capacity than higher DH, yet there is no consensus on the optimal DH values. Similar to an emulsion matrix, small peptides lack the structural strength to form a net around the air bubbles to be stabilized and could even form "noise" in the stabilization of the network. In fact, Olsen (1984) observed that removing small peptides from a hydrolyzed soy protein isolate by ultrafiltration further enhanced foam stability. The remaining high-molecular-weight peptides were stable enough to completely replace egg whites in a merengue formulation (Olsen, 1984). Some scientists have speculated an inverse relationship between foam capacity and foam stability. Ultimately, the peptides amino acid sequence and molecular weight distribution will govern these two parameters, and they themselves depend on the nature of the substrate and the specificity of the protease.

2.5.3: Solubility

The solubility of proteins refers to the ability to stay in an aqueous solution by interacting with water either by dipole moment, or hydrogen bonds. Their solubility profile will largely depend on the polarity of the amino acid residues exposed on the surface of the protein. Alternatively, electrostatic repulsions intermolecularly will enhance solubility, such is the case with casein, one of the main polypeptides in milk. The solubility of casein is attributed to the negatively charged,

macro-peptide region of κ- casein on the surface of the globular micelles (Lucey, 2011). These micelles exhibit intermolecular negative repulsion, thus stabilizing a colloidal dispersion between them. The solubility of a protein in an aqueous medium will tell us the degree of polar functional groups present in the exposed surface of this protein. Disruption of a proteins structure can significantly increase solubility. A study conducted by Hall. et al. (2017), showed an increased in solubility of tropical banded cricket (*Gryllodes sigillatus*) protein hydrolysates compared to whole crickets. The solubility of the peptides increased as the pH increased, and showed the lowest solubility in its unhydrolyzed form at around pH of 3, suggesting this could be the protein's isoelectric point (Hall. et al., 2017), since the isoelectric point is where a protein exhibits the lowest degree of solubility. Disrupting tertiary structure gives peptides greater flexibility to move, and ionizable groups may become exposed, allowing greater interactions with water. In that same study, enzymatic hydrolysis significantly improved solubility in all pH mediums, with the maximum solubility at a pH of 10 and with a 42% DH. Enzymatic cleavage of peptides is highly specific, thus peptides from different proteases will create different amino acid sequences each with unique solubility profiles. Figure 2.1 shows the increase in solubility of casein peptides at its isoelectric point (4.6 pH) and soy peptides cleaved by different enzymes. In this experiment, the peptides were hydrolyzed with the same DH, yet the difference in enzyme cleavage resulted in different solubility profiles. All enzymes used: Alcalase, Trypsin, and Sumizyme improved casein solubility by 50%, 45%, and 35%, respectively. Soy protein solubility was improved by hydrolysis with Alcalase, Trypsin, and Sumizyme, by 40%, 33%, and 35%, respectively.

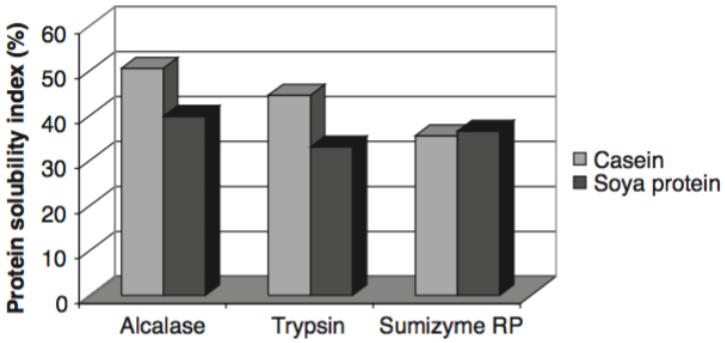

Figure 2.1: Protein solubility index of protein hydrolysate (casein) at isoelectric pH (4.5) (Whitehurst & Van Oort, 2009).

On the other hand, Figure 2.2 compares the solubility of the peptides with a 2% and a 6% DH (Whitehurst & Van Oort, 2009) hydrolyzed by the same enzyme. Figure 2.2 demonstrates that slight disruptions on the proteins sequence exposes functional groups able to interact with water, thus giving rise to higher degrees of solubility. However, the opposite could be true if high enough levels of hydrolysis are reached.

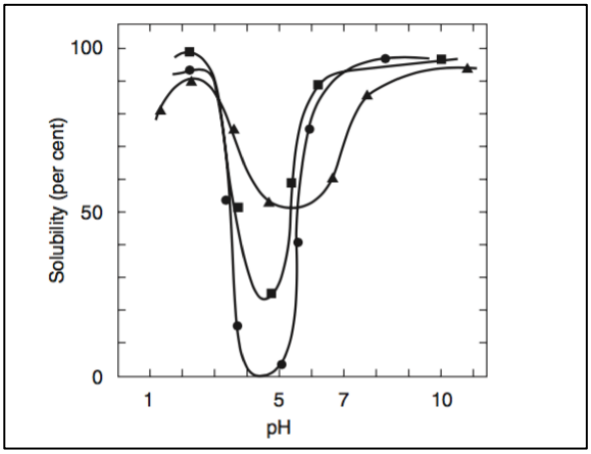

Figure 2.2: pH- solubility profiles of native casein and of Staphylococcus aureus Glu- specific V8 protease modified casein.

The solubility is expressed as per cent of total protein in solution. Circles - native casein; squares- DH= 2%, triangles – DH= 6.7%. (Whitehurst & Van Oort, 2009)

When hydrophobic interactions are the driving force in protein aggregation, then higher DH levels decrease solubility due to hydrophobic groups in the interior of the molecule becoming exposed. This causes the repulsion of water and favors intramolecular hydrophobic interactions (Tavano, 2013). The trend observed in Figure 2.2 suggests that the benchmark for maximum casein solubility was not surpassed by a DH of 6%. A study where glutelin was treated with Protamex with various DH (0-10%) showed an increase in surface hydrophobicity, yet there was significant increase in solubility mainly due to the rupturing of disulfide bonds (Zheng et al., 2015). This demonstrates that hydrophobic interactions were not the driving force in the matrix, rather the rupturing of covalent bonds (disulfide bridges) were the main factor in increasing the protein's solubility. There is no standard DH value to maximize the solubility profile of proteins because every proteins' unique amino acid sequence will yield hydrolyzed peptides with distinctive properties. We can conclude that slight DH disruptions can result in peptides with different solubility profiles, but the protease used along with the nature of the substrate will be the main elements that determine solubility index.

2.6: Rheology

Rheology is the study of deformation, in which a certain strain or stress is applied to a material, and its response measured, giving important information about the tested material. The definition of a viscoelastic material is one that exhibits elastic and viscous behavior. Elastic behavior relates to the ability of a material to resist deformation and regain its shape, while viscous relates to the ability of the material to resist flow (De Vicente, 2012). In general terms, elastic behavior related to the solid-like properties while the viscous behavior is related to the liquid-like properties. In rheology, either stress is applied (force per unit area) and strain measured or vice-versa. There are many types of rheological measurements, however one particularly important for this project is

oscillatory test. In this type of dynamic test an oscillatory strain ($\gamma(t) = \gamma_0 cost(\omega t)$) is applied to a sample and its sinusoidal stress response is measured ($\sigma(t) = \sigma_0 cost(\omega t + \delta)$). Hooke's law states that an ideal solid would respond to this applied strain amplitude with proportional stress, synchronizing the response and resulting in stress and strain in the same phase (Collyer, 1993). Newton's law states that an ideal liquid would respond to the strain rate with proportionality, this time the responses would be out of phase. Note that strain rate is the first derivate of strain. Because viscoelastic materials show both types of behavior (elastic and viscous) their responses will exhibit an angle shift (δ) (De Vicente, 2012). Separating elastic and viscous responses allows for the measurement of strain amplitude and strain rate at the same time. The following equation serves to define the elastic behavior or G', and the viscous behavior or G" (De Vicente, 2012).

$$G' = \frac{\sigma_0 \cos(\delta)}{\gamma_0} \; ; G'' = \frac{\sigma_0 \cos(\delta)}{\gamma_0}$$

For any polymeric material, the elastic strength will be directly affected by its structural features such as molecular weight and orientation of chains. Higher elastic strength is correlated with increasing mean molecular weight. For materials with very high molecular weights, the presence of smaller chains will have little to no effect on its viscoelastic response. On the other hand, for materials with an average of low and medium molecular weights, the weight range will have a greater influence on their response. Solid polymers with high strength are indicative of higher cross-linking activity and high mean molecular weight, one which may also be affected by the specific orientation of the polymeric units. Polymers with decreasing molecular weight gradually shift from a visco-elastic behavior to a visco-flowing behavior due to the failure of the smaller chains to resist deformation (Bartenev & Zuyev, 2013). In other words, small molecular weight polymers can have a plasticizing-like effect in the overall matrix given that their presence is

influential enough in the overall molecular weight distribution. These behavioral transformations happen at temperatures above the glass-transition temperature (T_g).

The T_g of an amorphous solid material is the event in which its mechanical properties transition from a glassy and rigid state to a rubbery, soft state (Gabbott, 2008). There are several factors affecting the T_g of a certain material, these can be extrinsically related, such as pressure, or intrinsically related, such as polymer branching. Analyzing T_g of polymers can provide valuable information in regard to structure. For polymers with rigid and branched chains, the T_g will be higher than one with a linear and flexible backbone. Similarly, high molecular weight polymers will have higher T_g than lower molecular weight polymers (Packham, 2005). All of these indicate that more energy is needed to start molecular mobility. Moisture content directly affects T_g as the absorption of water molecules will act as plasticizers. Higher moisture will allow more mobility, thus lowering the T_g (Packham, 2005). Only amorphous and semi-crystalline polymers possess a T_g, while crystalline solids exhibit a melting temperature (Gabbott, 2008).

2.7: Sensory Evaluation

Purified proteins are known to have very mild, if any, flavor. However, peptides are more reactive and tend to have flavor compounds “hidden” in their amino acid sequence (Whitehurst & Van Oort, 2009). Hydrolyzing a protein may also expose hydrophobic amino acids which have been known to create bitter tastes according to Adler-Nissen (Whitehurst & Van Oort, 2009), this creates a parameter to be measured between sets of hydrolysates. The % DH can be used as a good predictor of bitterness development as the relative amounts of amino groups become more readily available compared to the molecular weight distribution (Whitehurst & Van Oort, 2009). The

relationship between bitterness and % DH can be appreciated in Figure 2.3. Really high DH have been linked to lower bitter notes, but higher brothy and umami flavors (Whitehurst & Van Oort, 2009). Soy sauce is a good example of this, as the complex development of its flavor is a result of a 70% DH (Whitehurst & Van Oort, 2009), with high content of small peptides and free amino acids. Conversely, peptides with low DH tend to not exhibit bitter notes, given their physicochemical structure (Adler-Niseen, 1986).

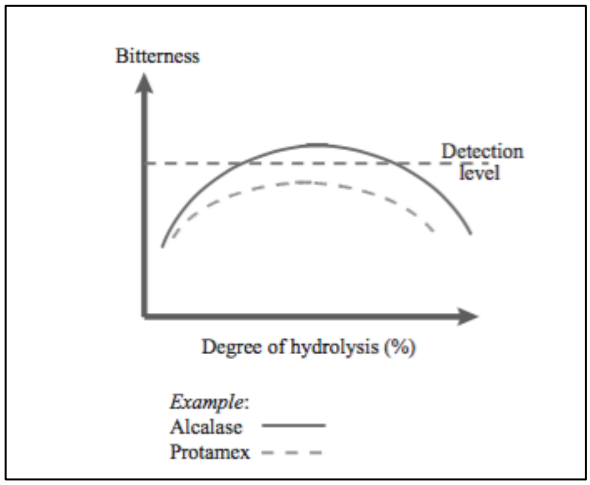

Figure 2.3: Qualitative description of the development of bitterness during hydrolysis of protein raw material using different proteases (Whitehurst & Van Oort, 2009).

According to Adler-Niseen (1986) peptides with low DH will be big enough to "hide" their hydrophobic groups through hydrophobic interactions, thus making them unavailable for taste receptors to perceive. Bitterness formation will also vary depending on the protease used because every enzyme will result in sets of peptides with various molecular weight distributions, and their specificity will govern the sequence of amino acids left in the peptide chain (Whitehurst & Van Oort, 2009). Alcalase is a protease widely used in the food industry for its high efficient performance, however the bitter notes from Alcalase with higher DH products are a main limitation

for the use of this enzyme (Whitehurst & Van Oort, 2009). Figure 2.3 also shows how the development of bitter notes changes depending on the protease used.

Studies show that certain enzymes will result in the development of superior flavor characteristics. For example, hydrolyzing casein with a proline-specific enzyme gave the lowest degree of bitterness compared to other enzymes used (Whitehurst & Van Oort, 2009). Flavor notes of mixed hydrolysates were also shown to have better overall palatability than a single protein source of peptides (Whitehurst & Van Oort, 2009). Endo- and exo-peptidases will result in different flavor profiles. We know that utilizing and enzyme that performs both cleavages, or using a mix of one endo- and one exo- peptidase, is more likely to give a lower bitterness because the exo- peptidase will cut the amino acids from the amino or the carboxyl terminal, which tend to be hydrophobic in nature. The new peptide formed has a lower degree of hydrophobicity, characteristic of a less bitter peptide, and the cleaved amino acid itself doesn't have a bitter taste either (Whitehurst & Van Oort, 2009).

Amino acids can elicit different tastes, depending on their size, hydrophobicity and chirality. Research on solutions with L- and D- enantiomer amino acids in their free form utilizing a set of trained panelists has shown opposing taste patterns with respect to chirality. Hydrophilic, L- small amino acids (Gly, Ala, Ser, and Thr) displayed sweet tastes, while hydrophobic L- large amino acids (Leu, Ile, Phe, and Trp,) displayed bitter notes. Amino acids with characteristics in between the latter two (Cys, Met, and Val) had sweet and bitter tastes intermixed (Kawai, Sekine-Hayakawa, Okiyama, & Ninomiya, 2012). Meanwhile, D-enantiomers had reverse tendencies. Umami notes has been well recorded in salts of Glu (monosodium L-glutamate) and Asp

(monosodium L-aspartate), and it was also noted in solutions with high concentration of amino acids Gly, Ala, Ser, Asn, and Gln (Kawai et al., 2012; Yamaguchi, Yoshikawa, Ikeda, & Ninomiya, 1971). In addition, these amino acids synergistically enhance the umami taste when 5'- inosine monophosphate is added. On the other hand, sour responses are elicited by Asp and Glu when presented in solutions in their free form (Kawai et al., 2012).

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CHAPTER 3: ENZYMATIC HYDROLYSIS OF CRICKET (*ACHETA DOMESTICUS*) PROTEIN TO GENERATE FUNCTIONAL PEPTIDES FOR THEIR USE IN A CORN TORTILLA FORMULATION

3.1: Abstract

Insects have the potential to be a viable protein alternative in a growing world, where traditional sources have been shown to be unsustainable. The successful incorporation of insects into our food chain will require their processing in order to enhance and expand their techno-functional properties. Protein hydrolysis is one of this kind that has been shown to improve insects' functional properties in the past, however there is a lack of research as to how these novel hydrolysates can be incorporated into a food product. In this work highly functional cricket (*Acheta domesticus*) protein hydrolysates (CPH) were generated using Alcalase (AL) and Flavourzyme (FL) proteases. *In vitro* functional parameters (solubility, emulsification and foaming) were studied, along with the physicochemical, and structural characteristics imposed when CPH replaced 20% corn flour in tortillas. Significant differences in functional properties were found between CPH produced with different proteases. AL-CPH powders had better emulsion and foaming results and higher water hydration capacity than FL-CPH powders, while the FL treatment resulted in more soluble peptides. In corn doughs, AL-CPH increased the elastic modulus, indicating significant cross-linking between polymers, while FL-CPH reduced the elastic modulus. Upon cooking the tortillas, AL-CPH resulted in matrices with low toughness and extensibility values, while FL-CPH created a strong and flexible structure, indicating differences in heat-induced intermolecular interactions between the AL- and FL-CPH and the tortilla matrix. Raman spectroscopy revealed a collapse of α - helical structure upon higher DH in both protease treatments. Peptides obtained via Alcalase treatment were shown to have protein-lipid interactions, affecting amphiphilicity properties.

Within the cooked tortilla matrix, the higher DH peptides created more ordered α -helices, and FL3 tortilla showed stronger disulfide bridge formations and several protein-starch interaction vibrations that could explain its superior rheological characteristics.

3.2: Introduction

Insects are considered potential sources of protein to alleviate some challenges associated with the growing demand for food (Van Huis et al., 2013). Nevertheless, insect consumption is limited in Western societies due to neophobia factors (Gmuer, Nuessli Guth, Hartmann, & Siegrist, 2016). Data suggest that incorporating insects as ground powders (flours) could relieve psychological strains (Gmuer et al., 2016); however, research has elucidated poor functional properties associated with these ground insect powders. For instance, low solubility was observed in mealworm larvae (*Tenebrio molitor)* and migratory locusts (*Locusta migratoria*) flours (Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016; Purschke, Tanzmeister, et al., 2018). Poor emulsion and foam formation were also reported for moth larvae (*Cirina forda*) flour (Omotoso, 2006).

Furthermore, studies incorporating insect flour into food matrices lack successful results due to insufficient insect quantities, palatable products, and comparable physicochemical properties to its' insect-free counterpart. For instance, replacing wheat flour with 5% insect flour from house cricket (*A. domesticus*), mealworm, and black soldier fly (*Hermetia illucens*), resulted in bread loafs with decreased water absorption and increased dough formation and stability (González, Garzón, & Rosell, 2018). Enriching wheat bread with 10% and 30% house cricket powder significantly increased bread firmness, darkened its color and gave poor overall sensory acceptability scores (Osimani et al., 2018).

To overcome the functional limitations of whole ground insects, protein hydrolysates produced from insects using commercial proteases have also been explored (Hall et al., 2017). Generally, physicochemical properties of protein hydrolysates are dependent on the type of protease used, nature of the native protein, molecular weight and degree of hydrolysis (DH) (Adler-Nissen, 1986). In a study by Hall et al. (2017), higher emulsion and foam capacities were obtained when tropical-banded crickets (*Gryllodes sigillatus*) were hydrolyzed with Alcalase. Better foaming was reported for migratory locust peptides derived from sequential protease treatment (Purschke, Meinlschmidt, Horn, Rieder, & Jäger, 2018). Similarly, a study performing alkaline proteolysis on *Hermetia illucens* protein showed improved amphiphilicity and solubility at every pH compared to its control (Mintah, He, Dabbour, Xiang, Agyekum, & Ma, 2019). Although there are published reports on functional traits of insect protein hydrolysates, to the best of our knowledge, no studies have been done regarding the functional and structural impact of insect protein hydrolysates on a food matrix. Tortillas, particularly tortilla chips, have become a popular snack in the U.S. over the past decade, with a market projected to be valued at USD $12,324.4 million by 2028 (Future Market Insights, 2018). In addition, the lower protein quality in corn tortillas makes it an ideal matrix to be fortified by the addition of protein hydrolysates (Landry & Moureaux, 1982; Valle & Pérez-Villaseñor, 1974).

The main objective of this study was to compare the efficacy of two proteases in generating an insect protein hydrolysate powder with useful functional properties that could be successfully incorporated into a popular snack, such as tortillas.

3.3: Materials and Methods

3.3.1: Materials

Alcalase (AL) (from *Bacillus lecheniformis,* > 2.4 U/g) and Flavourzyme (FL) (from *Aspergillus oryzae,* > 500 U/g) were purchased from Sigma Aldrich (St. Louis, MO, USA). Raw, frozen house crickets (*Acheta* domesticus) were obtained from a food-grade, cricket farm (Ovipost™, LaBelle, FL, USA). Raw yellow dent corn kernels (*Zea mays)* were purchased from CZ Grains (Iowa, USA) and used to make nixtamalized corn doughs (masa). All other reagents were obtained from Sigma Aldrich (St. Louis, MO, USA), VWR International (Radnor, PA, USA), Thermo Fischer Scientific (Waltham, MA, USA), and Bio-Check (UK).

3.3.2: Production of cricket protein hydrolysates (CPH)

Enzymatic hydrolysis was done following the method by Hall et al. (2017) with slight modifications. Whole crickets (100 g) were blended for 2 min in a commercial blender (Waring Commercial, CT, USA) with 250 mL of distilled water. The resulting slurry was pasteurized at 90°C for 15 min. The pH was adjusted with 5 M Na(OH) and the temperature set according to the enzyme's optimal conditions (pH 8 for Alcalase at 63°C, and pH 7 for Flavourzyme at 55°C). Enzyme concentration (% v/w) and hydrolysis time were determined in pre-screening experiments (Appendix B). Hydrolysis conditions and degree of hydrolysis (DH) used are shown in section 3.4.1.1, Table 3. Enzymatic hydrolysis was stopped by heating samples to 90°C for 15 min. Samples were cooled to 4°C and centrifuged for 15 min at 17,636 x *g* (Avanti J-26S Centrifuge, Beckmann- Coulter INC. CA, USA). Supernatant was lyophilized and stored at 4°C until further use. The control (cricket flour) was produced by thawing and dehydrating whole crickets in a

commercial dehydrator (Osten, Sunbeam Products, FL, USA) followed by grinding with a food processor (Waring Commercial, CT, USA) and stored at 4°C, until use.

The degree of hydrolysis (DH) for each CPH was measured according to the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979), with modifications by Liceaga-Gesualdo and Li-Chan (1999). After hydrolysis, a 1 mL aliquot of each trial was mixed with 1 mL of 24% TCA, and centrifuged (12,100 x *g* for 5 min). Next, a 0.2 mL aliquot was taken from the supernatant and added to 2 mL of 0.2 M sodium borate buffer (pH 9.2), to which 1 mL of TNBS (4 mM) was added. After a 30 min incubation, 1 mL of 2 M monosodium phosphate was added to stop the reaction. Light absorbance was measured (420 nm wavelength, 1 cm cuvette) with a UV-Visible spectrophotometer (Beckmann, Irvine, CA, USA). Degree of hydrolysis was determined using the following equation,

$$\% \text{ DH} = \frac{h}{h_{tot}} \text{ x } 100$$

where h= hydrolysis equivalents or number of peptide bonds cleaved obtained from a lysine standard curve, and h_{tot}= total number of peptide bonds per unit weight determined from the amino acid composition of the protein, as the sum of mmols of the individual amino acids per g protein (Adler-Nissen, 1979). For this study, the h_{tot} was experimentally determined to be 9.37 meq/g.

3.3.3: Characterization of CPH

3.3.3.1: Proximate Composition

Moisture, ash, crude fat, and crude protein content of CPH powders were determined using standard AOAC methods (950.46(b), 920.153, 960.39, 984.13 A-D), respectively. Total protein was calculated using standard conversion factor of 6.25. Protein yield was calculated based on the total protein content of *Acheta domesticus* crickets on an as is basis calculated based on data from Jonas-Levi, & Martinez (2017) in the initial batch by the total protein content in the hydrolysates.

3.3.3.2: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Molecular weight distribution was measured according to Hall, Johnson, & Liceaga (2018). Briefly, samples (2 mg/mL) were dissolved in the 2D-gel extraction buffer 50 mM Tri-HCl, pH 8.8, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 M urea, 2 M thiourea, 2% w/v 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 67mM dithiothreitol (DTT), then dissolved again (1:1) with 2X Laemmeli sample buffer and used immediately. Aliquots (10 μL) were added onto a 4-12% gradient gel (Bis Tris, NuPage, ThermoScientific, Waltham, MA) and run for 50 min at 200 V. Gels were stained overnight with Coomassie R-250 and distained with Coomassie brilliant blue R-250 distaining solution overnight. Precision Plus Protein TM Dual Xtra Prestained Protein Standards (Biorad, Hercules, Ca) were used as molecular weight standards.

3.3.3.3: Glass transition temperature (T_g)

The T_g of all CPH powders were measured with a differential scanning calorimeter (DSC) Q2000 (TA Instruments, New Castle, DE, USA). Prior to measurement, samples were weighted (0.5 $\pm$ 0.1 g) in aluminum pans and stored in a container with saturated salt solutions with the following relative humidity (RH): 11 (LiCl), 23 (KCH_3COO), 33 ($MgCl_2$), and 53 ($MgNO_3$) %. The

containers were then hermetically sealed. Containers were stored at 25°C for at least two weeks to allow complete passive diffusion of water. After the waiting period the aluminum pans were re-weighted and changes in mass were attributed to a gain or loss in total moisture content. The T_g was then measured following Thorat, Forny, Meunier, Taylor, & Mauer (2018) method, with modifications. Samples of 5 – 10 mg weight were measured on to aluminum DSC pans and sealed hermetically, followed by the programmed heating cycles:

AL samples with a_w of 0.11, 0.23, and 0.333 were cooled then heated from -10 to 80°C at 10°C/min, then cooled from 80 to -10°C at 50°C/min, held for 3 min at -10°C, and then heated again from -10 to 80°C at 10 °C/min. AL samples with a_w of 0.53 were cooled then heated from -20 to 70°C at 10 °C/min, then cooled from 70 to -20°C at 50°C/min, held for 3 min at -20°C, and then heated again from -20 to 70°C at 10 °C/min.

FL samples with a_w of 0.11 and 0.23 were cooled then heated from -20 to 80°C at 10°C/min, then cooled from 80 to -20°C at 50°C/min, held for 3 min at -20°C, and then heated again from -20°C to 70°C at 10 °C/min. FL samples with a_w of 0.33 were cooled then heated from -40 to 60°C at 10°C/min, then cooled from 60 to -40°C at 50°C/min, held for 3 min at -40 °C, and then heated again from -40°C to 60°C at 10°C/min. FL samples with a_w of 0.53 were cooled then heated from -60 to 40°C at 10 °C/min, then cooled from 40 to -60°C at 50°C/min, held for 3 min at -60°C, and then heated again from -60 to 40°C at 10°C/min.

The first heating cycle was meant to erase thermal history and allow for the solids to fully relax into its rubbery state. Measurements were taken on the second heating cycle, where T_g was taken where the temperature in which the endothermic baseline shift occurred.

3.3.4: Functional properties of CPH

3.3.4.1: Protein Solubility

Solubility was calculated following the Chobert, Bertrand-Harb, and Nicolas (1988) method with some modifications by (Hall, Jones, O'Haire, & Liceaga, 2017). A 200 mg sample of CPH was dispersed and stirred for 30 min in 20 mL buffer with a particular pH (tested pH: 3 (0.1 M $C_2H_3O_2Na$), 5 (0.1M $C_2H_3O_2Na$), 7 (0.1M Glycine-NaOH), and 9 (7.4 mM PO_4)). Next, samples were centrifuged (7500 x *g* at 4°C) for 15 min. The following equation was used to determine solubility (%):

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}} \text{ x } 100$$

Total protein content of CPH powder was calculated by a certified commercial laboratory (A&L Great Lakes, Fort Wayne, IN, USA) using the standard Kjeldahl method. Protein content in supernatant was quantified through the bicinchoninic acid protein assay (BCA) method, with bovine serum albumin used to calculate the standard curve.

3.3.4.2: Emulsion Capacity & Stability

Emulsification properties followed the Pearce and Kinsella (1978) method with modifications by Liceaga-Gesualdo and Li-Chan (1999). CPH was dispersed in 0.1 M phosphate buffer (pH 7) to obtain 0.5% (w/v), then 3 mL of this solution was added to 1 mL of 100% pure canola oil (Great

Value, Bentonville, AR, USA). This mixture was homogenized in a Sorvall Omni Mixer (Norwalk, CT, USA) at 18000 rpm for 1 min. A 25 μL aliquot was immediately diluted (200-fold) with 0.3 % sodium dodecyl sulphate (SDS) solution, and gently inverted 6 times. Turbidity was measured at 550 nm wavelength using a UV-Visible spectrophotomer (Beckmann, Irvine, CA, USA). The Emulsifying activity index (EAI) was calculated with the following equation:

$$\text{EAI} = \frac{(2T)(A)(df)}{(\phi)(c)(10{,}000)}$$

Where T is turbidity, A is absorbance at 550 nm and *df* is dilution factor (200-fold), ϕ is the volume of the oil phase (0.25) and c is for concentration of solids in solution.

Samples were left immobile and light absorbance was measured after 30, 60, and 90 min to calculate the Emulsion Stability Index (ESI) with the following equation:

$$\text{ESI (\%)} = \left(100 - \frac{\text{EAI}_0 - \text{EAI}_\text{T}}{\text{EAI}_0}\right) \text{ x } 100$$

Where EAI_0 equals the EAI (at time zero) and the EAI_T equals the EAI at time 30, 60, or 90 min.

3.3.4.3: Foaming Capacity & Stability

Foaming properties followed the methods by Waniska and Kinsella (1979) and Pacheco-Aguilar, Mazorra-Manzano, and Ramírez-Suárez (2008) with some modifications. A solution consisting of 0.75 g of CPH protein in 25 mL of distilled water was mixed thoroughly for 10 min with a magnetic stir bar at room temperature. A Sorvall Omni Mixer (Norwalk, CT, USA) was used to create the

foam by aerating at 18, 000 rpm for 1 min and volumes were recorded immediately after aeration and after resting periods (10, 30, 60, and 90 min). The following equation was used to calculate Foam Capacity (FC):

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

Foam Stability (% FS) was interpreted as foam remaining after immobile rest periods of 10, 30, 60, and 90 min.

3.3.4.4: Water Hydration Capacity (WHC)

The WHC is defined as the maximum amount of water that 1 g of material will retain under low-centrifugation conditions. WHC was measured following the American Association of Cereal Chemists (AACC) International method (56-30.01) with some modifications. Briefly, 2 g of CPH powder was added to a pre-weighted centrifuge tube, enough distilled water was added to thoroughly wet material and tube was vortexed for 5 min. Tube was centrifuged at 2000 x *g* for 10 min and supernatant discarded. Approximate WHC (mL/g) was calculated with the following equation:

$$\text{Approximate WHC} = \frac{(\text{weight of tube + sediment}) - (\text{weight of tube + 2})}{2}$$

The following formula was used to calculate amount of CPH (g) added to the following tubes:

$$\text{Weight of CPH (g)} = \frac{5}{\text{approximate WHC} + 1}$$

Four centrifuge tubes containing this amount of CPH were filled with distilled water by 1.5 and 0.5 mL more and 0.5 and 1.5 mL less than (5 – weight of CPH). Tubes were vortexed for 2 min and centrifuged at 2000 x *g* for 10 min. WHC was determined as the midpoint between the tube containing supernatant and the adjoining tube with no supernatant. WHC was calculated as the volume (mL) of water divided by g of CPH added.

3.3.5: Production of maize tortillas with CPH

Traditional nixtamalization was used to prepare corn doughs (masas) following a modified method from Rosales, Agama-Acevedo, Arturo Bello-Perez, Gutierrez-Dorado, and Palacios-Rojas (2016). Yellow dent corn (*Zea mays*) (700 g) was cooked at 80°C for 35 min with 3 L of reverse-osmosis water with 1.2% $Ca(OH)_2$, then left to steep for 16 hours at 25°C. The wastewater was discarded, and the corn washed three times with tap water and ground with a commercial stone grinder (Maquinas Gonzalez, Monterrey, MX) to form the dough. Dough was lyophilized and finely milled using an electric Cyclotec 1093 mill (Foss, Hillerod, Denmark). Corn tortillas were made with and without 20% CPH (w/w), and 1% (w/w) of sodium propionate, potassium sorbate, and sodium chloride, each. To this dry mixture, distilled water was added according to data obtained via mixograph-hydration tests (section 3.3.5.1, Table 2). Dough was mixed in a commercial electric mixer (Kitchen Aid, Benton Harbor, MI, USA) for 1 min, then 16 g dough balls were shaped into round disks (12 cm diameter) using a tortilla press. Tortillas were cooked on a hot electric griddle (Presto, WI, USA) at 250 $\pm$ 5°C for 2.50 min, flipping them every 30 seconds. Upon cooling, tortillas were packed in low-density polyethylene (LDPE) bags and frozen at –20°C unless otherwise specified.

3.3.5.1: Mixograph Hydration Tests

Maximum hydration was determined via mixograph tests. Briefly, 20 g of dried corn flour with and without 20% (w/w) CPH were added into the 35 g-Mixograph (National MFG, Lincoln, NE, USA) bowl and distilled water was slowly added. Mixing was done for 2 min and probe spring was set at position 2. Complete hydration of corn doughs was determined when the probe spring reached maximum (mm). Subsequently, water to solid ratios (termed water multiplier) were calculated at this time. In the case of AL-CPH corn doughs, the water multipliers were re-adjusted to facilitate their handling with the following formula:

$$\frac{50\ (M_o) + 6}{50} = M_n$$

Where M_o= old water multiplier and M_n= new water multiplier.

Water multipliers are listed in Table 2, below.

Table 2: Water Multipliers for corn doughs (raw) with 20% CPH

Trial code	Water (g) needed to reach max	Max reached (mm)	Water multiplier	New Water Multiplier
Control	29.5	64.0	1.48	Same
FL1	22.3	64.0	1.12	
FL2	21.2	75.0	1.06	
FL3	23.1	51.0	1.15	
AL1	18.6	117.0	0.93	1.05
AL2	17.7	106.0	0.88	1.01
AL3	17.9	100.0	0.89	1.00

Control = 100% corn dough.
Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.

3.3.6: Characterization of Tortillas and Tortilla Chips

3.3.6.1: Proximate Composition

Moisture, ash, crude fat, and crude protein content of CPH-tortillas were determined using standard AOAC methods (950.46(b), 920.153, 960.39, 984.13 (A-D), respectively. Total protein was calculated using standard conversion factor of 6.25.

3.3.6.2: Amino acid composition

Total amino acid analysis of CPH-tortillas and tortilla control was done by the Danforth Proteomics Center and Mass Spectrometry Facility (St. Louis, Missouri, USA). Samples were first pulverized with a mortar and pestle, then complete hydrolysis of protein was induced by suspending sample (1:10 w/v) directly in 12 M HCl overnight. Briefly, 40 μL of sample diluted in 30% d-water was heated to 30ºC for 2 hours to enable conversion of Cysteine to Cya and Methionine to MetS. Hydrolysis followed with 0.4% beta-mercaptoethanol (BME)/6 M HCl, then samples were dried and re-suspended in 200 μL of HCl (20 mM). Samples were then analyzed by UPLC Amino acid analysis solution using AccQTag Ultra Derivatization kit with UV detection.

3.3.6.3: Color

A Labscan XE Colorimeter (Hunter Associates Laboratory, VA, USA) was used to obtain the L (brightness), *a* (red-green), and *b* (yellow-blue) values of tortillas. Whole tortillas (dimensions?) were place directly on top of the reader using a 2-inch window tile in order to ensure no light entered the detector. Standard white and black tiles were used to calibrate colorimeter. Measurements were done in triplicate.

3.3.6.4: Water Activity

Water activity of CPH-doughs and control dough were measured with an Aqualab 4TE (Meter Group, Pullman, WA, USA) in triplicate at 25ºC. Aqualab was first calibrated with 0.5 m KCl (0.984 Aw) and 2.33 m NaCl (0.920 Aw) salts to obtain accurate measurements.

3.3.7: Structural Analysis

3.3.7.1: Rheology and Texture

Rheological properties of CPH-corn doughs and control dough were measured following (Guzmán, Flores, Escobedo, Guerrero, & Feria, 2009) with modifications. Small amplitude oscillatory strain (SAOS) tests were performed on 10 g of sample with a Rheometer DHR-3 (TA Instruments, Newcastle, DE), using a 40 mm hatched parallel plate system with a 1 mm sample gap. Amplitude sweeps were performed within a range of 0.01 to 1% at 1 Hz, to obtain a linear viscoelastic region. Frequency sweeps were then performed at a constant 0.1% strain with an angular frequency of 0.1 to 10 Hz. All measurements were run at 25°C in triplicate. The storage elastic modulus (G'), and the viscous modulus (G"), were obtained with the TRIOS software (TA Instruments, Newcastle, DE).

Toughness (g) and extensibility (mm) were obtained as described by (Ramírez-Wong et al., 2007) with some modifications. Whole tortillas were clamped to a ring attachment using a Texture Analyzer- *HD Plus* (TA Instruments, Newcastle, DE) and a biaxial compression force (1 mm/s, for 40 mm) was applied until rupture. Toughness (g) was recorded as the maximum force needed to rupture the tortilla, while extensibility (mm) was recorded as distance of probe travelled before rupture. Measurements were recorded after 0, 1, 5, and 10 days of preparation. Rollability was

determined via the dowel test following the methods by Suhendro, Almeida-Dominguez, Rooney, Waniska, and Moreira (1999), using an acrylic dowel (1.5 cm diameter) within 3 hours of making the tortillas, held at room temperature. The degree of rupturing was measured in a scale of 1-5, where 5= no cracking, 3= medium cracking, maintains structure, and 1= unrollable.

3.3.7.2: FT-Raman Spectroscopy

Raman spectra were obtained using a Bruker MultiRAM system (Bremen, Germany) with a 1064 nm laser source and a liquid nitrogen cooled Ge detector. Spectral data were collected over the range 400 – 3500 cm^{-1} at a resolution of 4 cm^{-1} under fixed measurement parameters. Laser power was adjusted according to optima resolution of samples, resulting in 100 mW, an average of 500 scans were taken. The OPUS software package provided by Bruker Optics (Billerica, MA, USA) was used for spectral acquisition, instrument control and preliminary file manipulation. Tortillas were pulverized using a mortar and pestle and spectra were taken directly of the powdered tortilla samples and powdered CPH. Spectra was baseline-corrected and normalized against the phenylalanine band at $1003 \pm 1\ cm^{-1}$.

3.3.8: Statistical Analysis

All analysis was done in triplicate and statistical significance (α = 5%) was calculated using Minitab 16® statistical software (State College, PA, USA). Analysis of variance (ANOVA) was carried out between trial measurements using a General Linear Model with Tukey's pairwise comparison to obtain 95% confidence level.

3.4: Results and Discussion

3.4.1: Characterization of CPH

3.4.1.1: Proximate Composition and SDS-PAGE

Proximate analysis and % DH of CPH are shown in Table 3. In order to create highly functional powders, the degree of enzymatic hydrolysis was kept below 20% in all trials (Dinakar Panyam, 1996). The enzymatic treatments were successful at creating a range of DH from low (8% DH), medium (11-12% DH), and high (14-14.5% DH). These values indicate a positive relationship between higher DH and increasing enzyme concentration and hydrolysis time.

In terms of proximate composition, significant differences were found between the cricket flour (CF) and the CPH, as well as between the CPH produced by the different proteases. The control, unhydrolyzed CF contained the lowest ($p < 0.05$) moisture and ash contents and highest crude fat content (Table 3). The higher ash content in CPH compared to the CF may be attributed to the sodium hydroxide used for pH adjustment during the production of CPH (Liceaga-Gesualdo & Li-Chan, 1999). The lower fat content in CPH compared to the CF likely resulted from the protease treatment and centrifugation, which solubilized CPH in the supernatant and seemingly left increasing amounts of lipid in the precipitate as the DH increased. AL-CPH had lower ash and higher crude fat contents than FL-CPH. Higher fat content in AL-CPH can be explained by Alcalase ability to release intracellular lipids more efficiently than Flavourzyme. This was previously demonstrated in a study observing the superior potential of Alcalase to obtain lard from porcine cells compared to other proteases, including Flavourzyme (Wang et al., 2016). Furthermore, Figure 3.1 A and B visually demonstrates the drastic changes in lipid release comparing Alcalase and Flavourzyme treatments, respectively. As expected, protein content in

CPH increased with higher % DH, indicating the ability of both proteases to release protein from the insoluble chitin in cricket flour, similar observations were reported by Hall et al. (2017, 2018); however these changes were not significantly different between treatments in the present work. Protein yield, based on the total protein content (as is) was the following: 51% for AL1, 55.2% for AL2, 60.2% for AL3, 21.8% for FL1, 23.1% for FL2, and 28.1% for FL3. These results demonstrate Alcalases' capacity to extract more protein than Flavourzyme, likewise an increase in enzymatic treatment time increased protein yield.

Results for the molecular weight distribution by SDS-PAGE revealed differences on peptide molecular weights between AL- and FL-CPH treatments (Figure 3.2). Bands can be observed at 120 kDa, however they are more pronounced in CF and AL-CPH treatments. In contrast, bands near 50 kDa are stronger in FL-CPH and CF. Bands with a molecular weight near 80 kDa are present primarily in AL-CPH, as well as a high number of medium-size peptides seen by a series of dark bands between 3.5-30 kDa. This type of "smeared" stains at low to medium molecular weight sizes (2 – 10 kDa) have been previously seen by Tan, Chang, and Meng (2019) in catfish peptides with similar DH (5 – 20%) in treatments with Alcalase and other endo-proteases. Alcalase's endo-protease activity naturally cuts internal peptide bonds (Walsh, 2002), therefore the hydrolysis range of AL1-3 (8-14% DH) resulted in soluble proteins composed mainly of high molecular weight peptides (120 kDa) and aggregated medium-size peptides. Conversely, Flavourzyme characteristic dual exo- and endo-protease activity produced several small molecular weight peptides and free amino acids (< 4.0 kDa). One free amino acid is approximately 110 Da, while small peptides may range from 200 – 2000 Da (Berg, Tymoczko, Stryer, 2002), placing them below the lower limits of the molecular weight standards in the gel herein used, therefore we

are unable to see them. Similar molecular weight distributions were seen by Celus, Brijs, & Delcour (2007), showing an overall predominance of intermediate molecular weight peptides (1.7-14.5 kDa) for Alcalase, while Flavourzyme exhibited a higher amount of lower molecular weight peptides (< 1.7 kDa) and higher weight peptides (> 14.5 kDa) at the same degree of hydrolysis.

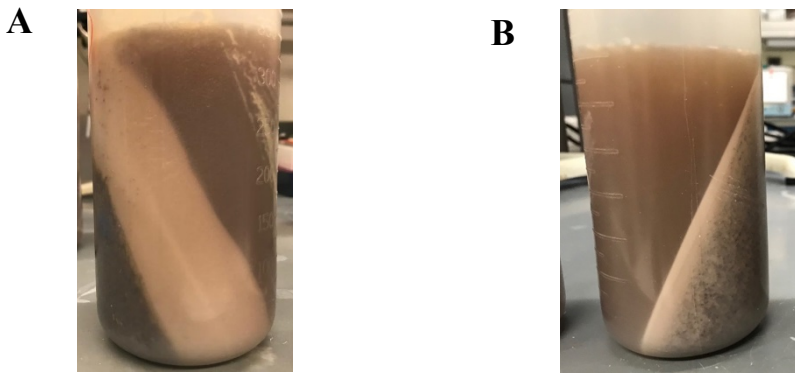

Figure 3.1: CPH samples hydrolyzed by (A) Alcalase and (B) Flavourzyme, after centrifugation at 17,636 × g for 15 min.

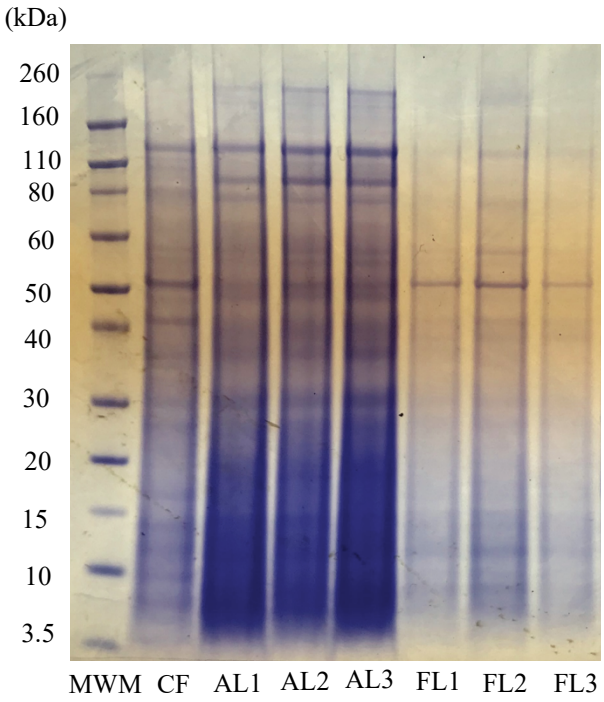

Figure 3.2: SDS PAGE of CPH trials and control (CF).

Trial codes: MWM: Molecular weight marker; CF: Cricket Flour; FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively; Control: corn tortilla control.

Table 3: Hydrolysis conditions, degree of hydrolysis (DH), and proximate composition of CPH trials and control

Trial code	E/S (%)	Hydrolysis Time (min)	DH (%)	Moisture (%)	Crude Protein[2] (%)	Ash[2] (%)	Crude Fat[2] (%)
FL1	0.5	10	8.21 ± 0.5d	12.0 ± 1.7a	70.6 ± 2.4a	17.4 ± 1.1a	6.1 ± 1.4cd
FL2	1.5	30	11.93 ± 0.4bc	10.3 ± 0.6ab	71.4 ± 4.1a	16.4 ± 0.6ab	6.2 ± 0.3cd
FL3	3.0	30	14.54 ± 0.9a	10.0 ± 1.7ab	72.8 ± 3.4a	13.7 ± 1.0b	3.8 ± 0.9d
AL1	0.1	5	8.11 ± 0.3d	10.7 ± 0.6ab	67.2 ± 1.5a	9.0 ± 2.0c	12.1 ± 0.6b
AL2	0.1	30	10.89 ± 0.7c	8.3 ± 0.6b	67.2 ± 1.5a	8.6 ± 0.6c	11.3 ± 0.7b
AL3	0.3	10	13.98 ± 1.7ab	12.0 ± 0.01a	69.7 ± 1.6a	9.5 ± 0.5c	7.1 ± 1.7c
Control (CF)	n/a	n/a	n/a	3.3 ± 0.6 c	67.4 ± 1.5a	4.0 ± 0.9d	23.2 ± 1.3a

CF = Cricket Flour; FL = Flavourzyme, AL = Alcalase; E/S = enzyme substrate ratio, percent (w/w) of enzyme added per g of protein content. Control was un-hydrolyzed cricket flour.
Values represent means ± SD of triplicate. Trials that do not share letters are significantly different ($p < 0.05$).
[2]Values listed are on a dry-basis.

3.4.1.2: Glass Transition temperatures (T_g)

The T_g of all CPH powders along a water activity range of 0.11 – 0.53 are listed on Table 4. Several differences between the enzymatic treatments are apparent based on their T_g values. Alcalase peptides have decreasing T_g with increasing water content, an expected relationship that indicates the role of water molecules acting as plasticizers, thus increasing molecular mobility (Packham, 2005). There was a lack of substantial change of Alcalase peptides' T_g between water activity 0.23 and 0.33 due to the insignificant increases in water content. This important distinction has been explained by others, concluding that total water content, as opposed to water activity, is a better indicator of the plasticizing effect within a matrix (Barbosa-Cánovas, Fontana, Schmidt, &

Labuza, 2007). This same relationship was observed in Flavourzyme peptides, although the increases in water content more severely affected the T_g compared to the pattern observed in Alcalase peptides. In fact, comparing the T_g of the highest and lowest water contents, the change in glass transition temperatures (°C) of Alcalase peptides and Flavourzyme peptides reached 29.5°C and 59.9°C, respectively. The glass transition temperatures of Alcalase peptides were significantly ($p < 0.05$) higher than those of Flavourzyme, indicating severe molecular weight differences among the two. It has been well established that an increase in mean molecular weight increases glass transition temperatures, because there is a general increase in energy required to reach translational mobility due to high density configurational arrangements (Fox & Flory, 1954; Packham, 2005). This indicates that Alcalase's peptides have an overall higher molecular weight than those of Flavourzyme, further supporting our assumptions that low molecular weight peptides (<3.5 kDa) from the Flavourzyme treatment were unable to be clearly seen in the SDS-PAGE. More importantly, these smaller peptides lowered the mean molecular weight of the overall collection of Flavourzyme peptides enough to lower the T_g to below ambient temperatures at all measured moisture contents. Within the different % DH, all the AL peptides exhibited similar T_g values, except at a_w 0.11, where AL3 showed a significantly ($p < 0.05$) smaller T_g than AL1 and AL2. This change in T_g indicates the presence of smaller peptides in the higher hydrolyzed treatment. Others have seen decreases in T_g in higher hydrolyzed wheat proteins as well (Zhou, Liu, Chen, Chen, & Labuza, 2014), attributing these observations to molecular weight differences. In addition, this study observed changes in microstructure via scanning electron microscopy (SEM) of higher % DH whey protein hydrolysates collapsing with lower RH and lower temperatures. The collapsing structures are said to be responsible for stickiness and caking. This observation is consistent with our current observations with respect to the texture of FL peptides

powders, likely due to the presence of more small molecular weight peptides and free amino acids. Within the different % DH all FL treatments seemed to have no significant changes in T_g at the measured water activities, except at 0.33, where higher % DH increased T_g steadily through the treatments. This increase in T_g may indicate presence of larger peptides, a result of the exo-peptidase activity giving off large fragments of the middle segments of the protein that had increased dispersibility with % DH, ending in the supernatant fractions upon protein extraction. The lower % DH within the FL treated-peptides did not have sufficiently dispersible large molecular weight fragments from the middle segments of the protein, ending in the pellet during protein isolation steps.

Table 4: Glass transition temperature (Tg) of all CPH trials

Trial	a_w	Moisture (%)	T_g (°C)
FL1	0.1	3.6	14.1 ± 0.7c
FL2		3.8	16.1 ± 2.4c
FL3		4.7	11.9 ± 0.1c
AL1		2.9	48.2 ± 0.7a
AL2		4.5	44.3 ± 2.6a
AL3		4.0	38.9 ± 1.6b
FL1	0.2	4.5	-2.9 ± 3.1b
FL2		5.5	-3.2 ± 3.4b
FL3		5.7	-0.3 ± 2.1b
AL1		4.5	40.2 ± 1.4a
AL2		5.4	38.9 ± 1.1a
AL3		5.7	37.1 ± 0.5a
FL1	0.3	7.5	-20.5 ± 2.3d
FL2		7.7	-15.4 ± 1.1c
FL3		8.0	-7.1 ± 1.3b
AL1		5.5	40.6 ± 0.7a
AL2		6.7	39.8 ± 0.4a
AL3		7.1	39.4 ± 0.2a
FL1	0.5	18.8	-48.9 ± 0.5b
FL2		19.3	-46.9 ± 0.7b
FL3		19.5	-45.8 ± 3.9b
AL1		12.3	19.6 ± 2.5a
AL2		11.7	20.0 ± 0.3a
AL3		13.2	18.7 ± 0.5a

Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.
Trials that do not share letters within the same water activity (A_w) column, are significantly different ($p < 0.05$). Values represent means ± SD of triplicate.

3.4.2: Functional Properties

3.4.2.1: Emulsifying Capacity & Stability

AL-CPH trials had significantly higher ($p < 0.05$) emulsion capacity (EAI) than CF, while FL-CPH trials had lower EAI (Table 5). Differences in the emulsion stability index (ESI) were also observed, with the highest ESI found in the AL-treatments at 30 min. Meanwhile, FL-trials did not improve stability compared to the control and were even less stable at some time points. All trials showed a decrease in stability after 60 min, with AL1 and AL2 remaining most stable throughout the 90 min. The molecular weight distribution of AL-peptides was larger than FL-peptides (Figure 3.2), which could be a reason for overall better emulsions. Similar observation occurred in milk peptides, where large peptides created more stable emulsions, while smaller peptides disrupted the emulsion interface, creating noise (Haque, 1993). Likewise, Tan et al. (2019) observed that larger molecular mass peptides displayed higher EAI and ESI compared to more hydrolyzed peptides. Another study on chickpea protein hydrolysates concluded that a 4% DH gave the highest EAI values compared to hydrolysates with higher % DH and un-hydrolyzed chickpea protein isolate (Ghribi et al., 2015). Similarly, others observed that EAI had a dependency on % DH when cricket (*G. sigillatus*) protein was treated with Alcalase, resulting in peptides with up to a 32 m^2/g EAI (26 – 29% DH) being significantly higher compared to its unhydrolyzed control (20 m^2/g) (Hall et al., 2017). Contrasting to the present study, emulsion activity of peptides from migratory locusts (*L. migratoria*), measured at pH 5 and 7 (EAI of 40%, and 50%, respectively) only improved when both, endo- and exo- proteases were used, compared to the unhydrolyzed control, but there was no improvement when only one of these proteases was used (Purschke et al., 2018). The stabilization of an emulsion is also dependent on the amphiphilic properties of the peptides, given that an emulsion requires oil droplets to suspend in an aqueous

medium. In fact, a study on the characterization of β – lactoglobulin peptides in the active sites of emulsion droplets concluded that amphiphilic properties were more important than peptide length in the formation of an emulsion (Rahali, Chobert, Haertle, & Gueguen, 2000). In the case that the lipids within the Alcalase pool of peptides possess surface activity, they would too affect EAI and ESI.

3.4.2.2: Foaming Capacity & Stability

All CPH trials had significantly better foam capacity than the CF, although different foam capacity trends were found between AL and FL trials. Increasing the % DH from 8% in AL1 and FL1 to 14% in AL3 and FL3, resulted in increasing foam capacity in the AL-CPH and decreased foam capacity in the FL-CPH. All CPH trials exhibited decreased foam stability over time, with greater decreases in the FL-CPH (FL2 and FL3). Studies have reported that hydrolyzing proteins can also improve foaming capacity and stability. For example, limited soy proteolysis with Alcalase improved emulsion and foam capacity (Whitehurst & Van Oort, 2009), and hydrolysis treatment (Neutrase then Flavourzyme) of locust peptides showed a significant increase ($p < 0.05$) in foam capacity (326%) at the protein's isoelectric point (pI) (pH 3) and increased foam stability at neutral and alkali pH (Purschke et al., 2018). Alcalase is an endo-protease with specificity for hydrophobic amino acids (Doucet, Otter, Gauthier, & Foegeding, 2003), explaining AL-CPH's ability to interact with non-polar surfaces at their terminal ends, while interacting with the aqueous environment with other hydrophilic residues, thereby increasing foaming capacity. The amphiphilicity needed to stabilize an emulsion is also needed to stabilize a foam, therefore the superior FC of AL-CPH compared to FL-CPH further shows the exposed amino acid differences between the two treatments. Furthermore, the exo-protease nature of Flavourzyme yields higher amounts of small terminal peptides and free amino acids. Previous research has shown that the

limiting size of hydrolysates is detrimental in the stabilization of emulsions and foams, outlining a surface-active peptide length of no less than 2 kDa (Dexter & Middelberg, 2008). However, size of the peptide may not be as relevant as the specificity of the protease, given the sequence of the parent protein.

3.4.2.3: Solubility

Protease treatments resulted in CPH that were significantly ($p < 0.05$) more soluble than CF at each pH tested. All trials exhibited the lowest solubility at pH 3, indicating the likelihood that pI was close to pH 3 for the CPH, similar to the pI of peptides sourced from tropical banded crickets (*G. sigillatus*) (Hall et al., 2017). Other studies on locust (*L. migratoria*) and mealworm (*T. molitor*) reported pI values for insect proteins around pH 4 (Purschke et al., 2018; Bußler et al., 2016). When comparing solubility between the protease types, FL-CPH tended to be more soluble than AL-CPH. The higher solubility of FL peptides might be related to its exo-peptidase activity, given that smaller MW peptides (< 3.5 kDa) present more ability to disperse in any medium due to an increase in polar and ionizable groups for enhanced interaction in water (Nguyen et al., 2017). Another possibility is FL-peptides' significant lower lipid content compared to AL-peptides and control. Alcalase has been shown to be an effective protease to extract porcine lard in comparison to other proteases, including Flavourzyme, suggesting its efficacy at breaking cellular structures to release lipids (Wang et al., 2016). In addition, Alcalase peptides likely have hydrophobic ending due to this protease's hydrophobic specificity (Doucet, Otter, Gauthier, & Foegeding, 2003), making them suitable to bind lipids. The increase in lipid content in AL-CPH trials decreased solubility as hydrophobic interactions entropically inhibit protein dispersion (Hayakawa & Nakai, 1985). Interestingly, the trend in solubility with % DH was opposite for the two protease types. AL-CPH tended to increase in solubility as % DH increased, while FL-CPH decreased in solubility

with increasing % DH. Increasing % DH in AL peptides, decreases amount of fat (section 3.4.1.1, Table 3), therefore decreasing an inhibition of protein dispersion and resulting in more soluble peptides. It is not clear why an increase in % DH in FL treatment decreases solubility, it could be due to the presence of large peptides that result from breaking the parent protein at its terminal ends that are neither amphiphilic nor have a significant amount of polar residues, therefore decreasing mean solubility. The creation of these large peptides more likely were left behind in the pellet upon centrifugation, although reaching a certain degree of dispersibility with incremental amount of % DH to be emulsified or dispersed in the supernatant. The indication of these large peptide fragments was noted by the increase of T_g values for the higher hydrolyzed FL peptides at a measured water activity of 0.33. In order to prove this theory, further experimentation would be needed. Increasing pH from 3 to 9 tended to increase the solubility of all CPH, achieving 100% solubility in FL1 and 76% solubility in AL3. Contrary to these results, Purschke et al. (2018) observed the lowest solubility of locust peptides after treatment with Flavourzyme, not significant to its un-hydrolyzed locust flour, and with a small increase in solubility (22%) at pH 9. In contrast, J. Wang, Y. Wang, Dang, Zheng, & Zhang (2013) observed almost 100% solubility at alkali pH for housefly larvae peptides, using a two-step hydrolysis procedure of Alcalase followed by Flavourzyme (60% DH).

Hall et al. (2017) created peptides with a range of % DH using Alcalase on tropical banded cricket protein and reported significant increase in solubility among all trials compared to its un-hydrolyzed control, reaching a maximum of 90% solubility at pH 9. Zielińska, Karaś, and Baraniak (2018) performed an alkaline protein extraction of tropical banded crickets and locusts observing a pI at pH 5 and having a characteristic “U” shape solubility curve throughout pH values 2 – 11.

The highest solubility was seen at pH 11 (96% crickets, and 90% locusts) but high solubility values were also seen at pH 2 (72% crickets, and 87% locusts). The overall increase in solubility of the peptides compared to the control may also be attributed to a separation of the protein from its chitin exo-skeleton, recognized to be an insoluble fiber. A protein's electronegative charge will affect protein-water interactions, therefore if a high pH environment imposes charges on the surface area of the peptides it would increase repulsive forces inter-molecularly, thus increasing solubility (Kramer, Shende, Motl, Pace, & Scholtz, 2012).

3.4.2.4: Water Hydration Capacity (WHC)

The most soluble CPH did not have the highest water hydration capacity (WHC, Table 5), but trends in WHC were consistent with the CPH trends in emulsifying ability. AL-CPH had a higher WHC than FL-CPH and CF, while Fl3-CPH trial had lower WHC than the CF. WHC has been defined differently throughout studies, therefore this study followed the official AACC definition stating that WHC will be "the maximum amount of water that 1 g of material may retain under low-speed centrifugation". The overall bounded water is composed of absorbed and retained water. The first relates to water tightly bound to the protein via chemical interactions (hydrogen bonding, electrostatic, etc.) while the latter refers to water immobilized by the establishment of networks formed by the polymer (Kneifel, Paquin, Abert, & Richard, 1991). In other words, total binding of water in a protein matrix will depend on the amount of water within and between particles. The amphiphilic properties of the AL peptides might be attributed to the hydrophilicity of the peptides themselves or naturally occurring lipids (high fat content in AL powders, section 3.4.1.1, Table 3) with surface activity (mono- and di- glycerides, phospholipids), thus still possessing water-binding properties. The lipids' surface activity is indicated by the emulsifying functionality of the CF and Alcalases' recorded potential to release fat from other animal cells (Wang et al., 2016). In addition,

interstitial water is increased with increasing polymer-polymer interactions, alluding to protein interactions between AL-CPH. The propensity of these large to medium size peptides to form entanglements was later confirmed in the small amplitude oscillatory shear (SAOS) rheological tests (section 3.4.4). Another study observed no differences in WHC when *A. domesticus* was submitted to defatting compared to a protein isolate, resulting in values comparable to FL-WHC of the present study (2.03 and 2.73mL/g, for defatted whole flour and protein isolate respectively) (Ndiritu, Kinyuru, Kenji, & Gichuhi, 2017). Notably, this study did not separate the chitin fiber from any of the samples due to a difference in protein isolation technique, which could attribute to some of the differences. In addition, the overall higher amount of polar amino acids (Appendix A) in AL peptides compared to the FL peptides would increase WHC, given that polar residues can bind more water molecules per residue compared to nonionized and hydrophobic side chains (Zayas, 2012).

Table 5: Functional properties of control flour and CPH trials.

	(min)	AL1	AL2	AL3	FL1	FL2	FL3	CF
EAI (m^2/g)	0	44.1 ± 1.2^{a}	43.6 ± 0.3^{a}	44.5 ± 0.6^{a}	21.8 ± 0.1^{c}	20.6 ± 0.01^{c}	16.1 ± 0.4^{d}	28.3 ± 1.2^{b}
ESI (%)	30	89.0 ± 3.2^{a}	82.2 ± 4.4^{a}	90.0 ± 3.0^{a}	38.1 ± 0.9^{bc}	31.4 ± 0.5^{c}	42.6 ± 6.3^{b}	37.8 ± 2.6^{bc}
	60	73.4 ± 2.5^{b}	81.1 ± 2.4^{a}	30.4 ± 1.2^{d}	28.4 ± 3.0^{d}	28.4 ± 0.6^{d}	33.8 ± 2.2^{cd}	39.1 ± 1.0^{c}
	90	32.9 ± 1.0^{a}	34.4 ± 1.9^{a}	24.0 ± 3.7^{b}	24.6 ± 1.5^{b}	23.3 ± 2.2^{b}	28.9 ± 1.6^{ab}	32.23 ± 4.6^{a}
FC (%)	0	82.2 ± 7.7^{c}	128.9 ± 3.8^{ab}	138.7 ± 9.2^{a}	120.9 ± 8.3^{b}	143.3 ± 3.3^{a}	68.9 ± 3.8^{c}	44.4 ± 3.8^{d}
FS (%)	10	50.93 ± 2.3^{c}	68.9 ± 1.7^{ab}	73.5 ± 3.3^{a}	59.8 ± 1.9^{bc}	64.2 ± 5.3^{ab}	39.2 ± 3.4^{d}	32.6 ± 4.1^{d}
	30	48.9 ± 2.5^{c}	59.2 ± 0.7^{ab}	62.0 ± 3.1^{a}	52.3 ± 3.1^{bc}	24.0 ± 3.4^{d}	30.7 ± 4.5^{d}	24.9 ± 4.8^{d}
	60	44.6 ± 3.2^{c}	55.3 ± 0.9^{ab}	58.3 ± 3.3^{a}	48.2 ± 3.5^{bc}	13.8 ± 0.8^{e}	16.0 ± 4.7^{de}	23.5 ± 3.9^{d}
	90	38.9 ± 6.5^{b}	52.3 ± 3.5^{a}	55.8 ± 2.6^{a}	45.5 ± 3.3^{ab}	7.6 ± 3.2^{d}	14.3 ± 4.3^{cd}	22.0 ± 4.6^{c}
Solubility	**pH**							
(%)	3	50.2 ± 2.3^{c}	50.3 ± 2.5^{c}	57.9 ± 0.1^{b}	68.7 ± 0.7^{a}	58.5 ± 0.4^{b}	52.0 ± 1.0^{c}	41.9 ± 3.6^{d}
	5	56.5 ± 1.6^{d}	51.8 ± 1.1^{e}	67.3 ± 0.4^{c}	94.5 ± 1.3^{a}	94.0 ± 0.2^{a}	83.9 ± 0.3^{b}	24.2 ± 0.1^{f}
	7	58.2 ± 0.8^{e}	56.9 ± 1.2^{e}	62.3 ± 1.0^{d}	98.8 ± 1.7^{a}	85.3 ± 0.8^{b}	74.7 ± 0.8^{c}	34.1 ± 0.2^{f}
	9	73.0 ± 0.2^{d}	76.6 ± 1.9^{d}	76.4 ± 3.0^{d}	100.0 ± 0.3^{a}	81.3 ± 0.5^{c}	86.7 ± 1.7^{b}	54.4 ± 0.5^{e}
WHC								
(mL/g)		5.7 ± 0.2^{b}	6.7 ± 0.1^{a}	6.0 ± 0.2^{ab}	3.3 ± 0.3^{cd}	3.6 ± 0.3^{cd}	2.7 ± 0.2^{d}	3.8 ± 0.3^{c}

EAI= Emulsion Activity Index; ESI= Emulsion Stability Index, FC= Foaming Capacity, FS= Foaming Stability, WHC= Water Hydration Capacity. Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively; CF: cricket flour. Values represent means $\pm$ SD of triplicate determinations. Values that do not share the same letter, in the same row, are significantly different ($p < 0.05$).

3.4.3: Characterization of Tortillas and Tortilla Chips

Moisture content of soft tortillas formulated with 20% CPH ranged between 30-35%, while control (100% corn tortilla, no CPH) had more moisture (44%) than all trials (Table 6). Ash content of soft tortillas ranged from 1.5–5.7%, with AL1 containing the least amount and FL1 containing the most. Higher ash values can be attributed to the initial high ash content of the protein powders (section 3.4.1.1, Table 3). As expected, protein content increased in all CPH-tortillas by more than two-fold compared to control. CPH-tortillas varied in protein from 18.2-19.6%, while corn control had 7.7% (Table 6). Fat content increased in all tortillas formulated with CPH-AL peptides (1.8-2.4%), while tortillas with FL1 peptides had a similar fat content (0.9-1.4%) compared to control (1.1%). The high fat content was expected given the initial content on the CPH-AL peptides (section 3.4.1.1, Table 3). Color development in tortillas varied. Control was significantly ($p < 0.05$) lighter than all CPH-tortillas, while these varied in L values from 56.0–62.0 (Table 6). Red hues showed all FL-tortillas were higher than control and AL-tortillas. Yellow hues (*b*) showed all CPH-tortillas had higher values compared to control, ranging from 31.8–33.7, but had no difference among them. Color psychology has been well researched, therefore the changes in color of a food product may attract or withdraw consumers from trying or buying that particular product (Ren & Chen, 2018). The alterations in color that CPH may impose on a food matrix must be considered. Sensory evaluation regarding consumer acceptability will be analyzed in the next chapter of this thesis.

Total essential amino acid composition of CPH-tortillas and control are listed in Table 6. Results show that CPH-tortillas contained more than double the amount of essential amino acids compared to the corn control tortilla. Lysine concentrations, a limiting amino acid in corn, increased on

average to 1.02 g/100g in AL-tortillas and 0.94g/100g in FL-tortillas, compared to 0.19 g/100g in the control tortilla. A 100 g sample of AL-tortillas represents 48% of the daily lysine requirement, while FL-tortillas would deliver 41%, according to the World Health Organization requirements of 2.1 g per day for a 70 kg adult (WHO, 2007). Total amino acid analysis can be found on Appendix A.

3.4.4: Structural Analysis

3.4.4.1: Rheology and Texture

The effects of formulation with the different CPH on the rheological properties (G' and G'') of the corn doughs (masas) were determined (Figure 3.3 and 3.4). The elastic modulus (G') of a system is the solid-like behavior, an indicator of the amount of energy stored elastically, while the viscous modulus (G") refers to the liquid-like properties, or the energy that is released through flow during oscillatory strain tests (Moura, Figueiredo, & Gil, 2007). The gel point of a network is a phenomenon that occurs when the G" surpasses G', thus changing its viscoelastic behavior from predominantly liquid to solid.

In Figure 3.3 amplitude sweeps show a viscoelastic linear region at the tested strain % range (0.01 to 1.00) for all tested CPH- doughs and corn control. The following angular frequency sweeps were performed at a strain of 0.1%, falling within the linear region.

Different rheological properties were found between the different protease treatments (Figure 3.4 A and B): G' and G" values for the FL-CPH doughs were lower than the control, while G' and G" of the AL-CPH were higher than the control. These results suggest FL peptides lack of cross-binding activity with other corn macromolecules within the raw dough matrix. The lower WHC of

FL peptides suggested the possibility of free water molecules acting as plasticizers in the FL-doughs; however, upon measuring the water activity (Table 7) we observed this was not the case. In fact, the water activity of the AL-doughs were slightly higher (0.97 ± 0.2) than the FL-doughs (0.96 ± 0.1), likely due to the re-adjustment in water addition from the mixograph-hydration tests, where formulation resulted in water content beyond that of full hydration of the dough. These re-adjustments were made evenly throughout all AL-dough preparations to assist with the malleability. The hydration tests indicated FL-corn doughs required more water than AL-corn doughs to achieve full hydration, resulting in FL-doughs likely having overall slightly higher moisture content. As previously stated, overall moisture content is a better indication of the extend of plasticizing effects by water, therefore this difference in water content between dough samples likely contributed to changes in rheological characteristics.

Table 6: Proximate composition, amino acid analysis and color values of tortillas formulated with and without 20% CPH.

Trial Codes[1]	Moisture (%)	Crude Protein[2] (%)	Ash[2] (%)	Crude Fat[2] (%)	Amino Acids g/100g sample		Color		
					EAA	Lysine	L	a	b
FL1	33.4 ±1.3^{b}	18.2 ± 0.1^{b}	5.7 ± 0.6^{a}	1.3 ± 0.2^{ab}	8.85	0.86	56.0 ± 0.8^{d}	9.3 ± 0.3^{a}	32.9 ± 1.7^{b}
FL2	32.0 ± 1.4^{b}	19.4 ± 0.2^{a}	4.1 ± 1.3^{ab}	0.9 ± 0.0^{b}	9.51	0.95	59.8± 0.7^{bc}	8.7 ± 0.3^{a}	35.8 ± 0.4^{b}
FL3	30.4 ± 1.1^{b}	19.3 ± 0.5^{ab}	5.4 ± 0.4^{a}	0.8 ± 0.2^{b}	7.38	1.01	56.7 ± 0.6^{d}	9.0 ± 0.1^{a}	36.3 ± 0.1^{b}
AL1	35.1 ± 4.2^{b}	18.5 ± 0.3^{ab}	1.5 ± 0.6^{c}	1.5 ± 0.4^{ab}	7.51	1.02	58.0±0.8^{cd}	7.5 ± 0.1^{b}	31.8 ± 0.5^{b}
AL2	33.6 ± 0.4^{b}	18.9 ± 0.5^{ab}	2.7 ± 1.1^{bc}	1.8 ± 0.7^{ab}	7.82	1.02	59.6 ±0.8^{c}	6.7 ± 0.2^{b}	32.3 ± 1.8^{b}
AL3	33.0 ± 1.3^{b}	18.5 ± 0.0^{ab}	3.6 ± 1.0^{abc}	2.7 ± 0.4^{a}	7.48	1.03	62.0 ± 0.9^{b}	7.0 ± 0.3^{b}	33.7 ± 1.6^{b}
C	44.4 ± 0.1^{a}	7.7 ± 0.3^{c}	2.0 ± 0.7^{bc}	0.3 ± 0.3^{b}	3.26	0.19	65.5 ± 0.9^{a}	6.8 ± 0.4^{b}	49.6 ± 1.3^{a}

EAA = Essential Amino Acids
L = lightness, *a* = red vs green, *b* = yellow vs blue
Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively; C: Control (corn tortilla).
[2]Values listed on a dry-basis. Values that do not share the same letter, in the same column, are significantly different ($p < 0.05$).

Table 7: Water Activity (Aw) for CPH- corn doughs (raw) and corn control dough (raw)

Trial Codes[1]	Aw
Control	0.987 ± 0.000a
FL1	0.965 ± 0.001c
FL2	0.961 ± 0.000d
FL3	0.961 ± 0.001d
AL1	0.971 ± 0.000b
AL2	0.967 ± 0.002c
AL3	0.972 ± 0.002b

[1]Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.
Values that do not share the same letter, are significantly different ($p < 0.05$).

Rheological differences were also affected by polymer size and intermolecular interactions. For FL1 and control, the G' > G" at the measured frequency (Figure 3.4 A), indicating a weak gel behavior, normal viscoelastic characteristic of corn dough (Santos et al., 2014). Doughs with AL-CPH showed opposing trends. The G' and the G" modulus increased, compared to control, for all AL-doughs, with AL2 and AL3 having the highest values ($4.48x10^4$- $5.98x10^4$ Pa), interpolating with each other (Figure 3.4 B). Overall, G' > G" for all AL-doughs, similar to control. The gel point of AL1 dough happened at the very beginning of the frequency sweep (0.1 Hz), while we are unable to see this event happening for either control, AL2, and AL3 doughs.

To best of our knowledge, there has been no other reports showing the pattern changes on rheological properties of nixtamalized corn dough by the addition of an edible insect protein. The high visco-elasticity behavior of all AL-doughs might be explained by the medium to high

molecular weight distribution seen by the SDS-PAGE (Figure 3.2). Weak linear polymer gels have been found to have a positive relationship between increasing molecular weight and elastic behavior due to high polymer entanglement, or a high number of cross-links. On the other hand, low molecular weight peptides may have a slipping motion inter-molecularly, therefore moving gradually from an elastic to a flowing behavior (Bartenev & Zuyev, 2013). This provides a possible explanation to the gel-point development for higher hydrolyzed (FL3) peptides (with more free-amino acids) (Figure 3.4 A). The viscoelastic behavior observed by all AL-doughs may not be a true depiction of chemical bonds formed within the matrix, because entanglement of high MW polymers could behave as topological cross-links only (Mitchell, 1980). Additionally, the fragility of the cooked AL-tortillas (Figure 3.6 B) indicates a lack of calcium bridge formation, thus stressing the changes in amino acids' availability to form bonds when proteins are denatured. The importance of protein interactions with starch and lipid complexes via calcium bridges has been previously illustrated by showing the decrease in G' and G" when corn was submitted to cooking in the absence of lime (Santos et al., 2014). This study emphasized that the network is not purely the result of gelatinized starch, but rather the interaction of key corn proteins with the other corn macromolecules. The traditional corn nixtamalization process, soaking and cooking corn kernels in $Ca(OH)_2$, creates negatively charged Asp and Glu residues. These residues, along with charged hydroxyl groups of starch, later participate in the formation of calcium bridges, essential to form a cohesive, strong, and flexible network (Miklus, 1999).

The effects of formulation with the different CPH on the texture traits (extensibility and toughness) of the cooked corn tortillas were determined (Figure 3.5 A and B). Tortilla toughness results show that tortillas formulated with FL3 and FL2 had the strongest network (toughness), compared to all

other treatments, which was not significantly ($p < 0.05$) different from the control (100% corn tortilla) (Figure 3.5 A). There was an increase in toughness for all CPH-tortillas and control after 5 days, likely due to the re-crystallization of amylose and amylopectin units as part of starch retrogradation. Results for AL3 at day 10 could not be gathered due to mold growth. Extensibility results (Figure 3.5 B) show that FL3-tortillas were equally flexible as the control, and significantly ($p < 0.05$) more flexible than all other treatments, except for FL2 at day 5 and 10. Extensibility for AL2 and AL3 tortillas were consistently the lowest at all time points. For both tests, increasing DH in FL-peptides resulted in stronger (tougher) and more flexible tortillas. In contrast, AL-peptides gave the lowest extensibility values, regardless of the DH. These results are further supported by the dowel test (Figure 3.6 A-C). Figure 4B illustrates the fragile matrix of AL-tortillas, all of which scored a 1 (unrollable) within the first 3 hours of making. Conversely, control tortillas and tortillas made with FL3 (Figure 3.6 B and C, respectively) scored a 5 (no cracking), while FL2 and FL1 scored a 4.5 due to slight breakage on the periphery of the tortillas. Argüello-García, Martínez-Herrera, Córdova-Téllez, Sánchez-Sánchez, & Corona-Torres (2017) showed that corn tortillas with 20% Barbados nut (*Jatropha curcas*) flour had > 50% decrease in hardness and deteriorated rollability compared to the control. Another study observed an inhibition to starch retrogradation by the addition of porcine plasma protein hydrolysates to a corn starch matrix, attributing this to an increase in water retention (Niu, Zhang, Xia, Liu, & Kong, 2018). This study correlates to AL-tortillas textural characteristics, noting that AL-CPH have higher water hydration capacity than FL-CPH (Table 5), which could help explain the textural differences between the trials. Additionally, another study showed that a 5% and a 10% soybean protein substitution in corn doughs resulted in tortillas that were significantly ($p < 0.05$) softer to rupture after 3 days of

storage, claiming starch inhibition by the higher water retention properties of the soy protein (Hernández-Reyes et al., 2018).

The lack of structure integrity in AL tortillas could be due to the higher fat content inhibiting protein-protein and protein-starch interactions, thus creating a fragile matrix. Another possibility is the shortage of calcium bridge formation due to differences in availability of Asp and Glu residues in the AL tortilla compared to the FL tortilla matrix. González et al. (2018) reported higher dough development and stability when 5% cricket (*A. domesticus*) flour replaced wheat flour in dough during bread making, suggesting a stronger network formation compared to control. Authors attributed these changes to protein composition. Meanwhile, the specific volume was similar to the control, a feature that represents gas-retention abilities by interacting with the other starch and gluten components in the dough. This last study was done with whole cricket flour, and while it does illustrate the potential of the *A. domesticus* proteins to form a network in a cereal product, our results highlight the impact on the functionality changes due to enzymatic hydrolysis of the protein.

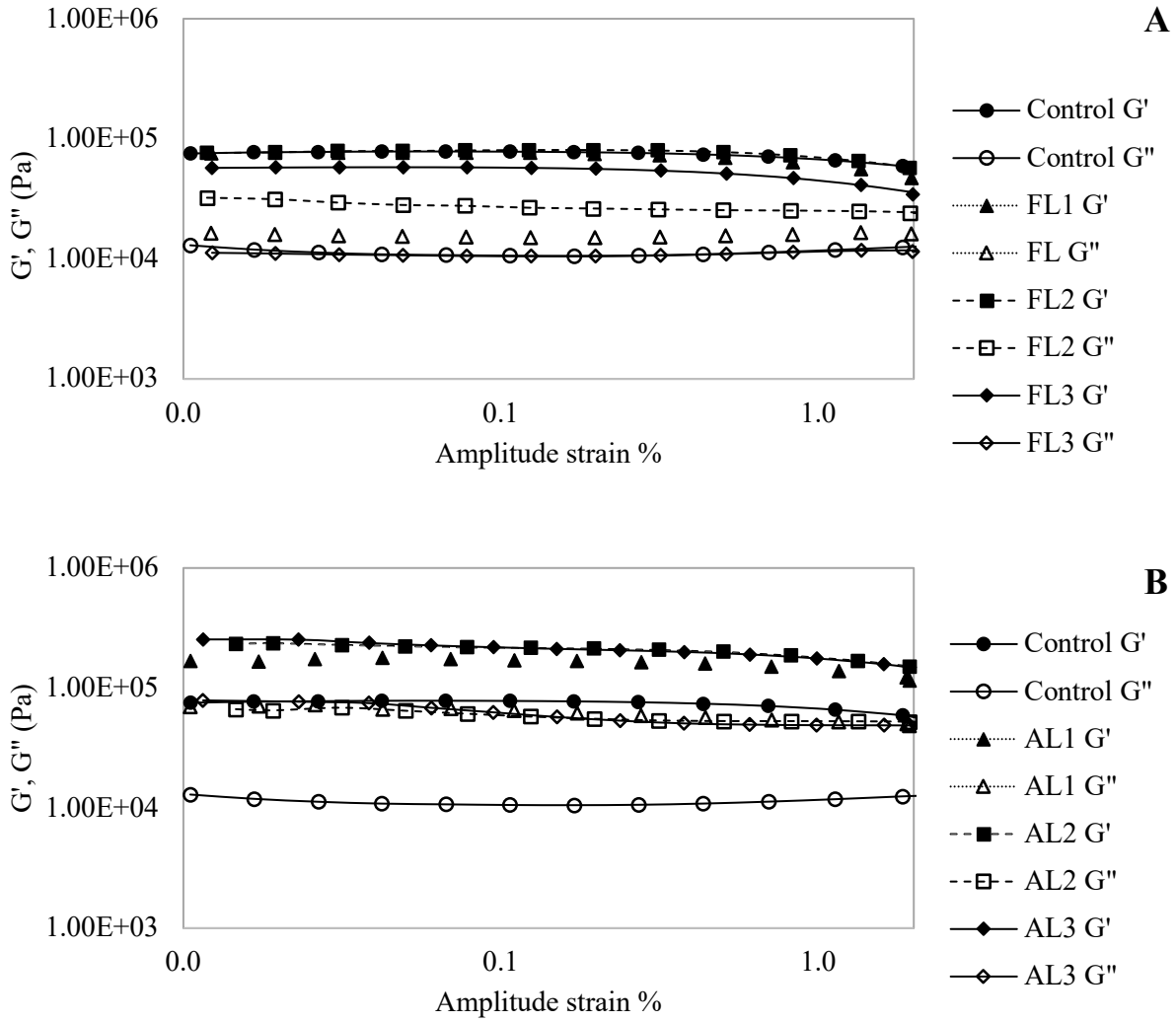

Figure 3.3: Amplitude sweeps of corn doughs with and without CPH. (A) Amplitude sweep for all FL-CPH doughs and control (B) Amplitude sweep for all AL-CPH doughs and control dough.

Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively; Control: corn tortilla without CPH.

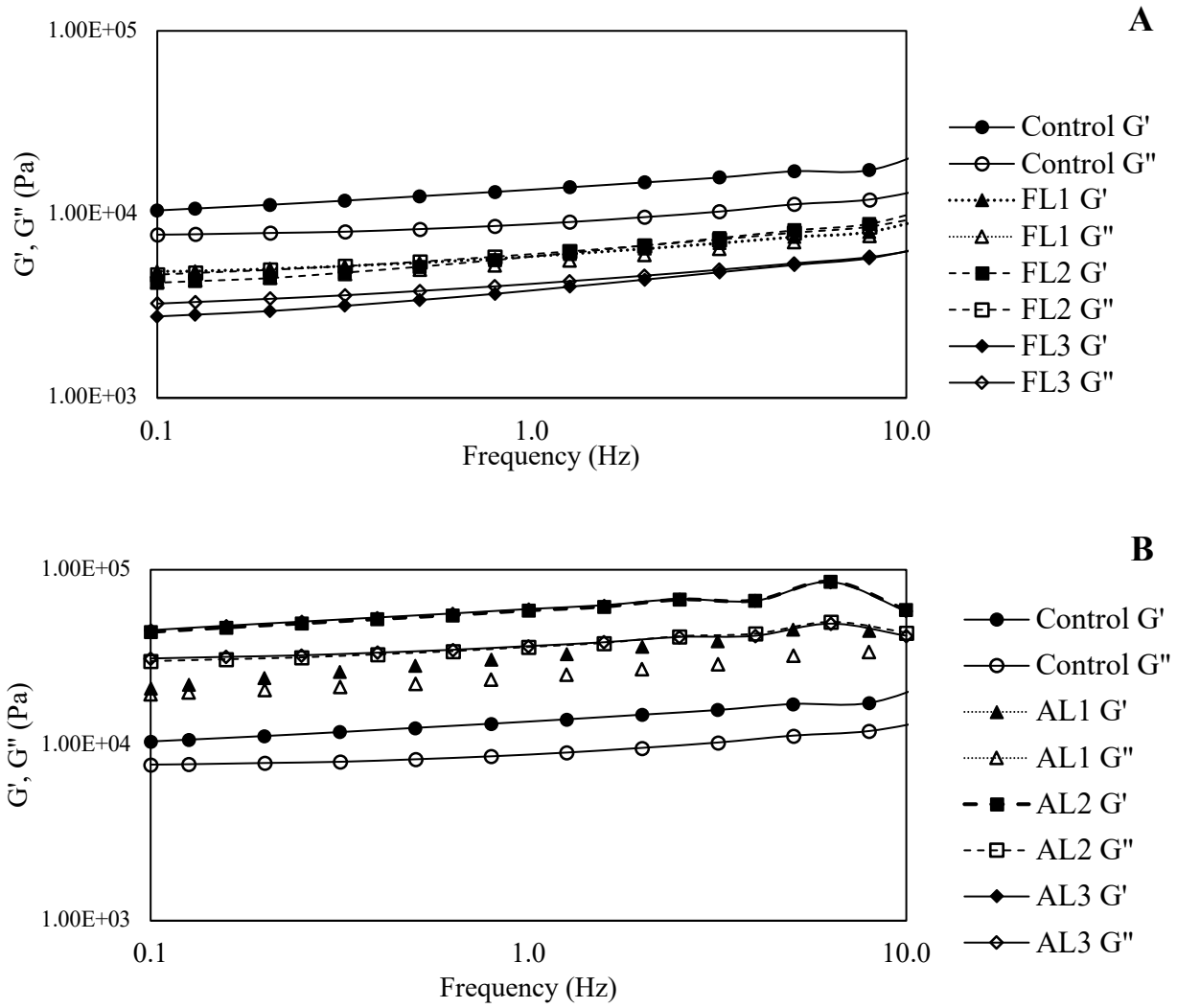

Figure 3.4: Frequency sweeps of corn doughs with and without CPH. (A) Frequency Sweep Tests for FL-CPH and control (B) Frequency Sweep Tests for AL-CPH trials and control.

Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively; Control: corn tortilla without CPH.

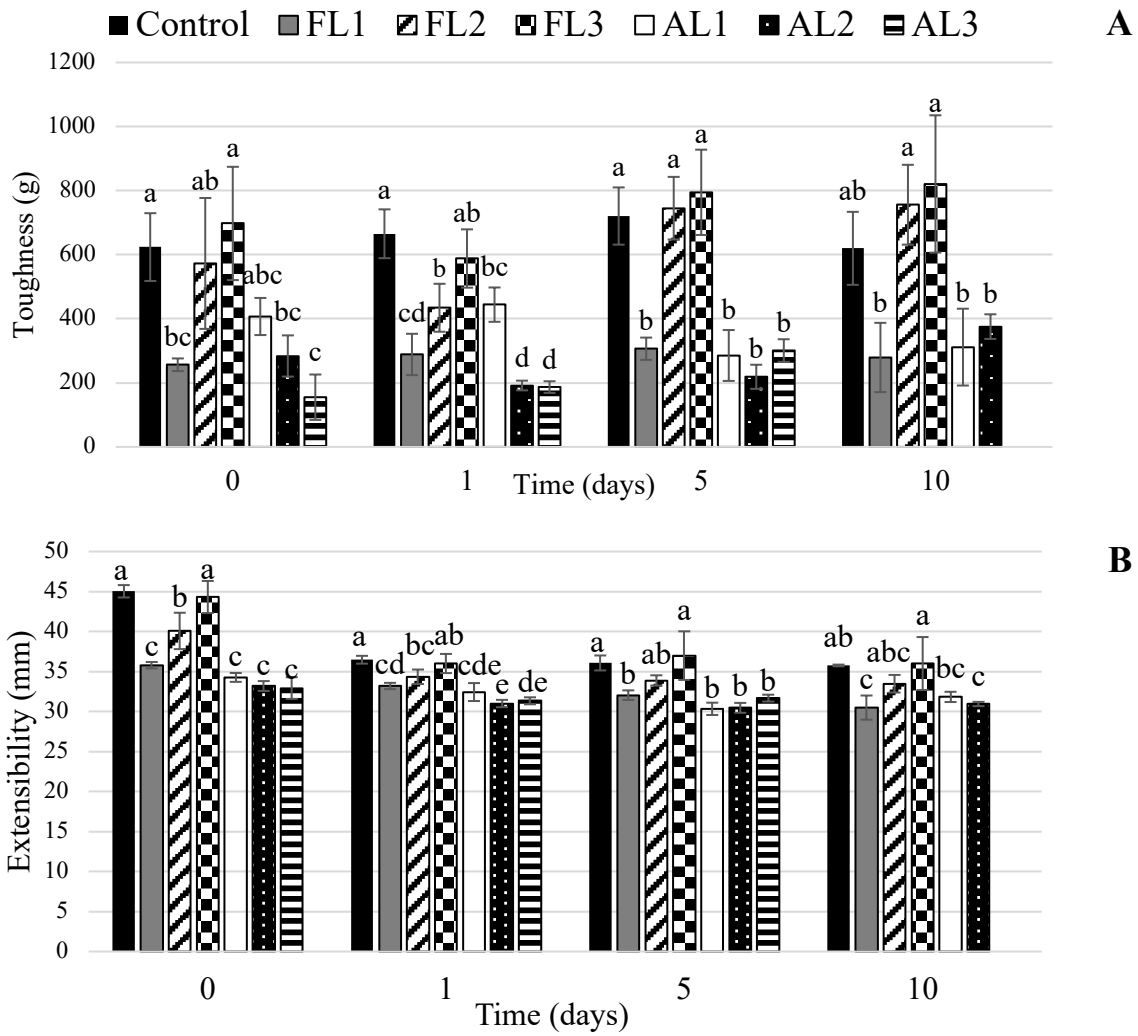

Figure 3.5: Texture analysis results for (A) Toughness and (B) Extensibility of CPH-tortillas, and control.

Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively; Control: corn tortilla without CPH. Statistical analysis was done comparing toughness of soft tortilla trials within the same day. Trials that do not share letters are significantly different ($p < 0.05$).

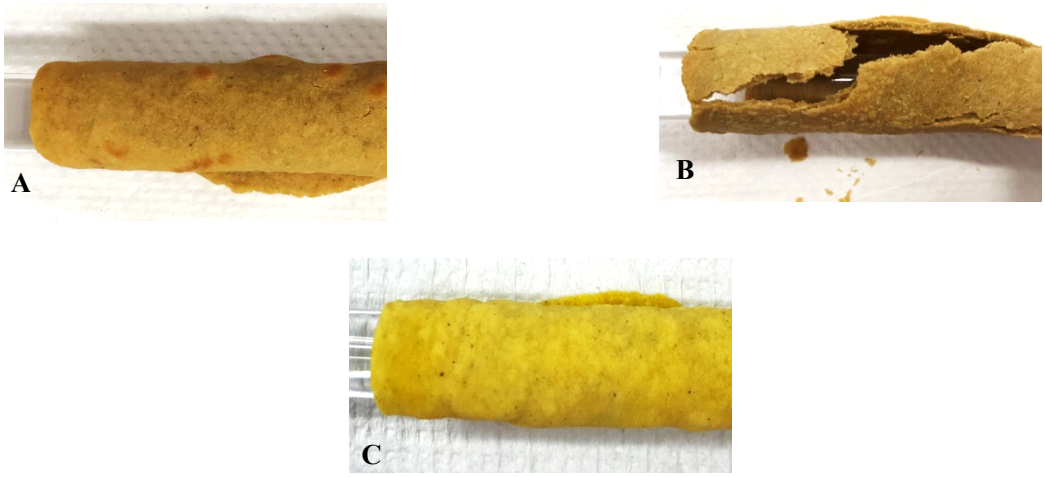

Figure 3.6: Rollability (Dowel) Test. **(A)** FL3- Tortilla **(B)** AL3- Tortilla **(C)** Control Tortilla
Trial codes: FL: Flavourzyme; FL 3: hydrolyzed with DH 14.5 %; AL: Alcalase; AL3: hydrolyzed with DH 14 %.

3.4.4.2: FT-Raman Spectroscopy

Vibrational modes of Raman spectroscopy can provide important structural information of molecules such as carbohydrates, proteins, lipids, and their interactions. Raman spectra relies on inelastic collisions of molecular vibrations and their radiation scattering, resulting in an excitation beam that provokes a band correlated with specific structural characteristics. Given this, Raman is an efficient tool to understand the distribution of food protein secondary structures and monitor changes upon treatment or processing (Li-Chan, Nakai, & Hirotsuka, 1994). In this work, we attempted to understand the major protein structure dynamics of cricket protein hydrolysates and modifications after being incorporated into a corn tortilla.

3100 – 2700 cm^{-1} region

Protein-lipid interactions can be studied via Raman spectra, seen around wavelength between 2850 -3000 cm^{-1}, displaying changes in the C-H stretching (Li-Chan et al., 1994). Specifically, the bands at 2850 and 2885 have been related to the presence of lipids and their relative intensity (I_{2850}/I_{2885}) increasing with "looseness" of hydrocarbon compactness. This translates to their physical structure, meaning a predominant 2850 cm^{-1} band is related to hydrocarbons in a liquid state, while a stronger 2885 cm^{-1} band suggests a crystalline structure (Li-Chan et al., 1994).

Spectra belonging to peptides powders hydrolyzed with Alcalase (Figure 3.7) show prominent shoulders around 2852 and 2886 cm^{-1}, representative of lipids interacting with protein bands seen at 2932 cm^{-1}. These protein-lipid interactions are not as prominent in Flavourzyme- hydrolyzed peptides (Figure 3.8), indicated by the relatively small lipid bands completely separate to the protein band at 2932 cm^{-1}. As mentioned in the functional properties section, Alcalases' ability to release intracellular phospholipids results in a pool of peptides with a higher amount of lipids (Table 3), which may also possess surface activity, thus affecting emulsifying and foaming properties. These bands further confirm protein-lipid interactions, an indication that these peptides may also lack the ability to interact strongly with starch in the tortilla, a reason why Alcalase-CPH did not form strong and flexible matrices in the cooked matrix.

In the spectra belonging to the cooked tortillas of both samples (Figure 3.9 and 3.10) there is a strong band at 2909 cm^{-1}. Bands in this region of starch-base systems denote C-1-H deformations coupled with CH_2 vibrations, both indicating changes in the structural backbone of starch (Flores-Morales, Jiménez-Estrada, & Mora-Escobedo, 2012). A study on retrograded starch from corn tortillas showed changes in intensity of a band at 2900 cm^{-1}, which could be an indication of an

opening of the glycosidic bond or changes in starch complexes with non-carbohydrate molecules (Flores-Morales et al., 2012). Comparing the spectra of tortillas with Flavourzyme peptides with those formulated with Alcalase, it is apparent that the Flavourzyme tortillas display a broader band. Based on the textural properties of tortillas with FL-peptides, we can assume these band changes are related to the structural differences on starch-protein interactions that took precedence with FL-peptides rather than with AL-peptides.

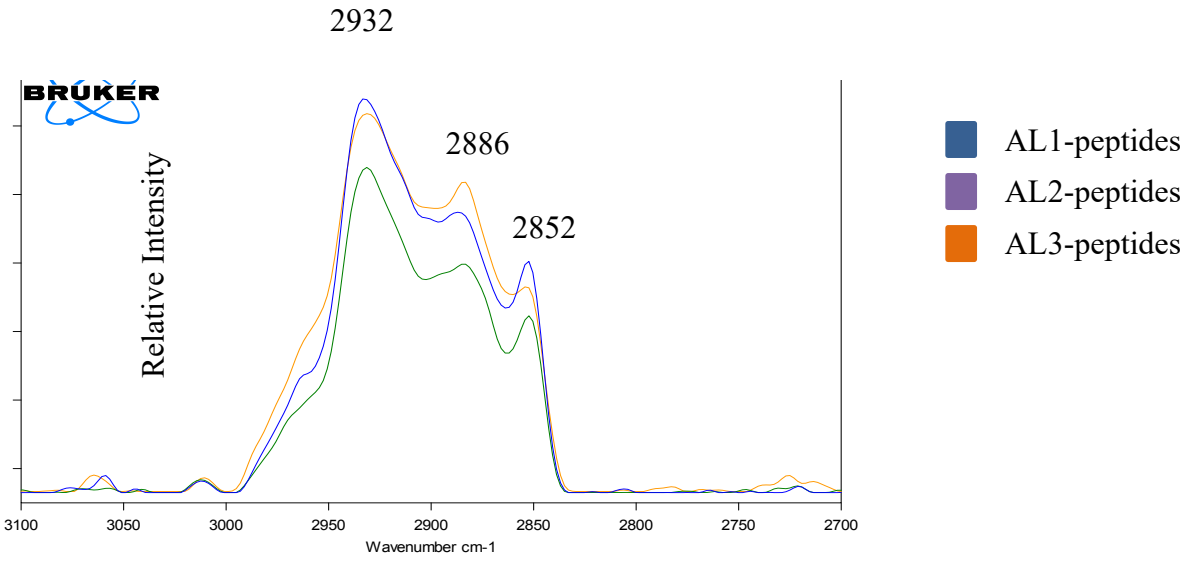

Figure 3.7: Raman spectra of Alcalase peptide powders (3100 cm^{-1} to 2700 cm^{-1}).
Trial codes: AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.

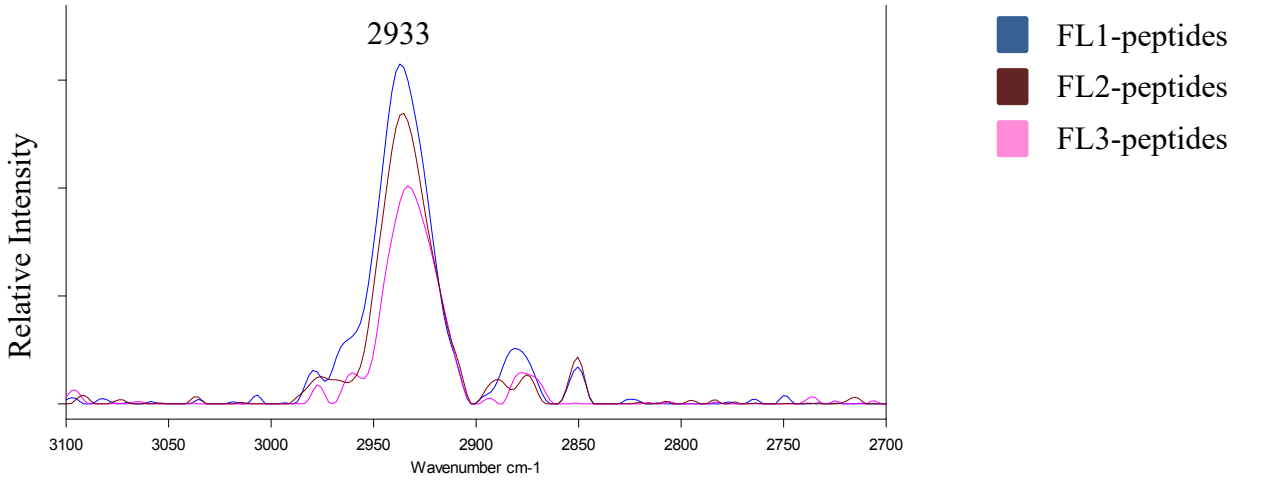

Figure 3.8: Raman spectra of Flavourzyme peptide powders (3100 cm^{-1} to 2700 cm^{-1}).

Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively.

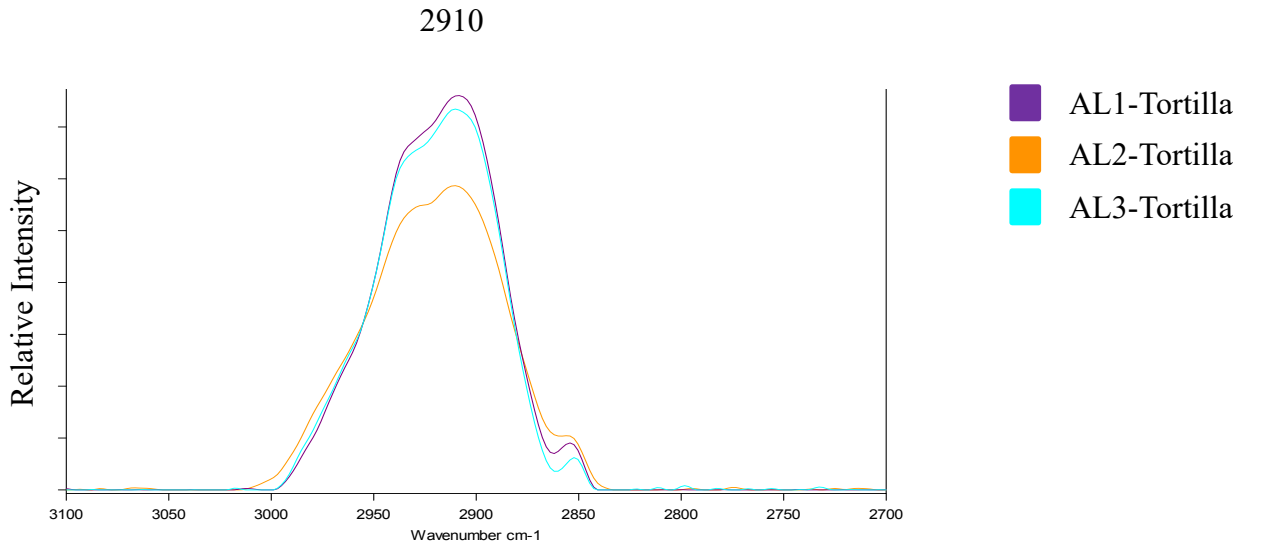

Figure 3.9: Raman spectra of tortillas formulated with 20% Alcalase-peptides (3100 cm-1 to 2700 cm-1).
Trial codes: AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.

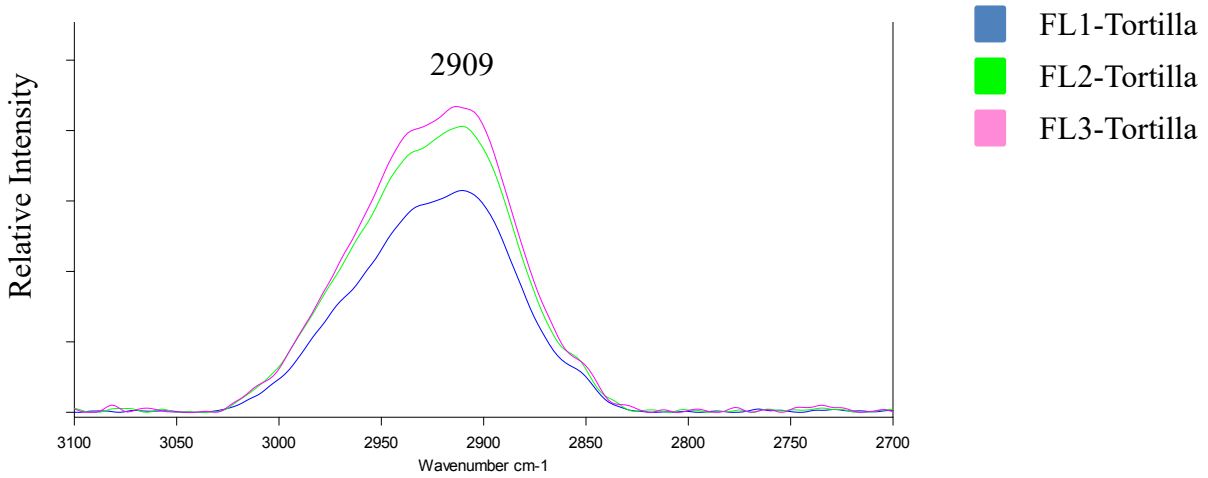

Figure 3.10: Raman spectra of tortillas formulated with 20% Flavourzyme-peptides (3100 cm-1 to 2700 cm1).

Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively.

1800-1200 cm^{-1} region

Amide I bands belong mostly to the peptide stretch of C=O vibrations and partially due to N-H bending vibrations. Bands in this region correspond to α- helical (1645 – 1657 cm^{-1}) and β- sheet (1665 – 1680 cm^{-1}) formation, whereas random coil structures elicit bands around 1660 cm^{-1} (Li-Chan et al., 1994). Amide III bands correspond to C-N stretching and N-H bending of the peptide bond. These bands are around 1260 – 1300 cm^{-1} for α- helices, 1238 – 1245 cm^{-1} for β- sheet formations, while random coils will appear closer to 12500 cm^{-1} (Li-Chan et al., 1994). The aliphatic rings of the aromatic side chains of Tryptophan are very sensitive to Raman spectroscopy and they can present bands around 544, 577, 761, 879, 1014, 1338, 1363, 1553, and 1582 cm^{-1} (Lord & Yu, 1970). The decrease in intensity in these bands has been correlated to a change in the polar environment of the rings. Intensity decreases of Trp bands denotes exposure of the aromatic

rings, previously hidden in the internal hydrophobic milieu of a polypeptide. Lastly, deformation twist of methylene (CH_2) elicit a band at 1453 cm^{-1}, indicating changes in environmental polarity of aliphatic residues (Rygula et al., 2013).

In the powdered protein hydrolysates, the spectra in Figures 3.11 and 3.12 compares Alcalase-CPH and Flavourzyme-CPH powders, respectively. These images show bands at 1659 cm^{-1}, suggesting primarily α- helical formation. Upon hydrolysis, the FL3 (14 %DH) shows a sharp drop in intensity, coupled with a broadening of the width and formation of two shoulders, which are an indication of a rupture of α-helices. One of the shoulders appears around 1675 cm^{-1}, a signature region for poly(L-proline) II (PPII) helix, known as a left-handed helix with a three-fold rotational symmetry (McColl, Blanch, Hecht, Kallenbach, & Barron, 2004). This type of conformation is typical in disordered and unfolded polypeptides, therefore showing the rupture of secondary structure caused by Flavourzymes' enzymatic treatment. Similarly, AL3 (14% DH) shows lower intensity at its amide I band compared to AL1 (8% DH) and AL2 (12% DH), although the changes for these peptides are not as intense as those presented for FL3. This information indicates that the higher degrees of hydrolysis severely disrupted secondary structures, but the changes were more severe for proteins hydrolyzed with Flavourzyme. In the presented spectra (Figure 3.12) the Amide III band of Flavourzyme peptides interpolates with that of the aliphatic residues of Trp, around 1340 cm^{-1}. There is a general broadening of bands and a decrease in intensity, indicating a less structure α- helix, that was previously seen by the Amide I band, and an increase in Trp exposure. In the case of Alcalase-CPH spectra, (Figure 3.11) there are clear bands at 1343 cm^{-1} (Trp) and 1302 cm^{-1} (amide III). These bands are much more pronounced compared to Flavourzyme-CPH spectra, suggesting different structural compositions between the two sets of peptides. Similar to

the Flavourzyme treatment, Alcalase hydrolysis disrupted α- helices and exposed Trp residues, seen once again by a decrease in their respective bands for the higher hydrolyzed AL3 (14 %DH). Overall, both spectra show that higher DH disrupted secondary structures, to the extent of exposing previously hidden aliphatic residues. Furthermore, both sets of spectra present strong bands at 1448 - 1453 cm^{-1} that decrease in intensity upon higher %DH, an indication of higher exposure of hydrophobic residues being exposed due to a rupture in structure.

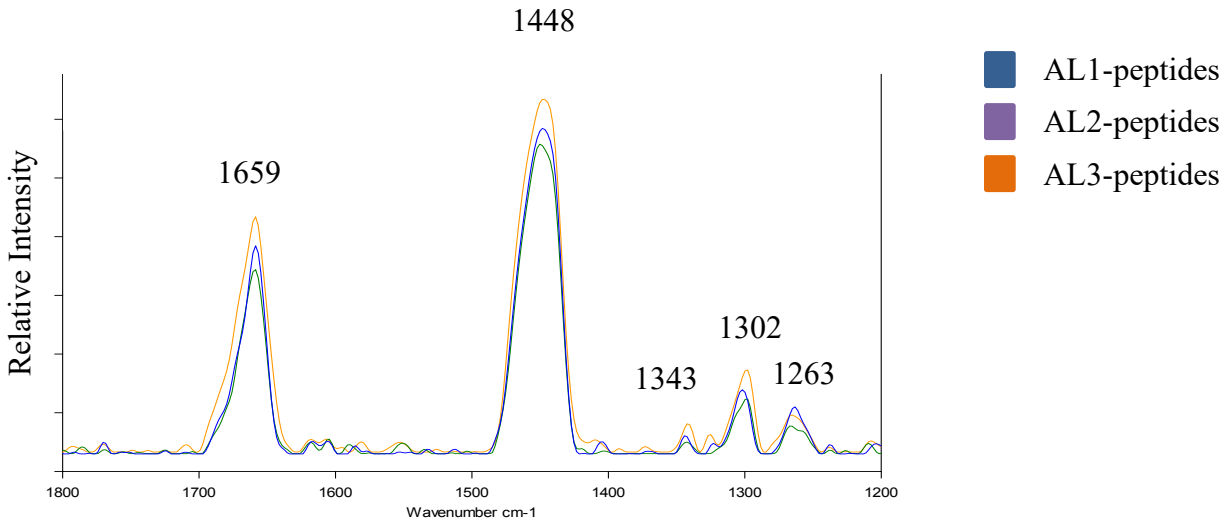

Figure 3.11: Raman spectra of Alcalase peptide powders (1800 cm^{-1} to 1200 cm^{-1}).

Trial codes: FL: AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.

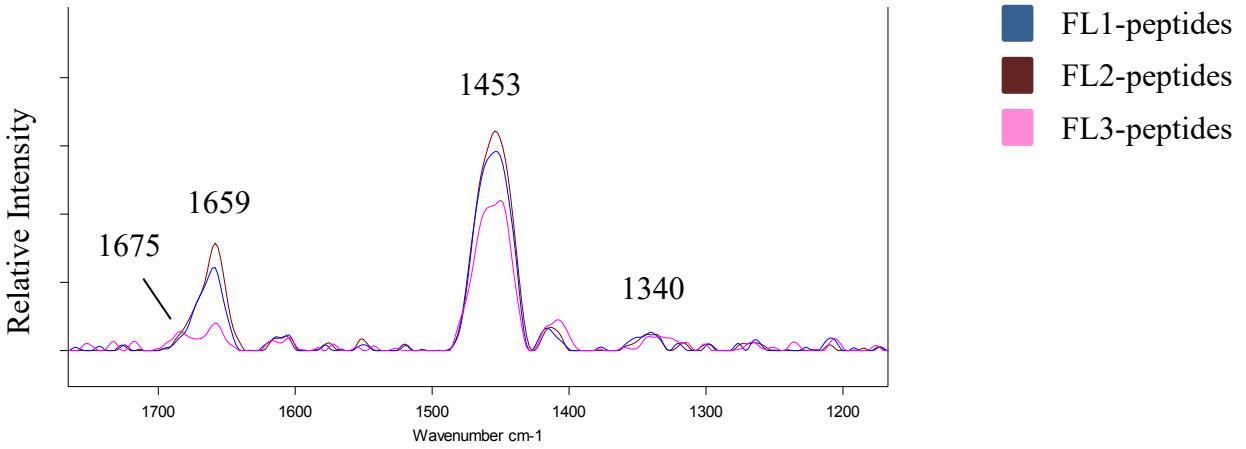

Figure 3.12: Raman spectra of Flavourzyme peptide powders (1800 cm-1 to 1200 cm-1). Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively.

Spectra of cooked tortillas made with FL-CPH (Figure 3.13) show a strong band at 1649 cm^{-1}, falling within the α- helical spectrum. This band becomes more pronounced with tortillas formulated with FL3 (14% DH), compared to FL1 and FL2 who had lower DH. This suggests that more ordered α- helical structures were formed within the tortilla matrix upon formulation with more hydrolyzed peptides. The amine I band for the cooked AL-CPH tortillas (Figure 3.14) appears at wavelength 1649 cm^{-1} as well. Similarly, the higher % DH of the peptides increase the intensity of this band, suggesting more ordered α- helical structures. The amide III band for FL-CPH and AL-CPH tortillas appears at 1262 and 1261 cm^{-1}, respectively. Both peaks increase upon formulation of tortillas with higher % DH peptides, denoting more α- helical structure formation. A major difference between Raman spectra of the cooked FL-CPH and the AL-CPH tortillas is the presence of a band in the former at 1603 cm^{-1}, intensifying with samples formulated with higher % DH peptides (FL3-CPH). This band is an indication of Tyrosine ring vibrational modes due to their sudden exposure (Takeuchi, 2011). The hydroxyl group of the Tyr residues may in that case act as hydrogen bond donor and acceptors, allowing for further interaction with protein and starch.

In starch-base systems, methylene vibrational modes are observed around wavelengths of 1300 – 1450 cm^{-1}. In the spectra of CPH-tortillas (Figure 3.13 and 3.14), the three major bands at 1336, 1383, and 1458 cm^{-1} correspond to CH_2 wagging, scissoring, and bending, respectively (Santha, Sudha, Vijayakumari, Nayar, & Moorthy, 1990; Vasko, Blackwell, & Koenig, 1972). These bands change in intensity with tortillas formulated with different % DH peptides. Similar to the bands belonging other starch-structural features, these increase upon usage of the highest % DH peptides to formulate the tortillas, highlighting differences in starch structure in all the sample matrices.

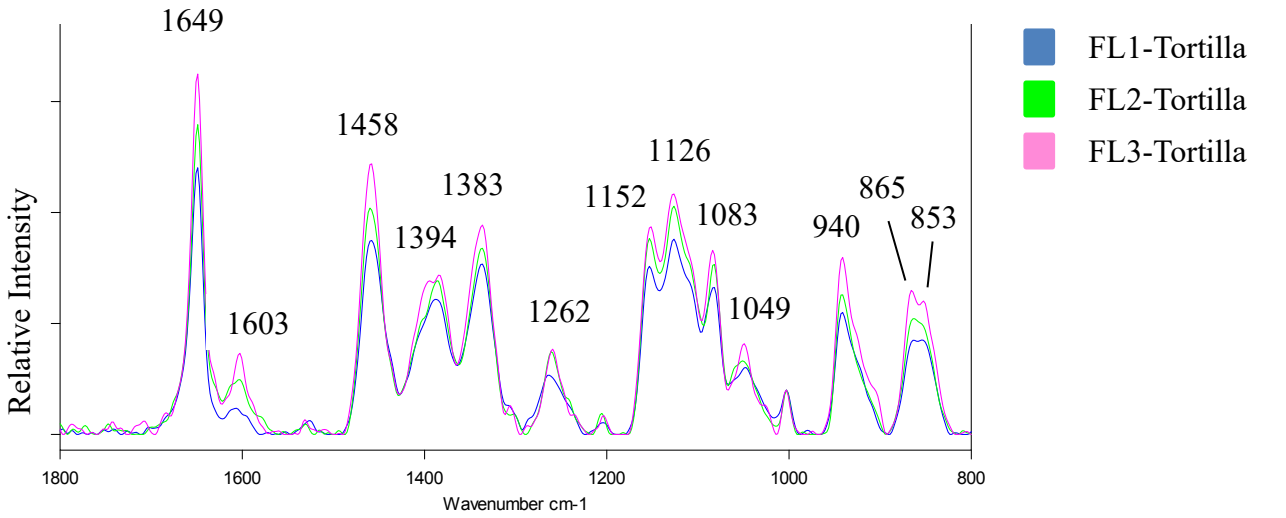

Figure 3.13: Raman spectra of tortillas formulated with 20% Flavourzyme-peptides (1800 cm-1 to 800 cm-1).

Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively.

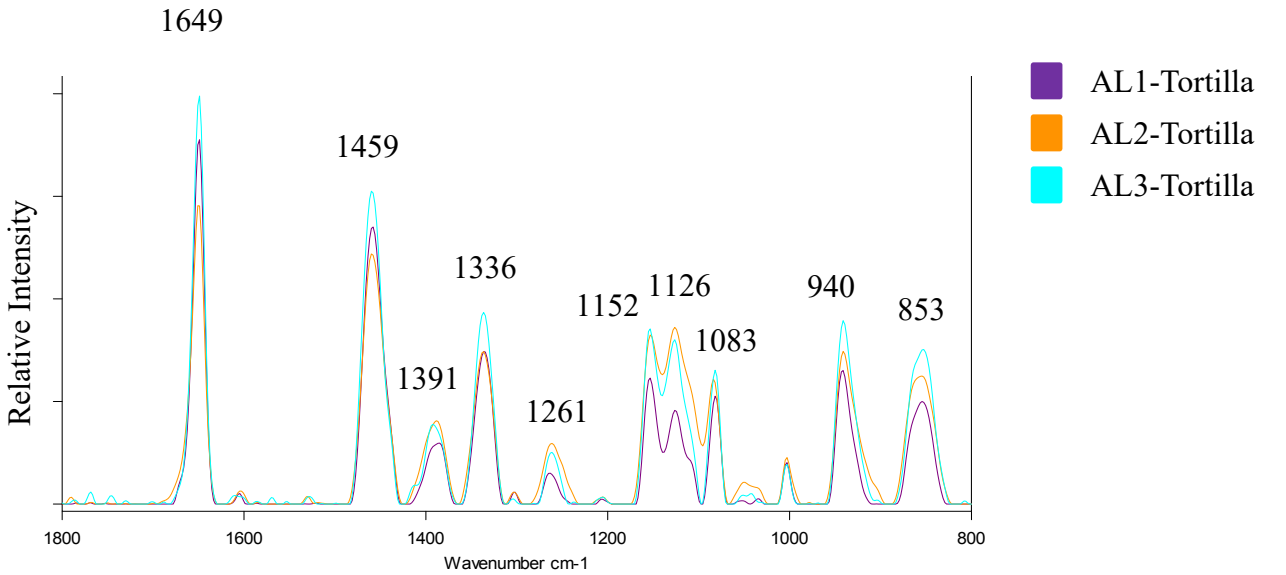

Figure 3.14: Raman spectra of tortillas with formulated 20% Alcalase-peptides (1800 cm^{-1} to 800 cm^{-1}).

Trial codes**:** AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.

1200 – 800 cm^{-1} region

This region has been called the "fingerprint" or the anomeric region of carbohydrates. The bands within this region are used to distinguish α vs β configurations within a polysaccharide matrix. The α -1,4 glycosidic linkage has been assigned as a strong band at 940 cm^{-1} and the presence β-anomers is seen as an increase in intensity of the band at 898 cm^{-1} (Vasko et al., 1972). Bands at 1152, 1126, 1083 and 1049 cm^{-1} are related to COH bending, and CC and CO stretching vibrations (Dudek et al., 2019; Nkhata, 2019).

In the spectra of the cooked Flavourzyme and Alcalase tortillas (Figure 3.13 and 3.14, respectively) all of the mentioned bands, except 898 cm^{-1}, are present and seem to increase in intensity for AL3, and FL3- CPH tortillas. These samples, containing the highest %DH peptides, provoke changes in the starch structure and orientation of glycosidic linkages, increasing band intensities. Changes on starch structure were also seen in the band located at 480 cm^{-1}, belonging to the pyranose ring vibrational mode, later discussed. In the Flavourzyme tortillas spectra (Figure 3.13) the band at 1049 cm^{-1} is significantly more pronounced than in the Alcalase tortillas spectra. This band sharpens with tortillas formulated with FL3 peptides, while almost flat in the Alcalase tortillas. The appearance of this sharp band could be an indication of protein-starch interactions that strengthened the structure of the cooked tortillas. This would further support the rheological tests that demonstrated the heat-induced intermolecular bonds within the FL3 matrix, alluding to protein-protein and protein-starch interactions.

Starch-based materials present a band around 850 cm^{-1}, related to the CH deformations and the C-O-C symmetric stretching of the glucose rings in amylose chains. The appearance of a doublet at 860 and 840 cm^{-1} is characteristic of an amylose-V complex, in which amylose forms helical structures with a complexing agent in the center, this highly order structures can form crystals. This type of doublet is absent in straight chain glucose oligomers, alluding to structural behavioral differences. The relative intensity (I_{840}/I_{860}) of the doublet increases with increasing crystalline order (Cael, Koenig, & Blackwell, 1975; Jane & Robyt, 1984).

Both sets of CPH-tortillas show a band at around 853 cm^{-1}, with increasing intensity upon formulation with higher %DH peptides (FL3 and AL3). This difference in intensity may be related to differences in complexation between CPH peptides and amylose chains. In addition, the FL3 tortillas band presents a doublet at 865 and 853 cm^{-1}, suggesting greater helical order within the FL3 amylose chain structure. This helical amylose complex is most likely an indication of protein-starch interaction, creating an ordered structure and providing an explanation of the superior strength and flexibility of the FL3 tortillas rheological properties.

800 – 400 cm^{-1} region

Disulfide bridge formation indicates creation of more ordered structures and they are strongly apparent in Raman spectroscopy around wavelengths between 500 – 550 cm^{-1}.The frequency of disulfide bridges will depend on the conformation of the CCSSCC moiety. The following bands have been well studied and summarize disulfide bond stretching from most to least stable: gauche-

gauche-gauche (508 – 512 cm^{-1}), gauche-gauche-trans (523 – 528 cm^{-1}), and trans-gauche-trans (540 – 545 cm^{-1}) (Wen, 2007).

There was a lack of bands in this region for the spectral reading belonging to either Flavourzyme or Alcalase protein powders, indicating an absence of disulfide bridges stabilizing the peptides structures. However, bands in this region were clear in the spectra within the cooked CPH-tortillas. The spectra of FL3-CPH tortillas (Figure 3.15) has a peak at 525 cm^{-1}, while this seems to collapse for tortillas formulated with lower % DH peptides (FL1 & FL2). The stronger peak formation denotes a larger amount of disulfide bridges in tortillas formulated with FL3-CPH. On the other hand, the AL-CPH tortillas have an absence in peak in this region (Figure 3.16) with no significant changes with the different enzymatic treatments, indicating no changes in disulfide bond formation. This chemical feature provides an explanation of the significantly stronger and flexible physical structure of the FL3-CPH tortillas seen by its rollability and rheological properties in comparison to the tortillas made with FL1- and FL2- CPH and those made with AL-CPH.

Starch presents a strong band at 480 cm^{-1} due to the glucose pyranose ring vibrations, this band has been used to quantify amylose and amylopectin in an organic sample. Researchers have correlated this band to structural features such as starch-phenolic acid interactions upon long storage times. A study on aging corn grains saw lower Raman bands at 1470 - 850 cm^{-1} and at 477 cm^{-1} compared to fresh corn grains, concluding on starch structure alterations due to complexations with phenolic acids (Nkhata, 2019). Others have seen that thermally treated corn starch has a decrease in intensity of the 477 cm^{-1} band, attributing this change to a general

disruption of the starch structural backbone (Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013).

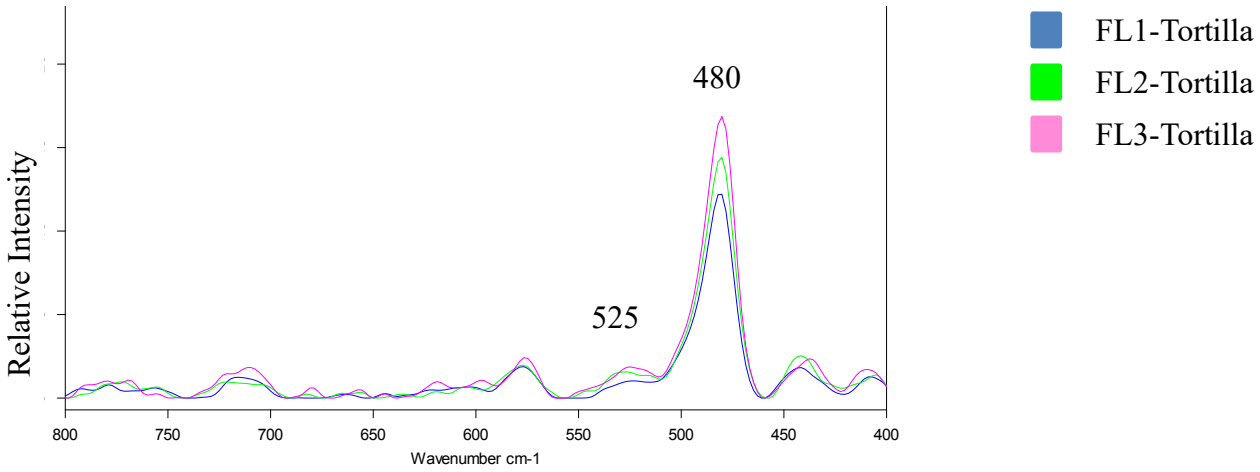

Figure 3.15: Raman spectra of tortillas formulated with 20% Flavourzyme-peptides (800 cm-1 to 400 cm-1).

Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively.

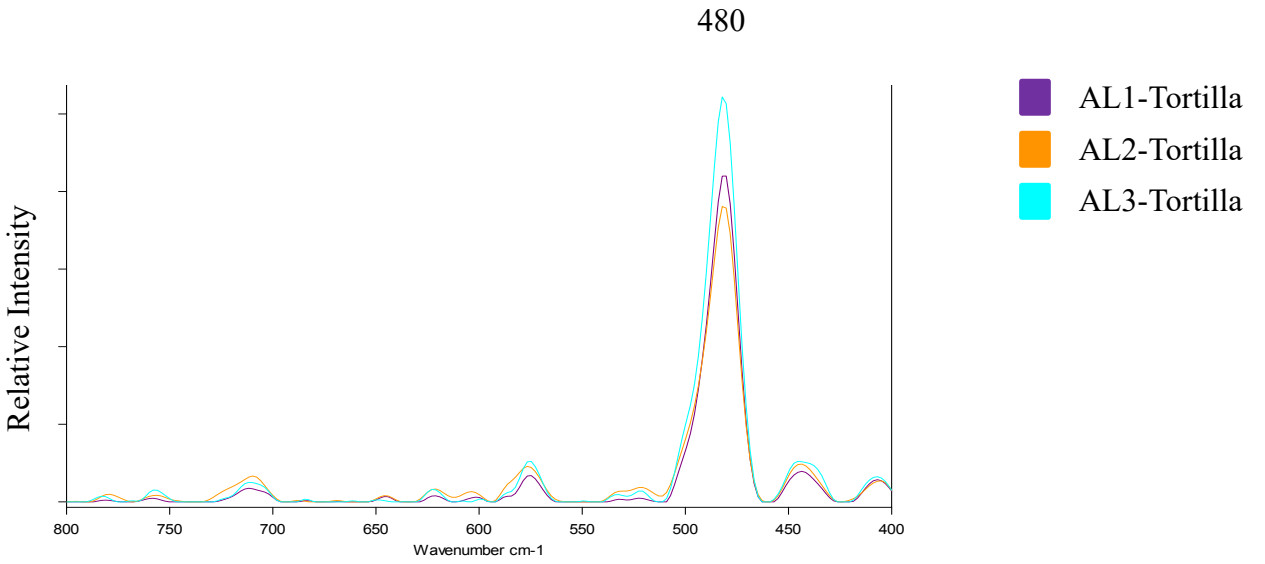

Figure 3.16: Raman spectra of tortillas formulated with 20% Alcalase-peptides (800 cm-1 to 400 cm-1).

Trial codes: AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.

Spectra of both cooked tortilla samples show a strong band at 480 cm^{-1}, indication of a starch-based matrix. Structural changes in starch are apparent by the decrease in intensity of this band for samples formulated with the lowest % DH peptides (AL1 & 2, and FL 1 & 2). As was the case for starch-phenolic complexation, we may deduce the changes in peak intensity can stem from protein-starch complexes. However, the starch-phenolic complexes were denoted as a decrease in peak intensity, while the rheological features of the tortillas formulated with the lower % DH within the FL sample did not outperform the strength and flexibility of the FL3-CPH tortillas, suggesting weaker protein-starch interactions. In addition, the starch-protein complexes were weak in all of AL tortillas, perhaps more so in the samples formulated with highest hydrolyzed (AL3) peptides, seen by the fragility of the structure in the rheological tests. We may deduce that the higher hydrolyzed peptides had different complexation with starch, thus increasing the peak at 480 cm^{-1}, however the extent of how this affected the rheological quality must be further studied.

3.5: Conclusion

The different proteolytic treatments resulted in two very distinct assortment of peptides. Overall, CPH displayed superior functional properties compared to the controlled (unhydrolyzed cricket flour). Alcalase treatments resulted in a set of peptides with higher lipid content, suspected of having surface-activity properties, overall resulting in superior emulsion, foaming and water hydration capacities. Flavourzyme hydrolysates resulted in more soluble peptides. Glass transition temperatures of FL-CPH were significantly lower than AL-CPH, indicating overall lower mean molecular weight. AL-CPH in the raw corn dough behaved as large aggregates cross-linking with each other; however, upon cooking they failed to form chemical bonds that contributes to the integrity of the tortilla. In contrast, FL-CPH raw corn dough had more small-size peptides, which contributed to a plasticizing effect in the matrix, lowering G'. Upon cooking the corn dough, the

FL-peptides were able to interact with other macromolecules and form a strong, flexile matrix. Raman spectroscopy was effective at showing collapses of α- helical structures in the CPH powders with increasing % DH in both enzymatic treatments. In addition, shoulder bands at 2850 – 2900 cm in AL-CPH powders are an indication of protein-lipid interactions, while these were absent in the FL-CPH powders. Lastly, several bands at 1049 – 1152 cm^{-1} and 1383- 1458 cm^{-1} have increasing intensities with different % DH in the CPH-tortillas spectra, denoting changes in the starch structure within the matrix. More importantly, the appearance of the band 1049 cm^{-1} can presumably correspond to protein-starch interactions, seen by a jump in intensity with the FL3-tortillas spectra, whose rheological properties had superior strength and flexibility.

Enzymatic proteolysis of whole crickets can be an effective tool to create protein/peptide ingredients with superior functionality and apt to be incorporated into a food product, such as a corn tortilla. These results highlight the potential food applications in which cricket protein hydrolysates may be utilized based on their proteolytic treatment. AL-CPH would be ideal for food matrices requiring amphiphilic proteins, such as mayonnaise, salad dressings, edible foams and high-fat beverages. Meanwhile, FL-CPH would be appropriate in matrices requiring solubility, starch interactions and cationic bridge formation, such as protein shakes, bakery items, gel systems, and meat analogues.

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CHAPTER 4: SENSORY ATTRIBUTES AND CONSUMER ACCEPTABILITY OF CORN CHIPS FORMULATED WITH CRICKET PROTEIN HYDROLYSATES

4.1: Abstract

Acceptance of insects as alternative proteins in the Western world is largely limited by feelings of disgust, however their potential to be used as part of an ingredient in a familiar food could relieve these psychological strains. In addition, their application into food systems is limited by the presence of insoluble chitin within insect powders. Subjecting these powders to controlled proteolysis can effectively isolate and induce conformational changes of the insect proteins, improving digestibility and enhancing techno-functional properties. In addition, proteolytic treatments can affect flavor. While *in vitro* assays have been performed, there is a general lack of research in the application of insect protein hydrolysates in food matrices. In this study cricket (*Acheta domesticus*) protein was hydrolyzed with Alcalase or Flavourzyme and the resulting peptides were used to formulate corn chips. Chips formulated with 20% peptides of varying degrees of hydrolysis were subjected to consumer acceptability evaluations, where they were scored as acceptable (score > 6.0). A set of 6 trained judges described flavor and aroma profiles of the chips, defining those formulated with peptides treated with Alcalase as having notes of shrimp, peanut, oily, and corn, while those treated with Flavourzyme were characterized as having tomato, ketchup, and French fry notes. Lastly, a preference test revealed significant preference for chips formulated with Alcalase. These results demonstrate an appropriate use of enzymatic hydrolysis to create palatable insect peptides for their application as novel ingredients in a food system.

4.2: Introduction

Insects have increasingly gained attention as alternative protein sources to alleviate current protein demands in a growing world population (Van Huis et al., 2013). The lower environmental impact associated with the farming of insects compared to traditional animal agriculture makes them key players in the development of more sustainable food products (Oonincx et al., 2010). The incorporation of these novel foods as viable ingredients will largely depend on consumer's perception and acceptance of products containing edible insects. Due to Westerns' food neophobic factors, edible insects have been seen with disgust (Gmuer, Nuessli Guth, Hartmann, & Siegrist, 2016; Yen, 2009), making consumer acceptability one major hurdle in the incorporation of insects into the Western diet.

Food neophobia refers to the fear of trying new foods. The two categorical groups include food neophobics, those with a negative disposition to try new foods, and food neophilics, those with a positive disposition to try new foods (Pliner & Hobden, 1992). Past studies have shown consumers categorized as neophobics have a low willingness to pay (WTP) for products containing insects compared to neophilics (Lombardi, Vecchio, Borrello, Caracciolo, & Cembalo, 2019); however, there is no evidence confirming neophobics will not accept these novel food products. Others have suggested incorporating insects as part of an ingredient in a familiar food may alleviate neophobic psychological strains (Gmuer et al., 2016), yet there is a lack of evidence of this concept containing real sensory evaluation studies. In fact, previous sensory evaluation tests on bread made with 10 and 30% cricket (*Acheta domesticus*) flour showed overall poor acceptability, scoring below 5 on a 9-point hedonic scale (Osimani et al., 2018). Flour in this document will refer to the grounding of the complete insect, an ingredient now commonly sold by entomophagy-driven companies.

Another study illustrated the off-flavor and off-texture cricket (*A. domesticus*) flour imposed on jelly, leading consumers to prefer jellies containing the entire cricket irrespective of the unfamiliarity with edible insects (Sogari, Menozzi, & Mora, 2018).

The presence of insoluble chitin in insect flours results in poor solubility, emulsion and foam capacity (Adebowale, Adebowale, & Oguntokun, 2005; Hall, Jones, O'Haire, & Liceaga, 2017; Purschke, Meinlschmidt, Horn, Rieder, & Jäger, 2018; Zielińska, Karaś, & Baraniak, 2018), among other functional parameters, making them difficult to incorporate in food formulations. In order to improve these functions Hall and others (Hall et al., 2017) applied enzymatic proteolysis, exposing previously buried amino acid residues, and separating protein from the chitin exoskeleton. They were successful at increasing solubility at every pH measured and improve emulsion capacity. Proteolysis is also known to give rise to unique sensory attributes, for instance the ripening of the Camembert cheese involves the enzymatic breakdown of milk proteins, fats and sugar that together make up its unique flavor and aroma (Štoudková & Zemanová, 2007). The application of enzymatic hydrolysis on insects may not only improve their functionality to enable the creation of novel food ingredients but could also create palatable sensory attributes that could facilitate their acceptability among consumers.

The objective of this study was to compare the sensory attributes and determine consumer acceptability of corn chips formulated with cricket (*A. domesticus*) peptides sourced from two different enzymatic treatments. Consumer acceptability across food neophobia groups was measured and descriptive analysis was used to determine flavor and aroma profiles.

4.3: Materials & Methods

4.3.1: Materials

Alcalase (AL) (from *Bacillus lecheniformis,* > 2.4 U/g) and Flavourzyme (FL) (from *Aspergillus oryzae,* > 500 U/g), were supplied by Sigma Aldrich (St. Louis, MO, USA). Raw, frozen *Acheta domesticus* crickets were obtained from a food-grade, cricket farm (Ovipost, LaBelle, FL, USA). Instant yellow corn flour (Maseca, Gruma, San Pedro Garza Garcia, MX) was purchased from a local grocery store.

4.3.2: Cricket Protein Hydrolysates-Chips (CPH-chips)

Cricket protein hydrolysates (CPH) were made following the method by Hall and others (Hall et al., 2017), with slight modifications. A slurry of crickets to water (1:2.5) was made using a commercial blender (Waring Commercial, CT, USA) and pasteurized by raising the temperature to 95°C for 15 min. Next, the pH and temperature of the mixture was adjusted to desire optimal enzymatic conditions (pH 8 for Alcalase at 63°C, and pH 7 for Flavourzyme at 55°C). Enzyme concentration (% v/w) and hydrolysis time were determined in pre-screening experiments (Appendix B). Time of hydrolysis and enzyme concentration for each treatment were as follows: for Alcalase: AL1, AL2, and AL3, had combinations of 5 min and 0.1%, 30 min and 0.1%, and 10 min and 0.3%, respectively. For Flavourzyme: FL1, FL2, and FL3, had combinations of 10 min and 0.5%, 30 min and 1.5%, and 30 min and 3.0%, respectively (section 3.4.1, Table 3).

The degrees of hydrolysis (DH) was measured following trinitrobenzenesulfonic acid (TNBS) by Adler-Nissen (Adler-Nissen, 1979) with modifications by Liceaga-Gesualdo and Li-Chan (1999). The DH % reached for AL1 and FL1, AL2 and FL2, and AL3 and FL3 was 8, 11, and 14%,

respectively. Enzymatic reaction was stopped by re-heating the mixture to 95°C for 15 min. Mixtures were cooled, and centrifuged (17,636 x *g* for 15 min, Avanti J-26S Centrifuge, Beckmann- Coulter INC. CA, USA). Supernatant was collected and lyophilized.

Tortillas chips were formulated by combining 20% CPH to 80% dried corn flour, followed by 1% sodium propionate and potassium sorbate as preservatives each and 1% sodium chloride for flavor. To this dry mixture, distilled water was added until optimal hydration was obtained. Liquid to dry proportions were determined via mixograph tests (section 3.3.5.1, Table 2). Doughs (16 g) were mixed for 1 min in a commercial electric mixer (Kitchen Aid, Benton Harbor, MI, USA) then pressed with a manual tortilla press (12 cm diameter) and cooked on an electric griddle (Presto, WI, USA) at 250 $\pm$ 5°C for 2.5 min, flipping them every 30 seconds. Once cooled, tortillas were cut into 8 triangles (base approx. 4 cm, length approx. 5 cm) and deep fried in an electric deep fryer (All-Clad, PA, USA) in 100% vegetable oil (Wesson, Conarga, IL, US) at 195ºC.

4.3.3: Proximate Composition

Moisture, ash, crude fat, and crude protein content of CPH powders were determined using standard AOAC methods (950.46(b), 920.153, 960.39, 984.13 A-D), respectively. Total protein was calculated using standard conversion factor of 6.25.

4.3.4: Color Measurement

A Labscan XE Colorimeter (Hunter Associates Laboratory, VA, USA) was used to obtain the L (brightness), *a* (red-green), and *b* (yellow-blue) values of CPH-chips. Standard white and black tiles were used to calibrate colorimeter. Measurements were done in triplicate.

4.3.5: Consumer Acceptability

Approval for the sensory study was obtained from the Institutional Review Board (IRB) at Purdue University. First, a food neophobia questionnaire (Pliner & Hobden, 1992) (Table 8) was sent to an online database of regular visitors of the Sensory Lab at Purdue University, upon which subjects were divided into two categories according to their responses; neophobic, and neophilic (Dine, 2009). From the neophobia survey, those that agreed to sample cricket protein were invited to participate in the tests. From those, 112 panelists were recruited for the AL-CPH chips test, and 95 panelists were recruited for the FL-CPH chips acceptability test. Panelists' demographics consisted of 59% female and 41% male with the following ages: 28% between 18–24, 45% between 24-34, 12% between 35-44, 6.5% between 45–54, and 4% between 55–64. The ethnicity distribution consisted of 57% Caucasian, 24% Asian, 12% Hispanic, and 6% African-American. The sensory evaluation test took place in a laboratory equipped with individual evaluation booths, with uniform neutral illumination, isolated from sources of acoustic contamination, ventilated, and conditioned to a temperature of 22 ± 2°C.

In the sensory acceptability test, panelists were asked to rate samples regarding appearance, aroma, flavor, and overall liking on a 9-point hedonic scale, where 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely. Panelists were also asked to score the flavor intensity using a 5-point just about right (JAR) scale, where 1= much too weak, 3= just about right (JAR), 5= much too strong, and to comment on what they liked/disliked about the samples. Panelists were given water to cleanse their palate between samples.

Table 8: Food Neophobia Questionnaire. Adapted from (Pliner & Hobden, 1992).

Food Neophobia Scale
I am constantly sampling new and different foods*.
I don't trust new foods.
If I don't know what is in a food, I won't try it.
I like foods from different countries*.
Ethnic food looks too weird to eat.
At dinner parties, I will try a new food*.
I am afraid to eat things I have never had before.
I am very particular about the food I will eat.
I will eat almost anything*.
I like to try new ethnic restaurants*.

*Questions with reverse scoring
Scoring: 1 = Strongly disagree, 7 = Strongly agree
Reverse scoring: 1 = Strongly agree, 7 = Strongly disagree

4.3.6: Descriptive Analysis

Descriptive analysis was performed according to the QDA™ method by Stone, Sidel, Oliver, Woolsey, and Singleton (2008) with modifications, using six trained judges that were pre-screened for sensory acuity of umami and bitter tastes. The screening consisted of duplicate triangle tests to recognize umami and bitter thresholds from solutions containing 0.07% mono-sodium glutamate

(MSG) (Accent Flavor Enhancer, B&G Foods Inc, Parsippany, NJ, USA) and 0.0035% Quinine monohydrate dihydrate (Sigma Aldrich, St Louis, MO, USA), respectively. Only samples AL1 and FL1 were analyzed due to their high scores in the consumer acceptability test. Judges had a total of 22 hours of training in which they started by developing a lexicon to describe flavors and aromas from the samples that were coded with three random number digits. Appropriate reference standards were obtained and defined, and their intensity rated using a 10 cm line scale, with anchored terms at both ends. Over the course of the training, the most relevant attribute terms were outlined and used to develop the lexicon. On the day of testing judges were given 2 triangular chips (base 4 cm, height 5 cm). Samples were presented in random order and evaluated individually by the trained judges using a 10 cm line scale.

4.3.7: Preference Test

The preference (paired-comparison) test was done by comparing AL1-CPH and FL1-CPH chips. Chips were made as stated in section 4.3.2, however precautions to avoid any burnt chips were taken in consideration resulting in time variations in frying between enzymatic treatment: 1 min 30 sec for AL1-CPH chips and 1 min 10 sec for FL1-CPH chips. Panelists were recruited from the same pool of subjects that had previously taken the food neophobia Questionnaire. The demographics for the N= 72 panelists consisted of 51% females and 49% males, with the following ages: 19% between 18 – 24, 51% between 25 – 34, 14% between 35 – 44, 7% between 45 – 54, 7% between 55 – 65, and 1% with 65 years old and above. The ethnicity demographics were: 50% Caucasian, 22% Asian, 12% Hispanic, 11% African American, and 4% other. The sensory evaluation test took place in a laboratory equipped with individual evaluation booths, with uniform neutral illumination, isolated from sources of acoustic contamination, ventilated, and conditioned to a temperature of 22 ± 2°C. A forced-choice preference test was used, where panelists were asked

to select which of the two samples they preferred and to explain why. In addition, panelists were also asked to rate overall liking and texture liking using a 9-point hedonic scale.

4.3.8: Statistical Analysis

Sensory evaluation data of degree of liking and preference test were collected and analyzed using the sensory evaluation software RedJade® (RedJade Software Solutions, LLC) at 95% confidence level. Descriptive analysis testing was done in duplicate and 2-way analysis of variance (ANOVA) with replication was performed using Excel (Version 16.26, Microsoft). Fisher's (protected) LSD multi-comparison test was used to determine significant difference ($p < 0.05$) among sample means.

4.4: Results & Discussion

4.4.1: Proximate Composition

Proximate composition results are listed in Table 9. Analysis revealed moisture contents slightly lower for FL-chips than for AL-chips, however the only significant difference ($p < 0.05$) among samples was between FL1 and AL3. Ash contents did not vary within enzymatic treatments, but AL2 and AL3 did show lower levels than FL-chips. A plausible explanation is the significant changes in compositions between CPH samples, where AL-CPH powders presented an overall higher percentage of fat and lower percentage of ash (Table 3). There were little deviations in fat content among samples and none showed significant differences. Protein content in chips ranged from 15.4 – 18.5 % and only AL2 was lower in protein content compared to FL-chips.

4.4.2: Color

Measurements of color revealed significant differences in brightness level (L) between the two sets of CPH-chips (Table 9). Values for FL-CPH ranged between 36.5 (FL1) to 37.5 (FL2) which were significantly ($p < 0.05$) darker than AL-CPH, whose values ranged from 41.8 (AL3) to 43.5 (AL1). The red (a-value) and yellow (b-value) hues did not differ significantly between sets of CPH-chips, ranging between 8.8 – 14.0 and 15.7 – 17.7, respectively. The darker color in FL-CPH chips could have been a contributor in its slight lower scoring in the overall liking tests (later discussed), however this attribute did not prevent either neophilics or neophobics from accepting these chips. The changes in appearance from these chips in comparison to a normal corn tortilla chip could have imposed expectations on consumers regarding sensory attributes upon completing the sensory test, since panelists were told they were performing sensory in a "corn tortilla chip formulated with cricket protein". In order to minimize this expectation factor, the term "tortilla chip" was changed to "corn snack" in the preference test.

Table 9: Proximate composition and color for chips with 20% CPH.

Trial Codes[1]	Moisture (%)	Ash[2] (%)	Crude Fat[2] (%)	Crude Protein[2] (%)	Color		
					L	a	b
FL1	2.7 ± 1.6[b]	5.2 ± 0.7[ab]	7.6 ± 3.4[a]	17.8 ± 1.1[ab]	36.5 ± 1.5[b]	10.6 ± 5.2[a]	15.7 ± 1.0[b]
FL2	1.8 ± 0.7[b]	5.6 ± 0.3[a]	5.8 ± 1.8[a]	18.2 ± 0.6[a]	37.5 ± 3.4[b]	14.0 ± 1.0[a]	16.4 ±1.6[b]
FL3	2.4 ± 0.6[b]	5.6 ± 0.8[a]	7.4 ± 3.7[a]	18.5 ± 0.7[a]	36.9 ± 1.1[b]	13.4 ± 1.2[a]	16.0 ± 0.8[b]
AL1	4.6 ± 1.5[ab]	4.1 ± 0.5[abc]	10.4 ± 3.6[a]	16.2 ± 0.6[ab]	43.5 ±0.2[a]	10.1 ± 0.2[a]	17.6 ± 0.1[b]
AL2	4.8 ± 2.3[ab]	3.1 ± 0.9[c]	11.3 ± 3.9[a]	15.4 ± 0.1[b]	41.5 ± 1.6[ab]	8.8 ± 1.5[a]	16.7 ± 1.1[b]
AL3	6.5 ± 0.8[a]	3.5 ± 0.1[bc]	9.15 ± 2.9[a]	16.4 ± 0.1[ab]	42.8 ± 1.9[a]	10.3 ± 0.3[a]	17.7 ± 0.9[b]

Color parameters, L = lightness, *a* = red vs green, *b* = yellow vs blue.
Trial codes: FL: Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL: Alcalase; AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.
[2]Values listed on a dry-basis. Values that do not share the same letter, in the same column, are significantly different ($p < 0.05$)

4.4.3: Consumer Acceptability

Food neophobia questionnaire revealed a population with a normal distribution, where most consumers either identified as intermediate neophilic or neophobic (Table 10). For the consumer acceptability test, intermediate and extreme neophilics were pooled together and termed simply "food neophilics", similarly; intermediate and extreme neophobic were pooled together and termed "food neophobics". Penalty analysis was done on scores from food neophilics and food neophobics (Figure 4.1 A and B, respectively) JAR scores for flavor intensity, and critical points were defined as penalties affecting 25% or more of consumers with penalty scores above 1 (Rothman & Parker, 2009).

Table 10: Food Neophobia Questionnaire Results (N = 201).

Food-Neophobia Questionnaire	**Extreme neophilics**	**Intermediate neophilics**	**Intermediate neophobics**	**Extreme neophobics**
Amount	37	93	49	22
Percent (%)	18.4	46.3	24.4	11.0
Score	10 - 13	14 - 21	22 - 28	29 - 53

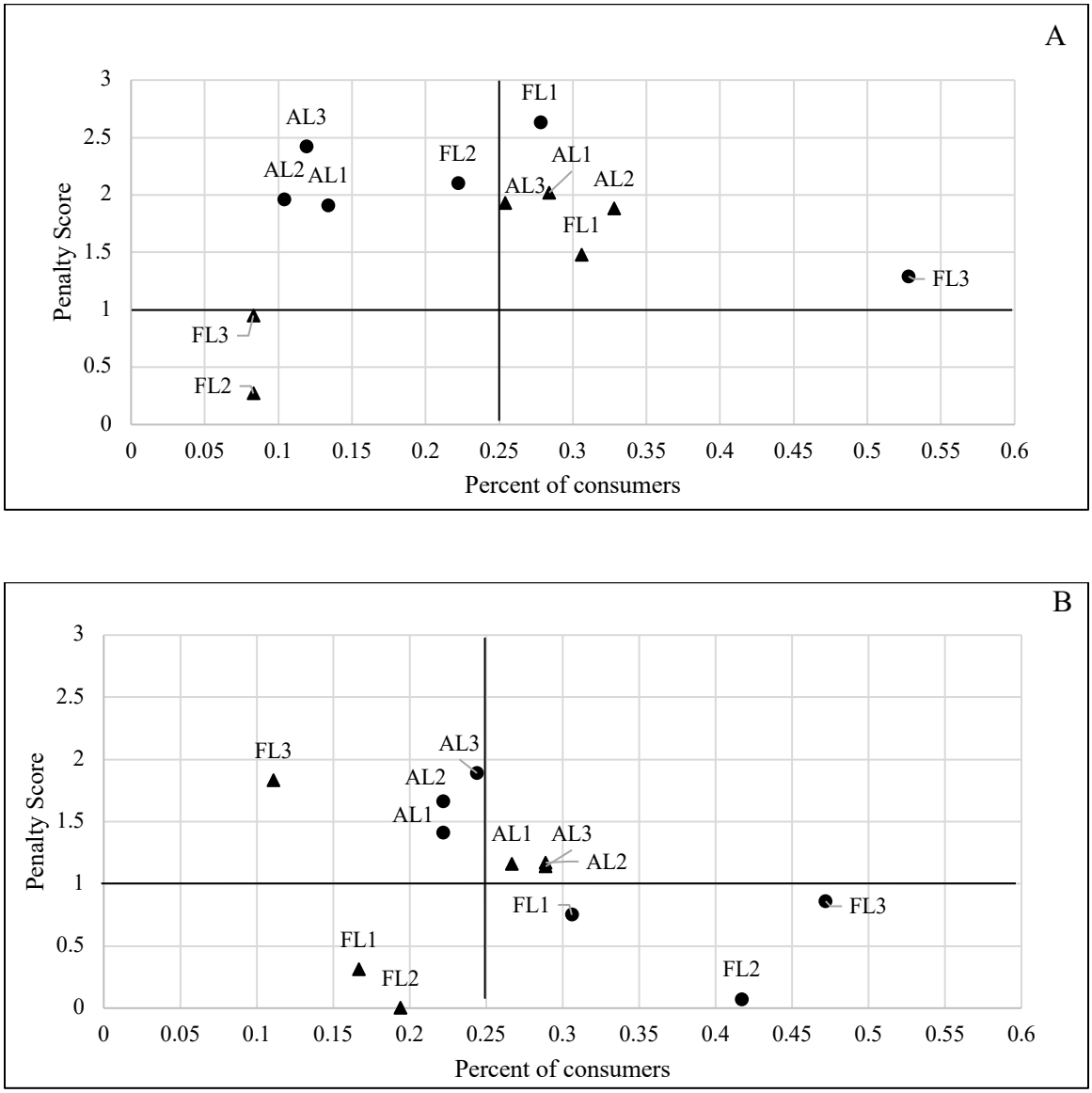


Figure 4.1: Penalty Analysis for CPH-Chips (A) Food Neophilics (B) Food Neophobics

Circles represent Flavor Intensity is Too Strong.
Triangles represent Flavor Intensity is Not Strong Enough.
Food neophilics = subjects with food neophobia scores between 10-21, Food neophobics = subjects with food neophobia scores between 22-53.

Sensory evaluation results are shown in Table 11. Results show overall acceptability (degree of liking scores $\geq$ 6.0) for all attributes for chips formulated with 20% AL-CPH chips. Overall degree of liking for aroma indicated high acceptability with overall scores ranging 6.56 to 6.75, while degree of liking for flavor ranged from 6.29 to 6.53. FL-CPH chips also scored as acceptable in all attributes, except for FL3 in overall liking, scoring significantly lower (5.60) than FL1 (6.09) and FL2 (6.00). Degree of liking for aroma in FL1 (6.63) and FL2 (6.80) were also highly acceptable and significantly higher than FL3.

Table 11: Degree of Liking (DOL) and Attribute Diagnostic Test (JAR) for all CPH chips.

	AL-CPH Chips Test N= 112			**FL-CPH Chips Test N= 95**		
	AL1	**AL2**	**AL3**	**FL1**	**FL2**	**FL3**
Overall Scores[1]						
Appearance	6.21a	6.04a	6.12a	6.31a	6.37a	6.12a
Aroma	6.56a	6.63a	6.75a	6.63ab	6.80a	6.38b
Flavor	6.53a	6.35a	6.29a	6.23a	6.33a	6.02a
Overall Liking	6.29a	6.23a	6.05a	6.09a	6.00a	5.60b
Attribute Intensity Score						
Flavor Intensity[2]	2.83	2.78	2.84	3.12	3.28	3.49
%Much too strong	2	2	1	5	7	11
%Slightly too strong	15	13	16	24	29	40
%JAR	55	54	56	49	52	40
%Slightly too weak	20	23	20	19	7	7
%Much too weak	8	8	7	2	4	2
Food Neophilics[2]						
Appearance	6.30a	6.16a	6.22a	6.39a	6.31a	6.10a
Aroma	6.43b	6.70ab	6.88a	6.54a	6.76a	6.37a
Flavor	6.54a	6.43a	6.45a	6.20a	6.34a	5.98a
Overall Liking	6.30a	6.28a	6.27a	6.03a	5.97ab	5.53b
Attribute Intensity Score						
Flavor Intensity[2]	2.82	2.72	2.78	3.1	3.32	3.54
%Much too strong	3	3	0	7	8	10
%Slightly too strong	11	8	12	22	25	42
%JAR	58	56	62	47	59	39
%Slightly too weak	23	24	17	22	3	8
%Much too weak	6	9	9	2	3	0
Food Neophobics[3]						
Appearance	6.09a	5.84a	5.96a	6.17a	6.47a	6.14a
Aroma	6.76a	6.53a	6.56a	6.78ab	6.86a	6.39b
Flavor	6.51a	6.22a	6.07a	6.28a	6.31a	6.08a

Table 11 continued

Overall Liking	6.29a	6.16a	5.73a	6.19a	6.06a	5.72a
Attribute Intensity Score						
Flavor Intensity[2]	2.84	2.87	2.93	3.14	3.22	3.42
%Much too strong	0	0	2	3	6	11
%Slightly too strong	22	22	22	28	36	36
%JAR	51	49	47	53	39	42
%Slightly too weak	16	22	24	14	14	6
%Much too weak	11	7	4	3	6	6

[1]Degree of Liking Scores are on a 9-point hedonic scale, where 1 = Dislike extremely, and 9 = Like Extremely.
[2]Flavor intensity scores using a Just About Right (JAR) scale, where 1 = much too weak, 3 = JAR, 5 = much too strong.
[3]Food neophilics = subjects with food neophobia scores between 10-21, [3]Food neophobics = subjects with food neophobia scores between 22-53.
Trial codes: FL = Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL = Alcalase AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.
Statistical analysis was done separately for each AL- and FL-CPH- chip test, respectively. Trials within each test that do not share the same letter are significantly different ($p < 0.05$).

Food neophilics scored all AL-CPH chips as acceptable (score > 6.0) in all attributes, including overall liking. Aroma was scored as highly acceptable (score > 6.5) with AL1 having significantly higher acceptability than AL3. Within this group, AL1-3 were penalized for “not having enough flavor”, resulting in critical points affecting overall-liking of the samples (Figure 4.1 A).

Food neophobics had acceptability of all AL-CPH chips in flavor and high acceptability in aroma. In terms of overall liking, AL1 and AL2 had acceptable scores, while AL3 did not, however there was no significant difference ($p < 0.05$) between them. Food neophobics also penalized AL1-3 samples for “not having enough flavor” (Figure 4.1 B), although penalty scores were lower than those of the food neophilics, suggesting this attribute did not affect overall liking of the samples as much as it did for neophilics.

Food neophilics scored all FL-CPH chips as highly acceptable in terms of aroma, and flavor. Scores for overall liking resulted in FL1 as acceptable and was significantly higher than FL3, which scored 5.53 (not acceptable). Surprisingly, FL1 and FL3 were both recognized as having critical points "too strong flavor" (Figure 4.1 A), with FL3 having overwhelmingly more than 50% of the consumers penalizing overall liking with this attribute. This contrasts with AL-CPH chips scores, indicating that FL-peptides gave the chips a stronger flavor, regardless of both sets containing 20% CPH. Meanwhile, FL1 also had "not enough flavor" as a critical point, however the penalty score was lower than its score for "too strong flavor".

Food neophobics scored FL-CPH chips as having highly acceptable aroma, with FL2 being significantly more acceptable than FL3. In terms of flavor, all FL-CPH chips were rated as acceptable, while in overall liking of FL1 and FL2 were acceptable and FL3 was not (score of 5.72), although this was not significantly different ($p > 0.05$) compared to the latter two. These scores differ from those of food neophilics, given that neophilics had significant difference in overall liking between samples. This suggest food neophobics were less stringent with their scoring compared to neophilics. Penalty analysis for food neophobics did not show flavor intensity as a critical point for any of the FL-CPH chips (Figure 4.1 B). Noteworthy, more than 25% of subjects within the group did stated "too strong flavor" as a flaw in all the FL-CPH chips but this did not penalize overall liking as much as it did for food neophilics.

Panelists' comments (Table 12) indicated that more than half of them viewed flavor as a positive attribute for AL1 and AL2 chips, while 36% mentioned texture as a positive attribute for AL1

chips. Comments on FL-chips indicated that 52% of consumers perceived flavor as a positive attribute for FL1 and FL2 chips, while 32.6% perceived texture as a positive attribute.

Decreasing amount of negative comments regarding a lack of flavor where seen with chips formulated with increasing % DH in both enzymatic treatments, but these also had an increasing amount of comments concerning “bad” or “bitter” tastes. The amount of this latter type of comments was more than twice in FL-CPH chips than in AL-CPH chips, reaching 70.5% and 28.6%, respectively. Contrasting with AL results, FL-samples had an abundance of negative comments regarding the texture, many times described as being “too hard”.

The commentary on texture differences were taken into consideration in the formulation of chips for the preference and descriptive analysis tests to avoid any burnt chips, while still cooking them fully. Furthermore, we decided to include a question on texture during the preference test.

Overall consumers liked the CPH-chips, regardless of their neophobia level, this is in accordance with previous studies claiming insects incorporated in an “invisible” format can increase consumer willingness to eat or buy these novel food products (Wendin et al., 2017). Consumers reaction to flavor intensity is more likely associated with the presence of volatile compounds present in the peptides due to the enzymatic procedure used. Protein hydrolysis occurred within a mixture of all insect compounds; including pheromones, which have been previously rendered as a main flavor source of insects (Ramos-Elorduy & Menzel, 1998). Hydrolysis induces conformational changes of the protein structure, resulting in changes in affinity for flavor compounds depending on the exposure of protein residues available for binding (Wang & Arntfield, 2016), a function related to

the type of enzymatic treatment. Ultimately this would give different flavor profiles and intensities depending on the enzyme used and extent of hydrolysis.

These results show that food neophobics can accept food products with edible insects knowingly of their presence in a food product but incorporated in the form of an ingredient. Previous studies on the consumption of crickets or cricket-formulated products have not had acceptable sensory evaluation results, scoring lower than mealworms and mealworm-formulated products (Caparros Megido et al., 2014; Osimani et al., 2018; Roncolini et al., 2019).

Table 12: Comments from Consumer Acceptability Tests.

	AL1	**AL2**	**AL3**	**FL1**	**FL2**	**FL3**
Total Number of Comments	112.0	112.0	112.0	95.0	95.0	95.0
Positive Comments						
Flavor %	53.6	58.9	48.2	50.5	53.7	37.9
Texture %	35.7	21.4	27.7	32.6	32.6	29.5
Negative Comments						
Flavor (lack) %	18.8	14.3	8.0	5.3	3.2	2.1
Flavor % (bad)	18.8	20.5	28.6	53.7	58.9	70.5
Texture % (not crunchy in AL chips; too crunchy in FL chips)	18.8	34.8	25.9	38.9	22.1	34.7

Trial codes: FL = Flavourzyme; FL 1, 2, 3: hydrolyzed with DH 8.2, 11.9, 14.5 %, respectively; AL = Alcalase AL 1, 2, 3: hydrolyzed with DH 8.1, 10.9, 14 %, respectively.

4.4.4: Descriptive analysis

Over the course of training sessions, judges developed appropriate vocabulary terms to define flavor and aroma attributes. Each term had a corresponding reference or standard, these are listed in Table 13.

The judges developed six terms to describe the aroma of all the samples, these were: corn, oily, peanut, French fries, tomato, and ketchup (Table 14). These same terms were used and re-defined to describe the flavor, however the term “oily” was excluded and replaced with “shrimp”. The two samples did not share any common descriptors, resulting in two completely different flavor and aroma profiles (Figure 4.2). The aroma profile of AL1-chips included the terms “corn”, “oily”, and “peanut”, while the flavor profile included the terms “corn”, “shrimp”, and “peanut”. On the other hand, the aroma profile of FL1-chips included the terms “French fries”, “tomato”, and “ketchup”, with these same descriptors utilized to define its flavor. The “oily” term was also used to describe the FL1-chips aroma, but to a lower degree than the other terms (Figure 4.2). Judges were also given solutions of Quinines (35 mg/ 1L) to identify bitter notes and results indicated negligible levels, therefore it was eliminated from the vocabulary.

Table 13: Lexicon definitions and references for CPH-Chips.

		Definition	Reference/Standards
Aroma	Corn	The aromatic associated with processed, fried corn chips	Corn Chips (Great Value®)
	Tomato	Sweet tomato aromatic associated with cooked/processed tomatoes	Hunt's® tomato sauce
	Ketchup	Sweet tomato combined with vinegar and spices	Heinz ® Ketchup
	French Fries	The aromatic associated with French fries, fried in peanut oil	French Fries (restaurant style)
	Oil	The aromatic associated with pure oil, somewhat nutty/earthy	Tahini oil, fresh vegetable oil
	Peanut	Aromatic associated with the natural volatiles of roasted peanuts	Roasted Peanuts (Store bought and roasted at 350°F for 5 min)
Flavor	Tomato Sauce	Mildly sweet tomato flavor	Hunt's® Tomato Sauce
	Ketchup	Very sweet tomato flavor, followed by vinegar and spices	Heinz® Ketchup
	Shrimp	Strong and dry shrimp/fishy flavor	Dry shrimp powder, Canned Shrimp
	Corn	Strong fried corn flavor, slightly toasted	Corn chips (Great Value®), Corn Nuts
	Roasted Peanut	Earthy flavor, associated with roasted peanuts	Roasted peanuts (roasted for 5 min)
	French Fries	Fried, oily, flavor associated with restaurant style French fries	French Fries (Five Guys ®)

The flavor profile herein developed suggests strong presence of the umami taste seen by the manifestation of tomato notes in FL1-CPH chips and shrimp notes in AL1-CPH chips. Tomatoes are among the vegetables with the highest amount of available glutamate, imposing umami taste in popular dishes (Mouritsen & Styrbaek, 2014). In addition, tomatoes also contain adenylate, a nucleotide that acts synergistically with glutamate to give umami notes. Moreover, the presence of "ketchup" notes and the use of tomato sauce to describe the "tomato" flavor indicates the complexity around these attributes. The umami notes of raw tomatoes are difficult to perceive over its dominant sweetness (Mouritsen & Styrbaek, 2014). The enhancement of umami from tomato comes from the maturing and cooking processes, therefore the usage of "ketchup" and tomato sauce to describe FL1-CPH suggests strong umami taste, an important attribute in the creation of palatable foods. The usage of "shrimp" to describe the AL1-CPH chips would also suggest umami notes, because shrimp is among the top shellfish products with free glutamate that reacts with inosinate and adenylate to synergistically give a strong umami taste (Mouritsen & Styrbaek, 2014). Crickets are within the phylum of arthropods as are shrimp and lobster, making them relatives of shellfish (Thorp, 2009).

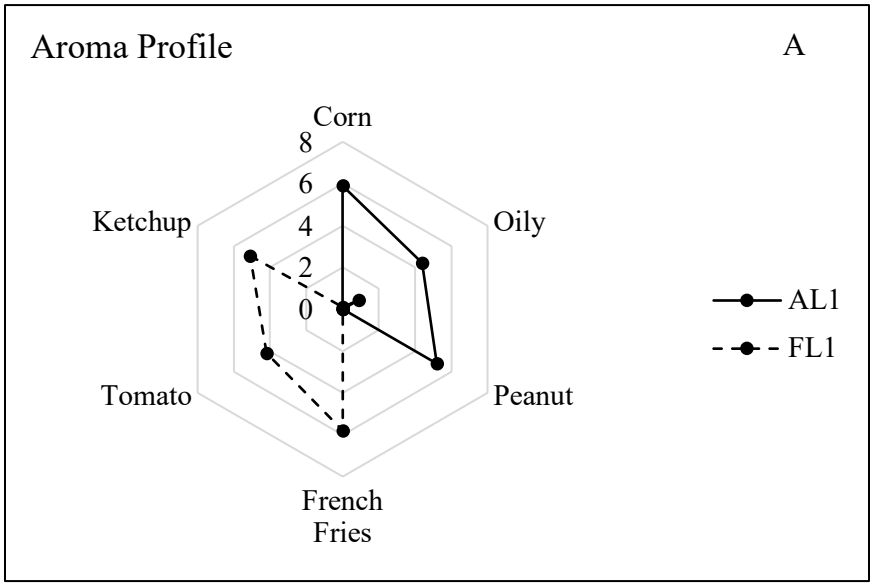
Crickets have been described as a source of umami taste before (Elhassan, Wendin, Olsson, & Langton, 2019); however, to the best of our knowledge, no other works have illustrated the degree of flavor diversity such as the presence of tomato, French fries, and peanut flavors in products formulated with cricket protein. The concept of food appropriateness, as it relates to the pairing of an insect flour or whole insects with an appropriate preparation thereof, has been previously studied to have a bigger impact in consumer acceptance than actual sensory liking tests. This research has illustrated the importance of incorporating insects into dishes with compatible food

carriers, such as savory meals as opposed to sweet desserts (Tan, van den Berg, & Stieger, 2016). The flavor notes that the CPH imposed on the fried corn tortilla chip may be appropriately associated with fried snacks (French fries with ketchup, and fried shellfish dishes), therefore the CPH had an appropriate carrier, aiding in the overall liking of the CPH-chips within the consumer acceptability testing. Moreover, the presence of flavor notes that have been identified with crickets (*Acheta domesticus*) before including "chicken", "nutty", and "cereal" (Elhassan et al., 2019), did not surface in the present study, demonstrating the influence of protein hydrolysis in unique flavor development.

Table 14: Descriptive Analysis Results on Aroma and Flavor Profiles for AL1- & FL1-CPH Chips.

	Aroma		**Flavor**	
	AL1	**FL1**	**AL1**	**FL1**
Corn	5.9 ± 1.2a	0.1 ± 0.3b	6.4 ± 0.8a	0.1 ± 0.3b
Oily	4.4 ± 1.0a	0.9 ± 0.7b	N/P	N/P
Shrimp	N/P	N/P	5.9 ± 0.4a	0 ± 0.0b
Peanut	5.2 ± 0.7a	0 ± 0.0b	6.0 ± 0.6a	0 ± 0.0b
French Fries	0 ± 0.0b	5.8 ± 1.0a	0.0 ± 0.0b	5.6 ± 0.9a
Tomato	0 ± 0.0b	4.2 ± 0.6a	0.0 ± 0.0b	4 ± 0.6a
Ketchup	0 ± 0.0b	5.1 ± 1.1a	0.0 ± 0.0b	5.2 ± 1.0a

Trial Codes: N/P= not perceived; AL = Alcalase; FL= Flavourzyme; AL1 and FL1: hydrolyzed with DH 8.1 and 8.2 %, respectively.
Statistical analysis was done separately for Aroma and Flavor, respectively. Trials that do not share the same letter in a row are significantly different ($p < 0.05$).



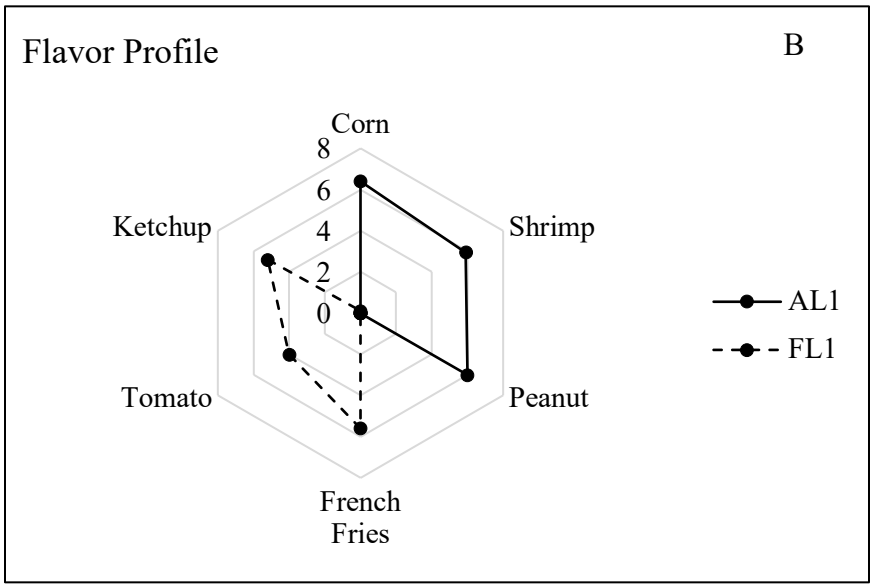


Figure 4.2: Descriptive Analysis results (A) Aroma Profile (B) Flavor Profile of AL1 and FL1 Tortilla Chips.

Trial Codes: AL = Alcalase; FL= Flavourzyme; AL1 and FL1: hydrolyzed with DH 8.1 and 8.2 %, respectively.

4.4.5: Preference Test

Results for the preference test are shown in Table 15. Results revealed significant ($p < 0.05$) preference of AL1-CPH chips (80.6%) over FL1-CPH chips (19.40%). This information was related to in their overall liking ratings, resulting in higher acceptability for AL1-CPH chips and acceptability for FL1-CPH chips, with scores of 6.94 and 6.08, respectively. These scores are above the overall liking results that were previously determined with the consumer acceptability tests with all the AL- and Fl-CPH chips. It must be noted that the change in label regarding the test from “tortilla chip” to “corn snack” could have nullified sensory expectations, thus improving overall liking scores. In addition, the panelists participating were already familiar with these chips, making them more accustomed to the idea of eating chips formulated with cricket protein powder. In terms of degree of liking for texture, FL1-CPH chips fell below the acceptability mark (5.28) while AL1-CPH chips were still regarded as highly acceptable (6.83).

The same preference, overall liking, and texture acceptance patterns were seen within the food neophilics’ scores, although ratings for FL1-CPH chips were slightly higher (5.70) in terms of texture. On the other hand, food neophobics were more severe with their scoring when comparing these two samples, rating FL1-CPH chips below the acceptability mark for overall liking (5.84) and for degree of liking for texture (4.75). Within this group, the scoring for AL1-CPH chips increased compared to the first time the panelists evaluated the sample (6.78 vs. 6.51) and lowered the score for FL1-CPH chips compared to their first evaluations (6.03 vs. 5.84), showing the impact of comparing two different samples.

Panelists' comments within this test are shown in Table 16. Data revealed that panelists preferring AL1-chips mentioned texture and flavor approximately 50% of the time as positive attributes, and none had negative comments regarding texture of AL1-chips. In contrast, panelists preferring FL1-chips mainly mentioned flavor (84.6%) as a positive attribute while texture was only mentioned as a positive attribute 38.5% of the total amount of comments. Within these same panelists, 30.8% of the comments had negative mentions of the texture of FL1-chips. Overall, negative comments on FL1-chips mainly revolved around "too hard" of a texture and "strong aftertaste" or "bitter taste". Because there were precautions in place to avoid burning the chips, the textural characteristics are related to the extent of protein interactions with other corn macromolecules. The textural properties induced by frying involves time of frying, moisture content and macromolecular interactions. Longer frying times has been shown to increase hardness in frying model systems (Ansarifar, Mohebbi, & Shahid, 2012). Our results reveal the opposite, given than FL1-chips were fried for 20 seconds less than AL1-chips, meaning other factors such as moisture content and protein configurations resulting in different protein-starch interactions are likely responsible for textural differences. The textural integrity of a corn tortilla is dependent on the creation of calcium bridges between negatively charged Asp and Glu residues with other negatively charged hydroxyl groups on starch and other ionized amino acids (Miklus, 1999). In addition, the creation of disulfide bridges would also affect the textural properties of the chips, as is the case in other food systems relying on disulfide bridge formation for proper gelation (Singh, 1991). These intermolecular interactions will differ depending on the availability of amino acid residues, a function directly related to the type of enzymatic treatment performed. Within the FL1-CPH chips network the hydrolysates seemed to have created strong intermolecular bonds that induced tougher textures while AL1-CPH did not. Strong aftertaste could stem from the presence

of small peptides in the FL pool, given that small peptides with hydrophobic residues are triggers of bitter taste. A study used sequential hydrolysis treatments of soy proteins with endo-proteases and Flavourzyme, claiming that the use of an exo-protease would reduce bitterness (Meinlschmidt, Schweiggert-Weisz, Brode, & Eisner, 2016). Paradoxically, they saw an increase in bitterness with increasing % DH when hydrolysis treatment was kept below 60 min, upon further hydrolysis the bitterness intensity decreased. Because our treatments were kept at low % DH (resulting from low hydrolysis time, < 60 min), the bitter taste of FL peptides could have increased with increasing % DH (seen by the comments in consumer acceptability tests, section 4.4.3, Table 12) and perhaps could have decreased upon further hydrolysis, however further experimentation would be needed. Further hydrolysis could result in lower bitterness depending on the parent protein amino acid sequence and size of resulting peptides, because it has been shown that at least a 3-carbon chain is needed to have any perceivable bitterness and only certain amino acid combinations would result in such tastes (Ishibashi et al., 1988). Nevertheless, descriptive analysis results neglected the use of "bitter" to characterize the flavor of FL1 chips, meaning the strong "aftertaste" that untrained panelists remarked upon may then stem from flavors not normally associated with corn chips, such as ketchup and tomato notes. Furthermore, research has shown that sweet, salty and sour are equally perceived throughout the tongue, but umami and bitter are perceived largely at the posterior part of the tongue. Because the anterior and posterior taste receptors are innervated with different cranial nerves, umami and bitter tastes could be triggering similar sensations (Feeney & Hayes, 2014). The lack of general knowledge around the word and meaning of "umami" and the unexpected sensation of this taste from a tortilla chip could have led panelists to simply to mistaken the sensation. Overall, the CPH herein studied are meant to be utilize as part of an ingredient in

any food matrix that the consumer would find appealing, therefore the utilization of FL-CPH in foods already associated with umami tastes may be more appropriate application.

Table 15: Degree of Liking (DOL) and Preference Test Results for AL1- and FL1-CPH Chips.

N = 72	AL1	FL1
Overall Scores		
Preference* (%)	80.60	19.40
Overall Liking[1]	6.94a	6.08b
Degree of Liking for Texture[1]	6.83a	5.28b
Food Neophilics[2]		
Preference* (%)	80.00	20.00
Overall Liking[1]	7.08a	6.28b
Degree of Liking for Texture[1]	6.85a	5.70b
Food Neophobics[2]		
Preference* (%)	81.30	18.80
Overall Liking[1]	6.78a	5.84b
Degree of Liking For Texture[1]	6.81a	4.75b

Food Neophobia Scores ranged from 10 to 53, average score= 21, standard deviation= 7.0.
[1]Degree of Liking Scores are on a 9-point hedonic scale, where 1 = Dislike extremely, and 9 = Like Extremely.
[2]Food Neophilics = subjects with food neophobia scores between 10-21, [2]Food Neophobics = subjects with food neophobia scores between 22-53.
*significant ($p < 0.001$).
Trial codes: FL = Flavourzyme; AL = Alcalase; FL 1: hydrolyzed with DH 8.2% AL 1: hydrolyzed with DH 8.1%. Trials that do not share the same letter in a row are significantly different ($p < 0.05$)

Table 16: Comments from the Preference Test.

	Comments on AL1-Chips from those preferring AL1-Chips	**Comments on FL1-Chips from those preferring FL1-Chips**
Total Number of Comments	56	13
Positive Comments (%)		
Texture	55.4	38.5
Flavor	48.2	84.6
Negative Comments (%)		
Texture	0.0	30.8

4.5: Conclusion

This study showed how enzymatic proteolysis can be used to create cricket protein powders palatable for their use in a food product, such as a corn tortilla chip. The use of enzymes to create emerging proteins more applicable in the food industry does not only translate into an enhancement in techno-functional properties compared to the use of whole insect flours, it also has significant impact in the sensorial attributes imposed. The degree of hydrolysis between trials had an imperceptible impact in the overall liking among samples formulated with AL-CPH (degree of liking stayed above 6.0), however consumers found the higher degree of hydrolysis of Flavourzyme peptides (FL3-CPH) detrimental enough to score poorly in overall liking. The descriptive analysis showed novel flavor and aroma attributes not previously associated with cricket-based products, as well as traces of umami taste from strong tomato and shrimp flavors. Lastly, the textural properties imposed by the enzymatic treatments were clearly seen in the final acceptability (degree of liking) test, were FL1-CPH had overall below acceptability levels for this attribute among all groups. Comments confirm the big textural differences between samples,

labeling FL1-CPH as “too hard” and with a strong “aftertaste” explain the strong preference for AL1-CPH chips. Textural differences are attributed to different macromolecular interactions while the origin of the “aftertaste” is still unclear. Lastly, the acceptance of cricket-based products on food neophobics was revealed. Our study demonstrated that enzymatic proteolysis may result in cricket protein powders that have overall acceptable sensorial attributes, opening a ray of possible incorporation of this new ingredient into other food matrices.

4.6: References

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CHAPTER 5: CONCLUSION AND FUTURE WORK

5.1: Conclusion

The lower environmental impact associated with the farming of insects has been gaining attention by the media, the food industry, and academia. This awareness has galvanized entomophagy in view of its potential as a protein alternative. However, the use of insect flours as a starting material is difficult due to a lack of techno-functional properties, limiting their application in food matrices. Researchers have shown the benefit of using enzymatic proteolysis to improve functional properties of insect proteins, yet there is a lack of evidence towards the applicability of these hydrolysates in a food product and their acceptance by consumers. This work attempted to further explore the functional property enhancement of cricket (*Acheta domesticus*) protein via different enzymatic treatments and their impact in a model food matrix. Enzymatic treatment with Alcalase resulted in cricket protein hydrolysates (CPH) with high emulsifying activity and stability, as well as foaming capacity and stability. This set of peptides could be suited to systems needing amphiphilicity properties such as mayonnaise, salad dressings, high fat protein beverages, and edible foams. Enzymatic treatment with Flavourzyme gave highly soluble CPH, significantly improving solubility at the isoelectric pH. These peptides could have applicability where solubility is needed such as beverages, broths, soups, and sauces. Subsequently, the application of the CPH in a nixtamalized corn dough provoked higher elastic behavior in doughs formulated with Alcalase CPH, while Flavourzyme CPH lowered elastic and increased viscous behavior. This rheological behavior is attributed to differences in mean molecular weights of the CPH. Larger mean molecular weight peptides from Alcalase treatments provoked topological entanglements, increasing elastic properties. Smaller mean molecular weight peptides from Flavourzyme treatments had a

plasticizing effect in the matrix, therefore moving gradually to a more viscous comportment. Upon cooking the CPH-corn doughs the Alcalase peptides failed to form sufficient intermolecular bonds to sustain the integrity of the tortilla, resulting in a weak and unrollable matrix. In contrast, the Flavourzyme peptides were able to form starch-protein interactions and disulfide bridges, necessary in the formation of a strong and flexible tortilla. Lastly, sensory evaluations revealed overall liking of corn chips formulated with CPH from both enzymatic treatments, however there was a preference for chips formulated with Alcalase peptides. This pattern was seen for the overall population, Food Neophilics and Neophobics. Studies in the past has shown that Neophobics have a tendency to dislike the idea of trying and buying insect-containing food products. This work illustrates how proper treatment of these novel proteins can lead to appropriate applications in food products that can lead to consumer acceptance across neophobia levels. Panelists' comments disclosed wide dislike for the texture of chips formulated with Flavourzyme peptides, describing them as "too hard". The intermolecular bonds formed within the corn tortillas with Flavourzyme peptides limited its expansion during frying and resulted in dense and tough chips. Descriptive analysis revealed a set of completely unique flavor and aroma profiles for both sets of CPH-chips. Chips with Flavourzyme peptides were characterized as having "tomato", "ketchup", and "French fry" notes, while those with Alcalase peptides had "corn", "oily", "peanut", and "shrimp" notes. The use of "tomato", "ketchup" and "shrimp" indicate strong umami taste from both sets of peptides and their association with fried savory foods indicates an appropriate use of these peptides in a fried corn chip formulation, likely aiding in their overall liking during hedonic tests.

The two chosen model systems, corn tortillas and tortilla chips, required different peptide characteristics for optimal physicochemical properties, illustrating the profound difference

enzymatic treatments have on peptide functionalities. Although more research is necessary on insect protein hydrolysates applicability in food systems, this work shows promising products that were accepted by a wide range of consumers, providing a stepping-stone in future food formulations.

5.2: Future Work

The continuation of this work could start by further characterizing the set of peptides and other macromolecules herein created in order to better understand their behavior. Starting with the isolation and characterization of lipid compounds within the Alcalase peptides via gas chromatography - mass spectrometry that would confirm the presence/absence of lipids with surface activity. Performing functionality assays that involve amphipathic properties with defatted hydrolysates could be a better indicator of the amphipathic properties of the peptides. Obtaining the molecular weight distribution of all peptide trials via high performance liquid chromatography would aid to support rheological and functionality theories. Furthermore, the use of the hydrolysates in this work in different food systems could demonstrate their capacity to interact with other macromolecules. For example, Flavourzyme peptides showed great capacity to interact with starch, providing the possibility of using these peptides in other bakery items. Alternatively, protein-protein interactions with the aid of cations could serve to show how either set of peptides could be used to form meat analogues, an important category of products that could directly replace traditional protein in a meal. Maximizing the content of insect in a food product and measure important physicochemical and structural thresholds would be beneficial in optimizing protein content. Digestibility studies of food matrices with insect protein hydrolysates and insect whole flour would also be important to observe the nutritional benefits of protein isolation and hydrolysis. The utilization of the other macromolecular compounds within insect such as the use of chitin in

organic by-products and the use of lipids in other food systems is an area essential to secure future sustainable agricultural systems. The use of these by-product in the creation of biodegradable plastics, fibrous materials for encapsulation, and the creation of oil-based products with essential fatty acids could even elevate the economic stance of using protein hydrolysis as a means to separate macromolecules. Likewise, the optimization of protein hydrolysis for its industrial scale via technological improvement would be essential to minimize ecological footprints. For instance, creating systems that use less water than the ratios stated in this work, having self-sufficient water cycles, and obtaining technology that could maximize protein yields. Lastly, the manipulation of the insect feed to observe the impact in its final physiological composition and its sensory attributes would give future guidelines on appropriate farming practices.

APPENDIX A. TOTAL AMINO ACID COMPOSITION OF CONTROL CORN TORTILLA, CONTROL CRICKET FLOUR AND CPH-TORTILLA

	Total Amino Acid g/ 100 g							
	Soft Tortillas with 20 % CPH							
Amino Acids	FL1	FL2	FL3	AL1	AL2	AL3	CF	C
Gly	0.92	1.01	0.95	0.76	0.81	0.80	3.41	0.25
Ala	1.63	1.69	1.64	1.39	1.41	1.45	6.28	0.58
Pro	1.40	1.46	1.37	1.19	1.24	1.23	3.63	0.64
Val	0.83	0.86	0.89	0.88	0.92	0.88	3.74	0.34
Ile	0.54	0.58	0.60	0.63	0.66	0.63	2.32	0.22
MetS	2.40	2.70	0.47	0.50	0.52	0.51	1.53	0.23
Leu	1.58	1.65	1.65	1.66	1.74	1.67	4.26	0.91
Phe	0.33	0.32	0.35	0.38	0.40	0.31	6.07	0.20
Asp	0.03	0.03	2.26	2.22	2.13	2.21	7.89	0.62
Glu	5.30	5.53	5.60	4.98	4.92	5.10	12.56	2.41
Lys	0.86	0.95	1.01	1.02	1.02	1.03	3.79	0.19
Arg	0.62	0.66	0.64	0.58	0.61	0.59	3.51	0.11
His	0.12	0.13	0.11	0.11	0.12	0.11	0.49	0.03
Ser	0.92	1.07	1.04	1.02	1.05	1.03	3.46	0.36
Cya	0.02	0.00	0.35	0.33	0.35	0.33	0.92	0.18
Thr	0.60	0.65	0.65	0.66	0.69	0.67	2.44	0.24
Tyr	0.01	0.00	0.01	0.01	0.01	0.01	0.03	0.01
HAA	9.63	10.28	7.92	7.40	7.70	7.48	31.24	3.37
NAA	5.33	5.56	7.86	7.20	7.05	7.31	20.45	3.03
PAA	1.60	1.74	1.77	1.71	1.76	1.73	7.79	0.33
POAA	1.55	1.72	2.05	2.02	2.10	2.05	6.85	0.79
EAA	8.85	9.51	7.38	7.51	7.82	7.48	28.90	3.26

CF = Cricket Flour, C = Corn Tortilla Control (no CPH), FL = Flavourzyme, AL = Alcalase
HAA: sum of hydrophobic amino acids; glycine, alanine, proline, valine, isoleucine, methionine, leucine, and phenylalanine; NAA: sum of negatively charges amino acids; aspartate, and glutamate; PAA: sum of positively charged amino acids; lysine, arginine and histidine; POAA: sum of polar amino acids; serine, cysteine, threonine, and tyrosine; EAA: sum of essential amino acids; valine, isoleucine, methionine, leucine, phenylalanine, lysine, histidine, and threonine

APPENDIX B. PRE-SCREENING HYDROLYSIS TREATMENTS

Enzyme	E/S	Time (min)	DH (%)
Alcalase	0.125	5	8.11 ± 0.3
	0.125	10	9.61 ± 0.1
	0.125	15	9.86 ± 0.9
	0.125	30	10.89 ± 0.7
	0.125	40	13.06 ± 0.9
	0.25	10	13.98 ± 1.8
Flavourzyme	0.5	10	8.21 ± 0.5
	1.5	10	10.43 ± 0.9
	1.5	30	11.93 ± 0.4
	1.5	60	13.52 ± 1.8
	3.0	10	12.31 ± 0.8
	3.0	20	12.86 ± 1.4
	3.0	30	14.54 ± 0.9

E/S: Enzyme to substrate ratio, DH (%): Degree of hydrolysis %