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**DEVELOPMENT OF VALUABLE AND MULTIFUNCTIONAL PROTEIN-BASED FOOD INGREDIENTS
FROM INVASIVE GREEN CRABS**

By

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A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Food and Nutrition Sciences)

The Graduate School

The University of Maine

August 2020

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Dissertation Advisors: Dr. Angela D. Myracle and Dr. Denise I. Skonberg

An Abstract of the Dissertation Presented
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
(in Food and Nutrition Sciences)
August 2020

European green crabs (*Carcinus maenas L.*) are an invasive species unintentionally introduced from Europe to the U.S. Their predation and burrowing activities have had detrimental effects on our marine habitats, aquaculture, and commercially important fisheries. Despite the large population of green crabs, they are not commercially utilized in North America as food products. The specific objectives of my research were to: 1) determine functional properties of protein recovered from green crab by isoelectric solubilization and precipitation (ISP) for potential food application, 2) investigate the application of enzymatic hydrolysis for deriving bioactive compounds from green crab proteins and examine the bioactivity changes of green crab protein derivatives after simulated human digestion, and 3) evaluate whether enzymatic hydrolysis by commercial proteases improves functional properties of green crab proteins in food formulations.

In the first study, the results showed that ISP processing can recover functional proteins from green crabs, and that the recovered proteins could potentially be successfully applied as ingredients in various food emulsions (e.g. sauces or chowders) and food gels (e.g. surimi or other meat analog products) to improve food product quality.

In the second study, enzymatic hydrolysis using commercially available proteases and health promoting effects of the hydrolysates were evaluated. Protein derivatives produced by Protamex had

the potential to alleviate type 2 diabetes by inhibiting carbohydrate digesting enzymes and secreting an insulin stimulating molecule. Also, no additional processing would be required to stabilize their anti-diabetic effects as food ingredients since they were stable to simulated human digestion.

The last study investigated the functional properties of protein derivatives obtained through enzymatic hydrolysis. Mild hydrolysis by Protamex and combination of Protamex and Flavourzyme for 15 min improved the foaming activity of the crab mince and combination of Protamex and Flavourzyme generated antioxidant hydrolysates that may contribute to extended shelf-life of food products.

These studies provide valuable information for the development of value-added food ingredients from green crab using ISP processing and enzymatic hydrolysis, and for the introduction of green crab proteins to the food industry, potentially contributing to food product development and the creation of new markets for this invasive species.

DEDICATION

This dissertation is wholeheartedly dedicated to my family, who have been my source of inspiration and provide me with unconditional support.

ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my co-advisors Dr. Angela Myracle and Dr. Denise Skonberg who gave me the opportunity to do valuable research, expertly guided me, and continuously provided me with emotional support. Their vision, sincerity, enthusiasm, motivation, and encouragement have deeply inspired me in the last 5 years. It was a great honor to have my committee members, Dr. Ian Bricknell, Dr. Jennifer Perry, and Dr. Rebecca Van Beneden on board and I am extremely grateful for their suggestions and feedback on my research goals.

I would like to thank the Sustainable Ecological Aquaculture Network (SEANET), the University of Maine Graduate School, and the School of Food and Agriculture for their financial support for completion of my PhD. Also, I would like to acknowledge Dr. Marissa McMahan at Manomet and Chad Coffin with the Maine Clammers Association for providing green crabs.

I am very grateful to Dr. Brian Perkins for providing me with scientific recommendations, Kathy Davis-Dentici for helping me unconditionally all the time, and Robert Dumas and Michael Murphy for training and helping me in the pilot plant.

I am very fortunate to have had a chance to work with lab members Amber, Alison, Charlie, Dhriti, Holly, Juliana, Sami, Samuel, Sarah, Teaka, Xue, and Zack - thank you all for helping me to accomplish this project. Also, I deeply appreciate Dr. Lee, Dr. Apostolidis, and Dr. Kwon for their continuous support and help. I also extend my gratitude and love to all my friends, Abby, Ade, Grape, Eunhye, Hyeweon, Jiwon, Panos, Praveen, Seulki, Surbhi, Tamanna, Weeam, and Youngeun for encouraging me with consistent support.

Special thanks to Dhriti, Surbhi, and Praveen, it is only because of you all that I will think of the beautiful memories created in Maine filled with fun potlucks, many late night experiments and crazy snow days!

I would like to thank my parents for their unconditional faith, love, support, patience, and sacrifices during my PhD. My brother and sister-in-law with my lovely niece and nephew, thank you for all your support. Lastly, many thanks and appreciation go to all my family and my uncle who is my role model as a scientist.

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CHAPTER 1

LITERATURE REVIEW

1.1. Introduction of Green Crabs

Green crabs (*Carcinus maenas* L.) are an invasive species unintentionally introduced from Europe to the United States around 1817 on Long Island by the water of ship ballast (Carlton & Cohen, 2003). Their secondary introduction to Newfoundland as a result of shipping movements and ballast water occurred in the 1900s. Now these crabs can be found from North Carolina to Nova Scotia. In 1951, green crabs first appeared on the west coast in Washington; however, they were not established at that time (Carlton & Cohen, 2003). In 1989, they were again introduced on the west coast in San Francisco Bay and have successfully settled north throughout parts of Oregon, Washington and even British Columbia. Green crabs are a successful marine invasive species in Maine. They migrated to Casco Bay and other parts of Maine in the early 20th century. They have also established populations in South Africa, Japan, Argentina, and Australia as well as North America (Carlton & Cohen, 2003).

1.1.1. General Characteristics

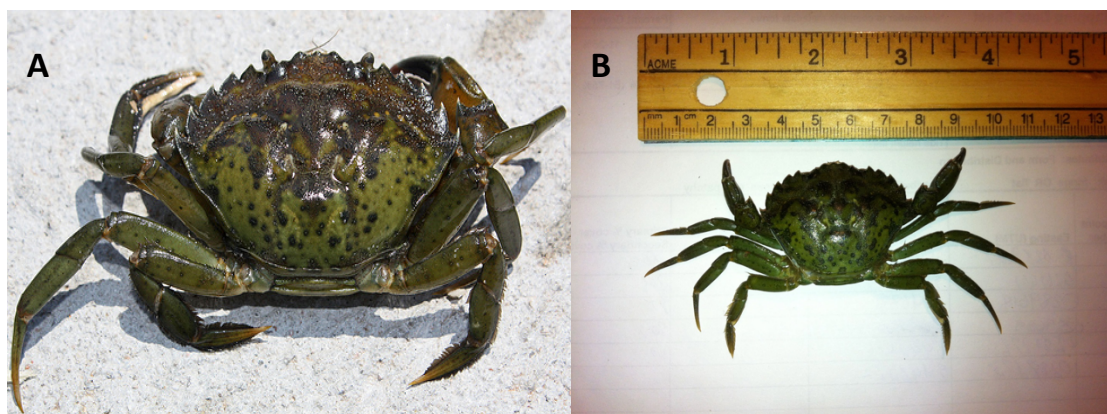


Figure 1.1. Green crab

[A source: steamboatisland.org and B source: haidanation.ca]

Green crabs are categorized as crustaceans, which are invertebrates with segmented bodies and chitin-based hard shells. The carapace width (CW) of green crabs is typically between 5-80 mm, much smaller than the average CW of commercially processed Dungeness (> 190 mm) and Jonah (> 222 mm) crab (Grosholz & Ruiz, 1996; Swiney et al., 2003; Robichaud & Frail, 2006). Green crabs have a set of five triangular spines on each side of the shell and three rounded lobes between the eyes (Figure 1.1). Their last pair of legs is flattened, and they show various colors such as green, red, brown, grey, and yellow (ODFW, 2020).

Optimal environmental temperatures for green crab range from 3 °C to 26 °C, but they can survive even at temperatures of less than 0 °C and higher than 35 °C. Although green crabs prefer salinities of 10-30 ‰, they can tolerate salinities ranging from 4 to 52 ‰ (MDMR, 2014). The physiology of green crabs is affected by environmental factors including temperature, salinity, and oxygen concentration of the ocean. However, they have a strong capability to survive a broad range of temperatures and salinities. Green crabs live along rocky shores (Bertness & Coverdale, 2013) in the interstices of cobbles and rocks (Ellis et al., 2007). They are also found in salt marshes (Konisky et al., 2006), eelgrass beds (Schmidt et al., 2011), and in unvegetated soft sediments (Gregory & Quijón, 2011).

1.1.2. Impact on Ecosystems and Local Society

Green crabs inhabit various marine habitats including rock, mud, sand, salt marshes and seagrasses (Konisky et al., 2006; Gregory & Quijón, 2011; Schmidt et al., 2011). It has been reported that a large green crab population can negatively affect eelgrass beds (Malyshev & Quijón, 2011). Eelgrass beds are critical habitats in Maine because they provide essential habitat, refuge, nursery and feeding grounds for other shellfish and finfish. However, green crabs have detrimentally affected the eelgrass by digging to find foods, thereby cutting roots of eelgrass (Malyshev & Quijón, 2011). Their digging activity in eelgrass beds destroys the eelgrass, and increases exposure of larval shellfish and fishes to their predators such as birds and other fishes.

Salt marshes play a significant role on the coastal Maine waterfront. They help absorb storm surges and reduce upland flooding, supporting high nutritional levels, building peat, and sequestering carbon (Giuliani & Bellucci, 2019). Also, saltmarshes are essential for the bluff erosion-stability cycle in Maine (MDA, 2011). Green crabs are commonly found in salt marshes and their aggressive burrowing activities can destroy the marshes. Marsh erosion by their burrowing could cause greater extents of bare bluff exposed to wave and ice erosion, which results in land loss.

Lastly, green crabs are well known as voracious predators. Most commercially significant fisheries and aquaculture species in Maine are favorite prey of green crabs. They like a diverse diet but prefer to prey on economically important seafoods such as clams, mussels, scallops, and lobsters, which are worth approximately \$25 million annually in Maine (Tyrrell et al., 2006; Sigurdsson & Rochette, 2013).

1.1.3. Green Crab Composition

Understanding green crab composition is critical for developing value added products. Fulton & Fairchild (2013) reported that whole green crab pulverized by a mill was composed of $12.3 \pm 0.3\%$ protein, $16.6 \pm 0.3\%$ ash, and $0.2 \pm 0.1\%$ lipid (wet weight basis) and contained all essential amino acids. The fatty acid profile of the whole green crab homogenate was composed of 68.0% unsaturated and 23.3% saturated, with 22.3% of eicosapentaenoic acid (EPA) and 12.84% of docosahexaenoic acid (DHA). According to Skonberg and Perkins (2002) and Naczek et al. (2004), crab meat contained 80.6-83.5% protein, 3.6-4.8% lipid, 5.1-19.2 mg% carotenoid, and 2.2% minerals (dry weight basis). The saturated and omega-3 fatty acids accounted for 19–20.7% and 37.4–40% of total fatty acids, respectively, with 16.5-22.3% of EPA and 9.3-13.4% of DHA. In addition, shell discards from green crabs were comprised of 12.6-14.5% chitin, 4.3-7% crude protein, 4.4-9.3% total carotenoids, and less than 1% lipids.

1.2. Industrial Utilization of Crabs

Crabs are commercially important seafood products in the U.S. and worldwide. Some commercially fished crabs found in the U.S. include Blue crab (*Callinectes sapidus* L.), Dungeness crab (*Metacarcinus magister* L.), Snow crab (*Chionoecetes opilio* L.), King crab (*Paralithodes* spp. L.), and Jonah crab (*Cancer borealis* L.). In 2017, U.S. landings of all species of crabs were approximately 125,000 metric tons having \$610.4 million of economic value (NOAA, 2018). More than 80% of these crabs were Blue crab, Dungeness crab, Snow crab, King crab and Jonah crab, and their utilization in edible fishery products, foreign trade, and processed products generated substantial economic profits (Table 1.1).

Table 1.1. US domestic landings of crabs in 2017 (NOAA, 2018)

Species	Metric tons	Economic value (million dollars)
Blue crab	56,290	189.9
Dungeness crab	21,805	212.7
Snow crab	9,661	81.3
King crab	5,851	57.9
Jonah crab	7,711	14
Green crab (harvested from CT, MA, RI)	1,045	< 1

On the other hand, landings of green crabs from Connecticut, Massachusetts, and Rhode Island in 2017 totalled about 1,045 metric tons and their value was less than \$1 million (NOAA, 2018), indicating that green crabs have a significantly low economic value as a commercial crab. The small size of green crab compared to other commercially popular crabs is a major obstacle to their utilization by the food industry. Crab meat is typically obtained manually from cooked crabs, and obtaining meat from green crab is very labor intensive and not cost-effective with low yield. The purpose of these landings was not clearly reported, however, there is a report that green crabs are used as a supplemental bait for fishing purposes (NCEE, 2008).

In order to create a commercial fishery for green crab, the value-adding process should be considered, thus increasing their economic value. The USDA defines value-adding as: 1) a change in the physical state or form of the product, 2) the production of a product in a manner that enhances its value, and 3) the physical segregation of an agricultural commodity or product in a manner that results in the enhancement of the value of that commodity or product.

1.2.1. Culinary Application

Crabs have a high culinary value due to their strong umami flavor generated by a high content of glutamate and aspartate. Whole crabs including King crab, Dungeness crab, Mud crab, Blue crab, and Jonah crab are widely used in many cuisines across the world. Many crab dishes including fried crab, black pepper crab, and chili crab are available in restaurants. Soft-shell crabs that have recently molted have a delicate texture and are rich in flavor, therefore, they are in high demand during the limited season in the spring and early summer (Manomet, 2020). In Asian countries, whole crabs and their mince are also widely used in seafood stocks or oyster and savory sauces via fermentation to improve their umami flavor.

1.2.2. Shell

Crustacean shells contain approximately 20-40% of protein, 20-50% of calcium carbonate, and 15-40% of chitin (Yan & Chen, 2015). Protein is a good source of nitrogen and can be utilized in fertilizers and animal feeds in the agricultural industry. Calcium carbonate has a wide range of applications in the pharmaceutical, agricultural, construction, and paper industries (Yan & Chen, 2015). Chitin is a polysaccharide consisting of a long chain of N-acetyl-D-glucosamine. The chitin in crab shells is used in several industries to produce chitosan and glucosamine when the other components of the shell are discarded as industrial waste. The chitin is typically transformed into chitosan via deacetylation by treatment with acid and base, and the chitosan comprises D-glucosamine chains (Yeul & Rayalu, 2013). Chitin and its derivatives such as chitosan and chitosan oligosaccharides have high potential as bioactive

materials due to their wound healing, antioxidant, anti-microbial, and anti-inflammatory (Khoushab & Yamabhai, 2010). In addition, they have heavy metal and other environmental pollutant removal activities (Khoushab & Yamabhai, 2010). Therefore, chitin and its derivatives can be utilized by food and nutrition (Kumar, 2000), pharmaceutical (Kato, Onishi, & Machida, 2003), biotechnological (Kim & Mendis, 2006), cosmetic (Muzzarelli et al., 2012), packaging (Leceta et al., 2013), textile, wastewater treatment (Kumar, 2000), and agricultural (Aklog et al., 2016) industries.

1.2.3. Lipids

In general, crabs have a low crude lipid content of less than 2% (wet weight basis), however, crab lipids contain appreciable proportions of omega-3 long-chain polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Gökođlu & Yerlikaya, 2003; Wu et al., 2010; Varadharajan & Soundarapandian, 2014). Generally, the contents of PUFA are higher than those of saturated fatty acids (SFAs) (Bergé & Barnathan 2005). The consumption of PUFA provides various health benefits that lowers the risks of inflammatory diseases (Calder, 2009), cardiovascular diseases (Harris, 2007), insulin resistance (Bellenger et al., 2011), and breast cancer (Wu et al., 2005). Therefore, crab lipids may be applicable in the food or dietary supplement industry.

1.2.4. Carotenoids

Crabs contain carotenoids including astaxanthin, lutein, zeaxanthin, and astacene (Naczka et al., 2004; Higuera-Ciapara et al., 2006). Astaxanthin is the major red-orange-colored carotenoid present in crab shells (Sachindra et al., 2005). Astaxanthin plays a vital role in the aquaculture industry since it helps growth and reproduction of fish as well as enhances pigmentation and consumer appeal. In addition, the high antioxidant properties of astaxanthin are comparable with those of β -carotene or even α -tocopherol (Miki, 1991). Due to its outstanding antioxidant activity astaxanthin has been attributed with potential for protecting humans against a wide range of ailments such as cardiovascular

problems, different types of cancer and some diseases associated with the immune system (Bennedsen et al., 2000; Naguib, 2000; Fasset & Coombes, 2011; Zhang & Wang, 2015).

1.2.5. Meat

Crab meat is reported to have 15-20% protein content (wet weight basis) and provide high quality protein including all essential amino acids (Gökođlu & Yerlikaya, 2003; Vilasoa-Martínez et al., 2007; Varadharajan & Soundarapandian, 2014). Also, high content of glutamate and aspartate in the meat contributes to its savory flavor. Therefore, in general, commercially extracted meat from Dungeness crab, Jonah crab, Blue crab, and King crab is added in many crab dishes including crab cakes, crab bisque, and crab Rangoon. Also, they are used in high-value products including pasteurized and/or canned crab meat. On the other hand, there are some crabs such as green crab and Asian shore crab, which do not have economic value due to their undesirable characteristics (e.g., small size, low meat yield, or appearance). Although these crabs are not currently processed in the U.S., they have proteins with high nutritional quality. Proteins and their fragments (peptides) from shellfish including crab, shrimp, mussels, and clams have been reported to have potential as value-added food ingredients to improve food product quality and provide health benefits. For example, peptides from snow crab byproducts have anti-cancer properties that may be used by the food and nutrition industries (Doyen et al., 2011). Antarctic krill (*Euphausia superba*) proteins could be utilized for gelation in surimi products (Chen & Jazynski, 2007). Also, proteins and peptides of blue mussels (*Mytilus edulis*) could be utilized as potent gel forming and anti-inflammatory ingredients by the food and dietary supplement industries (Vareltzis & Undeland, 2012; Kim et al., 2016).

1.3. Functional Properties of Proteins and Peptides

Food proteins in human diets are sourced from foods such as eggs, meats, milk, seafood, and soy. Their peptides are commonly derived from enzymatic processing of proteins. These dietary proteins and peptides perform a range of functions in humans. They provide essential materials to build

skin and muscle, and they play a critical role in essential biological processes. For instance, proteins contribute to the transport of important biological molecules such as oxygen and iron, protect the body against harmful substances such as pathogens, catalyze biological reactions, and deliver the messages from cells to cells (Alberts et al., 2008). Some peptides in our body act as hormones, which regulate physiological activities such as growth, appetite, reproduction, energy metabolism and stress response (Rose, 2019).

Protein is one of the essential macronutrients, along with carbohydrate and fat that are required to grow and maintain the human body. Proteins consist of essential and non-essential amino acids. Essential amino acids for humans include phenylalanine, valine, tryptophan, threonine, methionine, histidine (for children), isoleucine, leucine, and lysine, which cannot be synthesized by the human body (Damodaran et al., 2007). Therefore, they must be obtained from food sources. Animal sources including fish, meat, poultry, eggs, and dairy are considered to be complete protein sources since they supply all the essential amino acids that the human body needs. On the other hand, plant sources including legumes, wheat, and rice are considered as incomplete proteins sources because they provide limited essential amino acids (ASN, 2011). Although proteins are nutritionally important for humans, certain proteins in foods are undesirable since these can cause allergic reactions in some people. The eight major allergenic foods include milk, eggs, tree nuts, peanuts, shellfish, wheat, soy, and fish (Panel, 2010). Allergens in these foods can trigger an abnormal immune response that results in digestive problems, hives, and swollen airways (Panel, 2010).

Lastly, proteins and peptides play critical roles in formulated food products by improving qualities such as color, texture, and flavor. Some proteins and peptides derived from food sources have specific functional properties that make a positive contribution to food products. Functional properties of proteins are defined as the chemical and physical properties that affect their performance in food systems during preparation, processing, storage, and consumption and influence the quality and sensory

attributes of food products (Kinsella, 1976). For example, certain proteins can act as emulsifiers, foaming agents, buffering agents, and gel forming agents in food products. Peptides derived from proteins can also function as biofunctional ingredients such as antioxidants, anti-microbial agents, and cryoprotectants; these ingredients are added to food products to maintain desirable food quality and extend shelf-life during processing, freezing, and storage.

Functional properties of proteins and their derivatives are affected by intrinsic and extrinsic factors. The primary factors that affect protein functionalities are their amino acid composition and sequence, molecular weight, structure, food source, and classification such as sarcoplasmic proteins, myofibrillar proteins, and stroma proteins. Protein behavior in a food system is also dependent on extrinsic factors such as pH, temperature, solvent, ionic strength, and presence of other macronutrients including lipids and carbohydrates. The quality and stability of final food products are influenced by the ingredients added to improve functional properties (Onibala et al., 1997).

1.3.1. Solubility

Protein solubility is characterized as the dissolved protein content in a solution under specific conditions. It is affected by the amino acid composition and sequence, the conformation of amino acid sidechains, and the molecular weight of the protein. Protein hydrolysates produced from proteolysis have generally shown increased solubility compared to the intact protein. This is mainly due to the cleavage of large proteins into smaller peptide molecules and the increase in exposed polar groups available for interaction with water dipoles.

Environmental factors such as ionic strength, pH, solvent, temperature, and processing conditions also influence protein solubility. For example, changing the pH of a protein solution can impact solubility by inducing a change in the protein's net charge; subsequent electrostatic repulsion can increase protein solubility (Damodaran et al., 2007). According to Stefansson and Hultin (1994), myofibrillar proteins obtained from cod had high solubility when the ionic strength was less than 0.3

mM at both acidic and neutral pH. In addition, protein solubility is also dependent on temperature. Myofibrillar proteins started losing their solubility at 30-40 °C as they coagulated. At a temperature higher than 40 °C, protein denaturation is caused inducing a decrease of protein solubility, unfolding of proteins, and the formation of new electrostatic and hydrogen cross-linkages (Zayas, 2012).

In the development of functional protein ingredients, protein solubility is one of the vital functional properties determined first since it is an important physicochemical property that is associated with other functional properties such as emulsifying and gel forming activity (Hefnawy & Ramadan, 2011; Hayat et al, 2014). Also, evaluation of protein solubility is critical for the use of protein ingredients in food products since high solubility of functional proteins provides significantly expanded opportunities for potential food applications.

1.3.2. Emulsifying Activity

Emulsification is one of the most important processes in manufacturing formulated food products. Emulsification is defined as the process of dispersing one liquid in another immiscible liquid (e.g., oil and water) to form an emulsion (Tolve et al., 2016). Proteins act as emulsifiers and stabilizers of emulsion droplets by preventing structural changes such as coalescence, flocculation, creaming, or breaking (Zayas, 1997). The emulsifying properties of proteins are generally represented by two indices, namely emulsifying activity and emulsifying stability. The emulsifying activity is characterized as the amount of oil that can be emulsified per unit of protein, while the emulsifying stability represents the protein's ability to maintain the emulsion over a defined period of time (Boye et al., 2010).

Proteins with balanced hydrophilic and hydrophobic amino acids are able to form emulsions via a decrease in interfacial tension. The stabilization of emulsions in formulated foods such as dressings, mayonnaise, and sausages is necessary to prevent phase separation. The emulsifying activity and stability of proteins are affected by several factors such as protein source, type of proteins, concentration, solubility, molecular weight, temperature, pH, and ionic strength (Zayas, 1997). For

instance, myofibrillar proteins obtained from barracuda had decreased emulsifying activity as their concentration increased. However, emulsifying activity increased when the concentration of sarcoplasmic proteins was increased (Ramachandran et al., 2007). Only few studies have evaluated functional properties of proteins obtained from crustacean species. El-Beltagy and El-Sayed (2012) investigated emulsifying activity and stability of shrimp shell proteins recovered by pH shift processing. According to their results, acidic proteins showed a higher emulsification activity than alkaline proteins and their activity was correlated with protein solubility.

Some protein hydrolysates generated from fish muscle or by-products are also a good source of emulsifiers. Fish proteins from tuna (Nalinanon et al., 2011) and herring (Liceaga-Gesualdo & Li-Chen, 1999) showed improved emulsifying activity after hydrolysis due to their enhanced amphiphilic properties that enabled their absorption at the interphase between oil and water. Emulsifying activity of hydrolysates derived from shrimp muscle and crab shell was also evaluated. However, increasing the degree of hydrolysis by commercial enzyme treatment negatively influenced emulsifying activity and stability (Ketnawa et al. 2016; Jiang et al., 2017; Latorres et al., 2018). In order to possess high emulsifying activity, hydrolyzed proteins should contain at least 20 amino acids (Kristinsson & Rasco, 2000), therefore, a limited hydrolysis time is required to promote emulsifying activity.

1.3.3. Foaming Activity

Foams are defined as a two-phase system formed by a gas phase dispersed in a continuous liquid phase (Damodaran et al., 2007). In the foam formation process, air is diffused into the food system or protein solution by aeration, shaking, or stirring. Proteins can act as a soluble surfactant to decrease the surface tension of the continuous phase. According to Foegeding and Davis (2011), the foaming activity of protein depends on their ability to migrate to the interface of liquid and gas phases. Foaming stability is associated with the capacity of the protein to unfold at the interface and form a viscous interface by three-dimensional interactions with nearby protein molecules. The foaming activity

and stability are affected by several factors such as pH, protein concentration, type of proteins, and protein solubility (Damodaran et al., 2007). Myofibrillar proteins obtained from barracuda (*Sphyraena jello*) showed improved foaming activity and stability as the protein concentration was increased. In contrast, the sarcoplasmic proteins exhibited a lower foaming activity and stability than myofibrillar proteins (Ramachandran et al., 2007). A similar trend was observed with rohu (*Labeo rohita*) fish proteins (Mohan et al., 2006). Foaming activity of fish protein isolates from cod (*Gadus morhua*), herring (*Clupea harengus*), and salmon (*Salmo salar*) were comparable with soy protein isolate. Cod protein showed a higher foaming activity than salmon and herring proteins (Abdollahi & Undeland, 2018). In crustacean species, cooked whiteleg shrimp (*Penaeus vannamei*) hydrolyzed by giant catfish (*Pangasianodon gigas*) viscera proteases and commercial enzymes showed 52-100% foaming activity, which is comparable with the foaming activity of egg white (Ketnawa et al., 2016). Also, foaming activity and stability of raw white shrimp hydrolysates produced with Alcalase and Protamex proteases showed an increase in foaming activity at 10% degree of hydrolysis, indicating that high molecular weight peptides are more able to make a foam, since they enable the formation of a cohesive interfacial film capable of enveloping and retaining air (Latorres et al., 2018).

Foam stability is measured as the time required to lose 50% of the foam volume. Physical energy input that leads to flocculation, coalescence, or disproportion in air bubbles size can induce the destabilization of the foam. To avoid this, protein foaming agents are used with some polysaccharides such as pectins, guar, or xanthan gum to stabilize foams by increasing viscosity of the interface (Yada, 2017).

1.3.4. Gel Forming Activity

A gel is an intermediate status between a solid and a liquid. It is defined as “a substantially diluted cross-linked system that does not exhibit flow when in the steady-state” (Damodaran et al., 2007). It consists of polymers cross-linked via either covalent or noncovalent bonds, which form a

network that enables the entrapment of water. Gel forming activity is an important function in foods such as meat analog products, jellies, and puddings, and can also significantly affect their texture. Protein gel formation can be generated by heat, chemical, and enzyme treatments (Vasbinder et al., 2004; Nieto-Nieto et al., 2015; Tarhan et al., 2016). The gel forming ability of proteins can be described as the lowest protein concentration required to form a gel. There are many studies that have demonstrated the importance of myofibrillar proteins for gelation of seafood proteins (Yongsawatdigul & Park, 2004; Riebroy et al., 2008; Sun & Holley, 2011). Myofibrillar proteins are generally long and include myosin, actin, and regulatory proteins such as tropomyosin, troponin, and actinin. Myosin comprises 43-45 % of myofibrillar proteins in the muscle of mammals and fish, which form good quality gels (Sun & Holley, 2011). Also, it has been reported that actin enhanced the gel forming activity of myosin (Sun & Holley, 2011).

There are abundant studies demonstrating gel forming activity of finfish protein isolates. Recovered proteins from catfish, rockfish (*Sebastes flavidus*), bighead carp (*Aristichthys nobilis*), bigeye snapper (*Priacanthus tayenus*), and herring by the pH shift processing method showed decent gel forming activity and stability (Undeland et al., 2002; Chen et al., 2009; Chang et al., 2016; Panpipat & Chaijan 2017; Tan et al., 2019). In addition, gels made with alkali-extracted krill protein isolate exhibited a thermo-reversible characteristic under repeating heating/cooling cycles. Moreover, krill protein isolates could provide high quality gels without the addition of salt, unlike conventional surimi. Heat-induced gelation involves the induction of protein unfolding by denaturation. This leads to the exposure of hydrophobic amino acid residues. The unfolded molecules are rearranged and aggregated by disulfide bridges, hydrogen bonds, hydrophobic, and/or van der Waals interactions (Clark et al., 2001). The heat-induced gelation of proteins is affected by various factors including pH, type and source of muscle protein, molecular weight, temperature, protein concentration, and ionic strength (Sun & Holley, 2011).

1.3.5. Antioxidant Activity

Oxidative reactions are major contributors to quality deterioration in foods and lead to undesirable color, flavor, odor, and textural attributes (Estévez et al., 2005). The main targets of oxidative reactions that negatively affect foods are lipids. Oxidation of food lipids is a highly complex process that is dependent on various factors such as light, temperature, the proportion of polyunsaturated fatty acids (PUFA), the concentration of phospholipids, the composition of phospholipids, haem pigments, enzymes, the concentration of oxygen in the surrounding atmosphere, metal ions such as copper and iron, and mechanical processes (Biswas et al., 2012). The lipid oxidation process typically involves a free radical pathway (Figure 1.2). More specifically, in the initial stage of lipid oxidation, a free radical (R^*) is formed by removing an electron (H) from a fatty acid (usually a methylene group) by light, irradiation, or metals. During the propagation stage, this highly reactive free radical (R^*) removes a hydrogen from another fatty acid turning it into a free radical. The free radical is changed to a peroxy radical (ROO^*) by oxygen addition, and then the peroxy radical (ROO^*) can remove hydrogen (H) from another methylene group (RH). As a result, a hydroperoxide ($ROOH$) is formed, and a new free radical (R^*) is generated. This step is a chain reaction indicating that the reactive free radical reacts further and continuously produces oxidation products such as hydroperoxides, aldehydes, carboxylic acids, and alcohols (Fernandez et al., 1997; Laguerre et al., 2007). These oxidation products not only diminish quality attributes but can also decrease the shelf-life, nutritional quality, and safety of foods. The level of lipid oxidation can be indirectly determined by measuring oxidation products such as peroxides, thiobarbituric acid reactive substances (TBARS), and conjugated dienes (Laguerre et al., 2007).

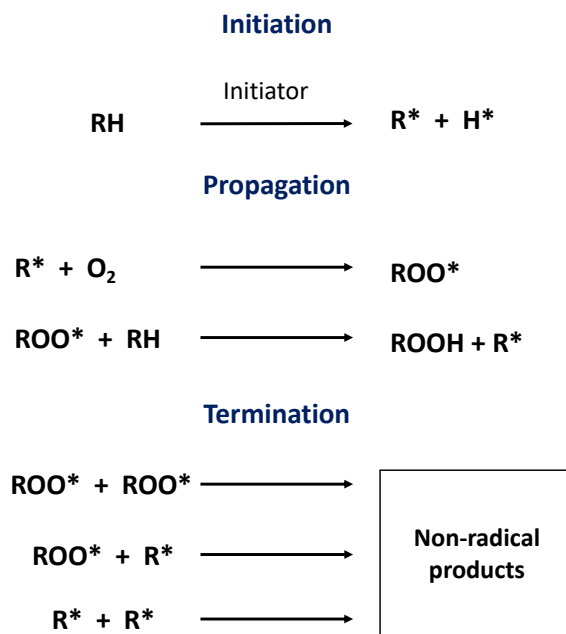


Figure 1.2. Mechanism of free radical lipid oxidation

In order to minimize the deterioration of food quality by lipid oxidation, antioxidant agents have been used for many decades. Antioxidant agents delay, control, and inhibit oxidative reactions as hydrogen donors or chelators. As hydrogen donors, antioxidants terminate the chain reaction of lipid oxidation by accepting free radicals. In the food industry, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) are commonly used as hydrogen donors. Ethylenediaminetetraacetic acid (EDTA) and organic acids are representative chelators that delay the onset of oxidation and slow the oxidation rate by chelating transition elements such as iron and copper that are able to contribute to producing free radicals (Shahidi, 2015). However, contemporary consumers' interests have focused on foods containing natural ingredients due to the potential toxicity and carcinogenic effects of some synthetic chemical antioxidants (Wang et al., 2013).

Many studies have shown that proteins and peptides obtained from food sources can be used as alternative antioxidant agents in food products (Sakanaka & Tachibana, 2006; Harnedy & FitzGerald,

2012; Nikoo et al., 2014). For instance, whey protein concentrate showed inhibitory activity against lipid oxidation in cooked beef (Shantha & Decker, 1995) and 2% of whey and soy proteins showed antioxidant activity in cooked pork patties (Peña-Ramos & Xiong, 2003). Peptides derived from seafood have also demonstrated the ability to inhibit oxidative reactions in food systems. Mahi mahi (*Coryphaena hippurus*) red muscle that was dipped in tilapia (*Oreochromis niloticus*) protein hydrolysates for 2 min showed a decreased peroxide value and TBARS over 90 h storage time (Dekkers et al., 2011). In addition, hydrolysates generated from pollock (*Theragra chalcogramma*) skin, silver carp (*Hypophthalmichthys molitrix*) muscle, amur sturgeon (*Acipenser schrenckii*) skin, and skipjack tuna (*Katsuwonus pelamis*) roe showed antioxidative activity in food models such as fish fillet, mince, and sausage (Sathivel, Huang, & Bechtel, 2008; Qiu, Chen, & Dong, 2014; Intarasirisawat et al., 2014; Nikoo et al., 2014; Nikoo et al., 2015). These functional peptides act as free radical scavengers and the majority of antioxidative peptides were relatively low molecular weight peptides ranging from 200-1800 Da (Wang, et al., 2013; Jiang et al., 2014; Aluko, 2015). Studies on the antioxidant activity of crustaceans including shrimp and crab have been focused on protein hydrolysates obtained from their byproducts (Khumallambam et al., 2011; Sila et al., 2014; Sowmya et al., 2014; Antunes-Valcareggi et al., 2017). Although studies have shown improved *in vitro* antioxidant activity after enzymatic hydrolysis of shell waste and other byproducts, no studies have reported on the antioxidant activity of crustacean hydrolysates in food models.

1.4. Bioactivities of Peptides

Bioactive peptides are unique protein fragments that not only act as sources of amino acids but also have various physiological functions that maintain human health. These peptides do not show any biological activity within the sequence of the parent protein. However, they become active in various physiological functions after being released from the protein by enzymatic hydrolysis during processing or digestion. Recent research has indicated that some peptides generated from dairy products, plants,

land animals, and seafood have a variety of bioactivities. For example, peptides derived from milk, salmon, egg yolk, and bean showed anti-hypertensive, anti-diabetic, or antioxidant activities (López-Fandiño et al., 2006; Li-Chan et al., 2012; Boutrou et al., 2013; Lacroix & Li-Chan, 2013). Further, some of these peptides show multifunctional properties; having more than one functionality and bioactivity in a food formula (Balti et al., 2015; Zambrowicz et al., 2015).

1.4.1. Oxidative Stress/Inflammation and Anti-Inflammatory Peptides

The top 10 non-infectious diseases causing the most deaths worldwide include chronic diseases such as heart disease, diabetes mellitus, cancer, and Alzheimer's disease (WHO, 2018). Chronic inflammation is significantly involved in the onset and development of these chronic diseases. Inflammation is a biological defense system that activates pro-inflammatory mediators such as tumor necrosis factor α (TNF- α), interleukin (IL)- 1, IL-6, and IL-8 to the infected site or injured tissue. Then, these mediators amplify the inflammatory response via cell signaling. However, excessive production of these mediators from immune cells results in tissue damage and abnormal immune function. There are two types of inflammatory response: acute and chronic. It has been reported that chronic inflammatory responses are related with diseases such as cancer, diabetes, cardiovascular disease, and Alzheimer's disease (Sies, 1997; Emerit et al., 2004; Valko et al., 2007; Cataldi, 2010; Mohamed, 2014). Therefore, alleviation of inflammation is very important to prevent and manage chronic disease. In order to reduce inflammation, systematic anti-inflammatory pharmaceutical products such as steroidal anti-inflammatory drugs (corticosteroids) and non-steroidal anti-inflammatory drugs (NSAID's) are commonly used, however, their side-effects including headaches, stomach pain, heartburn, stomach ulcers, dizziness, and liver or kidney problems remain as challenges to be solved (FDA, 2016). As an alternative to inflammation prevention and alleviation, bioactive peptides derived from food proteins could be considerable natural and nutraceutical products with minimal or no side effects.

Reactive oxygen species (ROS) are generated by living organisms under normal physiological conditions of the mitochondrial electron transport chain. ROS are unavoidable oxidants in the body, and they are produced as byproducts of the electron transport chain. ROS include superoxide (O⁻), peroxynitrite (ONOO⁻), hydrogen peroxide (H₂O₂), and nitric oxide (NO[·]). When superoxide and nitric oxide react with each other, the highly reactive peroxynitrite is generated, which accelerates peroxidation and protein nitration as well as lipid oxidation (Griendling & FitzGerald, 2003). Under normal conditions, oxidants are removed by various antioxidants. For example, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) are enzymatic antioxidants that function to suppress the formation of free radicals or ROS in cells (Birben et al., 2012). As non-enzymatic antioxidants, vitamin A, vitamin C, vitamin E, and β-carotene scavenge free radicals by donating an electron to them, therefore, they inhibit initiation and propagation of oxidative reaction (Birben et al., 2012). Oxidative stress is defined as imbalance between oxidation and antioxidants (Ighodaro & Akinloye, 2018). Under a chronic state of oxidative stress, oxidants can be excessively produced (Sies, 1997). Overproduction of oxidants can lead to physiological mutations, resulting in chronic diseases (Sies, 1997; Siti et al., 2015).

Oxidative stress and infection can share the same toll-like receptor 4 (TLR 4) signaling pathway (Gill et al., 2010). Once an inflammation inducer such as lipopolysaccharides (LPS) binds to the TLR 4 of macrophages, signal transduction is activated. As a result, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is released as a transcription factor. Then, the NF-κB activates the genes related to the transcription of inflammatory mediators such as cytokines including interleukins and tumor necrosis factor (TNF-α), and prostaglandins (PGs), and inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenases (COXs). The TLR signaling pathway also acts as a trigger of ROS formation and enhances ROS production (Friedman & Hughes, 2002; Proell et al., 2008; Rock & Kono, 2008; Kumar et al., 2012). Overproduction of inflammatory mediators and ROS can contribute to

the development of chronic diseases. Therefore, finding a strategy to prevent and reduce inflammation before it is further developed into a chronic disease is critically important.

Many recent studies indicate that bioactive peptides derived from dietary proteins can reduce oxidative stress by scavenging free radicals and alleviate inflammation by inhibiting inflammatory mediators and related enzymes. Peptides derived from hydrolysis of salmon, bean, camel milk, and egg exhibited anti-inflammatory activity in endothelial cells (Huang et al., 2010; Ahn, Je, & Cho, 2012a; Majumder, Chakrabarti, Davidge, & Wu, 2013; Vo & Kim, 2013). Blue mussel peptides obtained from enzymatic hydrolysis showed anti-inflammatory activity in a cell model. The peptides of >5 kDa inhibited nitric oxide production in LPS-induced RAW264.7 macrophages. Pretreatment with the >5 kDa peptide fraction markedly inhibited LPS-stimulated pro-inflammatory cytokines, iNOS, and COX2 protein (Kim, Ahn, & Je, 2016). These findings imply that various food-derived peptides could be applicable to the control of inflammation for human health.

1.4.2. Diabetes Mellitus and Anti-Hyperglycemic Peptides

Diabetes is a type of diseases caused by high levels of blood glucose resulting from defects in insulin production, insulin action, or both (CDC, 2020a). About 90% to 95% of all diagnosed cases of diabetes are in adults (CDC, 2020a). At least 366 million people worldwide have diabetes and this figure is likely to double by 2030 (WHO, 2004). In the United States, in 2018, 34.2 million people (10.5 % of American adults) had diabetes and by 2050 this figure is expected to increase by up to 33%, or one-third of all American adults (CDC, 2020a). In order to manage diabetes, Americans spent \$327 billion in 2017, and this figure is expected to rise rapidly based on the CDC's latest estimates (CDC, 2020b). In 2017, diabetes was the seventh leading cause of death in the United States with 83,564 deaths (CDC, 2020a).

Diabetes is classified into two main types, which are type 1 and type 2 diabetes. Type 2 diabetes is characterized by hyperglycemia, impaired insulin action, or insulin deficiency. An early abnormality of type 2 diabetes is insulin resistance characterized by insulin defect. Insulin resistance is a condition in

which the body secretes normal or elevated insulin levels but fails to use the insulin properly due to impaired insulin sensitivity. Insulin resistance leads to fat, muscle and liver cells not functioning effectively, thus decreasing glucose uptake and glycogen synthesis, and increasing intestinal glucose absorption (Cline et al., 1999). Further, insulin resistance leads to metabolic syndrome, which is a group of medical conditions associated with obesity that puts people at high risk for both cardiovascular disease and type 2 diabetes (Gungor et al., 2005). Prediabetes is a condition in which individuals have blood glucose levels higher than normal but not high enough to be classified as diabetes. Prediabetic people have an increased risk of developing type 2 diabetes, heart disease, and stroke (Coutinho et al., 1999; Meigs et al., 2002; Smith et al., 2002).

According to the Diabetes Prevention Program, a large prevention study on people at high risk for diabetes, lifestyle intervention reduced the development of diabetes by 58% during a 3-year period (Diabetes Prevention Program Research Group, 2003). A similar study found that lifestyle interventions to prevent or delay type 2 diabetes in individuals with prediabetes is feasible and more cost-effective than medications (Herman et al., 2008). It is important to recognize that optimal diet-induced prevention of type 2 diabetes onset could be an effective strategy to reduce the expected increases in morbidity and cost associated with diabetes treatment (Diabetes Prevention Program Research Group, 2003).

High blood glucose (hyperglycemia) is a symptom of diabetes. The major source of blood glucose is dietary carbohydrates that are hydrolyzed by pancreatic α -amylase, followed by α -glucosidase before being absorbed in the small intestine (Elsenhans & Caspary, 1987). Inhibition of carbohydrate hydrolyzing enzymes using α -glucosidase inhibitors such as acarbose, results in the reduction of elevated postprandial blood glucose levels and is an established strategy for managing type 2 diabetes (Krentz & Bailey, 2005). Additionally, inhibition of dipeptidyl peptidase IV (DPP-IV) to prevent the inactivation of glucagon-like peptide-1 (GLP-1), and stimulation of GLP-1 secretion could be another approach to

manage type-2 diabetes. GLP-1 is a metabolic hormone that has effects on glucose-mediated insulin secretion and insulin gene expression, however, it is rapidly inactivated by DPP-IV. Therefore, DPP-IV inhibition and GLP-1 secretion are applicable for the management of type 2 diabetes (Drucker, 2007). This strategy was demonstrated as effective by studies in diabetes patients in which a DPP-IV inhibitor showed improved metabolic control with reduced fasting and postprandial glucose levels (Ahrén et al., 2002), and the stimulation of GLP-1 secretion lowered plasma glucose levels (Toft-Nielsen et al., 2001).

Recent studies have showed that peptides derived from food proteins can act as natural inhibitors of α -glucosidase and DPP-IV with milder inhibitory activity compared to medications, which can be used to manage postprandial hyperglycemia with minimal side effects (Li-Chan et al., 2012; Lacroix & Li-Chan, 2012, 2013; Nongonierma et al., 2017). For instance, whey protein and β -lactoglobulin hydrolysates exhibited α -glucosidase and DPP-IV inhibitory activity having half maximal inhibitory concentrations (IC_{50}) of 3.5-4.5 mg/mL (Lacroix & Li-Chan, 2013). In addition, peptides derived from Atlantic salmon skin (Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala) by proteolysis had potential as a DPP-IV inhibitor (Li-Chan et al., 2012). Di- and tri-peptides obtained from blue whiting hydrolysis showed significant metabolic effects relevant to glucose control in a mouse model by stimulating GLP-1 release and inhibiting DPP-IV, and the anti-diabetic effects were not significantly changed after passing through a simulated gastrointestinal system (Harnedy et al., 2018).

1.5. Potential Methods to Obtain Functional and Bioactive Proteins/Peptides

Two common techniques used to obtain functional and bioactive proteins from underutilized proteinaceous raw materials include: 1) isoelectric solubilization/precipitation (ISP) and 2) enzymatic hydrolysis by proteases. The ISP technique is more widely used in the food industry than enzymatic hydrolysis for the recovery of proteins that have various functional characteristics since the hydrolysis technique can result in inconsistent functional properties depending on the processing parameters. However, enzymatic hydrolysis is commonly used to produce bioactive peptides, which need to be

released from the primary structure of food proteins, where they are bonded to other amino acids. This section focuses on the production of functional and bioactive proteins/peptides by both ISP processing and enzymatic hydrolysis.

1.5.1. Isoelectric Solubilization/Precipitation (ISP)

ISP processing is significantly related to the solubility of proteins at various pH levels. The solubility change of muscle proteins depending on pH was discovered by Meinke et al. (1972) as well as Meinke & Mattil (1973); however, separation of fish muscle proteins and lipids using ISP was proposed by Hultin and Kelleher (1999, 2000).

At the isoelectric point (pI), the net electrostatic charge of proteins becomes zero, and fish muscle proteins are precipitated since protein-water interactions of fish muscle proteins are minimized while protein-protein interaction via hydrophobic bonds is increased. However, the electrostatic charges of protein side chains can be different depending on the pH (Figure 1.3). For example, the addition of acid to a protein dispersion increases the positive charges on the proteins. Conversely, when sufficient base is added to a protein dispersion, the protein becomes negatively charged. The accumulated net positive or negative charge maximizes protein-water interactions and results in proteins' solubilization (Gehring et al., 2009).

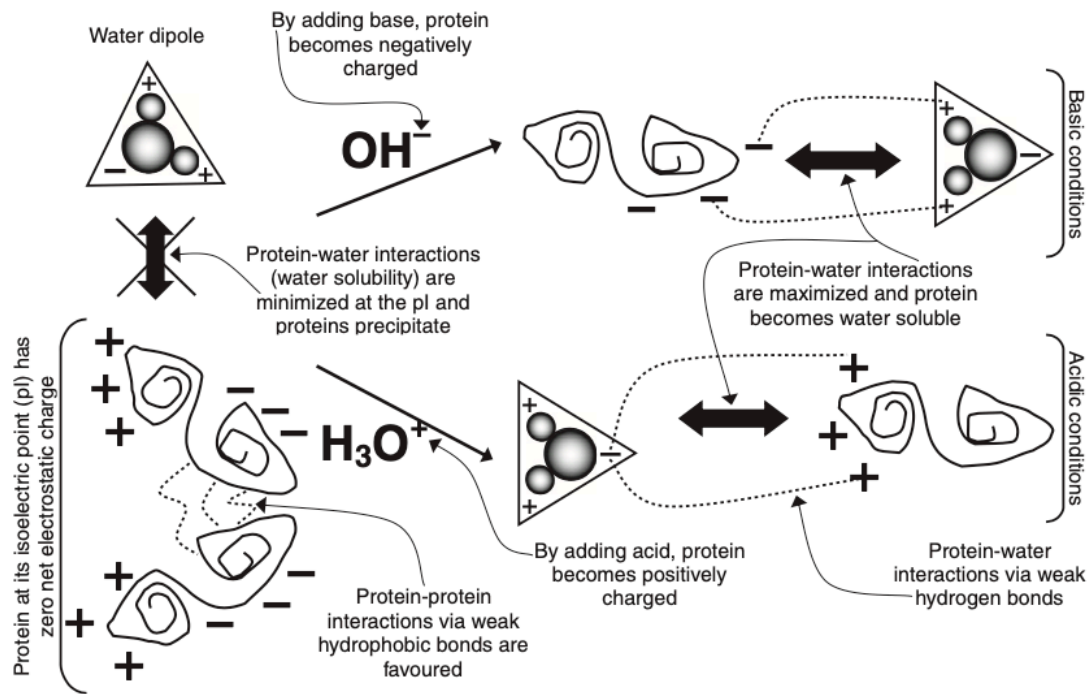


Figure 1.3. The biochemical principle of ISP processing [Source: Adapted from Gehring et al. 2009]

ISP processing is based on protein solubility and insolubility in water depending on the pH shift. During typical ISP processing, meat processing by-products are homogenized with water, and proteins are solubilized at either acidic or alkaline pH. Then, lipids and other insoluble materials including stromal proteins, bone, and skin are separated from the solubilized meat proteins through centrifugation. After the centrifugation, lipids are typically collected from the top layer, and the insoluble materials are separated by sedimentation in the bottom layer. The solubilized meat proteins in the middle layer are collected, and the pH is shifted to the pI (typically pH 5.5 for fish muscle proteins) of the meat proteins. At the pI, the protein solubility becomes poor; therefore, the proteins are precipitated and are typically collected by centrifugation (Figure 1.4).

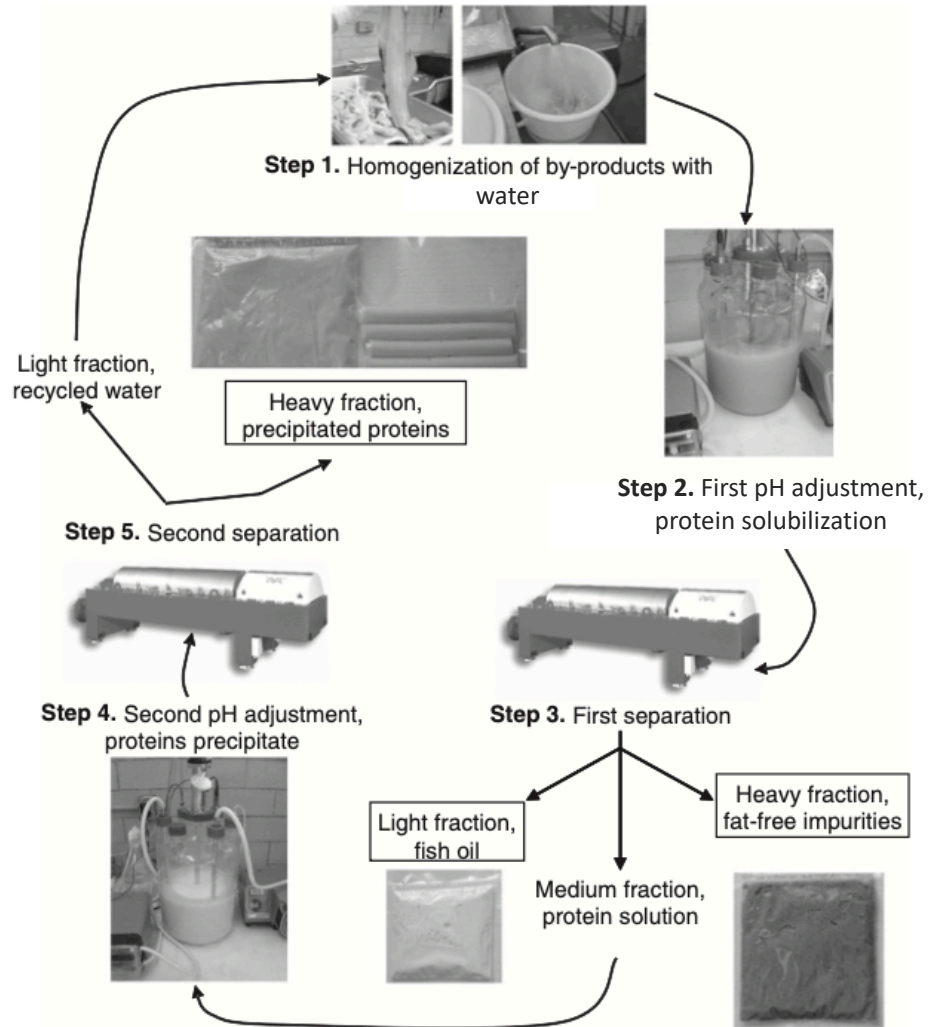


Figure 1.4. Diagram of ISP processing of fish processing by-product [Tahergorabi et al., 2011]

One of the advantages of ISP processing is a mild reduction of microbial growth through protein solubilization at very acidic or alkaline condition (Lansdowne et al., 2009a, 2009b). Also, ISP processing enables the continuous recycling of the processing water (Torres et al., 2007) and can be applied to recover functional proteins from low-value aquatic animals or processing by-products. Many studies have demonstrated that functional muscle proteins can be recovered from aquatic species (Undeland et al., 2002; Kristinsson & Liang 2006; Chen & Jaczynski, 2007; Mireles DeWitt et al., 2007; Rawdkuen et al., 2009). According to Panpipat and Chaijan (2017), protein isolates recovered from bigeye snapper head

by-product via solubilization at alkaline pH had a superior salt-solubility and gel forming ability while the acid protein isolate showed improved emulsifying activity. Also, scallop gonad protein isolates showed high solubility and foaming activity with high emulsifying activity (Han et al., 2019). The ISP process can be used to recover proteins containing essential amino acids and having high nutritional quality (Chen et al., 2009). Additionally, lipids and protein can be simultaneously isolated by the ISP process. Therefore, the ISP process is a useful technology to up-cycle the underutilized meat and processing by-products, and recovered proteins have the potential to be utilized as functional and nutritional ingredients in food products.

1.5.2. Protein hydrolysis (Proteolysis)

Proteolysis is a process widely used to develop bioactive and functional proteins/peptides from underutilized dietary proteins or meat processing by-products. This enzymatic process is catalyzed by proteases that cleave the peptide bonds between adjacent amino acids in proteins (Figure 1.5). A protease can be categorized based on its pattern of proteolysis as an exopeptidase, endopeptidase, or both exopeptidase and endopeptidase. Exopeptidases catalyze the removal of amino acids (or short peptides) from the terminal polypeptide bond; thus, single amino acids or dipeptides are released from proteins. On the other hand, endopeptidases cleave peptide bonds within a polypeptide or protein. Therefore, continuous proteolysis cleaves proteins into smaller molecular weight fragments such as peptides, peptones, and free amino acids (Donohue & Osna, 2003).

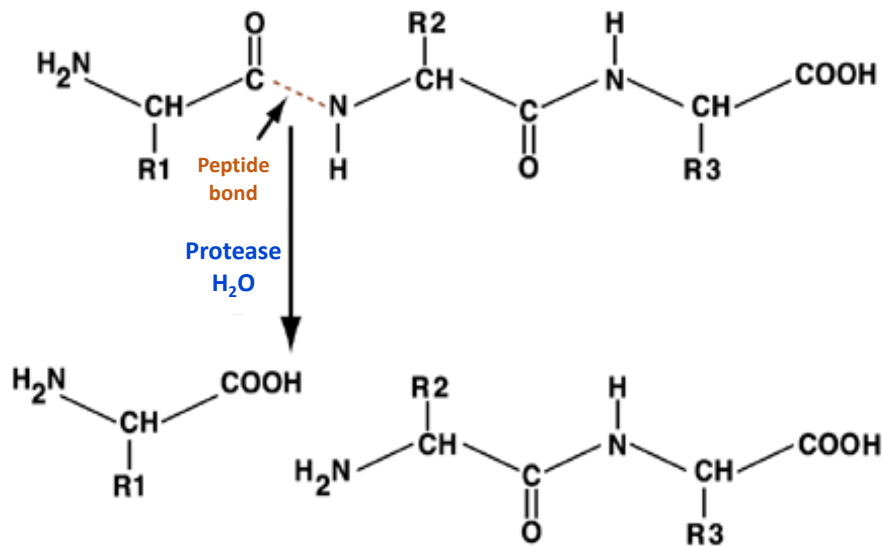


Figure 1.5. Scheme of enzymatic hydrolysis by protease [Modified from Donohue & Osna, 2003]

In order to produce functional/nutraceutical peptides, typical protein hydrolysis includes the following steps (Figure 1.6). First, the raw material is ground and homogenized with water. Next, the pH and temperature are adjusted using acid or base to the optimum conditions for the employed protease. After the enzyme addition, the enzymatic reaction is allowed to proceed for the target period. Subsequently, the enzyme is typically inactivated by heat treatment. Finally, the hydrolyzed material containing functional and bioactive peptides is collected through centrifugation or fractionation and subsequently stabilized by drying (Hajfathalian et al., 2018).

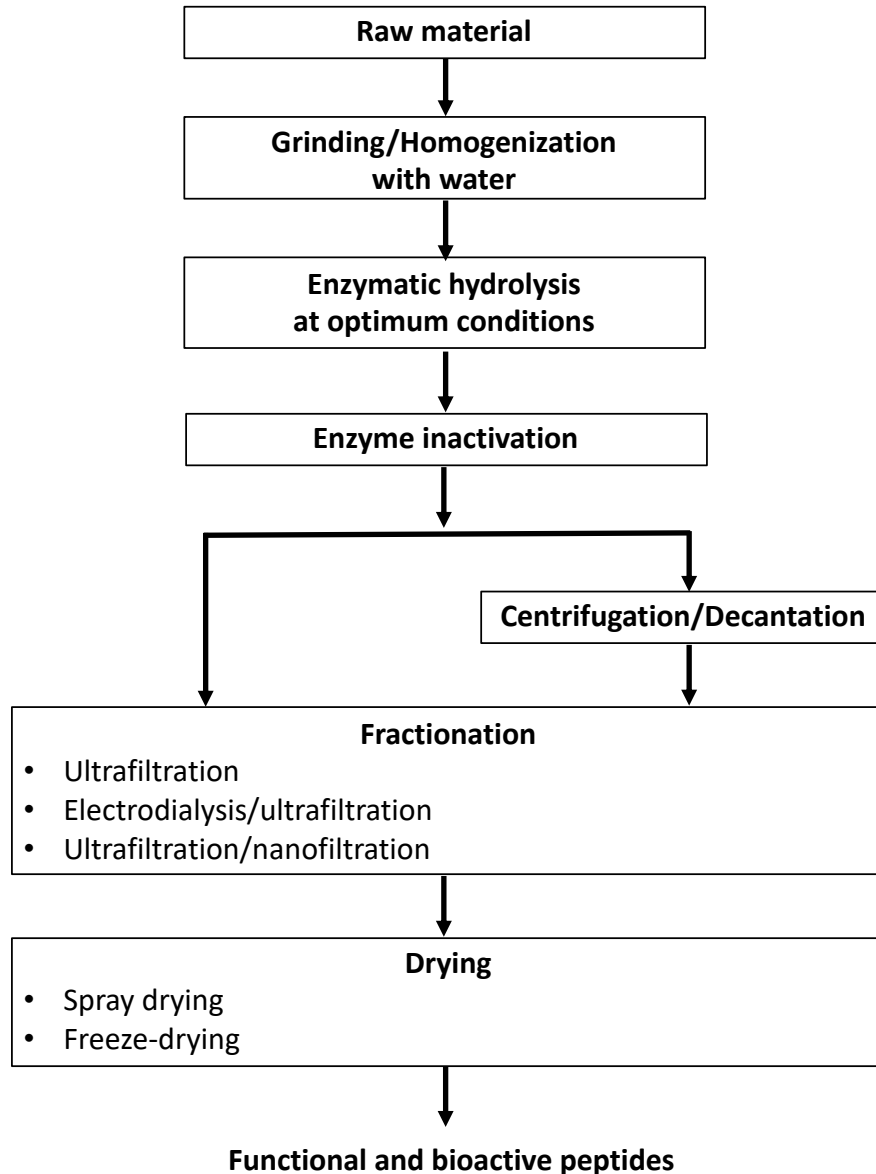


Figure 1.6. Flow diagram of protein hydrolysis using protease

The type of functional or bioactive peptides generated from enzymatic hydrolysis is dependent on several factors: 1) protein source, 2) specificity of the enzymes used, and 3) processing parameters such as temperature, pH, hydrolysis time, and the ratio of enzyme to substrate (Kristinsson, 2006). The food industry uses a wide variety of commercially available enzymes with varying characteristics (Table 1.2). The selection of appropriate protein source and enzyme type is very critical since those factors

remarkably affect the sequence and structure of produced peptides, which decide their functional and bioactive properties (López-Expósito & Recio, 2006; López-Fandiño et al., 2006). Processing conditions also influence the degree of hydrolysis (DH), a measure of the percentage of peptide bonds cleaved. Many studies have demonstrated that DH affects the functionality and bioactivity of peptides (Jamdar et al., 2010). Therefore, DH during enzymatic hydrolysis must be controlled to avoid excessive hydrolysis and to produce consistent functional and bioactive peptides.

Table 1.2. Commercially available proteases in the food industry

Enzyme	Origin	Specificity	Optimum pH	Optimum temperature (°C)
Alcalase	<i>Bacillus licheniformis</i>	Serine endopeptidase (mainly subtilisin A)	7-9	30-65
Flavourzyme	<i>Aspergillus oryzae</i>	Endo- and exo-peptidases (broad specificity)	5-7	50-55
Protamex	<i>Bacillus</i> spp.	Endopeptidase (broad specificity)	5.5-7.5	35-60
Papain	Papaya (<i>Carica papaya</i>) latex	Endopeptidase (broad specificity)	5.0-9.0	60-70
Neutrase	<i>Bacillus amyloliquefaciens</i>	Endopeptidase, metalloprotease	5.5-7.5	30-55
Bromelain	Pineapple fruit or stem	Cysteine endopeptidase (broad specificity)	6.0-8.5	50-60
Pepsin	Stomach	Endopeptidase, Leu, Phe, Trp or Tyr, unless preceded by Pro	1.5-2.5	37-42
Trypsin	Intestinal mucosa	Endopeptidase, Arg or Lys, unless followed by Pro.	8.0	37-42
Pancreatin	Pancreas	Broad specificity	8.0	37-42
α -Chymotrypsin	Intestinal mucosa	Endopeptidase, Phe, Trp, or Tyr, unless followed by Pro. Cuts more slowly after His, Met or Leu	7.8-8.0	37-42

Investigations on nutraceutical peptides obtained by enzymatic hydrolysis have been actively conducted for several decades. Briefly, some hydrolysates generated from dietary proteins and meat processing by-products have been shown to positively affect cardiovascular and gastrointestinal systems with anti-hypertensive, anti-diabetic, antithrombotic, anti-obesity, and prebiotic effects (Cudennec et al., 2008; Jung & Kim, 2009; Lee et al., 2010; Zhang et al., 2011; Prasanna et al., 2012; Boutrou et al., 2013; Balti et al., 2015; Oseguera-Toledo et al., 2015; Zambrowicz et al., 2015). Moreover, protein hydrolysis has been used to produce bioactive peptides having potential anti-inflammatory, antioxidant, antifungal, antimicrobial, antiviral, and anticancer activities (Lee & Maruyama, 1998; Osaki et al., 1999; Padhi & Verghese, 2008; Hsu et al., 2011; Juillerat-Jeanneret et al., 2011; Vo & Kim, 2013; Opheim et al., 2015).

On the other hand, the functional properties of proteins and peptides produced from enzymatic hydrolysis showed variable results in many studies. In general, the solubility of hydrolysates was increased due to an increase of low molecular weight peptides and ionic groups (Mutilangi et al., 1996), and antioxidant activity of hydrolysates was also improved after enzymatic hydrolysis (Samaranayaka & Li-Chan, 2011; Sila & Bougatef, 2016). However, regarding the emulsifying activity of hydrolysates, some studies showed decreased emulsifying activity for protein hydrolysates as compared to the untreated proteins (Turgeon et al., 1991; Balti et al., 2010). Conversely, some studies reported increased emulsifying activity of hydrolysates compared to untreated proteins (Taheri et al., 2014; Mune, 2015; Zou et al., 2016). Variable impacts of proteolysis on foaming activity of hydrolysates have also been reported. Some studies showed improved foaming activity (Souissi et al., 2007; Mune, 2015; Zou et al., 2016) whereas others showed that hydrolysates had decreased emulsifying activity (Balti et al., 2010; Mune, 2015) compared to that of the native protein. The decreased emulsifying and foaming activities were attributed to excessive hydrolysis, therefore, limited enzymatic hydrolysis is required to generate functional peptides.

Enzymatic hydrolysis has been actively applied to marine resources to improve their bioactive and functional properties. In shellfish, protein hydrolysates derived from clam, krill, mussel, oyster, and shrimp were demonstrated to have potential antihypertensive, anti-inflammatory, antioxidant, or antimicrobial effects (Liu et al., 2008; Tsai et al., 2008; Wang et al., 2008; Manni et al., 2010; Kim et al., 2016). Crab hydrolysates obtained from enzymatic hydrolysis were also reported to have antioxidant and anticancer activities, however, the studies were mainly focused on crab byproducts (Doyen et al., 2011; Antunes-Valcareggi et al., 2017; Jiang et al., 2017).

1.6. Research Needs

Despite the large population of invasive green crabs and their detrimental effects on fisheries and aquaculture, they are not commercially utilized in the U.S. as a human food resource. Obtaining lump crab meat from such a small crustacean would be very labor intensive and likely not economically viable. Mechanical separation using a piece of equipment known as a “deboner” is an efficient way to obtain raw or cooked minced meat from green crab, however, the mince has an unattractive appearance and slurry-like texture. Therefore, the development of value-added products from green crabs could be a strategy to generate economic value from this resource. Previous research has demonstrated that the ISP technique can be successfully applied to recover functional proteins from various marine animal resources and their byproducts. Also, many studies indicate that recovered proteins’ functional properties are dependent on the solubilization pH and protein source. However, there have been only limited reports on the application of the ISP technique to raw crabs and their byproducts. Moreover, there are no studies on the functionality evaluation of crab proteins recovered by ISP processing. Research is required to determine whether ISP processing of green crabs can be utilized to recover and improve the functional properties of green crab proteins.

Enzymatic hydrolysis is one of the commonly used techniques to improve the functional and bioactive properties of proteins. However, most studies have applied the hydrolysis technique to dairy

proteins and finfish products. Fewer studies have been conducted to evaluate the potential of enzymatic hydrolysis in deriving functional and bioactive peptides from shellfish proteins. Moreover, the majority of studies about the bioactivity of hydrolysates obtained from marine resources were primarily focused on their anti-hypertensive and antioxidant activities rather than their anti-diabetic properties. Research in this area is necessary to investigate the role of green crab hydrolysates as health promoting ingredients.

In the application of bioactive peptide ingredients to food products, the stability of the bioactivity is critical since the peptides could be further hydrolyzed by digestive enzymes and lose their activity during human digestion. Although there are reports that simulated gastrointestinal digestion can modify the bioactivities of peptides, there is a lack of information about bioactivity changes in crustacean hydrolysates after human digestion. It is essential to understand the impacts of digestion on bioactivity to demonstrate the stability of bioactive peptides obtained from green crab.

1.7. Objectives

The overall goal of this research was to develop multifunctional value-added protein ingredients from currently unutilized green crabs as a strategy to obtain economic benefits and potentially reduce their negative impacts on the marine ecosystem and shellfish aquaculture.

The specific objectives were as follows:

Objective 1: To investigate the application of the ISP method to obtain functional protein ingredients from green crab mince.

Objective 2: To evaluate the application of enzymatic hydrolysis to derive anti-inflammatory and anti-diabetic compounds from green crab mince, and to examine potential bioactivity changes in green crab protein hydrolysate after simulated human digestion.

Objective 3: To determine whether protein hydrolysis using commercially available enzymes improves the functional properties of green crab mince.

CHAPTER 2

POTENTIAL OF RECOVERED PROTEINS FROM INVASIVE GREEN CRABS (*CARCINUS MAENAS L.*) AS A FUNCTIONAL FOOD INGREDIENT

This chapter was published in Journal of the Science of Food and Agriculture and edited according to the dissertation format (Kang et al., 2019).

2.1 Chapter Abstract

Invasive green crabs contain high-quality proteins that have potential as functional ingredients in formulated foods. This study evaluated the functional properties and compositional characteristics of green crab proteins recovered by isoelectric solubilization/precipitation (ISP). Mechanically separated green crab mince (control) was solubilized at pH 2 (PP2) and pH 10 (PP10), then proteins were precipitated at pH 5.5 and subsequently freeze-dried. Yield of recovered protein powder was approximately 1.5 times higher for PP2 than for PP10. Compared with the control (230 g kg⁻¹), ash content was reduced in PP2 (54 g kg⁻¹) and PP10 (23 g kg⁻¹) samples. PP2 contained predominantly large-molecular-weight proteins, while small-molecular-weight proteins were distributed in PP10. With regard to functional properties, at pH 7 and 8, solubility of PP10 was significantly higher than that of PP2. At pH 7.5, PP10 exhibited significantly higher emulsifying activity (1482 m² g⁻¹) than PP2 (858 m² g⁻¹) and the control (958 m² g⁻¹). PP2 showed statistically higher gelation activity and had higher L* value than PP10 and the control. The results indicate that recovered green crab proteins have functional properties potentially useful for formulated foods, and that these functional properties can be modified by the solubilization pH during the recovery process.

2.2 Introduction

Green crabs (*Carcinus maenas L.*) are an invasive predatory species whose populations have increased dramatically along the U.S., Japanese and South African coasts (Leignel et al., 2014). The crabs consume commercially important marine resources such as scallops, lobsters and oysters not only in

North America and South Africa but in Europe as well (Sigurdsson et al., 2013; Leignel et al., 2014). Their burrowing activity destroys estuarine habitats, and their expansion has negatively influenced marine ecosystems, fisheries and aquaculture (Malyshev & Quijón, 2011). Increasing the commercial utilization of green crabs has the potential to reduce their detrimental effects on marine ecosystems. Green crabs have a carapace width typically between 5 and 80 mm, much smaller than Dungeness (>190 mm) and Jonah (>222 mm) crabs that have considerable economic value (Grosholz & Ruiz, 1996; Swiney et al., 2003). Various small crab species are popular in parts of Europe and in Asia, where they are generally consumed in whole form (Maine Sea Grant, 2017). However, green crabs are not harvested commercially in North America, primarily owing to their small size, which makes it difficult to efficiently process their meat.

Although their size causes a challenge in utilizing them as a human food, the shell and minced meat of green crabs have potential for utilization by the food industry. The crustacean exoskeleton contains chitin, which has a wide range of applications in industries such as food and agriculture, cosmetics, nutraceuticals, pharmaceuticals and textiles (Varshosaz et al., 2006; Kim & Thomas, 2007; Gao & Cranston, 2008; Morganti & Li, 2015; Boonlertnirun et al., 2017;). Many applications for crab shell derivatives have been reported in the scientific literature, whereas there are a limited number of studies on minced crab meat and its potential application in food products. Galetti et al. (2017) reported that cooked green crab was successfully processed by a mechanical separator to produce minced meat, which was then incorporated into a savory stuffed pastry. Green crab meat was reported to contain 806–835 g kg⁻¹ protein, 36–48 g kg⁻¹ lipid, 22 g kg⁻¹ total minerals and 0.051–0.192 g kg⁻¹ carotenoid on a dry weight basis and to provide nutritionally important amino acids and mono- and polyunsaturated fatty acids (Skonberg & Perkins, 2002; Naczek et al., 2004). In addition to their nutritional importance, green crab proteins may hold potential as functional ingredients in formulated food products. Functional proteins as food additives are added to a variety of foods to improve the quality of formulated products

including sausages, infant formulas, soups and sauces for their foaming, emulsifying and gelation properties (Puppo et al., 2005; Davis & Foegeding, 2007). However, most functional protein ingredients studied have been from plant and dairy sources as well as from fish by-products, and reports on the functional properties of crustacean proteins are extremely limited. Studies focused on the recovery and characterization of functional food proteins from green crab can contribute to the commercial utilization of this undervalued marine resource and provide a strategy to create novel functional protein ingredients for formulated foods.

As a method to obtain functional proteins for food ingredients, isoelectric solubilization/precipitation (ISP) is potentially applicable to minced crab meat. ISP is a process for protein recovery and concentration from a variety of raw materials. During ISP, proteins are solubilized at low or high pH and precipitated through a pH shift to their isoelectric point. This method has been applied to underutilized fish and to the wet-processing by-products of various fish species (Batista, 1999; Shi et al., 2017) However, ISP has been evaluated in only a few crustacean studies, namely whole Antarctic krill and dried crayfish meal (Chen et al., 2009; Romero et al., 2014). Compared with studies of fish and fish by-products, ISP processing of these crustaceans encountered some challenges and significantly different results in the yield and proximate composition of recovered proteins. These reports suggest that differences observed between fish and crustaceans are associated with the composition of the starting materials. To date, there are no reports on protein isolation from crustacean mince or on the functional properties of recovered crustacean proteins. Our study was designed to investigate the potential food application of protein powder recovered by ISP processing from green crabs. To achieve this, (i) physicochemical and functional properties of dried proteins recovered by ISP were characterized, (ii) the yield of green crab mince was quantified and (iii) recovered protein yields from low- and high-pH solubilizations were evaluated.

2.3 Materials and Methods

2.3.1. Mechanical Separation of Green Crab

Live green crabs (50 kg) were trapped in the Gulf of Maine in October 2015. The crabs were blast-frozen at $-30\text{ }^{\circ}\text{C}$ for 1 h, then stored at $-20\text{ }^{\circ}\text{C}$ until further use. To extract meat, 15 kg of frozen raw green crabs (carapace width 42–81 mm and weight 23–140 g) were stored overnight at $0\text{ }^{\circ}\text{C}$ and then processed using a mechanical separator (Paoli One-Step Mechanical Separator Model 22-849, Rockford, IL, USA). The separator works by crushing the crab starting material and forcing the soft tissue mince through the narrow microslits on the cylinder. The cylinder of the mechanical separator was intensively cleaned before use to obtain the maximum yield of crab mince. To clean the cylinder, it was pressure washed to remove as much material as possible. Then, the cylinder was placed in 12 L of 15% phosphoric acid at $70\text{--}80\text{ }^{\circ}\text{C}$ for 8 hr. Next, it was rinsed with water and any residual was physically removed from the microslits using a feeler gauge blade (0.006"). Then, any remaining residual was removed with the use of pressured air until no residual material came out of the cylinder microslits.

All parts of mechanical separator were sanitized with 10% bleach and re-assembled. The end cap was set at 1/3 open, and the breaker bar was set to 0.000 which allowed for the tightest grinding action. Then, partially thawed green crabs were processed through the mechanical separator. The minced meat was collected and blast-frozen at $-30\text{ }^{\circ}\text{C}$ for 1 h and stored at $-20\text{ }^{\circ}\text{C}$ until ISP processing.

2.3.2. Recovery of Green Crab Protein

Frozen mince was stored at $4\text{ }^{\circ}\text{C}$ overnight prior to ISP processing. The mince was diluted 1:9 (w/v) with cold deionized water and homogenized in a 1 L Waring blender (33BL79, New Hartford, CT, USA) for 1 min at maximum speed. The pH of the homogenate (initial pH 7.8) was adjusted to the desired acidic (pH 2) or alkaline (pH 10) endpoint with 6 M HCl or 6 NaOH, respectively, then stirred by an overhead stirrer (Fisher Scientific, Dubuque, IA, USA) for 30 min. To remove insoluble materials, the homogenate was centrifuged at $19,722 \times g$ for 20 min at $4\text{ }^{\circ}\text{C}$ (Beckman Coulter Avanti J-E, Fullerton, CA,

USA). The supernatant was collected and the pH was readjusted to 5.5. After stirring with a magnetic stirrer for 10 min, the solution was centrifuged at $19,722 \times g$ for 20 min at 4 °C (Beckman Coulter Avanti J-E, Fullerton, CA, USA) and the precipitate was collected. Both treatments (acidic, PP2 and alkaline, PP10) were processed in triplicate. The green crab mince (control) and precipitated proteins were blast-frozen, then freeze-dried (35 EL, VirTis Co. Inc., Gardiner, NY, USA). Samples were stored at -80 °C until use for physicochemical and functional protein analysis. Crude protein content in protein isolates and mince was determined based on the total nitrogen content and converted to protein content using a protein conversion factor of 6.25. The yield of recovered protein powder was calculated based on the following equation:

$$\text{protein recovery yield (g kg}^{-1}\text{)} = \frac{\text{recovered protein content in protein isolate (g)}}{\text{total protein content in mince (kg)}}$$

2.3.3. Proximate and Mineral Composition

Moisture, crude protein, fat, and ash were determined in the recovered proteins and the control mince using AOAC standard methods (2005). All sample powders were well ground using an electric chopper (Black & Decker, Towson, MD, USA). Sample powders were pooled from each replicate and proximate composition was determined by triplicate analyses.

2.3.3.1 Moisture Content

Moisture content was gravimetrically evaluated according to the AOAC 950.46 (2005) method by drying 1 g of sample in a pre-weighed aluminum pan in a 105 °C oven (VWR International, Radnor, PA) overnight. Then, the weight of pans containing samples was measured and the moisture content was calculated as follows:

$$\text{g/100g Moisture} = \frac{[\text{pan wt. (g)} + \text{initial sample wt. (g)}] - [\text{pan wt. (g)} + \text{dried sample wt. (g)}]}{\text{initial sample wt. (g)}} \times 100$$

2.3.3.2. Crude Protein Content

To determine crude protein content, any nitrogen contributed by chitin was quantified and then subtracted from the total nitrogen content. The nitrogen content in shell particles was determined by first deproteinizing the samples according to the method of Shahidi and Synowiecki (1991). Five grams of the sample were put into 100 mL of 2 % potassium hydroxide (KOH) solution and mixed for 30 sec. Then the mixture was incubated for 2 h at 90 °C. After cooling, the mixture was centrifuged at $19,722 \times g$ for 15 min at 4 °C (Beckman Coulter Avanti J-E, Fullerton, CA, USA) to separate insoluble and soluble materials. Then, the insoluble material was collected. Next, 100 mL of 5% KOH was added to the insoluble materials and the mixture was placed in a boiling water bath for 2 h. The mixture was chilled and centrifuged at $19,722 \times g$ for 15 min at 4 °C (Beckman Coulter Avanti J-E, Fullerton, CA, USA). The insoluble materials including shell particles were freeze-dried. Total nitrogen content in the control, recovered proteins, and deproteinized samples was analyzed by a combustion analyzer (TRU MAC CNS, LECO Corp., MI, USA) and shell nitrogen was subtracted to determine protein content of control and protein isolates using a protein conversion factor of 6.25.

2.3.3.3. Total Mineral Content (Ash Content)

Total mineral content was determined gravimetrically based on AOAC 938.03 (2005). One gram of oven-dried sample in a pre-weighted scintillation vial was heated in a muffle oven (Thermolyne Model F-A1730, Dubuque, IA) at 550 °C for 8 h (AOAC 938.03 2005). Then, the weight of scintillation vials containing samples was measured and the total mineral content was calculated as follows:

$$\text{g/100g ash (dwb)} = \frac{[\text{vial wt. (g)} + \text{ash wt. (g)}] - \text{vial wt. (g)}}{\text{dry sample wt. (g)}} \times 100$$

2.3.3.4. Selected Mineral Content

For mineral content determination, the ash was dissolved in 1 mL each of concentrated hydrochloric acid and nitric acid in scintillation vials. After 45 min, 10 mL of deionized water was added

and the samples were vortexed for 5 sec. Next, the samples in the vials were poured into a 100 mL volumetric flask and brought to volume with deionized water. After stirring, the mixtures in the volumetric flasks were allowed to settle overnight. Selected minerals including calcium, sodium and phosphorous in the solutions were analyzed by an inductively coupled plasma analyzer (Thermo Elemental IRIS Intrepid DUO ICP-OES, USA).

2.3.3.5 Crude Fat Content

The fat content was determined according to AOAC acid hydrolysis method AOAC 922.96 (AOAC 2005). Oven-dried samples (1 g) from each treatment were added to a French Square bottle with 10 mL of 8.1 N hydrochloric acid and were incubated in a water bath at 85-90 °C for 90 minutes. After cooling, 7 mL of ethanol was added and swirled for 15 sec. Then, 25 mL of ethyl ether was added to the sample and shaken vigorously for 60 sec. Next, 25 mL of petroleum ether was added to the sample and shaken for 60 sec, and the samples were allowed stand until the upper liquid (fat and ether) was practically clear. The upper layer (including fat and ether) was carefully removed using a glass pipette and transferred to a pre-weighed flat bottom beaker. Then, three more extractions were conducted using 15 mL each of ethyl ether and petroleum ether followed by shaking and removing the upper layer. The pooled ether and fat extracts were allowed to evaporate overnight under the chemical hood and then dried in a 105 °C the oven (VWR International, Radnor, PA) for 10 min. After cooling the beaker in a dessicator, final weight was recorded. The fat content (dwb) was calculated using the following formula:

$$\text{g/100g Crude Fat} = \frac{[(\text{flask (g)} + \text{fat weight (g)}) - \text{flask weight (g)}]}{\text{sample weight (g)}} \times 100$$

2.3.3.6 Amino Acid Analysis

Five milligrams of sample were hydrolyzed with 200 µL 6 N HCl with 1% phenol at 110 °C for 24 hours. Forty nmol/mL of norleucine were added to the hydrolyzed proteins as an internal standard since its signal is similar with the amino acid signal however, it also provides sufficient difference. Therefore,

the two signals could be easily distinguished by the amino acid analyzer. Amino acids were analyzed using a Na-based Hitachi 8800 (Tokyo, Japan) analyzer and sample was injected onto the ion-exchange column. Analytical standards were purchased from Sigma-Aldrich (St. Louis, Mo, USA) and all common amino acids were identified and quantified (g kg^{-1}) except for cysteine, methionine, and tryptophan. Samples were analyzed by John Schulze and the team at the Molecular Structure Facility (via Science Exchange) at the University of California, Davis.

2.3.4. Physicochemical Properties

2.3.4.1. Instrumental Color

Color differences among samples were assessed using a LabScan XE Hunter Lab colorimeter (Hunter Associates Laboratory, Reston, VA, USA). The colorimeter was allowed to warm up for 30 min before the colorimetric analysis. The colorimeter was standardized using black and white ceramic standard plates. Colorimetric analyses were conducted at a setting of 30.5 mm port size, 25.4 mm area view, and 10° illumination, and the disc with 5.1 cm diameter hole was used. One layer of sample was spread on the 50×9 mm polystyrene petri dish to cover the bottom of the petri dish. Instrumental color of samples was evaluated in triplicate. Each sample was analyzed three times following 120° rotation and L^* , a^* , and b^* values were averaged. L^* value is represented by a scale of dark (0) to light (100), a^* value is represented by a scale of green (-) to red (+), and b^* value is represented by a scale of blue (-) to yellow (+).

2.3.4.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Molecular weight distribution was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples (50 mg) were dissolved in 10 mL SDS-urea solution that contained 2% SDS, 8 M Urea, 50 mM Tris-HCl (pH 8.0), and 2% β -mercaptoethanol, then incubated in a 90-95 °C water bath for 5 min (Meyer & Lamberts, 1965; Yoshida et al., 2003). After cooling, the solubilized samples were centrifuged at $21,130 \times g$ for 5 min (Eppendorf 5242 R, Hamburg, Germany). Proteins were

separated on a 4% stacking and 10% separating gel (BioRad Laboratories, Hercules, CA) using a Bio-Rad Mini-PRO-TEAN III Cell (Bio-Rad Laboratories, Inc., Hercules, CA). Protein content in the loaded sample was adjusted to about 20 µg, and 10 µL of a protein molecular weight standard (Dual Color Standards 10-250 kD, Bio-Rad Laboratories, Inc., Hercules, CA) was loaded on the gel. Separated proteins were fixed in a 50% (v/v) ethanol and 10% (v/v) acetic acid gel-fixing solution for 1 h then washed with a solution of 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min. Following washing, the gel was stained using a staining solution containing 0.1% Coomassie blue R-250 (Bio-Rad Laboratories, Inc., UK), 40% methanol, and 10% acetic acid for 3 h, then the gel was destained with washing solution until the background color was removed.

2.3.5. Functional Properties of Recovered Proteins

2.3.5.1. Solubility

Solubility of the control and recovered proteins was determined at pH 3 through 11 according to the methods of Khaled et al. (2014). Samples (0.2 g) were mixed with 20 mL of deionized water and pH was adjusted to each appropriate point using 1 N HCl or 1 N NaOH. Following adjustment, each mixture was stirred with a magnetic stirrer for 30 min at room temperature. After centrifugation at $23,645 \times g$ for 15 min at 4 °C, the supernatant was collected and its protein content was evaluated by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. Ten microliters of supernatant was mixed with 200 µL of diluted Coomassie blue reagent (1:5 dilution with deionized water) (Bio-Rad Laboratories, Inc., Hercules, CA). After 5 min, the absorbance was measured at 595 nm. Protein solubility (%) was calculated based on the crude protein content of each sample, and all samples were analyzed in triplicate.

$$\text{Solubility (\%)} = \frac{\text{protein content (g) in supernatant} \times 100}{\text{total protein content (g) of sample}}$$

2.3.5.2. Emulsifying Activity and Stability

Emulsifying activity and stability were evaluated in triplicate using the methods of Tang et al. (2005). A 0.2% (w/v) sample solution was prepared by solubilizing 0.048 g of sample in 24 mL of deionized water. Then, 24 mL of sample solution was homogenized for 30 sec with 8 mL of vegetable oil (Hannaford Bros. Co., Scarborough, ME) in a 360 mL Waring blender. A 50 μ L aliquot of the emulsion was immediately transferred into 4.95 mL of 0.1% (w/v) sodium dodecyl sulfate solution at 0, 5, 10, 20, 30, 40, 50, and 60 min, and the absorbance of the diluted and vortexed solutions was read on a spectrometer (Beckman Du 530, Brea, CA, USA) set at 500 nm. Deionized water (3 mL) was used as a blank. Emulsifying activity (EA) was calculated by the following equation:

$$EA \text{ (m}^2\text{/g)} = \frac{2 \times 2.303 \times A(0) \times DF}{C \times OP \times 10,000}$$

A(0) = absorbance after 0 minutes

DF = dilution factor (100)

C = concentration of protein (g/mL)

OP = optical path (0.01 m)

2.3.5.3. Gel Forming Activity

Gel formation activity of the control and recovered proteins was determined in triplicate using the method of Sathe et al. (1982). Two milliliters of sample suspensions were prepared at 9%, 12%, 15%, 18%, 21%, 24%, and 27% concentrations (w/v) in 12 \times 125 mm glass test tubes. Each suspension was incubated in boiling water for 45 min and then rapidly cooled on ice. After 45 min, each test tube was inverted to determine the lowest concentration (%) at which the gelled suspension displayed no slipping or spilling in the tube.

2.3.5.4. Foaming Activity and Stability

Foaming activity and stability were evaluated according to Khaled et al. (2014). The sample was mixed in deionized water at a concentration of 0.1% (w/v), and then 20 mL of the solution was mixed by a rotor stator homogenizer (Brinkmann Instruments, Westbury, NY) for 1 min at room temperature. Following homogenization, the solution was immediately transferred into a 50 mL graduated cylinder and the total volume (mL) was measured at 0, 15, 30 and 45 min. Foam expansion was expressed as percentage of volume increase immediately after homogenization (0 min).

$$\text{Foam Expansion (\%)} = [(A-B)/B] \times 100$$

where A is the volume (mL) after homogenization and B is the volume (mL) before homogenization.

2.3.6. Statistical Analysis

Statistical differences among the three treatment means were determined using one-way analysis of variance (ANOVA) with a significance value of $p < 0.05$ followed by Tukey's HSD post hoc test (SPSS ver.23, IBM Corp., Armonk, NY). Also, correlation between solubility and emulsifying activity was determined by Pearson's correlation ($p < 0.05$, SPSS ver.23, IBM Corp., Armonk, NY).

2.4. Results and Discussion

2.4.1. Yield

The yield efficiency of seafood processing operations continues to receive attention due to its impact on economic and environmental sustainability. The mechanical separation method used in this study resulted in a 317 g kg⁻¹ yield of minced tissue from raw green crabs. Galetti et al. (2017) reported higher mince yields, of 447 g kg⁻¹ and 561 g kg⁻¹, for boiled and steamed green crabs, respectively. Additionally, steamed Jonah crabs yielded 533 g kg⁻¹ minced meat from mechanical separation (Gillman & Skonberg, 2002). These results indicate that thermal processing of crab, including boiling and steaming, may produce higher mince yields compared to raw crab. However, given the extensive denaturation and aggregation of crab proteins due to thermal processing, the mince derived from

cooked crab is more suitable for culinary applications rather than for producing functional protein additives.

Solubility of protein plays an important role in ISP processing since increased protein solubilization can lead to higher purity of the recovered protein (Kristinsson et al., 2005). Protein solubility increases as pH moves away from a protein's pI, and in this study, pH 2 and pH 10 were selected as representative acidic and alkaline pH values to obtain high solubility while simultaneously avoiding excessive denaturation of protein (Kristinsson & Liang, 2006). Green crab proteins solubilized at pH 2 and pH 10 exhibited significantly different yields, with a higher protein powder recovery at the lower pH, 453 g kg⁻¹ based on total protein content in the starting material, compared to 242 g kg⁻¹ at pH 10. A similar trend was shown by Kristinsson and Liang (2006) with Atlantic croaker where acidic (pH 2.5) solubilization resulted in a higher yield than alkaline (pH 11) solubilization. The yield of recovered protein powder is strongly associated with extent of solubilization, and the yield difference between PP2 and PP10 was likely due to the solubility differences of green crab proteins under acidic (pH 2) and alkaline (pH 10) conditions.

The yield of recovered protein powder was lower than expected, and likely related to the proximate composition of the starting material. Antarctic krill, having 765 g kg⁻¹ protein, 121 g kg⁻¹ fat, and 174 g kg⁻¹ ash (dwb), had a higher protein recovery (471- 500 g kg⁻¹) compared to the green crab (242-453 g kg⁻¹). The whole Antarctic krill had higher protein and lower ash content than the minced green crab (528 g kg⁻¹ protein, 77 g kg⁻¹ fat, and 230 g kg⁻¹ ash). For green crab mince, the crushing of the highly mineralized hard exoskeleton during the mechanical separation process contributed to the high ash content, which along with lower protein content were likely the primary factors affecting the protein recovery. Therefore, optimizing the mechanical separation of raw green crabs could increase mince yield and reduce shell particulates, thereby improving protein recovery. Additionally, increasing the ratio of water to crab mince during homogenization may increase the yield by facilitating increased

protein solubility. In this study, pH 5.5 was used for protein precipitation based on ISP of Antarctic krill (Chen et al., 2009). Optimizing the pH value for crab protein precipitation may contribute to enhanced yield of recovered protein, which could be achieved by assessment of crab protein solubility at various pH levels. Also, proteolytic enzymes present in the raw crab mince may have contributed to the generation of peptides that did not precipitate during protein recovery. Thus, the addition of a protein inhibitor to prevent proteolysis could be considered (Shi et al., 2017).

2.4.2. Proximate Composition

Proximate composition of recovered protein provides a means to evaluate the purity of recovered protein. In addition, protein content values are fundamental for measuring functional properties of green crab protein including solubility, gelation, and emulsifying activity/stability. In comparison with the protein content of the control (528 g kg⁻¹), both PP2 and PP10 had significantly higher values of 774 g kg⁻¹ and 700 g kg⁻¹, respectively (Table 2.1).

Table 2.1. Proximate composition (g kg⁻¹, mean ± SD) and selected mineral content (g kg⁻¹, mean ± SD) of the control and recovered proteins (dry weight basis)*

	Control	PP2	PP10
Crude protein (g kg ⁻¹)	528 ± 5.4	774 ± 0.1	700 ± 0.0
Crude fat (g kg ⁻¹)	77 ± 2.0	160 ± 6.0	238 ± 1.0
Ash (g kg ⁻¹)	230 ± 1.0	54 ± 2.0	23 ± 1.0
Minerals (g kg ⁻¹)			
Ca	58.4 ± 1.7	9.0 ± 0.2	1.5 ± 0.0
Na	19.5 ± 0.1	9.4 ± 0.2	6.9 ± 0.3
P	12.9 ± 0.1	6.6 ± 0.0	4.9 ± 0.1

* Each value is the mean ± S.D. of triplicate analyses (n=3) of composite samples.

Solubilization under acidic conditions resulted in a more concentrated protein product than under alkaline conditions. The amino acid composition of the proteins was dominated by glutamic acid + glutamine (63 – 106 g kg⁻¹), followed by aspartic acid + asparagine (44 – 85 g kg⁻¹) and leucine (37 – 64 g kg⁻¹) (Table 2.2). With few exceptions, ISP slightly increased the specific amino acid contents (g kg⁻¹ protein) in recovered protein powders. In contrast, histidine and glycine were significantly higher in the control mince. In comparison with PP10, PP2 had somewhat higher amounts of hydrophilic amino acids and lower amounts of hydrophobic amino acids, however the amino acid profiles were not substantially different.

The exoskeleton of hard shell crustaceans is highly mineralized. Previous studies indicated that mechanically separated crab mince contains a high content of minerals due to the incorporation of finely ground shell particles during mechanical processing (Gillman & Skonberg, 2002; Galetti et al., 2017). Therefore, reducing the high mineral content in green crab mince would play an important role in obtaining a high purity functional protein from green crab mince. Based on proximate analysis, the control had the highest ash content at 230 g kg⁻¹ followed by PP2 (54 g kg⁻¹) and PP10 (23 g kg⁻¹) (Table 2.1). PP2 had more than twice the ash content of PP10, due to extensive mineral solubilization under acidic conditions. During protein solubilization at pH 2, the pH was adjusted with hydrochloric acid, leading to dissolution of shell mineral, which was recovered with the protein precipitate. Calcium, phosphorous and magnesium are the primary minerals in the crustacean exoskeleton (Bosselmann et al., 2007), and their levels (Table 2.1) were consistent with values obtained for ash. Although there were differences between PP2 and PP10, the ash and selected mineral contents of both protein isolates were much lower compared to the control. The same mineral reduction effect of the protein recovery processing was shown in Antarctic krill (Shi et al., 2017). These results indicate that a portion of the minerals contributed by the green crab shell can be successfully removed by the ISP method.

Table 2.2. Amino acid composition * (g kg⁻¹) of the control and recovered proteins

Amino acid	(g kg ⁻¹ of sample weight)		
	Control	PP2	PP10
Aspartic acid + Asparagine	44.1	85.0	66.6
Threonine	21.7	38.9	34.0
Serine	19.2	34.2	28.4
Glutamic acid + Glutamine	63.0	105.8	82.1
Proline	24.2	30.1	24.8
Glycine	28.7	30.1	24.3
Alanine	27.0	38.1	30.6
Valine	22.8	40.3	33.4
Isoleucine	22.9	39.2	34.0
Leucine	36.7	63.9	55.0
Tyrosine	18.9	35.9	29.7
Phenylalanine	20.8	40.9	35.4
Histidine	29.0	26.7	23.2
Lysine	33.9	53.6	40.8
Arginine	20.2	48.2	34.7
Total	433.2	710.8	576.8

* Values represented from single analysis of pooled samples (n=1).

In contrast, the fat content was concentrated due to ISP processing. The fat content of the green crab control increased from 77 g kg⁻¹ to 160 g kg⁻¹ (PP2) and 238 g kg⁻¹ (PP10) after processing (Table 2.1). During protein recovery from finfish fillets or byproducts, the mixture separates into three distinct

layers: insoluble materials, soluble materials, and lipid, which facilitates the removal of the soluble protein. In the case of the green crab mince, the layers of insoluble and soluble materials were clearly distinguishable, however there was no lipid layer; rather, the lipid fraction was present as an emulsion with the soluble fraction. This was potentially due to a high phospholipids content of green crab. A similar result was found when isolating protein from Antarctic krill and menhaden (Shi et al., 2017); a clear lipid fraction was not observed due to the high levels of phospholipid. Phospholipids are polar emulsifiers, which led to their inclusion in the soluble protein fraction and subsequent concentration in the recovered green crab protein powder. Several approaches, including modifying the centrifugation conditions, reducing processing temperature, and/or adding sodium chloride should be explored to break or prevent formation of an emulsion. Alternatively, since polyunsaturated fats (PUFA) present in seafood are associated with various health benefits, the production of a PUFA-enriched protein powder may have potential as a bioactive ingredient for health-promoting food products.

2.4.3. Protein Molecular Weight (MW)

SDS-PAGE analyses indicate differences in molecular weight distributions of the control and recovered protein powders. In this study, a variety of different sized proteins were tentatively identified as myosin heavy chains (150-250 kD), actin (45 kD), tropomyosin (37 kD), troponin (23 kD) and myosin light chains (< 25 kD). Recovered protein powders (PP2 and PP10) showed different MW distributions compared to the control and to each other (Figure 2.1). In PP2, the content of larger MW proteins between 37 kD and 100 kD slightly increased in comparison to the control. This may have been due to protein aggregation after unfolding or to the concentrating effect of losing small MW proteins that did not precipitate at pH 5.5. PP10 samples contained small MW proteins of less than 37 kD, suggesting that extensive proteolysis occurred under the alkaline conditions (Figure 2.1). Some endogenous enzymes have an optimum activation pH under alkaline conditions, and the activation of these enzymes during solubilization at pH10 may have contributed to the proteolysis of PP10 (Shi et al., 2017). Differences in

protein size distribution among samples can affect functional properties of recovered protein powder since solubility, water holding capacity, and gelation are strongly associated with molecular weight. The larger MW proteins such as myosin heavy chains and actin in PP 2 are integral in gel formation, and the extent of their solubility contributes to protein gel characteristics.

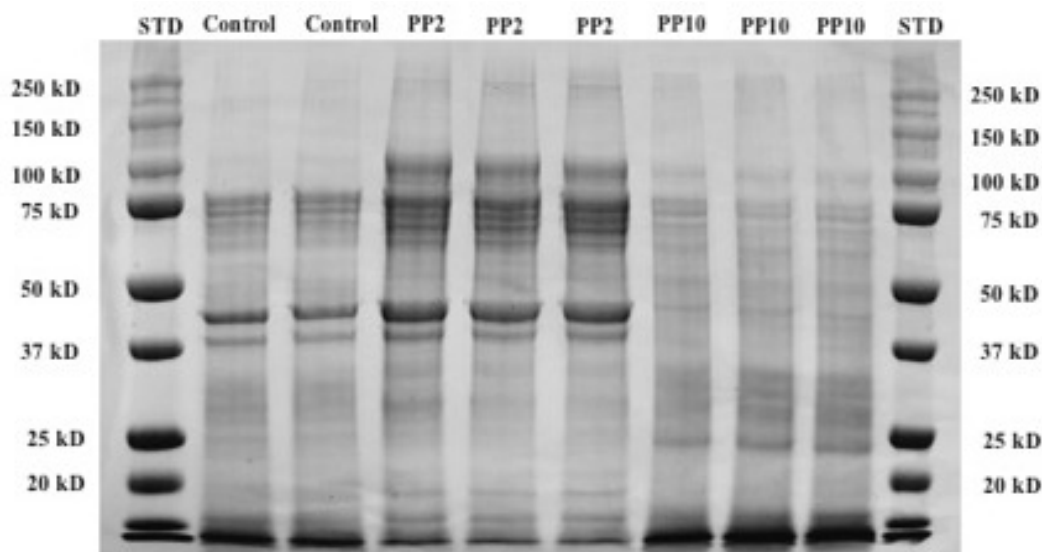


Figure 2.1. Molecular weight distribution of the control and recovered proteins (SDS-PAGE). Lanes 1 and 10 are the molecular weight standard. Lanes 2-3 indicate the crab mince control. Lanes 3-5 (PP2) are the recovered proteins from solubilization at pH 2. Lanes 6-9 (PP10) are the recovered proteins from solubilization at pH 10.

2.4.4. Functional Properties

While many studies have evaluated select functional properties of proteins recovered from fish, there are no previous reports on solubility, gelation, emulsifying properties, and foaming capacities of recovered crab proteins. Solubility is one of the most important properties of proteins since it is related to other key functional properties such as gelation, emulsification, and foaming capacity. Recovered protein powders showed different functional properties depending on solubilization pH. PP2 powders were the most soluble at pH 11 ($860 \pm 106 \text{ g kg}^{-1}$) whereas PP10 powders showed the highest solubility ($655 \pm 76 \text{ g kg}^{-1}$) at pH 3 (Figure 2.2). Both PP2 and PP10 were least soluble at pH 5, which was close to the pH value (5.5) used for precipitation during the recovery process. The control exhibited significantly

higher protein solubility from pH 4 through pH 7 than PP2 and PP10 but its solubility was less than 184 g kg⁻¹. PP10 exhibited higher protein solubility (132 g kg⁻¹ to 551 g kg⁻¹) from pH 7 to pH 10 in comparison with solubility of PP2 (27 g kg⁻¹ to 345 g kg⁻¹) at the same pH range. These differences in solubility between PP2, PP10, and the control were associated with their MW distribution (Pacheco-Aguilar et al., 2008). Compared to PP2, the higher solubility of PP10 at neutral pH can be explained by the larger amount of small MW proteins. Small MW proteins such as myosin light chains (< 25 kD) are associated with high solubility at neutral pH, due to their larger surface area.

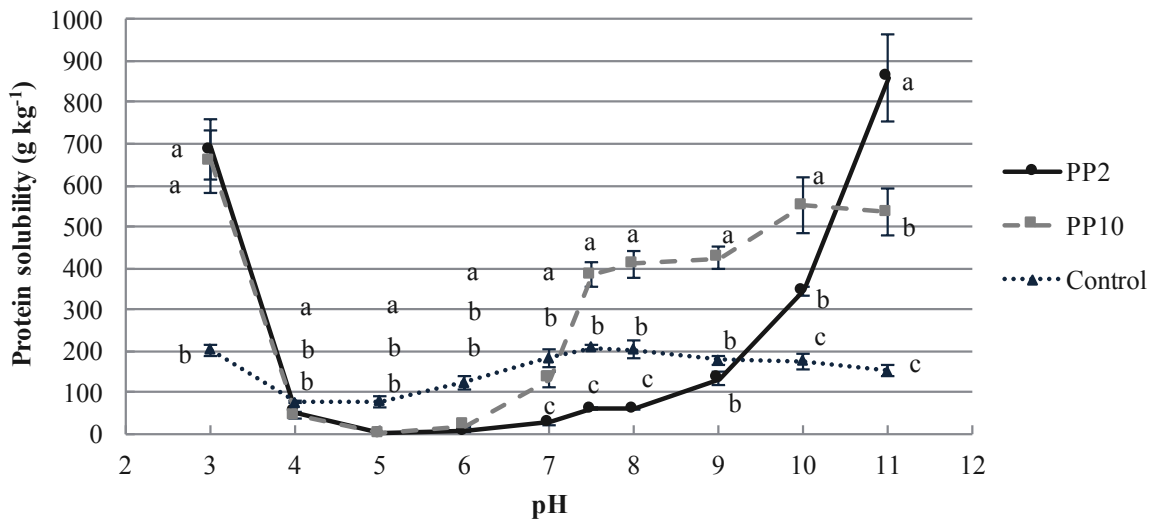


Figure 2.2. Protein solubility (g kg⁻¹) of the control and recovered proteins at different pH values. Values are means ± S.D. (n=3). Different letters indicate significant difference among treatments at given pH (p < 0.05) by ANOVA followed by Tukey's test.

The emulsification properties of the samples were significantly different and only PP10 demonstrated an emulsifying activity applicable to formulated food products. PP10 exhibited 1481 ± 11 m²/g of emulsifying activity at pH 7.5, which was significantly higher than PP2 (858 ± 20 m²/g) and the control (992 ± 11 m²/g) (Figure 2.3). A similar trend was observed in emulsifying stability. PP10 produced a more stable emulsion than PP2 and the control. The emulsifying activity of PP10 decreased by half after ~35 minutes whereas the emulsifying activities of PP2 and the control each disappeared

within 5 minutes. PP10 had a higher emulsifying capacity than some values reported for soy protein isolates, which are known to be good emulsifiers. For example, Chove et al. (2001) reported that soy protein isolates exhibited 250-350 m²/g of emulsifying activity at pH 7.0, as determined by the turbidimetric method. The trend of emulsifying capacity in the three treatments was consistent with the solubility trend observed at pH 7.5.

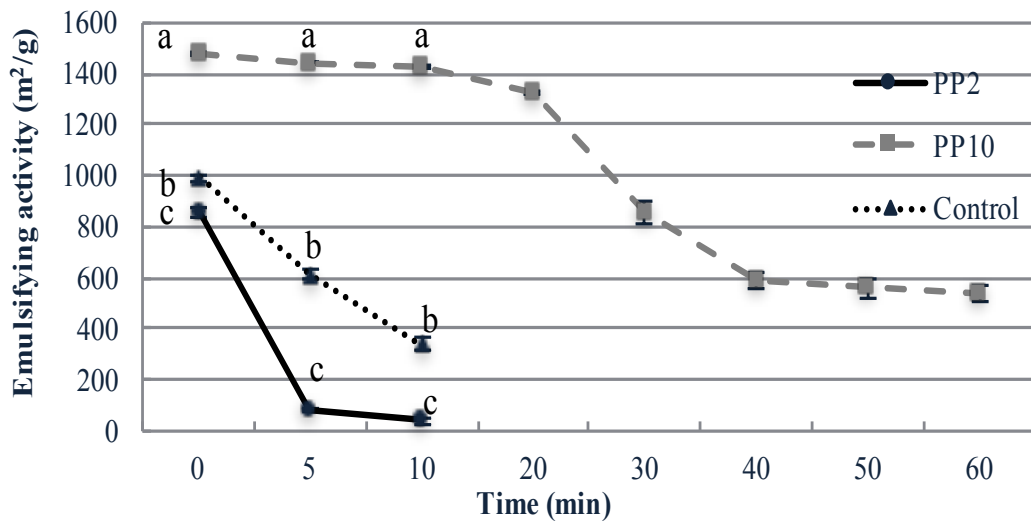


Figure 2.3. Emulsifying activity (m²/g) and emulsion stability of the control and recovered proteins. Values are means ± S.D. (n=3). Different letters indicate significant differences among treatments at given time (P < 0.05) based on ANOVA followed by Tukey's test.

High emulsifying activity of PP10 compared to control and PP2 is likely due to its solubility at pH 7.5. The correlation between emulsifying activity and protein solubility was $r = 0.956$ ($p < 0.05$), suggesting that emulsifying activity of proteins was dependent in part on their solubility. As previously reported, high solubility is important in an emulsion system since it enables a higher protein concentration at the interface between the oil and water phases, resulting in better emulsifying properties (McWatter & Holmes, 1979). In addition, the surface hydrophobicity of proteins is positively associated with emulsifying capacity (Kato & Nakai, 1980). Based on the amino acids analysis, PP10 was

slightly higher in hydrophobic amino acids content, which may also have positively contributed to its higher emulsifying properties, however its surface hydrophobicity was not analyzed.

Gelation properties of protein additives play an important role in formulated food products since protein gels entrap water, lipids, and other key ingredients (Kinsella, 1979). In this study, the lowest protein concentration required to maintain an intact gel in an inverted tube was used as an index of gel formation ability. The gelation properties of both protein isolates were improved compared to the control (Figure 2.4). PP2 (12%) had the strongest gelation activity followed by PP10 (21%) and the control (27%). Gel forming ability is interrelated with many factors such as protein concentration, pH, ionic strength, and divalent ions and sulfhydryl groups (Shimada & Matsushita, 1980). The higher gel forming ability exhibited by PP2 was likely due in part to its higher protein concentration (774 g kg^{-1}) compared to PP10 (700 g kg^{-1}) (Table 2.1). During gelation, heating causes denaturation of myosin and other large molecular weight proteins, and allows it to form a 3-dimensional network, which captures water in a low mobility state. Factors influencing gel forming capacity of proteins include amino acid composition, surface hydrophobicity, temperature, pH, ionic strength, salt, and pressure (Sun & Holley, 2011). However, the most significant contributor influencing gelation of ISP processed green crab proteins was differences in molecular weight. As described previously, PP10 samples had extremely low molecular weight proteins, which had diminished capacity to form sufficient protein networks and produce a rigid gel.

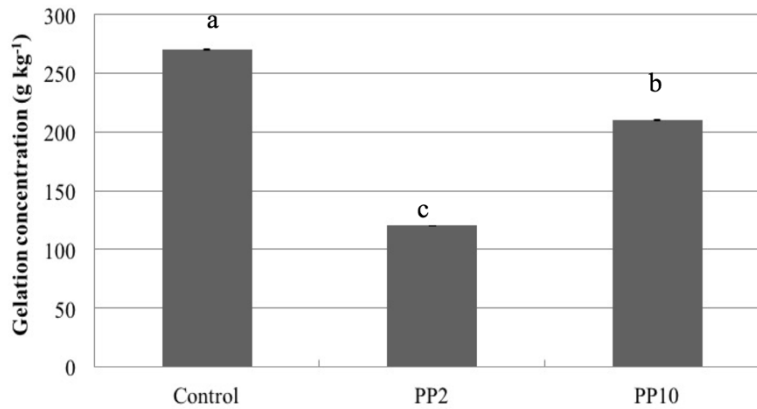


Figure 2.4. Minimum sample concentration (g kg⁻¹) required for gel formation. Values are means \pm S.D. (n=3). Different letters indicate significant difference among treatments ($P < 0.05$) based on ANOVA followed by Tukey's test.

In contrast to the other functional properties evaluated, foaming activity of green crab proteins was not enhanced by application of the ISP method. Although the foaming activities of PP10 (21%) and the control (20%) were significantly higher than that of PP2 (17%), all three treatments exhibited poor foaming activities (Figure 2.5) insufficient for use in food applications. Moreover, initial foaming activities of PP2 and PP10 were reduced by half after only 15 minutes. Foam formation and its stability are influenced by several factors including pH, salts, sugars, lipids and protein concentration (Damodaran et al., 2007), and protein-stabilized foams are most stable at their pI. For example, egg white, which has very high foaming capacity due to its ovalbumin content, exhibits good foaming properties at its pI (pH 4-5) and at pH 8-9. Also, a high protein content enables it to form a stiffer foam with enhanced viscosity, facilitating the formation of a cohesive protein film at the gas and liquid interface. However, lipids, especially phospholipids, reduce the foaming capacity of proteins remarkably. Thus, the phospholipids concentrated in the recovered protein powders may have more surface-activity than the proteins, possibly limiting absorption at the air-water interface by proteins.

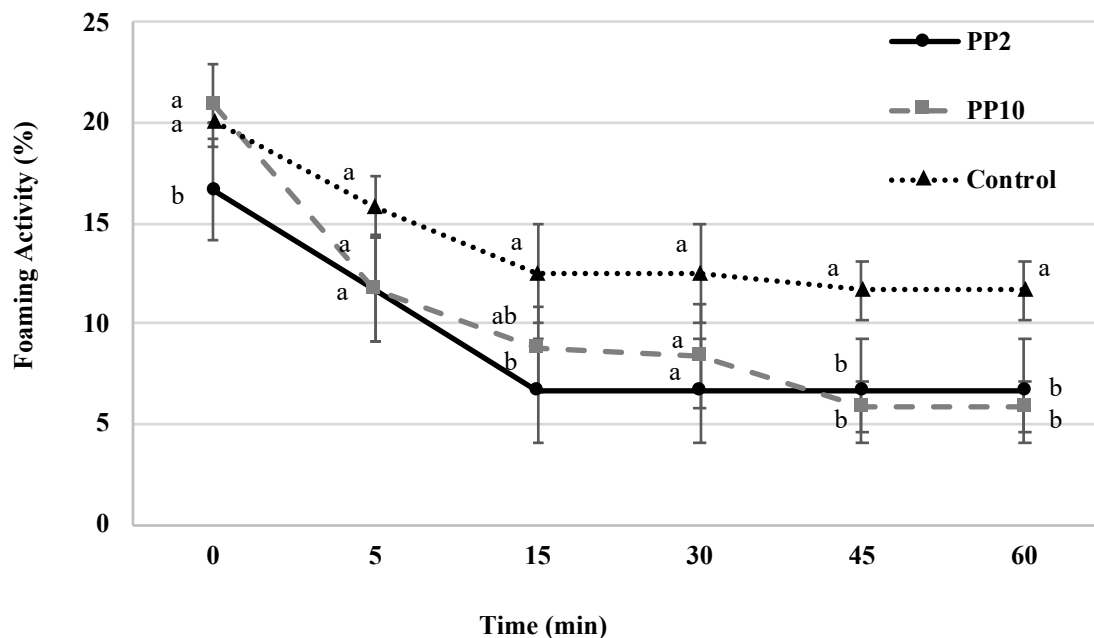


Figure 2.5. Foaming activity and stability of the control and recovered proteins. Values are means \pm S.D. (n=3). Different letters indicate significant differences among treatments at given time ($p < 0.05$) based on ANOVA followed by Tukey's test.

2.4.5. Instrumental Color

Color is also an important quality attribute of food ingredients, and significant differences in L^* , a^* , and b^* values were observed among the treatments (Figure 2.6). PP2 exhibited the highest L^* value (53.2 ± 0.3) followed by the control (51.1 ± 0.2) and PP10 (43.6 ± 0.3), indicating that PP2 had a much lighter color than the alkaline solubilized sample. Both a^* and b^* values significantly increased after the protein isolation. PP2 had significantly higher a^* and b^* values than PP10 and the control. In agreement with a^* and b^* values, PP2 appeared more red and more yellow compared to PP10 and the control (Figure 2.6). The higher a^* and b^* values in recovered protein powders compared to the control was partially a result of lipid concentration and pigment extraction due to ISP processing. Crab shells contain carotenoid pigments including lutein, β -carotene, astaxanthin, and canthaxanthin that have red and

orange colors (Acosta et al., 1993). Since these hydrophobic pigments are fat soluble, they were concentrated in the recovered protein powder along with lipid.

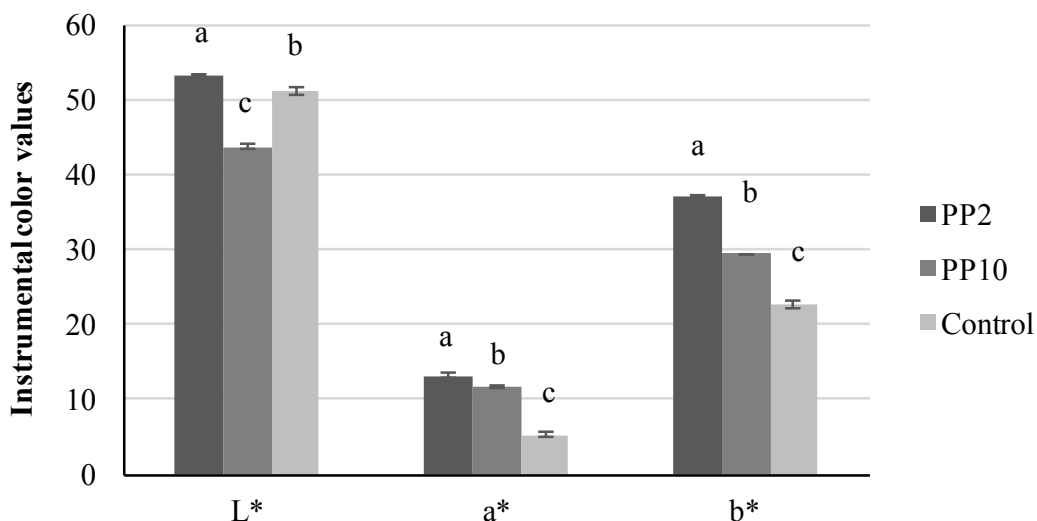


Figure 2.6. Instrumental color values (L*, a*, b*) of the control and recovered proteins. Values are the means \pm S.D. (n=3). Different letters indicate significant differences among treatments ($P < 0.05$) based on ANOVA followed by Tukey's test.

2.5. Conclusions

The ISP method was effective in recovering functional proteins from mechanically separated raw green crab. Recovered protein yield was lower than expected and further investigation is warranted to optimize the mechanical separation of raw green crab and the protein recovery method. The high ash content contributed by the crab exoskeleton was significantly reduced in recovered protein powder whereas its lipid content was concentrated. Nevertheless, commercial application of the recovered green crab protein in food products will require addressing economic feasibility, and a cost-benefit analysis of an optimized process is warranted. The functional properties of recovered protein powders were dependent on the solubilization pH during processing, and both the acid and alkaline solubilized green crab proteins have potential as functional food ingredients. PP2 may be most useful in protein gel-

based foods, such as lunchmeats, surimi, and other restructured meat products while PP10 would be appropriate for application in sauces, souffles, and chowders that require higher solubility and emulsifying activity at neutral pH. Future research should focus on the application of these recovered functional protein powders as ingredients in model food systems.

CHAPTER 3

ANTI-HYPERGLYCEMIC EFFECTS OF GREEN CRAB HYDROLYSATES DERIVED BY COMMERCIALLY AVAILABLE ENZYMES

This chapter was published in Foods and edited according to the dissertation format (Kang et al., 2020).

3.1 Chapter Abstract

The predation and burrowing activity of invasive green crabs have had detrimental effects on important marine resources and habitats. Our objective was to develop bioactive hydrolysates by enzymatic proteolysis of underutilized green crab. Mechanically separated mince was hydrolyzed with Alcalase, Protamex, Flavourzyme, and Papain (1%) for 60 min. Subsequently, the hydrolysates were introduced to a simulated gastrointestinal digestion model. Selected samples were fractionated by ultrafiltration, and their anti-hyperglycemic effects including α -glucosidase, α -amylase, and dipeptidyl peptidase-IV (DPP-IV) inhibitory activities and glucagon-like 1 (GLP-1) secretory activity were evaluated. The Protamex treatment showed the highest α -glucosidase inhibitory activity (IC_{50} 1.38 ± 0.19 mg/mL) compared to other enzyme treatments and the crab mince control, and its α -amylase inhibitory activity (IC_{50} 11.02 ± 0.69 mg/mL) was lower than its α -glucosidase inhibitory activity. Its GLP-1 secretory activity was approximately four times higher than the positive control (10 mM glutamine). The <3 kD fraction contributed significantly to the anti-hyperglycemic activity of Protamex-derived hydrolysates, and this activity was stable after simulated digestion. Our results suggest that green crab hydrolysates obtained by Protamex treatment have the potential for type 2 diabetes management and could be incorporated in food products as a health-promoting ingredient.

3.2. Introduction

In recent years, consumer interest in sustainability and healthy lifestyles has significantly increased, leading to higher demand for nutritious and environmentally-friendly food products by consumers and the food industry. The development of natural, bioactive food ingredients from currently underutilized food resources may positively contribute to satisfying this demand. Nutritional and bioactive food ingredients include proteins and peptides, polyunsaturated fatty acids (PUFAs), phytochemicals, fibers, probiotics, and prebiotics (Suleria et al., 2015). Marine organisms represent good sources for obtaining various bioactive compounds, and many of these bioactive compounds are derived from proteins (Kim & Wijesekara, 2010). Therefore, protein-rich marine organisms are an ideal raw starting material for the development of protein-derived bioactive compounds. European green crabs (*Carcinus maenas* L.) are an invasive species established in the U.S. that negatively influence fisheries, aquaculture, and marine habitats due to their high resilience, voracious predation, and strong burrowing activity (Grosholz & Ruiz, 1966; Malyshev & Quijón, 2011; Sigurdsson & Rochette, 2013; Leignel et al., 2014). Green crabs are very competitive predators that prefer juvenile clams, blue mussels, and lobster larvae, which are highly profitable for fisheries. Also, their borrowing activity has destroyed valuable marine habitats such as eelgrass beds and salt marshes (Malyshev & Quijón, 2011). Although there is a large population of green crabs in the U.S., they are not currently utilized by food industries due to their small size. To obtain the nutritious meat, significant labor and high costs would be required to remove the meat from the shell. However, our previous studies demonstrated that green crab mince obtained via mechanical separation can be used in the development of value-added food products (Galetti et al., 2017; Kang et al., 2019).

Green crab contains various nutritional and nutraceutical compounds such as chitin, carotenoids, essential amino acids, and PUFAs including eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA) (Naczek et al., 2004). Green crab meat has approximately 80% protein (Skonberg & Perkins, 2002); these proteins have the potential to be utilized as the initial material for the generation of multifunctional food ingredients. Some proteins and peptides extracted from protein-rich marine resources have been reported to act as functional and bioactive ingredients in food products (Intarasirisawat et al., 2014; Baharuddin et al., 2016). Enzymatic hydrolysis of a variety of protein sources has been extensively utilized as a method to generate bioactive peptides having antioxidant, anti-inflammatory, and anti-hypertensive effect (Ndiaye et al., 2012; Pan et al., 2012; Sánchez & Vázquez, 2017). Different types of proteases used for enzymatic hydrolysis have distinct specificity and produce peptides having different molecular weights and amino acid sequences, which may contribute to their diverse biological and physiochemical functional properties (Ahn et al., 2012a; Ahn et al., 2012b; Slizyte et al., 2016). Various studies have confirmed that commercially available proteases such as Alcalase, Protamex, Flavourzyme, and Papain have the potential to generate bioactive peptides from food proteins (Liu et al., 2013; Ovissipour et al., 2013). However, the majority of these studies were conducted with dairy proteins and finfish byproducts. Furthermore, the bioactivity of hydrolysates obtained from marine resources was studied extensively with regard to their anti-hypertensive and antioxidant activities rather than their anti-diabetic properties. Fewer studies have been conducted to assess the biofunctional activity of the valuable peptides derived from shellfish proteins. More specifically, there is a lack of research about the potential anti-diabetic and anti-inflammatory activities of crustacean and mollusk proteins and their hydrolysates.

Type 2 diabetes mellitus (T2DM) is one of the most prevalent chronic diseases in the world and ranks in the top 10 diseases causing the most deaths worldwide (WHO) (WHO, 2018). T2DM and associated complications result not only in health issues but also substantial direct and indirect costs (CDC, 2020b). During T2DM, excessive glucose is used in several mechanisms such as polyol, hexosamine, protein kinase C, and advanced glycation end products (AGE) pathways, which leads to

overproduction of reactive oxygen species (ROS) (Brownlee, 2005). Then, increased intracellular ROS stimulate a number of proinflammatory pathways, which results in persistent expression of proinflammatory genes even after the blood glucose is normalized. T2DM and its complications are further exacerbated by this persistent inflammatory stress (Brownlee, 2005). An optimal diet can help in the prevention of T2DM and is critical to reducing the attendant morbidity and cost associated with the treatment of T2DM and its complications (Diabetes Prevention Program Research Group, 2003; Steyn et al., 2004; Ley et al., 2014). The development of anti-hyperglycemic and anti-inflammatory ingredients from green crabs may contribute to the prevention of T2DM and chronic inflammation and reduce the use of pharmaceutical products and the risks associated with their side-effects (de Campos Zani et al., 2018). Furthermore, the development of natural and nutraceutical peptides generated from green crabs may encourage the development of a fishery for this invasive predator.

Therefore, the aim of our study was to investigate the anti-hyperglycemic and anti-inflammatory effects of green crab hydrolysates for potential use as a health-promoting food ingredient. To achieve this, (1) enzymatic hydrolysis using commercially available proteases was applied to mechanically separated green crab mince, (2) the potential anti-hyperglycemic and anti-inflammatory effects of green crab hydrolysates were investigated, (3) fractionation of hydrolysates using ultrafiltration was conducted, and (4) changes in anti-hyperglycemic and anti-inflammatory activities of green crab hydrolysates after simulated gastrointestinal digestion were investigated.

3.3. Materials and Methods

3.3.1. Materials and Reagents

Green crabs (carapace width: 40–85 mm and weight: 20–155 g) were harvested in 2018 on the Back River in Georgetown, Maine, USA. All of the chemicals and reagents that were used in this study were supplied by Fisher Scientific (Hampton, NH, USA), Thermo Fisher Scientific (Waltham, MA, USA), Bio-Rad Laboratories (Hercules, CA, USA), and Sigma-Aldrich (St. Louis, MO, USA) unless otherwise

described. Alcalase 2.4 L (AL), Protamex (PR), Flavourzyme (FL), and Papain (PA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat intestinal acetone powders of α -glucosidase (EC 3.2.1.20), porcine pancreatic α -amylase (EC 3.2.1.1), pepsin from porcine gastric mucosa (EC 3.4.23.1, >250 units/mg protein), pancreatin from porcine pancreas (EC 232.468.9, 8*USP), p-nitrophenyl- β -D-glucopyranoside (p-NPG), and Gly-Pro-p-nitroanilide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Porcine kidney dipeptidyl peptidase-IV (DPP-IV) (E.C. 3.4.14.5) and glucagon-like peptide 1 (GLP-1) ELISA kit were purchased from EMD Millipore (Billerica, MA, USA). Cell culture materials including penicillin-streptomycin (0.1g/L), Dulbecco's modified Eagle's medium (DMEM) containing low glucose, Glutamax, trypsin/EDTA (10X), and Poly D-lysine were obtained from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Minneapolis, MN, USA). Bovine serum albumin, lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Griess reagent, acarbose, sitagliptin, and glutamine were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.3.2. Preparation of Green Crab

Forty kilograms of harvested crabs were washed with tap water to remove sea water on them and then blast-frozen at -30°C for 1 h. Frozen crabs were stored at -20°C until further use. Partially thawed green crabs were processed through a mechanical separator (Paoli One-Step mechanical separator, Rockford, IL) to separate meat and shell streams, and then the minced meat was vacuum sealed in plastic bags (3 mil, $3.3\text{ cm}^3/100\text{ in}^2$ oxygen transmission rate, 80 microns, UltraSource, Kansas, MO, USA). The crab mince was blast-frozen at -30°C for 1 h and then stored at 20°C until further use (Figure 3.1).

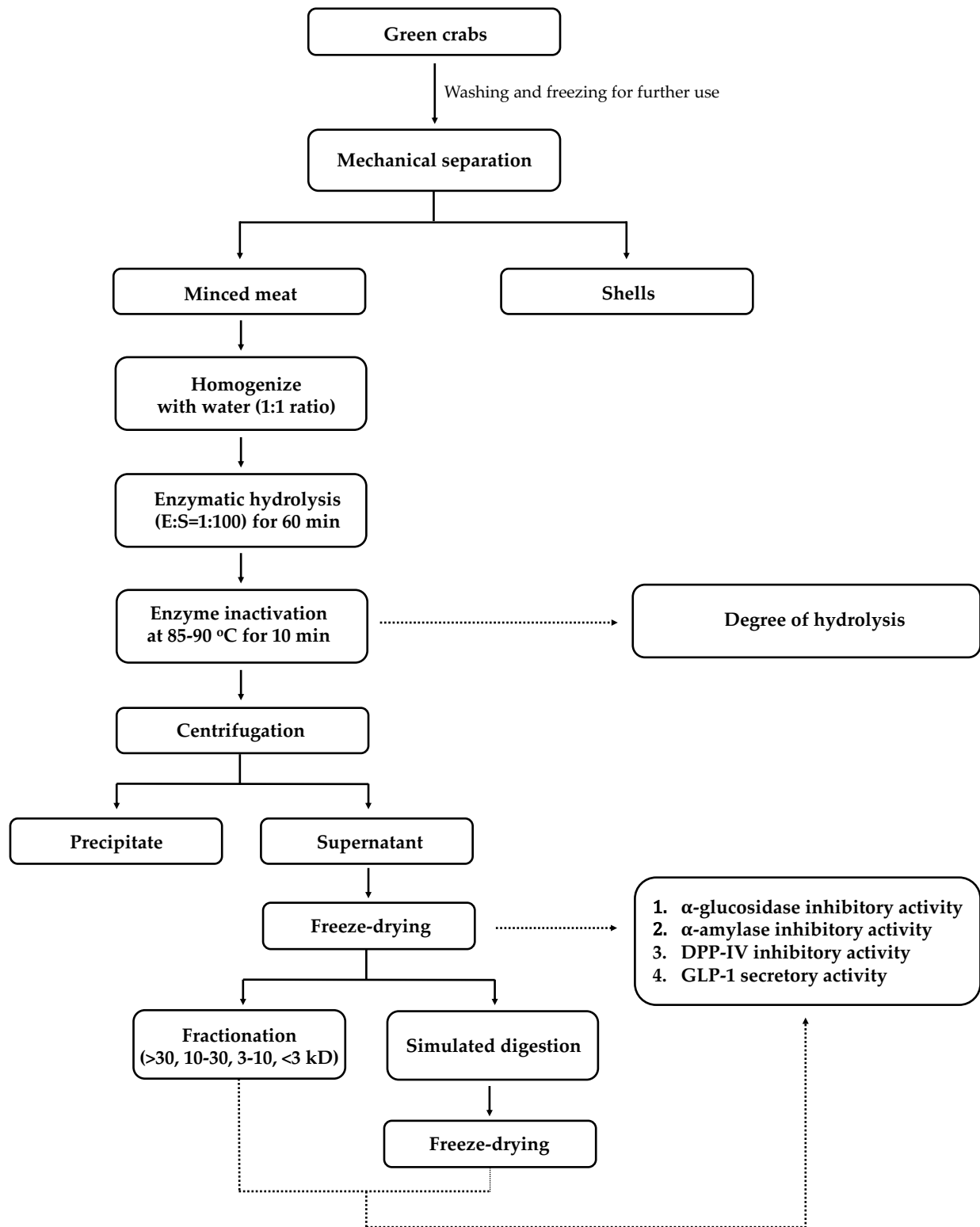


Figure 3.1. Process flow of sample preparation

3.3.3. Enzymatic Hydrolysis of Crab Mince

Enzymatic hydrolysis using commercially available proteases was conducted based on a modified protocol from Beaulieu et al. (2009). The raw crab mince (0.5 kg) was mixed with deionized water in a 1:1 (w/w) ratio, then homogenized for 1 min in a 1 L Waring blender (33BL79, New Hartford, CT, USA) at maximum speed. This step was conducted two times for each batch due to the limited volume of the blender. The enzymatic hydrolysis was conducted at the optimum pH and temperature of each enzyme including Alcalase (AL, 50 °C and pH 8), Protamex (PR, 50 °C and pH 7), Flavourzyme (FL, 50 °C and pH 7), and Papain (PA, 65 °C and pH 6). Protein content was calculated based on total nitrogen content as analyzed by a combustion analyzer (TRU MAC CNS, LECO Corp., St. Joseph, MI, USA) using a protein conversion factor of 6.25. Enzyme was added to the homogenate based on substrate (protein) mass (1:100 = E:S), and the mixture was hydrolyzed by an overhead stirrer (Fisher Scientific, Dubuque, IA, USA) for 60 min. During hydrolysis, the pH of the mixture was maintained using 6 N HCl or 6 N NaOH and temperature was monitored. After hydrolysis, the mixture was heated with stirring on a hot plate at 85–90 °C for 10 min to inactivate the enzyme and then immediately cooled on ice. For the crab mince control (CMC), mince was homogenized with water for 1 min and then immediately heated with stirring on a hot plate at 85–90 °C for 10 min without previous enzyme treatment. Subsampling was performed to evaluate the degree of hydrolysis, and then the mixtures were centrifuged at $19,722 \times g$ for 15 min at 4 °C, and the supernatants were collected. All of the treatments were processed in triplicate. The collected supernatants were blast-frozen at –30 °C for 1 h, then freeze-dried (35 EL, VirTis Co. Inc., Gardiner, NY, USA) at –30 to 25 °C under 250 mT for 10 days. All lyophilized supernatants were stored at –80 °C until further use.

3.3.4. Degree of Hydrolysis

Degree of hydrolysis was determined following the O-phthalaldehyde (OPA) method (Nielsen, 2001; Karayannakidis et al., 2014). OPA reagent was prepared with 375 mL of deionized water, 19.05 g

of sodium tetraborate decahydrate, 500 mg of sodium dodecyl sulfate (SDS), and 400 mg of 97% OPA in 10 mL of ethanol. After mixing, 440 mg of 99% dithiothreitol (DTT) was added to the solution and deionized water was added to achieve a final volume of 500 mL. For the sample preparation, the CMC and enzyme hydrolysates (1 g) were diluted (1:19 w/v) with 4% SDS solution. After centrifugation at $1100 \times g$ for 10 min, 4 mL of supernatant was collected. Subsequently, the supernatant was diluted to 50 mL with deionized water. Four mL of OPA reagent were mixed with 400 μ L of solubilized sample/standard (0.5 mg/mL serine) and the mixture was incubated at room temperature for 2 min. For a blank, 400 μ L of deionized water was used instead of sample/standard. Absorbance was measured at 340 nm and the degree of hydrolysis (%) was calculated based on the following three equations:

Serine-NH₂ (mequiv/g protein)

$$= \frac{((OD_{\text{sample}} - OD_{\text{blank}})) / ((OD_{\text{standard}} - OD_{\text{blank}})) \times 0.9516 \text{ mequiv/L} \times 0.05 \times 100}{(W \times V1 / V2 \times P)}$$

W = weight of sample (g);

P = protein (g/100 g sample);

V1 = volume of supernatant (4 mL);

V2 = volume of SDS solution (20 mL);

0.05 = sample volume in L.

$$h = \frac{((\text{Serine-NH}_2) - \beta)}{\alpha}$$

h = number of hydrolyzed peptide bonds;

$\alpha = 1.00$, $\beta = 0.40$ mequiv/g protein (α , β are constants given by Adler-Nissen, 1986);

$$DH (\%) = \frac{h \times 100}{h_{\text{total}}}$$

Total number of peptide bonds (h_{total}) of fish: 8.6 mequiv/g protein.

3.3.5. Simulated Gastrointestinal Digestion

Simulated gastrointestinal digestion of the control and crab mince hydrolysates was performed according to González-Montoya et al. (2018). The sample (25 g) was dissolved in deionized water (5% w/v) in a 500 mL Erlenmeyer flask, then pH was adjusted to pH 2 using 6 N HCl prior to heating to 37 °C. Pepsin was added to the sample mixture (4:100 = E:S protein/peptide mass) and stirred at 37 °C for 60 min. Then, pH was adjusted to 7.5 using 6 N NaOH. Subsequently, pancreatin (4:100 = E: S protein/peptide mass) was added for the intestinal digestion phase and the mixture was stirred at 37 °C for 2 h. To inactivate the digestive enzymes, the mixture was heated at 85–90 °C for 10 min. The mixture was freeze-dried (35 EL, VirTis Co. Inc., Gardiner, NY, USA) at –30 to 25 °C under 250 mT for 10 days and then stored at –80 °C until further use.

3.3.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Molecular weight distribution was determined by SDS-PAGE. All of the samples (500 mg) were dissolved in 5 mL of Laemmli sample buffer (Laemmli, 1970) containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 25% (v/v) glycerol, 0.01% bromophenol blue powder, and 5% β-mercaptoethanol, then heated in a 90–95 °C water bath for 5 min. Samples were separated on a 4% stacking and 16.5% separating gel (Bio-Rad Laboratories, Hercules, CA) using Tris/Glycine/SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) in a Bio-Rad Mini-PROTEAN III Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amount that was loaded to the wells was adjusted to 1 mg for the hydrolysates, 0.5 mg for the control, and 10 µL of a protein molecular weight standard (Precision Plus Protein™ Dual Xtra Prestained Protein Standards 2-250 kD, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Separated proteins were fixed for 1h in a gel fixing solution containing 50% (v/v) ethanol and 10% (v/v) acetic acid then washed for 30 min with a solution consisting of 50% (v/v) methanol and 10% (v/v) acetic acid. Following washing, gel staining was performed using Coomassie blue R-250 solution at room temperature for 3 h with gentle

agitation (Bio-Rad Laboratories, Inc., Hertfordshire, city, UK), then the gel was destained for approximately 3 h with washing solution until the background color was removed.

3.3.7. Fractionation by Ultrafiltration

Green crab hydrolysates were fractionated using ultrafiltration based on molecular weight. A volume of 15 mL of solubilized sample (50 mg/mL) was added to a 30 kD molecular weight cut-off (MWCO) filter device (Amicon® ultra-15 centrifugal filters, EMD Millipore, Burlington, MA, USA) for the first fraction, then centrifuged at $3234 \times g$ until the volume of retentate reached 250 μ L. After collecting the retentate, the <30 kD filtrate was transferred to a 10 kD MWCO filter device for the second fraction. After centrifugation at $3234 \times g$, 250 μ L of retentate was collected. The <10 kD filtrate was added to a 3 kD MWCO filter device for the third fractionation and was centrifuged at $3234 \times g$ until the retentate volume reached 250 μ L. Both the retentate and the <3 kD fraction were collected and all the hydrolysate fractions were stored at $-80 \text{ }^\circ\text{C}$ until used for further assays.

3.3.8. RAW 264.7 Cell Culture

RAW 264.7 macrophage cells (American Type Culture Collection ATCC, TIB 71, Rockville, MD, USA) that are widely used to measure nitric oxide production were used in this study. RAW 264.7 cells were cultured in 75 cm² plastic culture flasks with 15 mL of DMEM containing 25 mM glucose supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine (pH 7.4) at 5% CO₂ and 37 °C (370 Steri-Cycle CO₂ Water Jacketed Incubator, Thermo Fisher Scientific, Waltham, MA, USA). RAW 264.7 cells at 80% confluency were regularly scraped for subculture and 80% confluency was determined by observation using a microscope (CK40, Olympus, Center Valley, PA, USA).

3.3.9. RAW 264.7 Cell Viability Assay

Cell viability assay was performed to test the cytotoxicity of samples and determine the maximum concentration of samples suitable for assessment of the anti-inflammatory activity. Viability

of the RAW 264.7 cells was evaluated using the MTT assay. A density of 1×10^4 cells was seeded in each well of 96-well plates and incubated at 37 °C and 5% CO₂ for 24 h. After replacing old media with fresh DMEM, a volume of 20 µL sample solution (final concentration: 0.2–1 mg/mL) was added to the cells and incubated at 37 °C and 5% CO₂ for 24 h. Then, the mixture was replaced with 100 µL of 0.5 mg/mL MTT reagent and incubated at 37 °C and 5% CO₂ for 2 h. The MTT reagent was removed, then the precipitate was solubilized in 100 µL of DMSO. The absorbance was measured at 540 nm against ultrapure water by a microplate reader (Ex 808, Biotek, Winooski, VT, USA) and the cell viability (%) was calculated based on the negative control containing 20 µL of ultrapure water in place of the sample solution. For the assay blank, 20 µL of ultrapure water was treated without cells. The cell viability at different sample concentrations was calculated as follows:

$$\% \text{ cell viability} = \frac{(\text{Abs. sample} - \text{Abs. assay blank}) \times 100}{(\text{Abs. negative control} - \text{Abs. assay blank})}$$

3.3.10. Assay for Inhibition of Cellular Nitric Oxide Production

NO production was indirectly measured by nitrite content due to the fast oxidation of NO. RAW 264.7 cells obtained from ATCC were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine (pH 7.4) in a 5% CO₂ atmosphere at 37 °C. The cells were stimulated with LPS according to Tsai et al. (2014) with minor modification. Briefly, a density of 1×10^6 cells was plated in each well of 12-well plates and incubated at 37 °C and 5% CO₂ for 24h. After removing media, 880 µL of clear DMEM and 200 µL of sample solution (final concentration: 0.2-1 mg/mL) were added to the cells and different concentrations of samples were treated to observe a concentration dependency. Then, the cells were pre-incubated at 37 °C and 5% CO₂ for 1 h. Then, 120 µL of LPS (10 µg/mL) was added and incubated 37 °C and 5% CO₂ for 24 h. The media containing secreted NO was collected from each well. Nitrite content was measured by adding 100 µL of Griess reagent to 100 µL of collected media. After 15 min, the absorbance was measured at 540 nm against ultrapure

water by a microplate reader (Ex 808, Biotek, Winooski, VT, USA). Produced nitrite content was calculated based on the assay control containing 20 μL of ultrapure water in place of the sample solution. For the assay blank, 20 μL of ultrapure water was treated without cells. For a standard curve, nitrite solutions ranging from 0-130 μM were used.

$$\% \text{ NO production} = \frac{(\text{OD Sample} - \text{OD Assay blank}) \times 100}{(\text{OD Assay control} - \text{OD Assay blank})}$$

3.3.11. Rat Intestine α -Glucosidase Inhibition Assay

The rat intestine α -glucosidase inhibition assay was conducted according to the protocol in the Worthington Enzyme Manual (1993a) with modifications and Kwon et al. (2007). Crude enzyme was extracted from rat intestine acetone powder. For the extraction, 0.3 g of rat intestinal acetone powder was added to 12 mL of 0.1 M sodium phosphate buffer (pH 6.9 with 0.9% NaCl), then sonicated 12 times in 30 s pulses. After centrifugation at $10,000 \times g$ for 30 min at 4 $^{\circ}\text{C}$, the supernatant was used as the enzyme solution. A volume of 50 μL of solubilized sample (final concentration: 6-60 mg/mL) or acarbose (positive control) and 100 μL of enzyme solution were added in a 96 well plate then incubated at 37 $^{\circ}\text{C}$ for 10 min. Then, 50 μL of 5 mM p-NPG solution in 0.1 M phosphate buffer (pH 6.9 with 0.9% NaCl) was added and the mixture was incubated at 37 $^{\circ}\text{C}$ for 30 min. The absorbance was measured at 405 nm against ultrapure water by a microplate reader (Ex 808, Biotek, Winooski, VT, USA). The assay control contained 50 μL of 0.1 M sodium phosphate buffer in place of the sample. For the assay blank, substrate and sample were replaced with 0.1 M sodium phosphate buffer and ultrapure water, respectively. In the sample blank, 0.1 M sodium phosphate buffer was added instead of substrate. The α -glucosidase inhibitory activity was calculated as follows:

$$\% \text{ inhibition} = \frac{[(\text{Abs. assay control} - \text{Abs. assay blank}) - (\text{Abs. sample} - \text{Abs. sample blank})] \times 100}{(\text{Abs. assay control} - \text{Abs. assay blank})}$$

Sample concentration (mg/mL) was plotted vs. % inhibition, and the IC_{50} (the half maximal inhibitory concentration) values were calculated based on linear regression or log transformation.

3.3.12. Porcine Pancreatic α -Amylase Inhibition Assay

The α -amylase inhibitory activity was evaluated following a modified version of the assay described by the Worthington Enzyme Manual (1993b). Porcine pancreatic α -amylase solution (100 unit/mL) was prepared in 0.02 M sodium phosphate buffer (pH 6.9 with 6 mM NaCl). A volume of 100 μ L of solubilized sample (final concentration: 10-60 mg/mL) or acarbose (positive control) and 100 μ L of enzyme solution was incubated at 25 °C for 10 min, then 100 μ L of 1% soluble starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 6 mM NaCl) was added. After incubation at 25 °C for 10 min, 200 μ L of dinitrosalicylic acid color reagent was added, and, subsequently, the mixture was placed in a boiling water bath for 5 min. After cooling, 50 μ L of the mixture was diluted with 200 μ L of deionized water and the absorbance was measured at 540 nm against ultrapure water by a microplate reader (Ex 808, Biotek, Winooski, VT, USA). The assay control contained 100 μ L of 0.02 M sodium phosphate buffer in place of the sample. In the assay blank, 0.02 M sodium phosphate buffer and ultrapure water were added instead of the enzyme and sample. Enzyme was replaced with 0.02 M sodium phosphate buffer for the sample blank. The α -amylase inhibitory activity was calculated as follows:

$$\% \text{ inhibition} = \frac{[(\text{Abs. assay control} - \text{Abs. assay blank}) - (\text{Abs. sample} - \text{Abs. assay blank}) \times 100}{(\text{Abs. assay control} - \text{Abs. assay blank})}$$

Sample concentration (mg/mL) was plotted vs. % inhibition, and the IC₅₀ values were calculated based on linear regression or log transformation.

3.3.13. DPP-IV Inhibition Assay

DPP-IV impedes insulin secretion by inactivating GLP-1. In this study, the DPP-IV inhibitory activity was determined using a method from Li-Chan et al. (2012). DPP-IV enzyme was purchased from EMD Millipore (Burlington, MA, USA) and 0.01 unit/mL of enzyme solution was prepared in 100 mM Tris-HCl buffer (pH 8.0). A total of 25 μ L of sample (final concentration: 6-60 mg/mL) and sitagliptin (positive control) and 25 μ L of 1.59 mM Gly-Pro-p-nitroanilide solution in 100 mM Tris-HCl buffer (pH

8.0) was preincubated at 37 °C for 10 min, then 50 µL of enzyme solution was added. After incubation at 37 °C for 60 min, 100 µL of 1 M sodium acetate solution was added to stop the reaction. The absorbance was determined at 405 nm against ultrapure water by a microplate reader (Ex 808, Biotek, Winooski, VT, USA). The assay control contained 25 µL of 100 mM Tris-HCl buffer instead of the sample solution. For the assay blank, 100 mM Tris-HCl buffer and ultrapure water were added instead of the enzyme and sample. Enzyme was replaced with 100 mM Tris-HCl buffer in the sample blank. The DPP-IV inhibitory activity was expressed as percentage inhibition and was calculated as follows:

$$\% \text{ inhibition} = \frac{[(\text{Abs. assay control} - \text{Abs. assay blank}) - (\text{Abs. sample} - \text{Abs. assay blank}) \times 100}{(\text{Abs. assay control} - \text{Abs. assay blank})}$$

Sample concentration (mg/mL) was plotted vs. % inhibition, and IC₅₀ values were calculated based on linear regression or log transformation.

3.3.14. GLUTag Cell Culture

Murine GLUTag cells that are widely used for the stimulation of GLP-1 secretion were used in this study. GLUTag cells were gifted from Dr. D.J. Drucker (University of Toronto, Toronto, Canada) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 5.5 mM glucose (pH 7.4) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine in a 75 cm² culture flask at 5% CO₂ and 37 °C. GLUTag cells at 80% confluency were regularly trypsinized for subculture. Trypsin (3 mL) was treated to the cells for 7 min to detach the cells from the flask and collected cells were subcultured in new flasks. The 80% confluency was determined by observation using a microscope (CK40, Olympus, Center Valley, PA, USA).

3.3.15. GLUTag Cell Viability Assay

Cell viability assay was performed to examine the cytotoxicity of samples and determine the sample concentration for the GLP-1 secretion assay. Viability of the GLUTag cells was evaluated using the MTT assay. The surface of 96-well culture plates was coated with poly-D-lysine solution. A density of

2×10^4 cells was seeded in each well and incubated at 37 °C, 5% CO₂, and 80% humidity for 48 h. Sample solutions of different concentration (final concentration: 0.2–10 mg/mL) were added to the cells to observe the concentration dependency and incubated for 24 h. Then, the mixture was replaced with 100 µL of 0.5 mg/mL MTT reagent and incubated at 37 °C and 5% CO₂ for 4 h. The MTT reagent was removed by pipetting, then the precipitate was solubilized in 100 µL of DMSO with pipetting. The absorbance was measured at 540 nm against ultrapure water by a microplate reader (Ex 808, Biotek, Winooski, VT, USA). In the negative control, 20 µL of ultrapure water was replaced with the sample solution. For the assay blank, 20 µL of ultrapure water was treated without cells. The cell viability was calculated as follows:

$$\% \text{ cell viability} = \frac{(\text{Abs. sample} - \text{Abs. assay blank}) \times 100}{(\text{Abs. negative control} - \text{Abs. assay blank})}$$

3.3.16. GLP-1 Secretion Assay

GLP-1 secretory activity was evaluated by an assay modified from Ojo et al. (2013). GLUTag cells were seeded at a density of 2×10^5 in 24-well culture plates coated with poly-D-lysine and grown for 48 h in a 37 °C and 5% CO₂ incubator until the confluency reached 80%. The cells were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) by pipetting, then 1 mL of Krebs Ringer Buffer (KRB) containing 115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM HEPES, and 1M NaHCO₃ supplemented with 1% bovine serum albumin (BSA, fatty-acid-free) and 1 mM glucose (pH 7.4) was added. After pre-incubation at 37 °C and 5% CO₂ for 45 min, the KRB was replaced with 1 mL of KRB test solutions containing sample (final concentration: 0.5 g/mL) or 10 mM L-glutamine. L-glutamine (10 mM) was selected as a positive control since GLP-1 secretion activity by 10 mM L-glutamine was significantly higher than the activity by 10 mM glucose which is a major stimulating nutrient of GLP-1 (Reimann et al., 2006). All the KRB test solutions were prepared with supplementation of 1% BSA and 2 mM glucose (pH 7.4). The culture plates were incubated at 37 °C and 5% CO₂ for 2 h, then the mixture was collected. After centrifugation at 4 °C and 800× g for 5 min, the supernatant was collected and stored at –80 °C until further evaluation of GLP-1

concentration. Total GLP-1 concentration was determined based on the manufacturer's instructions in a commercial ELISA kit (GLP-1 Total ELISA, Millipore, Burlington, MA, USA). The GLP-1 secretory activity was expressed as a percentage (%) of the negative control (KRB buffer).

3.3.17. Statistical Analysis

The enzymatic hydrolysis process using each of the four commercial proteases was replicated three times and all of the assays were conducted in triplicate on each sample replicate. Statistical differences among the means of each treatment were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test, independent t-test, and paired t-test with a significance value of $p < 0.05$ (SPSS ver. 23, IBM Corp., Armonk, NY, USA). Correlations ($p < 0.05$) between the degree of hydrolysis (DH) and each biofunctional activity were analyzed using the Pearson coefficient (SPSS ver. 23, IBM Corp., Armonk, NY, USA).

3.4. Results and Discussion

3.4.1. Degree of Hydrolysis

In recent years, as interest in healthy lifestyles has increased, the demand for healthy diets, natural food ingredients, and bioactive compounds has intensified in consumers as well as the food industry. Some proteins and peptides are being used as nutraceutical ingredients in dietary supplements to enhance human health and product value. Many bioactive peptides having antioxidant, anti-hypertensive, and anti-hyperglycemic effects have been derived from enzymatic hydrolysis of protein sources including meat, dairy, fish, and their byproducts (Ndiaye et al., 2012; Pan et al., 2012; Lacroix & Li-Chan, 2013; Liu et al., 2013; Ovissipour et al., 2013; Chakka et al., 2015; Sánchez & Vázquez, 2017; Harnedy et al., 2018). However, there has been limited investigation into bioactive peptides derived from molluscan and crustacean resources.

The bioactive properties of peptides obtained by enzymatic hydrolysis are influenced by (1) the primary amino acid sequence of the protein source, (2) the specificity of the proteases applied, and (3) process parameters such as DH, hydrolysis duration, and enzyme:substrate ratio. As a primary protein source, underutilized green crab was used, which contains ~80% protein in its meat (Kang et al., 2019). Manual removal of green crab meat from the carapace is extremely labor-intensive because of the crab's very small size. Therefore, in this study, a mechanical separator was used to generate green crab meat mince that was subsequently used as the substrate for the enzymatic hydrolysis. To apply proteases with various specificity to the crab homogenate, four commercially available proteases that have two different modes of action were selected. AL, PR, and PA are endopeptidases, while FL has both endo- and exopeptidase characteristics. After hydrolysis, the DH was determined based on the number of cleaved peptide bonds over the total number of peptide bonds.

The degree of hydrolysis (DH) was determined by the OPA method with serine as a standard. DH was significantly increased after enzymatic hydrolysis for 60 min. DH of green crab hydrolysates ranged from 15.8% to 18.4%, while DH of the CMC (crab mince control) was 6.8%. Among the treatments, AL showed the highest DH ($18.4\% \pm 0.4\%$) followed by the PR ($17.1\% \pm 0.2\%$), FL ($16.5\% \pm 1.2\%$), and PA ($15.8\% \pm 0.3\%$) treatments, respectively (Figure 3.2). Based on these results, DH significantly increased in response to enzyme treatment. According to Jamdar et al. (2010), the bioactivity of protein hydrolysates was modified as DH was changed within the same enzyme treatment. Therefore, the determination of DH is necessary for controlling the enzymatic hydrolysis process to reproducibly obtain bioactive peptides.

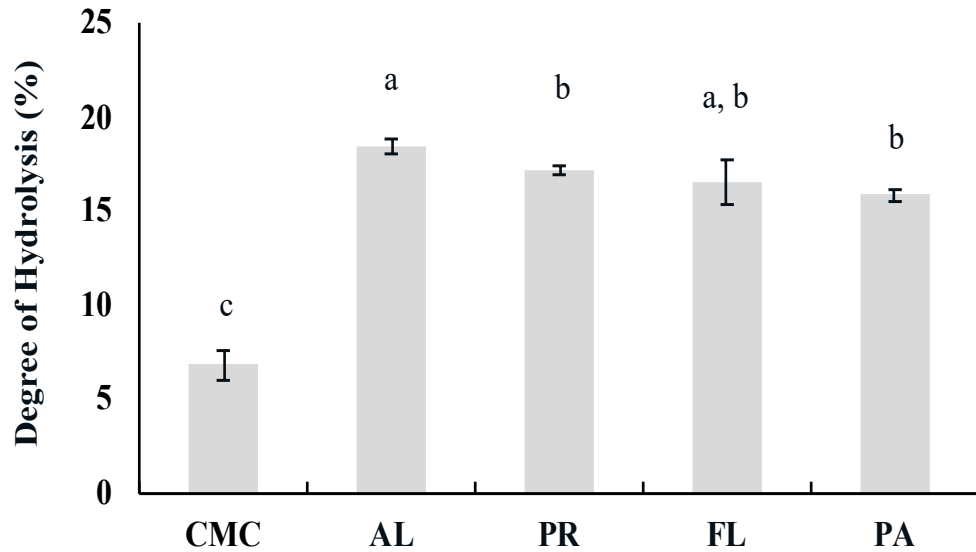


Figure 3.2. Degree of hydrolysis of green crab mince by enzyme type. CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation. Different letters on the bars indicate a significant difference ($p < 0.05$) among the treatments ($n = 3$).

3.4.2. Molecular Weight Distribution Using SDS-PAGE

The size distribution of proteins and peptides in the CMC and hydrolysates was determined using SDS-PAGE. Significant hydrolysis was observed in all of the treatments compared to the CMC (Figure 3.3a). Heavy chains in the 150–250 kD range were reduced by all the enzyme treatments. There were thick bands between 100 kD and 75 kD in the hydrolysates derived by FL and PA, and all of the treatments produced various bands between 20 kD and 37 kD. Similar band patterns were observed in the AL and PR treatments while FL and PA treatments resulted in similar molecular weight profiles. AL-derived hydrolysate showed the lowest intensity of bands between 2 and 10 kD compared to the CMC and other hydrolysates.

During simulated digestion, the CMC was hydrolyzed by digestive enzymes as compared to the process control that was applied to the simulated digestion with no digestive enzymes (Figure 3.3b). The simulated digestion process further hydrolyzed all the hydrolysates obtained from commercial enzyme

treatments. The intensity of bands above 15 kD decreased significantly as compared to before the simulated digestion, and the intensity of observed bands at <10 kD also decreased after the simulated digestion.

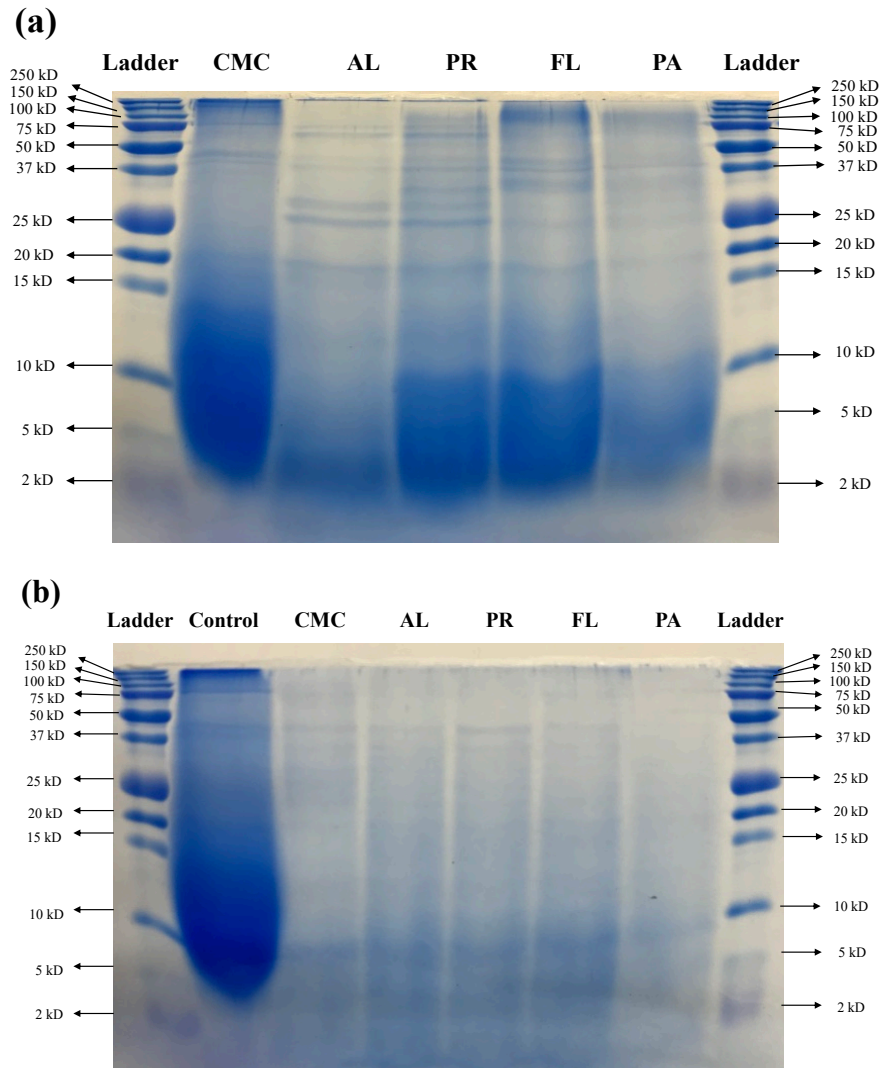


Figure 3.3. Molecular weight distribution (SDS-PAGE) of CMC and hydrolysates before and after simulated digestion. CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. **(a)** Before simulated digestion. Lane 1 and 7: molecular weight ladder. Lanes 2–6: CMC, AL-derived hydrolysate, PR-derived hydrolysate, FL-derived hydrolysate, and PA-derived hydrolysate, respectively. **(b)** After simulated digestion. Lanes 1 and 8: molecular weight ladder. Lanes 2–7: simulated digestion process control, CMC, AL-derived hydrolysate, PR-derived hydrolysate, FL-derived hydrolysate, and PA-derived hydrolysate, respectively.

In addition to the increase in DH, the molecular weight distribution of the CMC and hydrolysates before the simulated digestion also confirms that the enzymatic process was successful (Figure 3.3), and that different products were generated based on the commercial protease applied. The weak intensity of AL-derived hydrolysate bands between 2 and 10 kD may be associated with its DH. In many studies, AL showed an outstanding ability to hydrolyze large proteins to small peptides, and produced smaller peptides more rapidly compared to other commercially available proteases (Smyth & FitzGerald, 1998; Aspino et al., 2005). Therefore, the lower intensity of the 2–10 kD bands in the AL treatment was most likely due to a large number of peptides smaller than <2 kD passing through the gel during the SDS-PAGE. After the simulated digestion, the amount of larger size peptides was decreased, and the overall intensity of all the bands became weaker. This demonstrates that proteins and peptides in the CMC and hydrolysates were further hydrolyzed by the digestive enzymes and suggests that the smaller sized peptides may have passed through the gel during electrophoresis.

3.4.3. RAW 265.7 Cell Viability

Determination of cell viability is important since it evaluates the cytotoxicity of samples when the cells are seeded onto a plate. In the cell viability determination, the number of live cells is quantified and is usually represented as a percentage of the assay control which is not exposed to any treatment (King, 2000; Kroemer et al., 2009). The MTT assay developed by Mosmann (1983) has been widely used as a gold standard for determination of cell viability and proliferation. In this assay, the cell viability is measured based on reducing activity as enzymatic conversion of the tetrazolium compound to water insoluble formazan crystals by dehydrogenases produced in the mitochondria of living cells. To determine the cytotoxicity of CMC and hydrolysates, the cell viability after exposure to different concentrations of CMC and hydrolysates was evaluated on RAW 264.7 macrophage cells. RAW 264.7 cells were treated with green crab hydrolysates (1, 2, 4, 8, and 10 mg/mL). No cytotoxic effect of any of the samples was observed at a concentration of 1 mg/mL (Figure 3.4). Therefore, the inhibitory activity

of CMC and hydrolysates on nitric oxide production was determined at concentrations ≤ 1 mg/mL using RAW 264.7 cells.

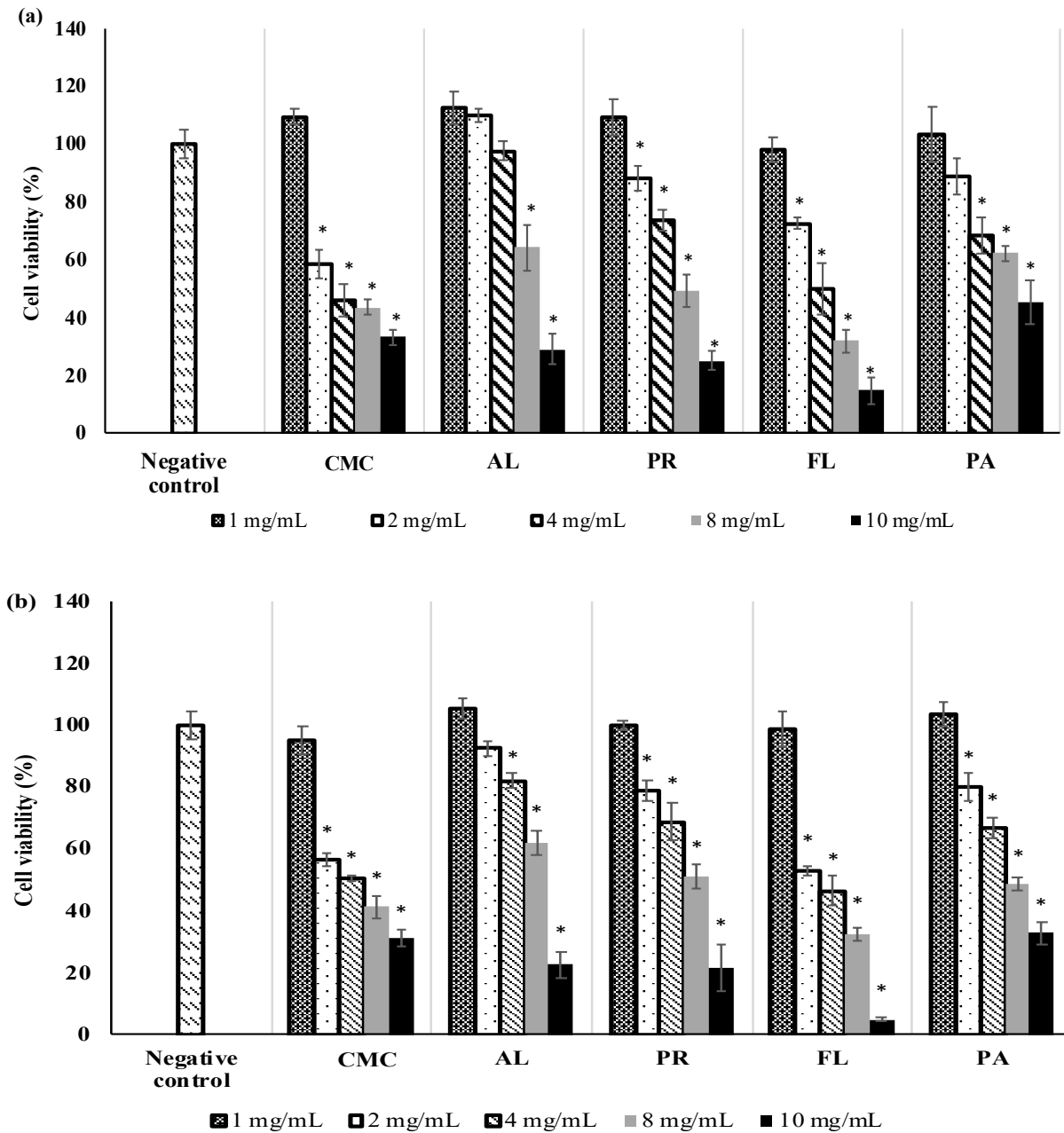
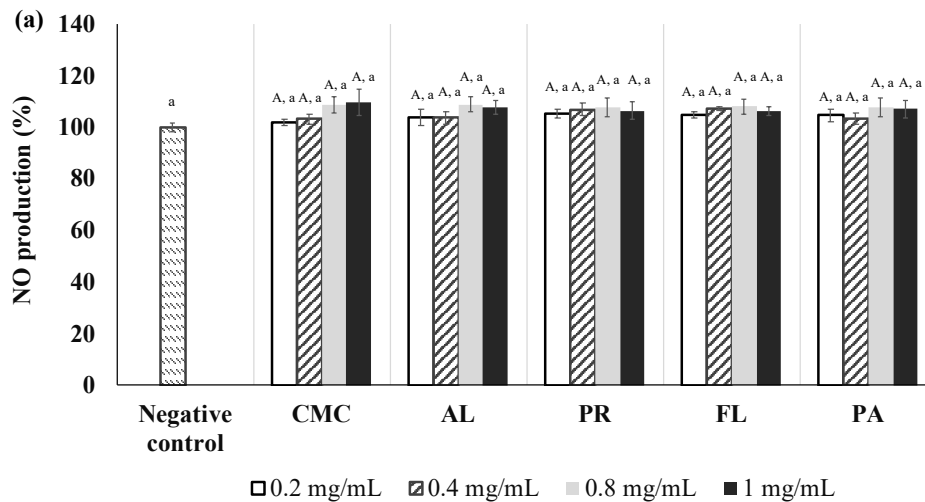


Figure 3.4. Cytotoxic effect of CMC and green crab hydrolysates before and after simulated digestion determined using RAW 264.7 cells. (a) Before simulated digestion (b) After simulated digestion. CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation (n = 3 treatment replicates). Asterisk (*) represents significant difference between negative control and each sample (p < 0.05).

3.4.4. Effect of the Green Crab Hydrolysates on NO Production

The RAW 264.7 cell line is available for an *in vitro* model of macrophage-mediated inflammatory studies. In this study, RAW 264.7 cells were used to evaluate the inhibitory activity of the green crab hydrolysates on NO synthesis. The inducible forms of nitric oxide synthase (iNOS) are critically involved in immune response. As proinflammatory enzymes, iNOS are responsible for NO production, and overproduced NO by iNOS is considered a cytotoxic molecule in inflammation. Thus, the NO production inhibitory activity of green crab hydrolysates was determined. However, NO in the biological system is an extremely unstable molecule that is rapidly oxidized to nitrite. Therefore, as an indirect method to measure NO production, the nitrite content is widely measured as an index of NO production.

NO production of RAW 264.7 cells was stimulated by LPS, then crab hydrolysates were applied to the cells. NO production by the treated cells was not significantly decreased (Figure 3.5). Therefore, enzymatic hydrolysis did not improve the anti-inflammatory activity of the crab mince as measured by NO production.



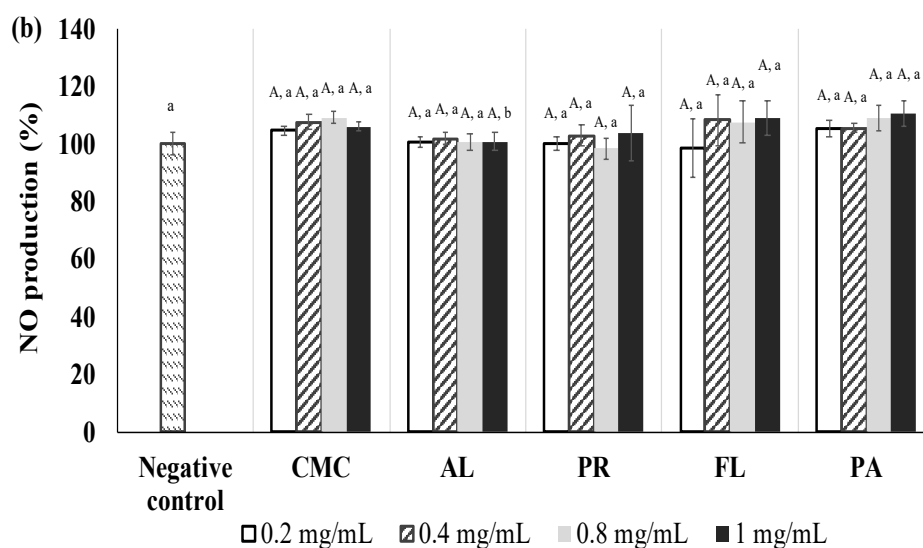


Figure 3.5. Effect of the green crab hydrolysates on NO Production using LPS-stimulated RAW 264.7 cells. CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation ($n = 3$ treatment replicates). Uppercase letters on the bars indicate a significant difference ($p < 0.05$) within each treatment. Lowercase letters on the bars indicate a significant difference ($p < 0.05$) between the negative control and each sample.

3.4.5. α -Glucosidase and Porcine α -Amylase Inhibitory Activities

The inhibitory activity of samples on rat intestinal α -glucosidase and porcine pancreatic α -amylase was assessed to evaluate the potential anti-hyperglycemic effect of the CMC and crab hydrolysates. IC_{50} values for α -glucosidase and α -amylase were determined to evaluate the efficacy of the inhibition. Acarbose was used as a positive control since it is a well-known inhibitor of carbohydrate hydrolyzing enzymes. Acarbose had IC_{50} values of 0.027 mg/mL and 0.84 mg/mL for α -glucosidase and α -amylase inhibitory activity, respectively. The α -glucosidase inhibitory activity of the CMC was significantly improved by the PR ($IC_{50} 1.38 \pm 0.19$ mg/mL) and PA ($IC_{50} 5.56 \pm 0.19$ mg/mL) treatments while the FL ($IC_{50} 20.24 \pm 0.19$ mg/mL) treatment showed a decrease in α -glucosidase inhibitory activity compared to the CMC ($IC_{50} 8.54 \pm 0.50$ mg/mL) (Figure 3.6a). Simulated digestion did not significantly affect the α -glucosidase inhibitory activity of hydrolysates obtained from the PR ($IC_{50} 1.49 \pm 0.08$ mg/mL)

and PA (IC_{50} 5.31 ± 0.51 mg/mL) treatments. However, the inhibitory activity of the FL-derived hydrolysate was significantly improved after the simulated digestion (IC_{50} 10.61 ± 1.90 mg/mL). The green crab hydrolysates obtained from PR showed the highest α -glucosidase inhibitory activity before and after simulated digestion and the activity was approximately 4–14 times higher compared to other enzyme treatments and CMC based on IC_{50} values.

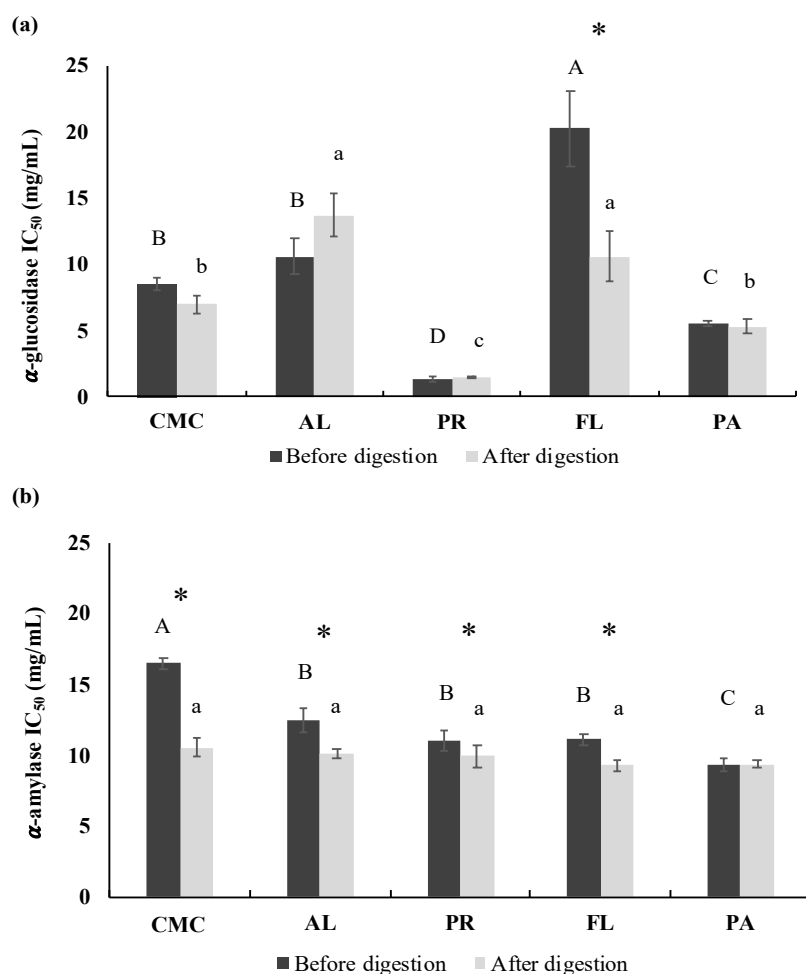


Figure 3.6. Rat intestinal α -glucosidase and porcine α -amylase inhibitory activities of CMC and hydrolysates before and after simulated digestion. **(a)** Rat intestinal α -glucosidase inhibitory activity. **(b)** Porcine α -amylase inhibitory activity. The IC_{50} values were represented by final assay concentration (protein and peptide basis). CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation ($n = 3$ treatment replicates). The letters on the bars indicate a significant difference ($p < 0.05$) among the treatments. An asterisk (*) represents significant difference after simulated digestion ($p < 0.05$) by paired t -test.

The porcine pancreatic α -amylase inhibitory activity of the CMC was statistically increased by enzymatic hydrolysis using commercial enzymes. Among the treatments, PA (IC_{50} 9.35 ± 0.42 mg/mL) exhibited the highest α -amylase inhibitory activity followed by PR (IC_{50} 11.02 ± 0.69 mg/mL), FL (IC_{50} 11.12 ± 0.37 mg/mL), AL (IC_{50} 12.53 ± 0.85 mg/mL), and the CMC (IC_{50} 16.49 ± 0.41 mg/mL), respectively (Figure 3.6b). Digestive enzymes including pepsin and pancreatin significantly improved the α -amylase inhibitory activity of CMC (IC_{50} 10.55 ± 0.68 mg/mL) and its hydrolysates treated with AL (IC_{50} 10.11 ± 0.34 mg/mL), PR (IC_{50} 9.93 ± 0.82 mg/mL), and FL (IC_{50} 9.31 ± 0.37 mg/mL), however, the inhibitory activity of PA-derived hydrolysate was not affected by simulated digestion. After simulated digestion, the α -amylase inhibitory activity among the treatments was not significantly different.

Pancreatic α -amylase and intestinal α -glucosidase are the two key enzymes involved in starch digestion, resulting in an increase in blood glucose levels. Therefore, the inhibition of both carbohydrases can be an indicator of potential anti-hyperglycemic effect (Hirsh et al., 1997). According to the DH and SDS-PAGE results, AL hydrolyzed the green crab protein to a greater degree compared to the other enzymes (Figures 3.2 and 3.3a). However, results indicate that PR generated the most effective α -glucosidase inhibitive hydrolysates (Figure 3.6a), and that the inhibitory activity was not statistically correlated ($p > 0.05$) with DH.

Based on the positive results of their α -glucosidase inhibitory and GLP-1 secretory activities, PR, FL, and PA were selected for subsequent fractionation. Figure 3.7 represents the α -glucosidase inhibitory activity of the fractions determined at 3.2 mg/mL. PR-treated fractions exhibited the highest α -glucosidase inhibitory activity among the treatments. Within the fractions, the highest α -glucosidase inhibitory activity was shown in the <3 kD fractions of all of the treatments. The <3 kD fraction of the PR-derived hydrolysate inhibited the α -glucosidase activity by 60% while the α -glucosidase inhibitory activity of other fractions was less than 50%. IC_{50} values could only be calculated for the <3 kD fraction

since the highest inhibitory activity of the >3 kD fractions was less than 50%. The PR treatment (IC_{50} 1.75 \pm 0.24 mg/mL) resulted in the highest α -glucosidase inhibitory effect followed by PA, CMC, and FL. The IC_{50} values of the control and the hydrolysates were strongly correlated with those of the <3 kD fractions ($r = 0.987$, $p < 0.05$), which indicates that the peptides in the <3 kD fraction were key contributors to the α -glucosidase inhibitory activity.

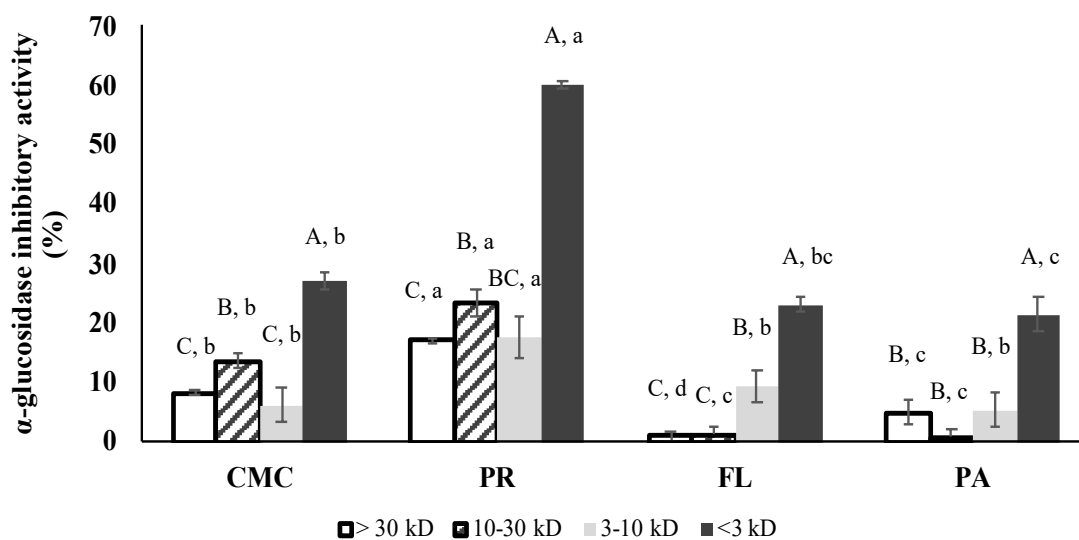


Figure 3.7. Rat intestinal α -glucosidase inhibitory activities of CMC and hydrolysates after fractionation (final assay concentration: 3.2 mg/mL protein and peptide basis). CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation ($n = 3$ treatment replicates). Uppercase letters on the bars indicate a significant difference ($p < 0.05$) within each treatment. Lowercase letters on the bars indicate a significant difference ($p < 0.05$) among the treatments.

The α -glucosidase inhibitory activity of PR-treated sample (IC_{50} 1.38 \pm 0.19 mg/mL) was remarkably higher than not only other treatments and the control but also the hydrolysates of sardine muscle (IC_{50} 48.7 mg/mL), whey protein isolate (IC_{50} 4.5 mg/mL), and edible insects ($IC_{50} > 2.0$ mg/mL) including mealworm larvae, crickets, and silkworm pupae (Matsui et al., 1999; Lacroix & Li-Chan, 2013; Yoon et al., 2019). In many studies, AL, FL, and digestive enzymes including pepsin and trypsin are commonly used to obtain bioactive peptides (Lacroix & Li-Chan, 2013; Ambigaipalan et al., 2015;

Harnedy et al., 2018; Yoon et al., 2019). Interestingly, in the current study, AL and FL treatment did not show improved inhibitory activity compared to the control. The high α -glucosidase inhibitory activity of the PR-treated sample emphasizes the importance of protein source and protease selection in obtaining bioactive peptides. A major concern regarding the use of bioactive peptides for human consumption and industrial application are the changes in biological activity as a result of further hydrolysis during gastrointestinal digestion and processing. However, simulated digestion did not affect the α -glucosidase inhibitory activities of PR and PA-treated samples, suggesting that their inhibitory activity was stable to pepsin and pancreatin action during the gastrointestinal digestion.

The enzymatic hydrolysis by commercial enzymes, and subsequently by digestive enzymes, improved the α -amylase inhibitory activity of the green crab proteins. The inhibitory activity was most likely primarily due to the <3 kD fractions (Figures 3.6b and 3.8) since those showed the highest inhibitory activity among the same concentration of fractions. The α -amylase inhibition of the PR-treated sample was weaker than its α -glucosidase inhibition which may contribute to reducing the side-effects that are frequently caused by inhibition of carbohydrate hydrolyzing enzymes. Acarbose, a synthetic pharmaceutical α -glucosidase and α -amylase inhibitor, has been commonly used to inhibit glucose absorption in diabetic patients. However, its strong α -amylase inhibitory activity ($IC_{50} < 1$ mg/mL) leads to the presence of non-digested polysaccharides in the large intestine, which causes side-effects including severe stomach pain, constipation, and diarrhea (Chiasson et al., 2002). On the other hand, since a strong α -glucosidase inhibitor that has weak α -amylase inhibitory activity could result in the presence of oligosaccharides or disaccharides digested by α -amylase in the large intestine, it might reduce the side-effects caused by non-digested polysaccharides. Therefore, the use of α -glucosidase and α -amylase inhibitors based on the PR-derived hydrolysates might be a good alternative to delay glucose absorption and help control blood glucose spikes. The inhibitory mechanism of both α -glucosidase and α -amylase by bioactive peptides has not been well characterized. However, recent studies have

reported that hydrophobic interactions of non-saccharide compounds that allow them to bind to the carbohydrase active site may contribute to their inhibitory activity (Bharatham et al., 2008).

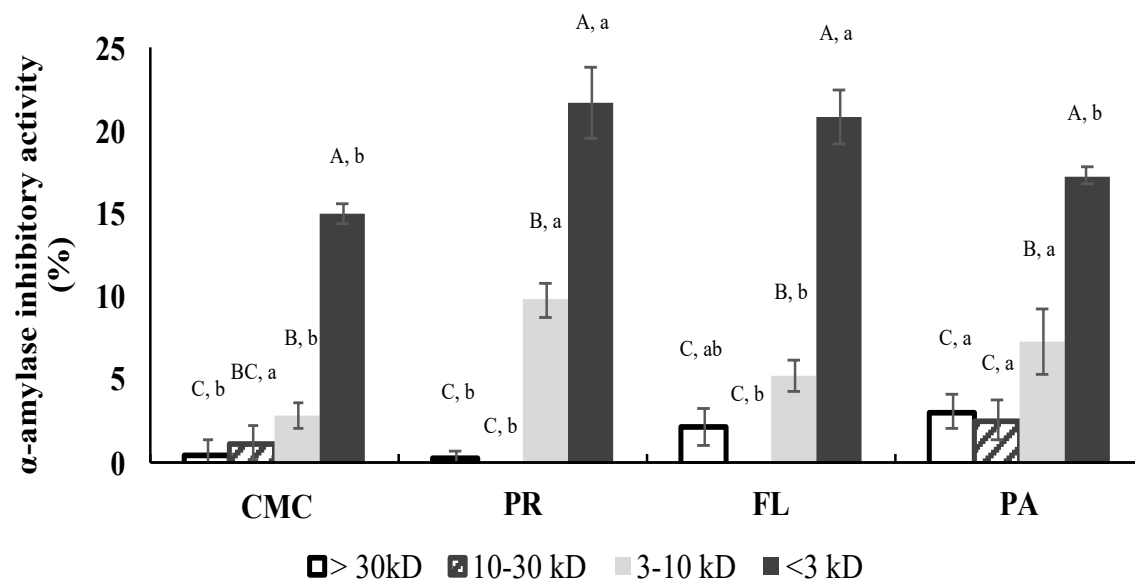


Figure 3.8. Porcine pancreatic α -amylase inhibitory activities of CMC and hydrolysates after fractionation (final assay concentration: 4.2 mg/mL protein and peptide basis). CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation ($n = 3$ treatment replicates). Uppercase letters on the bars indicate a significant difference ($p < 0.05$) within each treatment. Lowercase letters on the bars indicate a significant difference ($p < 0.05$) among the treatments.

3.4.6. DPP-IV Inhibitory Activity

Overall, DPP-IV inhibition by CMC ($IC_{50} 1.50 \pm 0.25$ mg/mL) was significantly improved by the enzymatic hydrolysis, and the DPP-IV inhibitory activity IC_{50} values of hydrolysates ranged from 0.56 mg/mL to 0.72 mg/mL (Figure 3.9). Sitagliptin was used as a positive control and had an IC_{50} value of 43.7 ng/mL. The simulated gastrointestinal digestion remarkably enhanced the DPP-IV inhibitory activity of CMC. However, the PR and PA treated samples after the simulated digestion showed significantly higher DPP-IV inhibitory activity than CMC.

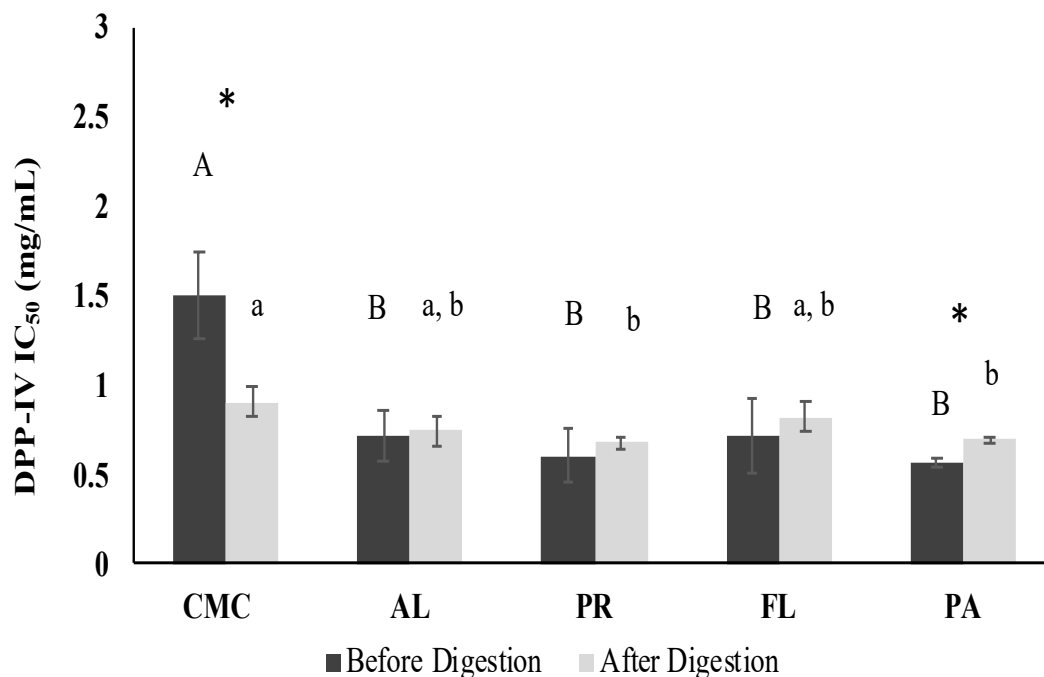


Figure 3.9. Dipeptidyl peptidase-IV (DPP-IV) IC_{50} values of CMC and hydrolysates. The IC_{50} values were represented by the final assay concentration (protein and peptide basis). CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation ($n = 3$ treatment replicates). The letters on the bars indicate a significant difference ($p < 0.05$) among the treatments. An asterisk (*) represents a significant difference after simulated digestion ($p < 0.05$) by paired t -test.

The DPP-IV inhibitory ability of the fractions obtained from the fractionation of the CMC and hydrolysates is shown in Figure 3.10. According to IC_{50} values, the <3 kD fraction (CMC: 1.39 ± 0.16 mg/mL, PR: 0.79 ± 0.05 mg/mL, FL: 0.88 ± 0.17 mg/mL, and PA: 0.93 ± 0.20 mg/mL) and 3–10 kD fraction (CMC: 1.68 ± 0.28 mg/mL, PR: 0.95 ± 0.06 mg/mL, FL: 1.15 ± 0.16 mg/mL, and PA: 0.88 ± 0.17 mg/mL) within each sample showed the highest DPP-IV inhibitory activity while the > 30 kD fraction (CMC: 4.80 ± 0.37 mg/mL, PR: 2.29 ± 0.40 mg/mL, FL: 2.70 ± 0.26 mg/mL, and PA: 2.21 ± 0.20 mg/mL) exhibited the lowest DPP-IV inhibitory activity within enzyme treatments. The DPP-IV inhibitory activity of all the fractions was improved after the commercial enzyme treatments; however, there was no significant difference among the treatments.

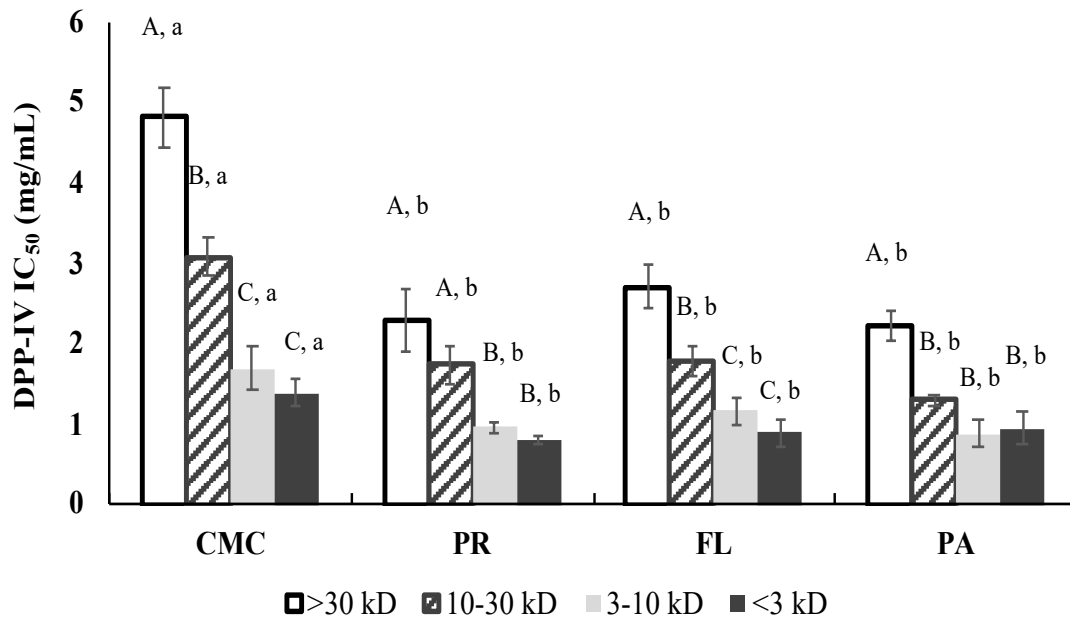


Figure 3.10. DPP-IV IC₅₀ values of CMC and hydrolysates after fractionation. The IC₅₀ values were represented by final assay concentration (protein and peptide basis). CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation ($n = 3$ treatment replicates). Uppercase letters on the bars indicate a significant difference ($p < 0.05$) within each treatment. Lowercase letters on the bars indicate a significant difference ($p < 0.05$) among the treatments.

DPP-IV is an enzyme that rapidly metabolizes incretins such as active GLP-1 and gastric inhibitory polypeptide (GIP) hormones. These incretins are important since the hormones help in blood glucose control by insulin secretion (Addison & Aguilar, 2011). DPP-IV as a postproline hydrolyzing enzyme cleaves dipeptides with X-Pro or X-Ala from the N-terminus of polypeptides (Blanco et al., 1998; Hildebrandt et al., 2000). In this study, the DPP-IV inhibitory activity of the CMC and hydrolysates was investigated as a potential strategy for T2DM management. Enzyme hydrolysis improved the DPP-IV inhibitory activity regardless of the protease applied, and the activity was not significantly ($p > 0.05$) correlated with DH. Recent studies on DPP-IV inhibitors derived from food sources reported that peptides containing Pro, Ala, and Gly at the P1-position and/or Trp at the N-terminal might have effective DPP-IV inhibitory activity (Lacroix & Li-Chan, 2012). The type of amino acid residues at the target cleavage site of peptides may significantly influence their DPP-IV inhibitory activity (Nongonierma

& FitzGerald, 2014). The <3 kD and 3–10 kD fractions showed similar IC₅₀ values to the hydrolysates before the simulated digestion (Figures 3.8 and 3.9). Thus, our study suggests that the fractions <10 kD are likely to play an important role in inhibiting the DPP-IV enzyme. However, overall DPP-IV inhibitory activity of the CMC and hydrolysates (IC₅₀ 0.56–1.5 mg/mL) was significantly lower than the medication sitagliptin (IC₅₀ 43.7 ng/mL) and other dairy protein and salmon byproduct hydrolysates (IC₅₀ <100 µg/mL) (Li-Chan et al., 2012; Lacroix & Li-Chan, 2013).

3.4.7. GLUTag Cell Viability

GLUTag cells were treated with green crab hydrolysates (0.125, 0.25, 0.5, 1.0 and 2.5 mg/mL) to determine their cytotoxic effects on the GLUTag cells. The cell viability after treatment with green crab hydrolysates significantly decreased at the concentrations of 1 mg/mL and 2.5 mg/mL (Figure 3.11). Therefore, total GLP-1 level secreted after treatment with green crab hydrolysates was evaluated at 0.5 mg/mL in GLUTag cells.

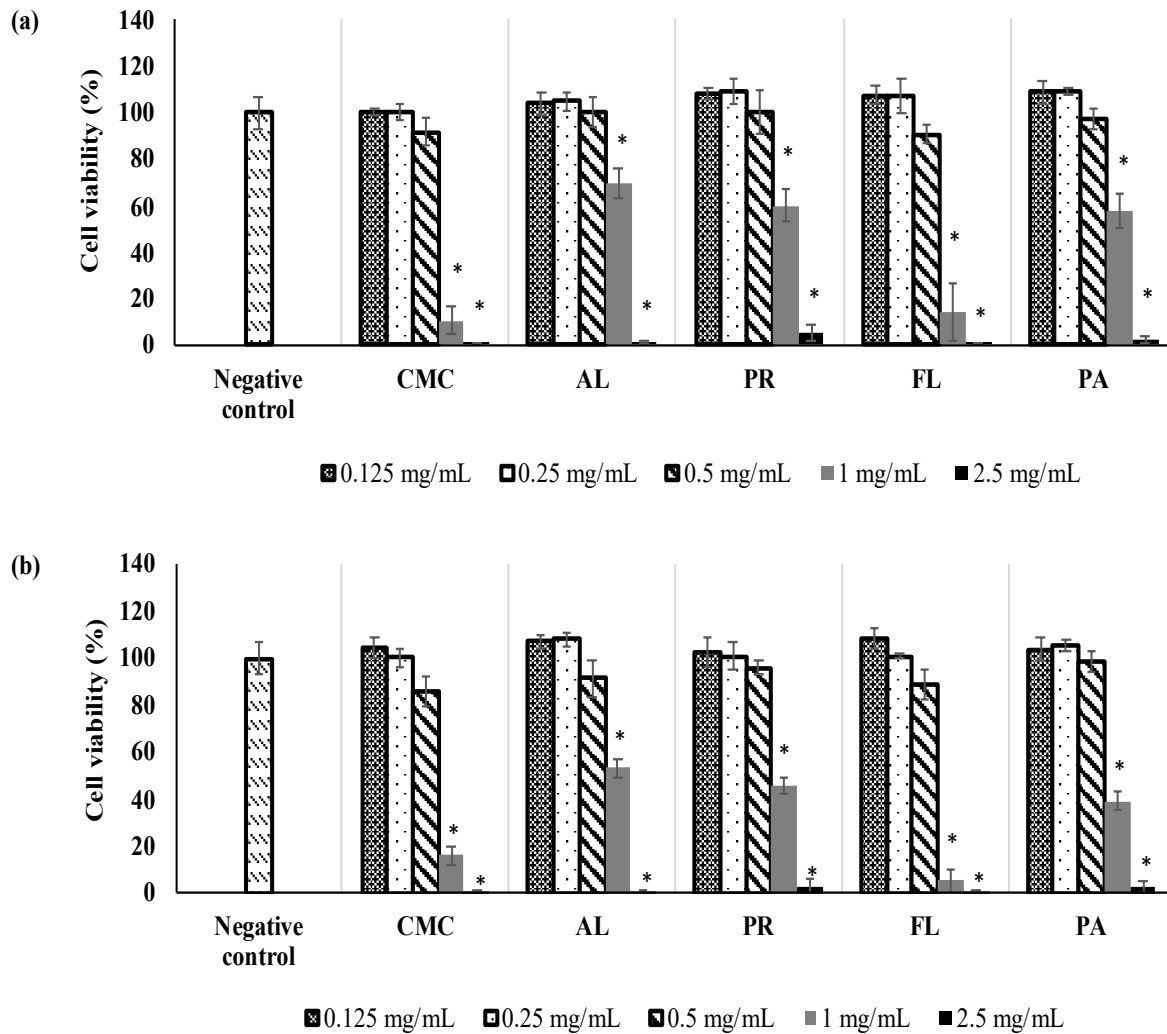


Figure 3.11. Cytotoxic effect of CMC and green crab hydrolysates before and after simulated digestion determined using GLUTag cells. (a) Before simulated digestion (b) After simulated digestion. CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation (n = 3 treatment replicates). Asterisk (*) represents significant difference between negative control and each sample (p < 0.05) by independent t-test.

3.4.8. GLP-1 Secretory Activity

The GLP-1 levels induced by the CMC and crab hydrolysate treatments were evaluated using the GLUTag cell model. Next, the results were compared with 10 mM glutamine as a positive control (Figure 3.12). The GLP-1 secretion induced by the CMC and hydrolysates was significantly higher than that by the positive control by 2.5–3.5 times. Among the samples, AL-derived hydrolysate showed the lowest

GLP-1 secretory activity, and the activity of all the samples was not significantly affected by simulated gastrointestinal digestion.

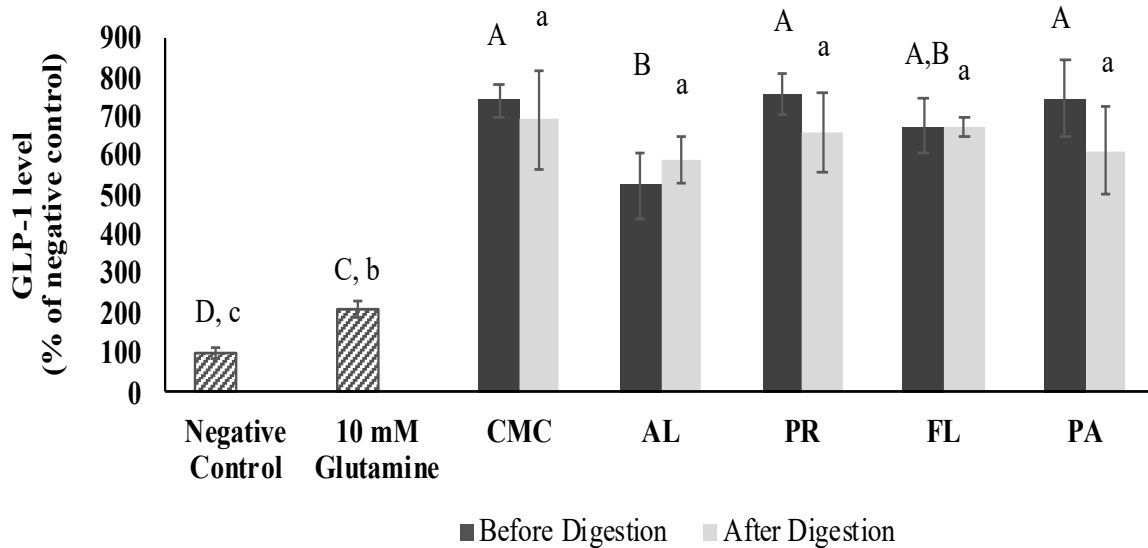


Figure 3.12. Glucagon-like 1 (GLP-1) level of CMC and hydrolysates before and after simulated digestion determined using GLUTag cells. The GLP-1 level was measured using 0.50 mg/mL of samples (final assay concentration, protein, and peptide basis). CMC: Crab Mince Control, AL: Alcalase, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation ($n = 3$ treatment replicates). The letters on the bars indicate a significant difference ($p < 0.05$) among the treatments.

After the fractionation, the total GLP-1 level induced by the CMC and crab hydrolysates (0.5 mg/mL) was evaluated using GLUTag cells. The GLP-1 secretory activity of fractions was improved by the enzymatic hydrolysis except for the >30 kD fraction of PA-derived hydrolysate and <3 kD fractions of FL and PA-derived hydrolysates (Figure 3.13). Glutamine (10 mM, positive control) increased GLP-1 level by $216\% \pm 13\%$ compared to a negative control (KRB buffer), and the <3 kD fractions had significantly higher GLP-1 levels than the positive control.

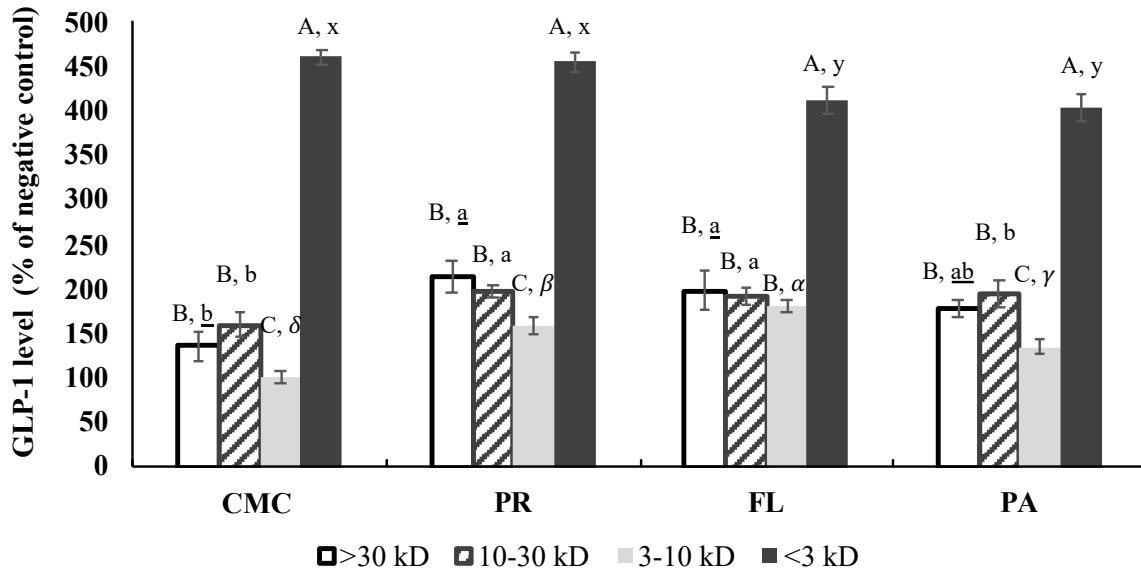


Figure 3.13. GLP-1 level of CMC and hydrolysates after fractionation (final assay concentration: 0.5 mg/mL protein and peptide basis). CMC: Crab Mince Control, PR: Protamex, FL: Flavourzyme, and PA: Papain. Each bar indicates the mean and standard deviation ($n = 3$ treatment replicates). Lowercase, lowercase letters with underline, and Greek letters on the bars indicate a significant difference ($p < 0.05$) among the treatments. Uppercase letters on the bars indicate a significant difference ($p < 0.05$) within each treatment.

GLP-1 hormone has the ability to reduce blood sugar levels by enhancing the secretion of insulin; however, it has a half-life of only ~ 1.5 min because of rapid inactivation by DPP-IV (Deacon et al., 1995). The GLP-1 content secreted from GLUTag cells was not different among the samples except for AL treatment that showed a lower GLP-1 level, and the GLP-1 secretory activity was not correlated with DH. In comparison with the positive control and hydrolysates from blue whiting and salmon skin, green crab hydrolysates released approximately 2.5–3.5 times higher GLP-1 levels at a lower sample concentration. Various amino acids including Gln, Glu, Ala, Ser, Leu, Gly, Asn, and Met have been demonstrated to stimulate GLP-1 secretory activity (Reimann et al., 2004; Gameiro et al., 2005; Reimer, 2006; Harnedy et al., 2018). According to Tolhurst et al. (2011), there are two major mechanisms involved in the stimulation of GLP-1 release by Gln on Glutag cells: (1) electrogenic sodium-coupled amino acid uptake resulting in a depolarization of membrane and activation of voltage-gated calcium

entry, and (2) increase of intracellular cAMP levels. Gameiro et al. (2005) reported that amino acids such as Ala and Gly activate ionotropic γ -aminobutyric acid (GABA) receptors, which respond to the various amino acids by generating chloride current. Our previous research showed that the green crab mince contains a high amount of Gln + Glu and Asn + Asp (Kang et al., 2019), which may have contributed to its higher GLP-1 secretory activity compared to the negative and positive controls. Small peptides including tri- and dipeptides (Leu-Gly-Gly, Gly-Leu, and Gly-Pro) are known to stimulate the secretion of GLP-1 from the *in vitro* cell model (Diakogiannaki et al., 2013; Harnedy et al., 2018). Therefore, the high GLP-1 content induced by the <3 kD fractions within treatments might be due to the concentration of amino acids and small di- or tripeptides after the fractionation.

3.5. Conclusions

Enzymatic hydrolysis was successfully applied to mechanically separated green crab mince to generate a potential anti-hyperglycemic food ingredient. These results suggest that the anti-hyperglycemic effects of green crab hydrolysates were dependent on the type of protease applied, and not on the degree of hydrolysis. Among the proteases evaluated, Protamex generated products having the highest α -glucosidase inhibitory activity. Furthermore, the fractionation study indicated that the <3 kD peptides primarily contributed to the bioactivities of the hydrolysates. Importantly for their prospective use in novel food products, the bioactivities of the hydrolysates were stable after the simulated gastrointestinal digestion. In conclusion, Protamex was the most effective protease for obtaining anti-hyperglycemic hydrolysates from green crab mince, and the development of bioactive hydrolysates may be a viable route for developing value-added food ingredients from this underutilized marine resource. However, substantial further research including identifying the bioactive compounds in the hydrolysates, evaluating their stability in a food model, and conducting *in vivo* assessment of their effectiveness is required for their future commercial application in health-promoting foods.

CHAPTER 4

FUNCTIONAL PROPERTIES OF GREEN CRAB HYDROLYSATES DERIVED FROM COMMERCIALLY AVAILABLE ENZYMES

4.1 Chapter Abstract

Invasive green crabs have diminished economically important marine resources and caused destruction of marine habitats. However, despite the high quality of protein in green crabs, they are underutilized as a food source due to their small size. The primary objective of this study was to develop functional hydrolysates through enzymatic hydrolysis of green crab. This study was designed to evaluate functional properties of green crab hydrolysates based on type of enzyme and duration of hydrolysis. Mechanically separated mince was homogenized with water (1:1), then hydrolyzed with 1% of Protamex (PR, pH 7), Flavourzyme (FL, pH 7), or a combination of both enzymes (CB, pH 7) at 50 °C for 0, 15, 30, 60, and 120 min. After enzyme inactivation at 85-90 °C for 10 min, the hydrolysates were lyophilized. Then, the degree of hydrolysis (DH) and functional properties including solubility, gel forming ability, antioxidant activity, and foaming and emulsifying activities were evaluated. Experiments were conducted in triplicate, and significant ($p < 0.05$) differences among treatments were determined by one-way and multi-way ANOVA. During hydrolysis, the DH of mince increased from 16.9% (non-hydrolyzed mince, NHM) to 35.6% (120 min) over time and CB showed the highest DH after 120 min hydrolysis. Soluble protein content at neutral pH and gel forming and emulsifying abilities were not significantly improved after hydrolysis. However, PR (34.9%) and CB (32.9%) treatments for 15 min significantly increased the foaming activity compared to NHM (25.8%). All enzyme treatments enhanced the ferric ion reducing antioxidant power over time, and hydrolysates derived from the CB treatment had the highest reducing power among the treatments. Results suggest that enzymatic hydrolysis could be used to improve the foaming activity and antioxidant activity of the crab mince and that these functional properties of the hydrolysates depend on the enzyme type and duration of hydrolysis.

4.2. Introduction

European green crabs (*Carcinus maenas* L.) are an invasive species in the U.S. and are commonly found in various marine habitats along Maine's diverse coastline of mud, rocks, saltmarshes and seagrasses (Grosholz & Ruiz, 1996; Tyrrell et al., 2006; Sigurdsson & Rochette, 2013). The crabs have a varied diet and prey on economically important species such as juvenile bivalve shellfish (eg. clams), urchins, scallops and lobsters. In addition, their aggressive burrowing activity can destroy habitats that are vital for commercially important crustaceans, mollusks and juvenile fish (Malyshev & Quijón, 2011). To date there is no efficient method to control green crab populations and decrease their detrimental effects on the marine ecosystem, fisheries, and shellfish aquaculture. Moreover, their small size does not allow them to be utilized as a convenient source of lump crab meat by the food industry.

Although their size makes traditional meat removal unfeasible, mechanically separated minced meat has potential for successful utilization by the food industry. Green crabs contain 80.6-83.5% protein (Skonberg & Perkins 2002; Nacz et al., 2004), which may have potential as a functional protein ingredient to improve the quality of food products. Many food proteins have various functional properties including gelation, emulsification and foaming (Puppo et al., 2005; Davis & Foegeding, 2007). Physicochemical properties of functional proteins influence their performance and behavior in food systems during preparation, processing, storage and consumption of food products. One of the processing methods to enhance the physicochemical properties of proteins is hydrolysis using food-grade proteases in controlled conditions. Many reports have demonstrated that enzymatic hydrolysis of seafood byproducts including sturgeon (Nikoo et al., 2014), tuna (Nalinanon et al., 2011), shrimp (Latorres et al., 2018), salmon (Sathivel et al., 2005), and herring (Sathivel et al., 2003) improved the functional properties such as solubility, water holding capacity, antioxidant, emulsifying, and foaming activities of extracted proteins.

Nevertheless, not much work has been done on crustacean species, and the majority of studies regarding functional properties of crustacean hydrolysates have focused on shrimp and its processing waste. Also, there is no report on the effects of enzymatic hydrolysis on the functional properties of raw crab proteins. Since the functional properties of protein hydrolysates are significantly influenced by many factors including enzyme specificity, hydrolysis time, substrate and enzyme concentrations, pH, temperature and presence of inhibitory substances (Panyam & Kilara, 1996), it is important to investigate the specific parameters to improve functional properties of proteins derived from different marine resources.

Therefore, the objectives of the present study were to investigate: 1) if the application of enzymatic hydrolysis using commercially available enzymes could create functional food ingredients from green crab, and 2) if the degree of enzymatic hydrolysis and enzyme type would influence the functional properties of hydrolysates obtained from green crab.

4.3. Materials and Methods

4.3.1. Preparation of Green Crab

Live green crabs (70 kg) were harvested in May and October 2019 on the Back River in Georgetown, Maine, USA. The crabs were washed with tap water and dead crabs were sorted out. The live crabs were blast-frozen at $-30\text{ }^{\circ}\text{C}$ for 1 h, then stored at $-20\text{ }^{\circ}\text{C}$ until further use. According to the protocol described in Chapter 2.3.2., 65 kg of frozen raw green crabs (carapace width 35–80 mm and weight 20–138 g) were stored overnight at $4\text{ }^{\circ}\text{C}$ and subsequently processed using a mechanical separator (Paoli One-Step Mechanical Separator Model 22-849, Rockford, IL, USA) to yield two streams of minced crab meat and shell. The minced meat was vacuum sealed in plastic bags (3 mil, $3.3\text{ cm}^3/100\text{ in}^2$ oxygen transmission rate, 80 microns, UltraSource, Kansas, MO, USA), and then blast-frozen at $-30\text{ }^{\circ}\text{C}$ for 1 hr and stored at $-20\text{ }^{\circ}\text{C}$ until enzymatic hydrolysis.

4.3.2. Enzymatic Hydrolysis of Green Crab Mince

Enzymatic hydrolysis was performed according to a modified method from Beaulieu et al. (2009). The frozen minced meat (25 kg) was thawed at 4 °C overnight until enzymatic hydrolysis the next day. The hydrolysis was conducted with three different enzymes including Protamex (PR), Flavourzyme (FL) and a combination of both PR and FL (CB) and processed in triplicate batches. For each batch, the mince (2 kg) was mixed with 70 °C deionized water in a 1:1 (w/v) ratio, and homogenized for 1 min using a hand blender (KHB1231CU0, KitchenAid, China) for 1 min. The pH and temperature of the homogenate were adjusted to pH 7 and 50 °C. Crude protein content in the mince was determined based on total nitrogen content. Total nitrogen content was analyzed by a combustion analyzer (TRU MAC CNS, LECO Corp., St. Joseph, MI, USA) and the crude protein content was calculated using a protein conversion factor of 6.25. The enzymes PR, FL, and CB were directly added to the homogenate based on protein content (1:100 = Enzyme:Substrate (w/w) for PR and FL, 0.5:100 = E:S (w/w) for each enzyme in CB). During hydrolysis, the mixtures were stirred by an overhead stirrer (Fisher Scientific, Dubuque, IA, USA). The pH of the mixtures was maintained using 6 N HCl or 6N NaOH and temperature was maintained in a water bath at 50 °C. For the crab mince control (CMC), the mince was homogenized with 70 °C deionized water in a 1:1 (w/v) ratio and the temperature and pH were adjusted to 50 °C and pH 7. Subsampling (800-1000 g) was performed at 0, 15, 30, 60, and 120 min and then the mixtures were heated at 85–90 °C for 10 min to inactivate enzymes and then immediately cooled on ice. All of the treatments were processed in triplicate. The collected samples were blast-frozen at –30 °C for 1 h, then freeze-dried (35 EL, VirTis Co. Inc., Gardiner, NY, USA) at –30 to 25 °C under 250 mT for 13 days. All dried samples were stored at –80 °C until analysis.

4.3.3. Degree of Hydrolysis

Degree of hydrolysis was determined according to the O-phthalaldehyde (OPA) method (Nielsen 2001; Karayannakidis et al., 2014). OPA reagent was prepared with 375 mL of deionized water, 19.05 g

of sodium tetraborate decahydrate, 500 mg of sodium dodecyl sulfate (SDS), and 400 mg of 97% OPA in 10 mL of ethanol. After mixing, 440 mg of 99% dithiothreitol (DTT) was added to the solution and deionized water was added to achieve a final volume of 500 mL. For the sample preparation, 1 g of the CMC and enzyme hydrolysates was diluted (1:19 w/v) with 4% SDS solution. After centrifugation at $1100 \times g$ for 10 min, 4 mL of supernatant was collected. Subsequently, the supernatant was diluted to 50 mL with deionized water. Four mL of OPA reagent were mixed with 400 μ L of solubilized sample/standard (0.5 mg/mL serine) and the mixture was incubated at room temperature for 2 min. As a blank, deionized water was used instead of sample/standard. Absorbance was measured at 340 nm and each determination was carried out in duplicate. The degree of hydrolysis was calculated based on the following three equations:

$$\begin{aligned} & \text{Serine-NH}_2 \text{ (mequiv/g protein)} \\ &= \frac{(((\text{Abs. sample} - \text{Abs. blank}))/((\text{Abs. standard} - \text{Abs. blank})) \times 0.9516 \text{ mequiv/L} \times 0.05 \times 100)}{(W \times V_1 / V_2 \times P)} \end{aligned}$$

W = weight of sample (g);

P = protein (g/100 g sample);

V1 = volume of supernatant (4 mL);

V2 = volume of SDS solution (20 mL);

0.05 = sample volume in L.

$$h = \frac{((\text{Serine-NH}_2) - \beta)}{\alpha}$$

h = number of hydrolyzed peptide bonds;

$\alpha = 1.00$, $\beta = 0.40$ mequiv/g protein;

$$DH = \frac{h \times 100}{h_{\text{total}}}$$

Total number of peptide bonds (h_{total}) of fish: 8.6 mequiv/g protein.

4.3.4. Functional Properties of Recovered Proteins

4.3.4.1. Solubility

Solubility of the control and recovered proteins was determined at pH 6, 7, 7.5, and 8 according to the methods of Khaled et al. (2014) since the pH values of many food products in the markets are in the neutral range. Samples (0.2 g) were mixed with 20 mL of deionized water and pH was adjusted to each appropriate point using 1 N HCl or NaOH. Following adjustment, each mixture was stirred with a magnetic stirrer for 30 min at room temperature to solubilize the protein. After centrifugation at 8000 × g for 15 min at 4 °C, the supernatant was collected and its protein content was evaluated by the Lowry method (Lowry et al., 1951). As a standard, hydrolyzed bovine serum albumin (BSA) was used. To hydrolyze BSA, 5 g of BSA was dissolved in 1 L of deionized water and the enzymatic hydrolysis was conducted at the optimum pH (pH 7) and temperature (50 °C) of PR and FL. PR (0.5:100 = E:S) and FL (0.5:100 = E:S) were added to the BSA solution based on BSA mass and the hydrolysis was conducted for 120 min. During hydrolysis, the pH of the mixture was maintained using 6N NaOH and temperature was monitored. After hydrolysis, the mixture was heated at 85–90 °C for 10 min to inactivate the enzymes and then immediately cooled on ice. Solution A (2% anhydrous sodium carbonate in 0.4% NaOH), Solution B (1% cupric sulfate-5H₂O), and Solution C (2.7% sodium potassium tartrate) were prepared for the Working Solution D (100 mL solution A + 1 mL solution B + 1 mL solution C). Five hundred microliters of sample solutions were added into glass test tubes and 5 mL of Working Solution D were added into the tubes and vortexed. After 10 min, 500 µL of Solution E (diluted 1N Folin-Ciocalteu phenol reagent) were added into the tubes which were vortexed and then the absorbance was measured against deionized water at 700 nm. Solubility was measured in duplicate and results were calculated based on the hydrolyzed BSA standard curve and expressed as mg solubilized protein/g sample.

$$\text{Solubility (\%)} = \frac{\text{protein content in supernatant (mg)}}{\text{mass of sample (g)}} \times 100$$

4.3.4.2. Emulsifying Activity and Stability

Emulsifying activity and stability were evaluated using the methods of Tang et al. (2005). Twenty four milliliters of 0.2% (w/v) sample solution was homogenized for 30 sec with 8 mL of vegetable oil (Hannaford Bros. Co., Scarborough, ME) in a 360 mL Waring blender at 70% power. A 50 μ L aliquot of the emulsion was immediately transferred into 4.95 mL of 0.1% (w/v) SDS solution at 0, 5, 10, 20, 30, 40, 50, and 60 min, and the absorbance of the diluted and vortexed solutions was read on a spectrometer (Beckman Du 530, Brea, CA, USA) set at 500 nm. As a positive control, whey protein isolate (Glanbia, Kilkenny, Ireland) was used and emulsifying activity (EA) was determined in duplicate and calculated by the following equation:

$$EA \text{ (m}^2\text{/g)} = \frac{2 \times 2.303 \times A(0) \times DF}{C \times OP \times 10,000}$$

A(0) = absorbance after 0 minutes

DF = dilution factor (100)

C = concentration of sample (g/mL)

OP = optical path (0.01 m)

4.3.4.3. Gel Forming Ability

Gel formation activity of the control and hydrolyzed proteins was determined using the method of Sathe et al. (1982). Two milliliters of sample suspensions were prepared at 30%, 35%, 40%, 45%, and 50% concentrations (w/v) in 12 \times 125 mm glass test tubes. Each mixture was incubated in boiling water for 45 min and then rapidly cooled on ice. After 45 min, each test tube was inverted to determine the lowest concentration at which the gelled suspension displayed no slipping or spilling in the tube. As a positive control, soy protein isolate was used (ADM, Chicago, IL, USA) and each determination was carried out in duplicate.

4.3.4.4. Foaming Activity and Stability

Foaming activity and stability were evaluated according to Khaled et al. (2014). The sample was mixed in deionized water at a concentration of 0.1% (w/v), and then 20 mL of the solution was homogenized (Brinkmann Instruments, Westbury, NY) for 1 min at room temperature to create a foam. Following homogenization, the solution was immediately transferred into a 50 mL graduated cylinder and the total volume (mL) was measured at 0, 15, 30, and 45 min. Pasteurized egg white powder (Just Whites, De-Bel, Elizabeth, NJ, USA) was used as a positive control. Foaming activity was measured in duplicate at each time period to assess foam stability. Foaming activity was expressed as foam expansion, the percentage of volume increase immediately after homogenization (0 min).

$$\text{Foam Expansion (FE, \%)} = [(A-B)/B] \times 100$$

where A is the volume (mL) after homogenization and B is the volume (mL) before homogenization.

4.3.4.5. Free Radical Scavenging Activity

Free radical scavenging activity was determined using free radicals of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (Re et al., 1999). Stock solutions of 7.4 mM ABTS^{•+} solution and 2.6 mM potassium persulfate solution were prepared. For the working solution, the two stock solutions were mixed in a 1:1 ratio and the working solution was allowed to react for 12-16 h at room temperature in the dark. The solution was diluted with deionized water until the absorbance reached 0.7 ± 0.02 at 734 nm. The working solution was freshly prepared and Trolox (50, 100, 250, 500 and 1000 $\mu\text{g/mL}$) was used as a positive control. Samples/Trolox (100 μL) were allowed to react with 2.9 mL of the working solution for 6 min and deionized water was used for a control. Then, the absorbance was measured at 734 nm against deionized water as a blank. Each determination was carried out in duplicate and % inhibition of ABTS^{•+} was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{(\text{Abs. Control} - \text{Abs. sample}) \times 100}{\text{Abs. Control}}$$

4.3.4.6. Ferric Ion Reducing Antioxidant Power (FRAP)

The FRAP assay was performed according to Benzie and Strain (1999) with some modifications. As stock solutions, Solution A (3.1 g sodium acetate trihydrate in 16 mL glacial acetic acid, pH 3.6), Solution B (10 mM 2, 4, 6- tripyridyl-s-triazine in 40 mM HCl), and Solution C (20 mM Iron (III) chloride hexahydrate) were prepared. The working solution was freshly prepared by mixing Solution A, B, and C in a ratio of 10:1:1, then warmed to 37 °C before using. For the standard curve, ferrous sulfate heptahydrate solutions ranging from 50-500 µM were used. Samples/standards (100 µL) were mixed with 3 mL of the working solution and incubated for 4 min and deionized water was used as a blank. Absorbance of the colored product (ferrous tripyridyltriazine complex) was determined at 593 nm and FRAP value (FeSO₄·7H₂O equivalents, mM) was determined in duplicate.

4.3.5. Statistical Analysis

The enzymatic hydrolysis process was replicated three times and all of the assays were conducted in duplicate on each sample replicate. Statistical differences among the means of each treatment were evaluated using one-way and multi-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test with a significance value of $p < 0.05$ (SPSS ver. 23, IBM Corp., Armonk, NY, USA). One-way ANOVA was used to assess differences among treatments at each time point and among time points within different enzyme treatments. Multi-way ANOVA was used to examine the influence of enzyme treatments and hydrolysis duration. Correlations between hydrolysis duration and each functional property and between DH and each functional property ($p < 0.05$) were analyzed through the Pearson coefficient (SPSS ver. 23, IBM Corp., Armonk, NY, USA).

4.4. Results and Discussion

4.4.1. Degree of Hydrolysis

DH is an important parameter that affects the functional properties of protein hydrolysates. DH is defined as the ratio of the number of hydrolyzed peptide bonds to the total number of peptide bonds

in the mass unit (Fernandes, 2016). DH influences the size and amino acid composition of the peptides, which can impact the functional properties and bioactivity of peptides generated during enzymatic hydrolysis. Figure 4.1 shows DH of the crab mince determined by the OPA method. During hydrolysis, both enzyme type and hydrolysis duration significantly affected DH of green crab hydrolysates ($p < 0.05$). DH significantly increased from 16.9% to 28.5-35.6% in all the treatments and it increased the fastest during the first 15 min. FL treatment exhibited the highest DH followed by CB, CMC, and PR. Interestingly, although hydrolysis of the CMC treatment lagged at first, it showed a similar final DH compared to PR and CB; this was likely due to endogenous proteolytic enzymes in the green crabs which were processed whole.

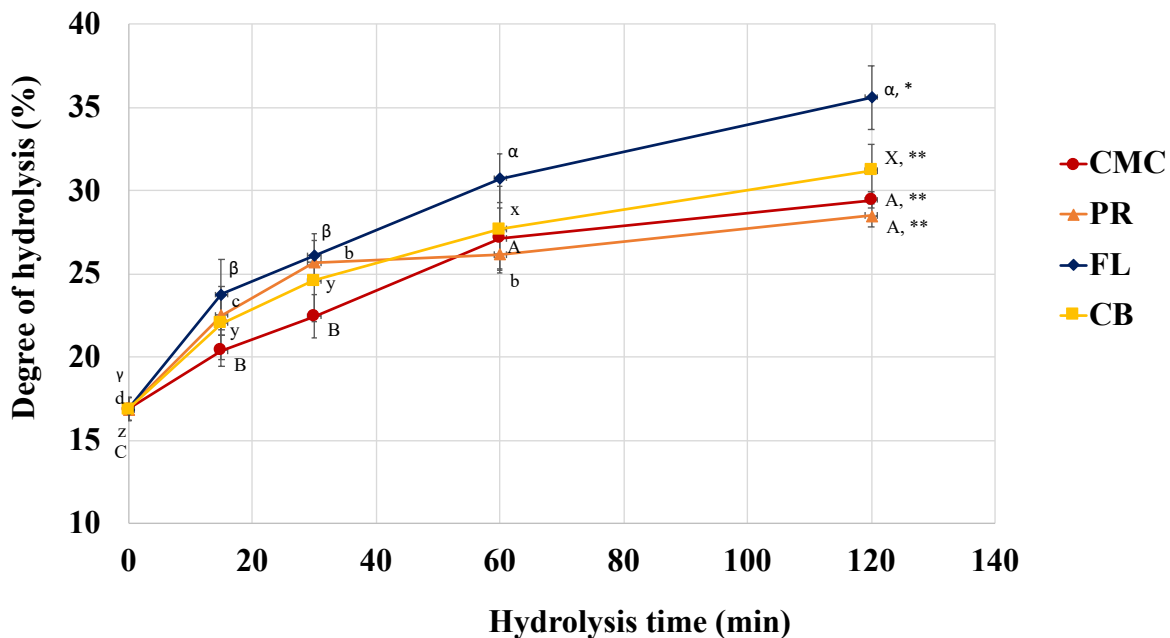
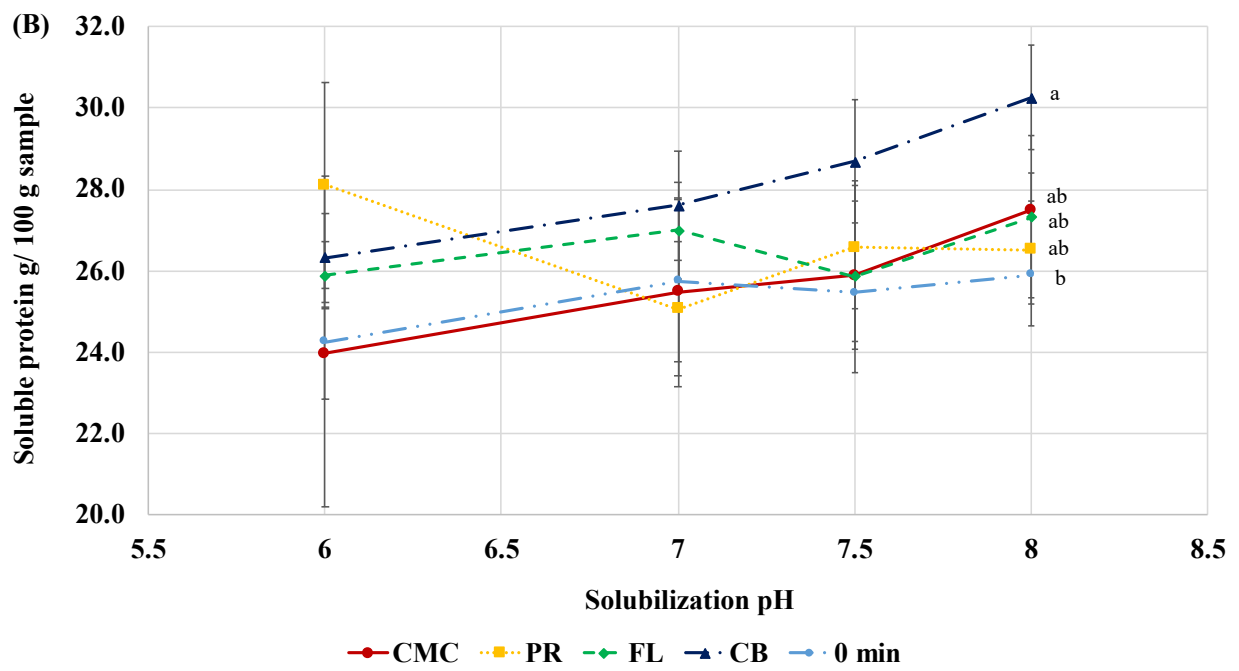
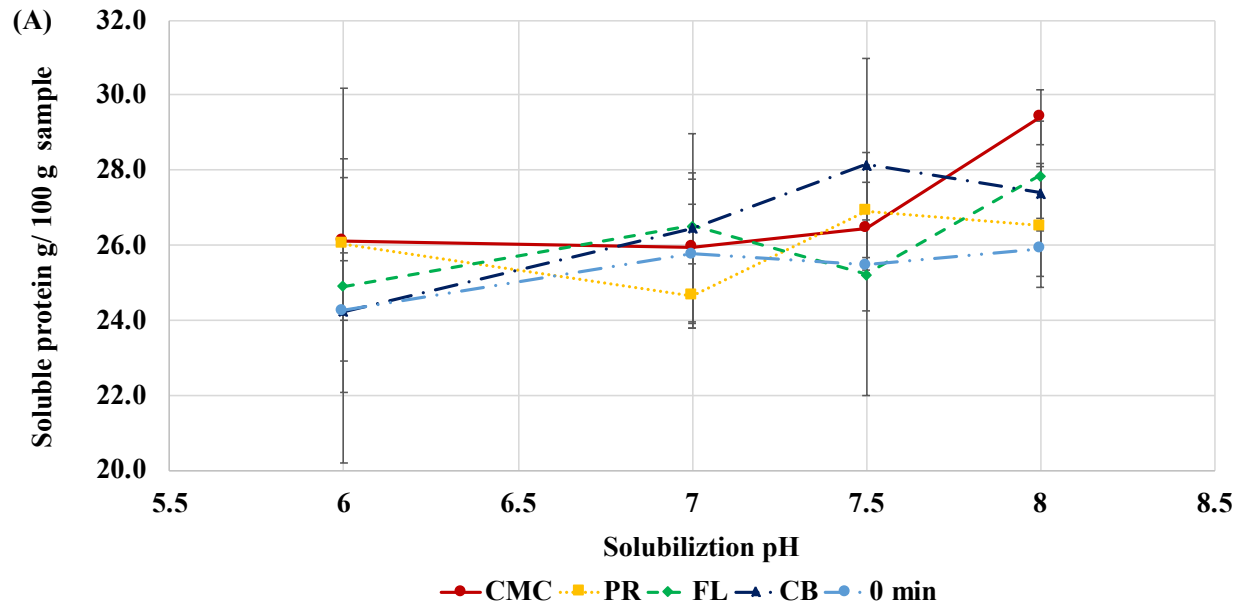


Figure 4.1. Degree of hydrolysis of green crab mince by enzyme type during hydrolysis. CMC: Crab Mince Control; PR: Protamex, FL: Flavourzyme; CB: Combination of PR and FL. Each data point indicates the mean and standard deviation ($n=3$). Uppercase, lowercase, and Greek letters on the graph indicate significant differences over time within treatment ($p < 0.05$) based on one-way ANOVA followed by Tukey's test. Asterisks (*) indicate a significant difference among the enzyme treatments at each time point ($p < 0.05$) based on one-way ANOVA followed by Tukey's test, and no asterisks mean no significant difference among the enzyme treatments at each time point.

4.4.2. Solubility

Solubility of protein hydrolysates is one of the most important characteristics for functionality since it is not only critical by itself, but also affects other functional properties such as emulsifying, foaming, and gel forming activities (Hefnawy & Ramada, 2011; Hayat et al., 2014). The soluble protein content of green crab hydrolysates was evaluated at pH 6-8 (Figure 4.2) because the pH of many commercially available food products is in the neutral range. According to statistical analysis, enzyme treatment and solubilization pH influenced the protein solubilization ($p < 0.05$). Non-hydrolyzed mince (NHM; CMC at time 0) showed relatively lower soluble protein content at each solubilization pH even though it was not statistically different. At pH 7 and pH 7.5, the CB treatment exhibited the highest soluble protein content among 30 min and 60 min hydrolyzed samples. Also, there was a trend for soluble protein content to increase as solubilization pH increased. The pI of seafood proteins ranges from pH 4.5-5.5. Therefore, the lower soluble protein content at pH 6 was likely due to the solubilization pH being so close to the pI. The soluble protein content of all the samples ranged from about 24%- 29%, and did not significantly increase with extended duration of hydrolysis. Many studies have reported that protein solubility is associated with DH (Jamdar et al., 2010). As DH is increased, protein solubility is also increased since the size of peptides is decreased. However, in this study, the soluble protein content at pH 6-8 was not correlated ($r = 0.071$ at pH 6, $r = 0.055$ at pH 7, $r = 0.088$ at pH 7.5, $r = -0.072$ at pH 8) with the DH, potentially due, in part, to unevenly distributed sample components. Although most of the shell was removed during the mechanical separation process, the initial protein content of the mince was 48.9% (dry weight basis) and there were shell particles evident in the mince. During hydrolysis, uneven sampling at each hydrolysis time point may have led to non-homogeneous sample composition, which could affect the soluble protein content resulting in high standard variations.



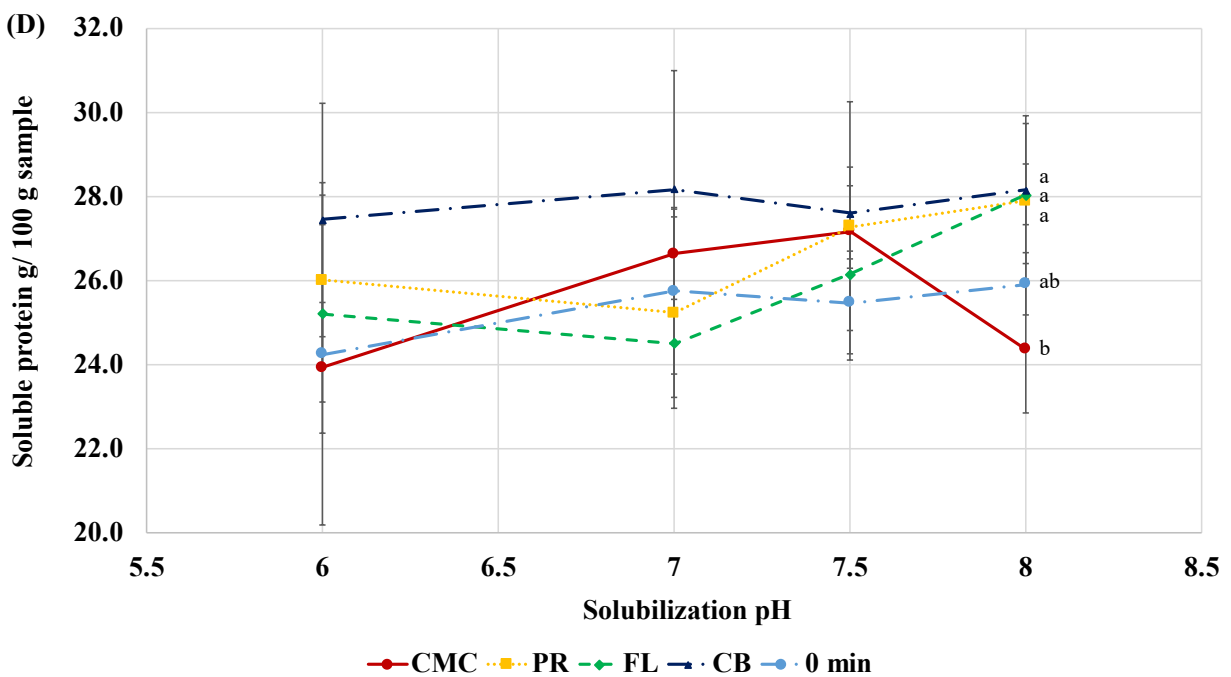
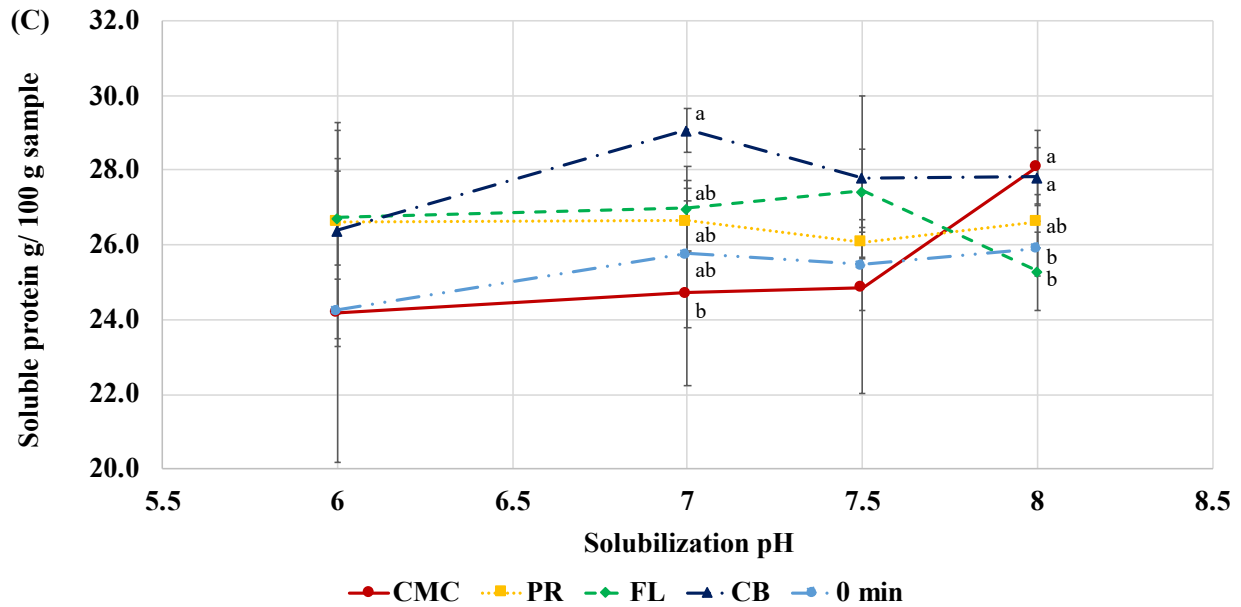
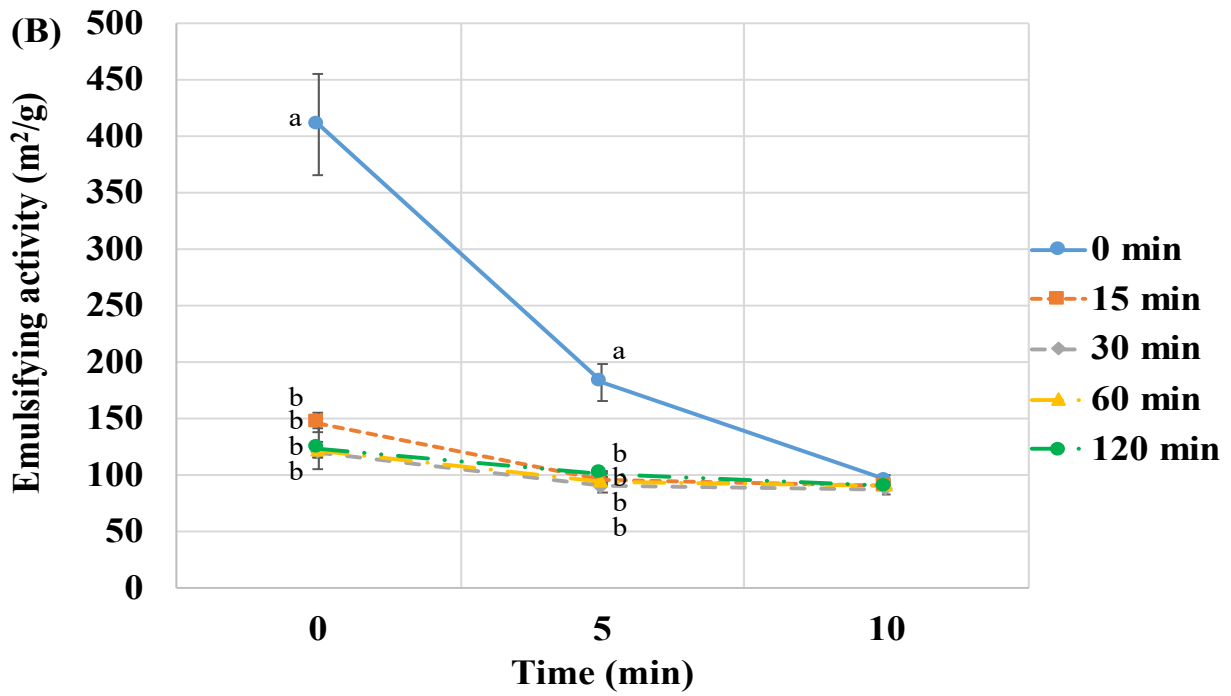
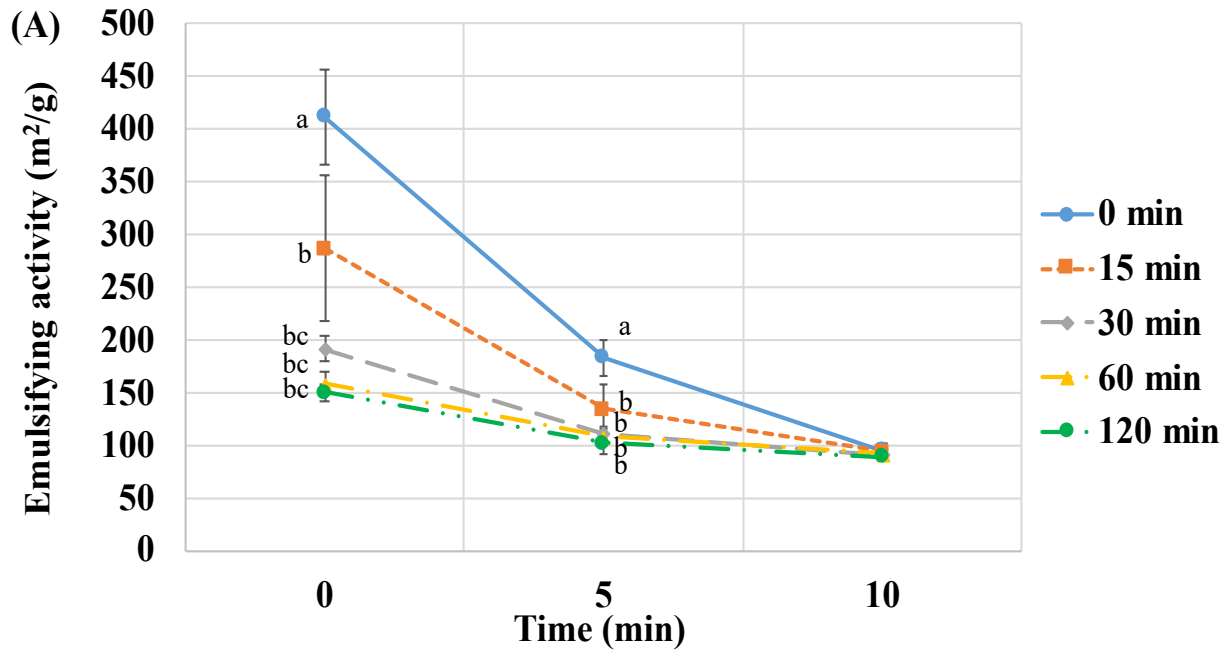


Figure 4.2. Solubilized protein content (g) per total mass of sample (100 g) at various pH values. (A) 15 min hydrolysis. (B) 30 min hydrolysis. (C) 60 min hydrolysis. (D) 120 min hydrolysis. CMC: Crab Mince Control; PR: Protamex, FL: Flavourzyme; CB: Combination of PR and FL; NHM: Non-hydrolyzed crab mince. Each data point indicates the mean and standard deviation (n=3). Different letters on the graph indicate significant differences among enzyme treatments at the same pH ($p < 0.05$) based on ANOVA followed by Tukey's test. No letters indicate no significant difference among the enzyme treatments.

4.4.3. Emulsifying Activity and Stability

In the study of emulsification activity, enzyme treatments, hydrolysis duration, and interaction of enzyme treatments and hydrolysis duration significantly affect the emulsifying activity of the hydrolysates ($p < 0.05$). The highest emulsifying activity ($411 \text{ m}^2/\text{g}$) was observed in the non-hydrolyzed mince (NHM) and hydrolysis by commercial or endogeneous proteolytic enzymes in the mince significantly decreased the emulsifying activity (Figure 4.3). Emulsifying activity of hydrolysates showed a reduced trend as duration of hydrolysis increased. In Chapter 2, green crab protein isolate obtained from solubilization at pH 10 ($1480 \text{ m}^2/\text{g}$) showed comparable emulsifying activity and stability with whey protein isolate ($1568 \text{ m}^2/\text{g}$) that is well known as a good emulsifier. On the other hand, NHM and hydrolysates obtained from enzyme treatment did not show substantial emulsifying activity and stability. Protein isolates obtained by ISP processing showed that the emulsifying activity was significantly associated with their solubility ($r = 0.956$, $p < 0.05$ in Chapter 2), likely because a high protein concentration at the interfacial surface between the oil and water layers results in good emulsification properties (Yatsumatsu et al., 1972; McWatters & Holmes, 1979). However, in this study, the emulsifying activity was not statistically associated with the soluble protein content of hydrolysates at pH 7.5 (pH of the emulsification assay) but the activity was inversely correlated with DH ($r < -0.7$, $p < 0.05$). An increase in DH reduced the emulsification properties of hydrolysates, probably due to a decrease in functional peptide chain length which diminished their capacity to reduce the interfacial tension (Kristinsson & Rasco, 2000).



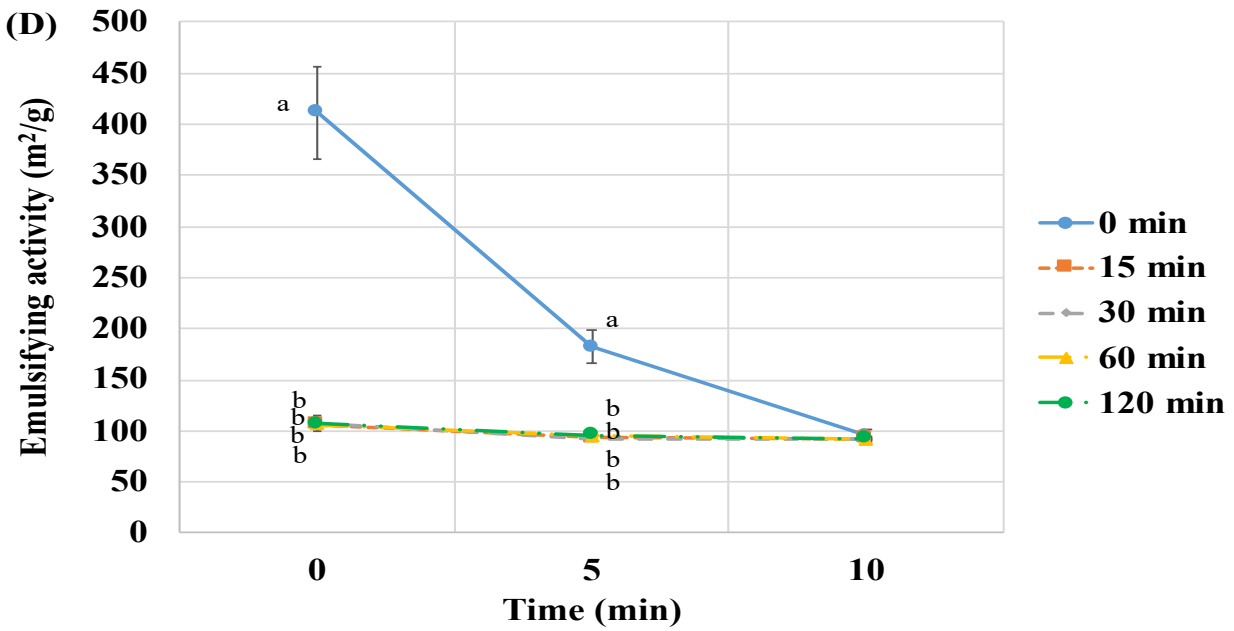
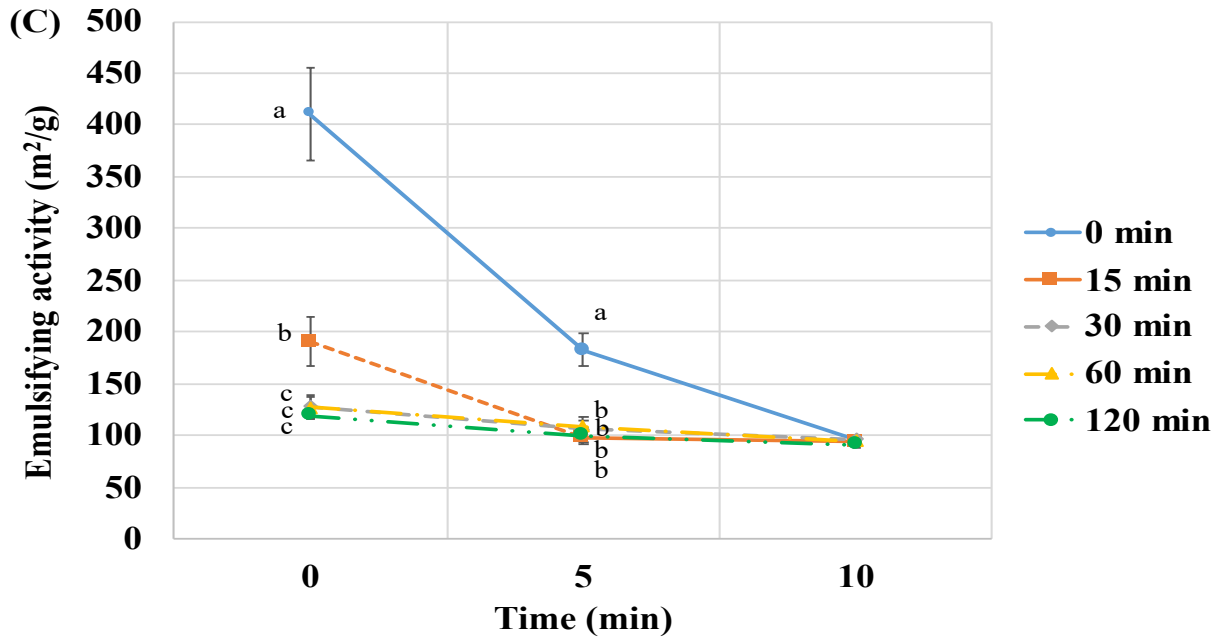
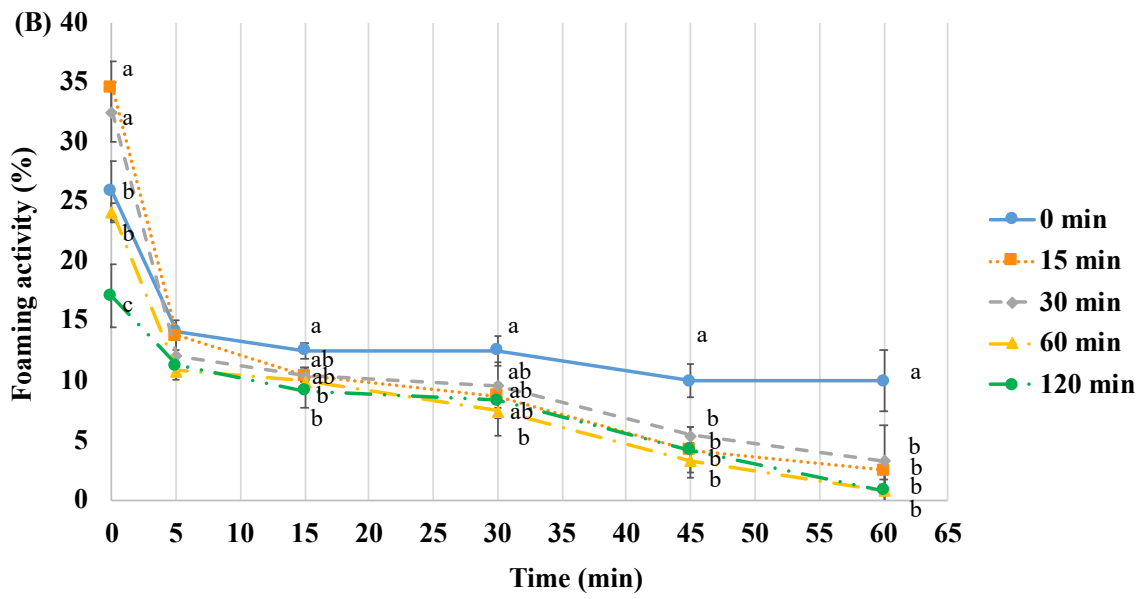
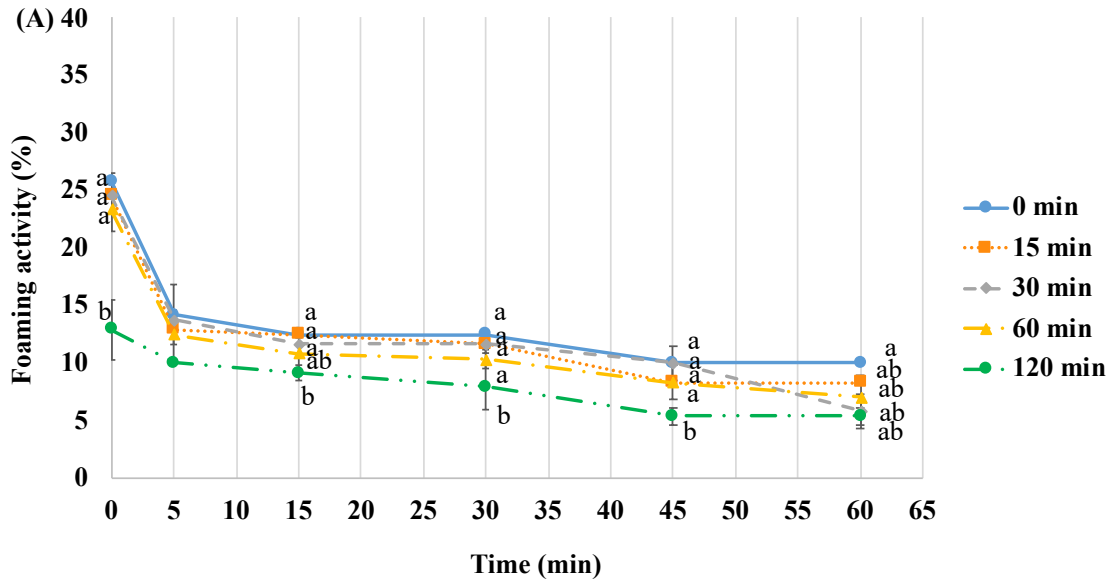


Figure 4.3. Emulsifying activity and stability of green crab hydrolysates. (A) CMC. (B) PR treatment. (C) FL treatment. (D) CB treatment. NHM: Non-hydrolyzed crab mince. Each data point indicates the mean and standard deviation ($n=3$). Different letters on the graph indicate significant differences among hydrolysis times ($p < 0.05$) based on ANOVA followed by Tukey's test. A lack of letters at a specific time point indicates no significant difference. Positive control: whey protein isolate (EA: $1568 \pm 8.7 \text{ m}^2/\text{g}$, 50% reduction of EA: at 60 min).

4.4.4. Foaming Activity and Stability

Foaming activity plays an important role in generating a foam in formulated food products such as meringue, whipped cream, and mousse, which also contributes to a light texture and mouthfeel. The foaming activity of hydrolysates was affected by enzyme type, hydrolysis time, and interaction of both enzyme type and hydrolysis time ($p < 0.05$). The activity was improved by PR (34.9%, Figure 4.4b) and CB (32.9%, Figure 4.4d) treatment for 15 min compared to NHM (25.8%). In the PR treatment, hydrolysis for 30 min also increased the foaming activity of the mince to 32.5% compared to NHM (25.8%). In Chapter 2, protein isolates showed less than 20% foaming activity. Although the foaming activity of these hydrolysates was significantly lower than that of egg white (representative foaming agent) which had 65.4% foaming activity (Figure 4.4), it was enhanced compared to protein isolates obtained by ISP processing. However, the initial foaming activities of these hydrolysates was significantly reduced after only 5 min, which indicates that the foam generated by hydrolysates was not stable and quickly lost foam volume. Excessive hydrolysis for more than 60 min decreased the foaming activity of hydrolysates. DH of hydrolysates was negatively correlated ($r = -0.740$) with the foaming activity ($p < 0.05$). The foaming activity of proteins and peptides is determined by many factors including solubility, amphiphilicity, segmental flexibility, and disposition of charged and polar groups (Panyam & Kilara, 1996). Therefore, only limited hydrolysis was required to improve the foaming activity rather than excessive hydrolysis which may have resulted in the loss of protein/peptide characteristics.



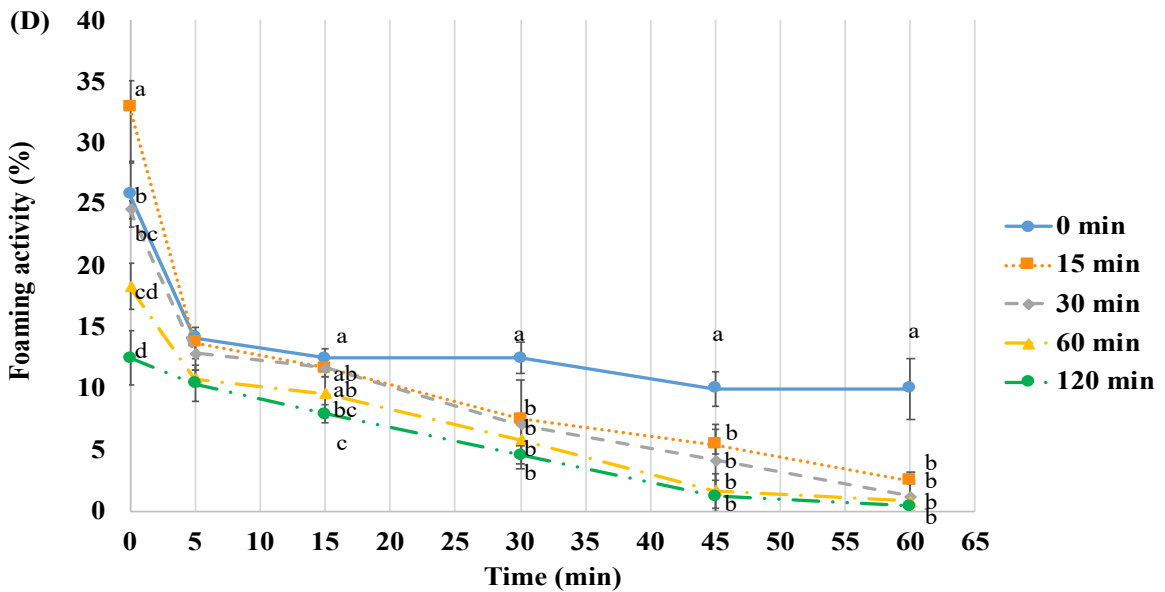
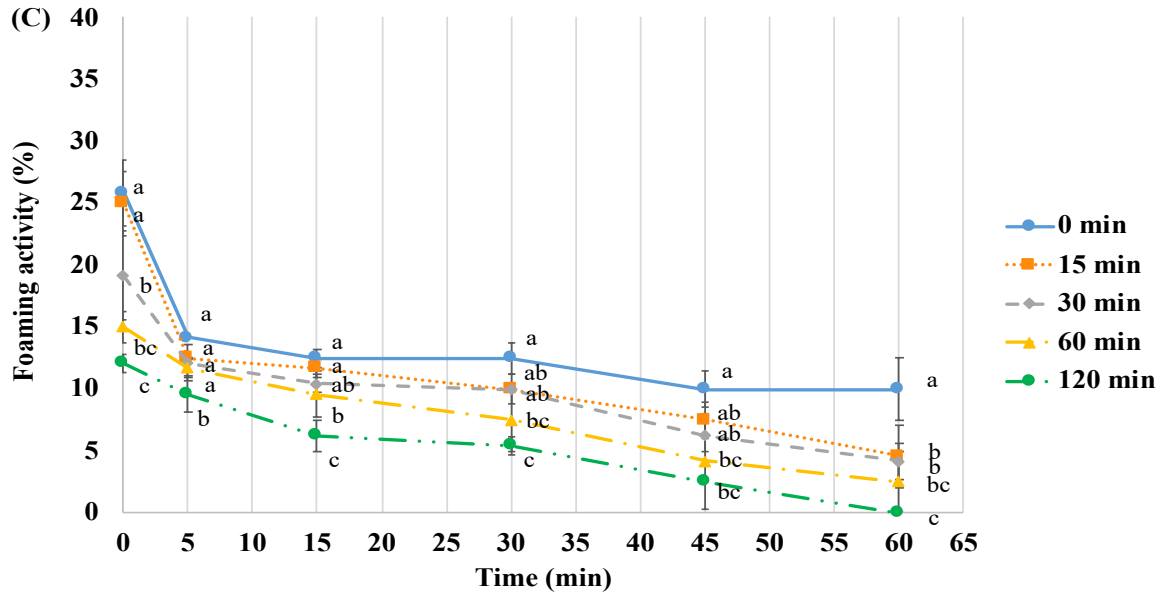


Figure 4.4. Foaming activity and stability of green crab hydrolysates. (A) CMC. (B) PR treatment. (C) FL treatment. (D) CB treatment. NHM: Non-hydrolyzed crab mince. Each data point indicates the mean and standard deviation ($n=3$). Different letters on the graph indicate significant differences among hydrolysis duration ($p < 0.05$) based on ANOVA followed by Tukey's test. The letters are not shared among different time points on x-axis, and no letter on each data point indicates no significant difference among hydrolysis duration. Positive control: egg white (FE: $65.4 \pm 1.4\%$, 50% reduction of FE: n.d.).

4.4.5. Gel Forming Activity

Protein additives are added to diverse formulated food products to improve the gelation and quality of food gels. In this study, gel forming activity was determined as the minimum required concentration for hydrolysates to form a gel. The gel forming activity was influenced by enzyme type, hydrolysis duration, and interaction of enzyme type and hydrolysis duration ($p < 0.05$). Overall, the PR treatment showed the lowest gel forming activity except for the 30 min hydrolyzed samples, whereas NHM (31.7%) and CMC 15 (31.7%) showed the best gel forming activity among the samples (Table 4.1). Soy protein isolate, which is well known to have good gel forming activity, required a 15% protein concentration to form a gel. In the crab ISP study, protein isolate obtained by solubilization at pH 2 showed a higher gel forming activity than soy protein isolates, requiring only a 12% protein concentration. On the other hand, overall gel forming activity of the mince and hydrolysates was very low for use in the food industry since they required a sample concentration of more than 30% to form a gel. The gel forming activity was also significantly dependent on hydrolysis time. As hydrolysis time increased, the gel forming activity decreased, and the activity was also associated with DH ($r = 0.847$, $p < 0.05$) of hydrolysates.

There are various internal and external factors that influence the gel forming activity of protein-based additives such as protein concentration, the molecular weight of proteins, hydrophobicity, ionic strength, and pH (Sun & Holley, 2011). It is generally considered that enzymatic hydrolysis of muscle proteins tends to decrease the gel forming activity since it reduces the size of the proteins and peptides and the introduction of charged groups increases the hydrophilicity of the proteins and peptides (Creusot & Gruppen, 2007). On the other hand, there are reports that a limited degree of hydrolysis increases the hydrophobicity of some globular proteins by exposure of buried non-polar residues, which contributes to the aggregation of peptides (Creusot & Gruppen, 2007). However, it is very critical to determine the specific combination of parameters including substrates, enzyme type, the ratio of

enzyme and substrate, and DH to enhance gel forming ability by protein hydrolysis (Creusot & Gruppen, 2007).

Table 4.1. Gel forming activity (%) of green crab hydrolysates

Hydrolysis time (min)	0	15	30	60	120
CMC	31.7±2.9 ^{A, c+}	31.7±2.9 ^{B, c}	39.3±2.9 ^{A, b}	45.0±0.0 ^{B, a}	50.0±0.0 ^{A, a}
PR	n.d	37.5±2.5 ^{A, c}	43.3±2.9 ^{A, b}	50.0±0.0 ^{A, a}	50.0±0.0 ^{A, a}
FL	n.d	35.0±0.0 ^{A, b}	46.7±2.9 ^{A, a}	48.3±2.9 ^{AB, a}	n.d.
CB	n.d	35.0±0.0 ^{A, c}	41.7±2.9 ^{A, b}	44.2±1.4 ^{B, b}	50.0±0.0 ^{A, a}

+ indicates NHM

CMC: Crab Mince Control; PR: Protamex, FL: Flavourzyme; CB: Combination of PR and FL; NHM: Non-hydrolyzed crab mince. Each value indicates the mean and standard deviation (n=3). Uppercase letters indicate significant differences among the treatments within same hydrolysis time while lowercase letters indicate significant differences among different hydrolysis times within the same enzyme treatment ($p < 0.05$) based on ANOVA followed by Tukey's test. Positive control: soy protein isolate (15%).

4.4.6. Free Radical Scavenging Activity

The free radical scavenging activity of hydrolysates was determined using the ABTS^{•+} assay. The results are represented by IC₅₀ values which are defined as the concentration required to scavenge 50% of the radical activity. The ABTS radical scavenging activity was influenced by hydrolysis time and interaction of enzyme type and hydrolysis time ($P < 0.05$). After 15 min hydrolysis, the CB treatment significantly improved the ABTS radical scavenging activity compared to NHM (Figure 4.5). Naturally present endogenous enzymes in the mince also significantly increased the antioxidant activity after 60 and 120 min hydrolysis whereas treatment by PR and FL did not improve the ABTS^{•+} scavenging activity.

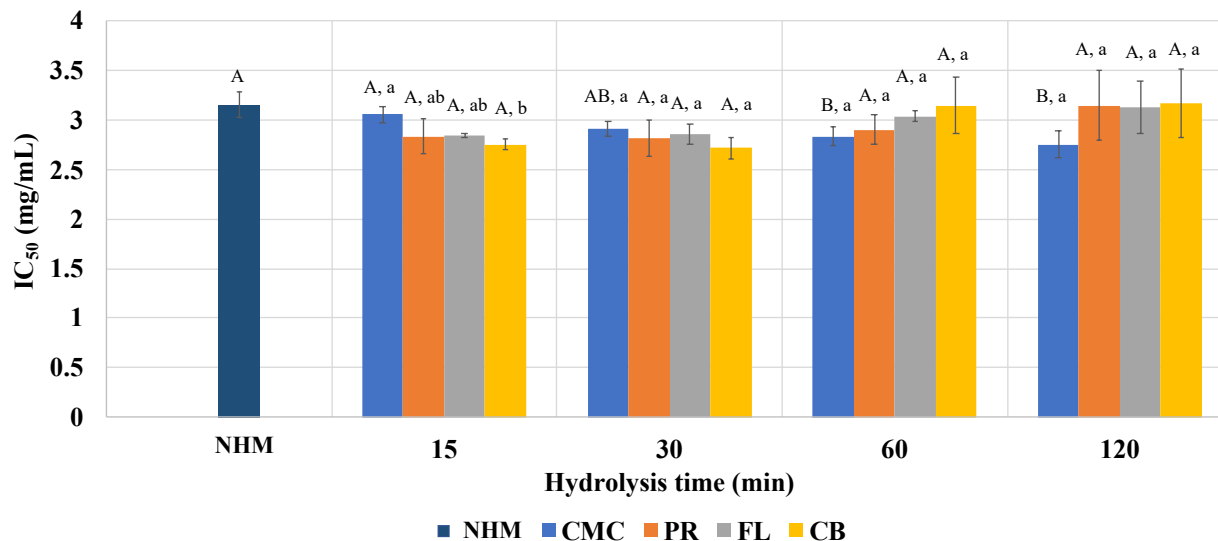


Figure 4.5. The ABTS radical scavenging activity of green crab hydrolysates. CMC: Crab Mince Control; PR: Protamex, FL: Flavourzyme; CB: Combination of PR and FL; NHM: Non-hydrolyzed crab mince. Each bar indicates the mean and standard deviation (n=3). Uppercase letters indicate significant differences among hydrolysis times within the same treatment (whereas lowercase letters indicate significant differences among different enzyme treatments within the same hydrolysis time based on ANOVA ($p < 0.05$) followed by Tukey's test. Positive control: Trolox ($IC_{50} 98.1 \pm 0.3 \mu\text{g/mL}$).

According to Sun et al. (2016), the higher DH samples which included small molecular weight peptides < 1 kD contributed the most to the free radical scavenging activity of Alaska pollock hydrolysates. However, in this study, the scavenging activity was not significantly associated with DH ($r = -0.181$), and this result was contrary to other antioxidant activity reports of protein hydrolysates (Jamdar et al., 2010; Huang et al., 2011). Although some hydrolysates obtained by treatment of CB and endogenous enzymes showed improved free radical scavenging activity, the activity was considerably lower than soluble hydrolysates obtained from shrimp processing byproducts ($IC_{50} 7.4 \mu\text{g/mL}$), sea squirt ($IC_{50} < 1\text{mg/mL}$), and pollock skin collagen ($IC_{50} < 0.5 \text{mg/mL}$) (Huang et al., 2011; Kim, 2011; Sun et al., 2016). In this study, whole hydrolysates including insoluble proteins and shells were collected and mixed with deionized water to assess the free radical scavenging activity, therefore the hydrolysates contained relatively lower concentrations of proteins and peptides compared to soluble hydrolysates used in other

studies. The lower content of peptides in the hydrolysates was likely related to the lower free radical scavenging activity observed in comparison with other seafood hydrolysates.

4.4.7. Ferric Ion Reducing Antioxidant Power (FRAP)

The FRAP method, which determines the reduction of complexes of 2, 4, 6-tripyridyl-s-triazine (TPTZ) with ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), is one of the most widely used methods to evaluate the antioxidant capacity of hydrolysates. Upon the reduction of TPTZ, its color changes from colorless to a blue ferrous complex. The antioxidant activity of hydrolysates measured by the FRAP assay indicated that the activity was influenced by enzyme type, hydrolysis time, and the interaction of enzyme and hydrolysis time ($p < 0.05$). The FRAP values were increased by the hydrolytic action of endogenous and commercial enzymes (Figure 4.5). Among the 15 and 30 min hydrolyzed samples, CB showed the highest FRAP values followed by FL, PR, and CMC. Although the DH was not strongly correlated with the FRAP values ($r = 0.529$), the FRAP values showed an increasing trend as the hydrolysis time increased. Many studies have reported that smaller size peptides generated by excessive hydrolysis had higher reducing power than large molecular weight fractions (Bougatef et al. 2009; Khantaphant et al., 2011; Le-Vo & Tran, 2017). The enhanced antioxidant activity by enzymatic hydrolysis may be due to released previously inactive peptides in the crab mince or production of antioxidant peptides produced depending on the specificity of each enzyme.

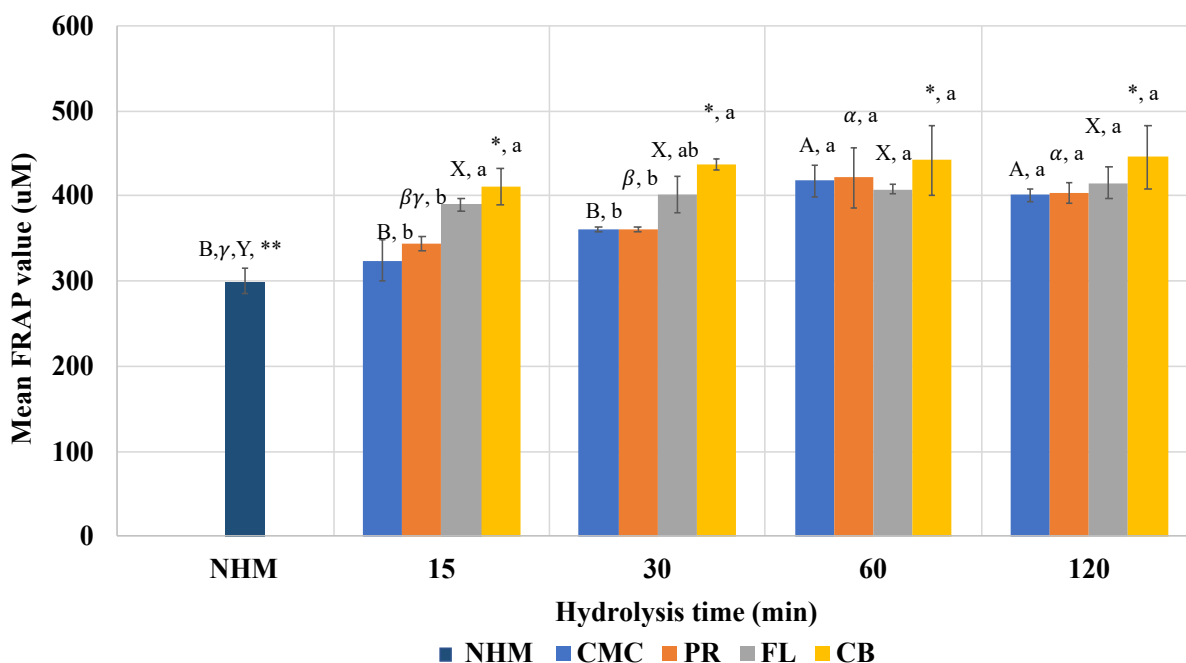


Figure 4.6. Ferric ion reducing antioxidant power of green crab hydrolysates. CMC: Crab Mince Control; PR: Protamex, FL: Flavourzyme; CB: Combination of PR and FL; NHM: Non-hydrolyzed crab mince. Each bar indicates the mean and standard deviation (n=3). Uppercase, Greek alphabet, and asterisk (*) on the graph indicate significant differences among hydrolysis times within enzyme treatment ($p < 0.05$) based on ANOVA followed by Tukey's test. Lowercase letters indicate significant differences among different enzyme treatments within the same hydrolysis time. Positive control: Trolox (62.6 $\mu\text{g/mL}$).

4.6. Conclusions

In this study, enzymatic hydrolysis using commercially available proteases was successfully applied to improve foaming activity and antioxidant activity of crab mince powder. Based on the results, hydrolysis for more than 60 min with commercial proteases is not recommended to obtain functional proteins from green crabs. Our results suggest the application of Protamex (1:100 =E:S) for 15 min to improve the foaming activity of the crab mince. Also, the combined treatment of Protamex and Flavourzyme (0.5:100 = E:S) for 15 min is recommended to enhance the foaming and free radical scavenging activities and reducing antioxidant power of the crab mince. However, due to the poor stability of their foams, crab mince hydrolysates may need to be used with synergistic foaming stabilizers or viscosifying agents. Our study demonstrated that the appropriate selection of specific

parameters in enzymatic hydrolysis is critical to obtain targeted functional protein ingredients from crab mince. However, further studies to determine molecular weight of hydrolysates, their antioxidant capacity in a food model, and improvement of foaming stability are required for future feasible application of green crab hydrolysates in formulated food products. Moreover, in order to increase functionality of the hydrolysates, separation of insoluble and soluble materials through centrifugation processing could be additionally applied to reduce the amount of non-protein materials in the hydrolysates.

CHAPTER 5

OVERALL CONCLUSIONS

This series of studies demonstrated that multi-functional proteins and peptides can be successfully derived from invasive green crabs through ISP and proteolytic processes. These studies were the first step to develop multifunctional protein ingredients derived from green crab and were designed as a proof of concept for deriving functional ingredients via ISP processing and enzymatic hydrolysis.

Functional and bioactive proteins/peptides derived from green crabs may be utilized by the food industry to improve the quality and formulation of commercial food products, prevent their deterioration, and provide health benefits that extend beyond their nutritional value. Protein isolates obtained from ISP processing could serve as natural emulsifying and gel forming agents, and bioactive and functional peptides generated from proteolysis may be applicable in food products and dietary supplements to alleviate type-2 diabetes and biological oxidation. Moreover, functional peptides produced by proteolysis can potentially be used to delay lipid oxidation and maintain food product quality. There is a growing interest among consumers in more natural food ingredients, and the development of functional ingredients from crab proteins can help meet consumer demands.

The first study confirmed that the mechanical separation of commercially valueless green crabs followed by ISP processing can recover protein ingredients potentially useful for formulated foods, and that their functional properties are highly dependent on pH during solubilization. This study is the first report on mechanical separation and ISP processing of raw crab starting material. Yield of protein recovered was higher under acidic (pH 2) than alkaline (pH 10) solubilization conditions. ISP processing considerably reduced the total mineral content of the crab mince which was mostly contributed from the crab shells. In contrast to results reported for finfish, lipids were concentrated 2-3 fold as a result of ISP processing presumably due to the higher phospholipid content in crustacean species. The pH 2

protein isolate contained predominantly larger molecular weight proteins (37 – 100 kD) while smaller molecular weight proteins (< 37kD) were distributed in the pH 10 protein isolate. These different physicochemical characteristics also affected their functional properties. The pH 10 protein isolate had significantly higher solubility at pH 7-8, higher emulsifying activity, and lower gel forming ability than the pH 2 protein isolate, and neither acid nor alkaline solubilization produced proteins with substantial foaming properties. The small molecular weight distribution of the pH 10 protein isolate contributed to higher solubility at neutral pH in comparison with the pH 2 protein isolate. Therefore, the pH 10 protein isolate may be useful in food products which require higher solubility and emulsifying activity at neutral pH such as sauces, chowders, souffles, or seafood sausages. Gel formation ability of the pH 2 protein isolate was strongly related to its large molecular weight proteins. The pH 2 protein isolate would be most useful in food gels, such as surimi, fishcakes, or lunchmeats.

However, there are some considerable challenges to improve the feasibility of recovering crab proteins for food applications. The most important issue is the low yield of recovered protein isolates. The yield of green crab protein isolates was less than 50%, which is significantly lower than the yield of commercially available soy and whey protein isolates (~70%). To improve the yield, the quality of the raw green mince derived from the mechanical separation process needs to be improved to increase its protein content and reduce the level of crab shell particulates. Mechanical separation using a deboner is influenced by various factors including equipment settings, cylinder condition, and extent of thawing of frozen crabs. Therefore, optimization of the mechanical separation process may enhance the amount and quality of the recovered mince and of the protein isolates derived from it. Also, results from this study indicate that the solubility of crab mince proteins is very low at pH 4.0-5.5. Thus, instead of precipitating proteins only at pH 5.5, serial protein precipitation at multiple pH values between 4.0 and 5.5 may improve the protein recovery.

As a method to increase the protein content in recovered proteins, defatting the crab mince using hexane could be considered. Unlike finfish, crustaceans contain a high amount of phospholipid that is not separated by centrifugation during ISP processing. Therefore, lipid removal of the initial material may help to increase the purity of protein isolates. Alternatively, methods to reduce or break the protein/phospholipid emulsion formed during the solubilization process could be explored.

The second study was conducted to evaluate the application of enzymatic hydrolysis to derive bioactive peptides from green crab mince and examine potential changes in the bioactivity of peptides after simulated human gastrointestinal digestion. This study demonstrated that enzymatic hydrolysis could improve anti-diabetic effects of green crab mince. Specifically, peptides generated by various enzyme (1:100=E:S ratio) treatments were evaluated for their capacity to prevent a blood glucose spike by inhibiting α -glucosidase digestive enzyme and stimulating a biological molecule (GLP-1) that helps insulin secretion. Anti-diabetic effects of green crab peptides were dependent on enzyme type, not degree of hydrolysis. Protamex had a higher potential to produce anti-diabetic ingredients than Alcalase, Flavourzyme, and Papain. The peptides generated by Protamex showed significantly higher α -glucosidase inhibitory activity compared to other dairy hydrolysates and the effect was not decreased by gastrointestinal digestion. Also, the strong α -glucosidase inhibition of the peptides with relatively lower α -amylase inhibition may contribute to minimize side-effects caused by non-digested polysaccharides. Both Alcalase and Flavourzyme are widely used in the seafood industry to upcycle seafood byproducts and generate bioactive protein derivatives. However, in this study, Alcalase and Flavourzyme treatment of the crab mince did not enhance either α -glucosidase inhibitory or anti-inflammatory activities, which demonstrated that the bioactivity of protein hydrolysates is dependent on enzyme type and protein source. The smallest peptides were most effective in all the different anti-diabetic mechanism assays, suggesting that the major contributor to anti-diabetic properties of green crab peptides is < 3 kD peptides.

In the MTT assay using RAW 264.7 cells, the results showed that the cell viability at 1 mg/mL sample concentration was not significantly different compared with the negative control. Therefore, green crab hydrolysates are nontoxic to RAW 264.7 cells at concentrations up to 1mg/mL. Green crab hydrolysates did not reduce NO production from inflammation induced macrophage cells. Therefore, the hydrolysates may not be able to inhibit the potential inflammatory mechanism associated with inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein and gene expression and suppress the production of pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukins.

Although this study demonstrated that enzymatic hydrolysis was effective in deriving bioactive peptides stable to simulated digestion, there is considerable room for improvement of the anti-diabetic effects of the hydrolysates. Unlike recovering proteins from ISP processing, enzymatic hydrolysis cannot concentrate proteins and peptides, therefore, the protein and peptide content in hydrolysates is lower than that of protein isolates. Therefore, to increase the protein and peptide content of the hydrolysates and reduce the interference with other non-protein based materials, protein isolation through ISP processing could be conducted prior to hydrolysis.

The last study was performed to determine whether protein hydrolysis using commercially available enzymes can improve the functional properties of green crab mince for food applications. Compared to the crab mince and protein isolates produced by ISP processing, treatments of Protamex (1:100=E:S) and the combination of Protamex and Flavourzyme (0.5:100= each E:S) for 15 min improved foaming activity, however, enzymatic hydrolysis of the crab mince decreased the emulsifying and gel forming activities. Interestingly, endogenous enzymes in the crabs contributed significantly to hydrolysis of the crab mince; hydrolysates generated by endogenous and commercial enzymes showed a comparable degree of hydrolysis after 120 min and the control hydrolysates showed the best free radical scavenging activity among all the hydrolysates. Enzymatic hydrolysis also significantly enhanced

ferric ion reducing antioxidant power. Peptides produced by the combination of Protamex and Flavourzyme treatment had significant potential as a natural antioxidant agent to prevent fat rancidity.

Even though enzymatic hydrolysis enhanced certain functional properties including foaming activity and antioxidant capacity of the crab mince, there were several challenges and limitations to the processing protocols. Unlike in the second study, the hydrolysates were not centrifuged after hydrolysis to reduce processing steps and production waste. Thus, shell particles and insoluble proteins were present in the hydrolysates, and it was difficult to grind the freeze-dried hydrolysates into a homogenous powder. Also, samples obtained after 120 min hydrolysis seemed to contain more insoluble residues in comparison with samples that were taken at 15, 30, and 60 min. This was most likely due to precipitated insoluble residues that were not evenly distributed among the samples. A better design for future research would be to perform each hydrolysis at different time points separately so that subsampling is not required. Also, milling of freeze-dried hydrolysates using a hammermill could be an option to yield a more homogeneous sample composition, which may contribute to lower variability in the solubility assay. Additional assessment at additional pH levels (pH 3-6) would help provide more comprehensive insight into solubility of the hydrolysates. Moreover, molecular weight distribution of hydrolysates should also be determined because functional properties including solubility, foaming/emulsifying activity, and gelation are influenced by the molecular weight of proteins.

The performed studies validated the development of multi-functional protein ingredients via ISP processing and enzymatic hydrolysis using commercially available proteases. In order for these ingredients from green crabs to become a commercial reality, their production process and functions should be optimized through further studies.

Peptides obtained from enzymatic hydrolysis should be tested for anti-diabetic efficacy through *in vivo* study to make sure that the activity is stable and that there are no potential side-effects (Table

5.1). *In vitro* assessments are performed with defined procedures in a controlled environment outside of a living organism. Therefore, the results observed from *in vitro* studies can be different from results obtained in living organisms. Also, identification of specific anti-diabetic peptides could provide useful information for other researchers developing anti-diabetic peptides from various food resources.

Table 5.1. Proposed future research for ongoing development of commercially viable protein ingredients from green crabs

- *In vivo* assessment of bioactive peptides for anti-diabetic effects and subsequent identification of the peptides
 - Evaluating the function and stability of proteins/peptides in food models
 - Shelf-life study of protein-based multifunctional food ingredients
 - Sensory and physicochemical evaluation of food products containing green crab protein-based ingredients
 - Economic feasibility assessment and cost–benefit analysis of optimized processes
-

The effect and stability of functional and bioactive proteins/peptides should be evaluated in food models since their activity and stability could be influenced by complicated food matrices, food processing, and interaction with other food components. For example, the pH 10 protein isolate could be applied to thermally processed clam chowder to evaluate if the protein isolate prevents phase separation during storage. Unlike the traditional surimi production process, ISP processing concentrates both myofibrillar and sarcoplasmic proteins, which help to improve the gel characteristics. The pH 2 protein isolate had higher gel forming activity with a larger amount of myofibrillar proteins compared to the pH 10 protein isolate. Therefore, the application of the pH 2 protein isolate in surimi or fishcake model might be a good strategy to evaluate the feasibility of the protein isolate in gel-based food products. Also, measurement of important properties including sensory attributes (texture, flavor, odor,

and color) and physicochemical characteristics (water-holding capacity and viscoelasticity) is recommended to evaluate the quality of the gel product. The potential of crab mince hydrolysates as an antioxidant agent could be examined in salmon patties by measuring lipid oxidation products such as hydroperoxides and aldehydes. Salmon contains a high amount of unsaturated fatty acids which are easily oxidized during refrigerated and frozen storage. Also, salmon represents one of the most popular fish species in the U.S. and is utilized in a wide variety of value-added products, therefore, it would be a good candidate food model for measuring antioxidant efficacy of the green crab hydrolysate.

The shelf-life of the functional and bioactive ingredients also should be assessed since the ingredients consist of proteins and peptides. Protein-based food ingredients could easily lose their functions, and their physicochemical characteristics can be affected by storage conditions including time, temperature, humidity, and packaging methods. Therefore, shelf-life evaluation of the protein isolates and hydrolysates under different storage conditions would provide information useful for the commercialization of these ingredients and their utility in food products.

The green crab protein isolates and hydrolysates produced in these studies had perceptible fishy odors and flavors. Therefore, sensory evaluation is important to evaluate whether these ingredients affect the odors and flavors of food products.

Also, shellfish is considered an allergenic food. The proteins including tropomyosin, myosin light chains, troponins, and some sarcoplasmic proteins are responsible for allergic reaction in people with allergies to crabs. In order to commercialize food ingredients derived from green crabs and use them in food products, the food label must clearly indicate that the ingredients are obtained from crab proteins since they can lead to allergic reactions.

Lastly, economic feasibility is a critical factor to be assessed to commercialize the functional and bioactive ingredients obtained from green crabs. Therefore, it is recommended to analyze the economic value of functional and bioactive ingredients. Assessment of the yield of obtained proteins/peptides and

the cost for harvesting green crabs, mechanical separating of shell and mince streams, all inputs during ISP and enzymatic hydrolysis process (chemicals and enzymes), and drying and packaging of obtained ingredients will help to determine their feasibility for industrial utilization.

In conclusion, the development of multi-functional food ingredients from the untapped green crab resource could be successfully achieved by ISP processing and proteolytic hydrolysis. The appropriate processing conditions need to be selected based on the intended purpose of the specific food ingredients. Utilization of functional and bioactive proteins/peptides derived from green crabs by the food industry would provide additional economic benefit to harvesters and the seafood industry by catching green crab as an incidental fishery. Therefore, value-addition of green crabs through functional protein isolation and enzymatic hydrolysis may help create profitable business opportunities for seafood harvesters and processors and the global food industry, and provide health benefits to consumers.

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