Protein Hydrolysate and Biocalcium from Salmon Frame: Preparation and their Fortification in Cracker

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ABSTRACT

Protein hydrolysates (PH) from two forms of salmon frame named 'chunk' and 'mince' were produced and characterized. Both samples were subjected to hydrolysis using alcalase and papain at 1-3% (w/w protein) for 0-240 min. PH prepared with either protease at 3% for 180 min had the solid yield of 24.05-26.39%. PH contained 79.20-82.01% proteins, 6.03-6.34% fat, 9.81-11.09% ash and 4.02-5.80% moisture. Amino acid profile showed that all PH had glutamic acid/glutamine (113.45-117.56 mg/g sample), glycine (77.86-86.18 mg/g sample), aspartic acid/asparagine (76.04-78.67 mg/g sample), lysine (61.97-65.99 mg/g sample) and leucine (54.30-57.31 mg/g sample) as the predominant amino acids. All PH possessed high solubility. The size distributions determined by gel filtration chromatography varied, depending on proteases and the form of frame used for the hydrolysis. Different PH showed varying antioxidant capacities.

Biocalcium powders, Bio-cal-A and Bio-cal-H, obtained from alkaline treated and non-alkaline treated salmon frame, a leftover from protein hydrolysis process, were characterized, in comparison with those calcined at 900°C for 6 and 9 h named Cal-A (6 h), Cal-H (6 h), Cal-A (9 h) and Cal-H (9 h). Calcium content in calcined bones (31.54-38.84%) was higher than those in biocalcium powders (27.32-30.88%). Also, phosphorus content was higher in calcined bones (15.16-18.11%), compared to those of biocalcium (13.22-14.40%). The variation of Ca/P ratios were observed among all samples, depending on chemical and heat treatment conditions. Mean particle size of all powders were 22.21-26.53 µm. Bio-cal-A had higher L*(lightness), b* (yellowness), ΔE* (total difference in color), ΔC* (chroma) values than others ($P<0.05$). X-ray diffraction showed a characteristic hydroxyapatite as the dominant phase in all samples and degree of crystallinity was obtained after calcination. Protein, fat, hydroxyproline and TBARS were detected in both biocalcium powders, but not detectable in calcined

bone powders. Pretreatment affected the amino acid compositions, abundance of volatile compounds and bioavailability of the biocalciums.

Whole wheat cracker fortified with BC and PH powders obtained from salmon frame are the sources of amino acids and calcium, respectively was developed as health promoting food. Fortification of BC and PH powders or their mixtures at different ratios (3:1, 1:1, 1:3) with total substitution of 16.67% based on whole wheat flour was carried out. Characteristics and nutritional value of resulting crackers were determined. Color, thickness, weight and textural properties of crackers varied with different ratios of BC and PH powders added. Incorporation of BC/PH (3:1) mixture showed no negative effect on sensory properties. Developed crackers possessed higher protein, fat, calcium, phosphorus, sodium and cholesterol but lower carbohydrate, sugar, fiber and energy value than the control. Crackers contained saturated fatty acid (0.31-0.38 mg/100g), monounsaturated fatty acid (0.083-0.16 mg/100g) and polyunsaturated fatty acid (0.026-0.045 mg/100g). Scanning electron microscopic images showed that the developed crackers were less porous and had a denser structure, compared to the control. Based on scanning electron microscopy energy dispersive x-ray spectroscopic (SEM-EDX) images, the crackers fortified with BC/PH (3:1) mixture had higher calcium and phosphorus distribution with higher intensity, compared to the control.

Overall, salmon frames, wastes from salmon processing industries could serve as an additional source of nutrients when processed into value-added product such as protein hydrolysate and biocalcium, which could be fortified into foods for enrichment of nutrients.

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Fish farming involves raising fish commercially in tanks or enclosures such as fish ponds. Fish farming industry plays a vital role in the social and economic well-being of nations in the world as well as in the feeding of a significant part of the world's population (Dekkers *et al.,* 2011). Hence, fish farming can be considered as one of the major occupations, which generates the income for farmers (Oosterveer 2008). As the population all over the world is increasing day by day, the demand for fish, chicken and meat is also increasing. The protein in fish is more valuable when compared to that of chicken and meat (Daram, 2016). Governments all over the world have been advertising and advising the people to include fish in their meal as it is rich in proteins and vitamins. The most important fish species produced worldwide in fish farming are carp, tilapia, salmon, and catfish etc (Ali-Arfat *et al.,* 2015). Some farmed fish, especially salmon, are rich in omega-3-fatty acids (Daram, 2016). They are commonly sold as whole fish or as fillets. Fish processing industry produces more than 60% as leftovers, which include head, skin, trimmings, fins, frames, viscera and roes, and only 40% fish products are for human consumption (Dekkers *et al.,* 2011). Without the appropriate treatment or management, pollution and disposal problems in both developed and developing countries have been faced. Waste generated from fish processing imposes a cost to dispose without gaining value (Bechtel 2003). Disposal or treatment has been identified to be cost-ineffective but also environmental unfriendly (Peter and Clive, 2006). However, these by-products contain considerable amounts of proteins, varying from 15 to 60% and are known to possess high nutritional value with respect to essential amino acids (Venugopal *et al.,* 1996; Arnesen and Gildberg 2006).

Production of protein hydrolysates has gained interest to offer solutions that reduce environmental problems (Harnedy and FitzGerald 2012). Protein hydrolysis is achieved by the cleavage of proteins to peptides by either enzymatic or chemical reactions. The most promising method is enzymatic hydrolysis, which is frequently

employed to produce protein hydrolysates that are highly functional and even nutritious (Kristinsson and Rasco 2000). Protein hydrolysis also produces several peptides with enhanced functional properties and bioactivities, compared to native protein (David and Katie 2003). Frames still have meat remaining, which contains proteins. Enzymatic hydrolysis has been implemented to recover protein in form of protein hydrolysate. Salmon frames without heads were hydrolyzed with the commercial protease called protamex and produced aqueous fraction rich in amino acid such as glutamic acid, aspartic acid, glycine and alanine (Liaset *et al.,* 2000). Je *et al.,* (2005) treated Alaska pollack frame with mackerel intestinal crude enzyme in order to produce protein hydrolysate. Yellowfin sole (*Limanda aspera*) frame containing considerable amount of protein was hydrolyzed by seven different proteases including alcalase, neutrase, pepsin, papain, ά -chymotrypsin, trypsin and tuna pyloric caeca crude enzyme (Rajapakse *et al.,* 2005). Different enzymes yielded hydrolysates with varying protein recovery and functional properties. Lee *et al.,* (2010) also hydrolyzed the tuna frame protein using alcalase, neutrase, pepsin, papain, ά-chymotrypsin and trypsin. Degree of hydrolysis (DH) after proteolytic digestion were 74.3%, 74.5% and 74.8% when ά-chymotrypsin, pepsin and neutrase were employed, respectively. However, other proteolytic enzymes showed DH lower than 60%. Different enzymes have different specificity and render different peptides. Hou *et al.,* (2011) obtained protein hydrolysates from Alaska pollock frame by hydrolyzing with ten different commercial proteases (alcalase, flavourzyme, protamex, trypsin, alkaline protease, mixed enzymes for animal proteolysis (MEAP), acid protease, neutral protease, bromelain and papain). It was observed that trypsin and MEAP gave the highest DH (25%), while other proteases gave a DH of about 15%. MEAP was shown to be the most effective protease. The main amino acids in Alaska pollock frame were glutamic acid and glycine. Alaska pollock hydrolysate could help to fulfil the requirement in human nutrition.

Calcium (Ca) is a crucial mineral to maintain human health. It has been identified as an essential element required for numerous physiological activities of human system, including maintaining nerve impulse transfer and heart rate, facilitating blood flow within capillaries, participating in blood coagulation and modulating muscle

function (Benjakul *et al.*, 2017). Deficiency of calcium has become a human problem, associated with rickets disease, osteoporosis as well as reduced bone mass (Benjakul *et al.,* 2017). This is due to inadequate calcium in most regular meals consumed by people. Tricalcium phosphate (TCP) and calcium carbonate ($CaCO_3$) have been used to supplement for calcium inadequacies (Zhao *et al.*, 2005). According to Trilaksani *et al.,* (2010), fish bone is one of the byproducts from the fish-processing industry, which contain calcium. The main elements of fish bones are calcium and phosphorus, especially in the form of calcium hydroxyapatite (Benjakul *et al.,* 2017). Calcination at high temperature has been implemented to produce hydroxyapatite (HA) (Piccirillo *et al.,* 2013). Therefore, fish bones still have the potential to be utilized in food products as a source of calcium. Benjakul *et al.,* (2017) produced biocalcium from precooked bone of skipjack, tongol (*Thunnus tonggol*) and yellow-fin (*Thunnus albacores*). They were rich in calcium and phosphorus along with collagenous protein. They could be used to alleviate calcium deficiency in foods. When calcination is implemented, organic matters such as proteins or peptides are removed. This may lead to poor solubility in gastrointestinal tract and thereafter low bioavailability. Calcium bioavailability as measured by the calcium transported across Caco-2 cell monolayer was reported (Perales *et al.*, 2005). The Caco-2 cell line is a human colon carcinoma cell line, which undergoes spontaneous differentiation in cell culture to form a polarized epithelial cell monolayer with many characteristics of enterocytes (Glahn *et al.,* 1996). Both *in vitro* simulated gastrointestinal digestion and Caco-2 cell monolayer have been applied to study calcium solubility and bioavailability in calcium containing foods (Perales *et al.,* 2005). Biocalcium from tuna showed high solubility in simulated gastrointestinal tract in comparison with calcium carbonate (Benjakul *et al.,* 2017). To improve calcium intake, several calcium-fortified products have been developed in the market such as biscuits fortified with tuna biocalcium (Benjakul and Karnjanapratum 2018). To serve the variety of nutritious foods for health care market, the fortification of health-promoting ingredients have been done intensively. Biscuits added with shrimp oil containing high amount of polyunsaturated fatty acids and astaxanthin was produced (Takeungwongtrakul and Benjakul 2017). Sponge cake added with cabbage leaf

powder with high fiber was also prepared (Prokopov *et al.,* 2015). Malted wheat biscuit fortified with cauliflower leaf powder were rich in β-carotene and iron (Wani and Sood 2014). Malted wheat flour blended cookies enriched with soybean protein was also produced (Bashir *et al.,* 2015). Whole wheat cracker is generally known as crunchy and hard snack based on flour. It is a popular food and its nutritive value can be improved by supplementation of target ingredients. In general, processed foods (i.e. snacks) rich in nutritive ingredients are required according to the consumer's preferences and needs.

Salmon *(Salmon salar)* is a fatty fish that contains high amount of omega-3 long chain polyunsaturated fatty acid and protein (Bell *et al.,* 1997). Salmon is rich in eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) omega- 3 fatty acid. These numerous compositions are of health benefits. Omega- 3 long chain fatty acids are essential for the function of the brain, cardiovascular well-being and fetal development. Apart from these, both EPA and DHA function in numerous parts of the body, such as in the cell membrane to provide fluidity and act in an anti-inflammatory capacity (Shearer *et al.,* 1994). Salmon meat has become a delicacy among the Thai people. Most of salmon are imported as frozen whole salmon, which needs filleting process. As a consequence, frame with meat remained can serve as the potential raw material for recovery of proteins or minerals. The utilization of frames under the concept of ‘zero waste’ is of essential demand, in which the value-added products can be obtained. Also, food products based on the recovered active ingredients from salmon frame can be produced to fulfil the increasing demands for ‘health care’ market.

1.2 Review of literature

1.2.1 Fish production

Fish is one of the most traded agricultural commodities and a major export for many developing countries, offering an opportunity for trade agreements, which contribute to the development of poor countries (FAO, 2013). Fish and fishery products are important sources of protein and essential micronutrients. According to Food and Agricultural Organization, fish accounted for 16.6% of the world's intake of animal protein and 6.5% of all the protein available in the world in year 2009 (FAO, 2013). This would make the fish production become a great economy contribution to the world. In year 2011, about 154 million tonnes of fish were produced with a value of 217.5 billion dollars. Approximately 131 million tonnes (85%) were directly utilized as food and the rest (15%) was under-utilized as live bait for fishing. Some were used as feed for carnivorous farmed species and marine worm ornamental products (FAO, 2013). There has been a sustained growth in the fish supply during the last 50 years with an average growth rate of 3.2% each year, which is higher than the growth rate of world's population (1.7%) (FAO, 2013). The list of top ten fish production and harvesting countries in the world is shown in Table 1. The production of fish in China, Indonesia, India and Russia has been increased continuously (FAO, 2013).

With the increasing world population, which will attain 9.8 billion by year 2050, the demand for aquatic food is set to increase further (United Nation, 2017). As a consequence, food production from fisheries and aquaculture will exceed the demand of 2014, which was about 160 million tons (FAO, 2013).

Table 1 World fish producer in year 2011

World	**Tonnes**	**(%)**
China	36,734,215	61.35
India	4,648,851	7.76
Vietnam	2,671,800	4.46
Indonesia	2,304,828	3.85
Bangladesh	1,308,515	2.19
Thailand	1,286,122	2.15
Norway	1,008,010	1.68
Egypt	919,585	1.54
Myanmar	850,697	1.42
Philippines	744,695	1.24
Other	7,395,281	12.35
Total	**59,872,600**	**100.00**

Source: Food and Agricultural organization (2013)

1.2.1.1 Fish processing

The term ‘fish processing’ refers to the processes associated with fish and fish products between the time fish are caught or harvested, and the time the final products are delivered to the customers (Pearson 1983). Practically, this term is extended to cover any aquatic organisms harvested for commercial purposes, whether caught in wild fisheries or harvested from aquaculture or fish farming. Most fish processing plants have used the following steps: stunning of fish, grading, removal of slime, scaling, washing, de-heading, gutting, cutting of fins, slicing into steaks, filleting, meat bone separation, packaging, labelling and distribution (Figure 1) (Ghaly *et al.*, 2013).

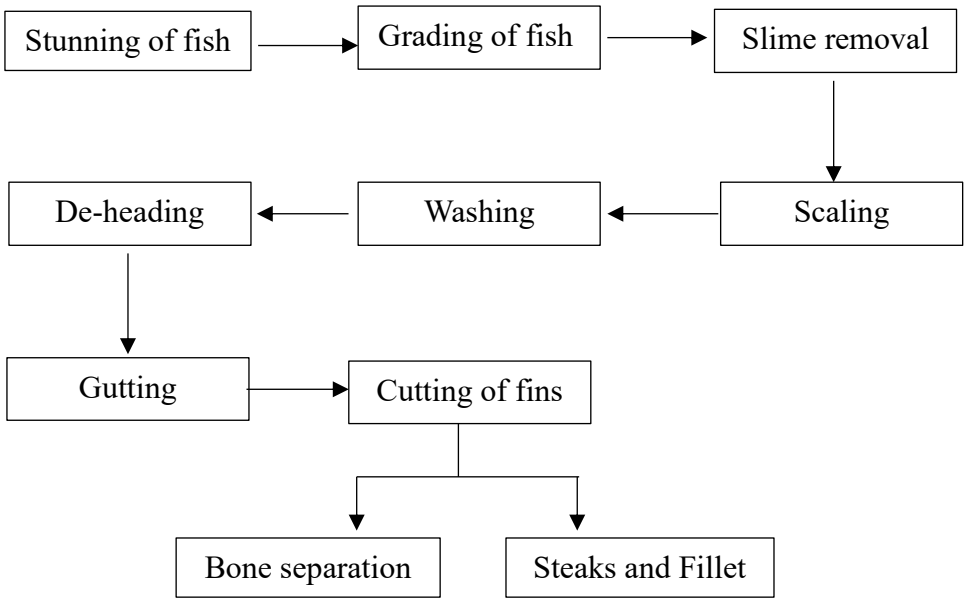

Figure 1 Steps for processing of fish

Source: Ghaly *et al.* (2013)

Stunning

The stunning of fish is the first and most critical step in the processing of fresh water and farmed fish because the prolonged agony experienced by the fish causes the formation of undesired substances in fish tissues. The oxygen deficiency in the blood and muscle causes accumulation of lactic acid and leads to paralysis of the neural system. Stunning is achieved by subjecting the fish to asphyxiation on board after capture until they die or by subjecting them to electric shock. (Bykowski and Dutkiewicz 1996; Borderías and Sánchez-Alonso 2011; Erikson *et al.*, 2012). Improper stunning or application of stress factors may influence the fish physiological reactions and the post mortem biochemical processes. Those changes affect the quality and durability of the final product (Thomas *et al.*, 1999; Parisi *et al.*, 2001).

Grading

The second step in fish processing is fish grading by species and size. Grading of fish can be done manually or by mechanical equipment. The mechanical grading is more precise for fish before or after rigor mortis than for fish in a state of rigor mortis.

The automated grading instruments are 6-10 times more efficient than manual grading (Ghaly *et al.,* 2013). The basic benefits of the automated system are low production costs and increased quality of fish products at the end of the processing chain (Tave and Tucker 1994).

Slime removal

Fish secretes slime on its surface as a protection mechanism against harmful conditions. The slime secretion stops before rigor mortis. Pseudomonas species are one of the potent spoilers, always present in the sea water, and fish slime provides them with a perfect environment to grow (Ghaly *et al.,* 2013). Anaerobic bacteria present during fish processing can produce hydrogen sulfide by taking up sulfur compounds from the slime, skin and flesh (Ghaly *et al.,* 2013). Therefore, the slime should be removed by continuous washing (Doyle 1995).

Scaling

The scales may harbour bacterial pathogens and the removal of scales is commonly done before filleting or slicing. The scaling can be done manually with a hard brush or mechanically using scaling blades. The scales of some fish such as perch, pike-perch, carp and bream are difficult to remove. These fish are first blanched in boiling water for 3-6 seconds and then scaled using mechanized hand-held scalers or an electrical scaler (Ghaly *et al.,* 2013). The electrical scalers are more efficient in completely eliminating scales than the manual tools and saves a lot of time (Borderías and Sánchez-Alonso 2011).

Washing

The primary goal of washing is to clean and remove the accumulated bacteria on the fish. The effective washing of fish depends upon the fish: water ratio, the quality of water and kinetic energy of the water stream. The washing time can be about 1-2 minutes and these mechanized washers can be used to process whole fish, de-headed and gutted fish as well as fish fillets (Ghaly *et al.,* 2013). Washing action should not cause any physical damage to the product. The washing process is always continuous and is accomplished by spraying pressurized water (Hossain *et al.,* 2004). Washing of the fish with clean water could reduce microbial load by 1.5 log CFU/g (Ahmed *et al.,*

2015). Contrary to this, a study in a Vietnamese processing company showed that the count of bacteria and coliforms in tap water increased from the time the fish entered the washing bath during washing process and stayed high during the washing process. It was reported specifically that the total plate count (TPC) rose sharply up to 5.6± 0.8 log CFU/100 ml. It could be a result of microbial proliferation in the wash water due to the presence of large amount of nutrients released from fish or the suspended matter (Ragaert *et al.,* 2007).

De-heading

Fish head constitutes up to 20% of its weight and it is usually considered as an inedible part (Ghaly *et al.,* 2013). The fish can be de-headed manually or mechanically. Manual cutting is easier for small fresh water fish (Ghaly *et al.,* 2010). Larger fish ranging from 20 to 40 cm can be de-headed using mechanical devices. Machines with a guillotine cutter are suitable for larger fish under-going round or contour cuts. Machines with a manually-operated circular saw are suitable for larger fish undergoing straight cuts. The amount of de-headed waste produced from fish processing is 27-32% (Arvanitoyannis 2010). De-heading of fish could extend shelf-life by 9 days when stored in ice, in comparison with the whole fish (Kristinsson and Rasco 2000). In order to reduce the bacterial load, immediately on the death, fish should be deheaded, gutted, washed and chilled in order to inhibit unfavourable enzymatic and microbiological processes (FAO, 2016).

Gutting

Gutting or evisceration of the fish is the removal of internal organs and optionally cleaning the body cavity of the peritoneum, kidney tissue and blood. For the gutting process, the fish is cut longitudinally to remove the internal organs on a table made of special material, which is easy to wash and does not absorb fluids. Mechanical gutting machines are used industrially for trout, eel and other fish, but their use increases the fish processing cost (Jonatansson and Randhawa 1986). Evisceration of fish could lower microbial load during the iced storage of fish. Quality deterioration was also retarded via evisceration (Liston 1980).

Cutting of fins

Fins are cut manually either by a knife or by mechanized rotating disc knives. This process is mostly carried out after de-heading and gutting. This process is difficult for cutting larger fish. The mechanical knives are provided with some slit openings, in which the fins are cut when the fish are passed through it manually (Mørkøre *et al.,* 2001).

Steaks and fillets

Fillets are pieces of meat containing only the dorsal and abdominal muscles. The fillets are processed manually or mechanically. Manual filleting is carried out in small fresh water fish industries and mechanical filleting is used for processing marine fish. Large fish such as cyprinids are sliced mechanically because of their solid and massive backbone (Hanson *et al.,* 2001). After filleting, backbone is generated as a byproduct (Ghaly *et al.,* 2013).

Deskinning

Some fish products are deskinned, in which skin is produced as leftover. However, skin contains collagenous proteins, which can be a starting material for collagen and gelatin extraction (Benjakul *et al.,* 2014). Collagen and gelatin have been extracted from skin of several fish. Properties are varied, depending on raw material, pre-treatment and extraction conditions (Sinthusamran *et al.,* 2013).

1.2.1.1.1 Uses of fish processing byproducts

The solid fish waste, which is often discarded, include head, tails, skin, gut, fins and frames (Table 2). These by-products of the fish processing industry can be a great source of value-added products such as proteins and amino acids, collagen and gelatin, oil and enzymes etc. (Disney *et al.,* 1977; Esteban *et al.,* 2007). These wastes are rich in protein (58%), fat (19%) and minerals.

Table 2 Composition of fish

Component	Average Weight (%)
Head	21
Gut	7
Liver	5
Roe	4
Backbone	14
Fins and lungs	10
Skin	3
Fillet and skinned	36

Source: Waterman (1979)

Since the volume of waste produced by processing plants is calculated to be about 50% of the total processed fish. Several efforts have been paid to exploit those byproducts such as production of fish silage, fish meal, fish sauce and hydrolysates (Portz and Cyrino 2004).

Fishmeal

Fishmeal is a dry powder prepared from whole fish or leftover from fish filleting process. The raw materials are transported to the processing factories either fresh or preserved (Ghaly *et al.*, 2013). The production of fish meal is carried out in six steps: heating, pressing, separation, evaporation, drying and grinding. When the fish is heated, the protein is coagulated and the fat deposits are ruptured. This liberates oil and water. The fish is then pressed, which removes large amounts of liquid from the raw material. The liquid is collected to separate oil from water. The water, also known as stick water, is evaporated to a thick syrup containing 30 to 40% solids (Ghaly *et al.*, 2013). Then it is subjected to drying using press cake method to obtain a stable meal. This meal is ground to the desired particle size. Fishmeal obtained from wild-harvested whole fish and shellfish currently makes up the major aquatic protein source available for animal feed (Barlow and Windsor 1984; Hussein and Jordan 1991).

Fish sauce

Fish sauce is made from small pelagic fish or by-products using salt fermentation. Fish are mixed with salt in the ratio of 3:1 at 30°C for six months and an amber protein solution is drained from the bottom of the tank. It can be used as a condiment on vegetable dishes and is very nutritious due to the presence of essential amino acids (Ghaly *et al.,* 2013). Fermented fish sauce has various biological activities including angiotensin I-converting enzyme (ACE) inhibitory activity and insulin secretion stimulating activity (Okamoto *et al.,* 1995; Ichimura *et al.,* 2003). Dissaraphong *et al.,* (2006) produced fish sauce from tuna internal organs by mixing organs with salt at a ratio of 9/9. The fermentation was performed up to 12 months. Fish sauce was brownish in color and rich in protein. Amino nitrogen content could be used as an indicator for degree of fermentation (Lopetcharat and Park 2002). The amino nitrogen contents were approximately 12.55-13.20 g N/I in all fish sauce samples after 12 months of fermentation.

Fish mince

Fish frame still has the meat left. This meat can be recovered and used as a fish mince. Fish mince from frame is a great source of proteins, minerals and fat with some other nutritional benefits (Ghaly *et al.,* 2013). On an industrial scale, fish mince is obtained from the separation of skin and bone using a mechanical bone separator. The coarseness, texture, color and yield of the mince is affected by the diameter of the perforations, pressure applied, and the raw material or trimmings used. The mince obtained from the nape and the frames are often darker in color than that from trimmings or cut-offs (Howgate *et al.,* 1992). Those minces are usually of low quality and have the limited use due to their accessibility to oxidation and off-flavor (Hsu *et al.,* 2009). The dark to reddish color obtained in frame mince comes mostly from the kidney of the fish located under the spinal cord or the backbone, which gets mixed into flesh with abrasive handling of the frame. Hence, water jet technology is applied for cleaning. The application of water jet technology has been used to clean the flesh from the backbone of round fish leaving the kidney intact by using high-pressure water jets to wash the meat off the bones. The production of fish mince has been increased since

there is an increase in fish consumption. The rough estimate of recoverable mince from leftover of round fish is shown in Table 3. This mince could be of value in fortification and development of new food product.

Fish protein isolate can be prepared from fish frame via acid or alkaline solubilization. Surasani *et al.,* (2017) obtained fish protein isolate from Pangas (*Pangasius pangasius*) fillet fish frame via acid or alkaline solubilization. The protein yield was found to be maximum at pH 2.0 in acid solubilization and at pH 13.0 in alkaline solubilization. The protein isolate obtained was rich in glutamic acid, aspartic acid, leucine and lysine. Protein isolate obtained using alkaline solubilization had higher amount of amino acids than that obtained by acid solubilization, mainly due to less pH-induced proteolysis in alkaline processing. Alkaline solubilization was found to be better for recovering protein from Pangas frame processing with good functionality and yields (Gehring *et al.,* 2011). Undeland *et al.,* (2002) obtained protein isolate from the frame of herring (*Clupea harengus*) by an acid or alkaline solubilization. The proteins were solubilized at pHs 2.7 and 10.8. Lipid content of the herring light muscle reduced from 0.13 to 0.043 g/g of protein in the final protein isolate. The lower lipid contents obtained by solubilizing the proteins could be as a result of the lipids becoming "liberated" as the muscle structure disintegrates. The protein isolates could be incorporated into surimi gels.

Table 3 Rough estimate of recoverable mince from leftover of fish

Recoverable mince	**Amount (%)**
Trimmings	3-4
Nape	4-5
Head	3-4
Belly flap	5-6
Frame	4-5
Total	15-18

Source: Oreopoulou and Russ (2007)

Several alternative uses of fish mince have been studied to lower wastage. Fish mince was recovered from round fish backbones and used as a value-added product in salted fish (Ghaly *et al.*, 2013). Mei *et al.*, (2003) reformed the mince from low value fish cut-offs and trimmings into fillet-like products with textural characteristics resembling intact fish flesh giving increased quality and added value to the products. Also, Yousefi and Moosavi-Nasab (2014) produced fish sausage from Talang queenfish (*Scomberoides commersonnianuus*) mince. The fish mince obtained had protein, fat, ash and moisture contents of 19.42, 19.12, 2.09 and 65.46%, respectively. However, the high fat content of the fish mince sausage showed that they are sensitive to oxidation, in which oxidation products such as peroxides and malonaldehyde were formed during the storage. Fish mince is generally washed with water to remove components such as pigments, trimethylamine oxide, lipids in the fish mince that can have negative effects on the quality and cause negative effect such as flavor, odor, stability and color problems (Kim and Park 2007). It has also been suggested that surimi can be used further in the preparation of a wide array of products, such as seafood analogues and fabricated meats (Kamal 1994) and other gel products (Hur *et al.*, 2011).

1.2.1.1.2 Proteolytic Enzymes

Enzyme acts as a catalyst that speeds up chemical reactions between organic components within the cells that would take an extremely long time to complete. Enzymes are used to perform desired functions in processing and analysis and to facilitate the conversions of raw materials into higher-quality desirable foodstuffs (Richardson *et al.*, 1987). Enzymes possess active site that is highly specific for certain substrates. Enzymes catalyze only one specific reaction and function by forming a complex with the substrate whose transformation takes place. Proteases are classified based on the specificity of the peptide bonds they attack (hydrolyze) and the mechanism by which they act (Lahl 1994; López-Otín and Bond 2008). There are four major known classes of proteases. They are classified according to their principal functional group in their active site: serine, thiol, carboxyl and metallo. They are also classified further based on the mechanism they use to hydrolyze into endopeptidases or exopeptidases (Tavano 2013). The function of endopeptidases is to cleave/hydrolyze the peptide

bonds within the protein molecules usually at specific residues to produce large peptides. The exopeptidases systematically remove amino acids from either the N terminus, called the aminopeptidases, or the C terminus, called carboxypeptidases, by hydrolyzing the terminal peptide bonds as shown in Figure 2. In protein hydrolysis, endopeptidases are always used, but sometimes endopeptidases are combined with exopeptidases to achieve a more complete degradation (Adler-Nissen 1986; Nunn *et al.,* 2003). The catalysis by proteases occurs primarily as three consecutive reactions: (1) the formation of the Michaelis complex between the original peptide chain and the enzyme, (2) cleavage of the peptide bond to liberate one of the two peptides, and (3) a nucleophilic attack on the remains of the complex to split off the other peptide and to reconstitute the free enzyme (Satake *et al.,* 2002). The hydrolysis of peptide bonds leads to an increase in the numbers of ionizable groups (NH_3^+ and COO),
with a concomitant increase in hydrophilicity and net charge, a decrease in molecular size of the polypeptide chain, and an alteration of the molecular structure. Nevertheless, the exposure of the buried hydrophobic interior to the aqueous environment also occurs (Mahmoud 1994; Bolen and Baskakov 2001).

Enzymatic hydrolysis breaks down fish protein using specific proteolytic enzymes, thus producing soluble and insoluble fractions (Hou *et al.,* 2011). Certain factors influence the hydrolysis of fish protein including raw material, enzyme used, conditions for hydrolysis and degree of hydrolysis (DH). Enzyme selection is made according to economics and efficacy of enzymes (Lahl, 1994). In general, microbial enzymes exhibit numerous advantages than plant and animal counterparts due to higher versatile catalytic activities and greater stability in wide pH range and temperatures in order to obtain shorter peptide bonds or cleavage (Guerard *et al.,* 2002). Several enzymes from plant (papain and ficin), animal (trypsin and pancreatic), or microbial (pronase and alcalase) sources have been employed (Loffler 1986). Besides that, autolysis, which uses endogenous enzymes within fish muscle and viscera, triggers the breakdown of proteins into smaller peptides (Prabha *et al.,* 2013). Preparation of fish protein hydrolysate requires proteolytic digestion of the byproducts at optimal pH and temperature required by the enzymes. The hydrolysis conditions for *Pellona ditchela*

with alcalase were optimized using response surface methodology (RSM) with three independent variables including pH (4-6), time (80-100 min) and temperature (40-60°C). From the RSM, the optimum condition required for maximum degree of hydrolysis 40.01%) was identified as 90 mins, pH 5 and 50°C. The hydrolysate obtained from *Pellona ditchela* had high amount of total amino acids such as threonine, methionine, isoleucine and phenylalanine.

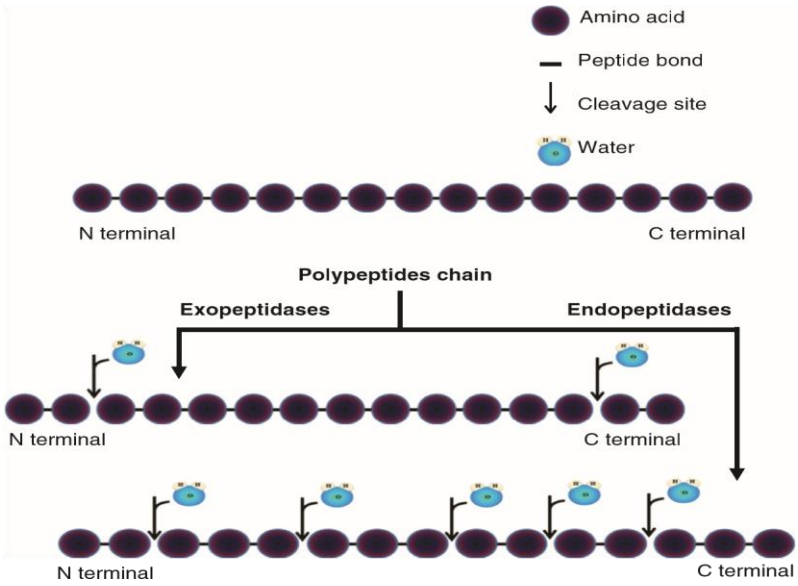

Figure 2 Enzymatic hydrolysis of proteins by endo- and exopeptidases

Source: Benjakul *et al.* (2014)

Viscera and digestive enzymes

Viscera and digestive tract of aquatic animals consist of various kinds of proteases. Despite the numerous proteases, the major ones are pepsin, trypsin and chymotrypsin. Pepsin is usually located in the stomach of fish. It belongs to the aspartic endopeptidase. This enzyme is majorly responsible for the digestion at acidic pH (De la Parra *et al.,* 2007). Trypsin is found in the spleen, caeca and pyloric (Kishimura *et al.,* 2005). It is noted that the variety of enzymes are found in different locations in the fish viscera and digestive tract. The enzymatic activity of trypsin and chymotrypsin in digestive tract and viscera from fish tends to be greatest at alkaline or neutral pH values (Bougatef *et al.,* 2010). In addition, the feeding as well as the season of capture will influence the enzyme activity. Heavily fed fish will generally deteriorate more rapidly

because of higher enzyme concentration in the digestive tract of the fish during feeding. The effect of season on enzyme activity varies with the feeding cycle, spawning cycle, water temperature and other variables. The thermal stability and activity of fish enzymes varies from one species to another. The activity and thermal stability of tryptic enzymes from horse mackerel (*Trachurus mediterraneus ponticus*) are higher than those from sprat (*Sprattus nostamus*). The pepsin from plaice (*Pleuronectes platessa*) is ten times more active than that from horse mackerel. The effect of digestive and viscera mass enzymes will depend on the method of dressing the fish, fish species, pH of the mixture and other factors. Since there is a large group of enzymes from these sources and each enzyme will require different conditions for optimal activity, it is difficult to predict the total effect in a specific situation. (Wheaton and Lawson 1985; Sattely *et al.,* 2008).

Muscle tissue enzymes

Muscle tissue enzymes are usually located within the cell. This resulted into more constraint of the muscle tissue enzymes and thus reduce their effectiveness for hydrolysis. Although muscle tissue enzymes have an optimal activity at neutral pH values, low pH also increases muscle tissue enzyme activity. For fish silage and some other products, most hydrolyzed fish products are prepared at pH values around 4 (Mackie 1982).

Plant enzymes

Papain from papaya latex, bromelain found pineapple juice and ficin from figs are well-known plant proteases. Papain is a traditional plant protease and has been used extensively for hydrolysis. It possessed broader specificity due to the presence of proteinase and peptidase isozymes. Papain is the best known cysteine protease. It has activity near neutral pH and is quite heat stable. It contains six sulfhydryl and one free cysteine, which is part of the active site. The seven site for recognizing substrate amino acid residues exist on the enzyme, all contributing to substrate specificity. It hydrolyzes amides of arginine, lysine readily and glutamine, histidine, glycine and tyrosine at reduced rate. Besides papain, papaya latex also contain chymopapain and caricain (Dubey *et al.,* 2007). All the three endopeptidases differ in primary structure but they

have very similar substrate specificities. The characteristics make them well suited to help hydrolyze protein in fish product conveniently (Wheaton and Lawson 1985). Cysteine proteases have relative molecular masses of 20-30 kDa. Cysteine protease activity depends on catalytic pair consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues differs (Dubey *et al.*, 2007). Generally, cysteine proteases are active only in the presence of reducing agents such as hydrogen cyanide (HCN) or cysteine. Cysteine protease catalyze the hydrolysis of carboxylic acid derivates through double displacement pathway involving general acid base formation of an acyl-thiol intermediate, the acyl-enzyme, resulting from nucleophilic attack of the active site thiol group on the carbonyl carbon of the scissile amide or ester bond of the bound substrate (Dubey *et al*., 2007). The first step in the reaction pathway corresponds to the association (or noncovalent binding) of the free enzyme and substrate to form the Micheaelis complex. Acylation of the enzyme, with formation and release of a first product follows this step. Acyl-enzyme reacts with a water molecule to form the second products (diacylation step). Release of this product results in the regeneration of the free enzyme (Rao *et al.,* 1998).

Microbial enzymes

Microbial enzymes are products from microorganisms. They are classified into various groups, dependent on whether they are active under acidic, neutral or alkaline conditions on the characteristics of the active site group of the enzyme, i.e. metallo- (EC.3.4.24), aspartic- (EC.3.4.23), cysteine- or sulphydryl- (EC.3.4.22), or serine-type (EC 3.4.21) (Rao *et al.,* 1998). Microorganisms are known for excretion of proteolytic enzymes capable of degrading proteins. Many types of microbes excrete proteolytic enzymes, including fungi (*Aspergillus oryzae*), bacteria (*Bacillus subtills*) and (*Bacillus licenformis*), actinomycetes (*Streptomyces griseus*) and yeast (*Saccharomyces spp*) (Mackie 1982). Careful selection and controlling of the growth environment within the hydrolysis chambers enable the desired microbes to flourish and produce significant quantities of proteolytic enzymes which help hydrolyze the fish protein (Wheaton and Lawson 1985). Bacterial alkaline proteases are characterized by their high activity at alkaline pH 8 to 10, and their broad specificity. Bacterial alkaline proteases either have

a serine center (serine protease) or metallo-type (metalloprotease). They have optimum temperature of around 60°C. These properties of bacterial alkaline proteases makes them suitable for various uses (Olivieri *et al.*, 2002). Also, bacterial alkaline proteases have different ranges of molecular masses such as 45 kDa, 36 kDa for the proteases from the wild strains and 40 kDa for other bacterial proteases (Gupta *et al.*, 2002).

Subtilisins are of Bacillus origin and they represent the second largest family of serine proteases. They are generally secreted extracellularly for the purpose of scavenging nutrients (Graycar 1999). This class of proteases is specific for aromatic or hydrophobic residues (at position P1), such as tyrosine, phenylalanine and leucine. Two different types of alkaline proteases, subtilisin Carlsberg (alcalase) and subtilisin Novo or bacterial protease Nagase (BPN) have been identified. Subtilisin Carlsberg produced by *Bacillus licheniformis,* while Subtilisin Novo produced *Bacillus amyloliquefaciens.* Both subtilisins have a molecular mass of 15-30 KDa but differ from each other by 58 amino acids. They have similar optimal temperature of 60°C and an optimal pH range of 8-10. Both enzymes exhibit a broad substrate specificity and does not depend on Ca^{2+} for its stability (Olivieri *et al.,* 2002).

1.2.3 Protein hydrolysate from fish processing byproducts

1.2.3.1 Production of fish protein hydrolysate

Hydrolysates are defined as proteins that are chemically or biologically broken down to peptides of varying sizes (Kristinsson and Rasco 2000). Protein hydrolysis decreases the peptide size. Hydrolysates contain the most available amino acids and their peptides possess various physiological functions of human body (Khantaphant and Benjakul 2008). Protein hydrolysates are used as readily available sources of protein for humans and animals and they have good functional properties (Neklyudov *et al.*, 2012).

Protein hydrolysis produces peptides with bioactivities (Kitts and Weiler 2003). Nowadays, research interest has grown in the production of protein hydrolysates because of their increasing significance as a potential ingredient for many health promoting functional foods (Cao *et al.,* 2009; Sae-leaw *et al.*, 2016). Several methods have been applied in the production of fish protein hydrolysate (FPH). These methods

include enzymatic hydrolysis, autolysis, thermal hydrolysis and bacterial fermentation. Enzymatic hydrolysis can be achieved with the aid of proteases. Endopeptidases can hydrolyze peptide bonds inside protein molecules. On the other hand, exopeptidases are able to hydrolyze peptide bonds from either C or N termini (Clemente 2000; Raksakulthai and Haard 2003). Hydrolysis of proteins into short chain peptides can augment functional and nutritional properties of foods (Kudo *et al.,* 2009). Individual protease possesses a specificity toward peptide bonds adjacent to particular amino acid residues. As a consequence, hydrolyze protein with the desired characteristics and properties can be manufactured (Wu *et al.,* 2003).

1.2.3.1.1 Hydrolysates obtained from different fish sources

A lot of the enzymes mentioned above have been used for effectively for hydrolysis of fish leftovers obtained after filleting process in the fish industries. Protein hydrolysates from heads of salmon (*Salmo salar*) and red salmon (*Oncorhynchus nerka*) were produced by alcalase. Optimized temperature, enzyme-to-substrate ratio, and pH using response surface methodology (RSM) were 49-61°C, 3.5-6.5 and 7.0-8.0, respectively. Hydrolysates obtained had protein content that varied between 78-85%; amino acid composition did not differ significantly between the crude protein sample before hydrolysis and the hydrolysates obtained. However, the amount of glycine appeared lower after hydrolysis with Alcalase. This was probably because of an incomplete hydrolysis of the connective tissue. The functional properties of fish protein hydrolysate could be enhanced by hydrolysis because the solubility of protein hydrolysates obtained was high at all pH values (Gbogouri *et al.,* 2004). In addition, Sathivel *et al.,* (2003) prepared protein hydrolysates from head, whole fish, body and gonad of herring (*Clupea harengus*) using alcalase for 75 min. Protein hydrolysates showed different functional property. The whole herring hydrolysate (WHH), herring body hydrolysate (HBH), and herring head hydrolysate (HHH) had high protein contents ranging from 84.4% to 87%, whereas herring gonad hydrolysate (HGH) had a significantly lower value (77%). All fish protein hydrolysates obtained from herrings had lower emulsifying capacities when compared with egg albumin and soy protein concentrate. Emulsifying stability of FPHs ranged from 48.6% to 54.2%, compared to

that of egg albumin (72.3%) and SPC (62.2%). Among herring FPHs, WHH and HBH had much higher antioxidant activities than HHH. Sathivel et al. (2003) produced the protein hydrolysates from red salmon (*Oncorhynchus nerka*) head using different proteolytic enzymes (alcalase, flavourzyme, palatase, protex, GC 106 and neutrase) for various reaction times (25, 50, 75 min). For all enzymes, a steady increase in % DH was observed with increased hydrolysis time. Samples with neutrase had the highest % DH at 15, 30, 45 and 60 min of hydrolysis time. However, the sample treated with alcalase had the highest overall % DH at 75 min of hydrolysis. The lowest % DH was observed for samples treated with the GC 106 enzyme. When hydrolysate was prepared with various enzymes for 75 min, lysine content of red salmon hydrolysates ranged from 71.4 to 83.2 (milligrams of amino acid per gram protein). Emulsifying stability of red salmon head hydrolysates ranged from 66.9% to 100%, and hydrolysate produced using flavourzyme had the highest emulsifying stability.

Fish frames having the remaining meat have been used for production of protein hydrolysate. Kim *et al.,* (1997) recovered proteins from cod frame using tuna pyloric caeca crude proteinase. Hydrolysis was carried out at 45-50°C for 12 h. The obtained hydrolysate were rich in glutamine, glycine, aspartine, alanine, lysine, serine, threonine and arginine. The hydrolysate could be used in the food processing industry as amino acid fortifying ingredients. Montecalvo Jr *et al.,* (1984) obtained protein hydrolysate from flounder frame using pepsin and ά- chymotrypsin at a pH of 5 and 7, respectively. The protein hydrolysates were rich in protein.

Apart from head or viscera, skins of fish have been used for hydrolysate preparation. Skin gelatin hydrolysate could be prepared with the aid of protease. Karnjanapratum *et al.,* (2016) produced gelatin hydrolysate from pre-treated non-swollen and swollen skin of unicorn leatherjacket skin using papaya latex. The DH varied depending on type of enzyme and hydrolysis time. All hydrolysates showed antioxidant activities with effective ability to retard lipid oxidation. Sae-leaw *et al.,* (2016) prepared gelatin hydrolysates from sea bass skin using alcalase. The immunodulatory potential of gelatin hydrolysates from seabass skins were determined by measuring their effect on the production of cytokines IL-6 and IL-1β in

lipopolysaccharide (LPS)-stimulated macrophage RAW264.7 cells. The result obtained showed that the seabass skin hydrolysates could significantly reduce interleukin-6 (IL-6) and IL-1β production in RAW264.7 cells.

Hydrolysates were also prepared from fish meat. Hydrolysate from yellow travelly meat was documented (Klompong *et al.*, 2007). Defatted mince and mince yellow stripe trevally (*S. leptolepsis*) was hydrolyzed with flavourzyme and alcalase respectively at various concentration levels of 0.25, 0.5, 1, 2.5, 5, 7.5 and 10% (w/w). The hydrolysate obtained from minced possessed a higher DH than those derived from defatted mince. With the same protein substrate and same amount of enzyme alcalase showed a higher DH than did flavourzyme over the entire hydrolysis period. Antioxidant activity of protein hydrolysates from yellow stripe trevally meat varied with DH and enzyme used. Emulsifying and foaming properties of protein hydrolysates were also dictated by both factors. Fresh round scad meat was hydrolyzed with different enzymes such as alcalase at pH 50°C, pH 9.5; neutral protease at 50°C and pH 7.0; papain, 55°C and pH 7.0; pepsin 37.5°C and pH 2.0 and trypsin of 37.5°C and pH 7.8 (Jiang *et al.*, 2014). Based on antioxidant capacities, all hydrolysates possessed similar reducing power, while hydrolysate prepared by neutral protease showed higher superoxide radical scavenger activity. Protein substrate from ornate threadfin bream prepared by an acid solubilization process was hydrolyzed with skipjack tuna pepsin (Nalinanon *et al.*, 2011). Hydrolysates obtained exerted good functionalities and antioxidant activities and could be incorporated as a multi-functional ingredient into foods.

In summary, hydrolysates from various fish waste exhibited several potential benefits. Hence products with various health-promoting properties from aquatic resources, especially fish protein hydrolysate, have a good chance of being successful in the market.

1.2.3.1.1.1 Amino acid composition of fish protein hydrolysate

Amino acids are the building blocks of proteins. They have wide nutritional value, taste, medicinal action and chemical properties (Ghaly *et al.*, 2013). Protein hydrolysates obtained after hydrolysis of proteins are composed of free amino acids and

short chain peptides exhibiting many advantages as nutraceuticals or functional foods (Chalamaiah *et al.,* 2012). The amino acid composition of any food proteins has significant role in various physiological activities of human body and affects either directly or indirectly in maintaining good health (Dos Santos *et al.,* 2011).

Fish protein hydrolysates have been reported to exhibit variation in their amino acid composition (Wasswa *et al.,* 2007; Bhaskar *et al.,* 2008; Ovissipour *et al.,* 2009). The variation in amino acid composition of different fish protein hydrolysates mainly depends on several factors such as raw material, enzyme source, and hydrolysis conditions (Klompong *et al.,* 2008; Klompong *et al.,* 2009). The essential amino acids required for maintaining of good health have been found abundantly in fish protein hydrolysates (Klompong *et al.,* 2009b; Sathivel *et al.,* 2003; Yin *et al.,* 2010). Among all the amino acids, aspartic acid and glutamic acid were found to be higher in most of the reported fish protein hydrolysates (Ghassem *et al.,* 2011; Hou *et al.,* 2011a; Klompong *et al.,* 2009a; Yin *et al.,* 2010). Fish protein hydrolysates contained all the essential and non-essential amino acids (Ghassem *et al.,* 2011; Khantaphant *et al.,* 2011; Klompong *et al.,* 2009a; Nakajima *et al.,* 2009; Wasswa *et al.,* 2007). Protein hydrolysates produced from fish processing byproducts of capelin (*Mallotus villosus*) (Shahidi *et al.,* 1995) had amino acid profiles similar to that of the original capelin, except for sensitive amino acids such as methionine and tryptophan which were affected to a relatively larger extent. The content of tryptophan was reduced by approximately 60% in the final product. In addition to muscle hydrolysates, head, skin and visceral hydrolysates were reported to contain all the essential and non-essential amino acids (Bhaskar *et al.,* 2008; Gimenez *et al.,* 2009; Ovissipour *et al.,* 2009). Aromatic amino acids were not reported in fish frame protein hydrolysates (Hou *et al.,* 2011b).

Protein hydrolysates produced from fish processing byproducts has shown to be rich in amino acid. Hydrolysate of Pacific whiting solid waste (*Merluccius productus*) (Benjakul and Morrissey, 1997) was prepared using alcalase and neutrase at 60°C and 50°C respectively. Amino acid compositions of freeze-dried hydrolysate were similar to those of Pacific whiting solid waste and Pacific whiting muscle. Hydrolysate obtained from Pacific whiting solid waste contained a lower amount of glutamic acid

and tryptophan than Pacific white solid waste and the muscle. Tryptophan in hydrolysate were reduced to 14.74-21.5% of that in Pacific white solid waste and muscle. Freeze-dried hydrolysate and Pacific whiting solid waste had a similar amount of glycine, which was approximately 2-fold higher than that of the muscle. Hence, the protein hydrolysates produced from different fish including byproducts from fish processing can be used as good source of essential amino acids.

1.2.3.1.1.2 Bio-activity of fish protein hydrolysate

Antioxidant

During cellular respiration in humans and other aerobic organisms, reactive oxygen species (ROS) and free radicals are generated. The ROS and free radicals play an important role in several diseases such as neurodegenerative disorders, hypertension, inflammation, cancer, diabetes, Alzheimer disease, Parkinson's disease and ageing problems (Bougatef *et al.,* 2009). The ROS and free radicals contain unpaired electrons in valency shell and attract electrons from other substances, thus making oxidative stress in the cells or tissues. These free radicals are unstable and react rapidly with the other substances or molecules in the body, leading to cell or tissue injury. In addition to the physiological production of ROS and free radicals, oxidation of fats and oils in food products during processing and storage also leads to production of undesirable radicals as well as the secondary lipid peroxidation products (Sarmadi and Ismail, 2010).

Lipid oxidation is one of the greatest concerns of the food industry and consumers because it leads to the development of undesirable off-flavors and potentially toxic reaction products (Arvanitoyannis et al. 2006). In order to prevent lipid peroxidation in food products, many synthetic antioxidants such as butylatedhydroxy toluene (BHT), butylatedhydroxy anisole (BHA), tertiarybutylhydroquinone (TBHQ), and propylgallate (PG) have been used (Kim and Wijesekara, 2010). Due to the potential health risks of synthetic antioxidants, the search for safe natural antioxidants is important for food industry.

An antioxidant is defined as any substance that considerably delays or inhibits the oxidation of a substance. Antioxidant in food play a significant role as health-benefiting factor that protects the body from oxidative stress. In recent years, fish

protein hydrolysates have gained much interest as potential source of antioxidative peptides. Selection of appropriate proteolytic enzyme is an important factor for the release of antioxidant peptides from fish proteins. Proteolytic enzymes such as alcalase, ά-chymotrypsin, neutrase, papain, pepsin, trypsin, pancreatin, flavourzyme, bromelain, pronase E, protamex, orientase, thermolysin, validase, protease A amano, protease N amano and cryotin F derived from plant, animal and microbial sources have been successfully used for the production of antioxidative peptides from fish protein sources (Batista *et al.,* 2010; Hsu, 2010; Nakajima *et al.,* 2009). Besides selection of appropriate proteolytic enzymes, the physico-chemical conditions of the process such as temperature and pH for optimal activity of enzyme and hydrolysis time are vital in the production of antioxidative protein hydrolysates or peptides with desirable functional properties (Samaranayaka and Li-chan, 2011). Several antioxidant peptides from these protein hydrolysates have been isolated (Bougatef *et al.,* 2010; Hsu 2010). These antioxidative peptides are inactive within the sequence of the precursor protein molecules but can be released after enzymatic hydrolysis. The antioxidative protein hydrolysates or peptides can be produced from fish protein sources by using various processes such as *in vitro* enzymatic hydrolysis, autolytic process using endogenous enzymes, microbial fermentation, and simulated gastric digestion (Bougatef *et al.,* 2010; Je *et al.,* 2007; Kim *et al.,* 2007). Among these methods, hydrolysis of fish proteins using exogenous proteolytic enzymes is widely applied process for the production of antioxidative fish protein hydrolysates or peptides. Active peptides capable of sequestering oxygen radicals, chelating prooxidant metal ions and inhibiting lipid peroxidation in food systems were documented (You *et al.,* 2010). Some antioxidative activities and peptide sequences of various fish protein hydrolysates from different fish species are shown below (Table 4).

Table 4 Antioxidative activities and peptide sequences of various fish protein hydrolysates (FPH) produced from different fish species

Source/Part used to prepare hydrolysate	**Enzyme**	**Antioxidative peptide**	**References**
Porcine skin collagen/Fish skin	Papain	Gln-Gly-Ala-Arg	Li *et al.*, 2007
Salmon/Protamine derived from fish milt	Pancreatin	Pro-Arg	Wang *et al.*, 2008
Thunnus tonggol/Cooking juice	Orientase	Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr	Hsu *et al.*, 2007
Theragra chalcogramma/Frame proteins	Crude proteinase from mackerel intestine	Leu-Pro-His-Ser-Gly-Tyr	Je *et al.*, 2005
Skate skin gelatin/Skate skin	Alcalase	Met-Val-Gly-Ser-Ala-Pro-Gly-Val-Leu-Leu-Gly-Pro-Leu-Gly-His-Gln	Ngo *et al.*, 2015
Skipjack (*Katsuwana pelamis*)/Roe	Alcalase	Met-Leu-Val-Phe-Ala-Val	Intarasirisawat *et al.*, 2012
Rockfish (*Sebastes hubbi*) skin/Fish skin	Alcalase	Tyr-Phe-Pro-Ala-His-Leu	Kim *et al.*, 2004
Skate skin/Skin	ά-chymotrypsin	Trp-Tyr-Phe-Leu-Met	Lee *et al.*, 2011
Sea bass skin (*Prionase glauca*)/Fish skin	Alcalase	-	Senphan and Benjakul 2014
Bluefin leatherjacket (Navodon septentrionalis)/Fish head	Papain	Gly-Pro-Pro	Chi *et al.*, 2015
Sea bream skin/Fish	Protease from bacillus	Val-Ile-Tyr	Fahmi *et al.*, 2004

1.2.3.1.1.3 Functional properties of fish protein hydrolysate

Solubility

One of the fundamental functional properties that determine the end use of hydrolysates is solubility. Good solubility is required for properties such as emulsification, foaming property and gelation. Changes in functional properties of native proteins are related to peptides and free amino acids produced by enzymatic hydrolysis. The controlled enzymatic hydrolysis of protein produces a series of smaller polypeptides with increased solubility and modified functional characteristics for different applications (Chalamaiah *et al.*, 2012). Hydrophobic and ionic interactions are the major factors that influence the solubility characteristics of proteins. Hydrophobic interactions promote protein-protein interactions and result in the decreased solubility, whereas ionic interactions promote protein-water interactions, resulting in an increased solubility (Kristinsson and Rasco 2000). It was reported that solubility of protein hydrolysates from red salmon reached 95% after being hydrolyzed by alcalase for 2 hr using an enzyme/substrate ratio of 5%, at 61°C at pH 7.5, whereas, solubility of raw red salmon protein was only 20% (Gbogouri *et al.*, 2004). The same trend was also observed in shark protein hydrolysates (Diniz and Martin 1997). Variation in solubility can be attributed to the net charge of peptides that increases as pH moves away from isoelectric point (pI and to surface hydrophobicity, which promotes aggregation via hydrophobic interactions (Taheri *et al.*, 2013). Nalinanon *et al.*, (2011) showed that ornate threadfin bream hydrolysate had its lowest solubility at pH 5, demonstrating that high molecular weight peptides precipitate when approaching their respective pIs. Longer processing times produced protein hydrolysate with smaller molecular weights, resulting in higher solubility. An increase in hydrophilic polar groups of hydrolysates leads to an increase in water-solubility (Kristinsson and Rasco, 2000). The improved solubility enables protein hydrolysates obtained from fish processing byproducts to be applied readily to formulate several food systems(Thiansilakul *et al.*, 2007).

Emulsifying capacity

Most processed foods contain oil, which exists as an emulsion together with other constituents. The most frequent emulsion is an oil- in-water emulsion in the form

of spread-texture food such as vinaigrette, mayonnaise and hollandaise sauce (Taheri *et al.,* 2013). Fish protein hydrolysates are good emulsifiers due to their improved amphiphilic nature, as they expose more hydrophilic and hydrophobic groups that enable orientation at the oil–water interface for more effective adsorption (Klompong *et al.,* 2007). The emulsifying capacity of rockfish protein hydrolysates, obtained by hydrolysis for 1 h with rhozyme, at an enzyme/substrate ratio of 1/75 and pH of 6.5–6.7, increased to 231 g oil/g protein from 145 g oil/g intact rockfish protein (Spinelli *et al.,* 1972). Similar results were also found with herring protein hydrolysates, obtained by hydrolysis for 1 h using alcalase at pH 8.0, temperature 50 °C and the enzyme was added at 0.5% (w/w) of the protein content in the mince. The emulsifying capacity of the herring protein hydrolysates increased to 12.16m^2/g solids from 10m^2/g solids (Liceaga-Gesualdo and Li-Chan 1999). However, the extent of hydrolysis has to be carefully controlled, as an excessive hydrolysis can decrease the emulsifying capacity of fish protein hydrolysates. The emulsifying capacity of rockfish protein hydrolysates dropped from 231 g oil/g protein to 224 g oil/g protein when the hydrolysis time was extended from 60 min to 90 min (Spinelli *et al.,* 1972). Protein hydrolysates of Pacific whiting (Pacheco-Aguilar *et al.,* 2008) and yellow stripe trevally (Klompong *et al.,* 2007) also showed the decrease in emulsifying capacity as the degree of hydrolysis increased. The reduced capacity is due to an excess of low molecular weight components, which lose the ability to orientate at the water–oil interface to stabilize the emulsion system (Klompong *et al.,* 2007). Kristinsson and Rasco (2000) reported that protein hydrolysates should consist of at least 20 amino acids to possess good emulsifying capacity. The emulsifying capacity of protein hydrolysates from fish processing byproducts was comparable to food-grade emulsifiers such as soy protein powder, casein protein powder and sodium caseinate powder (Taheri *et al.,* 2013). This indicates the strong potential of developing fish protein hydrolysates as alternative emulsifying agents for food formulations.

Oil binding capacity

Oil binding capacity is an important functionality used in meat and confectionery products (Sathivel *et al.,* 2003). This is attributed to the combination of

physical entrapment of oil and the hydrophobicity of the material. Hydrophobicity of fish protein hydrolysates was increased since hydrolysis cleaves the protein, leading to the exposure of hydrophobic groups (Kristinsson and Rasco 2000). Sathivel *et al.,* (2003) found that oil binding capacity of red salmon fish protein hydrolysates increased within a certain time of hydrolysis, whereas it decreased when hydrolysis was further extended. The maximum oil binding capacity (7.8 ml oil/g hydrolysate) was observed with a 50 min of hydrolysis using alcalase, at an enzyme/substrate ratio of 0.5% and 50 °C. It dropped to 4.3 ml of oil/g hydrolysate when hydrolysis time was extended to 75 min. The excessive hydrolysis compromises the integrity of the protein structure, and results in the degradation of the protein network, which can entrap oil. Fish protein hydrolysates from many fish species were found to have a superior oil binding capacity to commercial food-grade oil binders (Foh *et al.,* 2011) such as soy protein powder and casein (Foh *et al.,* 2011).

1.2.4 Biocalcium from fish bones

1.2.4.1 Calcium hydroxyapatite

Calcium contributes directly to human bone and teeth and is involved in several physiological activities including modulating muscle function, blood coagulation, and blood flow within capillaries. It also plays a major role in maintaining heart rate and controlling the nerve impulse transfer (Benjakul *et al.,* 2017). Generally, calcium is deficient in most of the regular diets. Hence an alternate means to improve availability is needed. The calcium deficiency is a severe problem causing osteoporosis as well as decreased bone mass (Cashman 2002). Nevertheless, the pure calcium has poorer absorption than calcium conjugated with peptides (Benjakul *et al.,* 2017). Ca chelated with peptides potentially prevents the precipitation of Ca in phosphate salt form, thus augmenting the amount of soluble Ca for absorption (June *et al.,* 2006). Calcium in most human diets is not sufficient, in which the lower level is found, compared to that required by a human body. The standards for daily minimum calcium intake have been issued in many countries. In a way to find alternative to this solution, studies on supplements containing calcium have been studied. Since fish bone is one of the numerous fish by- products, fish bone or skeleton is one of the valuable sources in

identifying health promoting components and a potential source of calcium and minerals (Larsen *et al.,* 2000).

Fish bones have been known as the excellent source of mineral, especially calcium. Fish bones contain 30 percent of collagen and are considered as an additional source of collagen along with fish skin. Fish bones consist of 60-70 percent minerals including calcium, phosphorous and hydroxyapatite (Kim 2005). Fish bones are a very good source of hydroxyapatite ($Ca_{10}(PO_4)6(OH)_2$), which can be used as a bone graft material in medical and dental applications. Hydroxyapatite is derived from natural materials such as coral and fish bone (Jensen *et al.,* 1996). The important properties of hydroxyapatite are as follows: it does not break under physiological conditions, it is thermodynamically stable at physiological pH and it plays an active role in bone binding. This property has been exploited for rapid bone repair after major trauma or surgery. Attempts have been taken to isolate fish bone derived hydroxyapatite and use them as an alternate for synthetic hydroxyapatite (Kim *et al.,* 1997; Ozawa and Suguru, 2002). Generally, very high heat treatment (1300°C) is used for production of hydroxyapatite from fish bone and this temperature gives a higher strength to hydroxyapatite structure (Choi *et al.,* 1999) and yields an excellent biocompatible inorganic substance (Park and Kim, 2001).

Casein phosphopeptides (CPP) attained after being intestinal digestion increased bone clarification (Jung *et al.,* 2006). CPP can bind Ca, in which the precipitation of Ca phosphate salts can be prevented. This leads to the increased soluble Ca availabe for absorption (Yuan and Kitts 1994). Since some consumers do not take milk and/or milk products because of lactose intolerance, peptides from aquatic sources can be used as a food supplement to enhance bioavailability of Ca. Bone oligopeptides from hoki bone having high affinity to calcium were produced using tuna intestinal crude enzyme. Such an enzyme was able to hydrolyze bone matrices consisting of collagen, non- collagenous proteins, carbohydrate, and minerals (Jung *et al.,* 2006). Fish bone phosphopeptides (FBP) (23.6% phosphorus) showing MW of 3.5 kDa increased solubility of Ca (Jung *et al.,* 2006). Hoki bone peptide II (FBP II) possessing a high ratio of phosphopeptides was able to prevent the formation of insoluble Ca salts. The

increased in levels of femoral total Ca, bone mineral density and strength were found in ovariectomized rats treated with FBP II (Jung *et al.*, 2006). Val–Leu–Ser–Gly–Gly–Thr–Thr–Met–Ala–Met–Ala–Met–Tyr–Thr– Leu–Val, with MW 1442 Da, from Alaska pollock backbone had the affinity to Ca ions localized on the surface of hydroxyapatite crystals. The peptide helped solubilize Ca similarly to CPP (Jung *et al.*, 2006).Val–Leu–Ser–Gly–Gly–Thr–Thr–Met–Tyr–Ala–Ser–Leu–Tyr–Ala–Glu, which is derived from pepsinolytic hydrolysate of hoki frame, had MW of 1561 Da (Jung and Kim 2007). Oligophosphopeptide from fish bone can be used as a nutraceutical to enhance the solubility and absorption of Ca.

1.2.4.1.1 Production of biocalcium

Fish bone, particularly from pre-cooking process, has the limited applications. This is mainly caused by undesirable dark color and strong fishy odor. The problematic fishy odor was more likely caused by the blood and lipids retained in the bone (Benjakul *et al.*, 2017). Additionally, undesirable darkish color, mainly triggered by blood oxidation and heat-mediated precipitation of blood in the bone, still exists. Therefore, appropriate treatment is a means to bring about biocalcium powder from fish bone having whiter color without fishy odor (Benjakul *et al.*, 2017). Washing with water could possibly help to obtain a clean bone, therefore reducing blood and lipids retained in the bone. Blood oxidation and heat-mediated precipitation of blood in the bone resulted in off color and off odor of bone. Non-collagenous protein can be removed by using NaOH, thus allowing it to stand for some period of time. Alkaline treatment will help to remove protein, but the excessive treatment might leach out collagen from bones. Lipid removal is done by using hexane to reduce oxidation and formation of offensive odor or rancidity and bleaching is performed to whiten the bone (Benjakul *et al*., 2017). The production of biocalcium powders has been studied and characterized (Benjakul *et al*., 2017). Biocalcium was prepared from pre-cooked skipjack tuna bone. The biocalcium obtained had a low amount of odorous compounds after different pre-treatment such as removal of non-collagenous protein and lipids, followed by bleaching. The biocalcium powder obtained had a moisture content, protein, fat, ash, hydroxyproline, phosphorus, calcium and iron content of 7.35%, 24.26%, 0.21%,

72.20%, 15.46%, 12.63%, 26.91% and 211.91 (ppm), respectively. It was observed that the biocalcium powder obtained had high iron content which might be as a result of abundance of Iron in tuna bone. High Iron content of fish bone from 15 species varied from 20 to 825 ppm (Hamada *et al*., 1995). The overall study showed that biocalcium can be processed efficiently from fish bones with improved whiteness when a combine treatment stated earlier was applied.

1.2.4.1.1.1 Characteristics of biocalcium

Characteristics of biocalcium powders obtained from pre-cooked tuna bones of tongol (*Thunnus tonggol*) and yellowfin (*Thunnus albacores*) were studied (Benjakul *et al*., 2017). Calcined bone powder (calcined at 900°C) and biocalcium powder were comparatively characterized. At 900 °C, calcination of bones can remove water retained in the bones as shown by moisture content close to zero. 0rganic matter such as fat and protein were decomposed at calcination temperature (900°C). Higher ash content was observed in calcined bone powders than those from biocalcium powders in yellowfin tuna pre-cooked and tongol bones. The increased in ash content of those calcined powders was as a result of the removal of organic matters at the selected calcination temperature. After removal of fat and protein, the content of inorganic compounds present in biocalcium was enhanced. Inorganic matters remained in calcined bone were related with high ash content (99.75–99.93%). Biocalcium powder from pre-cooked yellowfin and tongol bone had Ca contents of 26.76 and 26.73%. Calcined bone powder from pre-cooked tongol tuna bone and pre-cooked yellowfin tuna bone had Ca content of 40.47 and 40.13%, respectively. Also, the P contents (19.12– 19.15%) were increased in calcined bone higher, compared to those of biocalcium powders (12.7–12.74%). Based on total ash content, proportions of Ca (38.40–40.37% of ash content) and P (18.23–19.20% of ash content) were similar between biocalcium and calcined bone powder.

The diffraction patterns of both biocalcium and calcined bone powder showed that hydroxyapatite was present as the major phase in all powdered samples. Garner *et al.* (1996) documented that the inorganic matters from vertebrate bone contained hydroxyapatite (HA) crystals localized in collagen fibrils matrix. HA crystals contained

up to 60–65% of bone. Initial bone matrix transformation to a well-crystallized HA phase could be as a result of growth and nucleation of hexagonal di-pyramidal nanocrystals in powder particles at high temperature. Biocalcium and calcined bone powders from yellowfin tuna bone and pre-cooked tongol had Ca/P mole ratio that was relatively close to that of HA. Benjakul *et al.* (2017) showed that the high crystallinity and phase purity degree of the CB sample was due to alteration in initial bone matrix to a proper-crystallized HA phase under high temperature treatment. Therefore, it was exerted that biocalcium and calcined bone powder possessed varying chemical composition and crystallinity but exhibited similar ratio of Ca/P, corresponding to that of hydroxyapatite.

1.2.5 Biscuit

Biscuit is a type of snacks, the word "biscuit" is derived from Latin word "*Biscoctum"* means twice baked. Biscuits are one of the low-cost processed foods/snacks, which are most widely consumed (Jung and Kim, 2007). Biscuits convey the goodness of flour, fat and sugar in most acceptable and economical term (Sharma *et al*., 2003). They have a long shelf-life at ambient temperatures. Besides being a very palatable vehicle of nutrition and energy, biscuits constitute major component of human snacks in most part of the world. It is an unleavened crisp, sweet pastry made from wheat flour, shortening (hydrogenated fat) and sugar. It is usually made light by the addition of baking powder (a mixture of sodium carbonate, sodium bi-phosphate and cereal flour) (Sudha *et al*., 2007). Gluten protein forms elastic dough during baking and gives high organoleptic quality to the finished product (Sharma *et al*., 2003). Among many snack items, they have certain advantages such as being cheaper than the conventional snack items, available to buy at home or even during travel, seen in a variety of shops with various size, taste, packs and appeals to all groups of consumers.

1.2.5.1 Supplementation of food products

The world snack food market was valued at 66 billion USD in 2003 with baked goods, cookies and crackers, meat snacks, and popcorn accounting for about 22% of these sales (Hodgeon, 2004). The snack food industry includes manufacturers of potato chips, corn chips, popcorn, pretzels, extruded cheese snacks, seed snacks, mixed nuts,

peanuts and others. The market is relatively saturated. However, room remains for niche products high in nutrients such as fibre, protein, omega-3 fatty acids and gluten-free (Benjakul and Karnjanapratum 2018). With high demand for healthy products, several food products supplemented with nutritive ingredients have been prepared including biscuits fortified with shrimp oil to augment polyunsaturated fatty acid and astaxanthin contents (Takeungwongtrakul and Benjakul, 2017). Sponge cake made with cabbage leaf powder to increase fibre content was developed (Prokopov *et al*., 2015). Wheat biscuits fortified with cauliflower leaf powder were rich in β-carotene and iron (Wani and Sood, 2014). Wheat flour blended cookies fortified with malted soybean were developed to improve protein content (Bashir *et al*., 2015). Biocalcium (BC) was produced from egg-shell or fish bones (Hassan, 2015; Benjakul *et al*., 2017). Hassan (2015) added Bio-Ca powder from egg shell as dietary calcium in biscuits to increase their nutritive value. The calcium containing powder from fish bone could be used for the fortification of fish products such as surimi (Shungan, 1996). Before being fortified, bones should be converted into edible form by softening their structure with hot water treatment, hot acetic acid solutions or by superheated steam cooking (Ishikawa *et al*., 1990).

Whole wheat cracker, a flour-based snack product with the hard and crunchy texture, are common food, in which its nutritive value can be enhanced with the addition of salmon bone Bio-Ca powder. They are rich in fibre. Fibres possess a physiological effect on transit time and fecal bulk. Consumption of whole wheat products has been endorsed to be beneficial to health (Campbell *et al.,* 1991). Crackers made from whole wheat flour are usually crunchy and hard in texture. Recently, Benjakul and Karnjanapratum (2018) fortified biocalcium from skip tuna bone in whole wheat cracker. Biocalcium could be added up to 30% while acceptability was still obtained. The product consisted of Ca and P at 4.85 (g/100 g) and 2.02 (g/100 g), respectively. Texture became more compact and harder as a result of addition of tuna bone biocalcium powder, which could fill the void or gap in cracker crumb. The tuna bone biocalcium were substituted at different levels of 20-50% and there was no difference in all attributes tested up to 30% level of substitution. However, decreases in attributes

such as color, odor, texture, appearance, flavor, taste were observed at substitution level of 40-50%. High proportion of powder could dilute the flavoring ingredients such as butter, peanut, sugar and salt, thus reducing the intensity of those attributes in the finished product (Benjakul and Karnjanapratum, 2018). Biocalcium powder can be used a healthy food ingredient to fortify food product in the healthcare market.

Thus, with the increase in demand for healthy foods including snacks rich in nutrients, particularly functional ingredients, is increasing. Nevertheless, they are deficient in some essential amino acids as well as calcium. Addition of biocalcium and protein hydrolysate powders obtained from salmon frame could be a promising means to increase nutritive values of crackers.

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1.3 Objective

1 To optimize the production of protein hydrolysate through enzymatic hydrolysis of salmon frame and to study nutritive value and antioxidant properties of protein hydrolysate.

2 To produce and characterize biocalcium and calcined powders from salmon frame.

3 To develop cracker fortified with protein hydrolysate and biocalcium obtained from salmon frame.

CHAPTER 2
PRODUCTION OF PROTEIN HYDROLYSATE THROUGH ENZYMATIC HYDROLYSIS OF SALMON FRAME

2.1 Abstract

Protein hydrolysates from two forms of salmon frame named ‘chunk’ and ‘mince’ were produced and characterized. Both samples were subjected to hydrolysis using alcalase and papain at 1-3% (w/w protein) for 0-240 min. Hydrolysate prepared with either protease at 3% for 180 min had the solid yield of 24.05-26.39%. Hydrolysates contained 79.20-82.01% proteins, 6.03-6.34% fat, 9.81-11.09% ash, and 4.02-5.80% moisture. Amino acid profile showed that all hydrolysates had glutamic acid/glutamine (113.45-117.56 mg/g sample), glycine (77.86-86.18 mg/g sample), aspartic acid/asparagine (76.04-78.67 mg/ g sample), lysine (61.97-65.99 mg/g sample) and leucine (54.30-57.31 mg/g sample) as the predominant amino acids. All hydrolysates possessed high solubility. The size distributions determined by gel filtration chromatography varied, depending on proteases and the form of frame used for the hydrolysis. Different hydrolysates showed varying antioxidant capacities. Thus, protein hydrolysates from salmon frame could be used as a nutritive supplement in amino acid deficient foods.

2.2 Introduction

The demand for food including fish will increase with the drastic change in the world population, estimated to be 9.8 billion by 2050 (UN, 2017). Fish is one of the popular food commodities and is rich in nutrients such as proteins, minerals, omega-3 fatty acids and vitamins (Sidhu 2003; Lund 2013). During processing, more than 60% are generated as leftovers, but the amount of leftovers depends on the processes, the raw materials and the type of final products required (Ockerman and Basu 2014). Of all fish processed globally, salmon (*Salmo salar*) constitute a large portion. Salmon is also widely imported to Thailand where it is recognised as a delicacy among the Thai consumers. It is usually sold as a whole fish or as fillet. Leftovers derived from salmon processing include heads (containing the gills), trimmings (containing muscle, bone and skin), mince, frames and viscera (liver, kidney and roe) (See *et al.,* 2011). Also, their

value in the market is extremely low and are only considered useful in fertilizer production, ensilage or thrown away. Without the appropriate treatment or management, pollution and disposal problems occur. Nevertheless, these leftovers contain notable quantities of high value protein (15-60%) with respect to essential amino acids (Neves *et al.,* 2017). For the last two decades, those discards have been converted to an array of products, which includes collagen, gelatin, oils and hydrolysates. Protein hydrolysates produced from several fishery wastes have been studied. Some of which includes protein hydrolysate from muscle of ornate threadfin bream (Nalinanon *et al.,* 2011), round scad mackerel (Wu *et al.,* 2003), yellow travelly (Klompong *et al.,* 2007) and fish mince of Pacific hake (Jenkelunas and Li-Chan 2018). It has become a prospective ingredient in health promoting foods (Sae-leaw *et al.,* 2016). Simultaneously, production of protein hydrolysate is a cheaper way of reducing environmental problems, while gaining some value-added products.

To produce hydrolysate, fish wastes are subjected to hydrolysis, in which proteins are cleaved into smaller peptides with varying molecular weights by chemical or enzymatic reaction. Enzymatic hydrolysis could be achieved under controlled conditions such as temperature, pH and type of enzyme used. These determines the hydrophobicity, size and polarity of the resulting hydrolysate (Humiski and Aluko 2007). Protein hydrolysates possess interfacial properties and extremely good solubility, particularly with increased DHs (Klompong et al. 2007; Gbogouri et al. 2004). In general, enzymes such as papain, α-chymotrypsin, proteinase K, neutrase, flavourzyme, alcalase, trypsin, pepsin and protamex have been utilized to make hydrolysates from food proteins (Chi *et al.,* 2015). Alcalase is classified as an alkaline protease obtained from *Bacillus licheniformis*, while papain is a protease from the papaya latex (Aspmo *et al.,* 2005). To better exploit salmon frame, the production of protein hydrolysate could be a potential means. No information regarding the nutritional profile and antioxidant capacities of protein hydrolysate from salmon frame exists. This study was carried out to investigate the chemical composition as well as antioxidative activities of hydrolysates obtained from salmon frame using alcalase and papain.

2.3 Materials and methods

2.3.1. Chemicals

Alcalase from *Bacillus licheniformis* (2.4 L enzyme) and papain from the latex of *Carica papaya* were obtained from Novozymes (Bagsvaerd, Denmark). 2,4,6-tritrobenzenesulphonic acid (TNBS), 2,2-diphenyl-1picrylhydrazyl (DPPH), 2,2-azinobis (3ethylbenzothiaziline-6-sulphonic acid), diammonium salt (ABTS), 2,4,6-tripyridyltriazin (TPTZ), 6-hydroxy- 2,5,7,8-tetramethylchroman-2carboxylic acid (Trolox), 2,2′-azobis (2-methylpropionamidine) (AAPH) ethylenediaminetetraacetic acid (EDTA) and 3-(2-pyridyl)-5,6--diphenyl-1,2,4-triazine-4′,4″ disulfonic acid sodium salt (ferrozine) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Plasmid DNA (pUC 18) and SYBR™ Gold DNA Gel Stain were procured from Thermo Fisher Scientific Inc. (Waltham MA, USA). Sephadex^{TM} G-25, blue dextran and gel filtration calibration kit (vitamin B12, flavin, adenine dinucleotide and glycine-tyrosine) were obtained from GE Healthcare (Uppsala, Sweden). 1,1,3,3-tetramethoxypropane were procured from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

2.3.2 Raw material collection

Frames of salmon (*Salmo salar*) (30-35 cm in length) were obtained from Kingfisher holding Ltd Songkhla, Thailand. They were packed in a polyethylene bag, placed in a polystyrene box and embedded in ice. The samples were delivered to Seafood chemistry and biochemistry laboratory within 1 h and subsequently stored at -20°C.

2.3.3 Preparation and characterization of protein hydrolysate from salmon frames

2.3.3.1 Sample preparation

Frozen salmon frames were tempered overnight at 4°C and the size was reduced to 4-5 cm in length with the aid of electric cutting machine (W210E, Union Kitchen & Service, Bangkok, Thailand). Prepared samples were divided into 2 portions. The first portion was kept in a form named ‘chunk’. The second portion was further chopped with a blender (Phillips, Guangzhou, China) for 5 min to obtain the minced frame

termed ‘mince’. Both samples, chunk and mince, were stored in a refrigerator at 4°C until use but not longer than 6 h.

2.3.3.2 Chemical analysis of salmon frame

2.3.3.2.1 Proximate analysis

Proximate analysis of salmon frame was carried out using AOAC method (2000). Moisture, protein, lipid and ash contents were analyzed as per the method number No. 950.46, 920.153, 960.39 and 928.08 respectively (AOAC, 2000).

2.3.3.2.2 Determination of minerals

Minerals including Ca, P, Na, K, Zn, Fe and Cr was analyzed using the inductively coupled plasma optical emission spectrometer (ICP-OES) as described by Feist and Mikula (2014).

2.3.3.2.3 SDS-PAGE

Protein pattern of the salmon frame was determined using SDS-PAGE according to the method of Laemmli (1970) with 4.5% stacking gel and 10% separating gel according to method of (Klompong *et al.,* 2007). Minced frame was mixed with 5 % SDS solution at 85°C and incubated for 30 min. The mixture was centrifuged at 8500 xg for 5 min using (Beckman Coulter, Inc., Palo Alto, CA, USA). The supernatant was mixed with sample buffer (0.125 M Tris-HCl, pH 6.8 containing 4% SDS and 20% (v/v) glycerol) at a ratio of 1:1 (v/v). Proteins (15µg) were loaded onto the gel. The electrophoresis were run at a constant current of 15mA per gel by a Mini-protean II cell apparatus. The gels were fixed and stained with 0.05% (w/v) Coomassie brilliant blue R-250 in 15% methanol and 5% acetic acid and destained in 30% methanol and 10% acetic acid. The wide range molecular mass marker was used to estimate the molecular weight of proteins in salmon frame.

2.3.4 Production of protein hydrolysate

2.3.4.1 Preparation

Firstly, distilled water was added to either chunk or mince at room temperature. The mixtures was stirred gradually using an overhead stirrer equipped with a propeller at a speed of 9000 rpm for 2 min (mince only). Thereafter, the pH of both mixtures was adjusted to pH 8.0 using 0.1 M NaOH or 0.1 M HCl. The mixtures was incubated at

60°C for 15 min. The reaction was initiated by adding alcalase 2.4 L at various concentrations (1, 2 and 3 %, w/w protein) into the mixture. For hydrolysis using papain, the mixtures was firstly adjusted to pH 7.0, followed by incubation at 40°C for 15 min. Subsequently, papain were added to obtain different conditions (1, 2 and 3 %, w/w protein). The mixtures were stirred continuously. At designated times (0, 5, 10, 15, 20, 30, 40, 60, 90, 120, 180 and 240), 2 ml of the mixture were taken into a test tube and then placed into a water bath at 90°C for 15 min to terminate the enzymatic reaction. The samples were then solubilized by adding with hot SDS (5%) at a ratio of 1:1 (v/v). The mixtures was incubated at 85°C for 30 min, followed by centrifugation at 4000xg for 15 min using a centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA). The supernatant obtained was then subjected to the determination of degree of hydrolysis (DH).

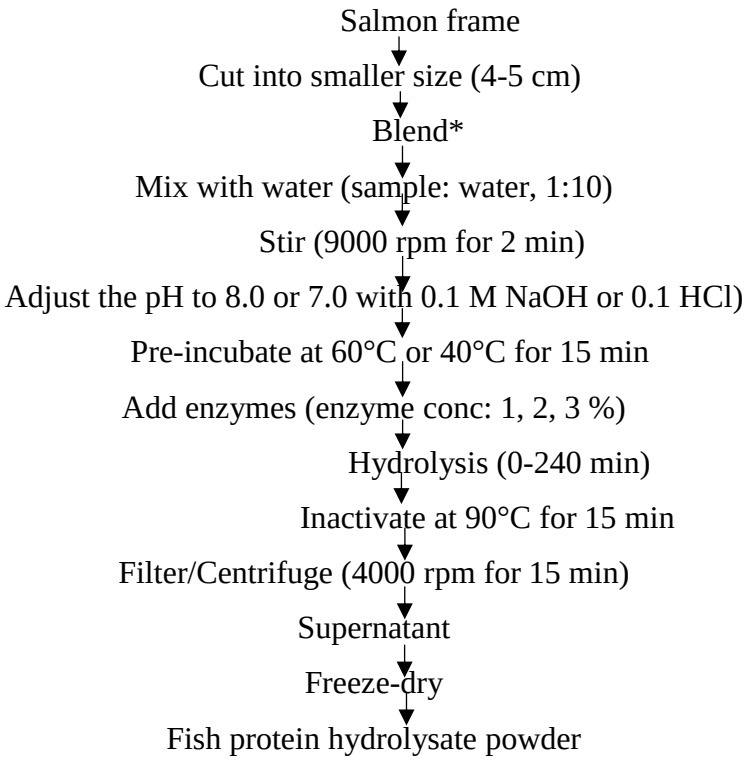
2.3.4.2 Determination of degree of hydrolysis (DH)

The free amino acid content was determined according to the method of Benjakul and Morrissey (1997). To properly diluted hydrolysate samples (125 µl), 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 60°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures was cooled at room temperature for 15 min. The absorbance will be read at 420 nm and ά-amino acid will be expressed in term of L-leucine. The degree of hydrolysis (DH) is defined as the percentage ratio of the number of peptide bonds broken to the total number of peptide bonds in the substrate. DH was calculated as follows:

$$DH=\left[\frac{L_H - L_0}{L_{max} - L_0}\right]\times 100$$

where L_H is the amount of ά-amino acid released at time t. L_0 is the amount of ά-amino acid in the original salmon mince homogenate. L_{max} is total ά-amino acid in the salmon mince obtained after acid hydrolysis with 6 N HCl at 100°C for 24 h. Hydrolysis time and enzyme concentration providing the high DH for the mince and chunk were selected for further study. The selected hydrolysates prepared from either chunk or

mince using alcalase or papain were centrifuged at 10,000 xg for 15 min. The supernatants were lyophilized and further subjected to analyses. Scheme for protein hydrolysate production from salmon frame are shown in Figure 3.



**Homogenization or blending will not be applied for the chunk*

Figure 3 Flow chart for production of protein hydrolysate powder from salmon frame

2.3.5. Characterization of the selected protein hydrolysates from salmon frame

2.3.5.1 Amino acid composition analysis

Amino acid composition were analyzed according to the method of Benjakul *et al.*, (2009). The hydrolysate samples were hydrolyzed under reduced pressure in 3 M mercaptoethane sulphonic acid in the presence of 2 mL/L (v/v) 3-2(2-aminoethyl) indole for 22 h at 110°C. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.1 ml were applied to an amino acid analyzer. The content were expressed as mg/g sample.

2.3.5.2 Determination of carotenoids

Total carotenoid content were determined in the hydrolysates according to the method of Senphan and Benjakul (2014) with a slight modification. Hydrolysates (1g) was homogenized in 25 ml of cold acetone (-20°C) for 2 minutes using a homogenizer at a speed of 13000 rpm and the homogenate was filtered through a Whatman No. 1 filter paper under vacuum. The filtrate was placed in a separator funnel and was partitioned with 25ml of petroleum ether. The separating funnel containing sample/solvent mixture was shaken gently and allowed to stand at room temperature (25°C) for 10 min. The lower layer was drain off. The top layer was washed twice with 25 ml of distilled water. The petroleum ether layer obtained was dried by occasional shaking with 15 g of anhydrous sodium sulphate for 30 min. The dried sample was filtered through a coarse sintered glass funnel. The residual sodium sulphate were washed with small volumes of petroleum ether for several times to remove all pigments. All petroleum ether fractions were pooled and evaporated under vacuum at 50°C using an evaporator. The residue was dissolved in petroleum ether and was made up to a final volume of 10 ml. After being appropriately diluted, the absorbance was read at 468 nm. The content of carotenoid were calculated using the equation given

$$\text{Total carotenoid (µg/g sample)} = \frac{A_{468} \times \text{volume of extract} \times \text{dilution factor}}{0.2 \times \text{weight of sample used in gram}}$$

where 0.2 is the A_{468} of 1 µg/ml standard astaxanthin, where A is the absorbance at 468 nm; V is the dilution volume (mL); W is the weight of sample used in gram.

2.3.5.3 Determination of antioxidative activities

Prior to analysis, freeze-dried hydrolysates were dissolved in distilled water to obtain the designated concentrations.

DPPH radical scavenging activity

DPPH radical scavenging activity were determined as described by Benjakul *et al.,* (2014) with a slight modification. Sample (5 mg/ ml; 1.5 ml) were added with 1.5 ml of 0.15 mM DPPH in 95% methanol. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. Blank was prepared in the same manner except that distilled water was used instead of the sample. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was prepared using Trolox in the range of 10–60 µM. The activity was expressed as µmol Trolox equivalents (TE)/g sample.

ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Binsan *et al*. (2008). The stock solutions include 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol to obtain A_{734} of 1.1 ± 0.02 using a spectrophotometer. Fresh ABTS solution was prepared daily.

Sample (1 mg/ml; 150 µl) was mixed with 2850 µl of ABTS solution and the mixture were left at room temperature for 2h in dark. The absorbance was measured at 734 nm using a spectrophotometer. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/g sample.

Chelating activity towards Fe^{2+}

Chelating activity towards Fe^{2+} was measured as according to Thiansilakul *et al.,* (2007). The sample (10 mg/ml; 4.7 ml) was mixed with 0.1 ml of 2 M $FeCl_2$ and 0.2 ml of 5 M ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature (26–28°C). The absorbance was then read at 562 nm using a

spectrophotometer. The blank was prepared in the same manner except that distilled water was used instead of the sample. The Fe^{2+} chelating activity was expressed as mmol EDTA equivalents (EE)/g sample. A standard curve of 0– 50 µM EDTA was prepared.

Ferric reducing antioxidant power (FRAP)

FRAP was determined according to the method of Benzie and Strain (1996). FRAP reagent was prepared by mixing acetate buffer (30 mM, pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportion of 10:1:1 (v/v). The sample solution (100 µl) was mixed with 3 ml of working FRAP reagent and incubated in dark condition at room temperature for 30 min. The absorbance of the reaction mixture was read at 593 nm using a spectrophotometer. The standard curve was prepared using Trolox ranging from 0 to 500 µM. The activity was expressed as µmol Trolox equivalents (TE)/g sample.

Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity (ORAC) was determined using the method of Madhujith and Shahidi (2007). The samples will be dissolved in 75 mM phosphate buffer (pH 7.0) to obtain a final concentration of 0.1 mg/ml. The prepared sample (20 µl) was loaded onto black polystyrene, non-treated 96-well microplate. The loaded microplate was inserted to microplate reader equipped. The samples were equilibrated at 37 C for 10 min. Then 200 µl of 0.11 µM fluorescein dissolved in 75 mM phosphate buffer (pH 7.0) were automatically injected to the sample at the first cycle. The reaction was initiated at the second cycle by automatic injection of 75 µl of 60 mM AAPH. The reaction was performed at 37°C. The fluorescence intensity was measured every 150 s for 62 cycles with excitation and emission filters of 485 and 520 nm, respectively. A gain adjustment was performed by pipetting 200 µl of fluorescein (0.11 µM) onto a designated well before starting the program to optimize signal amplification. The control was prepared in the same manner, except that 75 mM phosphate buffer (pH 7.0) was used instead of the sample. The kinetic curve (AUC) of the samples was plotted between fluorescence intensity and the number of cycles. Area below the curve directly

relates with the ORAC of sample. Trolox (0–50 µM) was used as the standard. The ORAC was expressed as µmol trolox equivalents (TE)/g sample.

2.3.5.4 Determination of solubility

To determine protein solubility, hydrolysate samples (10 mg) was dispersed in 8 ml of de-ionized water and pH of the mixture were adjusted to 3, 5, 7 and 9 with either 1 M HCl or 1 M NaOH. The mixture was stirred at room temperature for 30 min. The volume of solutions was made up to 10 ml by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 5000 xg for 15 min using a centrifuge at 4 ºC.

Protein content in the supernatant was determined by the Lowry method (Benjakul *et al.,* 2011), using bovine serum albumin as a standard. Total protein content in the sample was determined after solubilization of the sample in 0.5 M NaOH. Protein solubility was calculated as follows:

$$\text{Solubility} = \frac{\text{Protein content in the supernatant}}{\text{Total protein content in the sample}} \text{ X } 100$$

2.3.5.5 Molecular weight distribution

Molecular weight distribution of hydrolysate samples was carried out using a Sephadex G-25 gel filtration column. The sample (50 mg) was dissolved in distilled water (2 ml) and the mixture were loaded onto a column. After being loaded, the elution was performed using a chromatography system coupled with a fraction collector. Distilled water was used as an eluent at a flow rate of 0.5 ml/min. The fractions of 3 ml were collected, and their absorbance was recorded at 220 nm and 280 nm. Blue dextran (2,000,000 Da) was used for void volume measurement. The molecular weight (MW) markers, including insulin chain B (3495.89 Da), vitamin B12 (1355.4 Da), glycine–tyrosine (238.25 Da) and tyrosine (181.2 Da) was used. MW of fraction was estimated from the plot between available partition coefficient (K_{av}) and the logarithm of the molecular weight of the protein standards.

2.3.5.6 Color

All samples were be determined for L* (lightness), a* (redness/greenness) and b*(yellowness/blueness).

Color of all samples were measured by a Hunter lab colorimeter. Total difference in color (ΔE*) and color intensity (ΔC*) were calculated according to the following equation:

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

$$\Delta C = \sqrt{\Delta a^{*2} + \Delta b^{*2}}$$

where ΔL*, Δa* and Δb* are the differences between the corresponding color parameter of the sample and that of white standard (L* = 93.63, a* = −0.94 and b* = 0.40).

2.3.5.7 Bitterness

Bitterness of hydrolysates were examined by 10 panelists with the ages ranging from 25 to 35. The panelists were trained using a caffeine as a standard for a period of one month, twice a week. The standard solution was presented at different concentrations (0, 25, 50 and 75 ppm). Distilled water was used to represent score of 0, while 75 ppm caffeine represent the score of 15. For evaluation, 15-cm line scale anchored from “none” to “intense” was defined as tailored by (Nilsang *et al.*, 2005).

The hydrolysate samples, at a protein concentration of 2 g/100 ml, was served at ambient temperature coded with three-digital random number together with reference standard caffeine solution. Panelists will evaluate for bitterness of samples, compared to the reference using a 15-cm line scale. Between samples, panelists will be asked to eat a piece of un-salted cracker and rinse their mouths thoroughly with distilled water (Nilsang *et al*., 2005).

2.4 Experimental design and Statistical analysis

All the experiments were performed in triplicate with completely randomized design (CRD). Analysis of variance (ANOVA) was used for the data. Mean comparison was done using the Duncan’s multiple range test (Steel and Torrie 1980). The Statistical Package for Social Sciences (SPSS for windows, SPSS Inc., Chicago, IL, USA) was used.

2.5 Results and Discussion

2.5.1 Chemical compositions of salmon frame

Salmon frames had 62.90±0.93% moisture. Based on dry weight basis, salmon frame consisted of 36.08±0.44% protein, 38.51±1.70 fat and 24.60±0.29 ash. Thus, salmon frames were rich in protein, mainly from remaining meat and minerals were mostly from bones. This was in agreement with Harnedy *et al.,* (2018) who documented that salmon skin and trimmings derived during salmon filleting process, contained significant quantities of protein. Salmon frame showed higher ash content than Alaska pollock counterpart (14.99%) (Hou *et al.,* 2011). In addition, salmon frames also had high fat content. Salmon is a fatty fish that retains fat in the muscle (Toppe *et al.*, 2007). Fish bones were also reported to consist of high amount of oil (Phleger *et al.,* 1976).

Salmon frames had calcium (Ca) as the most abundant mineral (77.02 ± 0.4 g/kg), followed by phosphorus (P) (29.86 ± 0.51 g/kg). Potassium (K) and sodium (Na) were also found at levels of 9.55±0.1 and 2.81± 0.08 g/kg, respectively. Na and K were mostly the constituents found in the remaining meat of salmon frame. Zinc (Zn), iron (Fe) and chromium (Cr) were also detected in salmon frame at low levels (57.80±0.60, 19.98±0.32 and 1.56±0.08 mg/kg respectively). Fish bone generally has ca-hydroxyapatite as the major component along with collagen (Benjakul *et al.*, 2017). Ca-hydroxyapatite constitutes from 60 to 65% of bone. It has a crystalline in structure, $(Ca^{2+})_{10-x}(H_3O^{+})_{2-x}(PO_4^{3-})_6\ (OH^{-})_2$, having a Ca/P mole ratio of 1.67 (Hamada *et al.*, 1995). Hence, the fish bone can be referred to as a good source of mineral, which can boost the human well-being, most importantly to prevent osteoporosis (Benjakul *et al.*, 2018).

2.5.1.2 Protein pattern

For protein patterns, several protein bands were noticeable (Figure 4). Myosin heavy chain (MHC) (205 kDa) and actin (45 kDa) constituted as the vital protein bands. Both aforementioned proteins have been known as the main proteins in the fish meat retained along with frames. However, proteins with varying molecular weights were also attained. These might represent both myofibrillar proteins as well as sarcoplasmic

proteins in both bones or meat of the salmon frame. The result indicated that muscle proteins attached with frame could serve as proteinaceous substrate for preparing protein hydrolysate.

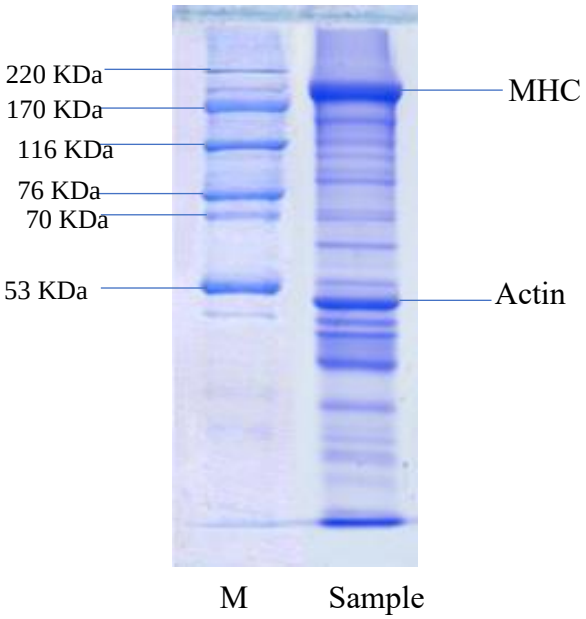


Figure 4 Protein pattern of salmon frame (M: Molecular weight marker; MHC: Myosin Heavy Chain).

2.5.2 Effect of protease types and forms of salmon frame on hydrolysis of proteins

During the hydrolysis of mince and chunk of salmon frame using alcalase and papain at various concentration (1-3%), degree of hydrolysis (DH) increased sharply within 10-20 min for mince, whereas the slower increase in DH was found in chunk. This indicated that mince with larger surface area provided the proteinaceous substrate more accessible to enzymatic hydrolysis than the chunk possessing the smaller surface. When the same enzyme level and hydrolysis time were used, alcalase yielded hydrolysates with higher DH, when compared to papain (Figure 5). Also, the higher the concentration of alcalase and papain used, the higher DH for both mince and chunk was attained. The result suggested that salmon frame proteins, mainly myofibrillar protein, were more preferable substrate for alcalase. During enzymatic hydrolysis, the cleavage

of proteins into short peptides took place. The efficiency of alcalase was reported for manufacturing fish protein hydrolysate from a variety of fish (Klompong *et al.,* 2007; Thiansilakul *et al.,* 2007). While protamex and flavourzyme could not hydrolyze roe proteins, alcalase was the only proteases that could hydrolyze defatted skipjack roe (Intarasirisawat *et al.,* 2012). With the same hydrolysis time, higher amount of enzyme resulted in greater DH ($P<0.05$). With 3% enzyme, DH of resulting hydrolysates was higher than those of hydrolysate prepared using 1 and 2% enzyme.

The form of the substrate also affected the degree of hydrolysis. For the chunk sample, the compact-structured proteins were less prone to hydrolysis, regardless of enzymes used. It was observed that DH was quite constant up to 140-160 min. It indicated that less peptides were cleaved. Conversely, proteins in mince were cleaved at a faster rate as indicated by higher DH, DH reached the plateau after 60 min when enzymes at high concentration (3%) was used. For chunk, the DH reached the constant value after 180 min when 3% enzyme was added. After a certain period of time, the availability of substrate for both enzymes, alcalase and papain, became less. Reduction in the rate of hydrolysis could be as a result of less availability of peptide bonds, cleaved by enzyme used. Furthermore, enzyme activity might be decreased and a product inhibition plausibly occurred (Intarasirisawat *et al.*, 2012).

The hydrolysis time of 180 min with protease (both alcalase and papain) concentration of 3% was selected for further study. Under aforementioned condition, there was no marked difference in DH between hydrolysates prepared from both forms of salmon frame. The mince hydrolyzed with 3% alcalase and 3% papain were referred to as “MA” and “MP”, respectively. Chunk hydrolyzed with 3% alcalase and 3% papain were regarded to as “CA” and “CP”, respectively.

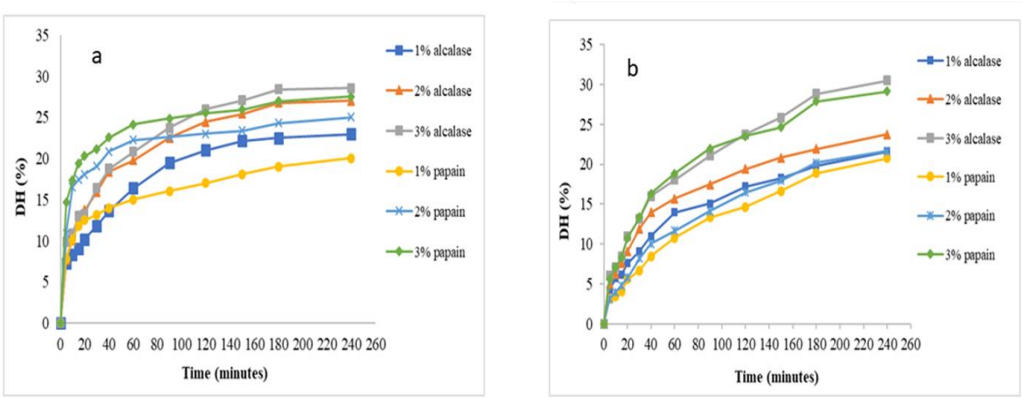


Figure 5 Degree of hydrolysis (DH) of hydrolysate prepared from mince (a) and chunk (b) of salmon frame using alcalase and papain at various concentration as a function of time. Bars represent the standard deviation (n=3)

2.5.3 Yield, characteristics and properties of protein hydrolysates from salmon frame with different forms prepared using different proteases

2.5.3.1 Yield of hydrolysates

Yields of hydrolysates obtained from mince and chunk of salmon frames using alcalase and papain are shown in Table 5. Yield of all hydrolysate samples were in the range of 24.05-26.39% (dry basis). The highest yield was found in CA sample ($P<0.05$). When the same form of frame was used, the hydrolysates produced from alcalase showed higher yield than that of papain ($P<0.05$). It was proposed that proteins in salmon frame could be hydrolyzed more effectively when alcalase was used. Alcalase are reported to be highly specific while papain possess broader specificity (Benjakul *et al.*, 2018). Alcalase was more specifically able to hydrolyze the chunk than the papain. In addition, the presence of endogenous proteases in raw material could aid commercial enzyme during hydrolysis (Mazorra-Manzano *et al.*, 2012). The higher yield of hydrolysate prepared with alcalase also suggested greater proteolytic efficacy during hydrolysis. Alkaline proteases like alcalase have been reported to exhibit higher proteolytic activities than acid or neutral ones such as pepsin or papain (Zhou *et al.*, 2012).

The result indicated that the form of frame used affected the yield of hydrolysates. It was noted that grinding of frame before hydrolysis might distribute fat

in the mince. Fat and other compounds inside the bone might be more released. Those compounds could be a barrier for protease to hydrolyze proteinaceous substrate. As a result, the slightly lower yield of MA and MP was obtained when the mince was used as a starting material for hydrolysis. Additionally, after removal of those components via centrifugation, the protein or peptide might be trapped to some extent in debris, especially those associated with fat. Hence, alcalase could effectively hydrolyze meat protein as well as collagen in bone. This plausibly led to the slightly higher yield of MA than MP (Figure 5b).

2.5.3.2 Proximate composition

Proximate compositions of hydrolysates obtained from salmon frame are shown in Table 1. Moisture contents of hydrolysates ranged from 4.02 to 5.80%. Hydrolysates were generally hygroscopic. After hydrolysis, the charged or polar residues were more exposed. This could influence the water binding capacity of hydrolysates. In general, low MW peptides show higher ability in holding water than the larger peptides (Cumby *et al.,* 2008). All hydrolysates were rich in protein (79.20-82.01%). There were no differences in protein content among different samples ($P>0.05$). High protein content observed among the samples was attributed to partial removal of lipids and insoluble undigested debris after hydrolysis (Thiansilakul *et al.,* 2007). Myofibrillar proteins in meat attached with frame were more likely hydrolyzed by proteases used. Furthermore, water soluble proteins could be liberated into hydrolysate. All hydrolysates had no difference in lipid content ($P>0.05$), which was in the range of 6.03-6.34%. The amount of lipids in hydrolysates could possibly affect its stability towards oxidation (Sila *et al.,* 2014). It was postulated that lipoproteins might constitute in hydrolysates. After hydrolysis, the separated lipids were skimmed. However, some lipids were still present in the hydrolysates.

Ash content of hydrolysates was in the range of 9.81-11.09%. High ash content in fish protein hydrolysates has been reported (Kristinsson and Rasco 2000). The high ash content of hydrolysates could possibly result from the release of mineral, especially from bones during the hydrolysis process. Salmon frame contained high amount of minerals, especially Ca and P.

2.5.3.3 Carotenoid content and color

Carotenoid contents of hydrolysates obtained from mince and chunk of salmon frame using 3% alcalase or 3% papain are shown in Table 5. Generally, low carotenoid content was observed for all hydrolysates ($P<0.05$). This could be as a result of long time for hydrolysis, which completely disrupted the protein-carotenoid complex, thus liberating more carotenoid. The protein-carotenoid complex were also disrupted by proteases, which led to an increase in the carotenoid recovered (Sowmya *et al.*, 2014). Those, free carotenoids could undergo oxidation during hydrolysis process at high temperature. Furthermore, carotenoids were more likely localized in lipid phase or pellet associated with lipids, thus decreasing the carotenoid recovery in hydrolysate. It has been recommended that at low temperature and short hydrolysis time, hydrolysate rich in carotenoid content was recovered to high extent (Sowmya *et al.,* 2014). Salmon meat was reported to contain a high amount of astaxanthin ranging from 0.36 -3.38 mg/kg (Yagiz *et al.,* 2010).

Lightness (L* value), redness/greenness (a* value) and yellowness/blueness (b* value) of the hydrolysates obtained from both mince and chunk of salmon frame using 3% alcalase or 3% papain are shown in Table 5. The highest redness index (a* value) was noticed in MA, followed by CA ($P<0.05$). Since MA and CA had higher redness (a*) than MP and CP ($P<0.05$), alcalase was likely able to cleave carotenoproteins more effectively than papain. For the same form of frame used, mince rendered the hydrolysates with redder color. This might be due to higher haemoglobin from backbone, when minced. Increase in hydrolysis time led to the enhanced non-enzymatic browning reaction. Non-enzymatic browning reaction more likely contributed to the dark appearance of hydrolysate with high DH (Wasswa *et al.,* 2007). When comparing lightness (L* value) of all the hydrolysates, those prepared using alcalase showed higher value ($P<0.05$) than those using papain. The b* values (yellowness) were also higher in hydrolysate prepared using mince than chunk. This was related with a* value. Thus, the form of frame and types of proteases were the factors governing color of resulting hydrolysates. However, all hydrolysate powder were yellowish pale in color. This coincided with the lower carotenoid content (Table 5).

2.5.3.4 Bitterness score

Different bitterness scores between hydrolysates were observed ($P<0.05$) (Table 5). CA showed higher score of bitterness than others ($P<0.05$). With same form of raw material, alcalase yielded the hydrolysate with higher bitterness than papain. It could be associated with peptides containing the bulky hydrophobic groups towards their C-terminal. Peptides possessing bulky hydrophobic groups of phenylalanine, isoleucine, tyrosine, valine, tryptophan and leucine at C-terminal had the bitterness (Yarnpakdee *et al.,* 2014). Higher hydrophobic amino acids obtained in the hydrolysate produced using alcalase (MA and CA) could be as a result of exposure of hidden hydrophobic peptides. This resulted in the enhanced sensation of bitter taste. In addition, proline localized internally in peptide chain was reported to be a vital distinct contributor to bitterness (Heinz Ney 1979). The bitterness was related well with the total hydrophobic amino acid (Table 6). The hydrolysates produced by alcalase showed higher content of hydrophobic amino acids than those prepared using papain. Additionally, hydrolysates produced from chunk had slightly higher bitterness score than those produced from mince when the same proteases was used. This was in agreement with the difference in hydrophobic amino acid between the samples. Bitter peptides containing valine, phenylalanine, isoleucine and glycine from ficin-treated fish concentrate were reported (Hevia and Olcott 1977). Bitterness could also be influenced by several other factors such as number of carbons in side chain, especially for branched chain amino acid, concentration and DH (Yarnpakdee *et al.,* 2015). The increased hydrolysis could possibly exposed more hidden hydrophobic amino acid residues. This occurrence could bring about an increase in bitterness of hydrolysate (Hou *et al.,* 2011). Both proteases used influenced the bitterness intensities of the hydrolysates differently.

Table 5 Yield, characteristics and property of hydrolysates from mince and chunk of salmon frame using alcalase and papain

Yield/Composition /Properties	**MA**	**MP**	**CA**	**CP**
Yield (%)	25.45±0.22^{c}	24.05±0.29^{a}	26.39±0.49^{d}	25.11±0.11^{b}
Moisture (%)	5.80±0.73^{b}	4.63±0.60a^{b}	4.18±0.00^{a}	4.02±0.02^{a}
Protein ** (%)	81.64±0.08^{a}	79.20±0.57^{a}	82.01±0.26^{a}	80.78±0.80^{a}
Fat ** (%)	6.34±0.29^{a}	6.14±0.42^{a}	6.28±0.15^{a}	6.03±0.34^{a}
Ash** (%)	11.09±0.50^{a}	10.65±0.28^{a}	9.81±0.36^{a}	9.84±0.72^{a}
Carotenoid (µg/g sample)	3.81±0.17^{b}	2.73±0.18^{a}	3.67±0.07^{b}	2.28±0.74^{a}
L*	87.11±0.01^{c}	85.43±0.00^{b}	87.47±0.02^{d}	84.92±0.00^{a}
a*	1.41±0.14^{d}	0.91±0.01^{b}	1.27±0.02^{c}	0.39±0.03^{a}
b*	15.65±0.09^{c}	17.37±0.00^{d}	15.28±0.00^{b}	14.45±0.01^{a}
ΔE*	18.06±0.09^{c}	20.63±0.00^{d}	17.49±0.00^{b}	17.03±0.01^{a}
Bitterness ^{Ⱡ}	4.27±1.41^{c}	3.97±1.20^{a}	5.08±1.05^{d}	4.02±1.59^{b}

MA; MP: hydrolysate from mince of salmon frame prepared using alcalase and papain, respectively. CA; CP: hydrolysate from chunk of salmon frame produced using alcalase and papain, respectively. Values are given as mean ± SD (n=3). Hydrolysis was conducted using 3% enzyme for 180 min. Different superscripts in the same row indicate significant differences ($P<0.05$). **Dry weight basis. ^{Ⱡ} Score are based on 15 cm- line scales (0: none and 15: intense).

2.5.4 Amino acid composition

Different amino acid compositions were found among hydrolysate samples (Table 6). Overall, glutamic acid/glutamine, aspartic acid/asparagine and glycine were dominant in all the samples. Glutamic acid and aspartic acid are notable to contribute to umami taste (Naknaen *et al.*, 2015). For glycine, it yields the sweet taste in seafoods (Aspevik *et al.*, 2016). In general, hydrolysates prepared by alcalase (MA and CA) possessed the higher contents of glutamic acid/glutamine, aspartic acid/ asparagine and glycine than those prepared by papain. Both enzymes, likely had different hydrolytic cleavage of peptide bonds. As a result, varying peptides were liberated and different amino acid compositions of hydrolysates were obtained.

The hydrolysates also consisted of hydrophobic amino acids including, leucine (54.30-57.31 mg/g sample), isoleucine (28.44-30.98 mg/g sample), phenylalanine (31.32-33.26 mg/g sample) and valine (34.33-37.65 mg/g sample). The hydrolysates contained proline content of (43.37-46.00 mg/g sample). This amount might result in the bitterness of protein hydrolysates (Thiansilakul *et al.*, 2007). It was noted that all samples contained hydroxyproline, indicating that collagen derivatives were present in hydrolysate. During hydrolysis at temperature higher than T_{max} of fish collagen, especially localized in bone or muscle, the collagen could be solubilized and hydrolyzed by both proteases used. Fish muscle is generally rich in essential amino acids as well as non-essential amino acids (Iwasaki and Harada 1985). The hydrolysates obtained could be used as a food supplement in order to compensate for imbalanced dietary protein. Lysine varied from 61.97 to 65.99 mg/g, while leucine constituted at 54.30-57.31 mg/g. Total essential amino acids were higher in hydrolysates prepared by alcalase than papain. Additionally, total amino acids was also higher in the resulting hydrolysates when alcalase was used. When comparing the forms used, chunk and mince, hydrolysates prepared using the same proteases showed similar amino acid compositions but might have some differences in the selected amino acids. Thus, hydrolysates from salmon frame had high content of amino acids and could serve as a supplement in food lacking proteins or amino acids.

Table 6 Amino acid composition of hydrolysates from mince and chunk of salmon frame using alcalase and papain

Amino acid compositions (mg/g sample)	**MA**	**MP**	**CA**	**CP**
Ala[B]	56.19	54.84	58.23	56.67
Arg[B]	53.28	54.41	55.88	53.48
Asp+Asn[B]	78.67	76.41	77.88	76.04
Cys[B]	0.18	0.13	0.19	0.07
Glu+Gln[B]	117.56	114.03	117.28	113.45
Gly[B]	77.86	78.78	83.82	86.18
His[A]	23.81	21.59	22.63	23.28
Ile[A]	30.44	29.72	30.98	28.44
Leu[A]	57.31	55.16	56.19	54.30
Lys[A]	65.33	64.77	65.99	61.97
Hyl[B]	3.35	3.45	3.76	3.84
Met[A]	25.96	25.12	26.89	24.99
Phe[A]	33.26	31.32	31.98	32.45
Hyp	16.37	17.92	18.69	19.96
Pro[B]	43.37	44.33	46.33	46.00
Ser[B]	38.24	37.65	38.78	38.16
Thr[A]	36.95	35.98	36.82	35.54
Trp[A]	4.89	5.25	4.86	4.82
Tyr[B]	25.35	24.80	24.64	23.83
Val[A]	36.56	35.69	37.65	34.33
Total amino acids	824.93	811.36	839.48	817.80
Hydrophobic amino acid	270.14	262.04	271.62	259.90
Total EAA	314.52	304.61	314.00	300.11
Total NEAA	494.04	488.83	506.80	497.73

Hydrolysis was conducted using 3% enzyme for 180 min. [A]Essential Amino acids (EAA). [B]Non Essential Amino Acids (NEAA). MA; MP: hydrolysate from mince of salmon frame prepared using alcalase and papain, respectively. CA; CP: hydrolysate from chunk of salmon frame produced using alcalase and papain, respectively.

2.5.5 Solubility of hydrolysates

Solubilities of hydrolysates from salmon frame are shown at different pHs in Figure 6. All hydrolysates were soluble over a wide range of PH range, in which more than 90% solubility was obtained. The hydrolysates were generally soluble in alkaline pH to a greater extent compared with the acidic pH. Similarly, protein hydrolysate from yellow stripe trevally (*Selaroides leptolepis*) meat, hydrolysed by alcalase and flavourzyme showed high solubility (>85%) in the pH range of 2-12 (Klompong *et al.*, 2008). The result suggested that proteins or peptides with high molecular weight (MW) remaining after hydrolysis were precipitated at this pH. In general, the degradation of proteins to smaller peptides leads to more soluble products (Gbogouri *et al.*, 2004). The smaller peptides from myofibrillar proteins are expected to have a proportionally more polar residues, with the ability to form hydrogen bonds with water and augment solubilities of hydrolysates. Also, enzymatic hydrolysis affects the molecular size and hydrophobicity, as well as polar and ionizable groups of protein hydrolysates (Mutilangi *et al.*, 1996). Therefore, the differences in solubility of hydrolysates with different DH might be determined by the size of peptides, the hydrophobic-hydrophilic balance, as well as the charge group of the peptides produced during the hydrolysis process.

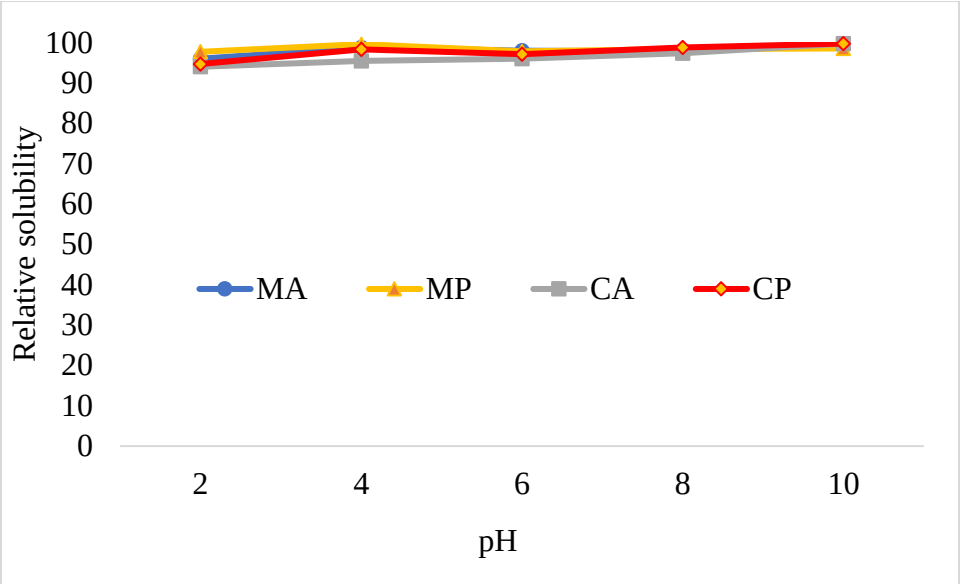


Figure 6 Solubilities of hydrolysates. MA; MP: hydrolysate from mince of salmon frame prepared using alcalase and papain, respectively. CA; CP: hydrolysate from chunk of salmon frame prepared using alcalase and papain, respectively. Bars represent the standard deviation (n=3).

2.5.6 Molecular weight distribution

Hydrolysates obtained from minced salmon frame using 3% alcalase or 3% papain (MA and MP) had two major peaks of both A_{220} and A_{280}, representing peptides having of 11860 and 3480 Da as shown in Figure 7. However, the peak height was slightly different, indicating the varying amount of peptides generated in both MA and MP. Minor peaks of A_{220} with molecular weight of 660, 496 and 248 Da were found in MA, while only 660 Da was found in MP. MA sample had peptides with higher peak area at MW of 660 Da than that of MP sample. MA had the peptides with MW of 660, 496 and 248 Da with higher peak area than MP. Due to the higher A_{280} of MA for peptides in the aforementioned MW, MA might contain more hydrophobic peptides. A_{220} is an indicator for peptide bonds, while A_{280} represents the peptides or proteins, mainly containing aromatic amino acids (Thiansilakul *et al.*, 2007; Karnjanapratum and Benjakul 2015). Amino acid sequence and peptide bonds in the substrate are factors that determines the compatibility with an enzyme active site (Aluko 2018). Higher peaks of low MW peptides in MA were in line with the more pronounced hydrolysis

with alcalase than papain. Higher DH was observed in MA than MP during the hydrolysis of mince sample. Moreover, the peak of A_{280} at MW of 248 Da was found only in MA sample.

When chunk sample was used for hydrolysis, it was found that both CA and CP had two major peaks of A_{220} and $A_{280,}$ consisting of 11860 and 3480 Da. Nevertheless, CA had higher peak of 3480 Da when compared to CP, which consisted of lower peak area of 3480 Da and higher peak of 11860 Da. Furthermore, Both CA and CP also showed the minor peaks at 29770, 660, 496 and 248 Da. However, CA had higher peak area at MW of 496 Da than that of CP. The result indicated that hydrolysate sample using Alcalase exhibited more effectiveness to cleave the protein from chunk salmon as evidenced by slightly higher DH (Figure 5). CA showed higher peak with MW of 660 Da than CP. Differences in A_{280} was also found between CA and CP. Amino acid sequence of proteins compatible with both proteases used could be different (Aluko 2018).

It could therefore be deduced that more aromatic compounds were present in MA and CA hydrolysate than that of MP and CP, respectively. This result correlated well with the total hydrophobic amino acid (Table 6) and bitterness (Table 5). Thus, the type of proteases had the marked impact on size distribution of peptides, in hydrolysate from salmon frame as indicated by different profiles (Figure 7). For the form of frame, it also affected the size distribution of peptides in hydrolysates to some degree. Those differences in size distribution could contribute to varying characteristics and properties of different hydrolysates.

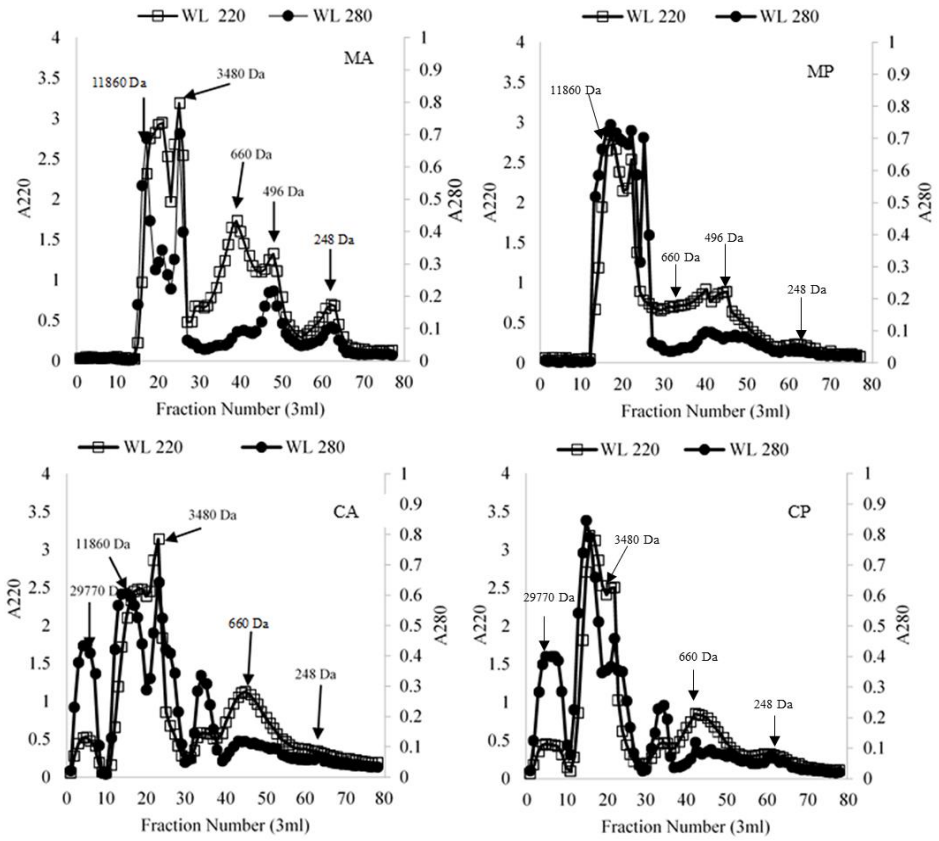


Figure 7 Elution profile by Sephadex G-25 size exclusion chromatography of hydrolysates from mince and chunk of salmon frame using alcalase and papain. Hydrolysis was conducted using 3% enzyme for 180 min. MA; MP: hydrolysate from mince of salmon frame prepared using alcalase and papain, respectively. CA; CP: hydrolysate from chunk of salmon frame prepared using alcalase and papain, respectively. Absorbance at 220 nm (rectangle), 280 nm (circle).

2.5.7 Antioxidative properties

2.5.7.1 DPPH radical scavenging activity

Different hydrolysates exhibited varying DPPH radical scavenging activity (Table 7). This assay has been employed to evaluate antioxidative properties of compounds as a hydrogen donor or free radical scavengers (Klompong *et al.,* 2007). When mince was used, MP had higher activity than MA ($P<0.05$), indicating higher hydrogen donating ability. Therefore, the free radicals were more scavenged. The result indicated that proteases with different specificity in cleavage of proteins or peptide bonds produced different peptides with various activities. This was reflected by different size distribution of various hydrolysates (Figure 7). Similar DPPH radical scavenging activity between the hydrolysate (CA and CP) was noted when prepared from the chunk using alcalase and papain ($P>0.05$). As the same proteases was used, no difference in DPPH radical scavenging activity between hydrolysates was found ($P>0.05$). Several factors including amino acid composition, side chain and chain length have been known to govern antioxidative activity (Klompong *et al.,* 2007; Intarasirisawat *et al.,* 2012). At 517 nm, DPPH shows maximal absorbance in ethanol as a stable free radical. When DPPH react with an antioxidant that has the ability to donate hydrogen, the radical is scavenged. Overall, all the hydrolysates from salmon frame were able to donate hydrogen atom toward radicals with coincidental formation of stable diamagnetic molecule. This could lead to the end of radical chain reaction (Binsan *et al.,* 2008). Nevertheless, activity was varied, depending on form of frame and proteases used for hydrolysates production.

2.5.7.2 ABTS radical scavenging ability

ABTS radical scavenging activities of all the hydrolysates are present in Table 7. MA showed the highest activity ($P<0.05$), followed by CA, CP and MP, respectively. This assay measures the capacity of antioxidants to donate a hydrogen atom or an electron to free radicals, in which a nonradical species is formed (Binsan *et al.,* 2008). ABTS assay is commonly used for both lipophilic and hydrophilic compounds, while DPPH assay is effective for lipophilic compounds (Re *et al.,* 1999). Higher antioxidative capacity of MA indicated the ability of peptides in MA in scavenging and

stabilizing the free radical, thereby retarding the chain reaction. This also confirmed the role of proteases in producing different antioxidative peptides. Nevertheless, ABTS radical scavenging activities between CA and CP were similar (P>0.05), reflecting similar ability in quenching ABTS radicals between both hydrolysates.

2.5.7.3 Ferrous reducing antioxidant power (FRAP)

Hydrolysates obtained from mince and chunk of salmon frame showed varying FRAP as presented in Table 7. In general, hydrolysates obtained using papain showed greater FRAP than those produced by alcalase (P<0.05). However, hydrolysates from chunk and mince exhibited similar FRAP when the same proteases was used. FRAP indicates the ability of tested compound in providing an electron to free radicals (Klompong *et al.*, 2007). FRAP is usually applied to quantify the ability of compound to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Binsan *et al.*, 2008). Hence, hydrolysates from salmon frame had FRAP, but the activity was affected by the proteases. However, the form of frame used as a starting material had no impact on FRAP of resulting hydrolysates.

2.5.7.4 Metal chelating activity

Metal chelating activities of MA and CP were highest (P<0.05), followed by CA and MP, respectively (Table 7). All the hydrolysates had peptides capable of chelating the prooxidative metals. The difference in metal ion chelating activity in different hydrolysates (HA, HP, CA and CP) might be due to differences in peptide chain length and varying amino acid sequences (Klompong et al. 2007). Histidine or histidine containing peptide possess the radical trapping and metal sequestering ability by the imidazole ring. The presence of transition metals, e.g. Co, Cu and Fe can accelerate both autoxidation and decomposition of hydroperoxide into volatiles (Senphan and Benjakul 2014). Thus, hydrolysates obtained from mince and chunk of salmon frame mostly likely acted as the secondary antioxidant, which was able to chelate the prooxidative metal ions. It was noted that both proteases and form of frame used for hydrolysis performed a profound role in metal chelating activity of resulting hydrolysates.

2.5.7.5 Oxygen reducing antioxidant capacity (ORAC)

ORAC of hydrolysates obtained from different forms using different proteases were 544.25-840.03 µmol TE/g sample (Table 7). CP sample showed the highest ORAC ($P<0.05$), while MA possessed the lowest ORAC value ($P<0.05$). Nonetheless, ORAC between MP and CA were not different ($P>0.05$). Peroxyl radical scavenging activity of compounds is determined using ORAC assay (Sae-leaw *et al.*, 2015). ORAC assay is confined for measuring chain breaking capacity against only peroxyl radicals (Sae-leaw *et al.*, 2015). When the same form of frame was used, hydrolysate prepared with the aid of papain exhibited the higher ORAC than those prepared by alcalase ($P<0.05$). Chunk provided the hydrolysate with higher ORAC than mince. Thus, both factors, proteases and form of salmon frames determined ORAC of resulting hydrolysates.

Overall, the result suggested that a wide variety of peptides having various modes of actions were liberated during hydrolysis. The exposure of free amino groups, size alteration, amino acid sequence generally determine the antioxidative activity of peptides (Sae-leaw *et al.*, 2015). The differences in antioxidative activity among all hydrolysates were governed by proteases used and the form of frames as raw material for hydrolysis.

Table 7 Antioxidative activities of protein hydrolysates from mince and chunk of salmon frame using alcalase and papain

Sample	DPPH radical scavenging activity (µmol TE/g sample)	ABTS radical scavenging activity (µmol TE/g sample)	FRAP (µmol TE/g sample)	Metal chelating activity (µmol EDTA/g sample)	ORAC (µmol TE/g sample)
MA	247.38±22.06^{a}	688.21±19.79^{c}	700.12±30.78^{a}	655.95±17.10^{c}	544.25±32.46^{a}
MP	293.12±11.74^{b}	349.21±23.42^{a}	909.85±17.86^{b}	435.74±45.47^{a}	663.12±26.36^{b}
CA	269.48±29.33^{ab}	444.23±25.89^{b}	738.18±15.54^{a}	532.92±29.92^{b}	693.94±31.90^{b}
CP	258.68±20.94^{ab}	413.22±33.78^{b}	861.85±35.03^{b}	646.40±36.73^{c}	840.03±4.79^{c}

Hydrolysis was conducted using 3% enzyme for 180 min. Values are given as mean ± SD (n=3)

Different superscripts in the same column indicate significant differences ($P<0.05$).

2.6 Conclusion

Salmon frame was composed of nutritional constituents. Calcium and phosphorus were the major mineral that could be obtained in salmon frame. Protein hydrolysate could be obtained from salmon frame and it could be used as a food supplement. The form of raw material as well as the proteases used greatly influenced the chemical composition, size distribution and antioxidant capacities of the hydrolysates. Mince form yielded the hydrolysates with higher antioxidant capacities. Alcalase rendered the higher yield but its hydrolysates had higher bitterness than papain. Nevertheless, to implement the “zero waste” concept, which involve complete utilization of salmon frame waste, the chunk form was preferred than the mince. High amount of bone residues was obtained after hydrolysis, in which its ease of operation and less energy required could be achieved. Thus, bone residues from chunk obtained after hydrolysis can be handled easily as a starting material, especially for biocalcium production.

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CHAPTER 3
PRODUCTION AND CHARACTERIZATION OF BIOCALCIUM AND CALCINED POWDERS FROM SALMON BONE

3.1 Abstract

Biocalcium powders, Bio-cal-A and Bio-cal-H, obtained from alkaline treated and non-alkaline treated salmon frame, a leftover from protein hydrolysis, were investigated, in comparison with those calcined at 900°C for 6 and 9 h named Cal-A (6 h), Cal-H (6 h), Cal-A (9 h) and Cal-H (9 h). Calcium content in calcined bones (31.54-38.84%) was higher than those in biocalcium powders (27.32-30.88%). Also, phosphorus content was higher in calcined bones (15.16-18.11%), compared to those of biocalcium (13.22-14.40%). Nonetheless, the mole Ca/P (1.60-1.66) ratios were observed among all samples depending on the chemical and heat treatment conditions. Mean particle size of all powders were 22.21-26.53 µm. Bio-cal-A had higher L*(lightness), b* (yellowness), ΔE* (total difference in color), ΔC* (chroma) values than others ($P<0.05$). X-ray diffraction showed a characteristic hydroxyapatite as the dominant phase in all samples with increase in degree of crystallinity after calcination. Protein, fat, hydroxyproline and TBARS were detected in both biocalcium powders, but not detectable in calcined bone powders. For *in-vitro* simulated gastrointestinal tract study, Bio-cal-A had higher solubility than Bio-cal-H and $CaCO_3$ ($P<0.05$). However, transportation of calcium across Caco-2 cell monolayer of Bio-cal-H was higher than Bio-cal-A and $CaCO_3$ ($P<0.05$). Thus, salmon frame could be used as potential source to produce high valuable products such as biocalcium for calcium supplement as well as hydroxyapatite for biomedical applications.

3.2 Introduction

Calcium has been identified as an essential element required for numerous physiological activities of human system, including maintaining nerve impulse transfer and heart rate, facilitating blood flow within capillaries, participating in blood coagulation and modulating muscle function (Benjakul *et al.*, 2017). Deficiency of calcium is a general problem associated with reduced bone mass and osteoporosis (Cashman 2002). This is due to inadequate calcium in most regular meals consumed by people. Tricalcium phosphate (TCP) and calcium carbonate ($CaCO_3$) have been used to supplement for calcium inadequacies (Zhao *et al.*, 2005). Another limitation of calcium intake in humans is unavailability of calcium, particularly inorganic calcium with poor adsorption. Recently, biocalcium containing peptides was produced from bones of tuna (Benjakul *et al.*, 2017). Peptides have the ability to chelate calcium, thereby preventing calcium precipitation in phosphate salt form (Benjakul *et al.*, 2017). Calcium bioavailability as measured by the calcium transported across Caco-2 cell monolayer have been reported (Perales *et al.*, 2005). The Caco-2 cell line is a human colon carcinoma cell line, which undergoes spontaneous differentiation in cell culture to form a polarized epithelial cell monolayer with many characteristics of enterocytes (Glahn *et al.*, 1996). Both *in vitro* simulated gastrointestinal digestion and Caco-2 cell monolayer have been applied to study calcium solubility and bioavailability in calcium containing foods (Perales *et al.*, 2005).

Fish consumption has been increased tremendously and its demand by year 2050 is estimated to be 9.8 billion (UN, 2017). During processing of fish, more than 60% are generated as leftovers including heads (containing the gills), trimmings (containing muscle, bone and skin), mince, frames and viscera (liver, kidney and roe) (See *et al.*, 2011). Salmon (*Salmo salar*) is served as the large portion globally due to its high market demand. It is widely imported to Thailand where it is largely recognized as delicacy among Thai consumers. It is usually sold as a whole or as a fillet, which often leads to generation of frames attached with remaining meat. Hydrolysis of salmon frame prepared in the mince and chunk form was done by Idowu et al. (2018). After hydrolysis, high amount of fish bones is remained as residues, particularly when chunk form was used. These residual bones could be used as a starting material for production of biocalcium. Fish bones are rich in calcium and phosphorus, especially in the form of

calcium hydroxyapatite (Benjakul *et al.,* 2017). Calcination at high temperature has been implemented to produce hydroxyapatite (HA) (Piccirillo *et al.,* 2013). When calcination is implemented, organic matters such as proteins or peptides are removed. This may lead to poor solubility of Ca in gastrointestinal tract, thus yielding low bioavailability. On the other hand, biocalcium contain proteins or peptides, which could enhance solubility in the gastrointestinal tract (Benjakul *et al.,* 2017). Biocalcium was produced from precooked bone of skipjack, tongol (*Thunnus tonggol*) and yellow-fin (*Thunnus albacores*) (Benjakul *et al.,* 2017). They were rich in calcium, phosphorus and collagenous protein. Biocalcium powders obtained from both pre-cooked tongol tuna bone and yellow tuna bone possessed higher solubility than that of their calcined counterpart (Benjakul *et al.,* 2017). Alkaline treatment has been implemented to remove proteinaceous substance. However, those proteins might help bind calcium, leading to the increased solubility. Thus, pre-treatment such as alkaline treatment might have the impact on property of biocalcium from fish bone. Thus, this study aimed at production and characterization of biocalcium powders from alkaline treated and non-alkaline treated salmon bone, obtained after protein hydrolysis, in comparison with their corresponding calcine counterparts.

3.3 Materials and Methods

3.3.1 Chemicals

Hexane was procured from LabScan (Bangkok, Thailand). Hydrogen peroxide and sodium hypochlorite were supplied from QReC (Auckland, New Zealand). Hydrogen peroxide and sodium hypochlorite were supplied from QReC (Auckland, New Zealand). Calcium carbonate, porcine pepsin, pancreatin and bile extract were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), nonessential amino acids, phosphate buffered solution (PBS) and trypsin-EDTA solution were obtained from Gibco (Scotland, UK).

3.3.2 Collection and preparation of bone from salmon frame

Frames of salmon (*Salmo salar*) (30-35 cm in length) were obtained from Kingfisher holding Ltd Songkhla, Thailand. The frames were cut into the length of 4-5 cm using a sawing machine. The prepared frames (chunks) were subjected to hydrolysis as detailed by Idowu et al. (2018). Bone residues obtained after hydrolysis were cleaned

using a high-pressure water jet cleaner (Model Andaman 120 bar, Zinsano, Bangkok, Thailand) to remove meat attached to the bones at a pressure of 120 bar. After cleaning, the bones were divided into two portions and kept at 4°C before use.

3.3.3 Pretreatment of bones

The first portion of prepared bones (50 g) were immersed in 2 M NaOH with a bone/solution ratio of 1:10 (w/v) at 50°C up to 120 min. Continuous stirring of the mixture was done at a speed of 150 rpm using an overhead stirrer attached to a propeller (Model RW 20n, IKA-Werke GmbH & CO.KG, Staufen, Germany). At different time (0, 10, 20, 30, 40, 50, 60, 90 and 120 min), 5 ml of the solution were taken for determination of total soluble protein content by the biuret method (Gornall *et al.,* 1949) and hydroxyproline (Bergman and Loxley 1963). The time rendering the solution with the highest soluble protein (non-collagenous) but the lowest hydroxyproline (a unique amino acid in collagen) content was selected for alkaline treatment. The second portion was not subjected to alkaline treatment. Both bones were dried separately with a laboratory scale rotary dryer (air velocity-1.5 m/s, temp-50°C, time-2 h). Dried samples were ground using a crushing mill (Model YCM1.1E, Yor Yong Hah Heng, Bangkok, Thailand) until particle sizes of approximately 3-4 mm were obtained.

3.3.4 Preparation of biocalcium and calcined bone

Both alkaline and non-alkaline treated samples were subjected to lipid removal, bleaching and grinding as detailed by Benjakul *et al.,* (2017). Biocalcium powder obtained from alkaline treated salmon bones and non-alkaline treated were termed Bio-cal-A and Bio-cal-H, respectively. Another portion of Bio-cal-A and Bio-cal-H was calcined using a muffle furnace (Model 320, P Nabertherm, Bremen, Germany) at 900°C for 6 h and 9 h and the resulting matters were named as Cal-A (6 h), Cal-A (9 h), Cal-H (6 h) and Cal-H (9 h), respectively. All the samples were ground to obtain fine particles using a ball mill (Model PM 100, Retsch GmbH, Haan, Germany) as illustrated by Benjakul *et al.,* (2017). All the samples were subjected to analyses.

3.3.4.1 Characterization of biocalcium and calcined bone powders

3.3.4.1.1 Chemical composition

Moisture, protein, fat and ash contents of all the samples were determined (AOAC, 2000). Inductively coupled plasma optical emission spectrometer (ICP-OES) (Model Optima 4300 DV, Perkin Elmer, Shelton, MA) was used for determination of

Ca and P in all the samples as per the method of Feist and Mikula (2014). Amino acid composition of biocalcium samples was determined as tailored by Chuaychan *et al.,* (2015). Volatile compounds in biocalcium samples were analyzed using a solid phase microextraction gas chromatography mass spectrometry (SPME– GC–MS) (Iglesias and Medina 2008).

3.3.4.1.2 Color

The color of samples were determined using a Hunter lab colorimeter (Colour Flex, Hunter Lab Inc., Reston, VA, USA). L*, a*, b*, ΔE* and ΔC* values were recorded.

3.3.4.1.3 Mean particle size

Mean particle size was determined following the method of Mad-Ali *et al.,* (2017) using a lazer particle size analyzer (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA).

3.3.4.1.4 Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (SEM-EDX)

SEM-EDX was used to measure the relative abundance of elements as detailed by Chuaychan *et al.*, (2015) using a field emission scanning electron microscope (FEI-XL30, FEI Company, Hillsboro, OR, USA) equipped with electron-dispersive X-ray spectroscope (EDX).

3.3.4.1.5 X- ray Diffraction analysis

The phase compositions of samples were determined by X-ray diffraction (XRD) using an X-ray Diffractometer (X' Pert MPD, PHILIPS, Eindhoven, the Netherlands) as described by Benjakul *et al.,* (2017).

3.3.4.1.6 Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) of biocalcium samples were determined as per the method of Benjakul *et al.,* (2017).

3.3.4.1.7 Solubility in simulated gastrointestinal tract

Solubility of calcium in biocalcium samples in comparison with $CaCO_3$ was determined using a gastrointestinal tract model system (GIMs) as tailored by Benjakul *et al.,* (2017). The samples (150 mg) were dispersed in 100 ml of 5 mM HCl-KCl buffer (pH 1.5). Subsequently, 5 ml of pepsin solution (32 U/ml) in 1 M HCl-KCl buffer (pH 1.5) were added. The mixture was mixed well and incubated at 37°C for 60 min

(stomach condition) with a continuous shaking using a Unimax 1010 shaker (Heidolph Model, Schwabach, Germany). Thereafter, the pH of the reaction mixture was adjusted to 6.8 with 1 M $NaHCO_3$. One milliliter of the enzyme mixture of bile and pancreatic juice containing pancreatin (10 mg/ml), trypsin (14,600 U/ml) and bile extract (13.5 mg/ml) in 10 mM Tris–HCl buffer (pH 8.2) were added to the mixture. The mixture was incubated at 37°C for 3 h (duodenal condition) with a continuous shaking. The termination of digestion was done by placing the mixture in a boiling water bath for 10 min. The final volume was adjusted to 150 ml before centrifugation at 7,000xg for 10 min. The supernatants (digests) were taken for calcium determination by atomic absorption (AA) (Perkin-Elmer, model 2380, Boston, MA). Calcium solubility was reported as % relative to total amount of calcium in the sample.

3.3.4.1.8 Bioavailability of calcium in Caco-2 monolayer

Calcium bioavailability of biocalcium samples in comparison with $CaCO_3$ was examined by monitoring the transportation of Ca across Caco-2 monolayer as described by O'Callaghan and O'Brien (2010). Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) non-essential amino acids. Thereafter, cells were incubated at 37°C in a humidified incubator with 5% CO_2, in the absence of antibiotics. Caco-2 intestinal model was established by seeding of the cells at a density of 5.0 x 10^4 cells /cm^2 on a transwell plate (6-well plate, 24 mm diameter, 0.4µm pore size membrane, Costar Corp., NY, USA). Media were changed every 2–3 days, over 21–25 days, to obtain a differentiated cell monolayer. Transepithelial electric resistance (TEER) measurements were performed twice weekly by a TEER Voltohmmeter (Millicell-ERS, Millipore, Ireland) to ensure that the monolayer was intact.

For each experiment, 1.5 ml of serum-free media and 50 µl of digest (obtained from *in vitro* simulated gastrointestinal tract) was added to the apical side of the transwell plate. Serum-free media (2 ml) was added to the basolateral chamber and the cells were incubated for 2 h at 37 °C in a humidified incubator with 5% CO_2. After incubation, the TEER was measured to ensure the monolayer remained intact. The media from both the apical and basolateral chambers were collected for determination of calcium content. Calcium content were analyzed using AA. Bioavailability of calcium was calculated as the following equation.

Calcium bioavailability (%) = calcium content in basolateral chamber/ calcium content of the digest added × 100

3.3.5 Experimental design and Statistical analysis

Experiments were run in triplicate with completely randomized design (CRD). Analysis of variance (ANOVA) was carried out. Means were compared using the Duncan's multiple range test. The Statistical Package for Social Science (SPSS 11.0 for Windows, SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

3.4 Results and Discussion

3.4.1 Total soluble protein and hydroxyproline content of salmon bone leached out during alkaline treatment

Total soluble protein content liberated into alkaline solution used for treatment of salmon bone, a leftover from hydrolysis process, was monitored as a function of time (Figure 8a). A sharp increase in extractable protein from salmon bones was observed up to 50 min of alkaline treatment. Thereafter, slight decrease in protein content was noticeable up to 120 min. Alkali was able to solubilize protein attached to the bone, remaining after enzymatic hydrolysis. This could lead to the removal of proteinaceous substances from the aforementioned bones. With continuous stirring at the operating temperature of 50°C, proteins likely underwent denaturation or unfolding. This resulted in an increase in mass diffusivity, which in turn accelerated the mass transfer and solubilization of denatured proteins from the bone matrix. The result was in line with Kumoro *et al.,* (2010) who reported the positive impact of high temperature on alkaline extraction of protein from chicken bone waste. Alkaline solutions have been reported to be effective in the removal of non- collagenous proteins from starting materials used for collagen or gelatin production (Benjakul *et al.,* 2018). After 50 min, no further increase in the total soluble protein was found. This could be a result of less availability of soluble proteins in the bone. Further degradation of peptides to free amino acids or dipeptides could result in the lower content of proteins detected by biuret method (Wu *et al.,* 2003).

Content of hydroxyproline, a distinct amino acid are present in collagen, was determined during alkaline treatment as shown in Figure 8b. Hydroxyproline in the bone matrix represents collagenous proteins. Collagenous proteins were released with continuous stirring as a result of softening and rupturing of the bones induced by

alkaline condition at high temperature. The release of these collagenous proteins occurred continuously up to 100 min. A decline in hydroxyproline content was found at 120 min. Collagenous proteins associated with ca-hydroxyapatite might not be able to be solubilized, thus still remaining in the bone matrix. Non-collagenous proteins such as fibronectin, proteoglycan, osteopontin, thrombospondin, osteonectin and osteocalcin could be removed by alkaline solution to some degree (Benjakul *et al.,* 2017). In the present study, alkaline treatment for 40 min was selected to remove non-collagenous proteins in the bone.

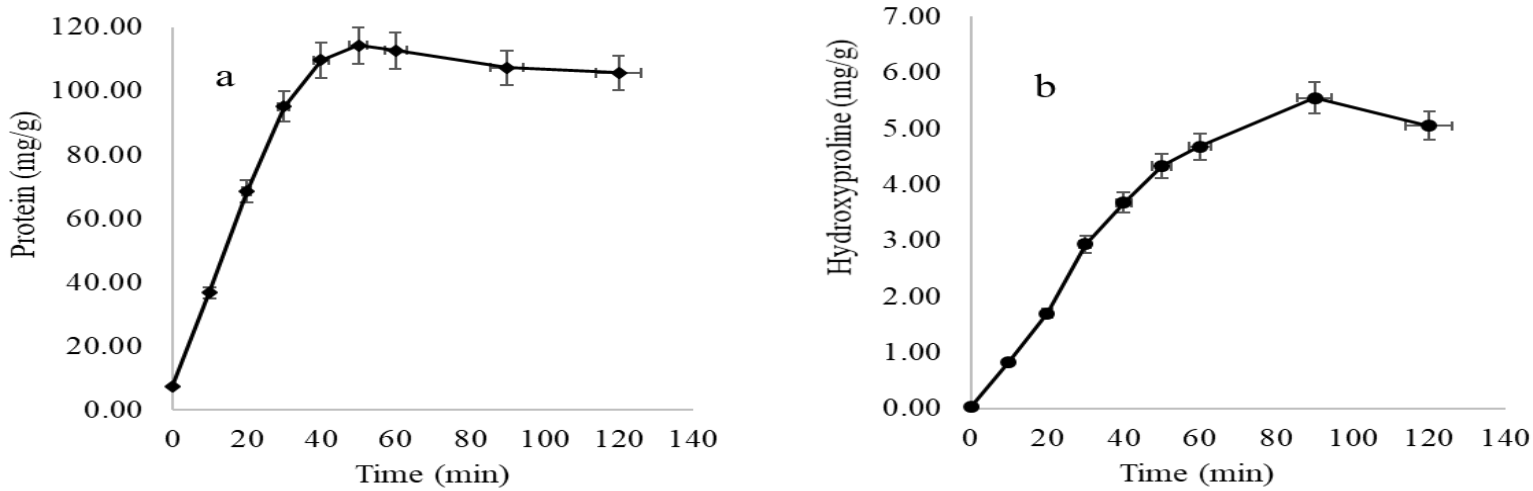

Figure 8 Total soluble protein (a) and hydroxyproline (b) contents as a function of time during for alkaline treatment of salmon bones. Bars represent the standard deviation (n=3).

3.4.2 Chemical compositions of biocalcium and calcined bone from salmon frame without and with alkaline treatment

3.4.2.1 Proximate composition and mole ratio

Chemical compositions of the biocalcium and calcined bone are shown in Table 8. On dry basis, bones calcined for 6 h had very low moisture content, of which those calcined for 9 h possessed no moisture. This could be as a result of high temperature treatment on bones during calcination, which removed all the organic compounds and water molecules. For biocalcium, Bio-cal-H had the higher moisture content than Bio-cal-A ($P<0.05$). Fluids or other proteinaceous residues might bind or complex with water in bone matrix as indicated by higher moisture content. The ash content of calcined powders were higher than their biocalcium counterparts ($P<0.05$). During calcination, all organic components were combusted. It was noted that calcination for a longer time yielded the powder with higher ash content. However, the lower ash content of the Cal-H powders could also be limited by the presence of some haem proteins as indicated in the color. Also, alkaline pretreatment rendered calcined bone (Bio-cal-A) with higher ash content than that without treatment (Bio-cal-H). Overall, high ash content of calcined powders (99.55-99.99%) were obtained.

Bio-cal-A possessed higher calcium and phosphorus contents than Bio-cal-H. This result was in line with ash content in the samples. Calcination for a longer time also provided the calcined bone with the increases in calcium and phosphorus contents. Fish bones are generally rich in minerals (Toppe *et al.*, 2007). The result suggested that the removal of non-collagenous protein with alkaline treatment could have led to higher ash content of Bio-cal-A. The Ca/P mole ratio of Bio-cal-A and Bio-cal H were 1.66 and 1.60, respectively. The calcined bones showed similar ratio, in which Cal-A (6h), Cal-H (6h), Cal-A (9h) and Cal-H (9h) had the ratio of 1.66, 1.61, 1.66 and 1.63, respectively. Garner and Anderson (2011) reported that vertebrate bone contains inorganic matter in the form of hydroxyapatite crystals deposited in the matrix of cross-linked collagen fibrils. The hydroxyapatite has a Ca/P mole ratio of 1.67. In the present study, the mole ratio of Ca/P from salmon bone (Table 8) was related closely to that of hydroxyapatite. Based on the Ca/P mole ratio, it could be proposed that hydroxyapatite was present as the dominant constituent in biocalcium and calcined powders from salmon bone, a residue from protein hydrolysis process.

3.4.2.2 Hydroxyproline content

Hydroxyproline was found only in Bio-cal-A and Bio-cal-H, while it was absent in the calcined bones, regardless of alkaline pretreatment or calcination temperature (Table 8). The presence of hydroxyproline indicated that collagenous protein constituted in the biocalcium, which correlated well with the protein content of biocalcium. Nevertheless, Bio-cal-H possessed higher hydroxyproline content than Bio-cal-A. Some collagenous proteins might be removed during alkaline treatment of salmon bones used for Bio-cal-A production. Calcination at high temperature directly combusted these collagenous proteins in the calcined samples ($P<0.05$). Therefore, hydroxyproline was absent in the calcined bones. In general, alkaline pre-treatment of salmon bone determined the characteristics of biocalcium, whereas calcination completely eliminated collagen from the bones.

3.4.2.3 Lipid oxidation

Lipid oxidation products of biocalcium samples, Bio-cal-A and Bio-cal-H, expressed as TBAR values are shown in Table 8. Bio-cal-H had the higher TBAR value than the Bio-cal-A. TBARS measurement is used to monitor the degradation of hydroperoxides, which leads to the formation of secondary oxidation products such as aldehydes (Sae-Leaw and Benjakul 2015). The decrease in TBARS value of Bio-cal-A could be as a result of alkaline treatment, which could remove pro-oxidant proteins such as haemoglobin. The result correlated well with differences in fat content of Bio-cal-A and Bio-cal-H as highlighted in Table 8. During alkaline treatment, the bone became soft and possibly led to the leaching out of some lipids trapped in the bone matrix. Subsequent treatment with hexane could contribute to the removal of lipid and lipid oxidation products to some extent. Oxidizing agents such as NaOCl and H_2O_2 plausibly disintegrate lipid oxidation product easily in Bio-cal A than Bio-cal-H as a result of the softness of the bones after alkaline treatment in the former. This plausibly facilitated the reduction in TBARS values in Bio-cal-A. In calcined samples, lipids were completely removed and the secondary oxidation products were not formed. Hence, TBAR values could not be detected for all calcined bones. Overall, various pretreatments and calcination process of fish bones could lower lipid oxidation in biocalcium and calcined powder, respectively.

Table 8 Chemical composition of biocalcium and calcined powders from salmon frame

Chemical composition	Bio-cal A	Bio-cal H	Cal-A (6hr)	Cal-H (6hr)	Cal-A (9hr)	Cal-H (9hr)
Moisture (%)	4.82 ± 0.07^{d}	7.81 ± 0.04^{e}	0.27 ± 0.00^{b}	0.45 ± 0.01^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Protein (%)*	12.07 ± 0.18^{b}	20.90 ± 0.06^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Fat (%)*	0.33 ± 0.01^{b}	1.70 ± 0.01^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Ash %)*	82.78 ± 0.25^{b}	69.59 ± 0.57^{a}	99.73 ± 0.05^{d}	99.55 ± 0.32^{c}	99.99 ± 0.00^{f}	99.97 ± 0.00^{e}
Hydroxyproline (mg/g)*	5.07 ± 0.01^{b}	14.79 ± 0.02^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
TBARS (mg malonaldehyde/kg sample)*	0.95 ± 0.00^{b}	3.34 ± 0.01^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Calcium (%)*	30.88 ± 0.33^{b}	27.32 ± 0.17^{a}	36.01 ± 0.26^{e}	31.54 ± 0.20^{c}	38.84 ± 0.55^{f}	34.24 ± 0.40^{d}
Phosphorus (%)*	14.40 ± 0.39^{b}	13.22 ± 0.48^{a}	16.79 ± 0.25^{e}	15.16 ± 0.32^{c}	18.11 ± 0.35^{f}	16.27 ± 0.45^{d}
Mole ratio Ca/P	1.66	1.60	1.66	1.61	1.66	1.63

Values are presented as mean ±SD (n=3). Different superscripts in the same row indicate significant difference ($P<0.05$).

*Dry weight basis

3.4.3 Amino acid composition of biocalcium powders

Amino acid compositions of biocalcium samples, expressed as residues per 1000 total residues, are shown in Table 9. Glycine was present as the major amino acid (324-327 residues/1000 residues). Glycine has been reported to comprise around 1/3 of total amino acids in collagen (Benjakul *et al.,* 2018). High level of glycine was reported in fish bone from horse mackerel, trout, salmon and cod (Toppe *et al.,* 2007). Glycine commonly occurs after every third position of ά-chain of collagen except for 14 amino acids from N-terminal and for 10 amino acids from C-terminal (Benjakul *et al.,* 2012). Other amino acids were found at varying proportions in the biocalciums. Alanine (98-101 residues/1000 residues), glutamic acid/glutamine (70-79 residues/1000 residues) and proline (78-94 residues/1000 residues) were also found at high levels. Imino acids present in both biocalciums are hydroxyproline and proline (45-69 and 78-94 residues/1000 residues, respectively). The imino acids of animal collagens correlated with their habitat (Foegeding 1996). Imino acids present in Bio-cal-H was higher than that present in Bio-cal-A. Fish bones used for processing of Bio-cal-A were subjected to alkaline treatment, which led to the rupturing of the bone matrix. This could result in the loss of imino acids present in bones as indicated by lower imino acids in Bio-cal-A. On the other hand, phenylalanine, threonine and tryptophan were higher in Bio-cal-A than Bio-cal-H. In general, amino acids are essential because of the physiological role they performed in the body (Chalamaiah *et al.,* 2012). The pretreatment thus affected amino acid residues in biocalcium samples.

Table 9 Amino acid composition of biocalcium powders from salmon frame as affected by alkaline pretreatment

Amino acids (residues/1000 residues)	Bio-cal-A	Bio-cal-H
Alanine	98	101
Arginine	40	46
Asparagine	49	50
Aspartic acid	0	0
Cysteine	30	11
Glutamic acid/Glutamine	70	79
Glycine	324	327
Histidine	11	8
Isoleucine	6	10
Leucine	22	25
Lysine	19	22
Hydroxylysine	8	10
Methionine	14	18
Phenylalanine	29	14
Hydroxyproline	45	69
Proline	78	94
Serine	60	53
Threonine	35	20
Tryptophan	26	14
Tyrosine	10	7
Valine	26	22
Total	1000	1000
Imino acids	123	163

Bio-cal-A: biocalcium powder from alkaline treated salmon frame. Bio-cal-H: biocalcium powder from non-alkaline treated salmon frame.

3.4.4 Volatile compounds of biocalcium powders

Volatile compounds of Bio-cal-A and Bio-cal-H are presented in Table 10. The most prevalent volatiles observed in both biocalcium samples were aldehydes. Aldehydes are used as indicator of lipid oxidation and they also contribute to off-flavor and off-odor (Benjakul *et al.*, 2017). Benzaldehyde, the most abundant aldehydic compound, was found in Bio-cal-H but not detected in Bio-cal-A. This could be as a result of lipid residue retained in the bone matrix of Bio-cal-H. Alkaline treatment of bone before production of Bio-cal-A could remove lipids residue to high extent. Hexanal and butanal were observed in Bio-cal-H than in Bio-cal-A. Heptanal, acetaldehyde and pentanal were not detected in Bio-cal-A but were observed in Bio-cal-H. Butanal and heptanal can be used as benchmark for flavor degradation in fish products (Iglesias and Medina 2008). Oxidation of the lipids retained at higher level such as n-6 polyunsaturated fatty acids (PUFA) during processing of biocalcium samples could have led to the higher generation of volatiles such as nonanal and octanal observed in Bio-cal-H than in Bio-cal-A.

In addition, ketones, alcohols, hydrocarbons, acids and other volatile compounds were also found in the biocalcium samples. Bio-cal-H possessed higher ketonic compounds such 2-octanone, 2-propanone, 2-hexanone and 2-butanone than those present in Bio-cal-A. With prior alkaline treatment, lesser amount of alcoholic compounds was found in Bio-cal-A, compared to that found in Bio-cal-H. These alcoholic compounds included 1-octanol, 1-hexanol and 1-hexadecane. Decomposition of hydroperoxides led to the formation of secondary products such as alcohols and ketones (Girard and Durance 2000). For hydrocarbons, tetradecane and hexadecane were observed in the powders. In addition, high proportion of volatile compounds such as acetonitrile was observed in Bio-cal-H, compared to Bio-cal-A. Butanenitrile and benzonitrile were observed in Bio-cal-H but were not detected in Bio-cal-A. Thus, alkaline treatment of bone before production of biocalcium could reduce the formation of volatiles in Bio-cal-A, compared to non-alkaline treated counterpart. Overall, the higher abundance of volatile compounds in Bio-cal-H than Bio-cal-A was most likely related to the higher lipids retained in the bones.

Table 10 Volatile compounds in biocalciums from salmon frame as affected by alkaline pretreatment

Volatile compounds	Bio-cal-A	Bio-cal-H
Aldehydes		
Butanal	2.57	28.00
Heptanal	ND	1.50
Octanal	0.32	0.86
Nonanal	0.36	0.97
Benzaldehyde	ND	202.32
Hexanal	12.99	49.34
Acetaldehyde	ND	4.05
Pentanal	ND	14.93
Ketones		
2-Octanone	2.80	2.91
2-Propanone	71.82	82.93
2-Hexanone	1.53	4.90
2-Butanone	24.21	26.97
Alcohols		
1-Octanol	1.17	1.36
1-Hexanol	0.99	1.03
1-Heptanol	0.45	0.56
Hydrocarbons		
Tetradecane	0.53	0.75
Hexadecane	1.49	2.19
Acids		
Hexanoic acid	0.18	0.99
Octanoic acid	ND	1.04
Pentanoic acid	0.32	0.61
Others		
Butanenitrile	ND	20.09
Acetonitrile	12.33	31.60
Benzonitrile	ND	2.20

Values are expressed as abundance ($x10^6$). ND not detectable. Bio-cal-A: biocalcium powder from alkaline treated salmon frame. Bio-cal-H: biocalcium powder from non-alkaline treated salmon frame.

3.4.5 Color of powder

L*, a*, b*, ΔE* and ΔC* of Bio-cal-A, Bio-cal-H and their calcined counterparts are stated in Table 11. Bio-cal-A possessed higher value of lightness (L*value) than Bio-cal-H and the calcined samples ($P<0.05$). The slightly higher creamy whitish color of Bio-cal-A could be as a result of removal of meat residue as well as blood during alkaline treatment of salmon bone. For Bio-cal-A and Bio-cal-H, during drying process, carbonyl compound-related products were formed via lipid oxidation and could undergo non-enzymatic browning reaction with the proteins, peptides and amino group of free amino acid retained in the bone powders. This more likely resulted in the increased yellow color obtained in the biocalcium powders. Both biocalciums had low redness (a* value). Similar trend was reported by Benjakul *et al.,* (2017), in which biocalcium powders from pre-cooked tongol (*Thunnus tonggol*) and yellowfin (*Thunnus albacores*) tuna bone had higher yellowness than calcined counterpart.

For the calcined samples, an increase in lightness (L*) was observed in both Cal-A and Cal-H when calcination time increased from 6 to 9 h. Similarly, the increases in *ΔC were also observed. For the same calcination time, Cal-A showed the higher L* but lower a* and b*-values than Cal-H ($P<0.05$). *ΔE-value was lower but ΔC*-value was higher for Cal-A, compared with Cal-H. Lower proteins retained in bones might decrease the combustion during calcination. The complexation of combusted substances could be lowered when blood, lipids and other haem residues were trapped in fish bones used for the processing of the calcined samples. The result illustrated the impact of pretreatment and calcination process on the color of the resulting bone powders, both biocalciums and calcined bones.

3.4.6 Mean diameters and particle size of biocalciums and calcine powders

Mean diameters and particle size distribution of Bio-cal-A and Bio-cal-H and their calcined counterparts with calcination time of 6 and 9 h are shown in Table 11 and Figure 9, respectively. All samples showed differences in the mean diameter (d_{43}), which ranged from 22.21 to 27.53μm. Bio-cal-A showed larger particle size than Bio-cal-H ($P<0.05$). For Cal-A, the higher calcination time resulted in the decrease in particle size. On the other hand, slightly higher size was found for Cal-H when calcination time was increased ($P<0.05$). Bio-cal-A showed a bi-modal distribution because of non-homogenous particles as indicated by the peak with the shoulder, while

Bio-cal-H showed a mono-modal distribution, indicating the presence of homogenous particles. The surface moisture, protein and fat concentration are the factors affecting the stickiness and agglomeration of particles, thus influencing the particle size distribution (Pısecký 1997). During alkaline treatment, collagen in the bone matrix, which tightly linked with ca-hydroxyapatite, might be removed. This could bring about the weakened matrix and the size could be reduced with ease.

For calcined samples, Cal-A and Cal-H had more ordered and compact form with uniform particle size distribution as shown by monomodal distribution. Also, the removal of organic compounds possibly resulted in the lower particle diameters due to more compactness of ca-hydroxyapatite. Therefore, the particle size distribution was greatly affected by the alkaline pre-treatment and calcination temperatures.

Table 11 Mean particle size and color values of biocalcium and calcined powders from salmon frame

Parameters	Bio-cal A	Bio-cal H	Cal-A (6 h)	Cal-H (6 h)	Cal-A (9 h)	Cal-H (9 h)
Mean particle size ($d_{43,}$ µm)	26.53 ± 3.49^{f}	24.05 ± 3.14^{d}	25.36 ± 2.78^{e}	22.21 ± 2.84^{a}	22.39 ± 2.64^{b}	23.12 ± 2.94^{c}
L*	95.29 ± 0.08^{f}	94.84 ± 0.08^{d}	93.61 ± 0.01^{c}	69.49 ± 0.04^{a}	95.01 ± 0.01^{e}	80.68 ± 0.01^{b}
a*	-0.60 ± 0.02^{c}	-0.31 ± 0.07^{d}	-1.59 ± 0.01^{b}	-0.23 ± 0.04^{e}	-2.63 ± 0.01^{a}	-0.36 ± 0.03^{d}
b*	7.13 ± 0.02^{d}	6.86 ± 0.14^{c}	-0.19 ± 0.02^{b}	-0.10 ± 0.07^{b}	-1.67 ± 0.01^{a}	-0.22 ± 0.01^{b}
ΔE*	6.94 ± 0.12^{d}	6.60 ± 0.08^{c}	0.87 ± 0.14^{a}	24.15 ± 0.15^{f}	3.00 ± 0.14^{b}	12.97 ± 0.09^{e}
ΔC*	7.15 ± 0.03^{f}	6.87 ± 0.14^{e}	1.60 ± 0.01^{c}	0.26 ± 0.06^{a}	3.11 ± 0.00^{d}	0.42 ± 0.01^{b}

Values are presented as mean ±SD (n=3). Different superscripts in the same row indicate significant difference ($P<0.05$).

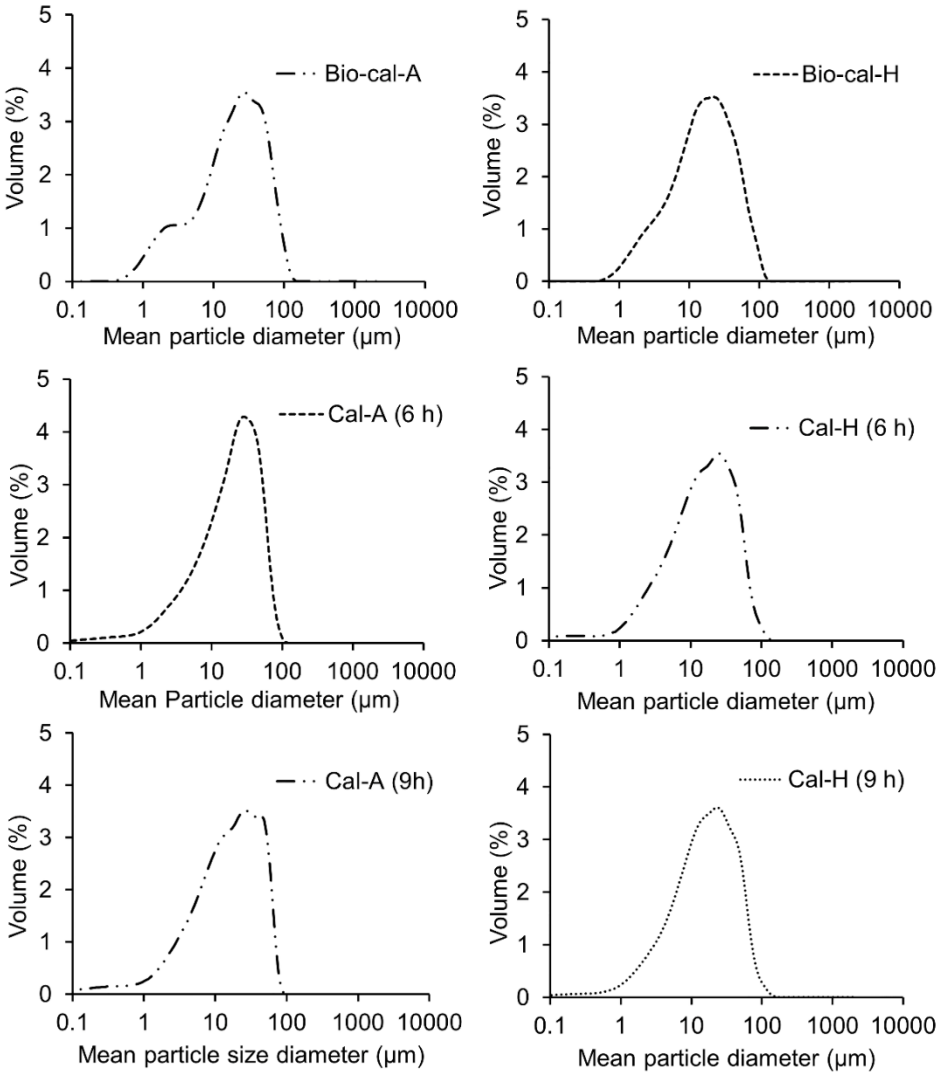

Figure 9 Particle size distribution of biocalcium and calcined powders from salmon bones. Bio-cal-A: biocalcium powder from alkaline treated salmon bone, Bio-cal-H: biocalcium powder from non-alkaline treated salmon bone, Cal-A (6 h): calcined bone powder obtained after 6 h of Bio-cal-A calcination, Cal-H (6 h): calcined bone powder obtained after 6 h of Bio-cal-H calcination, Cal-A (9 h): calcined bone powder obtained after 9 h of Bio-cal-A calcination, Cal-H (9 h): calcined bone powder obtained after 9 h of Bio-cal-H calcination.

3.4.7 Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (SEM-EDX)

Relative abundance of elements in all the samples were observed using SEM-EDX. Elements such as Ca and P along with other organic matters, including C and O were found in both biocalciums and calcined powders as depicted in Figure 10. It was noted that SEM-EDX has limitation (Choël *et al.*, 2005). Its detection limit, which varies from 1-10% (wt), resulted in the inability of other element present, particularly light elements in a compound to be measured. Consequently, elements with low content such as nitrogen could not be detected in the biocalciums. Fish bones usually contains collagen fibrils along with calcium phosphates, particularly hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ crystals. Alkaline treatment of fish bones led to an increase in Ca and P contents. This was in line with higher contents of Ca and P determined by ICP-OES (Table 8).

In all calcined samples, more pronounced and sharp peaks indicating higher intensity of Ca and P were observed. Simultaneously, the contents of C were decreased, re-affirming the removal of organic matters such as lipids and meat proteins as a result of calcination. Between the calcined samples, higher peaks were observed in Cal-A than in Cal-H when the same calcination time was used. This reconfirmed the impact of alkaline pre-treatment.

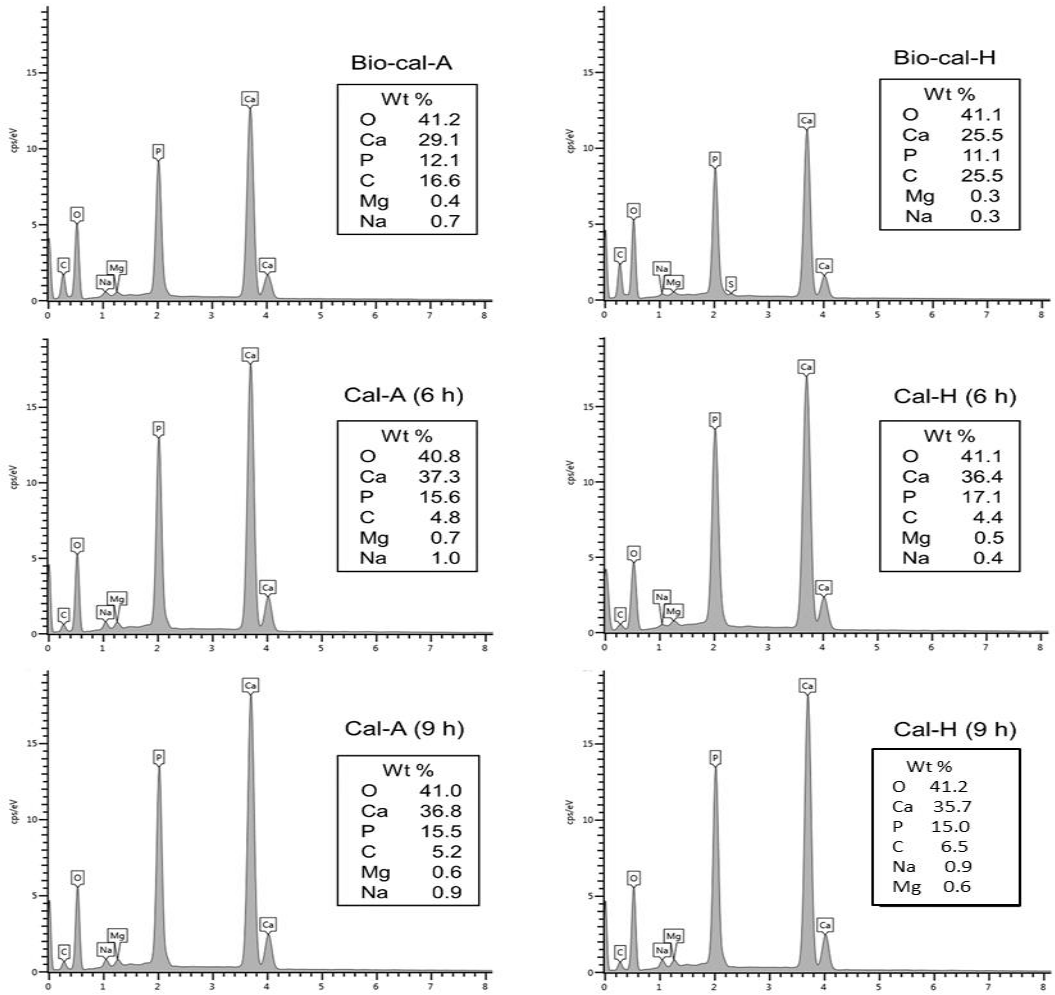

Figure 10 Elemental profile of biocalcium and calcined powders from salmon bones as analyzed by SEM-EDX. Bio-cal-A: biocalcium powder from alkaline treated salmon bone, Bio-cal-H: biocalcium powder from non-alkaline treated salmon bone, Cal-A (6 h): calcined bone powder obtained after 6 h of Bio-cal-A calcination, Cal-H (6 h): calcined bone powder obtained after 6 h of Bio-cal-H calcination, Cal-A (9 h): calcined bone powder obtained after 9 h of Bio-cal-A calcination, Cal-H (9 h): calcined bone powder obtained after 9 h of Bio-cal-H calcination.

3.4.8 X-ray Diffraction (XRD) patterns of powders

Diffraction patterns of both biocalciums and calcined powders from salmon bone showed that calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) phase (JCPDF:01-074-4172) was the dominant phase in all the samples (Figure 11). The crystallinity of the samples before calcination specifically Bio-cal-A and Bio-cal-H, were 54.56% and 49.18%, respectively. When calcination was implemented, the removal of organic matters and water with recrystallization occurred. Transformation of an initial bone matrix to a well-crystallized phases took place, leading to nucleation and growth of hydroxyapatite nanocrystals inside the powder particles under high temperature treatment (Pang and Bao 2003; Mobasherpour *et al.,* 2007). As a result, the diffraction peaks of calcined bones became more pronounced and their crystallinity increased from 54.56% to 89.85% and from 49.18% to 84.18% after 6 h of calcination for Cal-A and Cal-H, respectively. In addition, the crystallite sizes, calculated by using Scherrer's formula (Nasiri-Tabrizi *et al.,* 2014) of both calcine powders increased from about 15nm to 49nm. Under longer calcination time of 9 h, the crystallinity increased from 89.85 to 90.19% for Cal-A and from 84.18 to 87.72% for Cal-H while their crystallite sizes were similar to those of 6 h calcined samples. This corresponded with the increases in concentration of calcium and phosphorus as well as ash content shown in Table 8. It should be noted that the diffraction patterns of Cal-H powders showed a secondary phase of another apatite material in addition to the major phase of hydroxyapatite (Figure 11b). According to the peak profile fitting (JCPDF: 01-070-2064), this minor apatite phase could be identified as whitlockite ($Ca_{18}Mg_2(HPO_4)_2$-$(PO_4)_{12}$) compound. Bakry *et al.,* (2014) and Jang *et al.,* (2014) reported whitlockite as the second most abundant biomineral in hard tissues. Garavelli *et al.,* (1979) documented that annealing of fish bone could recrystallize to whitlockite in the abundance of an amorphous $Ca_3(PO_4)$-$_2$, which co-exist in an untreated bone tissue. In addition, Meinke *et al.,* (1979) reported that whitlockite could appear after fish bone pyrolysis when its Ca/P ratio was lower than 1.67. This was in good agreement to the Ca/P ratios shown in Table 8 where whitlockite phase was only observed in non-alkaline treated Cal-H powders which possessed Ca/P ratio of about 1.61-1.63. On the other hand, only a single phase of hydroxyapatite was observed in alkaline treated Cal-A samples with Ca/P of 1.66. Furthermore, Meinke *et al.,* (1979) also stated that the

presence of whitlockite in fish bone was related to the abundance of certain fluids in the spine, dermal and endochondral. It could be deduced that the presence of whitlockite was influenced by the presence of circulating fluid containing haem protein, etc in fish bone. Alkaline treatment could allow alkali to penetrate into the bone marrow and remove these fluids. Thus, the presence of whitlockite phases in Cal-A (6 h) and Cal-A (9 h) was not detected. Therefore, bone matrix composition as well as chemical and heat treatment conditions could impact on the crystallinity and phase composition of both biocalciums and calcined powders.

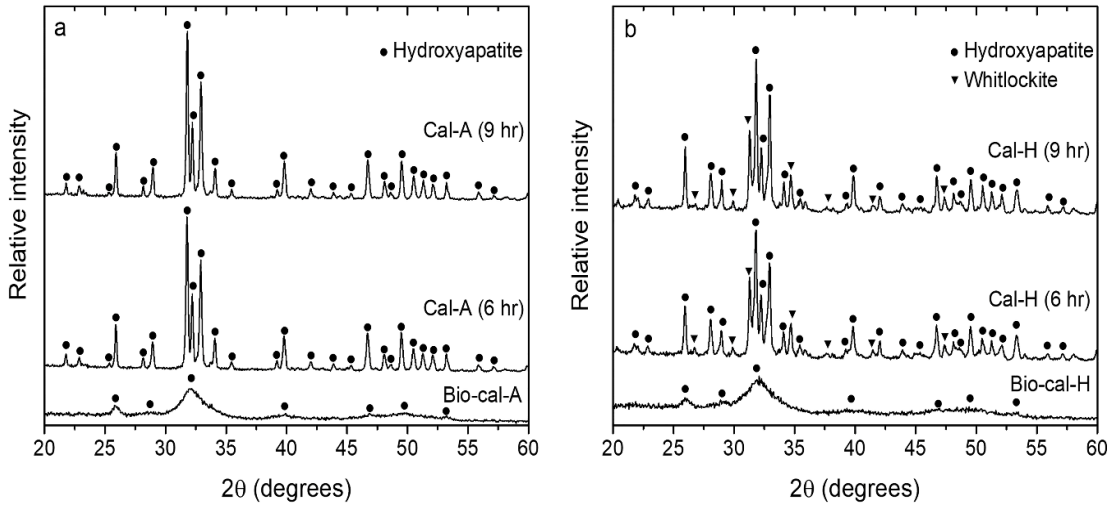

Figure 11 X-ray diffraction patterns of biocalcium and calcined bone powders from salmon bones Bio-cal-A: biocalcium powder from alkaline treated salmon bone, Bio-cal-H: biocalcium powder from non-alkaline treated salmon bone, Cal-A (6 h): calcined bone powder obtained after 6 h of Bio-cal-A calcination, Cal-H (6 h): calcined bone powder obtained after 6 h of Bio-cal-H calcination, Cal-A (9 h): calcined bone powder obtained after 9 h of Bio-cal-A calcination, Cal-H (9 h): calcined bone powder obtained after 9 h of Bio-cal-H calcination.

3.4.9 Bioavailability of biocalcium

3.4.9.1 *In-vitro* simulated gastrointestinal tract system

Ca-solubility of Bio-cal-H, Bio-cal-A and calcium carbonate were 7.65, 8.41 and 0.62%, respectively, using the gastrointestinal tract model (GIMs) (Table 12). The GIMs has been used to simulate the digestive system (Karnjanapratum and Benjakul 2015). It was observed that Ca-solubility in Bio-cal-A was higher than that of Bio-cal-H, whereas calcium carbonate showed the lowest solubility ($P<0.05$). After digestion, higher proportion of Bio-cal-A was still soluble than that of Bio-cal-H, thus preventing calcium from precipitating in the small intestine, where calcium is absorbed. Bicarbonate (HCO^-_3) is generally secreted into the intestine for neutralization of contents. Hence, the concentration of calcium available for absorption in the jejunum and ileum, which maintained an approximate pH of 7, is lower than that dissolved in the stomach (Goss *et al.,* 2010). The result suggested that calcium carbonate had very low solubility, while biocalcium showed higher solubility ($P<0.05$). The presence of peptides in biocalcium has been reported to act as an effective calcium carrier, thereby enhancing calcium absorption at small intestine (Malde *et al.,* 2010; Lu *et al.,* 2016). Higher calcium solubility of Bio-cal-A indicated that type of proteins retained in biocalcium played a role in solubility of Ca. The buffering capacity of protein toward the gastrointestinal contents could also be responsible for the solubility of calcium. Higher buffering effect of protein could allow more neutral chyme to withdraw from the stomach and stimulate less intestinal HCO^-_3 secretion, thus yielding less calcium precipitation (Benjakul *et al.,* 2017). This study confirmed the increased Ca-solubility of biocalcium containing peptides from salmon bone. Benjakul *et al.,* (2017) also documented that biocalciums from pre-cooked tongol (*Thunnus tonggol*) and yellowfin (*Thunnus albacores*) tuna bones had higher solubility than their calcined counterparts.

3.4.9.2 Transportation of calcium across Caco-2 monolayer

Bioavailability of calcium in Bio-cal-H, Bio-cal-A and $CaCO_3$ was 43.02, 38.18 and 21.48%, respectively, as indicated by the amount of calcium transported across the Caco-2 monolayer (Table 12). Bioavailability of Bio-cal-H was higher than Bio-cal-A and $CaCO_3$ ($P<0.05$). Pappenheimer and Reiss (1987) proposed that glucose and amino acids may induce substantial paracellular apical to basolateral transport and adsorptive transcytosis by solvent drag. Sodium-coupled active transport may furnish osmotic

force for convective paracellular flow. It was postulated that higher protein content (20.90%) of Bio-cal-H more likely possessed higher efficiency to induce paracellular transportation of calcium across the cell monolayer than Bio-cal-A (12.07% protein). This could lead to higher calcium bioavailability of Bio-cal-H than Bio-cal-A ($P<0.05$). Also, calcium with low content of peptides might have difficulty to pass through Caco-2 cells. This was due to physical limitation. Moreover, some peptides or proteins were degraded to nonactive fragments by brush border peptidases (Hidalgo *et al.,* 1989; Satake *et al.,* 2002). This could have contributed to the lower bioavailability of Bio-cal-A since it possessed lower peptides, compared to Bio-cal-H. Bioavailability of $CaCO_3$ was the lowest among the tested samples. Dispersal ability of $CaCO_3$ in Caco-2 monolayer was also reported (Hanzlik *et al.,* 2005). The human intestinal epithelial cell, Caco-2, is known to express a variety of small intestinal cell functions and has been used as a model of the small intestinal epithelium. (Hidalgo *et al.,* 1989; O'Callaghan and O'Brien 2010). Bioavailability of calcium from milk-based formulas and fruit juices containing milk and cereals was tested using Caco-2 cells (Perales *et al.,* 2005).

Bio-cal-A showed higher solubility, but it had less transportation across Caco-2 cell monolayer. There were several factors affecting the absorption, apart from the initial solubility. Absorption rate was not dependent on only solubility (Ekmekcioglu *et al.,* 1999; Perales *et al.,* 2005). Some factors have been highlighted to influence the differences in bioavailability of calcium. These include pH, lipophilicity, solubility of chemical compound, matrix composition of samples and the epithelial layer (Jovani *et al.,* 2001; Camara-Martos and Amaro-Lopez 2002; Perales *et al.,* 2005).

Table 12 Calcium solubility and bioavailability of biocalcium powders

Sample	% Ca-solubility	% Bioavailability*
Bio-cal-H	7.65 ± 0.08^{b}	43.02 ± 0.20^{c}
Bio-cal-A	8.41 ± 0.15^{c}	38.18 ± 0.10^{b}
$CaCO_3$	0.62 ± 0.13^{a}	21.48 ± 0.30^{a}

Bio-cal-H: biocalcium powder from non-alkaline treated salmon bone, Bio-cal-A: biocalcium powder from alkaline treated salmon bone.

*Bioavailability was determined by transportation of Ca across Caco-2 cell monolayer.

Values are presented as mean ±SD (n=3).

Different superscripts in the same column indicate significant differences ($P<0.05$).

3.5 Conclusion

Salmon frame, a residue from hydrolysate production, could be used as a starting material for the production of biocalcium or calcined powders. Biocalcium and calcined bones showed variation in chemical composition and crystallinity but possessed similar Ca/P ratio, which corresponded to hydroxyapatite as the dominant phase. Pre-treatment affected the chemical compositions such as amino acid composition and abundance of volatiles of biocalcium powders. Proteinaceous substances could assist calcium solubility as tested in GIMs and transportation through Caco-2 monolayer of biocalciums. Therefore, salmon frame could be used as a potential source of calcium for supplements.

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CHAPTER 4
CHARACTERISTICS AND NUTRITIONAL VALUE OF WHOLE WHEAT CRACKER FORTIFIED WITH BIOCALCIUM AND PROTEIN HYDROLYSATE POWDERS

4.1 Abstract

Whole wheat cracker fortified with biocalcium (BC) and protein hydrolysate (PH) powders obtained from salmon frame was developed as health promoting food. Fortification of BC and PH powders or their mixtures at different ratios (3:1, 1:1, 1:3) with total substitution of 16.67% based on whole wheat flour was carried out. Characteristics and nutritional value of resulting crackers were determined. Color, thickness, weight and textural properties of crackers varied with different ratios of BC and PH powders added. Incorporation of BC/PH (3:1) mixture showed no negative effect on sensory properties. Developed crackers possessed higher protein, fat, calcium, phosphorus, sodium and cholesterol but lower carbohydrate, sugar, fiber and energy value than the control. Crackers contained saturated fatty acid (0.31-0.38 mg/100g), monounsaturated fatty acid (0.083-0.16 mg/100g) and polyunsaturated fatty acid (0.026-0.045 mg/100g). Scanning electron microscopic images showed that the developed crackers were less porous and had a denser structure, compared to the control. Based on scanning electron microscopy-energy dispersive X-ray spectroscopic (SEM-EDX), the crackers fortified with BC/PH (3:1) mixture had higher calcium and phosphorus distribution with higher intensity, compared to the control.

4.2 Introduction

Baked foods including crackers account for about 22% of snack food in the market worldwide with estimated value of 66 billion USD in year 2003 (Han *et al.*, 2010). Snack foods mainly include corn chips, peanuts, potato chips, crackers, meat snacks and others. Although the market is relatively saturated, high demand for healthy food gives opportunities for snack foods with high nutrients to be developed. Crackers from whole wheat fortified with biocalcium (BC) from tuna bone (Benjakul and Karnjanapratum 2018), biscuits fortified with shrimp oil containing high polyunsaturated fatty acids and astaxanthin (Takeungwongtrakul and Benjakul 2017), sponge cake fortified with cabbage leaf powder rich in high fiber (Prokopov *et al.*, 2015), cookies from wheat flour blend enriched with protein from malted soybean

(Bashir *et al.,* 2015) and amaranth flour fortified wheat cookies (Sindhuja *et al.,* 2005) have been developed to serve for growing market of health promoting foods.

Leftovers from food processing industries such as egg shell and fish bones have been used to develop new products such as BC (Hassan 2015; Benjakul *et al.,* 2017). BC powder from egg shell of chicken was used as calcium supplement to nourish biscuits (Hassan 2015). Also, BC from tuna bone was used to fortify whole wheat crackers (Benjakul and Karnjanapratum 2018). It was discovered that BC obtained from fish processing wastes such as bones possessed high amount of phosphopeptides associated with high amounts of soluble Ca salts in gastrointestinal tract, thus increasing the intake of calcium (Jung and Kim 2007). This claim was in line with the study of Malde *et al.,* (2010), in which proteases treated salmon bone was used to feed piglets and it showed higher absorption than piglets fed with calcium carbonate. BC obtained from salmon frame showed high amount of minerals such as calcium and phosphorus. Thus, BC from fish processing waste such as salmon frame can be used to nourish foods, especially crackers. The protein hydrolysate (PH) from two forms of salmon frame named 'mince' and 'chunk' using proteases (alcalase and papain). The PH from the chunk using alcalase showed higher nutritive value, compared to its mince counterpart as indicated by higher amount of amino acids. PH could therefore be used as a supplement for amino acid deficient baked foods.

Demand for healthy foods including snacks rich in nutrients, particularly functional ingredients, is increasing. Among the health promoting foods, whole wheat food products are rich in fibre. Fibres possess a physiological effect on transit time and fecal bulk. Consumption of whole wheat products has been endorsed to be beneficial to health (Campbell *et al.,* 1991). Crackers made from whole wheat flour are usually crunchy and hard in texture. Nevertheless, they are lacking of some essential amino acids as well as calcium. Addition of BC and PH powders obtained from salmon frame could be a promising means to increase nutritive values of crackers. Nevertheless, the amount and ratios of BC and PH powders incorporated into crackers could affect their acceptability to consumers as well as their quality. This study was aimed to develop whole wheat cracker fortified with BC and PH powders from salmon frame at various substitution levels. Physical, textural, sensory and nutritional profiles of the crackers produced were examined and compared with the control.

4.3 Materials and methods

4.3.1 Materials

All ingredients such as unsalted butter (Orchid, Indofood Sukses Makmur Tbk, Jawa Tengah, Indonesia), peanut butter (Jif, J.M. Smucker Company, Ohio, USA), commercial whole wheat flour (Imperial, KCG Corporation Co., Ltd, Bangkok, Thailand), salt, baking powder and sugar were procured from a local market in Hat Yai, Songkhla, Thailand.

4.3.2 Preparation of protein hydrolysate (PH) powder

Frames of salmon (*Salmo salar*) (30-35 cm in length) were obtained from Kingfisher holdings Ltd Songkhla, Thailand. The frames were cut into 4-5 cm using an electric sawing machine. Prepared frames (chunks) were subjected to hydrolysis using proteases (alcalase and papain) as detailed by Idowu et al. (2018). After hydrolysis, the mixture was centrifuged at 4000xg for 15 min. Supernatant was freeze-dried using a freeze dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) and the PH powder was used for preparation of crackers.

4.3.3 Preparation of biocalcium (BC) powder

Fish bone residues obtained after hydrolysis were used as a starting raw material for production of BC powder. Bones were subjected to alkaline treatment (2 M NaOH, 50°C, 30 min). Bones were washed thoroughly as tailored by Benjakul *et al.*, (2017). Thereafter, bones were treated with hexane for defatting, sodium hypochlorite and hydrogen peroxide for bleaching. The samples were dried in a rotary tray dryer (temp-50°C, time-5 h) and ground into powder with the aid of a Ball Mill (PM 100, 127 Retsch GmbH, Haan, Germany). The BC powder obtained was sieved using a sieve shaker (Vibratory Sieve Shaker analysette3Pro, FRITSCHGmbH, Deutschland, Germany) to obtain particle size with less than 75µm. BC powder was used for fortification into crackers.

4.3.4 Study on the impact of BC and PH powders on characteristics and properties of whole wheat crackers

4.3.4.1 Preparation of whole wheat crackers

Traditional technology and formulation were used for the preparation of whole wheat crackers following the method of Benjakul and Karnjanapratum (2018) with a slight modification. Original dough formulation was used for production of whole wheat crackers based on dough weight: 1.4% salt, 2.1% baking powder, 2.6 % sugar, 6.0% peanut butter, 13.8% butter, 14.1% water and 60% whole wheat flour. For other formulations, wheat flour was substituted with BC, PH or their mixtures at a level of 16.67% of whole wheat flours (w/w) as shown in Table 13. Mixing of all ingredients was initiated in a dough mixer (KitchenAid casserole multifunctional 5k, KitchenAid, Benton Harbor, MC, USA) and mixing was performed for 3 min. The crackers dough was sheeted to a thickness of 0.3 mm with rectangular shape (2.4×7.0 cm^2). The shaped cracker dough was baked in an electric oven (Mamaru MR-1214, Mamaru Co., Ltd., Bangkok, Thailand) at 140°C for 40 min. Whole wheat crackers were allowed to cool after baking at room temperature for 1 h. Thereafter, they were subjected to analyses.

4.3.4.2 Analyses

4.3.4.2.1 Physical and textural properties

Physical parameters such as weight, length and thickness of the crackers were determined as stated by Saha *et al.*, (2011). Cutting force and fracturability of crackers were determined with the aid of texture analyzer (Stable Micro Systems, Godalming, Surrey, UK) as described by Benjakul and Karnjanapratum (2018). The color of samples were determined using a colorimeter (ColorFlex, Hunter Lab Reston, VA, USA) as tailored by Takeungwongtrakul *et al.*, (2015).

4.3.4.2.2 Water activity and moisture content

Water activity (aw) was measured using a water activity meter (4TEV, Aqualab, Pullman, WA, USA). Moisture content was determined (AOAC, 2000).

4.3.4.2.3 Sensory evaluation

Sensory evaluation was performed by 50 untrained panelists. They assessed the whole wheat crackers for appearance, color, odor, texture, taste, flavor and overall likeness using a nine-point hedonic scale (Benjakul and Karnjanapratum 2018). The samples were labelled with random three-digit codes. Panelists were instructed to rinse

their mouth with water after each sample evaluation and the order of presentation of samples was randomized (Carr *et al.*, 2001; Benjakul and Karnjanapratum 2018).

4.3.5 Characterization of the selected whole wheat crackers fortified with BC and PH powders

Crackers fortified with BC/PH (3:1) mixture at 16.67% substitution wheat flour were prepared as described previously. Control crackers were also prepared using typical formulation (without BC and PH powders incorporated). Both samples were subjected to analyses.

4.3.5.1 Chemical composition, energy value and mineral profile

Crackers were analyzed for protein, fat, ash, cholesterol, dietary fibre and total carbohydrate contents (AOAC, 2002). Inductively coupled plasma optical emission spectrometer (ICP-OES) (Model Optima 4300 DV, Perkin Elmer, Shelton, MA) was used for determination of Ca and P in crackers as per the method of Feist and Mikula (2014). For determination of fatty acid profile in the samples, lipids were firstly extracted from samples according to the method of Bligh and Dyer (1959). Fatty acids were determined as detailed by Muhammed *et al.*, (2015).

4.3.5.2 Scanning Electron Microscope (SEM)

Microstructure of crackers were visualized using a scanning electron microscopy (SEM) as described by Benjakul and Karnjanapratum (2018)

4.3.5.3 Scanning electron microscopy with energy dispersive x-ray spectroscopy (SEM-EDX)

Scanning electron microscopy with energy dispersive X-ray spectroscopy was used as detailed by Lin *et al.*, (2011). A field emission scanning electron microscope (FEI-XL30, FEI Company, Hillsboro, OR, USA) equipped with electron-dispersive X-ray spectroscope (EDX) was used. The samples were gold coated and observed with secondary electron mode at a 20kV accelerating voltage. Elemental analysis was conducted on the surface and cross section of the cracker by EDX to verify the distribution and quantity of the elements.

4.3.6 Experimental design and Statistical analysis

Experiments were run in triplicate with completely randomized design (CRD) for characteristics and textural properties of the crackers. T-test was used for the chemical composition and energy value of crackers. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by the Duncan's Multiple Range Test. Analysis was performed using the SPSS package (SPSS for windows, Inc., Chicago, IL, USA).

Table 13 Ingredients and formulation of whole wheat crackers fortified with BC, PH powders or their mixture at different ratios

Ingredients / **Formulation**	**Salt (g)**	**Baking powder (g)**	**Sugar (g)**	**Peanut butter (g)**	**Butter (g)**	**Water (g)**	**Whole wheat flour (g)**	**BC (g)**	**PH (g)**
BC	1.4	2.1	2.6	6.0	13.8	14.1	50.0	10	-
BC/PH (3:1)	1.4	2.1	2.6	6.0	13.8	14.1	50.0	7.5	2.5
BC/PH (1:1)	1.4	2.1	2.6	6.0	13.8	14.1	50.0	5.0	5.0
BC/PH (1:3)	1.4	2.1	2.6	6.0	13.8	14.1	50.0	2.5	7.5
PH	1.4	2.1	2.6	6.0	13.8	14.1	50.0	-	10
Control	1.4	2.1	2.6	6.0	13.8	14.1	60.0	-	-

*Substitution level was 16.67% of 60 g whole wheat flour.

4.3.7 Results and discussion

4.3.7.1 Characteristics and textural properties of whole wheat crackers fortified with BC and PH powders at different ratios

4.3.7.1.1 Weight and dimensions

Physical parameters such as weight, width, length and thickness of whole wheat crackers fortified with BC and PH powders from salmon frame at various ratios are shown in Table 14. Similar weights (4.05-4.36 g) were observed for all the whole wheat crackers ($P>0.05$). No difference in weight between the control and the crackers incorporated with BC and PH powders was obtained, regardless of BC/PH ratios ($P>0.05$). The incorporation of both powders (BC and PH) affected thickness differently, in which the addition of BC/PH (1:3) yielded the highest thickness ($P<0.05$). However, thickness of all crackers added with BC, PH and their mixtures were lower than that of the control ($P<0.05$). No difference existed between those added with BC or PH alone ($P>0.05$). In terms of length, incorporation of both powders led to a decrease in length ($P<0.05$), except for the sample added with BC/PH (1:3) mixture, which showed similar length to that of control ($P>0.05$). Widths of crackers fortified with BC, PH or the mixtures were lower than that of control ($P<0.05$). The substitution of wheat flour with both powders more likely led to the reduction in the amount of amylose and amylopectin, which are the major ingredients being puffed. Benjakul and Karnjanapratum (2018) reported that BC powder could be deposited within the starch gel matrix and possibly interfered the gel network to be puffed during baking. Thus, the air cell generated could not be trapped and held properly in the puffed crisp crackers. This resulted in the less raising. Similar finding was reported by Hassan (2015) for calcium enriched biscuit, in which chicken egg shell powder was incorporated. It was observed that the egg shell powder added to the formulation inhibited the formation of air cell in the puffed crisp biscuit. In this study, the incorporation of BC powder at increasing levels more likely resulted in the decrease in length and width of the resulting crackers. Similarly, PH powder also showed the interfering effect on the starch or wheat proteins, in which strong network could not be formed. PH powder was composed of small peptides obtained after cleavage of polypeptides by enzymatic hydrolysis. PH possessed high solubility, however it might have low ability absorbing at oil/water interface (Intarasirisawat *et al.*, 2012), in which lipid was added as ingredients in

cracker. Therefore, their contribution to the dough, particularly binding with lipids added in whole wheat crackers, was limited. BC and PH powders exhibited the combined effect on weakening wheat flour dough due to their interference in the normal sulphydryl/disulphide interchange reactions during the wheat flour dough development. Overall, both BC and PH powders affected anatomical parameters or dimension of whole wheat crackers.

4.3.7.1.2 Color

Surface colors of whole wheat crackers are shown in Table 14. Crackers fortified with BC powder possessed higher L* values than others ($P<0.05$). However, L* decreased with incorporation of mixed powders, especially with increasing levels of PH powder. Colored pigments in PH as well as caramelization of sugars upon baking mostly caused the decrease in lightness. PH was rich in free amino groups and the increase in levels of PH led to higher degree of non-enzymatic browning so called Maillard reaction. Similar observation was reported by Gani *et al.,* (2014) when whey and casein PH were incorporated into cookies. No difference in lightness was observed when BC/PH mixtures and PH powders were incorporated ($P>0.05$). Similar trend was found in a*(redness). For b* (yellowness), cracker added with BC powder had slightly higher value than that incorporated with BC/PH mixtures (3:1) ($P<0.05$). Decrease in b* (yellowness) values was observed with incorporation of BC powder. When PH powder was added, particularly at higher level, b* value was increased. Varying ΔE^* values were observed at different ratios of both powders. No difference in ΔC^* value between control, cracker added with BC/PH mixture (1:1) and BC powder ($P>0.05$). Overall, incorporation of BC and PH powders affected the color of cracker samples, depending on the ratios of BC/PH powders.

4.3.7.1.3 Water activity

The water activity and moisture content of whole wheat crackers are shown in Table 14. Water activity decreased with varying ratios of both powders. Increasing levels of BC powder could lower water activity ($P<0.05$). However, addition of PH powder had no impact on water activity of cracker. PH powder was hygroscopic in nature, which BC powder was dried and mainly contained inorganic matters. This result correlated well with the moisture content of the crackers. Similar observation was reported by Benjakul and Karnjanapratum (2018) when BC powder from precooked

tuna bone was added into whole wheat crackers. Moisture content has the impact on mouthfeel, while water activity has been identified as an index of shelf-life and storage stability of crackers (Cervenka *et al.*, 2006; O'brien 2008).

4.3.7.1.4 Cutting force and fracturability

Cutting force and fracturability are important textural properties of whole wheat crackers fortified with BC and PH powders obtained from salmon frame (Table 14). When molar teeth compress a food, the force applied is regarded to as cutting force. The ability to break food into pieces when it is bitten by incisors is regarded to as fracturability (Paula and Conti-Silva 2014). An increase in cutting force with coincidental decrease in fracturability of whole wheat crackers was observed as BC powder was added ($P<0.05$). However, the incorporation of PH powder slightly lowered cutting force, but slightly increased fracturability ($P<0.05$). The sample showed the highest cutting force but lowest fracturability of 20.62 N and 1.34 mm ($P<0.05$), respectively, when BC powder was incorporated. Benjakul and Karnjanapratum (2018) reported the effect of BC powder from precooked tuna bone when BC powder was incorporated in crackers at varying levels, whereby crackers obtained became more brittle and harder in texture with increasing substitution levels of BC powder. Therefore, crackers obtained became more compact in structure by filling of void or gap in cracker crumb by BC powder, particularly at higher level. Low amount of water used for the formulation led to the lower degree of gelatinization/swelling of amylose and amylopectin, which affected the formation of gel structure (Tako *et al.*, 2014). As a result, the BC powder might impede the formation of gel network and interfere the air bubble incorporation. This was evidenced by increased cutting force when BC powder was added. For whole wheat crackers incorporated with PH powder, cutting force of 10.89 N was obtained. It could be as a result of the weakening of the wheat flour dough due to interference by short chain peptides, which might impede sulphydryl/disulphide interchange reactions or disulphide bond formation occurred during wheat flour dough development. Increase in fracturability with PH powder addition than BC powder addition might be attributed to increased number of hydrophilic sites generated during hydrolysis, which were available to compete for the limited free water in dough. In general, fortification of BC and PH powders affected the cutting force and fracturability of crackers.

Table 14 Characteristics and textural properties of whole wheat crackers fortified with BC, PH powders or their mixture at different ratios

Parameters	Control	BC	BC/PH (3:1)	BC/PH (1:1)	BC/PH (1:3)	PH
L*	77.38±0.73^{d}	84.24±0.90^{e}	62.28±0.96^{c}	59.43±0.28^{b}	56.50±0.75^{a}	55.48±0.36^{a}
a*	8.32±0.75^{c}	4.98±0.24^{a}	4.77±0.50^{a}	6.89±0.46^{b}	11.28±0.37^{d}	10.63±0.05^{d}
b*	32.36±0.45^{cd}	29.82±0.87^{b}	25.91±0.47^{a}	31.60±0.83^{c}	33.08±0.84^{d}	32.86±0.91^{cd}
ΔE*	37.24±0.96^{b}	31.55±0.48^{a}	38.90±0.81^{c}	49.22±0.50^{e}	51.32±0.31^{f}	43.30±0.95^{d}
ΔC*	32.19±0.55^{c}	29.37±0.99^{b}	22.49±0.39^{a}	31.12±0.83^{c}	33.42±0.82^{d}	32.06±0.10^{c}
Water activity	0.29±0.00^{e}	0.21±0.00^{a}	0.24±0.00^{b}	0.26±0.00^{d}	0.27±0.00^{d}	0.25±0.01^{c}
Weight (g)	4.36±0.31^{a}	4.33±0.16^{a}	4.30±0.06^{a}	4.26±0.05^{a}	4.20±0.03^{a}	4.05±0.12^{a}
Moisture (%)	2.88±0.05^{f}	2.17±0.03^{a}	2.44±0.02^{b}	2.66±0.10^{d}	2.73±0.09^{e}	2.50±0.10^{c}
Length (cm)	7.30±0.07^{c}	7.13±0.01^{a}	7.15±0.03^{ab}	7.19±0.01^{ab}	7.23±0.03^{bc}	7.11±0.01^{a}
Thickness (mm)	0.41±0.31^{e}	0.31±0.14^{a}	0.33±0.25^{b}	0.35±0.17^{c}	038±0.11^{d}	0.30±0.01^{a}
Width (cm)	2.81±0.05^{c}	2.52±0.01^{a}	2.55±0.01^{ab}	2.62±0.05^{ab}	2.67±0.08^{b}	2.50±0.06^{a}
Cutting Force (N)	11.62±0.24^{b}	20.62±0.43^{f}	18.29±0.32^{e}	15.48±0.26^{d}	13.49±0.44^{c}	10.89±0.50^{a}
Fracturability (mm)	2.37±0.08^{e}	1.34±0.04^{a}	1.61±0.04^{b}	1.88±0.09^{c}	2.18±0.06^{d}	2.55±0.02^{f}

*Substitution level was 16.67% of whole wheat flour. Different superscripts in the same row indicate significant difference ($P<0.05$).

4.3.7.1.5 Sensory properties

Addition of BC and PH powders into whole wheat crackers at different levels affected likeness scores differently (Table 15). It was observed that incorporation of both powders particularly with increasing PH levels resulted in the decrease in all attributes tested including appearance, color, odor, texture, taste, flavor and overall acceptability of crackers. Generally, the lower levels of PH powder resulted in the higher likeness score, compared with higher levels ($P<0.05$). The highest level of PH powder incorporated resulted in the lowest likeness score for all the attributes. This could be as a result of bitterness of the hydrolysate. Idowu et al. (2018) reported the bitterness of hydrolysate obtained from salmon frame. Bitterness of hydrolysate occurred as a result of formation of peptides containing bulky hydrophobic groups towards their C-terminal. Peptides containing hydrophobic groups such as isoleucine, valine, tryptophan, phenylalanine, leucine and tyrosine at C-terminal contribute to the bitterness (Yarnpakdee *et al.,* 2014). Thus, the bitterness could be detected by the panelists, leading to the decreased likeness score of crackers fortified with PH powder. When BC powder was added, there were no effect on taste, appearance, color, odor and overall likeness ($P>0.05$), compared to the control. However, flavor and texture likeness scores were decreased when BC powder was added. Due to the similar sensory property between the control and that added with BC/PH powders (3:1), cracker was fortified with the mixture of BC and PH (3:1) and used for further study.

Table 15 Sensory properties of whole wheat crackers fortified with BC, PH powders or their mixture at different ratios

Attributes	Samples					
	Control	BC	BC/PH (3:1)	BC/PH (1:1)	BC/PH (1:3)	PH
Appearance	7.88 ± 0.72^{d}	7.85 ± 0.69^{d}	7.81 ± 0.52^{d}	6.92 ± 0.66^{c}	6.44 ± 0.48^{b}	5.98 ± 0.60^{a}
Color	7.55 ± 0.81^{d}	7.47 ± 0.76^{d}	7.42 ± 0.81^{d}	6.47 ± 0.79^{c}	6.21 ± 0.85^{b}	5.88 ± 0.77^{a}
Odor	7.27 ± 1.01^{e}	7.22 ± 0.99^{de}	7.18 ± 1.00^{d}	6.76 ± 1.03^{c}	6.26 ± 0.84^{b}	5.90 ± 0.82^{a}
Texture	7.44 ± 0.78^{e}	7.33 ± 0.84^{d}	7.35 ± 0.75^{d}	7.20 ± 0.74^{c}	7.03 ± 0.69^{b}	6.68 ± 0.60^{a}
Taste	7.71 ± 1.00^{d}	7.62 ± 1.01^{d}	7.60 ± 0.94^{d}	6.88 ± 0.90^{c}	5.82 ± 0.88^{b}	5.24 ± 0.80^{a}
Flavor	7.81 ± 0.81^{e}	7.71 ± 0.77^{d}	7.70 ± 0.84^{d}	6.81 ± 0.88^{c}	6.16 ± 0.80^{b}	5.86 ± 0.78^{a}
Overall acceptability/ Likeness	7.52 ± 0.91^{d}	7.46 ± 0.94^{d}	7.44 ± 0.89^{d}	6.86 ± 0.95^{c}	6.15 ± 0.88^{b}	5.87 ± 0.82^{a}

*Substitution level was 16.67% of whole wheat flour. Different superscripts in the same row indicate significant difference ($P<0.05$).

4.3.7.2 Chemical composition and nutritional value of whole wheat crackers fortified with BC/PH (3:1) mixture

4.3.7.2.1 Chemical compositions and energy values

The chemical compositions of whole wheat cracker fortified with BC/PH (3:1) mixture and the control cracker (without BC and PH powders) are shown in Table 4. Carbohydrate (66.67 g/100g), cholesterol (35.26 mg/100g), total fat (15.39 g/100g) and protein (11.88 g/100g) were the main constituents in the control. Whole wheat crackers fortified with BC and PH powder consisted of lower carbohydrate (61.00 g/100g) but higher cholesterol (36.93 g/100g), higher total fat (16.54 g/100g) and higher protein (12.24 g/100g). In addition, total sugar (6.24 g/100g) and total fibre (2.04 g/100g) in the control were higher than total sugar (5.18 g/100g) and total fiber (1.68 g/100g) in BC/PH fortified sample. Fortification of whole wheat cracker with BC and PH powders therefore had the impact on compositions, especially carbohydrate in dough as well as resulting cracker. This was indicated by the increases in protein, fat, ash and cholesterol. Calcium and phosphorus were increased by 17- and 8- fold, respectively, compared to those detected in the control. This correlated well with higher ash content of BC/PH fortified sample, compared to the control (Table 3). A slight increase in sodium (Na) was noticeable in BC/PH fortified sample ($P<0.05$). Generally, BC and PH powders were the rich sources of protein and minerals, respectively (Idowu et al. 2018; Benjakul et al. 2017). Thus, the marked increase in components such as proteins, Ca, P and Na contents were attained. The energy value of whole wheat cracker were calculated using the Atwater factor of 4, 9 and 4 kcal/g for protein, fat and carbohydrate, respectively (Prokopov *et al.*, 2015). The energy values BC/PH fortified sample was lower than that of the control ($P<0.05$). The incorporation of BC and PH powders in the formulation could dilute the carbohydrate. Thus, the energy became less, compared to the control. The results suggested that the addition of BC and PH powders in whole wheat cracker affected the chemical composition and lowered energy values of crackers.

Table 16 Chemical composition and energy value of whole wheat cracker and cracker fortified with BC/PH (3:1) mixture

Composition/energy value	Samples	
	Control	BC/PH fortified cracker
Protein (g/100g)	11.88[a]	12.24[b]
Total fat (g/100g)	15.39[a]	16.54[b]
Total carbohydrate (g/100g)	66.70[b]	61.00[a]
Total sugar (g/100g)	6.24[b]	5.18[a]
Total fibre (g/100g)	2.04[b]	1.68[a]
Ash (g/100g)	3.99[a]	8.54[b]
Calcium (g/100g)	0.12[a]	2.04[b]
Phosphorus (g/100g)	0.35[a]	1.00[b]
Sodium (g/100g)	1.05[a]	1.13[b]
Cholesterol (mg/100g)	35.26[a]	36.93[b]
Energy value (kcal/100g)	452.83	441.82

Different lowercase superscripts in the same row indicate significant difference ($P < 0.05$). [++] The conversion factor is 6.25.

4.3.7.2.2 Fatty acid composition

Fatty acid compositions of the control cracker and BC/PH fortified sample are shown in Table 17 and expressed in mg/100g total fatty acid. Saturated fatty acid (0.31-0.38 mg/100g) was observed as the dominant fatty acids in both samples. Saturated fatty acids (SFA) in the samples included butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0), undecanoic (C11:0), tridecanoic (C13:0) and heneicosanoic acid (C21: 0) in varying proportions. Capric, butyric, caproic and caprylic acids were dominant, while tridecanoic acid was found only in BC/PH fortified sample. Hu *et al.*, (1999) suggested that saturated fatty acids with chain length of C12:0-C16:0 are able to accelerate atherogenesis. Tricosanoic acid was found in both samples. For monounsaturated fatty acids, myristoleic, palmitoleic and cis-11-eicosenoic could not be detected in the control but was observed in varying amount in the BC/PH fortified sample. Oleic acid was the dominant monounsaturated fatty acid found in both samples. Natural occurring vegetable oils are rich in unsaturated fatty acids which contain only non-conjugated double bonds in the *cis* configuration. Since unsalted margarine and peanut butter were used as ingredients in crackers, unsaturated components could be isomerized to the *trans* form during baking or even extraction process as a result of oxidation, conversion during heating and by partial hydrogenation (Perez-Serradilla *et al.*, 2007). This could result in the formation of some t*rans* fatty acid observed in the both samples. Compared with the *cis* unsaturated fatty acids, the structure, physical properties, chemical stability and physiological effects (atherogenic effects) of *trans* fatty acids resemble those of the saturated fatty acids (Mensink and Katan 1990). Polyunsaturated fatty acid (PUFA) possessed biological and nutritional importance (Kandhro *et al.*, 2008). PUFA of both samples ranged from 0.026 to 0.045 (mg/100g). Both EPA and DHA were found in both samples, however slightly higher contents were obtained in BC/PH fortified samples. Similarly, the result corresponded with the higher fat and cholesterol observed in fortified cracker than the control as shown in Table 4. Thus, fortification of both BC and PH powders influenced the fatty acid profile of the resulting crackers.

Table 17 Fatty acid composition of whole wheat cracker and cracker fortified with BC/PH (3:1) mixture

Fatty acid (mg/100g)	Control	BC/PH fortified cracker
C4:0 (Butyric)	0.066	0.082
C6:0 (Caproic)	0.062	0.072
C8:0 (Caprylic)	0.045	0.052
C10:0 (Capric)	0.096	0.11
C11:0 (Undecanoic)	0.013	0.015
C13:0 (Tridecanoic)	ND	0.0072
C14:1 (Myristoleic)	ND	0.042
C16:1 (Palmitoleic)	ND	0.010
C17:1 cis 10 (Cis-10-Heptadecanoic)	0.0065	0.013
C18:1 cis 9 (Oleic)	0.077	0.091
C18:2 trans 9,12 (Linolaidic)	0.0074	0.0078
C18:2 cis 9,12 (Linoleic)	ND	0.012
C20:1 cis 11 (Cis-11-Eicosenoic)	ND	0.0083
(C21:0) Heneicosanoic	0.027	0.032
C20:3 cis 8,11,14 (Cis-8,11,14-Eicosatrienoic)	0.0061	0.0065
Cis-11, 14-Eicosadienoic	ND	0.0050
C23:0 (Tricosanoic)	0.0069	0.0072
C22:2 cis 13,16 (cis-13,16-Docosadienoic)	0.0058	0.0065
C20:5 cis 5,8,11,14,17 EPA (Cis-5,8,11,14,17-Eicosatrienoic)	0.0066	0.0069
Saturated fatty acid (SFA)	0.31	0.38
Monounsaturated fatty acid (MUFA)	0.083	0.16
Polyunsaturated fatty acid (PUFA)	0.026	0.045

ND-Not Detected

4.3.7.2.3 SEM images of whole wheat cracker

Scanning electron microscopic images of the surface and cross-sectional area of control and BC/PH fortified whole wheat crackers are shown in Figure 13. The structure of fortified sample showed a remarkable difference, compared with the control. The surface of the control (Figure 13a) showed a rough crumb, open structure with porous matrix with more gaps and air cells, compared with that of the BC/PH fortified sample (Figure 13b). The result correlated well with that of cross section of the control (Figure 13c) and the fortified counterparts (Figure 13d). Generally, volume of air could be entrapped and retained in the dough matrix of cracker, which resulted in the expansion with porous and open structure during baking. However, incorporated powders, particularly BC powder, filled the voids or air cells in the crumb of the cracker, hence yielding the dense structure. In addition, the dispersal of BC and PH powders throughout the dough matrix plausibly interrupted the aeration property of cracker dough during baking. Thus, physical and textural characteristics of crackers were determined by the internal structure.

4.3.7.2.4 SEM-EDX spectroscopy

Element distribution and their contents in the control (Figure 14 (a, b)) and BC/PH fortified whole wheat crackers (Figure 14 (c, d)) were evaluated by SEM-EDX. Based on element distribution and mapping, the samples were composed of carbon and oxygen as the most dominant elements in both samples. The higher amount with the higher intensity of inorganic elements, including Ca and P was observed in BC/PH fortified sample than that found in the control. Calcium was only identified in SEM-EDX image of fortified sample, which was in accordance with higher ash content (Table 16). SEM-EDX analytical technique has been used for qualitative measurement of elements in food products (Parween *et al.,* 2016). It was noted that SEM-EDX has limitation (Choël *et al.,* 2005). Its detection limit, which varies from 1-10% (wt), resulted in the inability of other element present in a compound to be measured. Consequently, elements with low content such as nitrogen could not be detected in the crackers. Overall, the results indicated that fortification of cracker with BC and PH powders affected the composition of crackers, especially elements.

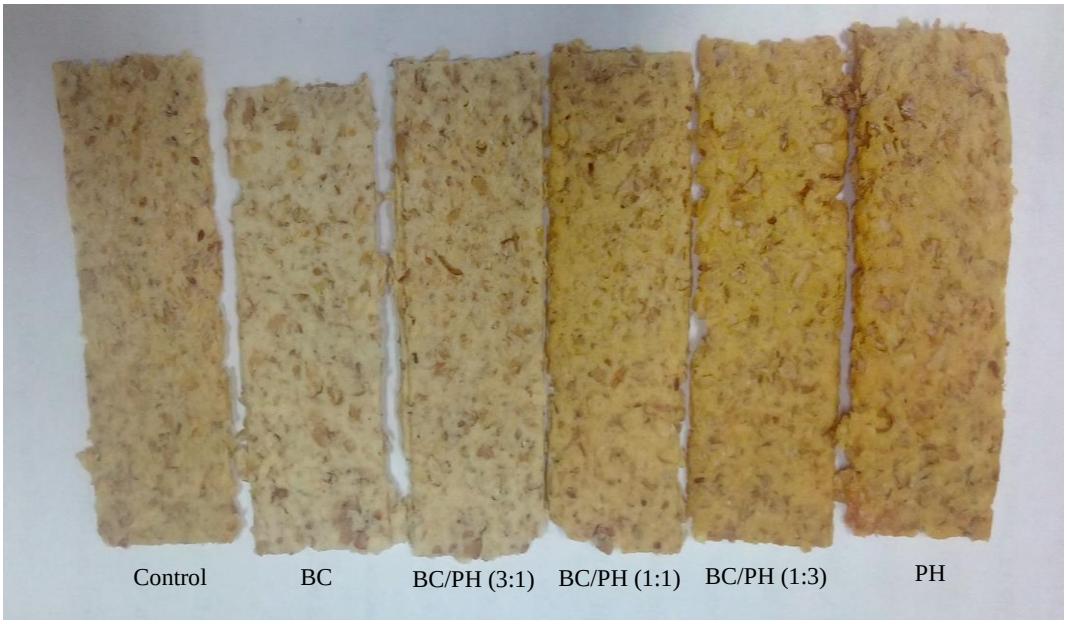

Figure 12 The photographs of whole wheat crackers fortified with BC, PH powders from salmon frame or their mixture at different substitution ratios. Powders were substituted at 16.67% of wheat flour.

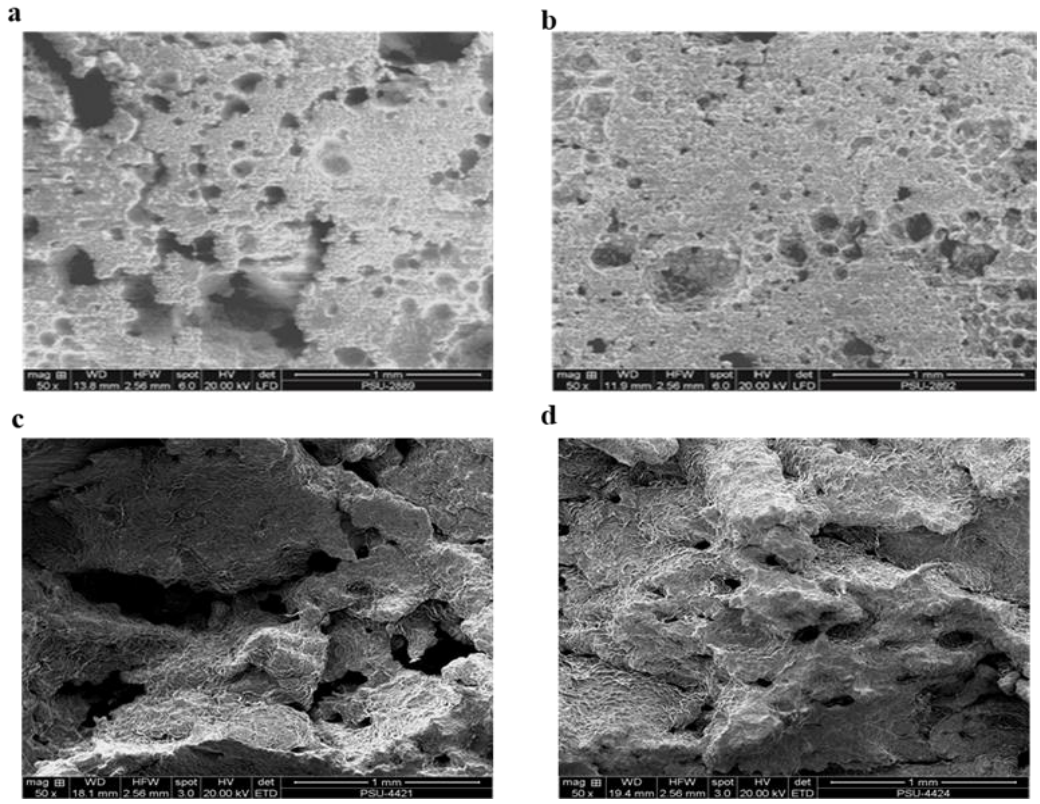

Figure 13 Scanning electron microscopic photographs of surface (a and b) and cross-section (c and d) of whole wheat cracker and BC/PH (3:1) fortified crackers. a and b: 50 x magnification, c and d: 50 x magnification.

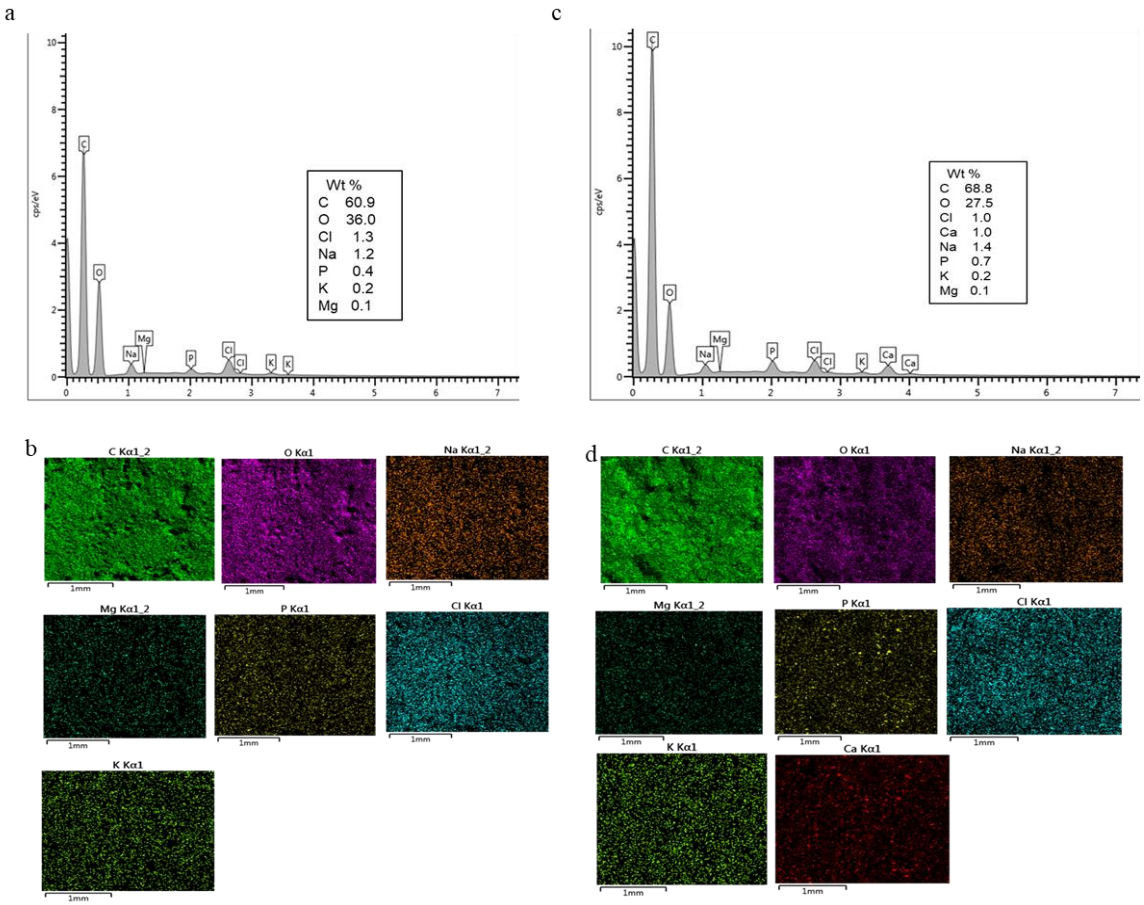

Figure 14 Elemental profile (a and c) and mappings of different elements (b and d) on cross-section of whole wheat cracker and BC/PH (3:1) fortified cracker as analyzed by SEM-EDX.

4.3.8 Conclusion

Incorporation of BC and PH powders obtained from salmon frames into whole wheat cracker increased Ca, P and protein contents. In general, qualities depended on the substitution ratios of individual powder. Mixtures of BC/PH (3:1) was recommended as the appropriate ratio for fortification, in which 16.67% substitution of whole wheat flour was used. No adverse effect on sensory attributes, color and texture was obtained in the final product. BC and PH could be used to fortify foods with inadequate nutrients.

4.3.9 References

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CHAPTER 5
SUMMARY AND FUTURE WORKS

5.1 Summary

1. Protein hydrolysate (PH) could be obtained from salmon frame and it could be used as a food supplement. The form of raw material as well as the proteases used greatly influenced the chemical composition, size distribution and antioxidant capacities of the hydrolysates. Hydrolysate from mince showed higher antioxidant ability, while that from chunk showed higher bitterness and amino acid profile. However, high amounts of residues were obtained from chunk, which could be used as a starting material for biocalcium production.

2. Biocalcium (BC) and calcined bones showed variation in chemical composition and crystallinity but possessed similar Ca/P ratio, which corresponded to hydroxyapatite as the dominant phase. Alkaline pre-treatment affected the chemical compositions such as amino acid composition, abundance of volatiles as well as bioavailability of biocalcium powders.

3. Incorporation of BC and PH powders obtained from salmon frames into whole wheat cracker increased Ca, P and protein contents. Addition of 7.5% BC and 2.5% PH was able to fortify the crackers without any adverse effect on sensory attributes, color and texture of the fortified crackers.

5.2 Future works

1. Removal of bitterness from hydrolysate, while maintaining functional properties should be studied.

2. Clarification and purification of skimmed oil from hydrolysate must be conducted and refined oil can be used for incorporation into foods.

3. Increase in bioavailability of biocalcium powders should be investigated.

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List of Publication and Proceeding

Publications

Idowu, A.T., Benjakul, S., Sinthusamran, S., Sookchoo, P. and Kishimura, H. (2018). Protein hydrolysate from salmon frames: Production, characteristics and antioxidative activity. Journal of Food Biochemistry. DOI: 10.1111/jfbc.12734.

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