Value-added Products from Squid (*Loligo formosana*) Ovary and Pen: Characterization and Application in Food Systems

Avtar Singh

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Science and Technology

Prince of Songkla University

2018

Value-added Products from Squid (*Loligo formosan*a) Ovary and Pen: Characterization and Application in Food Systems

Avtar Singh

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Science and Technology

Prince of Songkla University

2018

Thesis Title	Value-added Products from Squid (*Loligo formosana*) Ovary and Pen: Characterization and Application in Food Systems.
Author	Mr. Avtar Singh
Major Program	Food Science and Technology

Major Advisor

…….…….....
(Prof. Dr. Soottawat Benjakul)

Examining Committee:

…….……..….....Chairperson
(Asst. Prof. Dr. Manee Vittayanont)

…….…..…....Committee
(Prof. Dr. Soottawat Benjakul)

…….…….....Committee
(Assoc. Prof. Dr. Sappasith Klomklao)

…….……...Committee
(Asst. Prof. Dr. Kongkarn Kijroongrojana)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Food Science and Technology.

……..…….…….....
(Prof. Dr. Damrongsak Faroongsarng)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

…….…….Signature
(Prof. Dr. Soottawat Benjakul)
Advisor

…….…….Signature
(Mr. Avtar Singh)
Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

…….…….Signature

(Mr. Avtar Singh)

Candidate

Thesis Title Value-added Products from Squid (*Loligo formosana*) Ovary and Pen: Characterization and Application in Food Systems.

Author Mr. Avtar Singh

Major Program Food Science and Technology

Academic Year 2018

ABSTRACT

Characteristics and functional properties of squid (*Loligo formosana)* ovary (SO) were studied. Protein (18.64±0.51%) and carbohydrate (7.44±0.2%) were found as the major constituents. Albumin (79.02±0.79%) was the dominant protein of SO, followed by glutelin-1 (8.31±0.62%) and globulin (6.68±0.08%). Nevertheless, prolamin and glutelin-2 constituted approximately 1% of total proteins. SO was rich in non-essential amino acids (52.26%) and polyunsaturated fatty acid (PUFA, 43.76±0.84%). SO lipids had high amount of docosahexaenoic acid (C22:6) (28.59%). When SO powder (SOP) was prepared and tested, foaming capacity (FC) and stability (FS) of foam from SOP were increased with increasing concentration up to 8% ($p < 0.05$). When partial hydrolysis of SO using 1% Alcalase or 1% papain at different hydrolysis times was conducted to improve the foaming properties, SO hydrolysate (SOH) prepared by Alcalase for 30 min (SOH-Al-30) showed the highest FC (236%). The SOH-Al-30 powder showed high solubility and surface hydrophobicity with slightly larger particle size than that of SOP. SOH-Al-30 preheated at 60 ºC showed the highest FC (300%). Therefore, foaming properties of SOP could be enhanced with partial hydrolysis and appropriate pre-treatment.

Ultrasonication at amplitude 70% for 30 min was the optimized condition using central composite design (CCD) in combination with response surface methodology (RSM). Surface hydrophobicity of ultrasonicated SOP (USOP) was increased by 28%. Solubility, total sulfhydryl group and mean particle size of SOP was decreased after ultrasonication. FC of USOP was increased by 83.3%, compared to the control and the lowest liquid drainage was attained. USOP foam showed uniformly distributed smaller sized bubbles with a thick lamella. Thus, ultrasonication at appropriate condition enhanced foaming properties SOP. The addition of USOP to

replace the egg white powder (EWP) at different substitution levels (0-100%) in cake was studied. Highest elastic modulus (G´) and average bubble size of batter was obtained with the addition of 100% USOP. Cake added with 100% USOP showed higher volume and lower baking loss, when compared to the control cake (100% EWP). The lowest values of hardness, gumminess and chewiness were noticeable for cake containing 100% USOP ($p<0.05$), in which oil phase was distributed in gluten matrix uniformly. Cake added with 100% USOP had higher overall likeness score ($p<0.05$). Thus, addition of 100% USOP resulted in the production of cake with superior quality and increased overall acceptance by consumers.

Serine protease inhibitors from SO (SOSPI) were extracted and characterize. Optimal extraction condition included 0.45 M NaCl for 1 h. After heat treatment at 70 °C for 10 min, the highest specific activity was obtained. The major SOSPIs were present as monomer with molecular weight of 9.10 and 10.27 kDa. Autolysis study of bigeye snapper surimi revealed that myosin heavy chain was more retained with coincidentally lower trichloroacetic acid-soluble peptide content as the level of SOSPI increased. Surimi added with 1% SOSPI showed the highest gelling property with increased water holding capacity. Breaking force of gel from surimi from Indian mackerel increased when SOSPI levels increased up to 2%. However, EWP showed higher efficiency in increasing breaking force of resulting surimi gel. TCA-soluble peptide content and expressible moisture content of surimi gel decreased when the levels of SOSPI and EWP increased ($p<0.05$) but resulted in lower whiteness. Highly connected and denser microstructure was observed for surimi gel added with 2% EWP as compared to that containing 2% SOSPI. The SOSPI had no negative effect on sensory attributes and could serve as the alternative protein additive to improve gel strength of surimi.

Chitosan from squid (*Loligo formosana*) pens was also prepared and characterized. Firstly, ultrasonication condition was optimized for deproteinization of squid pen using CCD. Squid pen ultrasonicated at amplitude 69% for 41.46 min at the solid/solvent ratio of 1: 18 yielded 34% (w/w) chitin with the lowest remaining protein. When the resultant chitin was subjected to deacetylation at different temperatures and times, yield and degree of deacetylation (DDA) of chitosan was in the range of 65-50%

(w/w) and 78-90%. Intrinsic viscosity and molecular weight (MW) of chitosan was in the range of 3.2-6.52 dL/g and 1.2 x105-3.2 x105 Dalton, respectively. DDAs of chitosan produced by deacetylation at 130 °C for 2 h (CH130-2) determined using FTIR and ^{1}H-NMR were nearly same (87 and 89%, respectively). CH130-2 stabilized the emulsion under the mimicked pH condition of gastrointestinal tract. It could form gel entrapping oil in simulated *in vitro* gastrointestinal tract. Therefore, it could be used as dietary fiber to control the adsorption of fat/oil in the human digestive tract.

Chitooligosaccharide (COS) produced using various non-specific enzymes named lipase, pepsin and amylase was characterized. COS produced by 8% (w/w) lipase (COS-L) showed the maximum FRAP and ABTS radical scavenging activity than those prepared using other enzymes. COS-L had the average MW of 79 kDa, intrinsic viscosity of 0.41 dL/g and water solubility of 49%. COS-L showed antibacterial activity against both pathogenic and food born bacteria. When COS-L was incorporated into sardine surimi gel at various concentrations (0-3%), the highest breaking force and deformation of gel were obtained at 1% COS-L ($p<0.05$). WHC of gel was increased as COS-L was added up to 2% ($p<0.05$). Gel added with 1% COS-L had the denser network with higher connectivity than the control. COS-L increased likeness score for all sensory attributes of gel as compared to control. When gel incorporated with 1% COS-L was stored at 4 °C, textural properties and whiteness were maintained and the lower PV, TBARS and microbial growth were obtained in surimi gel during 10 days of storage at 4 °C. Thus, incorporation of 1% COS-L could improve gel properties of sardine surimi gel as well as preserved the gel during extended storage.

Overall, squid processing waste, squid pen and ovary, could be used to extract various value-added bioactive compounds. They might be used to improve the quality and retard quality loss of various food products during extended storage.

ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my advisor, Prof. Dr. Soottawat Benjakul of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University for his patience, motivation, co-operation, kindness, immense knowledge and constructive criticism. His generous contribution in my academic career especially, working hard throughout research period, preparation of manuscripts and thesis dissertation are deeply appreciated. I am grateful for his dedication in creating an excellent working atmosphere. It's a great privilege and honor to work and study under his supervision. His determination and perseverance to train me to be a good researcher with responsibility, vigilance and honesty will always be acknowledged.

I am grateful to my examining committee, Assoc. Prof. Dr. Sappasith Klomklao of Department of Food Science and Technology, Faculty of Agro- and Bio-Industry, Thaksin University, Phatthalung, Thailand as well as Asst. Prof. Dr. Manee Vittayanont and Asst. Prof. Dr. Kongkarn Kijroongrojana of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University for the valuable time devoted to my thesis and their kindness, comments and helpful suggestion.

I would like express my sincere thanks and best wishes to Dr. Prabjit Singh. My deep gratitude is also dedicated to my dear friends and colleagues of Fish Chemistry and Biochemistry lab (2205) members who shared the hard time and helping hand with me. It was great experience with all of you during my study.

All above, I am grateful to my Father S. Surjit Singh, Mother S. Swarn Kaur and brothers, who supported me emotionally and financially throughout my study. I always knew that you believed in me and wanted the best for me.

I also would like to thanks all faculty members of Agro-Industry and its staff, students for their kind co-operation during my study period. Thailand's Education Hub for Southern Region of ASEAN Countries (TEH-AC, 2015) scholarship from Graduate School of Prince of Songkla University, Hat Yai, Thailand also acknowledged for the financial support.

Finally, many thanks to all the Thai and International friends who have supported me directly or indirectly to complete the research work.

Avtar Singh

CONTENTS

CONTENTS (Continued)

Chapter

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Thailand is one of major marine capture producing countries with the estimated amount of 1,630,047 tones (FAO, 2014). Thailand and other Southeast Asian countries are the major exporters for the marine fisheries, specially squid and cuttlefish (Hoque, Benjakul and Prodpran, 2010). For marine capture, 78% of catch are for human consumption and the rest (22%) is trash fish, which is discarded or used for non-food purposes such as fishmeal production, etc. During processing, low market value viscera is generated as a by-product which can be serious cause for ecological problems and environmental pollution, especially when the appropriate management is not implemented. Nevertheless, these discards can be the potential sources of beneficial compounds (Morrissey and Okada, 2007). Natural bioactive substances have the least quantum of side effects when compared with the synthetic counterparts. Due to the huge diversity of marine bioactive compounds with respect to their chemical structure, mode of action and applicability, the extreme concern has been increased towards the screening of marine natural products for their biomedical potential and other applications including food ingredients (McConnell, Longley and Koehn, 1994).

In squid processing industry, visceral organs such as ovary, nidamental glands, pen or gladius, etc. is mostly discarded. Squid ovary has the accountable percentage with respect to the body weight. Ovary may contain egg, which can serve as the nutrient for human consumption. Fish roe or fish egg are considered as the important source for the bioactive compounds, which exhibit antioxidant, antibacterial, immunomodulatory activities, etc. Fish roe is well known as a nutritive source due to presence of polyunsaturated fatty acids (PUFAs) (Intarasirisawat, Benjakul and Visessanguan, 2011). Additionally, products derived from fish roe, e.g. hydrolysate, etc. has been reported to possess the improved functional properties. Protein hydrolysates from underutilized merigala (*Cirrhinus mrigala*) fish egg had good fat adsorption capacity, foam capacity and emulsifying capacity (Chalamaiah *et al.*, 2015). Proteins are surface- active agents and therefore exhibit excellent

interfacial properties. Liquid egg white (EW), egg white powder (EWP), gelatins, casein, other milk proteins, soy proteins and gluten have been used as foaming agents in the food industries (Zayas, 1997). EW is most widely used foaming agent due to its superior foaming properties than the other proteins. Egg white powder is another form, which can be used in bakery product because of the long shelf-life and the ease of transportation. Song *et al.* (2009) observed that the quality of angel cake was enhanced with the addition of irradiated EWP. However, high cost, egg like flavor and allergy limit the use of EW in the food. Other food grade foaming agents are still in demand by the food industry and have been searched as processing aid for manufacturing foods and products with prime quality and acceptability.

Among food products, gelling products, especially from muscle proteins, have become popular owing to high nutritive value with the unique property. Surimi, is a stabilized myofibrillar protein obtained from fish flesh, which is washed with water to remove lipids, blood, enzymes and sarcoplasmic proteins. Nevertheless, surimi from some fish species suffers with the protein degradation termed 'modori', which is mediated by indigenous proteases. Heat stable indigenous proteolytic enzymes varies from species to species e.g. arrowtooth flounder (Wasson, Babbitt and French, 1993), Pacific whiting (Seymour *et al.*, 1994), lizardfish and bigeye snapper (Benjakul, Visessanguan and Leelapongwattana, 2003; Benjakul, Visessanguan and Tueksuban, 2003). These proteases show activity between 50 to 70 °C, which results in the proteolysis of protein network during the setting of gels associated with weak gel (Park, 2000). To solve this problem, food-grade protease inhibitors have been employed. However, those inhibitors are still costly and some additives such as plasma proteins are of religion constraint. Egg white may cause allergy and contribute to egg like smell in surimi gel. In addition to protease inhibitors, a variety of additives with different functions have been incorporated into surimi. Some of them induce protein cross-linking (Sae-Leaw *et al.*, 2018; Vate *et al.*, 2015). Phenolic compounds or hydrocolloids such as gellan have been used to enhance gel properties surimi (Buamard *et al.*, 2017; Petcharat and Benjakul, 2018). Recently, Quan and Benjakul (2017) reported that duck and hen albumens inhibited proteolysis and enhanced breaking force and deformation of surimi from sardine. Nevertheless, some

additives impose the adverse effect on gel properties and religious constraints limits their applications. Therefore, surimi industry still requires the alternative food grade additives.

Squid pen is another byproduct, which can be converted to marketable products, particularly chitin and chitooligosaccharides. β-chitin from squid pen has high reactivity toward various solvents due to its looser confirmations than α-chitin (Lavall, Assis and Campana-Filho, 2007). β-chitin can be transformed to chitosan or COS with appropriate processes (Elieh-Ali-Komi and Hamblin, 2016). Chitosan is biodegradable, biocompatible and less toxic. Therefore, it has been widely utilized in various applications, especially in medical and pharmaceutical areas (Panith *et al.*, 2016). It has been used in different forms such as solution, gel, film and fiber (Rinaudo, 2006). Chitosan has resistance towards digestive enzymes, which allow it to use as a dietary fiber to improve human health. Chitosan and its carboxymethylated derivative showed potential to bind to bile acid, thus possessing cholesterol lowering ability (Zhao *et al.*, 2011; Huang *et al.*, 2012). Chitosan has been used to improve the gelling properties as well as extended the shel-life of surimi gel (Amiza and Kang, 2013; Mao and Wu, 2007). Due to low water solubility of chitosan, its applications are limited. Therefore, hydrolyzed product of chitosan, so called chitooligosaccharide, is of great interest, owing to high solubility of COS in comparison with chitosan. COS has been known to possess antibacterial activity (Olatunde and Benjakul, 2018) and antioxidant activity (Laokuldilok *et al.*, 2017; Lodhi *et al.*, 2014).

Squid ovary and value-added products from squid pen can be an alternative ingredient with particular functionality and can be used to improve the quality of foods. Squid ovary can act as the promising foaming agent and also can serves as the food grade protease inhibitor to alleviate the gel weakening phenomenon. Chitosan from squid pen can serve as fat binding agent, whereas chitooligosaccharide (COS) can be used to improve gel properties and increase the shelf-life of surimi gel. As a whole, squid pen and ovary can be better exploited, in which novel food ingredients could be manufactured.

1.2 Literature Reviews

1.2.1 Squid

1.2.1.1 Classification

Kingdom : Animalia
Phylum : Mollusca
Class : Cephalopoda
Order : Teuthida
Family : Loliginidae
Genus : *Loligo*
Species : *formosana*

Squid is a cephalopod, which is one of three groups of mollusks. Mollusks such as cockles and whelks have shells, but in the squid, the shell is modified and consists of a strip of cartilage, known as the pen or gladius, buried in the flesh (Hanlon and Messenger, 1996). There are almost 1000 species of cephalopods, however the species commercially caught are squid, cuttlefish, and octopus. The most commercially important group of all cephalopods is squid. Squid have a distinct head, bilateral symmetry, a mantle, and arms. Squid, like cuttlefish, have eight arms arranged in pairs and two, usually longer are named tentacles. Squid are strong swimmers and certain species can "fly" for short distances out of the water. Movement is by jet propulsion. Water is taken into the mantle cavity and forcibly expelled through a siphon, which can be rotated. Thus, the animal can move forwards or backwards with great rapidity (Hanlon and Messenger, 1996). Cephalopods have well developed eyes and many are rapacious predators. All cephalopods have ink sac or reservoir containing a brown or black viscous fluid, which is ejected through the siphon when animal is alarmed (Hanlon and Messenger, 1996). The resulting cloud of ink forms an effective screen behind when animal can escape.

1.2.1.2 Squid ovary

The cone-like structure attached to the dorsal part of the sexual coelom by the connective tissue layer-mesentery, running from the stomach to the posterior

end of the coelom is called ovary (Nigmatullin *et al.,* 1991). In cephalopods, the egg is protected by the gelatin-like mass, which is secreted by nidamental glands. Nidamental glands are large, paired glandular structures found in the mantle cavity (Nair, *et al.,* 2011). Accessory nidamental glands may also be present (Nair *et al.,* 2011). Nidamental glands are composed of lamellae and are involved in the secretion of egg cases or the gelatinous substance comprising egg masses (Young *et al.,* 1999). The main peak in spawning occurs between October and January with a secondary peak between July and August. An analysis of the seasonal cycle in spawning using maturity indices gave similar results and indicated the presence of two size groups with different maturity stages. Females were always more numerous than males during the study period and showed the highest incidence of mating in spring (September–November) (Tafur *et al.,* 2001). Ripe egg size of *Loligo* sp. ranged from 1.4 × 1.9 to 1.8 × 2.8 mm with average diameters on random axes of 1.66–2.39 mm. The egg size of autumn-winter spawners was larger than that of spring-summer spawners and varies with the female size in the same cohort. During maturation, the initially unimodal size distribution of oocytes in the ovary becomes bimodal due to the formation of a batch of ripe eggs (540–13,820 oocytes). The total yolk oocyte stock ranges from 1,100 to 21,000 (Laptikhovsky *et al.,* 2002). Additionally, the ovaries of mature females contained a range of oocyte sizes with discrete peaks, indicating a continued production and development of oocyte cohorts (McGrath and Jackson, 2002).

1.2.1.3 Squid pen

Pen is a cartilaginous hard part found in many cephalopods, particularly squids. It is also called gladius due to its resemblance to the Roman short sword ‘Gladius’ (Toll, 1998). It is located dorsally within the mantle and usually extends for its entire length and provide support to the whole body (Toll, 1998; Young and Harman, 1998). Squid pens contain chitin about 35% of the dry weight (Brine and Austin, 1981; Chandumpai *et al.*, 2004). Squid pen chitin has different structure from crustaceous chitin, which has α-structure in which the polymer chains are arranged in an anti-parallel fashion with strong inter-molecular hydrogen bonding. β-chitin found in squid pen is characterized as a loose-packing parallel fashion with

weak inter-molecular interactions (Kurita *et al.*, 1993; Kurita *et al.*, 1994; Kurita *et al.*, 2000).

1.2.1.4 Status and production/import/export of squid

Cephalopods are marketed in various forms including fresh, frozen, canned, dried, salted, and smoked (Sikorski, 1990). In 2010, the world catch of cephalopods was 3.65 million metric tons (MT), of which 2.25 million MT were produced in China, Japan, Vietnam, South Korea, Indonesia, Thailand, India, Malaysia, the Philippines and Taiwan. An estimated 1.5 million MT (USD 4.3 billion) of squid, cuttlefish and octopus were reported in the international market in 2009 (Urch, 2012). Asian countries imported 734,000 MT with the value of USD 1.8 billion, which showed the dominance in the global cephalopod trade. In 2009, a total of 1.5 million MT of squid, cuttlefish and octopus were exported globally with a reported value of USD 4.0 billion. Of this, Asian countries exported 823,000 MT with the value of USD 2.2 billion (Urch, 2012). Imports are dominated by squid and cuttlefish, amounting to more than 1 million MT in 2011, and almost every market in South East Asia has increased its imports in recent years. Major shares of domestic landings in Japan, China, South Korea, Malaysia and Taiwan are also marketed locally as fresh and dried (mainly squid) products (FAO, 2017).

From 2000 to 2014, global annual squid landings varied between 2.7 million tons and 3.5 million tons. In 2014 and 2015, global landings were about 3.0 – 3.2 million tons annually. In 2015, Argentine Loligo landings hit a record 358, 000 tons, which were 306, 000 tons in 2014. Japanese squid imports fell year on year by 5.2% in 2015, to 79, 000 tons. US squid imports have been stable at around 70, 000 tons for some years. In 2015, there was a slight increase in imports (+4.6%). China remains the major supplier, accounting for 56.8% of total imports, and in 2015. China was the main supplier and managed to export about the same amount as in 2014, while imports from Peru and Argentina were down by 13.8% and 32%, respectively. Similarly, squid import in Spain also reduced from 92, 500 tons in 2014 to 87, 500 tons in 2015 (-5.4%). A massive reduction (-25.5%) in import has been reported in Falklands (Malvinas) (FAO, 2017).

1.2.1.5 Utilization of squid processing byproducts

During processing of squid, a wide range of byproducts including, skin, viscera, pen, etc. is generated. Skin and ink, has been utilized and incorporated into the food system. Gelatin was extracted from the skin of splendid squid at different temperatures (50-80 °C) and the maximum yield (45.3%) was obtained at 80 °C. The gelatin extracted at 80 °C showed the decrease in emulsion activity index, whereas emulsion stability index, foam expansion and stability increased as the concentration (1–3%) increased (Nagarajan *et al.*, 2012). Additionally, squid gelatin was used as biopolymer for film making. Nagarajan *et al.* (2012) prepared film from the gelatin extracted from the squid skin at different temperatures. Decreases in tensile strength (TS) and elongation at break (EAB) of films, but water vapor permeability (WVP) increased as the extraction temperature increased. Nagarajan *et al.* (2013) also reported that squid skin gelatin could yield the film with the improved property when skin was bleached using 2% H_2O_2 prior to gelatin extraction.

Ink has been used in several foods, especially pasta. Due to the dark color associated with melanin, the removal of melanin could bring about the liquid containing active components named ‘Melanin free ink’ (MFI). MFI from splendid ink possessed DPPH, ABTS radical scavenging, metal chelating activities, and ferric reducing antioxidant power (FRAP) and prevented the oxidation of β-carotene-linoleic acid system. MFI was also reported to retard the lipid oxidation in mackerel mince during ice storage for 15 days (Vate *et al.*, 2013). MFI at 0.08% increased the breaking force and deformation of sardine surimi gel by 23% and 12% and also improved water holding capacity (WHC) of surimi gel (Vate *et al.*, 2015). Squid ink was also used to improve the growth performance, antioxidant ability and immune functions of growing broiler chickens and has been employed in the development of feed additives for animals (Liu, 2011).

Viscera of squid are known as the excellent source of protease and other enzymes (Uddin *et al.*, 2009). Chymotrypsin (MW 31 kDa) from jumbo squid hepatopancrease was purified (Marquez-Rios *et al.*, 2016). Furthermore, Kishimura *et al.* (2001) purified trypsin inhibitor from the hepatopancreas of squid (*Todarodes*

pacificus) with an estimated MW of approximately 6300 Da. The squid trypsin inhibitor was acid- and heat-stable, and active against trypsins from the pyloric ceca of starfish (*Asterias amurensis*) and saury (*Cololabis saira*) and porcine pancreatic trypsin. It also inhibited the breakdown of walleye pollock myofibrillar proteins.

Squid head of high protein content contains the abundant sweet-umami amino acid, glutamic acid. Its dried form was rich in glutamic acid. The major volatiles found are trimethylamine and toluene along with the 15 important compounds exhibiting dried squid flavor. Hydrolysate from squid has been produced and used as the flavorant. The freeze dried sweet-umami hydrolysate prepared by Flavourzyme contained protein at 76%, and emulsifying activity index (EAI) (22). For the foam-mat dried sample, the highest emulsion stability index (15) and foaming stability (29) were obtained. The antioxidant property of the freeze-dried sample was higher than that of the foam-mat dried sample (Sukkhown *et al.*, 2017). Moreover, Sudhakar and Nazeer, (2015) reported that the peptide derived from squid mantle protein through α-chymotrypsin hydrolysis exhibited high antioxidant activity tested by DPPH radical scavenging, hydroxyl radical scavenging and superoxide radical scavenging assays.

1.2.2 Chitin

Chitin is a natural polysaccharide, consisting of 2-acetamido 2-deoxy-β-d-glucose through a β-(1-4) linkage. It is mainly found in invertebrates, vertebrates as well as some species of plants (Lodhi *et al.*, 2014) (Figure 1). It is the second largest biopolymer found in nature after cellulose (Abdou, Nagy and Elsabee, 2008). This structural biopolymer plays a similar role like collagen in the higher animals and cellulose in terrestrial plants (Pillai, Paul and Sharma, 2009). It is an ordered crystalline microfibrils, which are the main structural components of the exoskeleton of arthropods or in the cell walls of fungi and yeast. Crustaceans chitin is a fibrous material embedded in a six stranded protein helix (Pillai *et al.*, 2009). Chitin occurs as α and β-forms, depending on the sources. Moreover, γ-chitin, showing similar morphology to α and β-forms, has also been reported in fungi and yeast (Rudall and Kenchington, 1973; Blackwell, 1973; Rudall, 1969; Atkins, 1985). Crab, shrimp and

krill shells mainly consist of α-chitin, whereas β-chitin is mainly present in squid pens (El Knidri *et al.*, 2016). In addition, Kumari *et al.* (2015) successfully extracted chitin from fish scales from *Labeo rohita.* α-chitin has antiparallel structure with hydrogen bonds, which limits its reactivity with other solvents. Both forms of chitin are highly hydrophobic and only soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjugation with aqueous solutions of mineral acids and dimethylacetamide containing 5% lithium chloride (Kumar and Majeti, 2000). Therefore, it is commonly transformed into its derivative specially chitosan, which possesses various applications due to its solubility in aqueous acid solutions.

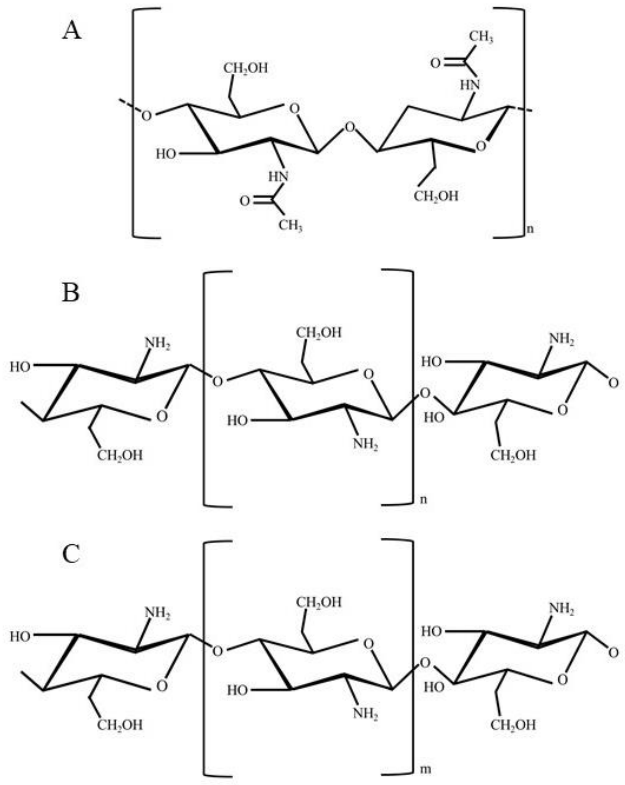

Figure 1. Chitin (A), chitosan (B) and chitooligosaccharide (C).

1.2.2.1 Squid pen chitin

Squid pens have been reported to be a main source for the production of β-chitin. This chitin has a higher solubility, reactivity and affinity towards solvents than α-chitin. This is more likely due to parallel structure and absence of inter-

hydrogen bonding (Jung and Zhao, 2011). α-chitin is dominated by strong C–O–NH hydrogen bonding which maintained the chains at a distance of about 0.47 nm and β-chitin does not contained such kind of bonding which make it more susceptible to intra-crystalline swelling than α-chitin (Blackwell, 1969; Pillai et al., 2009). Therefore, β-chitin possessed important functions with different applications (Bautista-Baños *et al.*, 2006; Abdou *et al.*, 2008; Cuong *et al.*, 2016). Moreover, β-chitin is more susceptible to deacetylation to form chitosan due its looser confirmation. Squid pens contained around 31–49% chitin, 46-74% protein and had a low amount of minerals (0.03–1.9 %) (Lavall *et al.*, 2007; Cuong *et al.*, 2016). Therefore, extraction of squid pen chitin is mainly involved deproteinizations, due to the negligible amount of minerals and absence of pigments (Lavall *et al.*, 2007). A variety of squid pens has been used as starting material including *Loligo chenesis, Ommastrephes bartrami , Doryteuthis sibogae, Illex argentines, Todarodes pacifica and Dosidicus gigas* (Cuong *et al.,* 2016). Due to the absence of severe conditions of demineralization, β-chitin has a higher molecular weight (MW) (5.300-11.680 kDa) than α-chitin (650-1.036 kDa) (Chaussard and Domard, 2004). β-chitin is white in color and does not contain heavy metals such as Cu, Pb, Fe, Cr, As. Thus, β-chitin can be used for particular applications requiring a high MW and high purity (Chaussard and Domard, 2004; Lavall *et al.*, 2007; Cuong *et al.*, 2016; El Knidri *et al.*, 2016).

1.2.2.2 Extraction of β-chitin from squid pen

Squid pen contains lower amount of minerals and looser structure. Its extraction at mild conditions as compared to shells of shrimp, crab, krill, etc. It was reported that prolonged alkaline treatment under at high concentration and temperature causes extensive deacetylation of β-chitin (Kurita *et al.*, 1993). Lavall *et al.* (2007) observed the destruction of native structure of β-chitin, when it was extracted using demineralization followed by deproteinization. Moreover, prolonged and harsh conditions for deproteinization also resulted in dissolution of chitinous material along with the protein, which lowered the overall yield. Therefore, it was suggested that β-chitin should extracted without demineralization under mild deproteinization condition. In general, 40-42% of β-chitin can be recovered from squid pen (Kurita *et al.*, 1993; Tolaimate *et al.*, 2000; Tolaimate *et al.*, 2003;

Chaussard and Domard, 2004; Lavall *et al.*, 2007). However, lower yields 36–37% were reported of β-chitin from the pens of *Loligo lessoniana* and *Loligo formosana* using 0.3 M NaOH at 80–85°C. The extraction was repeated twice for 1 h (Chandumpai *et al.*, 2004). This might result in the loss of chitin during repeated process. The amount of β-chitin extracted from the pens of different species of squids does not vary widely. Squid pen chitin was deproteinized using 4% NaOH at 80 °C for 10 h and remaining protein content was observed at 0.63%. The obtained chitin had high MW ($8.5 \pm 0.1 \times 10^3$ kDa) and negligible amount of minerals (Cuong *et al.*, 2016). Lavall *et al.* (2007) extracted chitin from squid pens from *Loligo sanpaulensis* and *Loligo plei* with high DA (95%) when both demineralization and deproteinization were employed. However, low DA (90%) was obtained when β-chitin was extracted by deproteinizations without demineralization. Overall, various extraction conditions have been used to deproteinize chitin from squid pen, resulting in chitin with different physicochemical and functional properties of chitin. Therefore, optimization of extraction conditions is required to obtain chitin and its derivatives of desired properties.

Recently, ultrasonication has been widely used for the extraction of numerous polysaccharides including pectin, hemicellulose and starch from various sources. Ultrasonication has been documented to reduce the processing cost and time. Chitin has been extracted from Atlantic shrimp and freshwater prawn using ultrasonication (Kjartansson *et al.*, 2006; Kjartansson *et al.*, 2006) .

1.2.3 Chitosan

Chitosan is a semi-crystalline polymer produced by deacetylation of chitin, in which acetyl groups are removed with help of alkali and high temperature (Figure 1). Generally, chitosan is soluble in aqueous acidic medium when degree of deacetylation (DDA) reached up to 50%. Chitosan act as polyelectrolyte in acidic media and increased its solubility by protonation of the $-NH_2$ on the C-2 of glucosamine repeat unit (Rinaudo, 2006). Behavior of chitosan in solution depends not only on MW and DDA but also on the distribution of acetyl group along the chain (Kubota and Eguchi, 1997; Rinaudo, 2006). Therefore, chitosan produced with

appropriate MW and DDA could enhance it solubility and their use in the various fields.

1.2.3.1 Preparation of chitosan

The conversion of chitin to chitosan mainly involved enzymatic and chemical methods (Younes and Rinaudo, 2015). However, due to low cost and ease for commercial level production chemical methods have been used widely for the deacetylation of chitin. Deacetylation involved the removal of acetyl groups using high concentration of alkali at higher temperature. Deacetylation of chitin is performed in two ways 1) heterogeneously and 2) homogenously (Sannan, Kurita and Iwakura, 1976; Chang *et al.*, 1997). For heterogenous deacetylation, chitin is heated with concentrated NaOH for few hours, resulting in DDA of 85–99%. For homogeneous method, alkali chitin is prepared after dispersion of chitin in concentrated NaOH (30g NaOH/45g H_2O/ 3g Chitin) at 25 °C for 3 h or more, followed by dissolution in crushed ice around 0 °C. This method produce soluble chitosan with an average DDA of 48–55% (Younes and Rinaudo, 2015). Heterogenous deacetylation causes an irregular distribution of N-acetyl-D-glucosamine and D-glucosamine residues. This might block some acetyl group distribution along polymeric chains (Aiba, 1991) and yield different physicochemical properties than the chitosan produced by homogenous deacetylation. The MW and DDA of chitosan are mainly affected by NaOH concentration, reaction time, temperature and repetition of alkaline steps (Younes and Rinaudo, 2015). Tsaih and Chen (2003) observed that rate of deacetylation was decreased over deacetylation time. The DDA of chitosan increased at higher rate at the beginning of reaction process and, then slowed over time (Tsaih and Chen, 2003). It was postulated that high temperature and concentration of alkali enhanced the diffusion of NaOH into the chitin matrix (Chang *et al.,* 1997). Abdou *et al.* (2008) reported that DDA of chitosan from different sources increased up to 80-90% with increasing NaOH concentration from 10 to 40%. The highest DDA of the resulting chitosan after alkali deacetylation at 99 and 140 °C were 92 and 95%, respectively. Considering the impact and role of each factors on DDA and MW, deacetylations condition was optimized using various models such as response surface methodology (RSM) etc. Deacetylation reaction

were optimized using RSM for preparation of chitosan from shrimp waste. MW of 150 kDa, and a deacetylation degree of 90% were observed at a temperature of 130 °C for 90 min (Weska *et al.*, 2007). In another study, Hwang *et al.* (2002) controlled the MW and DDA of chitosan using RSM and wide range of MW (100-1100 kDa) with varying DDA (67 to 96%) were obtained using alkaline treatment. The drastic decrease in MW and the increases in DDA of chitosan were obtained with increasing temperature, reaction time, and NaOH concentration (Hwang *et al.*, 2002).

Chitosan from β-chitin can be extracted using mild conditions for deacetylation due to looser structure and higher affinity towards solvents. Abdou *et al.* (2008) deacetylated β-chitin to obtain DDA up to 90% DDA using 30% NaOH without pretreatment with NaOH. When squid pen chitin was heated at 80°C with 40% aqueous sodium hydroxide for 3 h, 67% of chitosan with DDA of 80% was obtained (Kurita *et al.*, 1993). A linear regression model was used to predict the effect of different deacetylation conditions on DDA of squid pen chitin. It was reported that average 90% DDA was attained for all combinations. The extraction conditions were varied in the range of 20-60%, 40-100 °C and 15-120 min for NaOH concentration, temperature and time, respectively. The solid-solvent ration was ranged between 1/10 or 1/20 (Methacanon *et al.*, 2003). Therefore, chitosan from β-chitin can be produced using milder extraction conditions than that from α-chitin. Chitosan of high DDA and low MW with higher solubility is generally produced from β-chitin. Additionally, it can result in lower production cost and generation of chemical waste. Thus, MW and DDA of chitosan and their applications are varied with the reaction conditions.

1.2.4 Chitooligosaccharide

Chitooligosaccharides (COS) are the depolymerized products of chitosan (Xu *et al.*, 2008) (Figure 1). Chitooligosaccharide (COS) has been gaining interest in pharmaceutical, medicinal and food applications due to their non-toxicity and high solubility (Lodhi *et al.*, 2014). COS is the hydrolyzed product of chitosan with varying MW. COS is generally produced by physical, chemical or enzymatic methods, however physical and chemical methods impair physicochemical and functional properties of COS (Liang, Sun and Dai, 2018; Lodhi *et al.*, 2014).

Hydrolysis of chitosan can be performed using acid (HCl), or mixture of acid and electrolytes such as nitrous acid, phosphoric acid and hydrofluoric acid, etc. Moreover, hydrolysis was performed by oxidative reductive methods with hydrogen peroxide or persulfate (Mourya, Inamdar and Choudhari, 2011). Lactic acid, trichloroacetic acid, formic acid, and acetic acid were also used for hydrolytic reaction (Mourya *et al.*, 2011). Due to safety concern and uncontrolled hydrolysis, chemical methods are limited. In addition, formation of secondary products during the hydrolysis are difficult to remove from the mixture (Lodhi *et al.*, 2014). Physical methods, such as irradiation and ultrasonication are unable to reduce the MW of chitosan efficiently (Mourya *et al.*, 2011). Therefore, enzymatic method has been employed more widely to produce low MW COS with the controlled degree of depolymerization (DDP) (Lee, Xia and Zhang, 2008; Lodhi *et al.*, 2014).

1.2.4.1 Enzymatic hydrolysis of chitosan

Enzymatic depolymerization of chitin and chitosan with specific enzymes such as chitinases and chitosanases has been documented, respectively (Aam *et al.*, 2010). However, high cost and less availability limits their use for COS production. Therefore, chitosan has been commonly hydrolyzed using non-specific enzymes such as pectinase, lipase, proteases, amylase and cellulase (Mourya *et al.*, 2011; Lodhi *et al.*, 2014). The hydrolytic susceptibility of chitosan to a wide range of enzymes, including 10 kinds of glycanases, 21 different types of protease, five lipases and a tannase derived from various sources has been reported (Mourya *et al.*, 2011).

In general, COS are produced by solubilizing chitosan by acidification, mainly acetic acid, incubating the chitosan solution with enzyme, and terminating the enzymatic reaction by heating at 100°C (Lodhi *et al.*, 2014). COS of different low MW have been produced using non-specific enzymes by varying enzyme concentration and incubation period. Chitosan consist four different types of glycosidic bonds in their structures. These include linkages between two N-acetylated units (A–A), acetylated and deacetylated units (A–D), deacetylated and acetylated units (D–A) and two deacetylated units (D–D). Enzyme can cleave more than one type of these linkages owing to different affinities of enzyme towards different

linkages. This led to varying cleavage rates (Roncal *et al.*, 2007). Therefore, DDP of chitosan generally varied with the type and source of enzyme.

DDP of chitosan not only depend on the enzymes and hydrolytic conditions but also on distribution of acetyl group in chitosan (Anthonsen, Vårum and Smidsrød, 1993). Among the various non-specific enzyme used for chitosan hydrolysis, amylase showed high affinity towards chitosan. This is probably due to the presence of β-1, 4 bond between glucosamine, which was similar to that found in amylose (Rokhati *et al.*, 2013). The pH of the chitosan solution is adjusted to around 5, more likely due to the precipitation of chitosan at neutral and alkaline pH. Therefore, enzyme with optimum activity near pH 5-6 can produce COS with high DDP. Pan and Wu (2011) documented the optimum condition for hydrolysis of chitosan from shrimp shell by using glucoamylase, at pH 4.5 and 55 °C for 24 h. Optimum hydrolysis of chitosan was conducted using α-amylase (pH 5; 50 °C) for 6 h (Wu, 2011). Lipase exhibited higher activity towards chitosan from shrimp waste, yielding DDA of 83% and 73%, when hydrolysis was carried out at 37 °C for longer than 6 h. The maximum COS yield of 24 h hydrolysis at 37 °C was obtained around 94% (Lee, Xia and Zhang, 2008). DDA and average MW of chitosan also influenced the susceptibility towards the different enzymes. Zhang and Neau (2001) stated that lower MW and lower DDA chitosan sample possessed a higher affinity for the β-glucosidase associated with higher catalytic efficiency and higher degradation of chitosan. Nevertheless, Kittur *et al.* (2003) observed substrate inhibition against pectinase isozyme at chitosan concentrations greater than 12 mg/mL. Hence, hydrolysis conditions and molecular property of chitosan determine MW and application of COS.

1.2.5 Food application of chitin and its derivatives

Chitin and its derivatives (chitosan and COS) possessed a wide range of applications in medical, pharmaceutical, and food industries. With various biological activities including antioxidant effects, antimicrobial effects, and other properties. They could be used in the food industry to improve food safety, quality, and shelf-life (Hamed, Özogul and Regenstein, 2016).

1.2.5.1 Antioxidative activity

Lipid oxidation resulted in off-flavor and off-odors of fatty foods, especially fatty fish due to oxidative deterioration of polyunsaturated fatty acid, thereby shorten shelf-life (Mao and Wu, 2007). Antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole have been employed to inhibit lipid oxidation in the various foods. However, concerning to health related issue with the synthetic antioxidants, food industries are focusing on natural antioxidants (Hamed *et al.*, 2016). Chitosan and COS are non-toxic natural antioxidant. Free amino group of glucosamine can form ammonium group by taking a hydrogen ion from the system, which later can react with radicals (Senphan and Benjakul, 2014; Laokuldilok *et al.*, 2017). Chitosan, which has strong intra-molecular hydrogen bonding, showed lower antioxidant activity (Tomida *et al.*, 2009). Kim and Thomas (2007) stated that chitosan with MW 30 kDa had the higher DPPH radical scavenging activity as compared to those of 90 and 120 kDa chitosan. This is more likely due to less availability of free amino group to act as hydrogen donor. In addition, metal chelating ability of chitosan might be helpful to entrap free iron that is released from the heme proteins of meat during heat processing (Tharanathan and Kittur, 2003). Chitosan (2%) coated oyster had lower lipid oxidation and microbial load than the control (Rong *et al.*, 2010). When silver carp was coated with chitosan (2%) dissolved in 1% acetic acid, shelf-life at –3 °C was extended to 30 days, compared to 20 days for the control. COS contain more hydroxyl groups after hydrolysis and can function as hydrogen donor to radicals. Therefore, COS shows more potential to inhibit the lipid oxidation than chitosan. Hence, both chitosan and COS with appropriate MW could be used to increase the shelf-life of food products stored at refrigerated temperature.

1.2.5.2 Antibacterial activity

Chitosan and COS are known to possess antibacterial activity and have used widely to preserve seafood or their products (No *et al.*, 2002). In general, antimicrobial activity of chitosan and COS is more likely because of the inhibition of mRNA and protein translation. Additionally, chelation of essential nutrients and metals are well-known mechanisms advocating antimicrobial activity. The

antibacterial activity of COS and chitosan is mainly depending on MW. *Bacillus subtilis, Pseudomonas aureofaciens, Bifidobacterium bifidum 791* and *Enterobacter agglomerans* were resistant to chitosan with an average MW of 5 to 27 kDa (DDA-85%), while *Escherichia coli* was more sensitive to the 5-kDa chitosan (Gerasimenko *et al.*, 2004). Gerasimenko *et al.* (2004) also reported the sensitivity of *Candida krusei, Staphylococcus aureus* and *Bifidobacterium bifidum* ATCC 14893 against chitosan with different MW, which were varied between 5 and 27 kDa. Park, Lee and Kim (2004) studied the effect of chitosan of DDA 90%, 75% and 50% and their COS of varying MWs on 32 strains of *V. parahaemolyticus.* In addition to antibacterial activity, chitosan also possessed antifungal activity. For antifungal activity, they might penetrate to fungal cell and caused structural and molecular changes in the cells (Younes *et al.*, 2014). Amiza and Kang (2013) studied the effect of food grade chitosan (MW-10 kDa, DDA-95%) on gel from catfish surimi. Surimi gels added with 2% chitosan showed lower microbial counts than the commercial and control gel during storage at 4 °C. Moreover, Muzzarelli and Muzzarelli (2005) stated that chitosan can be used as edible film to extend the shelf-life of food. TBARS, TVB-N content, LAB count, *Pseudomonas* count, and mesophilic bacteria count were lower in brown trout when coated with chitosan prepared in 1.5% acetic acid than 1.5% lactic acid. The shelf-life was extended to 9 and 12 days for samples coated with chitosan dissolved in lactic acid and acetic acid, respectively, compared to the control (6 days) (Alak, 2012). Chitosan has been combined with other preservatives or treatments to enhance its preservative effect. Shrimps coated with chitosan dissolved in 1% acetic acid in the presence of 1.5% garlic oil had an extended shelf-life (15 days) when compared to the control (5 days) (Aşik and Candoğan, 2014). There is no information about the extension of shelf-life of seafood using chitooligosaccharide (Olatunde and Benjakul, 2018). However, it can be used to enhance the shelf-life of various kind of food products.

1.2.5.3 Fat binding capacity

Chitosan has resistance towards digestive enzymes, which allow it to use as a dietary fiber to improve human health. Sugano *et al.* (1988) reported that chitosan has the potential to bind cholesterol and fat in the digestive tract. Its fat

binding property can help protect fat/oil to be digested by lipase. Chitosan and its carboxymethylated derivative showed potential to bind to bile acid, implying they had cholesterol lowering ability (Huang *et al.,* 2012; Zhao *et al.,* 2011). Under acidic aqueous condition (*in vitro* human gastro-intestinal), chitosan was hydrated and dispersed throughout the medium. Protonation of amino group in chitosan molecule provided strongly positive charge, which was able to attract negatively charged particle including oils, fat containing phospholipids, etc. (Panith *et al.,* 2016). This caused the formation of fat emulsion complex. Under duodenal conditions such as high pH and presence of bile salt causes formation of gel due to the precipitation of chitosan. It was reported that those gel like structure can entrap oil and bile salt, resulting in the lower fat absorption and cholesterol production (Helgason *et al.*, 2008). Chitosan with high MW showed the highest ability to bind fat under *in vitro* gastrointestinal tract (Panith *et al.,* 2016). Sugano *et al.* (1988) reported the loss in the weight of rat fed with 5% chitosan as compared to those fed with cellulose. Additionally, Thongngam and McClements (2005) noticed the potential of chitosan to bind bile acid under *in vitro* condition, resulting in inhibition of the absorption and enterohepatic circulation of bile acids. This led to leading to a decrease in plasma cholesterol level. Therefore, chitosan can be utilized as a dietary fiber, which reduces fat digestion or absorption and lowers the cholesterol levels.

1.2.6 Food proteins and their foaming properties

Proteins are widely used to stabilize the foam for the production of variety of foods. Foam is a two-phase system consisting of air cells separated by a thin continuous liquid layer called the lamellar phase (Figure 2A) (Damodaran, Parkin and Fennema, 2008). Food foams are usually very complex systems, including a mixture of gases, liquids, solids, and surfactants. Air bubbles distribution and structure in foam influences the foam appearance and textural properties. Products containing foams with a uniform distribution of small air bubbles are smooth and light (Damodaran *et al.* 2008). The most widely used protein foaming agents are egg white (EW), gelatins, casein, other milk proteins, soy proteins, and gluten (Zayas, 1997).

1.2.6.1 Mechanism of foam formation

Generally, bubbling, whipping, or shaking of protein solution creates protein-stabilized foams. The foam formations involve three major steps (Zayas, 1997) as follows:

1). Diffusion of soluble globular proteins to the air/water interface to reduce the surface tension.

2). Unfolding and rearrangements of proteins at the interface.

3). Interaction of the polypeptide/protein to form film around the bubble.

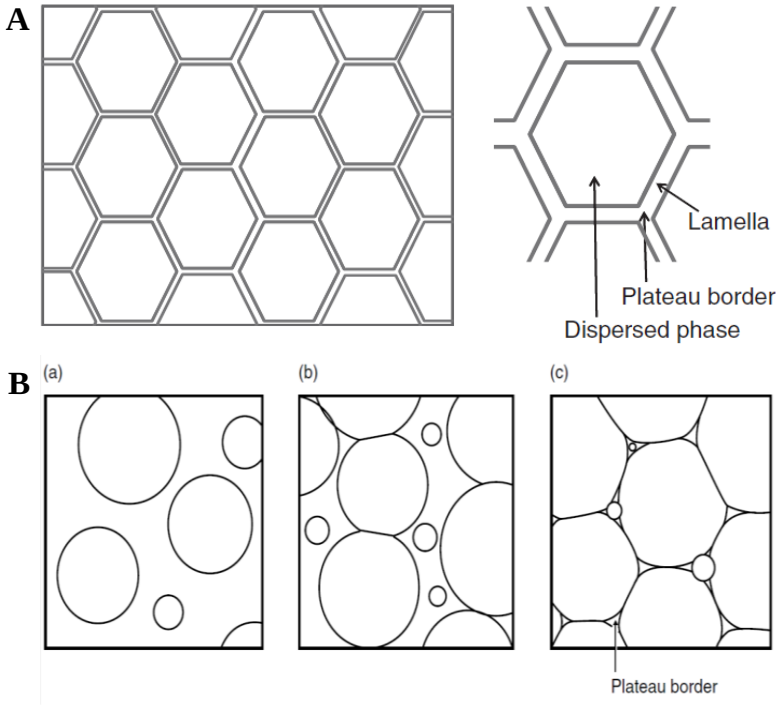

Figure 2. Illustration of structure of foam showing lamella and plateau border (A) and Foam structure at different stages during foam formation (a-c), (a) whipping stage and (b-c) formation of lamellae (B).

Source: Damodaran, Parkin and Fennema (2008)

Proteins are amphiphilic molecules and surface-active agents and they reduce the surface tension at the air/water interface, which results in the formation of stable foam by forming strong films at the interface (Zayas, 1997). The migration of proteins from bulk liquid to an interface indicates that proteins possess low free energy at interface than the bulk aqueous phase (Damodaran *et al.*,2008). During the interfacial film formation, proteins migrate to the interface by diffusion and adsorption, followed by the penetration into the surface. It depends on the unfolding and rearrangement of the protein molecules with the polar groups towards the water and non-polar groups facing to air. Thereafter, continuous film is formed at the surface through protein-protein, electrostatic, hydrophobic interactions and hydrogen bonding (Zayas, 1997). Figure 2B showed the foam formation after the creation of initial bubbles. As soon as whipping stops, bubbles rise rapidly and form a foam layer. The buoyancy force soon is sufficient to cause mutual deformation of bubbles, causing the formation of flat lamellae between them. Further drainage of interstitial liquid causes the bubbles to attain a polyhedral shape.

Proteins form a highly viscoelastic film at an interface, which has the ability to withstand mechanical shocks during storage and handling. Thus, protein-stabilized foams and emulsions are more stable than those prepared with small molecule surfactants, and because of this, proteins are extensively used for these purposes (Damodaran *et al.*, 2008).

1.2.6.2 Foam stability

Foam are kinetic stable colloidal dispersion and undergoes destabilization over certain time period. The instability of foam is occurred by three major mechanisms: Liquid drainage, bubble coalescence and disproportionation of bubbles (Cascão *et al.*, 2003; Martinez *et al.*, 2005; Sarkar and Singh, 2016). Lamellar liquids in the foam tend to drain. When the space between films decreases to 50 Å, there is the gradual collapse of the foam (Kinsella, 1981). Liquid drains under the influence of gravity and results in gradual rise of bubbles through the foam phase. Aqueous phase from the colloidal system drains through the lamellae and the plateau

borders between the foam bubbles under the influence of gravity. The foam stability is influenced by film thickness, mechanical strength, protein-protein interactions and environmental factors such as pH and temperature (Kinsella and Whitehead, 1989). Liquid drainage from foam can be reduced by incorporating polar side chains of protein polypeptides, which interact with water and retains water in lamella (Zayas, 1997).

Coalescence involves thinning and rupturing of the isolated liquid interfacial films separating two neighboring bubbles (Damodaran *et al.,* 2008). Foam bubbles are stabilized against coalescence by the generation of strong colloidal forces that act between the film surfaces and the adsorption of surface-active molecules such as proteins to form a dense film. The third type of foam destabilization is disproportionation, which involves bubble coarsening because of the diffusion of gas through the foam films, from the smaller bubbles to the larger bubbles (Sarkar and Singh, 2016).

1.2.7 Factors affecting the foaming properties

1.2.7.1 pH of medium

pH of the proteins plays an important role in foaming properties. At isoelectric point (pI), the net charge on the protein is zero, hence the electrostatic attractions are maximum (Zayas, 1997). Therefore, more protein will adsorb at the interface and form thick layer at interface, which form stable foam. At pI, foam formed shows maximum viscosity, rigidity and stability against the gravitational force (Zayas, 1997). Kim and Kinsella (1985) observed that the surface pressure was maximum near the pI of bovine serum albumin (BSA) and decreased with the pH and above pH 6. The other factor affected by the pI is solubility. Lack of repulsion between the proteins molecules leads to the lowest solubility. Therefore, at pI, only soluble fractions will participate in foam formations which results in decreased foaming capacity. Proteins with pH away from the pI, exhibits good foaming capacity but lower foam stability (Damodaran *et al.*, 2008). BSA (pI = 4.9) showed maximum foam stability in the pH 5-6 (Kim and Kinsella, 1985). β-lactoglobulin showed rapid adsorption at pH 5.3 and surface viscosity was decreased when pH was increased to 7

from 5-6 (Waniska and Kinsella, 1985). Protein isolate extracted from *Arthrospira platensis* by isoelectric precipitation showed the maximum foaming capacity (275%) at pH 10. For foam stability, foam volume recorded after 30 min at pH 10 (56.5%) was higher than that at pH 7 (36%) and at pH 3 (2%) (Benelhadj *et al.,* 2016). Similarly, effect of pH and ionic strength on the stability of foams prepared with amaranth protein isolate was analyzed by Bolontrade, Scilingo and Añón (2016). Foams prepared at acidic pH were more stable than alkaline pH. At pH 2, the foams were present for longer times. This behavior is consistent with the characteristics of the interfacial film, which showed a higher viscoelasticity and a greater flexibility at acidic pH than alkaline pH value. However, the enhanced foam stability was achieved by increasing the concentration of proteins in the foaming solution (Bolontrade *et al.*, 2016). Soluble aggregates of pure β-lactoglobulin (median diameter of 50 nm) showed the maximum foam capacity at pH 5 (228%) in comparison with those at pH 3 and 6.8. For native- lactoglobulin, the maximum foaming capacity (230%) was also observed at pH 5 (Dombrowski, Gschwendtner and Kulozik, 2017). Miquelim, Lannes and Mezzenga (2010) investigated the interactions of egg albumin with different types of polysaccharides and their effect on the surface tension at different pHs. At pH 4, the surface tension was decreased from 70 mN/m to 42 mN/m by the egg albumin, and from 70 mN/m to 43 mN/m, 40 mN/m and 38 mN/m by subsequent addition of xanthan, guar gum and κ-carrageenan, respectively. At pH 7.5, surface tension was decreased from 70 mN/m to 43 mN/m by the simultaneous presence of the protein and κ-carrageenan. However, the higher surface tension of 48 and 50 mN/m was found when xanthan and guar gum were added, respectively when compared with carrageenan addition (Miquelim *et al.,* 2010).

1.2.7.2 Protein concentration

The foaming properties can be improved with increasing concentration of proteins due to the formation of thick interfacial film with smaller and dense bubbles. The maximum overrun was obtained at protein concentration 2-8%, which results in the optimum liquid viscosity and thickness of film (Zayas, 1997). Vani and Zayas (1995) observed that foam expansion was increased with increasing concentration of EW, nonfat dry milk (NFDM), corn germ protein flour (CGPF), and

wheat germ protein flour (WGPF). However, foam capacity of soy flour (SF) was decreased at higher concentrations. At the higher concentration, protein solubility decreased and impeded the migration of proteins to the interface. Due to the high viscosity, the incorporation of air is reduced, therefore lowering the foam expansion. Foam from sodium caseinate solution expanded in the range of 100-300%. At high concentration of sodium caseinate (8%), the reduced foam capacity (<300%) was observed in comparison with the lower concentration (1-4%). Insoluble material is believed to interfere with the interfacial properties (Britten and Lavoie, 1992). Foaming capacity of sodium caseinate, WPI and ovalbumin depended on protein concentration (Britten and Lavoie, 1992). Protein from alfalfa leaf was dissolved in distilled water and final concentration was adjusted to 10mg/L at different pHs (2, 7 and 10). The maximum foaming capacity was observed at pH 2 (117 mL), followed by pH 7 (19 mL) and 10 (15 mL). For foaming stability, at pH 7 and 10, all foam samples were collapsed after 15 min of incubation, whereas the highest stability (90%) was found at pH 2.

1.2.7.3 Presence of other biomolecules and salt

Foaming properties of protein are affected by the presence of lipids and sugars. Addition of sucrose, lactose and other sugars, generally improves the foam stability of protein but decreases the foaming ability. The improved foaming stability is due to the increase in viscosity of foaming solution, which lowers the liquid drainage (Damodaran *et al.*, 2008). The addition of pectin or guar gum with increasing concentration decreased the foam volume (ErÇElebi and IbanoĞLu, 2009). Similarly, soy protein foams with the addition of xanthan gum (Carp *et al.,* 1997) or kappa-carrageenan (Carp *et al.,* 2004) or sunflower protein isolate foams added with xanthan, guar gum and k-carrageenan had the decreases in the foam volume (Martinez *et al.,* 2005). When whey protein isolate (WPI) model systems without and with the addition of glucose, sucrose, starch and inulin were tested, foaming capacity of 286., 299, 307, 280 and 287% and foaming stability (after 15 min) of 115, 143, 141, 135 and 137 were obtained. Thus, foaming capacity was not affected much by the addition of carbohydrates, whereas foaming stability was much improved (Herceg *et al.,* 2007). Sadahira *et al.* (2014) studied the effect of protein–pectin electrostatic

interaction on the foam stability mechanism of EW. Foams produced with protein: pectin ratio 15:1 showed the lowest bubble growth rate and the greatest drainage, whereas protein: pectin ratio 55:1 presented the lowest drainage. Larger particles seemed to build an interfacial viscoelastic network at the air–water interface with reduced gas permeability, leading to the higher stability via lowering the disproportionation. Soluble complexes of smaller sizes increased viscosity, leading to a low drainage of liquid and inhibiting the bubbles coalescence. The foaming capacity was increased with increasing WPC concentration. When xanthan gum (XG) at different levels was present, small differences in foaming capacities between samples added with 0.05 and 0.15% XG was noticeable, in which the values of 600-738% and 550-700%, respectively were obtained (Martínez-Padilla *et al.*, 2015). Similarly, Martínez-Padilla *et al.* (2014) found high foaming capacity of WPC enriched milk (380-534%), prepared with skim milk powder (10%) and WPC (5-15%). Pernell *et al.* (2002) reported the foaming capacity between 554% and 797% when prepared with WPI (20% protein) and sugar (16.2%). Mixing xanthan gum and carrageenan to EW and whey protein resulted in the formation of stable viscoelastic and rheological properties (Liszka-Skoczylas, Ptaszek and Żmudziński, 2014; Żmudziński *et al.*, 2014)

Lipids, especially phospholipids, also affects the foaming properties of proteins when present at concentration >0.5%. Lipids are more surface-active agents than the proteins, which make them to adsorb faster than proteins. This results in the formation of less stable foam due to the lack of viscoelastic characteristics of lipid (Damodaran *et al.*, 2008). Yolk phospholipid lecithin exhibits strong antifoaming capacity. Yolk at a level 0.03% in albumen can completely inhibit foaming capacity of egg white (Bergquist, 1986). However, BSA can enhance foaming stability in the systems containing lipids (Poole, West and Fry, 1986). Gamboa and Barraquio (2012) studied the foaming properties of milk containing fat at different levels (0-3.95%). Foaming capacity and foam stability of 12% reconstituted skim milk were significantly higher than those of whole milk (3.00-3.95% fat) and 1.5% fat, 9% milk solids nonfat milk. Fats reduced foam ability due to their competition with proteins at the interface (Gamboa and Barraquio, 2012).

Salts affect the solubility of proteins, hence influencing foaming properties. Generally, salts at optimum concentration increase the solubility of proteins. Kinsella and Melachouris (1976) reported that NaCl added to soy protein suspensions increased FC, however decreased FS. The increase in foaming capacity was more likely due to higher protein solubility, in which more proteins are available to form film at air/water. However, NaCl affected the partial denaturation of polypeptides of proteins that is necessary for protein-protein interaction and stability. Additionally, NaCl can change protein conformation and solubility, depending on the ions and proteins involved. Divalent cations, such as Ca^{2+} and Mg^{2+}, improve both foam ability and foam stability at 0.02–0.4 M. This is primarily due to cross-linking of protein molecules and creation of films with better viscoelastic properties (Zhu and Damodaran, 1994a). Addition of 1.8 % NaCl increased foaming ability of water-soluble pork liver protein and mixture of water and salt soluble liver proteins, while foam stability was not affected. Further increase in NaCl (3.4 %) decreased both foaming ability and foam stability. Refolding of the protein molecules because of the higher level of hydrophobic groups with 3.4 % NaCl might occur (Steen *et al.,* 2016). Similarly, Zouari *et al.* (2011) reported that NaCl (pH 6.0) up to 1 % improved foam capacity of turkey liver proteins, while a higher NaCl concentration (2%) resulted in a decrease in foaming capacity.

1.2.8 Foaming agents

1.2.8.1 Milk proteins

Milk proteins are known to possess water binding, foaming, emulsifying, and gelling properties (Jyotsna *et al.,* 2007). Meringue, angel food cake or nougat, are stabilized by the whey proteins during the processing conditions, such as mixing, heating and drying (Foegeding, Luck and Davis, 2006). Whey proteins shows the excellent foaming properties because they are soluble and stabilize foam by increased viscosity. Whey proteins are considered as substitute for egg white proteins due to high contents of sulfhydryl and disulfide groups, heat coagulation and foaming properties (Zayas, 1997). Kappa-casein is good foaming agent due to its ability to reduce surface tension rapidly and form large foam volume with large air cells and

foaming capacity ranged from 300 to 2000% (Hailing and Walstra, 1981). Tornberg, Granfeldt and Håkansson (1982) reported that caseins were better surface tension depressors than whey proteins in their native state. Whey protein concentrate (WPC) was spray-dried at different temperature with varying concentrations of Tween 60 to obtain a WPC used in hot beverages. Spray drying at 210 °C with 1.50% Tween 60 improved foaming stability (Osorio *et al.,* 2014). As bovine β-lactoglobulin was heated at 80 °C for 90 min at different pHs (6.8 or 8) and NaCl concentration (0–130 mM), the formation of soluble aggregates with median particle diameters (2.5 nm to 1.1 μm) produced stable foam and stability was increased with increasing aggregate size (Dombrowski *et al.,* 2016).

1.2.8.2 Egg proteins

Egg contained mainly two major types of proteins; egg white (EW) protein and yolk protein. EW constitutes about 40 different proteins which make 10.5% of total EW and the rest is water (85%) and carbohydrates, ash, and trace amounts of lipids constitutes around 1% of total egg (Booth, 2003). Ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), lysozyme (3.5%), and ovomucin (3.5%) are considered as the main proteins and avidin (0.05%), cystatin (0.05%), ovomacroglobulin (0.5%), ovoflavoprotein (0.8%), ovoglycoprotein (1.0%), and ovoinhibitor (1.5%) are the minor proteins found in egg white (Kovacs-Nolan, Phillips and Mine, 2005). Proteins are distributed among EW and yolk, whereas lipids are mainly concentrated in the yolk. EW has high viscosity, which decreases with aging due to the breakdown of ovomucin-lysozyme complex. EW is the most widely used foaming agent, and due to unique combinations of proteins, the EW produces large volume foams stable to heating (Zayas, 1997).

Egg albumen is a heterogeneous protein system and basic function of albumen in foam is to provide the stability i.e. retention of a large volume of gas. Native albumen contains water-soluble, surface-active proteins that can migrate to the air/water interface (Hailing and Walstra, 1981). Denaturation causes the unfolding of egg proteins at the air/water surface and proteins with highly hydrophobic secondary structure shows rapid denaturation at the interface. Foaming properties of albumen are

related to surface denaturation of the globulin fraction of albumen followed by protein-protein interactions (Townsend and Nakai, 1983). Ovalbumin is highly structured globular protein (Ip, 4.6-4.8) constituted around 54% of total egg albumen. It is a phosphoglycerate, in which phosphate and carbohydrate are attached to the polypeptide chain. Carbohydrate increases film viscosity and foam stability (Kitabatake and Doi, 1987). Ovalbumin molecules are absorbed at the air-water interface with hydrophobic portion facing toward the air, thus exposing cysteinyl residues (Kitabatake and Doi, 1987).

Ovomucoid, which makes up about 11% of the total egg albumen solids, is a glycoprotein and contains about 20-25% carbohydrate which make it as an effective foaming agent with high foaming stability and resistant to heat in acid and alkaline media. Ovomucin stabilizes foams formed during short whipping time (Johnson and Zabik, 1981a). Improvement of foaming capacity in ovomucin solution is mostly due to increased viscosity. Protein solutions with high ovomucin content and without lysozyme showed good foaming capacity but produced cake of small size (Johnson and Zabik, 1981a). This effect of ovomucin is related to its insolubilization at the air-albumen interface, thus facilitating the formation of stable foams with lower expansion.

Ovomucin fraction, isolated from egg albumen by isoelectric precipitation, was subjected to enzymatic hydrolysis with four proteases (pronase E, alcalase, flavourzyme, neutrase) for 0–24 h. Foaming capacity reached at its optimum when degree of hydrolysis (DH) increased to 40%, resulting in lower initial surface tension. Enzymatic hydrolysis had non-significant effect on the foam stability (Hammershøj, Nebel and Carstens, 2008). Liang and Kristinsson (2007) reported that pH 12.5 induced unfolding, improved overrun, stability and reduced liquid drainage of egg albumen as compared to untreated control. Egg white exposed to pulsed light produced foams with higher stability. This effect was attributed to jamming of protein aggregates and fragments in the fluid interstices between bubbles (Manzocco, Panozzo and Nicoli, 2013).

Lysozyme has the highest isoelectric point (pH -10.7) of all the other albumen proteins with the lowest MW. Lysozyme forms viscous films and enhances

foam stability (Zayas, 1997). Lysozyme influenced the foaming properties of other egg proteins. Lysozyme, especially at higher concentrations, depressed the foaming capacity of globulins, conalbumin, and ovalbumin. The negative effect of lysozyme on foam ability may be related to the increase in the rate of formation of an ovomucin-lysozyme complex (Johnson and Zabik, 1981a). Lysozyme exhibited the improved foaming power in the absence of ovomucin. The foaming index of mixtures of egg proteins was reduced when lysozyme was added at pH 5.3 and an ionic strength of 0.20. This was due to a specific interaction between egg proteins, ovomucin and lysozyme. Johnson and Zabik (1981a) reported that the most desirable foams and cake volumes were prepared with the following protein levels: 0.2-1.0% ovomucin, 0.-0.8% lysozyme, and 12.2-14.8% globulins. Lysozyme interacts with other egg proteins with conalbumin, ovalbumin, and ovomucoid, and this interaction may influence the chemical and physical properties of egg white. Carbohydrate components of ovomucin participated in electrostatic associations with lysozyme. The formation of the ovomucin-lysozyme electrostatic complex may be sufficient to produce a change in foam stability. The formation of a damaged network of the ovomucin-lysozyme complex produces a dry foam that can be damaged by mechanical action. Globulin and ovalbumin films looked very thin; conalbumin film seemed slightly thicker and less flexible while lysozyme film was considerably thicker. (Johnson and Zabik, 1981b).

During the storage, the percentages of α-helix and β-turn of egg lysozyme declined. Conversely the β-sheet and random coil increased but the polarity of microenvironment around tryptophan residue gradually decreased. The results suggested that the conformation of lysozyme became more flexible during egg storage. Lysozyme activity was decreased but emulsion stability index and foaming ability of lysozyme rose up with the storage duration (Sheng *et al.*, 2016).

1.2.9 Modification of proteins to improve the foaming properties

Common food proteins possess good functional properties including solubility, gelation, emulsification and foaming. The functional properties of proteins are impaired near their pI. This can be formed in most acidic foods. Modification of

food proteins by physical and chemical means can enhance their functional properties over a wide pH range, and other processing conditions. The modifications are done to improve the solubility, flexibility and alteration of proteins structure etc. Kim and Kinsella (1986) reduced the disulfide bonds in soy protein to increase the molecular flexibility and foaming capacity.

1.2.9.1 Preheating

Increased unfolding of the polypeptides at the interface and facilitation of the hydrophobic associations contributes to the improved foaming properties. The moderate heating at 70-80 °C may increase unfolding of soy protein at the air-water interface during foam formation and enhance intermolecular interaction to produce a stable film. Zhu and Damodaran, (1994b) reported that heating of WPI at 70 ºC for 1 min improved the foaming properties, whereas heating at 90 ºC for 5 min impaired it. Partial heating of protein solution leads to the unfolding of protein molecules, in which the hydrophobic domains are exposed. This results in the more protein-protein interaction. As a result, stronger and more stable film is formed at the interface. The decrease in foaming properties of WPI is most probably due to the polymerization of protein via disulfide bond, which was unable to adsorb at interfacial layer. Foaming properties of unheated (native) or heated WPI dispersions of three different concentrations (1, 2 or 3%) at pH 2 and 7 were studied. Heating at 80 ºC for 20 h and at pH 7.0 led to the formation of globular aggregates. Dispersion, fibrils (2–3 nm height and up to 15 μm length) were formed at pH 2. Maximum foam overrun was obtained for all concentrations of WPI fibrillar dispersions at pH 7, while the minimum value was obtained in 1% native WPI solution at pH 7 with the exception at 3% WPI in the unheated state, where the foam overrun for pH 7 was larger than solution of pH 2 (Oboroceanu *et al.,* 2014). Pre-heating of skimmed milk was performed by high-temperature-short-time (HTST), high temperature (HT)- or ultra-high-temperature (UHT)-heating. Foam density was decreased in a continuous fashion from unheated to UHT-heated milk. Foam from unheated milk was the most stable. The viscosity of unheated milk samples was slightly higher (1.170.1 mPa. s) than that of heat-treated milk samples (0.970.1 mPa. s). The surface tension values of the milk samples and the average bubble diameter in the corresponding milk foams increased

slightly from non-heated to high-temperature heated (HT, 90 ºC, 20 s) milk and then decreased with the further application of heat. The foam density, however, decreased continuously from non-heated to UHT-heated milk. The foams prepared from UHT-heated skimmed milk drained off within 20 min. Foams prepared from unheated or HT-heated skimmed milk showed comparable drainage values between 70% and 80% after 10 and 20 min, respectively (Borcherding *et al.*, 2008). WPI exhibited a similar foaming capability than that of control mixtures (WPI: Maltodextrin, Mixture 1:1 and Mixture 1:2) and WPI treated at 50 ºC, 80% relative humidity (RH) for 48 h. WPI treated at 50 ºC, 50% RH for 24 h, showed a higher foaming capability, whereas WPI treated at 60 ºC, 80% RH for 48 h presented the lowest foaming capability. However, WPI sample treated at 60 ºC and 80% RH for 48 h showed lowest foaming capability which could be due to its low solubility (Martinez-Alvarenga *et al.*, 2014).

1.2.9.2 Partial hydrolysis

Chemical and biological methods are the most widely used for protein hydrolysis, in which chemical hydrolysis is used more commonly in industrial practices. Biological processes using added enzymes are employed more frequently, and enzyme hydrolysis holds the most promise (Shahidi, Han and Synowiecki, 1995). Protein hydrolysates are produced for a wide range of uses in the food industry, including milk replacers, protein supplements, stabilizers in beverages and flavor enhancers in confectionery products (Kristinsson and Rasco, 2000).

Enzymatic hydrolysis of proteins is a complex process because of several peptide bonds and their specific accessibility to enzymatic reactions (Linder *et al.*, 1995). The specificity of enzymes is not the only factor that affects the peptide profile of the final product. Environmental factors such as temperature and pH play an important role. Both temperature and pH can greatly affect the enzyme reaction kinetics, and the effect of these factors is different for each enzyme. Generally, there is an optimum combination of both pH and temperature, where an enzyme is the most active. Temperature and pH extremes deactivate the enzymes by denaturing them (Kristinsson and Rasco, 2000).

Acid hydrolysis was employed to improve foaming capacity of legume proteins. Soybean and amaranth proteins functionality were improved through an acid treatment followed by neutralization. Amaranth proteins were more efficient than soybean protein in decreasing the surface tension. The acid treatment improved the foam formation capacity of all samples. Foam stabilization was also enhanced by the acid treatment, however hydrolysate form soy was better foam stabilizers than amaranth protein hydrolysates (Ventureira, Martínez and Añón, 2012).

Enzymatic hydrolysis change protein conformation and structure and consequently physicochemical and functional properties of proteins. It improved the foaming properties by increasing absorption at the interface and by reducing the peptide size (van der Ven *et al.*, 2002). Jeewanthi, Lee and Paik (2015) reported that Alcalase treated WPH-35 resulted in the highest foaming expansion capacity of 288% after 5 h hydrolysis with 9.5% DH, compared to trypsin, pepsin, protease A, and protease M hydrolysates. Davis, Doucet and Foegeding (2005a) also reported on the improvement of forming ability of Alcalase 2.4L and pepsin hydrolysates of β-lactoglobulin (β-LG), compared to trypsin. Native β-LG is resistant to hydrolysis by pepsin, which has a broad specificity with a preference for cleaving after hydrophobic residues. Excessively hydrolyzed WPHs were shown to have poor foaming stability due to very short peptides production (Kilara and Panyam, 2003; Ye and Singh, 2006).

The overall highest foam overrun values were gained for ovomucin hydrolyzed by Alcalase for 1 h and by flavourzyme for 6–24 h with capacities of ∼1.5 L foam/L liquid (Hammershøj *et al.*, 2008). Foam overrun and stability of WPI was improved by limited hydrolysis with alcalase to a DH of 3% (Althouse, Dinakar and Kilara, 1995). However, the foam overrun and foam stability of β-lactoglobulin was enhanced after extensive hydrolysis by a *Bacillus licheniformis* protease with DH of 19% as indicated by the highest foam overrun. Hydrolysis to a DH of 86% resulted in increased foam stability (Ipsen *et al.*,2001). Hydrolyzed β-lactoglobulin (DH ~ 15%) had high values of elastic modulus and essentially no viscous modulus, meaning that increased foam stabilization is promoted by an elastic interface of adsorbed proteins and peptides between the air and water phases (Davis, Doucet and Foegeding, 2005b).

A small DH as 0.4% greatly improved foam overrun of soy protein that increased from 120% to 250%. As degree of hydrolysis increased to 5.0 or 5.2%, foam overrun decreased (Martínez, Farías and Pilosof, 2011).

1.2.10 Ultrasonication

1.2.10.1 Principle

Ultrasound is an acoustic wave with a frequency greater than 20 kHz, the threshold for human auditory detection (O'Sullivan *et al.*, 2016). Ultrasound can be classified in two distinct categories based on the frequency range, high frequency (100 kHz to 1 MHz) low power (<1 W cm^{-2}) ultrasound, utilized most commonly for the analytical evaluation of the physicochemical properties of food (Chemat, Zille and Khan, 2011), and low frequency (20–100 kHz) high power (10–1000 W cm^{-2}) ultrasound recently employed for the alteration of foods, either physically or chemically (McClements, 1995). Application of ultrasound to liquid systems causes

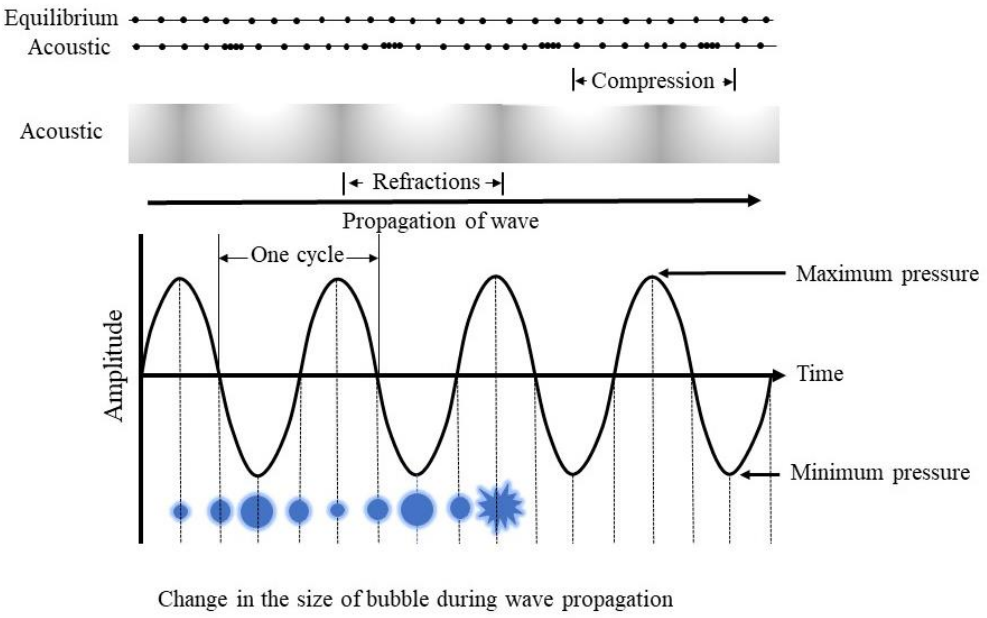

Figure 3. Cavitation caused by ultrasonication.

Source: Soria and Villamiel (2010)

acoustic cavitation which is the phenomenon of generation, growing and eventual collapse of the bubbles (Figure 3). As ultrasound waves propagate, the bubbles oscillate and collapse, causing the thermal, mechanical, and chemical effects. Mechanical effects include collapse pressure, turbulences, and shear stresses (Yusaf and Al-Juboori, 2014), while the chemical effects include generation of free radicals (Lateef, Oloke and Prapulla, 2007). The effects in the cavitation zone generate extremely high temperatures (5,000 K) and pressures (1,000 atm) (Soria and Villamiel, 2010). Depending on the frequency of the ultrasound, locally produced alternating positive and negative pressures cause expansion or compression of the material, resulting in cell rupture (Majid, Nayik and Nanda, 2015). Ultrasound causes hydrolysis of water inside the oscillating bubbles, leading to the formation of H+ and OH− free radicals that can be captured in some chemical reactions e.g. free radicals can be scavenged by amino acids of the enzymes involved in structure stability, substrate binding, or catalytic functions. This disruption effect of sonication is significantly resisted by homogenous liquids (Ercan and Soysal, 2011). During sonication treatment, bubbles produced are divided into two types on the basis of their structure: (1) Non-linear, forming large bubble clouds with equilibrium size during pressure cycles are known as stable cavitations bubbles. (2) Non-stable, rapidly collapsing and disintegrating into smaller bubbles are known as internal (transient) cavitations bubbles. These small bubbles quickly dissolve, but during bubble stretching, the mass-transfer boundary layer is thinner and the interfacial area is greater than during bubble collapse. Thus, more air transfers into the bubble during the stretching phase than leaks out during the collapse phase (Tiwari and Mason, 2012).

For liquids containing solids, similar phenomena may occur when exposed to ultrasound. Once cavitation occurs near an extended solid surface, cavity collapse is non-spherical and drives high-speed jets of liquid to the surface (Leighton, 1994). These jets and associated shock waves can damage the highly heated surface. Liquid-powder suspensions produce high velocity interparticle collisions. These collisions can change the surface morphology, composition, and reactivity (Zhang *et al.*, 2014). Ultrasound can greatly enhance chemical reactivity in a number of systems

by as much as a million-fold (Kumar and Maurya, 2008), thus effectively acting as a catalyst by exciting the atomic and molecular modes of the system (such as the vibrational, rotational, and translational modes). In addition, ultrasound breaks up the solid pieces from the energy released from the bubbles created by cavitation collapsing through them. This gives the solid reactant a larger surface area for the reaction to proceed over, increasing the observed rate of reaction (Patel *et al.*, 2010).

1.2.10.2 Application of ultrasonication in food proteins

Application of ultrasound in protein modification has received ample attention in recent years either as pretreatment in order to enhance modification or chemical reaction of protein by changing its physical and functional attributes such as, gelation, foam ability, emulsification, and solubility. The high-intensity ultrasound enhances protein solubility by changing protein conformation and structure in the way that hydrophilic parts of amino acids from inside are opened toward water (Jambrak *et al.*, 2008; Moulton and Wang, 1982). This treatment also decreases protein MW,

implying that larger area of protein is covered by molecules of water (Jambrak *et al.*, 2008). The increased temperature after treatment also contribute to enhanced solubility since in general protein solubility increases with temperature between 40 ºC and 50 ºC which is the case for using ultrasound horn (probe) (Jambrak *et al.*, 2008).

For ultrasonic emulsification, viscosity decreases and lesser particle size distribution in sub-micron oil-droplets emulsions is obtained. However, change in sonication parameters caused remarkable change in stability and oil droplet size of the emulsion formed (Kaltsa *et al.*, 2013). Ultrasound treatment of food proteins has been related to affect the physicochemical properties of a number of protein sources including soy protein isolate/concentrate (Arzeni *et al.*, 2012; Hu *et al.*, 2013) and egg white protein (Arzeni *et al.*, 2012; Carolina *et al.* 2012). Increases in the hydrophobicity and emulsion stability of ultrasound treated egg white protein (EWP) were found in comparison to untreated EWP (Arzeni *et al.*, 2012a; Arzeni *et al.*, 2012b). In addition, Arzeni *et al.* (2012) described a significant reduction in protein aggregate size for soy protein isolate (SPI) when ultrasound was used. High intensity ultrasonication (HIUS) treatment (20 min) and HIUS with temperature increased the

foaming capacity 228% by alteration of particle size. However, foam stability was not improved significantly (Morales *et al.*, 2015). Similarly, foaming properties of SPI and egg yolk were improved by modifying isoelectric point to 10, and sonication was used to increase protein dispersibility after the ethyl esterification reaction. However, only the addition of sonicated and modified SPI (SMSPI) showed improvement of foaming in the 5% egg protein model system with 0.4% yolk addition (Wang *et al.*, 2012). Foam capacities and foam stabilities of whey proteins were also improved after ultrasound treatments at 20 kHz and 40 kHz (Jambrak *et al.*, 2008). Foam capacities WPI and WPC were increased after ultrasonication treatment for 15 min at 20 kHz probe treatment, and 40 kHz bath (132% to 235% and 220% and 124% to 221% and 209%, respectively) (Jambrak *et al.*, 2008). Foam stabilities were improved for all samples after 20 kHz probe, having the highest increase for WPI (68.3–98.4 min) and WPC (55.6–89.5 min) samples, and after 40 kHz bath treatments having the highest increase for HWP (65.7–85.6 min) samples (Jambrak *et al.*, 2008). The foaming properties of wheat gluten was improved to the highest value of 160% from 70% after treatment with ultrasonication power of 900 W (Zhang *et al.*, 2011). Recently, Xiong *et al.* (2018) observed the increase in FC of pea protein isolated from 145.6% to 200.0% with increasing amplitude from 30 to 90% for 30 min.

1.2.11 Muffins and cake-like system

The matrix of muffins or cakes is a solid foam. Its aerated structure is formed by incorporating bubbles into the batter during mixing and baking. These bubbles eventually become interconnected gas cells in the crumb of the final (Tan *et al.*, 2015). For most muffins or cakes, a desirable eating quality entails high volume and a light, open, fine texture. Proteins are the essential building component of these aerated structures (Davis, Foegeding and Hansen, 2004). Final quality is dependent on the original foam, such as high-ratio cake formulations as it required to withstand considerable stresses during processing and drying and remain aerated (Chesterton *et al.*, 2015). EWP is one of most reliable foaming agents. Whey protein has been studied as potential replacers, however the lack of stability is a major problem (Foegeding *et al.*, 2002). Pea protein isolate (PPI) with non-allergic nature and high nutritional value make it a good candidate for food foaming applications

(Gharsallaoui *et al.,* 2009). Muffins and cake-like products are made up of moderate to high sugar content and variable levels of flour, eggs and fat. Other commonly used ingredients are baking powders, emulsifiers and milk. These ingredients cause the bubble formation in the batter, which provide the desired texture to product.

Rise in temperature in the oven gelatinizes the starch and coagulates the proteins increase the viscosity of the batter (Delcour and Hoseney, 2010). Baking process leads to the formation of solid structure by starch and protein, which is strong enough to provide strength to the product. The firm structure is due to the combined effect of the gelled starch and the gel network of coagulated egg proteins. Starch granules have two main functions: they swell to form the 'building bricks' of the final crumb and as they swell, they bind the excess water in the system (Donovan, 1977). The most widely used flour is wheat. The way the batter behaves during baking depends on the size of the starch granules, their gelatinization temperature and the amount of water they are capable of absorbing (Kilcast, 2013). These properties also vary according to the moisture and sugar content. The sum of all these factors determines the final volume of the baked cake (Kilcast, 2013).

Cakes prepared with nonfat dry milk (NFDM) and soy products were softer than those prepared without protein sources. NFDM also increased weight and volume index (117.24 g and 157.75) of cake than the control (119.48 g and 154.00). NFDM reduces the moisture loss during baking and maintains the moisture content during storage (Alp and Bilgiçli, 2008). Flour from chickpea with different varieties (Andaluz, Pedrosillano, Lechoso and Sinaloa varieties) and the kind of flour used (white or whole) determined the characteristics of product. Volume, symmetry, chroma, and crust and crumb L* diminished when increasing the amount of chickpea flour. The replacement of wheat flour by chickpea flour also induced an increase in the initial firmness but cohesiveness and resilience diminished. Pedrosillano and Sinaloa produced cakes with the highest volume and gave layer cakes with the lowest firmness, gumminess and chewiness (Gómez *et al.,* 2008).

1.2.12 Surimi

Surimi is washed fish mince rich in myofibrillar proteins, which are solubilized at high ionic strength. It has unique gelling properties and can be used for manufacturing of several products. The main steps in surimi preparation include heading, gutting and deboning; mincing; washing and dewatering; refining; screw press; stabilizing surimi with cryoprotectants followed by freezing and frozen storage (Park *et al.,* 2013). Surimi possesses the functionalities including gelling, water and fat binding and emulsifying properties and can be used as a functional protein ingredient in several products

Generally, lean fish are commonly used for surimi preparation due to the superior gelation and white color of resulting gels. In addition, dark fleshed fish had darker color and exhibited poor gelling property. Apart from its higher lipid content and susceptibility to oxidation, higher concentrations of trimethylamine oxide (TMAO), sarcoplasmic proteins and protease activity are associated with poor quality of gels (Eymard *et al.,* 2005; Shimizu, Toyohara and Lanier, 1992). Myoglobin and hemoglobin are responsible for the red color of dark muscle but they also promote lipid oxidation (Eymard *et al.,* 2005). Nevertheless, the role of sarcoplasmic proteins such as myoglobin is controversial. Those components hamper myosin cross-linking during the gelation process (Park and Park, 2007). Murakawa *et al.* (2003) reported that oxidized lipids can interact with proteins, induce denaturation, and polymerization, thereby showing an adverse effect on the quality of surimi gels.

1.2.12.1 Surimi gelation

Gelation is an aggregation of proteins, forming a three-dimensional network, which entraps water (Pomeranz, 1991). Myofibrillar proteins (myosin and actin mainly) are responsible for the gelation. Myofibrillar proteins are firstly solubilized by salt to obtain the paste. Subsequently, the paste is generally subjected to two steps heating (40/60 and 90 ºC). The gel network is formed when sufficient intermolecular bonds occur (Figure 4). It is stabilized by ionic linkages, hydrophobic interactions, covalent bonds (disulfide bonds and covalent cross-linking), and hydrogen bonds (Lanier, Carvajal and Yongsawatdigul, 2005). Myosin is the most

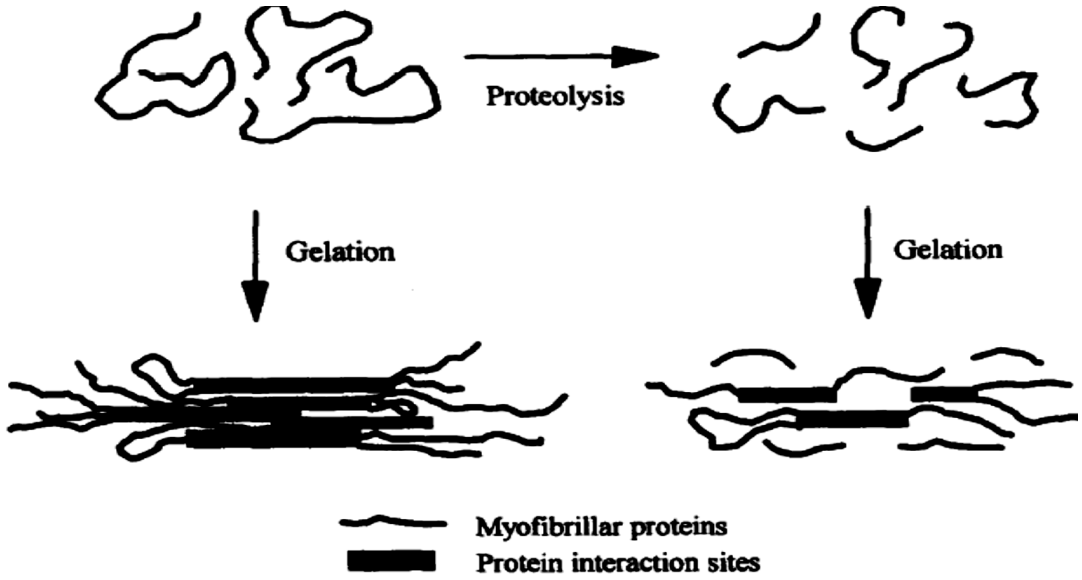

Figure 4. Surimi gelation and proteolysis of myofibrillar proteins.

Source: An, Peters and Seymour (1996)

important component for adequate gel formation in fish gel products. Sano *et al.* (1988) reported that the gelation of carp actomyosin occurred in two stages: at temperature ranges of 30-41 °C and 51-80 °C. First stage of gel development was due to interactions among the tail portions of myosin molecules, followed by the second stage, which was attributed to hydrophobic interactions among the head portions of myosin (Sano *et al.*, 1990). Samejima, Ishioroshi and Yasui (1981) also proposed that the heat-induced gelation of myosin occurred in two steps: (1) aggregation of the globular head segments of the myosin molecules, which is closely associated with the oxidation of sulfhydryl groups and (2) network formation resulting from the unfolding of the helical tail segment. Protein conformation changes during heating and the hydrophobic amino acids, which are found mainly in the head portion, become more exposed (Sano *et al.*, 1990). Gelation is affected by the myosin/actin ratio, determining the synergistic or antagonistic effect of actin on myosin gelation (Matsumoto, 1980). Surimi product from pre-rigor mortis tilapia showed the better gel properties than that from post-rigor fish. However, rigor mortis had a little effect on the gelation of fish protein isolate (FPI). The inclusion of 2% salt into FPI improved gel strength, although the addition of 3% salt into FPI decreased G′ as well as gel strength (Kobayashi, Mayer and Park, 2017). However, the addition of salt did

not enhance gelation properties in tilapia surimi, possibly due to the chopping conditions. Therefore, the degree of protein unfolding prior to gelation is an important factor to improve gel qualities. Additionally, some additives have been known to affect gelling properties of surimi. Cando *et al.* (2016), reported that cystine (0.1%), tetra-sodium pyrophosphate (0.05%) and lysine (0.1%) improved the textural and rheological properties of surimi from *Alaska Pollock*. This was more likely due to the induction of myofibrillar protein unfolding by these additives, thus facilitating the formation of the types of bonds needed to establish an appropriate gel network (Cando *et al.*, 2016). Ohmic heating was reported to minimize proteolysis of surimi from lizardfish and goatfish as compared to the water bath heating. Ohmic heating increased breaking force and deformation of thread bream and bigeye snapper surimi by 1.3 and 1.6 times, respectively, as compared to water bath heating. Gels heated ohmically had the greater extent of disulfide bond formation as compared to gels heated in water bath at 90 °C. In addition, rapid heating method with shorter heating time improved water holding capacity and whiteness of tropical surimi gels when compared to water bath heating (Tadpitchayangkoon, Park and Yongsawatdigul, 2012).

1.2.12.2 Setting phenomenon (Suwari)

Setting is a phenomenon explaining the increased textural properties of surimi gels after pre-incubation at a certain temperature below 40 °C for a specific period of time prior to cooking (Lanier, Carvajal and Yongsawatdigul, 2000). Setting involves gel network formation of muscle proteins triggered by protein unfolding. Setting temperature is dependent on the fish species, habitat temperature and heat stability of myosin of respective species (Morales *et al.*, 2001). Setting can be carried out at low (0-4 °C), medium (25 °C), and high (40 °C) temperatures (Lanier *et al.*, 2000). Setting at different temperatures may lead to different gel characteristics, especially with different fish species. Since setting at low temperature takes a longer time, it is not commonly implemented in the industries (Benjakul, Chantarasuwan and Visessanguan, 2003). So far, high-temperature settings are more widely used, due to the shorter time required, but protein degradation, induced by modori-inducing proteinase, generally active at 50-60 °C, can occur (Benjakul *et al.*, 2003). Therefore,

medium temperature setting can be an alternative for the manufacturer to obtain a better gel quality without severe proteolytic degradation. Setting of surimi from cold water fish such as Alaska pollock from Bering sea and Pacific whiting surimi was achieved at 4-5 and 25 °C (Kim *et al.*, 1993). High temperature setting at 40 °C has been applied for surimi from tropical or warm water fish species (Lee and Park, 1998; Morales *et al.*, 2001; Yongsawatdigul, Worratao and Park, 2002). Heating process induced protein aggregation of proteins, which is stabilized by hydrophobic interaction and disulfide bonds (Benjakul *et al.*, 2003). Formation of large aggregates is presumably a prerequisite for formation of a good elastic gel (Chan, Gill and Paulson, 1992). Due to instability of hydrogen bonds during the heating process, the alpha helix unfolds, exposing hydrophobic amino acids, which further undergo hydrophobic interaction (Benjakul *et al.*, 2003). Alvarez and Tejada (1997) reported that sardine kamaboko gels had much higher gel strength than the corresponding suwari gels, indicating that protein–protein interactions were established at cooking temperature, which strengthened the network previously formed by setting.

Transglutaminase (TGase) has been reported to be the major enzyme involving polymerization of myosin during setting (Kamath *et al.*, 1992). It helps in the formation of cross-links between myosin heavy chain (MHC) by covalent ε-(γ-glutamyl) lysine cross-linkage (Benjakul, Visessanguan and Chantarasuwan, 2004). The subsequent setting of surimi at appropriate temperature aims to improve the gel strength by activating TGase (Benjakul *et al.*, 2004; Moreno, Carballo and Borderías, 2008). Ca^{2+} is required for the catalytic activity of tissue or indigenous TGases (Yongsawatdigul *et al.*, 2002) and play an important role in gel strengthening via cross-linking of MHC (Kumazawa *et al.*, 1995). Ca^{2+} has been reported to improve textural properties of surimi from threadfin bream after setting at either low or high temperatures but breaking force value of gels set at 40 °C was higher than at 25 °C (Yongsawatdigul *et al.*, 2002). Incorporation of $CaCl_2$ at 50 mM/kg in combination with 3% WPC effectively improved the gel strength of goatfish kamaboko gel (Benjakul *et al.*, 2010). Since surimi from some species has poor setting phenomenon, external TGase, especially microbial TGase, has been employed. Duangmal and Taluengphol (2010) reported that breaking force of red tilapia surimi gel was

enhanced by 240% fold with addition of 2g/kg MTGas/g surimi. Kudre and Benjakul (2013) found the improved gel properties of sardine surimi with the addition of MTGase in combination with Bambara groundnut protein isolate (BGPI). Kumazawa *et al.* (1995) suggested that setting phenomenon was suppressed in the presence of TGase inhibitors, such as NH_4Cl and EDTA. Kaewudom, Benjakul and Kijroongrojana (2013) incorporated fish gelatin up to 10% in combination with 1.2 units MTGase/g surimi to obtained the surimi with grade AA grade. This combination increased the breaking force by 200% as compared to the control. Furthermore, MTGase addition at 0.8% with and without high pressure (80 MPa) increased the breaking force by 748.05% and 226.37%, respectively, in surimi from flying fish (Herranz *et al.*, 2013).

1.2.12.3 Gel weakening (modori)

Degradation of muscle proteins occurred due to the endogenous heat-activated proteinases (An *et al.*, 1996; Benjakul *et al.*, 1997), which results in the formation of weak or soft gel. Gel softening or gel weakening is termed "modori" (Morrissey *et al.*, 1993; Benjakul *et al.*, 2004). Proteolytic activity in muscle is high at temperature above 50 °C and causes the rapid and severe degradation of myofibrillar proteins, particularly myosin (Wasson *et al.*, 1993). Proteinases associated with modori can be categorized into two major groups: cathepsin (Seymour *et al.*, 1994) and heat-stable alkaline proteinase (Wasson *et al.*, 1993). High level of cysteine proteinase activity mediated by cathepsin B, H, and L was found in Pacific whiting and arrowtooth flounder (Wasson *et al.*, 1993), chum salmon and mackerel (Lee, Chen and Jiang, 1993) during spawning migration (Yamashita and Konagaya, 1990). Softening of arrowtooth flounder gel is due to a cysteine proteinase that has the maximum autolytic activity at 50-60°C (Greene and Babbitt, 1990). Klomklao, Kishimura and Benjakul (2008) reported the presence of proteinases in sardine (*Sardinop*s *melanosticus*). Cathepsins B and H are easily washed off during surimi processing, whereas cathepsin L remains in the muscle tissue (An *et al.*, 1994; Park and Morrissey, 2000). Cathepsin L has an optimum temperature of 55 °C and causes textural deterioration when the Pacific whiting surimi paste is slowly heated (An *et al.*, 1994). Benjakul *et al.* (2003) compared the autolysis of two species of bigeye

snapper and found that *P. macracanthus* had higher degradation of myosin heavy chain in both mince and washed mince than those from *P. tayenus*, especially when the incubation time was increased. During setting, proteolysis takes place to some degree and may affect the properties of resulting gels. Benjakul *et al.* (2004a) reported that suwari gel from some tropical fish, prepared by setting at 25°C showed lower degradation although the setting time increased up to 8 h, compared with setting at 40 °C. Kamath *et al.* (1992) found that proteolysis in croaker paste increased with increasing temperature of setting, especially in the temperature range of 40-50 °C. Proteolytic degradation of surimi gels is increased at temperatures above 50 °C with the rapid and severe degradation of myofibrillar proteins, particularly myosin (Jiang *et al.*, 2000). Tachasirinukun, Chaijan and Riebroy (2016) also reported that gels from spotted featherback (SF) muscle set at 4 °C/18 h and 60 °C/30 min exhibited higher proteolytic degradation than setting at 25 °C/30 min, 25 °C/2 h and 40 °C/30 min. Kudre, Benjakul and Kishimura (2013) found that sardine surimi exhibited maximum hydrolysis at 65 °C, however proteolysis markedly decreased when incubated above 65 °C, probably due to thermal denaturation of endogenous proteases. Similarly, surimi from *Sardinella gibbosa* showed the highest autolysis temperature at 70 °C (Rawdkuen *et al.*, 2007). Mince from scad had higher proteolytic activity than washed mince. The highest autolysis was observed at 65 and 60°C for mince and washed mince, respectively. Both mince and washed mince showed the optimum pH for autolysis at 9.0, and their activities decreased with increasing NaCl concentration (0–3.5%) (Wongwichian *et al.*, 2016).

1.2.13 Inhibition of modori

To tackle modori or weakening of surimi, numerous food-grade protease inhibitors have been widely used in surimi (Benjakul *et al.*, 2004a). The most widely used inhibitors are egg white and WPC (Choi, Lanier and Park, 2005). Nevertheless, some disadvantages arise with the addition of protein additives. Egg white makes more elastic and stretchable gel after partially heat. Ovalbumin caused aggregation during heating due to hydrophobically driven protein–protein interactions. Egg white added at 10–30 g/kg improved the mechanical properties of red tilapia surimi gels obtained by setting at 40 °C for 90 min, followed by heating at

90 °C for 30 min, as well as for surimi gels from arabesque greenling (*P. azonus*) and walleye pollock (*T. chalcogramma*), incubated at 25 °C for up to 15 h before cooking at 90 °C for 30 min (Duangmal and Taluengphol, 2010; Kato *et al.,* 2010). EWP contained several trypsin inhibitors and ovoinhibitor was the most active trypsin inhibitor in EWP (Weerasinghe *et al.,* 1996). Ovastatin with MW of 780 kDa has a potential to inhibit trypsin and is homologous to α_2-macroglobulin (α_2M) in molecular structure, function, and inhibition mechanism (Nagase and Harris, 1983). Cystatin, a cysteine proteinase inhibitor, was also found in egg white (Anastasi *et al*., 1983). Apart from their protease inhibitory activities, EW was able to form gel, thus having the filler effect or binding ability to strengthen the surimi gel matrix (Anastasi *et al*., 1983). Lu and Chen (1999) reported that the EWP also functioned as a binder in meat. The water holding capacity (WHC) of Alaska pollock and Pacific whiting surimi gels was affected by potato starch (4%) and/or egg white (1%). The gels were treated with ultra-high pressure or heat (90°C, 40 min). For Alaska pollock surimi gels, WHC varied from 90.6 g/100 g to 96.6 g/100 g, and from 92.7 g/100 g to 96.6 g/100 g in Pacific whiting (Tabilo-Munizaga and Barbosa-Cánovas, 2005). Hunt, Park and Handa (2009) studied the effect of different types of egg white protein including regular dried egg white (REW), special dried egg white (SEW), liquid egg white (LEW) on Pacific whiting and Alaska pollock surimi. It was suggested that 2% to 3% SEW improved textural properties of Pacific whiting and Alaska pollock (Hunt *et al.*, 2009).

WPC increases the shear strain of surimi gels prepared from Pacific whiting and Alaska pollock (Park, 1994; Piyachomkwan and Penner, 1995; Weerasinghe *et al.,* 1996b). Rawdkuen and Benjakul (2008) reported that breaking force and deformation of kamaboko gels of all surimi increased as the levels of WPC added increased (0-3%). WHC of kamaboko gels was improved with increasing concentrations of WPC. However, WPC at 3% (w/w) significantly decreased the whiteness of the gels. The microstructure of surimi gels generally became denser with the addition of WPC. Addition of 3% WPC in goatfish surimi increased the breaking force by 50 and 116% for kamaboko and modori gel containing $CaCl_2$ (50 mM/kg surimi), respectively, and by 45 and 157% for kamaboko and modori gel without

$CaCl_2$, respectively. In addition the water holding capacity was also increased by the addition of 3% WPC (Benjakul *et al.*, 2010).

Chicken, porcine and beef plasma proteins have been used (Benjakul *et al.*, 2004a; Park, 2005), but in all cases some disadvantages arise. Morrissey *et al.* (1993) reported that BPP showed the highest inhibitory activity in Pacific whiting surimi as compared to egg white and potato extract. Yada (2004) also reported that BPP exhibits a remarkable capability of inhibiting modori, or gel weakening, during the cooking of surimi. The addition of BPP up to 3% increased gel strength of lizardfish surimi more effectively, but resulted in a lower whiteness (Benjakul *et al.*, 2004a). Similar results were obtained for surimi gels from arabesque greenling (*Pleurogrammus azonus*) and walleye pollock (*Theragra chalcogramma*) added with BPP (Kato *et al.*, 2010). Rawdkuen *et al.* (2007) reported that cysteine proteinase inhibitor containing fraction from chicken plasma could inhibit autolysis and improve gelation of Pacific whiting surimi. Li, Lin and Kim (2008) found that the addition of rainbow trout plasma protein improved whiteness, gel texture, and water-holding capacity of Alaska pollock surimi. Fowler and Park (2015) studied the effect of salmon blood plasma (SPP) on the Pacific whiting surimi and salmon mince and observed the inhibition of autolysis of surimi from Pacific whiting and salmon mince. SPP exhibited strong inhibition towards cysteine and serine proteases. SPP significantly increased gel strength of Pacific whiting surimi, heated ohmically to and held at 60° for 30 min, followed by heating ohmically to 90 °C. SPP at a level of 1% was also found to increase gel strength in gels held at 25 °C for 2 h prior to ohmic heating (Fowler and Park, 2015).

Protein isolate or protein concentrate from plant origin such as legume seed, etc. have been used as a source of protease inhibitors. Klomklao and Benjakul (2015) observed the increases in breaking force and deformation of kamaboko gel with addition of trypsin inhibitor from adzuki beans. The modori gels from sardine surimi added with protein isolates from Thai legumes had the increased gel strength since the protein isolates inhibited the protease activity in the surimi (Kudre, Benjakul and Kishimura, 2013). Bambara groundnut protein isolate with trypsin inhibitory activity increased breaking force and deformation of gels prepared from threadfin

bream surimi (Oujifard *et al.*, 2012). Bambara groundnut and mungbean protein isolate enhanced the gel strength of both modori and kamaboko gel from sardine surimi due to the presence of trypsin inhibitors in the protein isolates from these two beans. Bambara groundnut was also reported to contain a high amount of trypsin inhibitor (Kudre and Benjakul, 2013; Benjakul *et al.*, 2000). Furthermore, Minor-Pérez (2017) observed the reduction in the proteolytic activity in myofibrillar proteins from jumbo squid mantle with the addition of soybean flour in a dose dependent manner during the storage at 4 and 10 ºC.

Recently, protease inhibitor from fish roe has been extracted and used in surimi. Klomklao *et al.* (2016) reported that isolated trypsin inhibitor from the roe of yellowfin tuna (*Thunnus albacores*) (TIYTR). TIYTR showed inhibitory activity against proteolysis in kamaboko (40/90 °C) and modori (60/90 °C) gels from bigeye snapper surimi in a concentration-dependent manner and retained MHC in both gels when the level of TIYTR increased up to 3.0%. Additionally, breaking force of both kamoboko and modori gels containing TIYTR increased as the amount of TIYTR increased.

1.3 Objectives

1) To characterize and study the foaming properties of squid ovary powder (SOP).

2) To improve foaming property of squid ovary with partial enzymatic hydrolysis.

3) To investigate the effect of ultrasonication under various conditions on the foaming properties of SOP.

4) To replace the EWP with USOP in cake and to characterize the properties of resulting cake.

5) To extract and partially purify serine protease inhibitor from squid ovary (SOSPI) and to study the effect of SOSPI on gel properties of suwari and kamaboko gel from bigeye snapper surimi.

6) To study the effect of SOSPI on the gel and sensory properties of surimi from Indian mackerel.

7) To extract chitin using ultrasonication from squid pen and to investigate the effect of different deacetylation condition on molecular and fat binding properties of chitosan.

8) To prepare chitooligosaccharide (COS) from squid pen chitosan using different enzymes and to investigate its impact on properties and storage stability of sardine surimi gel stored at 4 °C.

1.4 References

Aam, B. B., Heggset, E. B., Norberg, A. L., Sørlie, M., Vårum, K. M. and Eijsink, V. G. H. 2010. Production of chitooligosaccharides and their potential applications in medicine. Marine Drugs. 8: 1482-1517.

Abdou, E. S., Nagy, K. S. A. and Elsabee, M. Z. 2008. Extraction and characterization of chitin and chitosan from local sources. Bioresource Technology. 99: 1359-1367.

Aiba, S. 1991. Studies on chitosan: 3. Evidence for the presence of random and block copolymer structures in partially N-acetylated chitosans. International Journal of Biological Macromolecules. 13: 40-44.

Alak, G. 2012. The effect of chitosan prepared in different solvents on the quality parameters of Brown Trout Fillets (*Salmo trutta* fario). Food and Nutrition Sciences. 3: 1303.

Alvarez, C. and Tejada, M. 1997. Influence of texture of suwari gels on kamaboko gels made from sardine (*Sardina pilchardus*) surimi. Journal of the Science of Food and Agriculture. 75: 472-480.

Amiza, M. and Kang, W. 2013. Effect of chitosan on gelling properties, lipid oxidation, and microbial load of surimi gel made from African catfish (*Clarias gariepinus*). International Food Research Journal. 20: 1585.

An, H., Peters, M. Y. and Seymour, T. A. 1996. Roles of endogenous enzymes in surimi gelation. Trends in Food Science and Technology. 7: 321-327.

An, H., Weerasinghe, V., Seymour, T. A. and Morrissey, M. T. 1994. Cathepsin degradation of Pacific whiting surimi proteins. Journal of Food Science. 59: 1013-1017.

Anthonsen, M. W., Vårum, K. M. and Smidsrød, O. 1993. Solution properties of chitosans: conformation and chain stiffness of chitosans with different degrees of N-acetylation. Carbohydrate Polymers. 22: 193-201.

Aşik, E. and Candoğan, K. 2014. Effects of chitosan coatings incorporated with garlic oil on quality characteristics of shrimp. Journal of Food Quality. 37: 237-246.

Bautista-Baños, S., Hernández-Lauzardo, A. N., Velázquez-del Valle, M. G., Hernández-López, M., Ait Barka, E., Bosquez-Molina, E. and Wilson, C. L.

2006. Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. Crop Protection. 25: 108-118.

Benjakul, S., Chantarasuwan, C. and Visessanguan, W. 2003. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chemistry. 82: 567-574.

Benjakul, S., Seymour, T. A., Morrissey, M. T. and An, H. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced Storage. Journal of Food Science. 62: 729-733.

Benjakul, S., Visessanguan, W. and Chantarasuwan, C. 2004. Cross-linking activity of sarcoplasmic fraction from bigeye snapper (*Priacanthus tayenus*) muscle. LWT - Food Science and Technology. 37: 79-85.

Benjakul, S., Visessanguan, W. and Leelapongwattana, K. 2003. Purification and characterization of heat-stable alkaline proteinase from bigeye snapper (*Priacanthus macracanthus*) muscle. Comparative Biochemistry and Physiology. Part B: Biochemistry and Molecular Biology. 134: 579-591.

Benjakul, S., Visessanguan, W. and Tueksuban, J. 2003. Changes in physico-chemical properties and gel-forming ability of lizardfish (*Saurida tumbil*) during post-mortem storage in ice. Food Chemistry. 80: 535-544.

Benjakul, S., Visessanguan, W., Tueksuban, J. and Tanaka, M. 2004a. Effect of some protein additives on proteolysis and gel-forming ability of lizardfish (*Saurida tumbil*). Food Hydrocolloids. 18: 395-401.

Benjakul, S., Yarnpakdee, S., Visessanguan, W. and Phatcharat, S. 2010. Combination effects of whey protein concentrate and calcium chloride on the properties of goatfish surimi gel. Journal Texture Studies. 41: 341-357.

Brine, C. J. and Austin, P. R. 1981. Chitin variability with species and method of preparation. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry. 69: 283-286.

Cando, D., Herranz, B., Borderías, A. J. and Moreno, H. M. 2016. Different additives to enhance the gelation of surimi gel with reduced sodium content. Food Chemistry. 196: 791-799.

Chalamaiah, M., Jyothirmayi, T., Diwan, P. V. and Dinesh Kumar, B. 2015. Antioxidant activity and functional properties of enzymatic protein

hydrolysates from common carp (*Cyprinus carpio*) roe (egg). Journal of Food Science and Technology. 52: 5817-5825.

Chan, J. K., Gill, T. A. and Paulson, A. T. 1992. Cross-linking of myosin heavy chains from cod, herring and silver hake during thermal setting. Journal of Food Science. 57: 906-912.

Chandumpai, A., Singhpibulporn, N., Faroongsarng, D. and Sornprasit, P. 2004. Preparation and physico-chemical characterization of chitin and chitosan from the pens of the squid species, *Loligo lessoniana* and *Loligo formosana*. Carbohydrate Polymers. 58: 467-474.

Chang, K. L. B., Tsai, G., Lee, J. and Fu, W.-R. 1997. Heterogeneous N-deacetylation of chitin in alkaline solution. Carbohydrate Research. 303: 327-332.

Chaussard, G. and Domard, A. 2004. New aspects of the extraction of chitin from squid pens. Biomacromolecules. 5: 559-564.

Choi, Y. J., Lanier, T. C. and Park, J. W. 2005. Proteolytic enzymes and control in surimi. *In* Surimi and surimi seafood. (Park, J. W., ed.). p. 227-277. Taylor and Francis group. Boca Raton, Fla.

Cuong, H. N., Minh, N. C., Van Hoa, N. and Trung, T. S. 2016. Preparation and characterization of high purity β-chitin from squid pens (*Loligo chenisis*). International Journal of Biological Macromolecules. 93: 442-447.

Damodaran, S., Parkin, K. L. and Fennema, O. R. 2008. Fennema's Food Chemistry. CRC Press/Taylor and Francis. Boca Raton.

Duangmal, K. and Taluengphol, A. 2010. Effect of protein additives, sodium ascorbate, and microbial transglutaminase on the texture and colour of red tilapia surimi gel. International Journal of Food Science and Technology. 45: 48-55.

El Knidri, H., El Khalfaouy, R., Laajeb, A., Addaou, A. and Lahsini, A. 2016. Eco-friendly extraction and characterization of chitin and chitosan from the shrimp shell waste via microwave irradiation. Process Safety and Environmental Protection. 104: 395-405.

FAO. 2014. The state of world fisheries and aquaculture. *In* Food and Agriculture Organization. United Nations, Rome. .

Fowler, M. R. and Park, J. W. 2015. Effect of salmon plasma protein on Pacific whiting surimi gelation under various ohmic heating conditions. LWT - Food Science and Technology. 61: 309-315.

Fowler, M. R. and Park, J. W. 2015. Salmon blood plasma: Effective inhibitor of protease-laden Pacific whiting surimi and salmon mince. Food Chemistry. 176: 448-454.

Gerasimenko, D. V., Avdienko, I. D., Bannikova, G. E., Zueva, O. and Varlamov, V. P. 2004. [Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms]. Prikladnaya Biokhimiya i Mikrobiologiya. 40: 301-306.

Greene, D. H. and Babbitt, J. K. 1990. Control of muscle softening and protease-parasite interactions in arrowtooth flounder *Atheresthes stomias*. Journal of Food Science. 55: 579-580.

Hamed, I., Özogul, F. and Regenstein, J. M. 2016. Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. Trends in Food Science and Technology. 48: 40-50.

Helgason, T., Weiss, J., McClements, D. J., Gislason, J., Einarsson, J. M., Thormodsson, F. R. and Kristbergsson, K. 2008. Examination of the interaction of chitosan and oil-in-water emulsions under conditions simulating the digestive system using confocal microscopy. Journal of Aquatic Food Product Technology. 17: 216-233.

Herranz, B., Tovar, C. A., Borderias, A. J. and Moreno, H. M. 2013. Effect of high-pressure and/or microbial transglutaminase on physicochemical, rheological and microstructural properties of flying fish surimi. Innovative Food Science and Emerging Technologies. 20: 24-33.

Hoque, M. S., Benjakul, S. and Prodpran, T. 2010. Effect of heat treatment of film-forming solution on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. Journal of Food Engineering. 96: 66-73.

Huang, J., Zhao, D., Hu, S., Mao, J. and Mei, L. 2012. Biochemical activities of low molecular weight chitosans derived from squid pens. Carbohydrate Polymers. 87: 2231-2236.

Hunt, A., Park, J. W. and Handa, A. 2009. Effect of various types of egg white on characteristics and gelation of fish myofibrillar proteins. Journal of Food Science. 74: C683-692.

Hwang, K. T., Jung, S. T., Lee, G. D., Chinnan, M. S., Park, Y. S. and Park, H. J. 2002. Controlling molecular weight and degree of deacetylation of chitosan by response surface methodology. Journal of Agricultural and Food Chemistry. 50: 1876-1882.

Intarasirisawat, R., Benjakul, S. and Visessanguan, W. 2011. Chemical compositions of the roes from skipjack, tongol and bonito. Food Chemistry. 124: 1328-1334.

Jiang, S. T., Hsieh, J. F., Ho, M. L. and Chung, Y. C. 2000. Microbial transglutaminase affects gel properties of golden threadfin-bream and Pollack surimi. Journal of Food Science. 65: 694-699.

Kaewudom, P., Benjakul, S. and Kijroongrojana, K. 2013. Properties of surimi gel as influenced by fish gelatin and microbial transglutaminase. Journal of Food Bioscience. 1: 39-47.

Kamath, G. G., Lanier, T. C., Foegeding, E. A. and Hamann, D. D. 1992. Nondisulfide covalent cross-linking of myosin heavy chain in “setting” of Alaska pollock and Atlantic croaker surimi. Journal of Food Biochemistry. 16: 151-172.

Kim, K. W. and Thomas, R. L. 2007. Antioxidative activity of chitosans with varying molecular weights. Food Chemistry. 101: 308-313.

Kim, S. H., Carpenter, J. A., Lanier, T. C. and Wicker, L. 1993. Setting response of Alaska pollock surimi compared with beef myofibrils. Journal of Food Science. 58: 531-534.

Kittur, F. S., Kumar, A. B. V., Gowda, L. R. and Tharanathan, R. N. 2003. Chitosanolysis by a pectinase isozyme of *Aspergillus niger*—A non-specific activity. Carbohydrate Polymers. 53: 191-196.

Kjartansson, G. T., Zivanovic, S., Kristbergsson, K. and Weiss, J. 2006. Sonication-assisted extraction of chitin from North Atlantic shrimps (*Pandalus borealis*). Journal of Agricultural and Food Chemistry. 54: 5894-5902.

Kjartansson, G. T., Zivanovic, S., Kristbergsson, K. and Weiss, J. 2006. Sonication-assisted extraction of chitin from shells of fresh water prawns (*Macrobrachium rosenbergii*). Journal of Agricultural and Food Chemistry. 54: 3317-3323.

Klomklao, S., Benjakul, S., Kishimura, H., Osako, K. and Simpson, B. K. 2016. Trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe: Effects on gel properties of surimi from bigeye snapper (*Priacanthus macracanthus*). LWT - Food Science and Technology. 65: 122-127.

Klomklao, S., Kishimura, H. and Benjakul, S. 2008. Endogenous proteinases in true sardine (*Sardinops melanostictus*). Food Chemistry. 107: 213-220.

Kobayashi, Y., Mayer, S. G. and Park, J. W. 2017. Gelation properties of tilapia fish protein isolate and surimi pre- and post-rigor: Rigor condition of tilapia FPI and surimi. Journal of Food Bioscience. 17: 17-23.

Kubota, N. and Eguchi, Y. 1997. Facile preparation of water-soluble N-acetylated chitosan and molecular weight dependence of its water-solubility. Polymer Journal. 29: 123.

Kudre, T., Benjakul, S. and Kishimura, H. 2013. Effects of protein isolates from black bean and mungbean on proteolysis and gel properties of surimi from sardine (*Sardinella albella*). LWT - Food Science and Technology. 50: 511-518.

Kudre, T. G. and Benjakul, S. 2013. Combining effect of microbial transglutaminase and bambara groundnut protein isolate on gel properties of surimi from sardine (*Sardinella albella*). Food Biophysics. 8: 240-249.

Kudre, T. G., Benjakul, S. and Kishimura, H. 2013. Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. Journal of the Science of Food and Agriculture. 93: 2429-2436.

Kumar, R. and Majeti, N. V. 2000. A review of chitin and chitosan applications. Reactive and Functional Polymers. 46: 1-27.

Kumari, S., Rath, P., Sri Hari Kumar, A. and Tiwari, T. N. 2015. Extraction and characterization of chitin and chitosan from fishery waste by chemical method. Environmental Technology and Innovation. 3: 77-85.

Kumazawa, Y., Numazawa, T., Seguro, K. and Motoki, M. 1995. Suppression of surimi gel setting by transglutaminase inhibitors. Journal of Food Science. 60: 715-717.

Kurita, K., Ishii, S., Tomita, K., Nishimura, S.-I. and Shimoda, K. 1994. Reactivity characteristics of squid β-chitin as compared with those of shrimp chitin: High potentials of squid chitin as a starting material for facile chemical modifications. Journal of Polymer Science Part A: Polymer Chemistry. 32: 1027-1032.

Kurita, K., Kaji, Y., Mori, T. and Nishiyama, Y. 2000. Enzymatic degradation of β-chitin: susceptibility and the influence of deacetylation. Carbohydrate Polymers. 42: 19-21.

Kurita, K., Tomita, K., Tada, T., Ishii, S., Nishimura, S.-I. and Shimoda, K. 1993. Squid chitin as a potential alternative chitin source: Deacetylation behavior and characteristic properties. Journal of Polymer Science Part A: Polymer Chemistry. 31: 485-491.

Kurita, K., Tomita, K., Tada, T., Ishii, S., Nishimura, S. I. and Shimoda, K. 1993. Squid chitin as a potential alternative chitin source: deacetylation behavior and characteristic properties. Journal of Polymer Science Part A: Polymer Chemistry. 31: 485-491.

Lanier, T. C., Carvajal, P. and Yongsawatdigul, J. 2000. Surimi and surimi seafood. *In* Surimi Technology. (Park, J. W., ed.). p. 237-265. Marcel Dekker Inc. New York.

Lanier, T. C., Carvajal, P. A. and Yongsawatdigul, J. 2005. Surimi gelation chemistry. *In* Surimi and Surimi Seafood. (Park, J. W., ed.). p. 435–489. Taylor and Francis Group. Boca Raton.

Laokuldilok, T., Potivas, T., Kanha, N., Surawang, S., Seesuriyachan, P., Wangtueai, S., Phimolsiripol, Y. and Regenstein, J. M. 2017. Physicochemical, antioxidant, and antimicrobial properties of chitooligosaccharides produced using three different enzyme treatments. Food Bioscience. 18: 28-33.

Lavall, R. L., Assis, O. B. G. and Campana-Filho, S. P. 2007. β-Chitin from the pens of *Loligo* sp.: Extraction and characterization. Bioresource Technology. 98: 2465-2472.

Lee, D.-X., Xia, W.-S. and Zhang, J.-L. 2008. Enzymatic preparation of chitooligosaccharides by commercial lipase. Food Chemistry. 111: 291-295.

Lee, J. J., Chen, H. C. and Jiang, S. T. 1993. Control of muscle softening and proteases identified as cathepsins L and L-like (58 kDa) proteinase from mackerel (*Scomber australasicus*). Bioscience, Biotechnology, and Biochemistry. 57: 1470-1476.

Lee, N. and Park, J. W. 1998. Calcium compounds to improve gel functionality of Pacific whiting and Alaska pollock surimi. Journal of Food Science. 63: 969-974.

Li, D. K., Lin, H. and Kim, S. M. 2008. Effect of rainbow trout (*Oncorhynchus mykiss*) plasma protein on the gelation of Alaska pollock (*Theragra chalcogramma*) Surimi. Journal of Food Science. 73: C227-234.

Lodhi, G., Kim, Y.-S., Hwang, J.-W., Kim, S.-K., Jeon, Y.-J., Je, J.-Y., Ahn, C.-B., Moon, S.-H., Jeon, B.-T. and Park, P.-J. 2014. Chitooligosaccharide and its derivatives: preparation and biological applications. BioMed Research International. 2014:

Mao, L. and Wu, T. 2007. Gelling properties and lipid oxidation of kamaboko gels from grass carp (*Ctenopharyngodon idellus*) influenced by chitosan. Journal of Food Engineering. 82: 128-134.

Matsumoto, J. J. 1980. Chemical deterioration of muscle proteins during frozen storage. *In* Chemical Deterioration of Proteins. (Whitaker, J. K. and Fujimaki, J. R., eds.). p. 95-124. ACS Symposium Series. Washington D.C.

McConnell, O. J., Longley, R. E. and Koehn, E. 1994. The discovery of natural products with therapeutic potential. *In* The Discovery of Marine Natural Products with Therapeutic Potential. (Gullo, V. P., ed.). p. 109-174. Butterworth-Heinemann. Boston

Methacanon, P., Prasitsilp, M., Pothsree, T. and Pattaraarchachai, J. 2003. Heterogeneous N-deacetylation of squid chitin in alkaline solution. Carbohydrate Polymers. 52: 119-123.

Minor-Pérez, H. 2017. Effect of soybean flour (*Glycine max*) and its inhibitors enzyme on endogenous protease activity in surimi-like gel elaborated with

jumbo squid (*Dosidicus gigas*) during the storage at 4° C and 10ºC. Ibero-American Journal of Agricultural and Biological Sciences. 6: 39-56.

Morales, O. G., Ramírez, J. A., Vivanco, D. I. and Vázquez, M. 2001. Surimi of fish species from the Gulf of Mexico: evaluation of the setting phenomenon. Food Chemistry. 75: 43-48.

Moreno, H. M., Carballo, J. and Borderías, A. J. 2008. Influence of alginate and microbial transglutaminase as binding ingredients on restructured fish muscle processed at low temperature. Journal of the Science of Food and Agriculture. 88: 1529-1536.

Morrissey, M. T. and Okada, T. 2007. Maximizing the value of marine by-product *In* (Shahidi, F., ed.). Woodhead Publishing Ltd., CRC Press. Cambridge.

Morrissey, M. T., Wu, J. W., Lin, D. and An, H. 1993. Protease inhibitor effects on torsion measurements and autolysis of Pacific whiting surimi. Journal of Food Science. 58: 1050-1054.

Mourya, V. K., Inamdar, N. N. and Choudhari, Y. M. 2011. Chitooligosaccharides: Synthesis, characterization and applications. Polymer Science Series A. 53: 583-612.

Muzzarelli, R. A. A. and Muzzarelli, C. 2005. Chitosan Chemistry: Relevance to the Biomedical Sciences. *In* Polysaccharides I: Structure, Characterization and Use. (Heinze, T., ed.). p. 151-209. Springer Berlin Heidelberg, Heidelberg.

No, H. K., Young Park, N., Ho Lee, S. and Meyers, S. P. 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. International Journal of Food Microbiology. 74: 65-72.

Olatunde, O. O. and Benjakul, S. 2018. Natural preservatives for extending the shelf-life of seafood: a revisit. Comprehensive Reviews in Food Science and Food Safety. 17: 1595-1612.

Oujifard, A., Benjakul, S., Ahmad, M. and Seyfabadi, J. 2012. Effect of bambara groundnut protein isolate on autolysis and gel properties of surimi from threadfin bream (*Nemipterus bleekeri*). LWT - Food Science and Technology. 47: 261-266.

Pan, S. and Wu, S. 2011. Preparation of water-soluble chitosan by hydrolysis with commercial glucoamylase containing chitosanase activity. European Food Research and Technology. 233: 325-329.

Panith, N., Wichaphon, J., Lertsiri, S. and Niamsiri, N. 2016. Effect of physical and physicochemical characteristics of chitosan on fat-binding capacities under in vitro gastrointestinal conditions. LWT - Food Science and Technology. 71: 25-32.

Park, J. W. 2000. Ingredient technology and formulation development. *In* Surimi and Surimi Seafood. (Park, J. W., ed.). p. 343-392. Marcel Dekker. New York.

Park, J. W. 2005. Surimi seafood: products, market, and manufacturing. *In* Surimi and Surimi Seafood. (Park, J. W., ed.). p. 375-433. Taylor and Francis. Boca Raton, Fla.

Park, J. W. and Morrissey, M. T. 2000. Manufacturing of surimi from light muscle fish. *In* Surimi and Surimi Seafood. (Park, J. W., ed.). p. 23-58. Marcel Dekker Inc. New York.

Park, P.-J., Lee, H.-K. and Kim, S.-K. 2004. Preparation of hetero-chitooligosaccharides and their antimicrobial activity on Vibrio parahaemolyticus. Journal of Microbiology and Biotechnology. 14: 41-47.

Pillai, C. K. S., Paul, W. and Sharma, C. P. 2009. Chitin and chitosan polymers: Chemistry, solubility and fiber formation. Progress in Polymer Science. 34: 641-678.

Pomeranz, Y. 1991. Proteins: General. *In* Functional Properties of Food Components. (Pomeranz, Y., ed.). p. 147-189. Academic Press. Sandigo.

Quan, T. H. and Benjakul, S. 2017. Comparative study on the effect of duck and hen egg albumens on proteolysis and gel property of sardine surimi. International Journal of Food Properties. 20: S2786-S2797.

Rawdkuen, S., Benjakul, S., Visessanguan, W. and Lanier, T. C. 2007. Effect of chicken plasma protein and some protein additives on proteolysis and gel-forming ability of sardine (*Sardinella gibbosa*) surimi. Journal of Food Processing and Preservation. 31: 492-516.

Rinaudo, M. 2006. Chitin and chitosan: Properties and applications. Progress in Polymer Science. 31: 603-632.

Rokhati, N., Widjajanti, P., Pramudono, B. and Susanto, H. 2013. Performance comparison of α-and β-amylases on chitosan hydrolysis. ISRN Chemical Engineering. 2013:

Roncal, T., Oviedo, A., de Armentia, I. L., Fernández, L. and Villarán, M. C. 2007. High yield production of monomer-free chitosan oligosaccharides by pepsin catalyzed hydrolysis of a high deacetylation degree chitosan. Carbohydrate Research. 342: 2750-2756.

Rong, C., Qi, L., Bang-zhong, Y. and Lan-lan, Z. 2010. Combined effect of ozonated water and chitosan on the shelf-life of Pacific oyster (*Crassostrea gigas*). Innovative Food Science and Emerging Technologies. 11: 108-112.

Samejima, K., Ishioroshi, M. and Yasui, T. 1981. Relative roles of the head and tail portions of the molecule in heat-induced gelation of myosin. Journal of Food Science. 46: 1412-1418.

Sannan, T., Kurita, K. and Iwakura, Y. 1976. Studies on chitin, 2. Effect of deacetylation on solubility. Die Makromolekulare Chemie. 177: 3589-3600.

Sano, T., Noguchi, S. F., Matsumoto, J. J. and Tsuchiya, T. 1990. Thermal gelation characteristics of myosin subfragments. Journal of Food Science. 55: 55-58.

Sano, T., Noguchi, S. F., Tsuchiya, T. and Matsumoto, J. J. 1988. Dynamic viscoelastic behavior of natural actomyosin and myosin during thermal gelation. Journal of Food Science. 53: 924-928.

Senphan, T. and Benjakul, S. 2014. Antioxidative activities of hydrolysates from seabass skin prepared using protease from hepatopancreas of Pacific white shrimp. Journal of Functional Foods. 6: 147-156.

Seymour, T. A., Morrissey, M. T., Peters, M. Y. and An, H. 1994. Purification and characterization of Pacific whiting proteases. Journal of Agricultural and Food Chemistry. 42: 2421-2427.

Song, H.-P., Kim, B., Choe, J.-H., Jung, S., Kim, K.-S., Kim, D.-H. and Jo, C. 2009. Improvement of foaming ability of egg white product by irradiation and its application. Radiation Physics and Chemistry. 78: 217-221.

Soria, A. C. and Villamiel, M. 2010. Effect of ultrasound on the technological properties and bioactivity of food: a review. Trends in Food Science and Technology. 21: 323-331.

Sugano, M., Watanabe, S., Kishi, A., Izume, M. and Ohtakara, A. 1988. Hypocholesterolemic action of chitosans with different viscosity in rats. Lipids. 23: 187-191.

Tabilo-Munizaga, G. and Barbosa-Cánovas, G. V. 2005. Pressurized and heat-treated surimi gels as affected by potato starch and egg white: microstructure and water-holding capacity. LWT - Food Science and Technology. 38: 47-57.

Tachasirinukun, P., Chaijan, M. and Riebroy, S. 2016. Effect of setting conditions on proteolysis and gelling properties of spotted featherback (*Chitala ornata*) muscle. LWT - Food Science and Technology. 66: 318-323.

Tadpitchayangkoon, P., Park, J. W. and Yongsawatdigul, J. 2012. Gelation characteristics of tropical surimi under water bath and ohmic heating. LWT - Food Science and Technology 46: 97-103.

Tharanathan, R. N. and Kittur, F. S. 2003. Chitin — The Undisputed Biomolecule of Great Potential. Critical Reviews in Food Science and Nutrition. 43: 61-87.

Thongngam, M. and McClements, D. J. 2005. Isothermal titration calorimetry study of the interactions between chitosan and a bile salt (sodium taurocholate). Food Hydrocolloids. 19: 813-819.

Tolaimate, A., Desbrieres, J., Rhazi, M. and Alagui, A. 2003. Contribution to the preparation of chitins and chitosans with controlled physico-chemical properties. Polymer. 44: 7939-7952.

Tolaimate, A., Desbrières, J., Rhazi, M., Alagui, A., Vincendon, M. and Vottero, P. 2000. On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. Polymer. 41: 2463-2469.

Tomida, H., Fujii, T., Furutani, N., Michihara, A., Yasufuku, T., Akasaki, K., Maruyama, T., Otagiri, M., Gebicki, J. M. and Anraku, M. 2009. Antioxidant properties of some different molecular weight chitosans. Carbohydrate Research. 344: 1690-1696.

Tsaih, M. L. and Chen, R. H. 2003. The effect of reaction time and temperature during heterogenous alkali deacetylation on degree of deacetylation and molecular weight of resulting chitosan. Journal of Applied Polymer Science. 88: 2917-2923.

Wasson, D. H., Babbitt, J. K. and French, J. S. 1993. Characterization of a heat stable protease from arrowtooth flounder. Journal of Aquatic Food Product Technology. 1: 167-182.

Weska, R. F., Moura, J. M., Batista, L. M., Rizzi, J. and Pinto, L. A. A. 2007. Optimization of deacetylation in the production of chitosan from shrimp wastes: Use of response surface methodology. Journal of Food Engineering. 80: 749-753.

Wongwichian, C., Chaijan, M., Panpipat, W., Klomklao, S. and Benjakul, S. 2016. Autolysis and characterization of sarcoplasmic and myofibril associated proteinases of oxeye scad (*Selar boops*) muscle. Journal of Aquatic Food Product Technology. 25: 1132-1143.

Wu, S. 2011. Preparation of water soluble chitosan by hydrolysis with commercial α-amylase containing chitosanase activity. Food Chemistry. 128: 769-772.

Xiong, T., Xiong, W., Ge, M., Xia, J., Li, B. and Chen, Y. 2018. Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. Food Research International. 109: 260-267.

Xu, Q., Dou, J., Wei, P., Tan, C., Yun, X., Wu, Y., Bai, X., Ma, X. and Du, Y. 2008. Chitooligosaccharides induce apoptosis of human hepatocellular carcinoma cells via up-regulation of Bax. Carbohydrate Polymers. 71: 509-514.

Yamashita, M. and Konagaya, S. 1990. High activities of cathepsins B, D, H, and L in the white muscle of chum salmon in spawning migration. Comparative Biochemistry and Physiology. B: Comparative Biochemistry. 95: 149-152.

Yongsawatdigul, J., Worratao, A. and Park, J. W. 2002. Effect of endogenous transglutaminase on threadfin bream surimi gelation. Journal of Food Science. 67: 3258-3263.

Younes, I. and Rinaudo, M. 2015. Chitin and chitosan preparation from marine sources. Structure, properties and applications. Marine Drugs. 13: 1133-1174.

Younes, I., Sellimi, S., Rinaudo, M., Jellouli, K. and Nasri, M. 2014. Influence of acetylation degree and molecular weight of homogeneous chitosans on antibacterial and antifungal activities. International Journal of Food Microbiology. 185: 57-63.

Zhang, H. and Neau, S. H. 2001. In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation. Biomaterials. 22: 1653-1658.

Zhao, D., Huang, J., Hu, S., Mao, J. and Mei, L. 2011. Biochemical activities of N, O-carboxymethyl chitosan from squid cartilage. Carbohydrate Polymers. 85: 832-837.

CHAPTER 2
CHARACTERISTICS AND FUNCTIONAL PROPERTIES OF OVARY FROM SQUID *LOLIGO FORMOSANA*

2.1 Abstract

Characteristics and functional properties of the ovary from *Loligo formosana* were studied. Moisture (72.07±0.24%) was dominant, followed by protein (18.64±0.51%) and carbohydrate (7.44±0.2%). Ash (1.39±0.03%) and lipids (0.46±0.5%) were found as the minor constituents. Albumin (79.02±0.79%) was the major protein of the squid ovary, followed by glutelin-1 (8.31±0.62%) and globulin (6.68±0.08%). Nevertheless, prolamin and glutelin-2 constituted approximately 1% of total proteins. Based on electrophoretic study, albumin showed the largest band intensity. The squid ovary was rich in non-essential amino acids (52.26%) and showed high hydrophobic amino acids (48.03%). The squid ovary was rich in polyunsaturated fatty acid (PUFA, 43.76±0.84%), followed by saturated fatty acid (SFA, 39.36±0.12%) and monounsaturated fatty acid (MUFA, 12.94±0.55%). Ovary lipids had high amount of docosahexaenoic acid (C22:6) (28.59%). At pH 3, the squid ovary powder (SOP) had the maximum solubility (96.39%), whereas the lowest solubility (38.33%) was observed at pH 9. The foaming capacity and stability of foam from SOP were increased with increasing concentration up to 8% ($p<0.05$). Globulin fraction showed the higher foaming capacity, as compared to albumin and glutelin-1 fractions. The squid ovary had good nutritional value and possessed the excellent foaming properties. Therefore, it could serve as a novel food additive or ingredient.

2.2 Introduction

Global marine production was 82.6 and 79.7 million tons in 2011 and 2012, respectively. The cephalopods (squid, cuttlefish and octopus) contributed around 3% of total world trade in 2012 (FAO, 2014). Spain, Italy and Japan are the largest consumers and importers of these species. Squid is one of popular marine product for consumption in Asian countries (FAO, 1989). In Asian continent, Thailand is the largest exporter of squid and cuttlefish.

Squid (*Loligo formasana*), a mollusc, belongs to class Cephalopoda with family Loliginidae. It is neritic species with a maximum mantle length of 30 cm. Some squids spawn throughout the year. The peak spawning period is spring and fall, in which ovary is fully matured. For squid processing, only mantle or tentacles are required as food and the rest including viscera, ovary, skin and pen etc. are discarded. Those internal organs can serve as the potential sources of beneficial compounds (Morrissey and Okada, 2007). Squid ink has been reported to exhibited antioxidative activity (Vate and Benjakul, 2013) and melanin-free-ink was also utilized to enhance the gel properties of sardine (Vate and Benjakul, 2016).

In female squid, the ovary constitutes around 10-15% of total body weight and is generally discarded by squid processing industry. As a consequence, ovary has not been fully exploited. Nevertheless, the information of squid ovary as potential food ingredient, especially for human consumption, has not been reported.

2.3 Objective

To investigate chemical composition and functional properties of squid ovary from squid *Loligo formosana*.

2.4 Materials and methods

2.4.1 Chemicals

All chemicals used were of analytical grade. High and low molecular weight protein markers were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bis-acrylamide was procured from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate (SDS), N, N, N´, N´-tetramethylethylenediamine (TEMED) and Coomassie Blue R-250 were purchased from Merck (Darmstadt, Germany).

2.4.2 Squid ovary

Squid (*Loligo formosana*) viscera were gifted from Sea Wealth Frozen Food, Songkhla, Thailand. The ovary was manually separated from other internal organs. The obtained ovary was washed with tap water and subsequently chopped using a blender to attain homogeneity. Fresh samples were used for proximate

analysis, fatty acid profile analysis and protein fractionation. Another portion of prepared sample was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The freeze-dried squid ovary powder (SOP) was sieved using stainless screen (0.4 mm mesh). The powder was packaged in a polyethylene bag at -40 °C until use.

2.4.3 Analyses

2.4.3.1 Proximate analysis

Moisture, protein, fat and ash contents of the squid ovary were determined according to the methods of AOAC (2000). Carbohydrate content was determined by subtracting from the sum of moisture, protein, fat and ash contents. The data were expressed, based on both wet and dry weight of sample.

2.4.4 Amino acid analysis

Amino acid composition of SOP was analyzed using an amino acid analyzer (Sinthusamran *et al.,* 2014). Under the reducing pressure condition, the samples were hydrolyzed in 4 M methanesulphonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115 °C for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.04 mL was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

2.4.5 Fatty acid profile analysis

Lipid was extracted from squid ovary as per the method of Bligh and Dyer (1959). Fatty acid methyl ester (FAME) was prepared from the lipid extracted from squid ovary according to the method of AOAC (2000). The prepared methyl ester was injected to the GC (Shimadzu, Kyoto, Japan) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column ($30\,m \times 0.25\,mm$), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250 °C and detector temperature of 270 °C. The oven was set at 170 to 225 °C at a rate of 1 °C/min without the initial or final holding. Retention times of FAME standards were used to

identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g lipid.

2.4.6 Protein fractionation

Fractionation of squid ovary proteins was carried out according to the method of Kwon *et al.* (1996) with a slight modification. Five different solvents including deionized water, 0.5 M NaCl, 70% 2-propanol (IPA), 50% glacial acetic acid, and 0.1 M NaOH were used to extract different proteins present in squid ovary, based on their solubility. Squid ovary was stirred with the different solvents at a matter/solvent ratio of 1:10 (w/v) for 14-16 h at 4 °C. The mixtures were then subjected to centrifugation at 20000 ×g for 30 min using a HITACHI CR22N centrifuge (Hitachi Koki Co., Ltd. Tokyo, Japan). Extraction with each solvent was repeated twice. The supernatants were pooled and then dialyzed against deionized water at a ratio of 1:20 (sample: deionized water). Dialysates were freeze-dried, and the resulting protein powders were stored in polyethylene bag and kept at -40 °C until use. The protein content of individual fraction was determined by the Kjeldahl method. Protein fractionated using water, NaCl, IPA, acetic acid, and NaOH solvents represented albumin, globulin, prolamin, glutelin-1 and glutelin-2, respectively.

Protein patterns of SOP and protein fractions were determined by SDS-PAGE according to a method of Laemmli (1970). Samples (2 g) were homogenized using an IKA homogenizer (IKA, Model T25, Selangor, Malaysia) with 18 mL of 5% SDS solution at a speed of 11,000 rpm. The mixtures were then heated at 85 °C for 1 h, followed by centrifugation at 8500 ×g for 5 min. Supernatants were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% SDS and 20% glycerol and β-mercaptoethanol). Samples (15 µg proteins) were loaded onto a polyacrylamide gel and subjected to electrophoresis. After electrophoresis, gels were stained with a mixture of 0.05% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with a mixture of 30% (v/v) methanol and 10% (v/v) acetic acid.

2.4.7 Solubility study

Solubility of SOP at different pHs (1-12) was determined by the method of Kittiphattanabawon *et al.* (2012). The SOP (10 g/L) was dissolved in distilled water and homogenized for 1 min at 5,000 rpm. Homogenate was stirred at room temperature (26-28 °C) for 1 h. The pH of SOP solution (8 mL) was adjusted with 6 M NaOH or 6 M HCl to obtain the final pHs ranging from 1 to 12 and the volume of SOP solutions was adjusted 10 mL with distilled water. The pH was readjusted to the designated pHs. The SOP solutions were stirred for 1 h at room temperature. The SOP solutions were centrifuged at 8,000 ×g at room temperature for 10 min using a centrifuge (MIKRO20, Hettich Zentrifugan, Germany). Protein content in the supernatant was determined by the Biuret method (Robinson and Hogden, 1940). Protein solubility was calculated and compared with a total protein content of the sample, which was determined after complete solubilization of the sample using 0.5 M NaOH.

2.4.8 Study on foaming properties

2.4.8.1 Impact of SOP concentration

Foaming of SOP was evaluated by whipping 2, 4, 6, 8 and 10% (w/v) SOP suspension using an IKA homogenizer at 13,400 rpm for 1 min following the method of Akintayo *et al.* (1999) with slight modifications. Volumes were recorded before and after whipping and the percentage of increased volume was calculated according to the following equation:

Foam capacity (FC)=A-B/C x 100

where: A; total volume after whipping, B; the volume of liquid after whipping, C; the volume of liquid before whipping.

Foam stability was determined by the following equation at different times after whipping (0, 30, 60 and 90 min).

Foam stability (FS)=At-B/C x 100

where: At; total volume at different times.

2.4.8.2 Impact of different protein fractions

Different freeze-dried powder of major fractions including albumin, globulin and glutelin-1 at a concentration of 2% (w/v) were subjected to whipping as previously described. FC and FS were then determined.

2.4.9 Statistical Analysis

All experiments were run in triplicate and each value represents the mean of three measurements. Data were subjected to analysis of variance. Comparison of means was carried out by the Duncan's multiple range tests (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL).

2.5 Results and discussion

2.5.1 Proximate composition of squid ovary

Squid ovary showed high moisture content (72.07±0.24%), followed by protein (18.64±0.51%). Ash (1.39±0.03%) and lipid (0.46±0.05%) were found at low content. Carbohydrate (7.44±0.2%) constituted at high level. Based on dry weight basis, protein (66.73±0.51%) was found as the major component of squid ovary, whereas carbohydrate (26.64±0.30%) was the second major constituent, followed by ash (4.97±0.03%). Very low lipid (1.64±0.50%) content was present in squid ovary. In general, the most cephalopods have 20% more protein, 80% less ash, 50-100% less lipid and 50-100% less carbohydrate, when compared to fish. The high protein content and low ash content differentiate cephalopods from the other marine organisms (Lee, 1995). Additionally, Clarke *et al.* (1994) reported that the chemical composition of female squid varied with the seasons, particularly ovary, which serve as the rich source of protein.

2.5.2 Amino acid profile

Amino acid compositions expressed as gram of amino acid per 100 gram of SOP are shown in Table 1. Glutamic acid/glutamine, aspartic acid/asparagine,

alanine, arginine, glycine, serine, tyrosine and proline were the major non-essential amino acids, which constituted 52.26 g/100 g. Glutamic acid/glutamine (12.39 g/100 g) and aspartic acid/asparagine (10.39 g/100 g) were the major non-essential amino acids found in SOP. Furthermore, they were the dominant hydrophilic amino acids in SOP. For essential amino acids, leucine (9.88 g/100 g) and lysine (8.86 g/100 g) were the predominant amino acids, followed by isoleucine, threonine, valine, phenylalanine and methionine (6.58, 6.21, 5.97, 4.08 and 2.61 g/100 g, respectively). It was noted that leucine was the most prevalent hydrophobic amino acid in SOP. Histidine (2.53 g/100 g) and tryptophan (0.98 g/100 g) were present at a low content, whereas cysteine was found at a negligible amount. In addition, the SOP had a slightly higher percentage of hydrophilic amino acids (51.95 g/100 g) than that of hydrophobic counterpart (48.03 g/100 g). The ratio of hydrophobic amino acids to hydrophilic amino acids was 0.92. The proteins with high hydrophobic amino acid (more than 30 g/100 g) are surface active proteins, which can exhibit the good interfacial properties including foaming and emulsifying agents (Zayas, 1997).

Table 1. Amino acid compositions of squid ovary powder (expressed as g/100 g sample)

Essential amino acids	g/100 g	Non-essential amino acids	g/100 g
Histidine	2.53	Alanine	4.28
Isoleucine	6.58	Arginine	6.62
Leucine	9.88	Aspartic acid/asparagine	10.39
Lysine	8.86	Cysteine	0.03
Methionine	2.61	Glutamine/glutamic acid	12.39
Phenylalanine	4.08	Glycine	4.20
Threonine	6.21	Proline	4.70
Tryptophan	0.98	Serine	4.92
Valine	5.97	Tyrosine	4.75
Total	47.7		52.28

*Hydrophobic/hydrophilic ratio - 0.92

2.5.3 Fatty acid profile

Fatty acid compositions of lipid extracted from the squid ovary are shown in Table 2. Squid ovary showed the higher proportion of polyunsaturated fatty acid (PUFA, 43.76±0.84%), followed by saturated fatty acid (SFA, 39.36±0.12%) and monounsaturated fatty acid (MUFA, 12.94±0.55%). Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) was the most prevalent PUFA present in the squid ovary, which accounted for 28.59±0.10% of total fatty acid. Cis-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) and Cis-5, 8, 11, 14-eicosatetraenoic acid (ARA) constituted about 8.49±0.04 and 5.01±0.02% of total fatty acid, respectively. Squid ovary contained the higher amount of n-3 PUFA than n-6 PUFA, which showed approximately 85.26 and 14.62% of PUFA content. Additionally, the high n-3/n-6 PUFA ratio (5.83) indicated the high proportion of essential n-3 fatty acids in squid ovary (Table 2). High levels of n-3 PUFA (37.31% of total fatty acids) reflected nutritional benefits for human health (Karnjanapratum *et al.,* 2013).

SFAs were the second major fatty acids present in the squid ovary. Palmitic acid (19.54±0.01%) was predominant SFA, followed by stearic acid (14.21±0.05%), myristic acid (2.86±0.00) and lignoceric acid (1.27±0.00). Other SFAs were found at the low amount (individually less than 1%). Palmitic acid is the major SFA present in plants, animals and micro-organisms, whereas stearic acid is ubiquitous and its amount varied according to the sources (Gunstone *et al.,* 2007). MUFA of squid ovary contained cis-9-octadecenoic acid (4.11±0.00%) as major fatty acid and gadoleic acid, cis-vaccenic acid, heptadecenoic acid and cis-11-eicosenoic acid were found at 2.14±0.01, 1.25±0.00, 1.06±0.00 and 1.02±0.01% of total fatty acid, respectively. The high content of DHA, especially n-3 PUFA, in diet could lower the cardiovascular disease (Karnjanapratum *et al.,* 2013; Kris-Etherton *et al.*, 1999).

Table 2. Fatty acid profile of squid ovary powder.

Saturated fatty acid (% of total fatty acid)	
Arachidic acid (C20:0)	0.72±0.01
Caprylic acid (C8:0)	0.04±0.00
Capric acid (C10:0)	0.04±0.00
Lauric acid (C12:0)	0.36±0.01
Lignoceric acid (C24:0)	1.27±0.00
Myristic acid (C14:0)	2.86±0.00
Pentadecanoic acid (C15:0)	0.26±0.00
Palmitic acid (C16:0)	19.54±0.01
Stearic acid (C18:0)	14.21±0.05
Behenic acid (C20:0)	0.06±0.00
Total saturated fatty acid	39.36±0.12
Monounsaturated fatty acid (% of total fatty acid)	
Myristoleic acid (C14:1)	0.05±0.00
Nervonic acid (C24:1)	0.05±0.00
Cis-10-pentadecenoic acid (C15:1 n-11)	0.96±0.01
Palmitelaidic acid (C16:1 t9)	0.10±0.00
Palmitoleic acid (C16:1 n-7)	0.77±0.00
Heptadecenoic acid (C17:0)	1.06±0.00
Cis-9-octadecenoic acid (C18:1 n-9)	4.11±0.00
Cis-vaccenic acid (C18:1 n-7)	1.25±0.00
Trans-elaidic acid (C18:1 n-9 trans)	0.16±0.03
(C18:1 n-1 trans)	0.15±0.00
Paullinic acid (C20:1 n-7)	0.16±0.00

Gadoleic acid (C20:1 n-11)	2.14±0.01
Cis-11-eicosenoic acid (C20:1 n-9)	1.02±0.01
Total monounsaturated fatty acid	12.94±0.55
Polyunsaturated fatty acid (% of total fatty acid)	
Cis-5, 8, 11, 14-eicosatetraenoic acid (C20:4 n-6, ARA)	5.01±0.02
Cis-9,12,15-octadecatrienoic acid (C18:3 n-3, ALA)	0.11±0.00
Cis-4,7,10,13,16,19-docosahexaenoic acid (C22:6 n-3, DHA)	28.59±0.10
Trans-octadecadienoic acid (C18:2 t9t12)	0.05±0.00
Moroctic acid (C18:4 n-3)	0.07±0.00
Cis-11, 14-eicosadienoic acid (C20:2 n-6)	0.22±0.00
Cis-9,12-octadecadienoic acid (C18:2 n-6)	1.17±0.00
Cis-5, 8, 11, 14, 17-eicosapentaenoic acid (C20:5 n-3, EPA)	8.49±0.04
Cis-11, 14, 17-eicosatrienoic acid (C20:3 n-3)	0.05±0.00
Total polyunsaturated fatty acid	43.76±0.84
Total n-3 polyunsaturated fatty acid	37.31±0.09
Total n-6 polyunsaturated fatty acid	6.4±0.01
Ratio n-3/n-6	5.83±0.00
Unidentified peak	4.92±0.06

Value are presented as mean ± SD (n=3)

2.5.1 Protein distribution/composition

Squid ovary contained varying proteins at different levels, based on solubility as shown in Table 3. It was observed that albumin was the most predominant protein (79.02±0.79%) present in the squid ovary. Glutelin-1 (8.31±0.62%) and globulin (6.68±0.08%) were also found in squid ovary. Glutelin-2 (0.99±0.04%) was extracted by NaOH, whereas prolamin (0.19±0.03%) was extracted using 70% isopropyl alcohol. The sum of proteins extracted by five different solvents accounted for 95.19% (w/w) of the total protein content. This suggested that some proteins, which might be associated tightly with other constituents, could not be fully extracted from squid ovary. However, those proteins were lower than 5% of total proteins. Different proteins in squid ovary with varying compositions/proportions, therefore affected functional properties differently.

Table 3. Distribution of different proteins in squid ovary

Fractions	Solvents	Protein Recovery (%)
Albumin	Deionized water	79.02±0.79
Globulin	0.5 M NaCl	6.68±0.08
Prolamin	70% Isopropyl alcohol	0.19±0.03
Glutelin-1	50% acetic acid	8.31±0.62
Glutelin-2	0.1M NaOH	0.99±0.04

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

Protein patterns of different protein fractions isolated from squid ovary under reducing conditions are shown in Figure 5. Squid ovary showed two major bands with MW of 162.9 and 121.7 kDa. Additionally, proteins with MW of 13.19 and 9.94 kDa were found. Similarly, albumin (168.08 and 152.96 kDa) and globulin (173.4 and 139.2 kDa) also showed two major bands. However, protein with MW of 13.19 kDa was found only in both albumin and globulin. Two protein bands with MW of 179.0 and 148.2 kDa were also observed in a glutelin-1 fraction but they were found at very low content. In squid ovary, proteins with MW of 86.9 and 92.5 kDa

were also noticeable. Similarly, albumin possessed protein bands of MW 89.65 and 92.51 kDa. For both glutelin-1 and 2, the protein with MW of 98.5 kDa was found. Additionally, both glutelin-1 and 2 had protein with MW lower than 30 kDa, including 19.2 and 20.5 kDa. For prolamin, only protein band with MW of 215.6 kDa

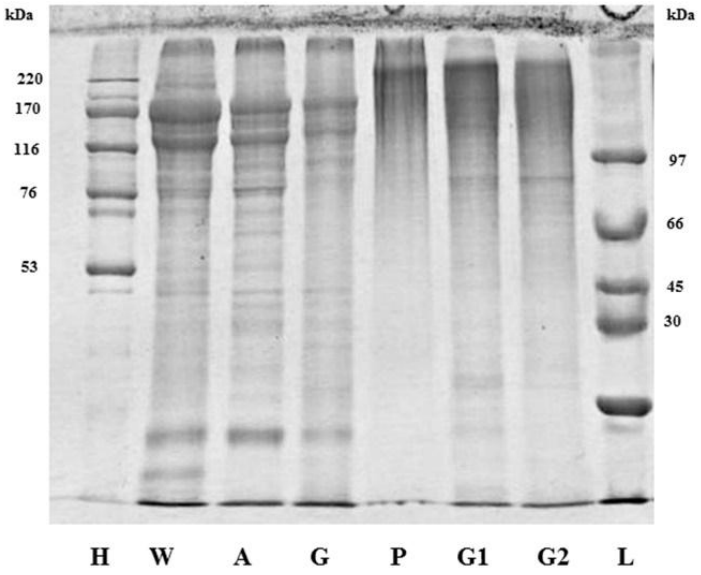

Figure 5. SDS–PAGE patterns of squid ovary powder and its fractions under reducing condition. W, A, G, P, G1 and G2 denoted whole sample, albumin, globulin, prolamin, glutelin-1 and glutelin-2, respectively. L and H denoted low and high MW standards, respectively.

was observed as dominant. No small MW proteins were detected. The result suggested that squid ovary more likely contained globulin and albumin as major proteins in comparison with those obtained in whole sample. However, it contained low amount of prolamin, which is alcohol soluble protein. Additionally, low affinity of prolamin with the Coomassie Blue dye was reported (Kwon *et al.*, 1996). Therefore, squid ovary had varying protein compositions, in which albumin constituted as the dominant protein.

2.5.2 Solubility

Solubility of SOP at different pHs is depicted in Figure 6. Solubility is one of important functionalities, affecting interfacial properties of protein in the

colloidal system (Thiansilakul *et al.*, 2007). SOP showed high solubility from pH 1 to 3 (91.46 to 96.39 %). At pH 3, SOP showed the highest solubility ($p < 0.05$). With increasing pH from 4 to 9, solubility decreased continuously and reached the lowest value (38.33%) at pH 9. The increase in solubility was observed when pH was increased from 10 to 11. Thereafter, the slight decrease (85.53%) was found when pH was 12. Generally, the minimum solubility was observed at the isoelectric point (pI)

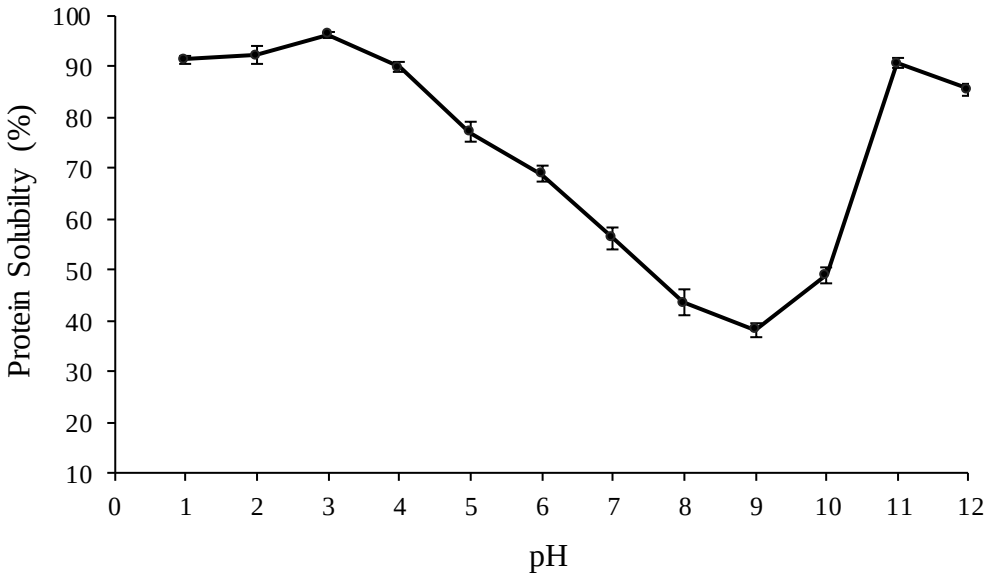

Figure 6. Protein solubility of squid ovary powder at different pHs (1-12). Bars represent the standard deviation (n=3).

of protein, where the net charge becomes zero. As a result, the electrostatic repulsion is minimum and aggregation and precipitation via hydrophobic interactions were enhanced (Sae-leaw *et al.*, 2016). Based on solubility, pH 9 was assumed to be pI of SOP proteins. SOP generally showed high solubility in acidic and alkaline conditions, which were more likely due to the increased repulsion between the protein molecules with dominant positively charged and negatively charged domains, respectively.

2.5.3 Foaming properties of SOP at different levels

FC and FS as affected by SOP at different concentrations (1, 2, 4, 6, 8 and 10%, w/v) are shown in Figure 7A. FC of SOP was increased in a dose dependent

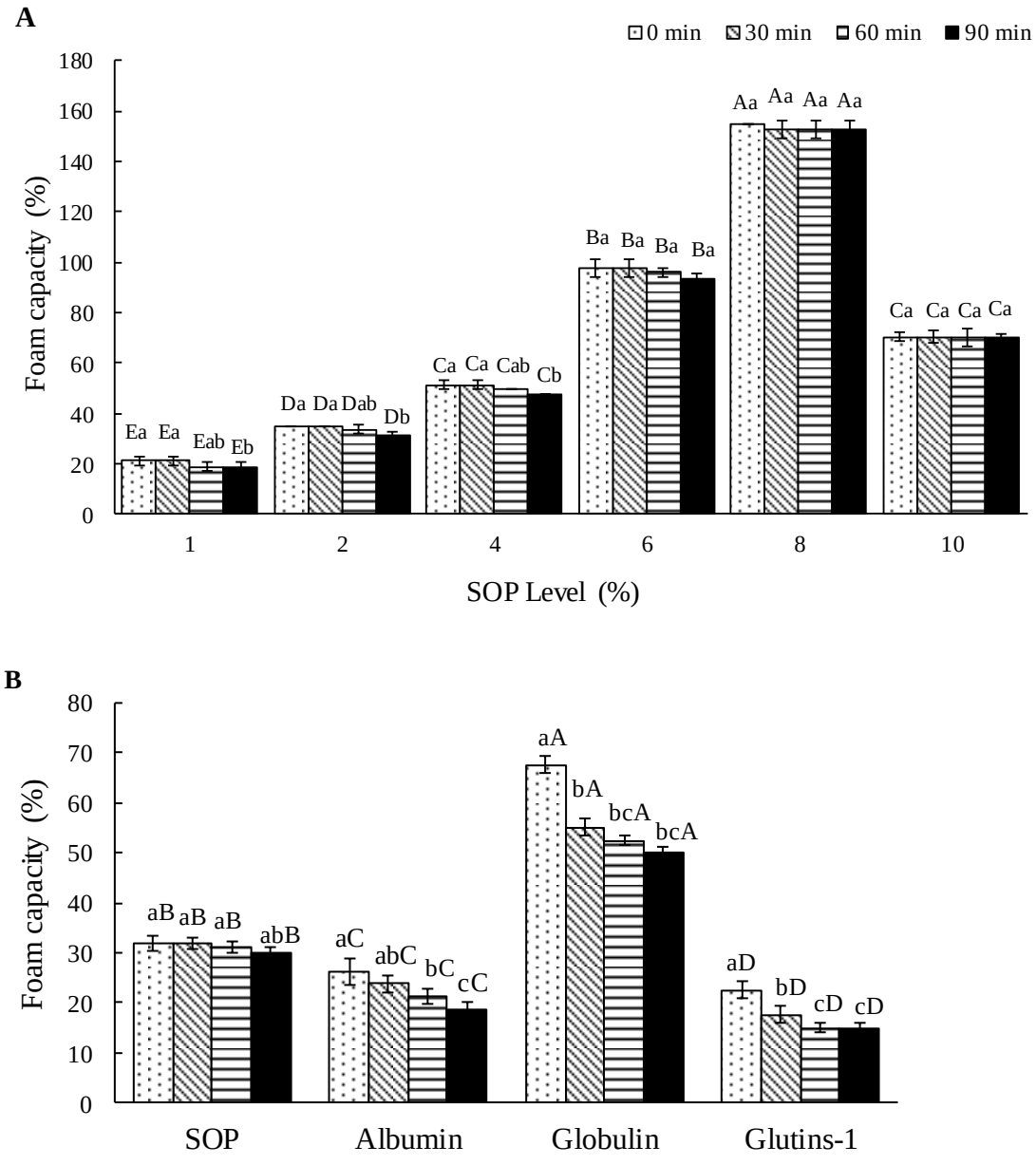

Figure 7. Effect of squid ovary powder at different levels (A) and different squid ovary protein fractions (2%) (B) on foaming properties. Values are given as mean ± SD (n=3). Different uppercase letters on the bars within same aging time indicate the significant difference ($p<0.05$). Different lowercase letters on the bars within the same SOP concentration (A) or within the same fraction (B) indicate the significant difference ($p<0.05$).

manner up to 8% ($p<0.05$). FC was increased from 21.25 to 155% as SOP concentration increased from 1 to 8% ($p<0.05$). However, FC was decreased by 54.51% in the presence of 10% SOP ($p<0.05$). At higher concentration, the increased viscosity of SOP suspension might impede the migration of protein in SOP to air-water interface. Moreover, high viscosity of mixture more likely reduced the air incorporation, thus lowering the foaming capacity. Foam from sodium caseinate solution expanded in the range of 100-300%. At high concentration (8%), the reduced foam capacity (<300%) was observed in comparison with the lower concentration (1-4%). Insoluble material is believed to interfere with the interfacial properties (Britten and Lavoie, 1992). For FS, SOP at 1, 2 and 4% showed slight foam destruction when the foam was allowed to stand from 60 and 90 min ($p < 0.05$). Nevertheless, no foam destruction was observed for 6 and 8% SOP with increasing foam aging times. This was more likely due to the formation of a thick and flexible interfacial layer of protein at the interface of air and water (Zayas, 1997). The flexibility of proteins is an important factor in the reduction of surface tension, while protein-protein interactions are required for the formation of highly viscoelastic films for foam stability (Kim and Kinsella, 1985). Foam capacity of porcine serum and porcine serum albumin was increased when concentration increased up to 8%. At 10%, no significant difference was observed in foam capacity (Ramos-Clamont and Vázquez-Moreno, 2006). Hence, SOP at 8% appeared to be the optimum concentration for foam formation.

2.5.4 Foaming properties of different protein fractions of squid ovary

FC and FS of SOP and different major protein fractions (albumins, globulin and glutelin-1) are shown in Figure 7B. Globulin proteins showed the higher FC (67.5%) than SOP (32%) ($p<0.05$). However, albumin (26.25%) and glutelin-1 (22.5%) proteins showed the lower FC, compared to SOP ($p<0.05$). The highest FC of globulin fraction might be due to the high solubility and unfolding of protein molecule in the salt solution (0.5 M NaCl). Conversely, glutelin-1, with the lower FC, was plausibly denatured to some degree by acetic acid (50%, v/v) used for fractionation. For FS, foam destruction of 22.22, 19.08 and 9.52% was observed in glutelin-1, globulin and albumin proteins after 30 min, respectively. On the other hand, SOP showed foam stability up to 90 min at room temperature ($p>0.05$). On the

other hand, at 60 and 90 min, albumin showed the enhanced foam destruction ($p<0.05$). No marked difference was observed between globulin and glutelin-1 during 60 and 90 min ($p>0.05$). The higher FS of SOP was most probably due to the presence of other biomolecules (carbohydrate and lipids) along with the protein. Carbohydrates are known to enhance the foam stability by increasing the viscosity and thickness of interfacial film (Zayas, 1997). With the sequential protein extraction, carbohydrate might be removed. As a consequence, the stability of foam might be decreased in some protein fractions.

2.6 Conclusion

Squid ovary, a byproduct obtained from the squid processing industry, was a good source of protein. The squid ovary was rich in amino acids. Lipid of squid ovary was rich in DHA and other n-3 PUFA. The squid ovary showed the excellent foaming ability. It had high solubility in acidic pH range. Therefore, squid ovary could serve as proteinaceous materials and could be used as an alternative foaming agent.

2.7 References

Akintayo, E. T., Oshodi, A. A., and Esuoso, K. O. 1999. Effects of NaCl, ionic strength and pH on the foaming and gelation of pigeon pea (*Cajanus cajan*) protein concentrates. Food Chemistry. 66: 51-56.

AOAC 2000. Official Methods of Analysis. Association of Official Analytical Chemists, Rockville, USA.

Bligh, E. G., and Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. 37: 911-917.

Britten, M., and Lavoie, L. 1992. Foaming properties of proteins as affected by concentration. Journal of Food Science. 57: 1219-1241.

Clarke, A., Rodhouse, P. G., and Gore, D. J. 1994. Biochemical composition in relation to the energetics of growth and sexual maturation in the ommastrephid Squid *Illex argentinus*. Philosophical Transactions of The Royal Society B Biological Science. 344: 201-212.

FAO 1989. A regional survey of the aquaculture sector in east Asia. *In*: Food and Agriculture Organization. United Nations, Rome.

FAO 2014. The state of world fisheries and aquaculture. *In*: Food and Agriculture Organization. United Nations, Rome.

Gunstone, F. D., Harwood, J. L., and Dijkstra, A. J. 2007. The Lipid Handbook. CRC Press Taylor and Francis Group. Boca Raton, United States.

Karnjanapratum, S., Benjakul, S., Kishimura, H., and Tsai, Y.-H. 2013. Chemical compositions and nutritional value of Asian hard clam (*Meretrix lusoria*) from the coast of Andaman Sea. Food Chemistry. 141: 4138-4145.

Kim, S. H., and Kinsella, J. E. 1985. Surface activity of food proteins: relationships between surface pressure development, viscoelasticity of interfacial films and foam stability of bovine serum albumin. Journal of Food Science. 50: 1526-1530.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., and Shahidi, F. 2012. Effect of extraction temperature on functional properties and antioxidative activities of gelatin from shark skin. Food and Bioprocess Technology. 5: 2646-2654.

Kris-Etherton, P. M., Pearson, T. A., Wan, Y., Hargrove, R. L., Moriarty, K., Fishell, V., and Etherton, T. D. 1999. High–monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. The American Journal of Clinical Nutrition. 70: 1009-1015.

Kwon, K., Park, K. H., and Rhee, K. C. 1996. Fractionation and characterization of proteins from coconut (*Cocos nucifera* L.). Journal of Agricultural and Food Chemistry. 44: 1741-1745.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227: 680-685.

Lee, P. G. 1995. Nutrition of cephalopods: fueling the system. Marine and Freshwater Behaviour and Physiology. 25: 35-51.

Morrissey, M. T., and Okada, T. 2007. Marine enzymes from seafood by-product *In* Maximizing the Value of Marine By-Product (Shahidi, F., Ed.). p. 374-396.Woodhead Publishing Ltd., Cambridge.

Ramos-Clamont, M. G., and Vázquez-Moreno, L. 2006. Foaming properties of porcine serum and porcine serum albumin. Ciência e Tecnologia de Alimentos. 5: 105-111.

Robinson, H. W., and Hogden, C. G. 1940. The biuret reaction in the determination of serum proteins: I. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. The Journal of Biological Chemistry. 135: 707.

Sae-leaw, T., Benjakul, S., O'Brien, N. M., and Kishimura, H. 2016. Characteristics and functional properties of gelatin from seabass skin as influenced by defatting. International Journal of Food Science and Technology. 51: 1204-1211.

Sinthusamran, S., Benjakul, S., and Kishimura, H. 2014. Characteristics and gel properties of gelatin from skin of seabass (*Lates calcarifer*) as influenced by extraction conditions. Food Chemistry. 152: 276-284.

Steel, R. G. D., and Torrie, J. H. 1980. Principles and procedures of statistics: A biometrical approach. McGraw-Hill, New York.

Thiansilakul, Y., Benjakul, S., and Shahidi, F. 2007. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). Food Chemistry. 103: 1385-1394.

Vate, N. K., and Benjakul, S. 2013. Antioxidative activity of melanin-free ink from splendid squid (*Loligo formosana*). International Aquatic Research. 5: 1-12.

Vate, N. K., and Benjakul, S. 2016. Effect of the mixtures of squid ink tyrosinase and tannic acid on properties of sardine surimi gel. Journal of Food Science and Technology. 53: 411-420.

Zayas, J. F. 1997. Foaming properties of proteins. *In* Functionality of Proteins in Food. p. 260-309. Springer Berlin Heidelberg, Heidelberg.

CHAPTER 3
EFFECT OF ULTRASONICATION ON PHYSICOCHEMICAL AND FOAMING PROPERTIES OF SQUID OVARY POWDER

3.1 Abstract

Central composite design (CCD) in conjunction with response surface methodology (RSM) was used to optimize the ultrasonication conditions for the improvement of foaming properties of squid ovary powder (SOP). Ultrasonication at amplitude 70% for time 30 min was the optimized condition based on predictive models. Ultrasonication affected physicochemical properties and structure of SOP. Surface hydrophobicity of ultrasonicated SOP was increased by 28%. Solubility was decreased slightly with coincidental decrease in total sulfhydryl group. Nevertheless, ultrasonication had no impact on protein pattern. Mean particle size was reduced by 64.1% of SOP after ultrasonication. Differential scanning calorimetric study revealed that ultrasonication affected transition temperature of proteins in SOP. Foaming capacity of ultrasonicated SOP was increased by 83.3%, compared to the control. When effect of various whipping times on foam properties was examined, the maximum FC (320%) and the lowest liquid drainage was attained when the ultrasonicated SOP was whipped for 3 min. Ultrasonication also improved the foam properties of SOP by increasing G´ and G´´. Microstructure study revealed that smaller sized bubbles with uniform distribution and thicker lamella were obtained in foam of ultrasonicated SOP. Additionally, foam of ultrasonicated SOP was more stable after 60 min. Thus, ultrasonication under the optimal condition along with appropriate whipping time yielded the foam from SOP with increased foaming capacity and stability.

3.2 Introduction

Foam is a two-phase system consisting of air cells separated by a continuous liquid layer called the lamellar phase (Damodaran, Parkin and Fennema, 2008). Food foams are the complex system, including a mixture of gases, liquids, solids, and surfactants. Proteins are widely used to stabilize the foam for the production of variety of foods. The most widely used protein foaming agents are egg

and milk proteins (Zayas, 1997). Different proteins show varying foaming properties. In general, whey proteins (lactalbumin) are more stable foaming agent than sodium caseinate (Marinova *et al.*, 2009; Martínez-Padilla *et al.*, 2014). Modifications are required to enhance foaming properties of some proteins or hydrolysates such as in wheat gluten (Popineau *et al.*, 2002; Wang *et al.*, 2006). Proteins are amphiphilic molecules, which act as surface active agents. They are able to reduce the surface tension at the air/water interface and play a role in forming strong films at the interface (Damodaran *et al.*, 2008). Proteins possess several functional properties which are governed by intrinsic and extrinsic factors (Speroni *et al.*, 2009; Phillips, 2013). Modification of food proteins by physical and chemical means has been employed to improve their functional properties. Enzymatic hydrolysis, deamidation, cross-linking using transglutaminase or Maillard reaction have been used to modify the functional properties of proteins (Brownlee, 1995; Klompong *et al.*, 2007; Mirmoghtadaie, Kadivar and Shahedi, 2009). However, chemical modifications may impair nutritional value of products and could cause the adverse health effects. Therefore, the physical modifications of protein structure by heating, freezing or extrusion have been widely implicated to improve the functional properties of proteins (Camire, 1991; Bhargava and Jelen, 1995; Bußler *et al.*, 2015).

Ultrasound has recently gained significant interest as it possesses a wide range of applications including extraction, preservation and modification of proteins or other biomolecules. Ultrasound is a sound wave with a frequency greater than the threshold for human auditory detection (Chemat and Khan, 2011; O'Sullivan *et al.*, 2014). Ultrasound is classified into 2 categories: 1) high frequency (100 kHz to 1 MHz) low power (<1 W cm^{-2}) and 2) low frequency (20–100 kHz) high power (10–1000 W cm^{-2}). High frequency ultrasound changed the physicochemical properties of soy proteins (soy protein isolate/concentrate and flakes), egg white, whey protein concentrates, etc. (Jambrak *et al.*, 2009; Karki *et al.*, 2009; Arzeni *et al.*, 2012). Increased hydrophobicity and reduced protein aggregate were observed in soy protein isolate after treatment with high frequency ultrasound (Arzeni *et al.*, 2012). Jambrak *et al.* (2011) reported the improved solubility, emulsifying and foaming properties, rheological and thermophysical properties of milk powder subjected to high power

ultrasonication (30 kHz frequency). Low frequency ultrasound is introduced for alteration of food structure by lowering the surface tension and increasing the rate of adsorption of proteins. The use of ultrasound in liquid-solid systems causes acoustic cavitation due to generation, growing and eventual collapse of the bubbles. As ultrasound waves propagate, the bubbles oscillate and collapse, causing the thermal, mechanical, and chemical effects. Mechanical effects include collapse pressure, turbulences, and shear stresses (Chemat and Khan, 2011), while the chemical effects include the generation of free radicals (Lateef, Oloke and Prapulla, 2007). The high-intensity ultrasound enhances protein solubility by changing protein conformation and structure in the way that hydrophilic parts of amino acids from inside are opened toward water (Jambrak *et al.*, 2008). However, during ultrasonication, the buried hydrophobic domains are exposed. Protein-protein interactions can be enhanced and result in the formation of aggregates, which lower the solubility (Arzeni *et al.*, 2012). This treatment also decreases molecular weight of proteins, implying that larger area of protein is covered by molecules of water (Jambrak *et al.*, 2008). Bovine serum albumin (BSA) had the increased intramolecular mobility and surface activity when treated with ultrasound (20 W cm^{-2}) for 15-45 min at 85 ºC (Güzey *et al.*, 2006). Foam capacities and foam stabilities of alpha-lactalbumin were increased after ultrasound treatments at 20 kHz and 40 kHz (Jambrak *et al.*, 2010).

Fish roe or fish egg are considered as the important source for the alternative ingredient with bioactivity e. g. antioxidant, antibacterial, immunomodulatory activities, etc. High amount of polyunsaturated fatty acid (PUFAs) was found in fish roe (33.38–39.68%) and squid ovary (43.76%). Both sources are therefore nutritive for humans (Intarasirisawat, Benjakul and Visessanguan, 2011; Singh, Benjakul and Kishimura, 2017). Protein hydrolysates from underutilized merigala (*Cirrhinus mrigala*) fish egg had good fat adsorption capacity, foam capacity and emulsifying capacity (Chalamaiah *et al.*, 2010). Recently, reported that squid ovary possessed excellent foaming properties and it could be used as a new foaming agent in foods. Ovary from female squid has the accountable percentage (10-15%) with respect to the body weight. It is mostly discarded by the squid processing industries. To better exploit squid ovary, the

improvement of its foaming ability is required. The use of ultrasound under the appropriate conditions could be a promising means to modify proteins in squid ovary in the way which favors their foam formation. Thus, squid ovary could be used to replace egg white (EW), commonly used as foaming agent. EW has undesirable flavor and allergy, thus limiting its use in the food. Nevertheless, no information on the impact of ultrasound treatment on foaming ability of squid ovary exists.

3.3 Objective

To optimize the operation conditions of ultrasonic treatment to maximize the foaming properties of squid ovary powder (SOP).

3.4 Material and methods

3.4.1 Chemicals

All chemicals were of analytical grade. High and low molecular weight (MW) protein markers, 1-anilinonaphthalene-8-sulfonic acid (ANS) and DTNB (5,5-dithio-bis-(2-nitrobenzoic acid) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS), and Coomassie Blue R-250 and β-Mercaptoethanol (βME) were purchased from Merck (Darmstadt, Germany).

3.4.2 Collection and preparation of squid ovary powder (SOP)

The frozen squid (*Loligo formosana*) viscera were obtained from Sea Wealth Frozen Food, Songkhla, Thailand. The ovary was manually separated and chopped using a blender (Panasonic, MX-898N, Malaysia) at 250 rpm for 2 min to attain homogeneity, followed by freeze-drying using a freeze-drier (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The freeze-dried powder was defined as SOP. SOP was transferred into polyethylene bag and stored at -40 °C until use.

3.4.3 Optimization of ultrasonication conditions for SOP treatment

Response surface methodology was employed for the optimization of ultrasonication conditions. A CCD with two independent variables including ultrasonication amplitude (X_1, %) and time (X_2, min) at five code levels was

performed. The central value of all variables was coded as zero. The minimum and maximum ranges of all variables were used, and full experimental plan with regards to their actual and code is provided in Table 4. Five replicates (run order 9-13) at the center point were used to estimate a pure error sum of squares. The dependent variables included foaming capacity (FC), foaming stability (FS) and surface hydrophobicity. The experimental data were fitted to a polynomial model. Generally, a second-order model was applied in RSM:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_1 X_1^2 + \beta_2 X_2^2 + \beta_{12} X_1 X_2$$

where Y is the dependent variables (FC and surface hydrophobicity); X_1 and X_2 are the independent variables; β_0 is the model constant; β_1 is the linear coefficient and β_{12} is the interaction coefficient.

The optimum conditions were determined by superimposing maximum areas of all responses representing all combinations of independent variables using the Design-Expert Statistical package version (Statease, Inc Minneapolis, Minn, USA). The fitted polynomial equations were then expressed in the form of 3-D plot, in order to illustrate the relationship between dependent variables and the experimental variables tested.

SOP (16 g) was transferred into 500 mL beaker and the final volume was raised to 400 mL using distilled water, in which the concentration of 4% was obtained. The mixture was subjected to ultrasonication (Sonics, Model VC750, Sonic & Materials, Inc., Newtown, USA) with operating frequency of 20 kHz ± 50 Hz and high intensity power of 750 W under different conditions (Table 4). The temperature was controlled at 25-30 ºC during operation using the iced bath. All ultrasound treated solutions were subjected to analyses.

3.4.4 Analyses

3.4.4.1 Determination of foaming capacity and stability

Foaming capacity (FC) and stability (FS) of treated solutions were determined as described in section 2.4.8.1.

3.4.4.2 Surface hydrophobicity

Protein surface hydrophobicity was determined by the method of Kittiphattanabawon *et al.* (2012). All treated solutions were mixed with 10 mM phosphate buffer (pH 6.0) containing 0.6 mM NaCl to obtain a final protein concentration of 5 g/ L. The samples were diluted again to 0.125, 0.25, 0.5, and 1 g/ L using the same buffer. The diluted samples (4 mL) were well mixed with 20 μL of 8 mM ANS in 0.1 M phosphate buffer (pH 7.0). The relative fluorescence intensity of ANS-protein conjugates was measured using a spectrofluorometer (RF-15001, Shimadzu, Kyoto, Japan) at the excitation wavelength of 374 nm and the emission wavelength of 485 nm. Protein surface hydrophobicity was calculated from initial slopes of plots of relative fluorescence intensity versus protein concentration (g/ L). The initial slope was referred to as S_oANS.

3.4.5 Validation of predicted model

FC, FS and surface hydrophobicity were determined for SOP ultrasonicated under the optimized condition. The experimental errors for the models were determined by comparing observed values with predicted values.

3.4.6 Characterization of SOP and ultrasound treated SOP (USOP)

SOP was subjected to ultrasonication under optimized condition as described previously. Thereafter, the treated sample was freeze-dried using a freeze drier. Powder referred to as ‘ultrasonicated SOP, USOP’ was further analyzed, in comparison with SOP.

3.4.6.1 Solubility

SOP and USOP were dispersed in deionized water to obtain a concentration of 1% (w/v). The pH was adjusted to pH 7 with either 6 N NaOH or 6 N HCl. Suspensions were centrifuged at 20,000 xg for 15 min at room temperature (25 ºC) using a centrifuge (Allegra 25R centrifuge, Beckman Coulter, Palo Alto, CA, USA). Protein content in supernatant was measured using the Biuret method (Gornall, Bardawill and David, 1949). For total protein, the samples were solubilized with 0.5

M NaOH (Kittiphattanabawon *et al.*, 2012). The solubility was reported as percentage relative to total protein content of SOP (5.67 mg/mL) and USOP (6.73 mg/mL).

3.4.6.2 Total sulfhydryl group

Total sulfhydryl group content was measured according to the method described by Benjakul *et al.* (2001). To 2.5 mL of SOP and USOP solutions (1%, w/v), 3 mL of 0.2 M Tris–HCl buffer, pH 6.8, containing 8 M urea, 2% SDS and 10 mM EDTA, were added. The mixture was then added with 0.25 mL of 10 mM DTNB in 0.2 M Tris–HCl buffer (pH 6.8) and incubated at 40 °C for 20 min. The absorbance at 412 nm was measured using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Sample blank was prepared in the same manner, except that 0.2 M Tris–HCl (pH 6.8) was used instead of DTNB solution. Sulfhydryl group content was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$.

3.4.6.3 SDS – Polyacrylamide gel electrophoresis

Protein patterns of SOP and USOP were determined by SDS–PAGE as described in section 2.4.6.

3.4.6.4 Particle size measurement

The particle size of hydrated SOP and USOP was measured by the method described by Aewsiri *et al.* (2011). The particle size of samples dispersed in 10 mM sodium phosphate buffer (pH 7.0) with the final concentration of 1 mg protein/mL was measured for dynamic light scattering at room temperature. The measurements were performed by a Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK). A refractive index of 1.42 was used for dispersed phase of all protein solutions.

3.4.6.5 Differential scanning calorimetry (DSC)

Thermal properties of SOP and USOP were determined using a differential scanning calorimeter (PerkinElmer, Model DSC-7, Norwalk, CT, USA). The DSC instrument was calibrated with indium (m.p. 156.6°C, ΔHf = 28.45 J/g).

Samples (10–12 mg) were weighed into aluminum pans and were hermetically sealed. Prior to analysis of samples, the baseline was obtained from an empty hermetically sealed aluminum pan. The scanning temperature was raised from 20 to 100 ºC with the heating rate of 10 ºC/min. T_{onset}, T_{max} and T_{end} of samples were determined.

3.4.7 Study on foaming properties of SOP and USOP under optimized conditions

3.4.7.1 Effect of whipping time on foaming properties

The effect of whipping time was studied by whipping the solution of SOP and USOP (4%, w/v) for whipping times (1, 2, 3 and 4 min). FC was determined as previously described.

Liquid drainage of foam, representing foam stability, was determined immediately after foam formation. The 50 mL of foam made from 4% SOP and USOP solutions were transferred to a glass funnel. Opening of funnel was closed with the glass wool to provide support to foam and easy drainage of liquid. The liquid drained was collected and measured after 60 min. Whipping time yielding the highest FC and lowest drainage was used for further study.

3.4.7.2 Rheological properties of foam

A rheometer (HAAKE RheoStress 1, Thermo Fisher Scientific, Karlsruhe, Germany) with cone and plate geometry (4º angle, 35 mm diameter) was used to monitor foam viscoelastic properties. A fresh sample was used in frequency sweeps. Immediately after whipping of 4% SOP and USOP solutions, foams were allowed to stand for 2 min. The foams were gently placed in the center of the lower plate and the upper geometry was gently descended to a gap of 3 mm. The excessive foams were carefully removed, and the samples were allowed to stand for 1 min before testing. The adhesive nature of SOP foam and small gap between the two plates allowed sample to form an adhesive and thin layer, which did not allow the slippage of sample during measurements. Logarithmic amplitude sweep in the range of 0.001% to 10% strain at a frequency of 0.5 Hz and 25 °C was applied to obtain the

linear viscoelastic region (LVR). Frequency sweep from 0.1 to 10 Hz was performed at 0.5% strain and 25 °C. Elastic (G´) and viscous (G´´) moduli were recorded.

3.4.7.3 Confocal laser scanning microscopy (CLSM)

Foam microstructure was visualized by a confocal laser scanning microscope (Olympus, Tokyo, Japan). Rhodamine B, as a specific dye for protein, was added to each sample prior to foam formation (0.2 mL of 0.1% w/w for 1 g protein). After whipping, a small amount of foams was gently loaded into a single-welled microscope slides (with 1 to 1.2 mm concavity). An excitation wavelength of 533 nm and the emission wavelength of 630 nm with a helium–neon red laser were used for foam structure analysis. CLSM images were recorded after 5 and 60 min after foam formation.

3.4.8 Statistical Analysis

Optimization of extraction was analyzed using CCD as mentioned above. All experiments were run in triplicate. Data were subjected to analysis of variance. Comparison of means was carried out by the Duncan's multiple range tests (Steel and Torrie, 1980). T-test was used for pair comparison. Analysis was performed using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL, USA).

3.5 Results and discussion

3.5.1 Optimization of ultrasonication of SOP using RSM

Thirteen treatments were derived from the CCD using two independent variables including amplitude (X_1) and ultrasonication time (X_2) (Table 4). Non-significant model and significant lack of fit were observed for FS. It was suggested that both independent variables had no significant effect on FS. Therefore, the predicted model for FS could not be used to optimize the ultrasonication conditions.

Table 4. Central composite design for ultrasonication of squid ovary powder using RSM and experimental data for foaming capacity (FC), surface hydrophobicity of SOP under the different ultrasonication treatments.

Std.	Coded levels		Actual levels		FC (%)	FS (%)	Hydrophobicity
	Amplitude (%)	Time (min)	Amplitude (%)	Time (min)			
1	30.00	10.00	-1	-1	162.2±13.9	17.7±1.7	537.0±2.3
2	70.00	10.00	1	-1	180.8±9.6	14.0±1.6	512.2±4.3
3	30.00	30.00	-1	1	166.7±6.7	17.2±2.5	522.1±6.2
4	70.00	30.00	1	1	220.0±11.5	14.4±1.9	585.4±6.0
5	21.72	20.00	-1.414	0	161.0±11.5	14.8±1.3	499.7±3.0
6	78.28	20.00	1.414	0	204.0±3.8	14.8±0.2	555.4±3.0
7	50.00	5.86	0	-1.414	141.1±6.9	14.4±1.7	536.6±5.0
8	50.00	34.14	0	1.414	173.3±12.0	14.1±4.4	550.9±6.2
9	50.00	20.00	0	0	166.6±12.5	13.0±0.6	501.3±9.2
10	50.00	20.00	0	0	176.1±10.3	13.1±1.0	505.3±1.0
11	50.00	20.00	0	0	168.0±8.9	14.0±1.9	510.9±3.7
12	50.00	20.00	0	0	170.9±6.3	12.4±0.6	497.6±3.3
13	50.00	20.00	0	0	166.1±5.6	12.9±0.8	504.3±2.3

Hence, FC and surface hydrophobicity as the dependent variables were used to optimize the ultrasonication for SOP treatment. The ANOVA results indicated that both parameters of the model could explain response variables as shown by significant F-value and p value for the model (Table 5). Lack of fit was also given in Table 5. Based on the result, lack of fit was not significant, confirming the validation of the fitted models. R^2 for FC and surface hydrophobicity were 0.91 and 0.96, respectively. For a good fit of all models, the R^2 values had to be higher than 0.75 (Table 5). This result indicated that the data fit the statistical model very well (Joglekar and May, 1987).

Table 5. Regression coefficients of the predicted second-order polynomial models for FC and surface hydrophobicity of squid ovary powder subjected to ultrasonication.

Regression coefficients (β)	***Foaming capacity***	***Hydrophobicity***
Intercept		
β_0	168.0	503.87
Linear		
β_1	16.59	14.67
β_2	11.15	9.8
Cross product		
β_{12}	8.67	22.0
Quadratic		
β_1	1.39	12.71
β_2	-2.25	- 20.82
R^2	0.91	0.96
Lack of Fit	0.066	0.065
p-value	< 0.0001	< 0.0001

3.5.1.1 Effect of independent variables on FC

The effect of the ultrasonication conditions (amplitude and sonication time) on FC was studied. F-values and p values were used to determine the significance of each coefficient. Both independent variables (amplitude and sonication time) had significant effect on FC (Figure 8A). The value of FC was in range of 161 to 220%. Among all the variables, FC was highly influenced by linear term of amplitude (X_1) ($p<0.0001$). However, interaction between amplitude and sonication time had non-significant effect on FC ($p>0.05$). Amplitude (X_1) and sonication time (X_2) showed the positive effect on FC as tested by regression as shown below:

$$FC= 168+16.59X_1 + 11.15\ X_2 +8.67\ X_1\ X_2 +1.39\ X_1^2-2.25\ X_2^2$$

With increasing amplitude and sonication time, FC generally increased. Increase in FC of SOP treated with ultrasonication was more likely due to the partial unfolding of the protein molecules (Table 4). Furthermore, the flexibility and surface activity of protein molecules could be enhanced. As a result, the faster adsorption at the air-water interface could be achieved (Dukhin, Kretzschmar and Miller, 1995; Gülseren *et al.*, 2007). However, slight decrease in FC was observed when amplitude was increased to 78% (Table 4). Under the harsh conditions, unfolded protein molecules plausibly underwent protein-protein aggregation, thereby lowering solubility and foaming ability (Table 4). When ultrasonication was applied, unfolding of protein molecules took place. Subsequently, hydrophobic group exposed might favorably undergo protein-protein interaction, leading to the formation of aggregates. The large aggregates lowered their solubility (Table 4). SOP without ultrasonication showed lower FC (118%) than sodium caseinate (275%), whey protein isolate (230%) and ovalbumin (200%) at the same concentration (4%) (Britten and Lavoie, 1992). In another study, foam expansion of milks (pH 6.6) reconstituted from low-heat powders and high heat powders was 760–860% and 790–920%, respectively (Augustin and Clarke, 2008). Ultrasound conditions have been known to influence foaming properties of proteins. Foam capacity of alpha-lactalbumin was improved after ultrasound treatments for 15 min at 20 kHz (145%) and 40 kHz treatments (141%) than the control (113%) (Jambrak *et al.*, 2010). Similarly, FC was increased

to 123 from 110%, when soy protein isolate was treated with ultrasound for 30 min at 20 kHz (Jambrak *et al.*, 2009). The regression equation obtained from ANOVA with R^2 value of 0.91 (Table 5) revealed that the model was able to explain the variation in responses. The p value of models ($p<0.0001$) and non-significant lack of fit (0.066) indicated that the obtained experimental data could fit well with the model.

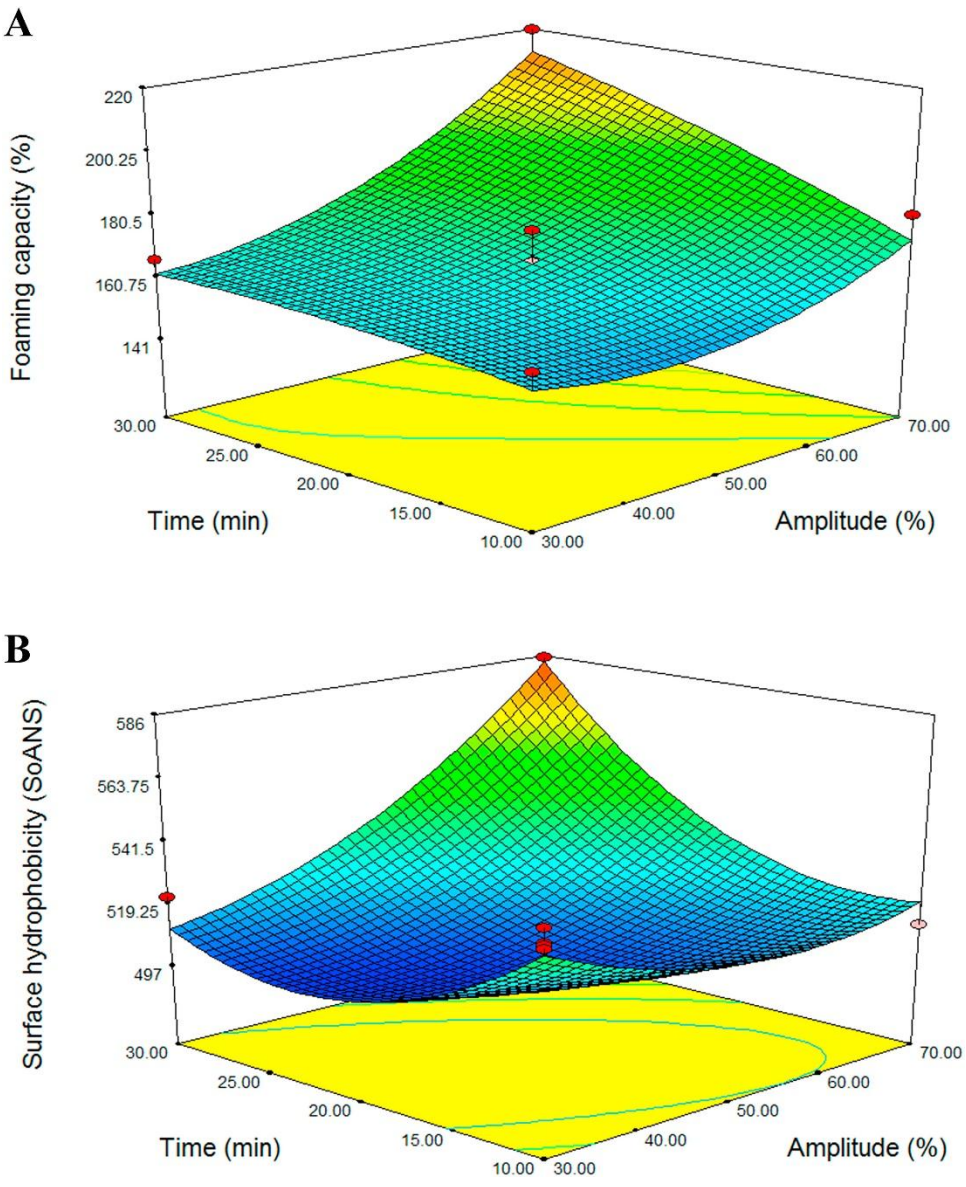

Figure 8. Three-dimensional response surface for the ultrasonication under various conditions on foaming properties of SOP. FC (A) and hydrophobicity (B).

3.5.1.2 Effect of independent variables on surface hydrophobicity

Effect of independent variables on surface hydrophobicity is shown in Table 5 and Figure 8B. Surface hydrophobicity was in range of 498 to 585. Among all the variables, surface hydrophobicity was highly influenced by quadratic term of sonication time (X_2) ($p<0.0001$). Linear term of amplitude (X_1), interaction term of both variables and quadratic term of amplitude (X_1) showed similar effect on surface hydrophobicity. Surface hydrophobicity was also influenced by linear term of sonication time (X_2). Effect of amplitude (X_1) and sonication time (X_2) showed as regression equation and indicated the positive effect of all linear variable on surface hydrophobicity with the following equation:

$$\text{Surface hydrophobicity} = 503.87+14.67X_1 + 9.8\ X_2 +22\ X_1\ X_2 +12.71\ X_1^2 - 20.82\ X_2^2$$

As the amplitude increased, the surface hydrophobicity of SOP increased. These results suggested that the ultrasonication induced the certain degree of unfolding of protein molecules and exposed the hydrophobic groups and region buried inside the molecules to the polar environment. The regression equation obtained from ANOVA with R^2 value of 0.96 (Table 5) revealed that the model could be used to explain the variation in responses. With significant p value of models ($p<0.0001$) and non-significant lack of fit (0.065), the obtained experimental data could fit well with the model.

3.5.2 Optimization of the ultrasonication and the model validation

Ultrasonic conditions were optimized using FC and surface hydrophobicity as dependent variables to enhance the foaming properties of SOP. The optimized condition was: amplitude of 70% with sonication time of 30 min. The validation of the statistical model and regression equation of optimal condition were examined. FC and surface hydrophobicity were determined from SOP treated with ultrasound under the optimized condition. Similar predicted value and observed values with the minimum percentage of error (FC: 0.81% and surface hydrophobicity 0.21%) were obtained. The result reconfirmed the validity and acceptability of the

statistical model for the optimization of ultrasonication condition. Therefore, optimum ultrasonication could be used to treat SOP in order to improve foaming properties.

3.5.3 Characteristics of SOP and USOP

3.5.3.1 Solubility

Solubility of SOP and USOP is shown in Table 6. Solubility of USOP (66.16%) was lower than that of SOP (72.2%). When ultrasonication was applied, unfolding of protein molecules took place. Subsequently, hydrophobic group exposed might favorably undergo protein-protein interaction, leading to the formation of aggregates. The large aggregates lowered their solubility. Ultrasonication with high amplitude and sonication time yielded USOP with decreased solubility as compared to SOP treated at lower amplitude for the shorter time (data not shown). Arzeni *et al.* (2012) also observed the decrease in solubility, when EW was subjected to ultrasonication. However, Jambrak *et al.* (2009) reported the increase in solubility when soy protein was treated with ultrasonication (20 kHz, 30 min). Ultrasonication resulted in the reduction of particle size of SOP (Table 6). Smaller particles possessed the increased surface area, which could be more reactive or get involved in reactions such as oxidation or protein aggregation between particles. Formation of intermolecular disulfide bonds contributed to the loss in protein solubility (Shimada and Cheftel, 1988). Furthermore, Singh *et al.* (2017) reported that squid ovary had high percentage of carbohydrates, which might play a role in lowering solubility of SOP via the interaction between protein and carbohydrates. When ultrasonication was implemented, the interactions between all constituents might be favored. Nevertheless, lactose in WPC had no influence on solubility due to presence of lactose which act as preservative for WPC during pasteurization (Arzeni *et al.*, 2012). Thus, several factors were involved in lowering the solubility of SOP when ultrasound was applied.

Table 6. Solubility, surface hydrophobicity, total sulfhydryl group, particle size and transition temperatures of SOP and USOP

		SOP	USOP
Solubility (%)		72.2 ± 0.4[a]	66.2 ± 0.1[b]
Hydrophobicity		421.4±9.7[b]	585.4±7.2[a]
Total SH group (mole/10^5 g protein)		424.8 ± 8.1[a]	396.3± 1.1[b]
Mean particle size (µm)		17.1 ± 1.4 [a]	6.15 ± 0.3 [b]
Particle size (µm)			
<10%[A]		0.88 ± 0.0 [a]	0.71 ± 0.0 [b]
<50%[B]		12.72 ± 0.1 [a]	1.83 ± 0.0 [b]
<90%[C]		42.99 ± 2.0 [a]	20.40 ± 1.5 [b]
Transition temperature (°C)			
First peak	T_{onset}	21.3 ± 1.0 [a]	24.0 ± 1.0 [b]
	T_{max}	24.3 ± 1.9 [a]	31.8 ± 0.9 [b]
	T_{end}	28.2 ±1.1 [a]	38.9 ± 1.6 [b]
Second peak	T_{onset}	50.3 ± 2.7[*]	ND
	T_{max}	62.7 ± 1.9[*]	ND
	T_{end}	75.4 ± 2.0[*]	ND

Values are mean ± SD (n=3). ND: Not detected. Different superscripts in the same row denote significant difference ($p<0.05$)

*No data was available for comparison with counterpart.

[A] Using Mie's theory it is determined that 10% particles have diameter less that stated.

[B] Using Mie's theory it is determined that 50% particles have diameter less that stated.

[C] Using Mie's theory it is determined that 90% particles have diameter less that stated.

3.5.3.2 Surface hydrophobicity

Surface hydrophobicity was increased significantly when SOP was treated with ultrasound under optimized condition ($p<0.05$) (Table 6). Surface hydrophobicity is one of characteristics which affects the structural and functional properties of protein (Kato and Nakai, 1980). USOP showed the higher surface hydrophobicity (585.36) than SOP (421.4). Cavitation effect of ultrasound applied was plausibly associated with the unfolding of protein molecules, in which

hydrophobic domains became more exposed. Similarly, surface hydrophobicity of reconstituted milk protein concentrate increased as ultrasound treatment was applied for 0.5 min. (Yanjun *et al.*, 2014) also reported the increase in surface hydrophobicity, when WPC, SPI and EW were treated with high intensity ultrasound (net power output, 750 W at an amplitude of 20%) for 20 min. In general, hydrophobic proteins orient more readily than less hydrophobic ones at interface, when with their polar groups directed toward the aqueous phase and their hydrophobic groups toward the non-aqueous phase to lower surface free energy (Kato and Nakai, 1980). The increased surface hydrophobicity results in higher flexibility, faster unfolding and more interaction at the interface, which results in the formation of strong and elastic film with higher FC (Lomakina and Mikova, 2006).

3.5.3.3 Total sulfhydryl group

The lower sulfhydryl group content of SOP was observed after the ultrasonication under the optimized condition (Table 6). Total sulfhydryl group content was significantly decreased from 424.8 to 369.3 when treated with ultrasound ($p<0.05$). Ultrasound may produce transient radical species ($OH^{\bullet}$, $H^{\bullet}$) in the gas phase of the cavities that cross-react to produce hydrogen peroxide. The reaction of hydrogen peroxide with BSA molecules may alter its chemical structure (Gülseren *et al.*, 2007). Gülseren *et al.* (2007) and Taylor and Richardson (1980) also observed the decrease in sulfhydryl groups content in BSA and skim milk, respectively after ultrasonication. Hydrogen peroxide generated during ultrasonication might induce the oxidation of SOP protein, in which disulfide bonds were formed. This was evidenced by the decrease in free sulfhydryl group. Additionally, the unfolding of protein might favor the availability of sulfhydryl groups to reactive species. As a result, sulfhydryl content of SOP was decreased. The decreases in sulfhydryl group were coincidental with the lower solubility of USOP, compared to SOP. It was suggested that oxidation of sulfhydryl groups might contribute to the decrease in solubility of SOP.

3.5.3.4 Protein pattern

SDS-PAGE was used to monitor polymerization or degradation of protein caused by the ultrasonication. Under non-reducing condition, SOP showed the major bands with MW of 255.7, 136.2, 90.4 and 13.7 kDa. However, in the presence of β-ME, SOP had the different protein patterns, in which the bands having MW of 165.5, 120.5 and 22.55 kDa were observed. Additionally, a large number of proteins with MW in the range of 15.49 kDa and lower were found (Figure 9). The result suggested that SOP proteins were stabilized by disulfide bond. In general, USOP showed the similar protein patterns to SOP when the same condition, either reducing or non-reducing, was used. This result confirmed that the molecular weight of the proteins was not altered by ultrasonication under the optimal condition used in the present study. Similar results were observed by Yanjun *et al.* (2014), who treated

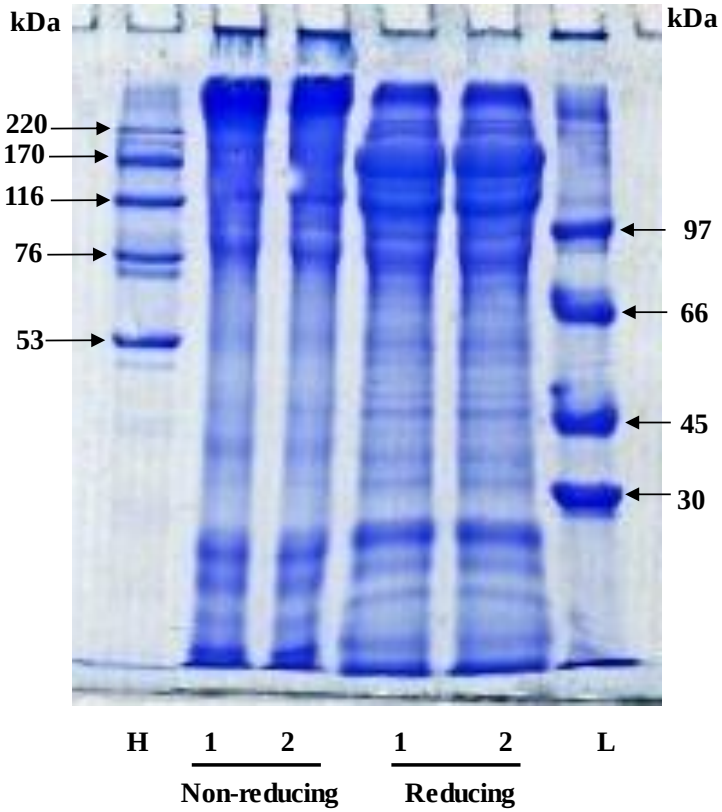

Figure 9. SDS–PAGE patterns of SOP and USOP under reducing and non-reducing conditions. Lane number 1 and 2 denoted SOP and USOP, respectively. L and H denoted low and high MW standards, respectively.

reconstituted milk protein concentrate with power ultrasound (12.50 ± 0.31W and 50% amplitude) for 0.5-5 min. No difference in protein pattern between the control and the ultrasonicated BSA was found and no dimerization occurred (Gülseren *et al.*, 2007). Thus, ultrasonication had no influence on protein pattern of SOP. Nevertheless, it showed the impact on physicochemical properties and conformation of protein in SOP.

3.5.3.5 Particle size distribution

Particle size distribution of SOP and USOP is shown in Table 6. Significant reduction in particle size after the ultrasonication was noticeable ($p<0.05$). The mean particle size of SOP was decreased from 17.14 to 6.15 µm after treated with ultrasound. It was noted that the particle size distribution was narrow (Table 6). The reduction in the particle size was more likely due to cavitational effect produced by ultrasonic probe, which dissociated the large particle into smaller one (Morales *et al.*, 2015). Ultrasonication produced shear stress, which increased the aggregation and collision between the aggregates and resulted in the reduced particle size (Jambrak *et al.*, 2008). Jambrak *et al.* (2008) also observed the reduced particle size distribution of whey protein after treatment with an ultrasonic probe of 20 kHz. Conversely, Arzeni *et al.* (2012) observed the increase in particle size, when EW was treated with high intensity ultrasound (amplitude: 20%; sonication time: 20 min). Overall, reduction in the particle size could improve the foaming properties of proteins. Small aggregates (< 70 nm) of beta-lactoglobulin showed better foaming properties than the beta-lactoglobulin with large aggregates (> 120 nm) (Rullier, Novales and Axelos, 2008). The lower particle size of USOP might help in migration of proteins to air-water interface. This was evidenced by the increased FC.

3.5.3.6 Differential scanning calorimetry (DSC)

Transition temperatures of SOP were analyzed by heating SOP and USOP from 20 to 100 ºC (Table 6). Native SOP showed two transition peaks. The first peak had T_{onset}, T_{max} and T_{end} of 21.28, 24.33 and 62.67 ºC, respectively. The second peak showed T_{onset}, T_{max} and T_{end} of 50.33, 62.67 and 75.37 ºC, respectively.

After the ultrasonication of SOP, the first peak was slightly shifted to the higher temperatures (T_{onset}, T_{max} and T_{end}; 24.04, 31.38 and 39.98 ºC). This was more likely due to the partial denaturation of proteins in SOP as induced by ultrasound used. The association of new intermolecular bonds between the denatured molecules might increase thermal stability of proteins as indicated by the shift to higher temperature (Chandrapala *et al.*, 2011). However, the second peak completely disappeared, indicating the denaturation of some proteins in SOP after the ultrasonication. Denaturation involves dissociation of intramolecular non-covalent and covalent bond (Chandrapala *et al.*, 2011). Disruption of bonding involved in the stability of tertiary and/or secondary protein structures might lead to the complete denaturation of proteins (Relkin, 1994). Therefore, DSC results suggested that ultrasonication directly affected thermal properties of SOP via inducing denaturation or aggregation of proteins.

3.5.4 Foaming properties of SOP and USOP

3.5.4.1 Effect of whipping time on FC

Effect of whipping time (1-4 min) on the foaming capacity of 4% (w/v) SOP and USOP solutions is shown in Figure 10. FC was increased with increasing whipping time for both SOP and USOP (Figure 10A). USOP showed the higher FC than SOP for all whipping time used ($p<0.05$). USOP showed the higher FC (320%) than the SOP (244%), when the whipping time of 4 min was used ($p<0.05$). With increased unfolding and surface hydrophobicity induced by ultrasound, proteins could migrate to the air-water interface more efficiently, occupy and form the film surrounding air bubbles. This was shown by the higher FC of USOP. It was noted that the solution of USOP showed higher FC (220%) then corresponding USOP after freeze-drying (158.9%). This was more likely due to aggregation of the exposed hydrophobic domains during the freeze-drying process, leading to lowered solubility with decreased FC.

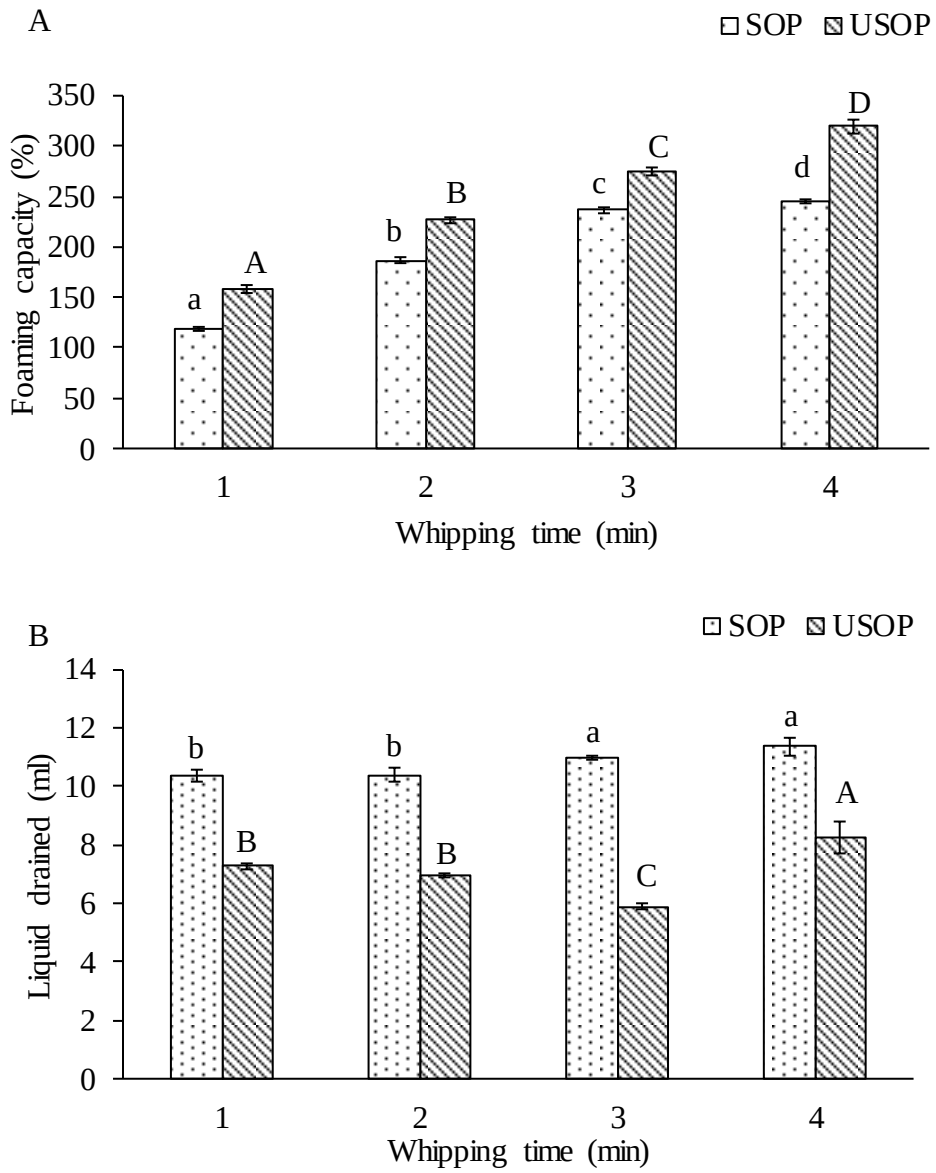

Figure 10. Effect of whipping time on foaming capacity (A) and liquid drainage (B) of foam prepared from 4% SOP and 4% USOP solutions. Bars represent the standard deviation (n = 3). Different lowercase or uppercase letters on the bars under same sample indicates significant difference ($p<0.05$).

FS of SOP and USOP solutions were determined in term of liquid drainage (Figure 10B). Foam of USOP solution showed the lower liquid drainage at all whipping times (1-4 min), as compared to that prepared from SOP solution. This indicated the higher foaming stability of foam from USOP. Generally, foam with the lower liquid drainage is more stable. Drainage of liquid from lamellar is associated

with the collapse of air bubbles, in which liquid is pooled and drained (Narsimhan and Ruckenstein, 1986). Liquid drainage was decreased when whipping time increased up to 3 min for the foam prepared from USOP solution *(*$p<0.05$). Nevertheless, liquid drainage was increased when the longer whipping time (4 min) was implemented ($p<0.05$). Excessive whipping might enhance the aggregation of unfolded proteins induced by ultrasound. As a consequence, the poorer solubility was obtained, resulting in the increased drainage from lamella. No difference in liquid drainage between foam with whipping time of 1 and 2 min ($p<0.05$). For foam prepared from SOP solution, the increases in liquid drainage were obtained with increasing whipping time up to 4 min ($p<0.05$), indicating lower foam stability. It was noted that increasing FC of SOP solution was related with lower stability. Sample with high FC might exert greater pressures inside bubbles. As a result, liquid drainage was induced (German, O'Neill and Kinsella, 1985). Additionally, higher liquid drainage might be due to the complete denaturation of protein due to the excessive whipping. Singh *et al.* (2017) reported that squid ovary contained high amount of carbohydrate (26.64%), which might play an important role in stabilizing foam by increasing the viscosity of mixture. Jambrak *et al.* (2010) reported that mechanical homogenization by ultrasonication, which usually disperses the protein, carbohydrates and fat particles more evenly, could improve the foaming properties. Thus, ultrasonication of SOP along with the appropriate whipping time could improve both foaming capacity and stability.

3.5.4.2 Rheology of foam

Storage (G´) and loss (G´´) moduli of SOP and USOP as function of frequency are shown in Figure 11A and B, respectively. Higher G´ and G´´ were observed for USOP, in comparison with those of SOP at all frequencies tested. Higher G´ indicated that foam was solid-elastic, whereas higher G´´ implied that foam became more liquid viscous. The results suggested that ultrasonication increased the viscoelastic properties of foam from SOP. Cavitational effect of ultrasonication most likely altered the structure of proteins in SOP via unfolding. Unfolded proteins with more exposed hydrophobic domains could form hydrophobic-hydrophobic interactions, in which strong and viscoelastic film could be formed at air-water inter.

Entanglement of unfolded proteins could increase viscoelastic property of USOP solution as indicated by the higher G´ and G´´ than SOP. Elastic films surrounding air

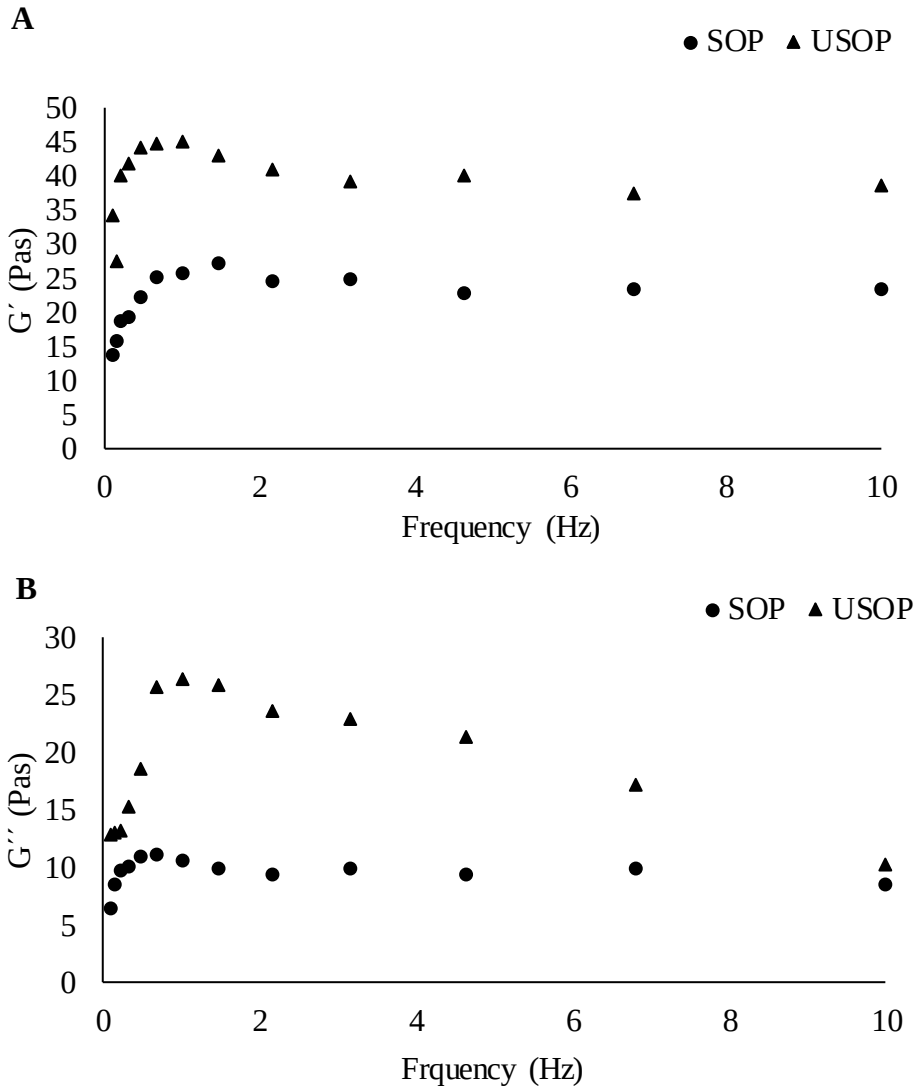

Figure 11. Storage modulus (A) and loss modulus (B) of foam prepared from 4% SOP and 4% USOP solutions. Whipping time of 3 min was used for foam preparation.

bubbles more likely contributed to the increased foam stability of USOP. Tan *et al.* (2015) also observed the increased G´ and G´´ of WPC when ultrasound amplitude (20-60%) and treatment time (5-25 min) increased. The result reconfirmed that ultrasonication was able to increase the foam viscoelastic properties of SOP in the

fashion, which favored the formation of viscous and strong films at air-water interface.

3.5.4.3 Confocal laser scanning microscopy (CLSM)

Microstructures of foam prepared from SOP and USOP solutions visualized by CLSM are shown in Figure 12. Freshly prepared foam (5 min) from SOP showed a larger air bubble with the thinner lamella (Figure 12A), whereas foam prepared from USOP showed relatively smaller bubbles with thicker lamella (Figure 12B). USOP plausibly had the modified proteins, which were able to migrate into the interface rapidly. As a result, the small air bubbles were formed and stabilized by protein films. In the present study, the reduction in particle size of USOP was noted, in comparison with SOP (Table 6). Smaller molecules could move to air-water

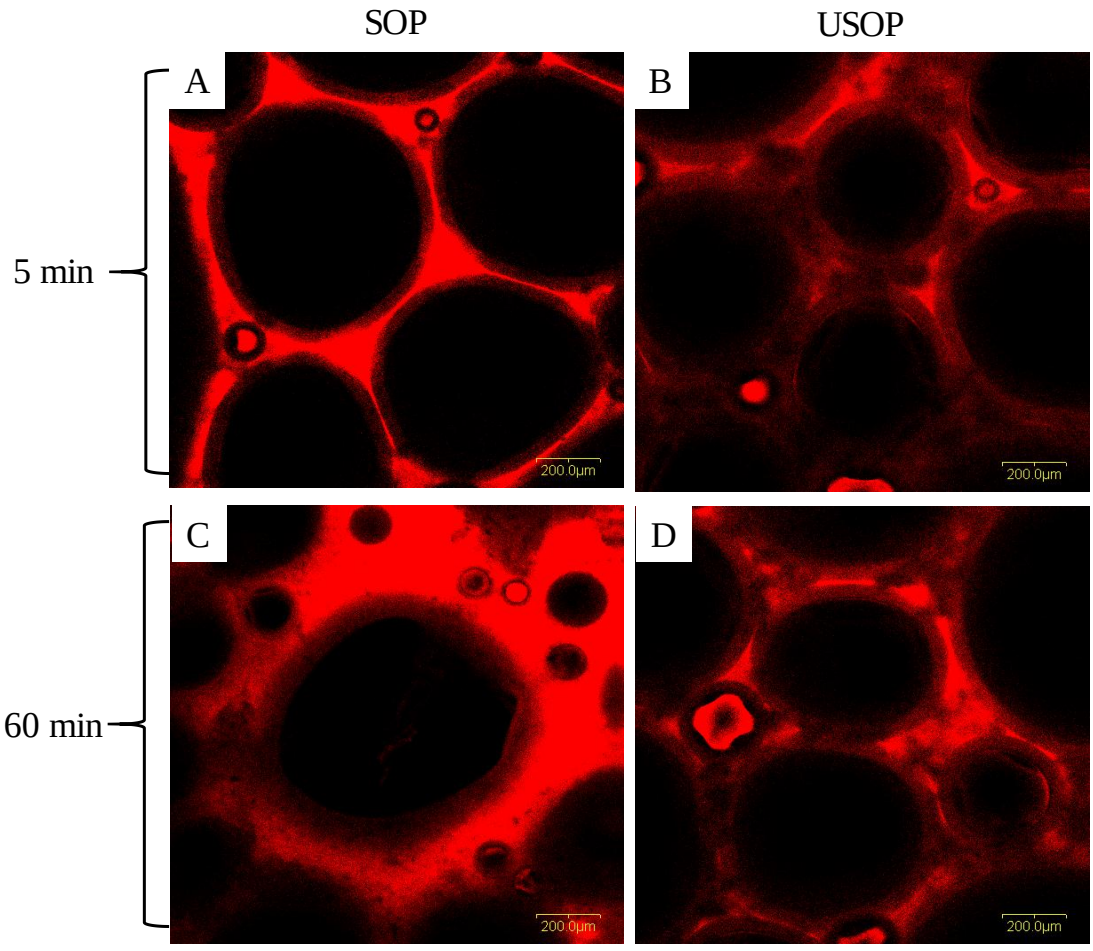

Figure 12. Confocal microscopy image of foam prepared from 4% SOP and 4% USOP solutions at 5 min (A, B) and 60 min (C, D) after whipping. Whipping time of 3 min was used for foam preparation.

interface with ease at the faster rate. The result coincided with higher FC of USOP than SOP. Figure 12C and D showed the microstructure of foam from SOP and USOP solutions after the 60 min of whipping of solutions. Bubbles of foam prepared from SOP solution disappeared after 60 min, mainly due to the collapse of foam associated with the liquid drainage (Figure 12C). However, foam from USOP solution still contained air bubbles but slight increase in the size of the bubble was observed (Figure 12D). Uniform distribution of bubbles in the USOP foam than SOP foam supported the higher stability of foam (Zayas, 1997). This result was coincidental with the lower liquid drainage or high foam stability of foam from USOP than SOP (Figure 10). Liquid drainage decrease the thickness of lamella, which results in the coalescences of bubbles (Zayas, 1997). Thus, ultrasonication was a means to improve foam stability of SOP.

3.6 Conclusion

Ultrasonication conditions (amplitude and sonication time) were optimized using RSM to improve the foaming properties of SOP. Ultrasonication of frequency 20 kHz for 30 min at an amplitude 70% increased foaming capacity of SOP. Whipping time of SOP and USOP up to 4 min increased FC. Highest foaming stability of USOP was obtained when whipping time of 3 min was used. Rheological properties of foam were also improved when SOP was subjected to ultrasonication. Ultrasonication increased surface hydrophobicity, however it slightly decreased solubility and total sulfhydryl group content. Transition temperatures were altered after ultrasonication. Ultrasonication had no impact on protein pattern of SOP. Overall, foam became more stable with decreased liquid drainage when ultrasonication was used for pretreatment of SOP.

3.7 References

Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P. A. and Gruppen, H. 2011. Surface activity and molecular characteristics of cuttlefish skin gelatin modified by oxidized linoleic acid. International Journal of Biological Macromolecules. 48: 650-660.

Arzeni, C., Martínez, K., Zema, P., Arias, A., Pérez, O. and Pilosof, A. 2012. Comparative study of high intensity ultrasound effects on food proteins functionality. Journal of Food Engineering. 108: 463-472.

Augustin, M. A. and Clarke, P. T. 2008. Skim milk powders with enhanced foaming and steam-frothing properties. Dairy Science and Technology. 88: 149-161.

Benjakul, S., Visessanguan, W., Ishizaki, S. and Tanaka, M. 2001. Differences in gelation characteristics of natural actomyosin from two species of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. Journal of Food Science. 66: 1311-1318.

Bhargava, A. and Jelen, P. 1995. Freezing of whey protein concentrate solutions and its effect on protein functionality indicators. International Dairy Journal. 5: 533-541.

Britten, M. and Lavoie, L. 1992. Foaming properties of proteins as affected by concentration. Journal of Food Science. 57: 1219-1241.

Brownlee, M., Michael. 1995. Advanced protein glycosylation in diabetes and aging. Annual Review of Medicine. 46: 223-234.

Bußler, S., Steins, V., Ehlbeck, J. and Schlüter, O. 2015. Impact of thermal treatment versus cold atmospheric plasma processing on the techno-functional protein properties from *Pisum sativum* 'Salamanca'. Journal of Food Engineering. 167(B): 166-174.

Camire, M. E. 1991. Protein functionality modification by extrusion cooking. Journal of the American Oil Chemists Society. 68: 200-205.

Chalamaiah, M., Rao, G. N., Rao, D. G. and Jyothirmayi, T. 2010. Protein hydrolysates from meriga (*Cirrhinus mrigala*) egg and evaluation of their functional properties. Food Chemistry. 120: 652-657.

Chandrapala, J., Zisu, B., Palmer, M., Kentish, S. and Ashokkumar, M. 2011. Effects of ultrasound on the thermal and structural characteristics of proteins in reconstituted whey protein concentrate. Ultrasonics Sonochemistry. 18: 951-957.

Chemat, F. and Khan, M. K. 2011. Applications of ultrasound in food technology: processing, preservation and extraction. Ultrasonics Sonochemistry. 18: 813-835.

Chemat, F. and Khan, M. K. 2011. Applications of ultrasound in food technology: processing, preservation and extraction. Ultrasonics Sonochemistry. 18: 813-835.

Damodaran, S., Parkin, K. L. and Fennema, O. R. 2008. Fennema's Food Chemistry. CRC Press/Taylor and Francis. Boca Raton.

Dukhin, S. S., Kretzschmar, G. and Miller, R. 1995. Dynamics of Adsorption at Liquid Interfaces: Theory, Experiment, Application. Vol. 1. Elsevier.

German, J., O'Neill, T. and Kinsella, J. 1985. Film forming and foaming behavior of food proteins. Journal of the American Oil Chemists Society. 62: 1358-1366.

Gornall, A. G., Bardawill, C. J. and David, M. M. 1949. Determination of serum proteins by means of the Biuret reaction. The Journal of Biological Chemistry. 177: 751-766.

Gülseren, İ., Güzey, D., Bruce, B. D. and Weiss, J. 2007. Structural and functional changes in ultrasonicated bovine serum albumin solutions. Ultrasonics Sonochemistry. 14: 173-183.

Güzey, D., Gülseren, İ., Bruce, B. and Weiss, J. 2006. Interfacial properties and structural conformation of thermosonicated bovine serum albumin. Food Hydrocolloids. 20: 669-677.

Intarasirisawat, R., Benjakul, S. and Visessanguan, W. 2011. Chemical compositions of the roes from skipjack, tongol and bonito. Food Chemistry. 124: 1328-1334.

Jambrak, A., Lelas, V., Mason, T. J., Krešić, G. and Badanjak, M. 2009. Physical properties of ultrasound treated soy proteins. Journal of Food Engineering. 93: 386-393.

Jambrak, A., Mason, T. J., Lelas, V., Herceg, Z. and Herceg, I. L. 2008. Effect of ultrasound treatment on solubility and foaming properties of whey protein suspensions. Journal of Food Engineering. 86: 281-287.

Jambrak, A. R., Lelas, V., Mason, T. J., Krešić, G. and Badanjak, M. 2009. Physical properties of ultrasound treated soy proteins. Journal of Food Engineering. 93: 386-393.

Jambrak, A. R., Mason, T. J., Lelas, V., Herceg, Z. and Herceg, I. L. 2008. Effect of ultrasound treatment on solubility and foaming properties of whey protein suspensions. Journal of Food Engineering. 86: 281-287.

Jambrak, A. R., Mason, T. J., Lelas, V. and Krešić, G. 2010. Ultrasonic effect on physicochemical and functional properties of α-lactalbumin. LWT-Food Science and Technology. 43: 254-262.

Joglekar, A. M. and May, A. T. 1987. Product excellence through design of experiments. Cereal Foods World. 32: 857-868.

Karki, B., Lamsal, B. P., Grewell, D., Pometto, A. L., Van Leeuwen, J., Khanal, S. K. and Jung, S. 2009. Functional properties of soy protein isolates produced from ultrasonicated defatted soy flakes. Journal of the American Oil Chemists' Society. 86: 1021-1028.

Kato, A. and Nakai, S. 1980. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. Biochimica et Biophysica Acta (BBA) - Protein Structure. 624: 13-20.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2012. Effect of extraction temperature on functional properties and antioxidative activities of gelatin from shark skin. Food Bioprocess Technology. 5: 2646-2654.

Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chemistry. 102: 1317-1327.

Lateef, A., Oloke, J. K. and Prapulla, S. G. 2007. The effect of ultrasonication on the release of fructosyltransferase from *Aureobasidium pullulans* CFR 77. Enzyme and Microbial Technology. 40: 1067-1070.

Lomakina, K. and Mikova, K. 2006. A study of the factors affecting the foaming properties of egg white-a review. Czech Journal of Food Sciences. 24: 110-118.

Marinova, K. G., Basheva, E. S., Nenova, B., Temelska, M., Mirarefi, A. Y., Campbell, B. and Ivanov, I. B. 2009. Physico-chemical factors controlling the foamability and foam stability of milk proteins: sodium caseinate and whey protein concentrates. Food Hydrocolloids. 23: 1864-1876.

Martínez-Padilla, L., García-Mena, V., Casas-Alencáster, N. and Sosa-Herrera, M. 2014. Foaming properties of skim milk powder fortified with milk proteins. International Dairy Journal. 36: 21-28.

Mirmoghtadaie, L., Kadivar, M. and Shahedi, M. 2009. Effects of succinylation and deamidation on functional properties of oat protein isolate. Food Chemistry. 114: 127-131.

Morales, R., Martínez, K. D., Pizones Ruiz-Henestrosa, V. M. and Pilosof, A. M. R. 2015. Modification of foaming properties of soy protein isolate by high ultrasound intensity: particle size effect. Ultrasonics Sonochemistry. 26: 48-55.

Narsimhan, G. and Ruckenstein, E. 1986. Hydrodynamics, enrichment, and collapse in foams. Langmuir. 2: 230-238.

O'Sullivan, J., Arellano, M., Pichot, R. and Norton, I. 2014. The effect of ultrasound treatment on the structural, physical and emulsifying properties of dairy proteins. Food Hydrocolloids. 42: 386-396.

Phillips, L. G. 2013. Structure-Function Properties of Food Proteins. Academic Press. Cambridge, Massachusetts, United States.

Popineau, Y., Huchet, B., Larré, C. and Bérot, S. 2002. Foaming and emulsifying properties of fractions of gluten peptides obtained by limited enzymatic hydrolysis and ultrafiltration. Journal of Cereal Science. 35: 327-335.

Relkin, P. 1994. Differential scanning calorimetry: A useful tool for studying protein denaturation. Thermochimica Acta. 246: 371-386.

Rullier, B., Novales, B. and Axelos, M. A. 2008. Effect of protein aggregates on foaming properties of β-lactoglobulin. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 330: 96-102.

Shimada, K. and Cheftel, J. C. 1988. Texture characteristics, protein solubility, and sulfhydryl group/disulfide bond contents of heat-induced gels of whey protein isolate. Journal of Agricultural and Food Chemistry. 36: 1018-1025.

Singh, A., Benjakul, S. and Kishimura, H. 2017. Characteristics and functional properties of ovary from squid *Loligo formosana*. Journal of Aquatic Food Product Technology. 26: 1083-1092.

Speroni, F., Beaumal, V., de Lamballerie, M., Anton, M., Añón, M. C. and Puppo, M. C. 2009. Gelation of soybean proteins induced by sequential high-pressure and thermal treatments. Food Hydrocolloids. 23: 1433-1442.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and Procedures of Statistics: A Biometrical Approach. 2nd Ed. McGraw-Hill. New York.

Tan, M. C., Chin, N. L., Yusof, Y. A., Taip, F. S. and Abdullah, J. 2015. Characterisation of improved foam aeration and rheological properties of ultrasonically treated whey protein suspension. International Dairy Journal. 43: 7-14.

Taylor, M. and Richardson, T. 1980. Antioxidant activity of skim milk: effect of sonication. Journal of Dairy Science. 63: 1938-1942.

Wang, J., Zhao, M., Yang, X. and Jiang, Y. 2006. Improvement on functional properties of wheat gluten by enzymatic hydrolysis and ultrafiltration. Journal of Cereal Science. 44: 93-100.

Yanjun, S., Jianhang, C., Shuwen, Z., Hongjuan, L., Jing, L., Lu, L., Uluko, H., Yanling, S., Wenming, C., Wupeng, G. and Jiaping, L. 2014. Effect of power ultrasound pre-treatment on the physical and functional properties of reconstituted milk protein concentrate. Journal of Food Engineering. 124: 11-18.

Zayas, J. F. 1997. Foaming Properties of Proteins. *In* Functionality of Proteins in Food. p. 260-309. Springer Berlin Heidelberg, Heidelberg.

CHAPTER 4
EFFECT OF PARTIAL ENZYMATIC HYDROLYSIS ON PHYSICOCHEMICAL AND FOAMING PROPERTIES OF OVARY FROM SQUID *LOLIGO FORMOSANA*

4.1 Abstract

Squid ovary (SO) was treated with 1% Alcalase or 1% papain at different hydrolysis times and foaming properties of resulting hydrolysates were investigated. SO hydrolysate (SOH) prepared by Alcalase for 30 min (SOH-Al-30) with degree of hydrolysis (DH) of 1.79% showed the highest foaming capacity (FC) (236%). For SOH prepared by papain for 15 min with DH of 0.21%, FC of 221% was attained. Foams from both SOH were collapsed by 31 and 26% when incubated at 25 ºC for 60 min, respectively. The SOH-Al-30 powder showed high solubility and surface hydrophobicity. Particle size of SOH-Al-30 powder was slightly higher than that of squid ovary powder (SOP). FTIR spectra indicated that hydrolysis slightly affected the conformation and interaction of protein molecules. SOH-Al-30 preheated at 60 ºC showed the highest FC (300%) and had the lowest liquid drainage when whipped for 4 min. Viscoelastic properties and microstructure of foam from pre-heated SOH-Al-30 were much improved than those from SOP. Thus, enzymatic hydrolysis having the controlled DH, followed by pre-heating at optimum temperature with the appropriate whipping time could improve foaming properties of squid ovary.

4.2 Introduction

Proteins are surface-active agents due to amphiphilic nature which helps to reduce the surface tension at the interface, thus resulting in the formation of stable foam by forming strong interfacial film. The commonly used foaming agents in the food systems are egg white and milk proteins (Zayas, 1997). However, numerous factors such as chemical composition, temperature, pH, etc. impaired the functional properties of proteins such as solubility, viscoelasticity and foaming properties. Therefore, modification of proteins has been implemented to alter their native structure to improve or enhance their functional properties. Partial enzymatic hydrolysis is an efficient method for improvement of protein’s functional properties

(Guo *et al.*, 2013; Zeng *et al.*, 2013). Modified protein conformation consequently affect functional and physicochemical properties of proteins (van der Ven *et al.*, 2002). Jeewanthi, Lee and Paik (2015) observed that Alcalase treated whey protein hydrolysate with DH of 9.5% exhibited the highest foaming capacity of 287%, compared to those prepared by pepsin, trypsin, protease M and protease A. Davis, Doucet and Foegeding (2005) also documented the higher improvement of foaming ability of hydrolysates from β-lactoglobulin (β-LG) prepared using Alcalase and pepsin, compared to trypsin. Excessive hydrolysis of whey protein lowered foaming stability due to the formation of very short peptides (Kilara and Panyam, 2003; Ye and Singh, 2006).

Thailand and other Southeast Asian countries are the foremost exporters for the marine fisheries, specially squid and cuttlefish (Hoque, Benjakul and Prodpran, 2010). During squid processing, low market valued viscera is generated as a by-product. Among them, squid ovary constitutes around 10-15% of total body weight and is known as an alternative source of nutrients. Squid ovary is a good source of protein, amino acids, docosahexaenoic acid and other n-3 polyunsaturated fatty acid (Singh, Benjakul and Kishimura, 2017). Squid ovary possessed high foaming stability but had low foaming ability than the commonly used foaming agent such as egg white powder (Singh *et al.*, 2017). Singh, Benjakul and Kijroongrojana (2017) successfully enhanced the foaming properties with the aid of ultrasonication. Nevertheless, no data related to the effect of enzymatic hydrolysis on the foaming properties of squid ovary has been found.

4.3 Objective

To study was to investigate the impact of hydrolysis using Alcalase and papain on physicochemical and foaming properties of squid ovary. The impact of preheating and whipping times on foaming property of the selected squid ovary hydrolysate was also examined.

4.4 Materials and methods

4.4.1 Chemicals/enzymes

All chemicals were of analytical grade. Rhodamine B, Sodium dodecyl sulphate (SDS), trinitrobenzenesulfonic acid (TNBS), Coomassie Blue R-250 and high and low molecular weight (MW) protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). β-Mercaptoethanol (βME) and 1-anilinonaphthalene-8-sulfonic acid (ANS) were obtained from Merck (Darmstadt, Germany).

Frozen squid (*Loligo formosana*) viscera were gifted from the Sea Wealth Frozen Food, Songkhla, Thailand. Food grade Alcalase (15 unit/mL) was obtained from Novozyme (Bagsvaerd, Denmark). Papain (10 unit/mL) were gifted from Siam Victory Chemicals Co., Ltd. (Bangkok, Thailand).

4.4.2 Collection and preparation of squid ovary

Ovaries having weight in the range of 12-14 g were manually separated from other organs, placed in a polythene bag, subsequently frozen and stored at -20 ºC. The ovaries were utilized within two months. Squid ovary is rich in protein (18.64%) followed by carbohydrate (7.44 %). Ash (1.39%) and lipids (0.46%) were found as the minor constituents (Singh *et al.*, 2017). Before use, frozen ovaries were thawed using running water until the core temperature reached 0-2 ºC. Thawed samples were subjected to chopping using a Panasonic blender (Model MX-898N, Malaysia) for 2 min at 250 rpm. One portion was freeze-dried using a freeze dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The other portion was reserved for use in the preparation of squid ovary hydrolysate (SOH). The resulting freeze-dried sample was blended and sieved through 0.4 mm siever. The powder obtained was referred to as squid ovary powder (SOP).

4.4.3 Effect of hydrolysis on characteristics and properties of squid ovary

4.4.3.1 Preparation of squid ovary hydrolysates

Chopped squid ovaries which was prepared as described in Section 3.4.2 were homogenized at 8,000 rpm for 2 min with an IKA homogenizer (IKA, Labortechnik homogenizer Selangor, Malaysia) in the presence of distilled water and final solid content was adjusted to 5% (w/v). Homogenate (pH: 6.5) was preincubated at 60 and 38 ºC for 10 min to reach the optimum temperature of Alcalase and papain, respectively. Alcalase or papain at 1% (w/w, solid content) was added into pre-incubated mixture and hydrolysis was carried out for 5, 15, 30 and 60 min. Based on our preliminary work, enzyme above 1% did not increase the hydrolysis efficacy. The mixture was stirred continuously during the hydrolysis process. The reaction was terminated by boiling the mixtures at 95 ºC for 10 min. The hydrolysates were cooled in ice bath and termed as squid ovary hydrolysate (SOH). SOH was subjected to analysis.

4.4.4 Analysis

4.4.4.1 Determination of degree of hydrolysis (DH)

SOH was determined for α-amino group content as described by Benjakul and Morrissey (1997) using TNBS. The DH was calculated from following equation:

$$DH = [(L - L_0)/ (L_{max} - L_0)] \times 100$$

where L is the amount of α-amino groups of hydrolysate sample. L_0 is the amount of α-amino groups in SO. L_{max} is the total α-amino groups in SO obtained after acid hydrolysis (6 M HCl at 100 °C for 24 h).

4.4.4.2 Determination of foaming capacity and stability

Foaming capacity and stability were determined as described in section 2.4.8.1.

4.4.4.3 Protein pattern

Protein patterns of SO and SOHs prepared using Alcalase or papain were determined as described in section 2.4.6.

4.4.5 Characterization of squid ovary powder and the selected squid ovary hydrolysate

SOH prepared using Alcalase for 30 min, showing the highest FC and FS was freeze-dried using a freeze dryer. Powder obtained named 'SOH-Al-30' was further analyzed in comparison with SOP.

4.4.5.1 Solubility

Protein content of SOP and SOH-Al-30 powder (1%, w/v) was determined using biuret method (Gornall, Bardawill and David, 1949). Soluble protein content of SOP and SOH-Al-30 was 4.69 and 2.13 mg/mL, respectively. The solubility was reported as the percentage relative to total protein content of SOP (7.41 mg/mL) and SOH-Al-30 (2.83 mg/mL) as following the method described by Kittiphattanabawon *et al.* (2012).

4.4.5.2 Surface hydrophobicity

Surface hydrophobicity of SOP and SOH-Al-30 powder was calculated as described in section 3.4.4.2.

4.4.5.3 Fourier Transform Infrared (FTIR) Spectroscopic Analysis

FTIR spectra of SOP and SOH-Al-30 powder were recorded in mid-IR region using a FTIR spectrometer (EQUINOX 55, Bruker, Ettlingen, Germany) as per the method described by Ali, Kishimura and Benjakul (2018).

4.4.5.4 Particle size measurement

The particle size of SOP and SOH-Al-30 powder was measured as described in section 3.4.6.4.

4.4.5.5 Foaming properties

Since SOH-Al-30 powder showed higher foaming properties than SOP, only SOH-Al-30 powder was used for pre-heating study.

4.4.5.6 Effect of pre-heating on foaming properties of SOH-Al-30

Effect of pre-heat treatment on foaming capacity and foam stability of SOH-Al-30 powder was determined. SOH-Al-30 powder was dissolved in distilled water to obtained final concentration of 4% (w/v). The solution was heated at different temperatures (50, 60, 70 and 80 ºC) for 10 min in a temperature-controlled water bath. Subsequently, the samples were cooled down suddenly using iced water. Temperature of the samples were brought to the room temperature and FC and FS were determined as described previously. Preheating at temperature showing the highest FC and FS was used for further study.

4.4.5.7 Effect of whipping time on foaming properties of SOP and preheated SOH-Al-30

To investigate the effect of whipping time on foaming properties, SOP and preheated SOH-Al-30 solutions (4%) were whipped for different whipping times (1, 2, 3 and 4 min). FC and liquid drainage were determined as previously described in sections 2.4.6 and 3.4.7.1, respectively.

4.4.6 Characterization of foams from squid ovary powder and the selected squid ovary hydrolysate

Foams from solutions of SOP and preheated SOH-Al-30 powder (4%) with appropriate whipping time were used for further characterization.

4.4.7 Foam rheology

Rheology of foams from solutions of SOP and preheated SOH-Al-30 powder (4%) was measured as described in section 3.4.7.2.

4.4.8 Confocal laser scanning microscopy (CLSM)

Microstructure of foam whipped from SOP and pre-heated SOH-Al-30 was measured as described in previous section 3.4.7.3.

4.4.9 Statistical analysis

All experiments were repeated three times. Data were subjected to analysis of variance. Comparison of means was carried out by the Duncan's multiple range tests (Steel and Torrie, 1986). T-test was used for pair comparison. Analysis was performed using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL, USA).

4.5 Results and discussion

4.5.1 Effect of hydrolysis on characteristics and foaming properties of squid ovary

Characteristics of squid ovary hydrolysates (SOH) prepared by Alcalase or papain named ‘SOH-Al’ and ‘SOH-Pa’, respectively, as a function of hydrolysis time are presented in Table 7. DH of both SOHs increased with increasing hydrolysis time ($p<0.05$). At the same hydrolysis time, higher DH was obtained in SOH-Al ($p<0.05$). The results indicated that Alcalase was more effective in hydrolysis of peptide bonds in squid ovary, in comparison with papain. Alcalase has been used widely to hydrolyze proteins from fish (Benjakul, Karnjanapratum and Visessanguan, 2017; Klomklao and Benjakul, 2018) and plants (Zhu, Zhou and Qian, 2006; Zeng *et al.*, 2013).

FC of squid ovary was improved significantly when the hydrolysis by both enzymes was employed ($p<0.05$). SOH-Al showed the highest FC (237%) at 30 min of hydrolysis ($p<0.05$). Slight decrease in FC of SOH-Al was noted with hydrolysis of 60 min ($p<0.05$). This might be related with shorter peptides, which showed the lower ability to form strong film at the air-water interface. The maximum FC (221%) was obtained in SOH-Pa with hydrolysis time of 15 min, having DH of 0.21%. Increase in FC was more likely related to the increase in solubility, which

caused fast diffusion of proteins to air-water interface (Mune-Mune, 2015). Hydrolysis of globular protein molecules into the smaller molecules increased the number of smaller polypeptide chains, thus facilitating the diffusion of peptides to the

Table 7. Degree of hydrolysis (DH), foaming capacity and foaming stability of squid ovary and squid ovary hydrolysates prepared by Alcalase and papain with different hydrolysis time.

	Hydrolysis Time (min)	DH (%)	Foaming capacity (%)	Foaming stability (%)
Squid ovary	0	0	86.67 ± 3.85^{cdE}	7.19 ± 0.94^{dcE}
Alcalase	5	0.91±0.15	220.00 ± 7.70^{bB}	25.21 ± 0.63^{cD}
	15	1.35±0.14	227.44 ± 7.68^{bAB}	26.25 ± 0.64^{bC}
	30	1.78±0.17	236.67 ± 10.76^{aA}	27.25 ± 0.49^{bC}
	60	2.30±0.03	224.44 ± 8.82^{bB}	30.05 ± 1.59^{aA}
Papain	5	0.14±0.19	217.78 ± 6.39^{abB}	26.56 ± 1.40^{aC}
	15	0.21±0.02	221.11 ± 10.72^{aB}	26.79 ± 1.89^{aC}
	30	1.59±0.12	206.67 ± 6.67^{bC}	26.31 ± 1.62^{aC}
	60	2.14±0.01	155.56 ± 1.95^{cD}	29.15 ± 1.43^{bB}

Values are mean ± SD (n = 3). Different uppercase superscripts in the same column denote significant difference ($p < 0.05$). Different lowercase superscripts in the same column under the same enzyme used including that of squid ovary denoted significant difference ($p<0.05$).

interface. Kong, Zhou and Qian (2007) documented the increased surface activity with increasing number of polypeptide chains from wheat gluten hydrolysates produced using commercial proteases. Similarly, FC of soy protein isolate was improved by partial hydrolysis using Alcalase, pancreatin and papain (Zeng *et al.*, 2013). For SOH-Pa samples, FC was increased up to 15 min of hydrolysis.

Subsequently, FC decreased to the minimum (156%), when hydrolysis time was increased to 60 min ($p<0.05$). Peptides or polypeptides with short chain might be unable to form the strong interfacial layer around the air bubble. DHs of SOH were varied between 0.9 to 2.2% for SOH prepared by both enzymes. Partial hydrolysis with low DH (1–10%) was generally used to improve functional properties, mainly foaming and emulsifying properties of several proteins (Panyam and Kilara, 1996; Foegeding *et al.*, 2002).

For FS, both SOH-Al and SOH-Pa showed the lower FS as compared to SO (Table 7). SO had the lowest reduction in foam volume (7.19%), whereas volumes of foam from SOH-Al and SOH-Pa were reduced in the range of 25-30% and 26-29%, respectively. High FS generally resulted from protein molecules with large size, which form thick and strong film at air-water interface (Zayas, 1997). Hydrolysis produced short peptide chains, which might not be able to form thick and strong interfacial layer at the interface. FS of SOH-Al decreased with increasing DH and showed the highest reduction (30%) in foam volume when hydrolyzed for 60 min ($p<0.05$). Nevertheless, SOH-Pa showed no significant difference in FS up to 30 min of hydrolysis ($p>0.05$). However, FS decreased as hydrolysis time of 60 min was used ($p<0.05$). In general, high FC observed in both SOHs indicated higher pressure at the interfacial layer than the lower FC obtained in SO. This could result in the more destruction associated with more liquid drainage as influenced by gravity (German, O'Neill and Kinsella, 1985). Hence, SOH-Al and SOH-Pa showed the lower FS than that of SO.

4.5.2 Protein pattern

Protein patterns of SO in comparison with SOH-Al and SOH-Pa with different hydrolysis times are shown in Figure 13. SO contained the major bands with MW of 255.7, 136.2, 90.4 and 13.7 kDa under the non-reducing conditions (Singh *et al.*, 2017). However, in the presence of β-ME, squid ovary had the different protein patterns, in which the major bands having MW of 220.32, 213.76, 160.9 and 133.6 kDa were observed. Additionally, a large number of proteins with MW in the range of 132.3–15.49 kDa were found (Figure 13). The result indicated that squid ovary

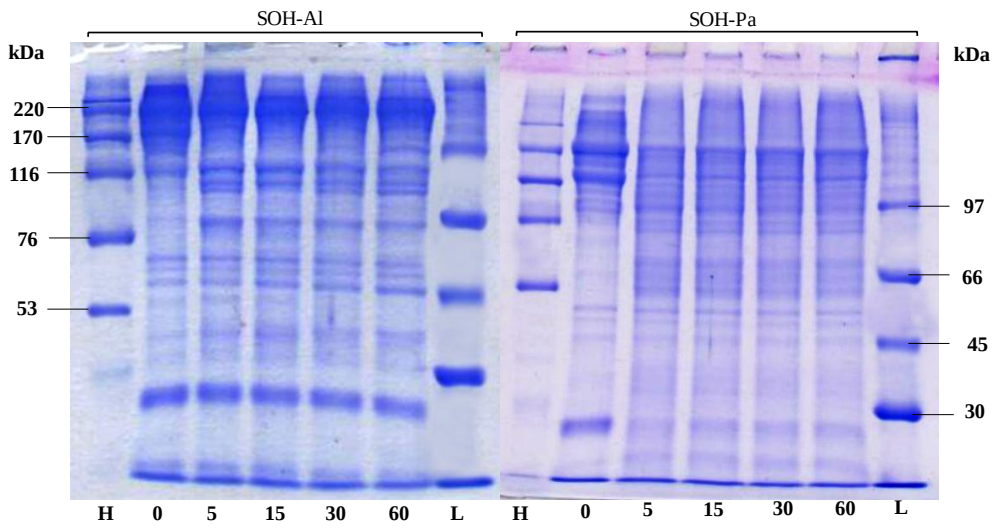

Figure 13. SDS–PAGE patterns of squid ovary, squid ovary hydrolysates (SOH) prepared using Alcalase (SOH-Al) and papain (SOH-Pa). Number denoted as hydrolysis time (min). L and H denoted low and high MW standards, respectively.

proteins were stabilized by disulfide bond. Protein band with MW of 213.76 kDa disappeared when hydrolyzed by Alcalase. Coincidently, protein bands with MW in the range of 150-170 kDa were detected. Intensity of protein band with MW of 122.3 was slightly decreased with increasing hydrolysis time. Appearance of two bands with MW of 96.6 and 89.7 kDa was observed in the Alcalase treated samples. With increasing hydrolysis time, the intensity of bands with MW in the range of 30-50 kDa was increased. As a consequences, smaller proteins or peptides were formed, which efficiently adsorbed at the air/water interface as evidenced by the increased FC. Protein pattern was obtained for SOH-Pa showed higher degradation of SOP proteins as indicated by lower band intensities as compared to SOH-Al. Major bands with MW of 211-225 kDa disappeared to higher degree as compared to those of SOH-Al. Papain generally hydrolyzed peptide bonds in a broad range, regardless of amino acids at P and P′ position (Mamboya, 2012). Generally, SOH-Pa contained proteins or peptides with MW of more than the 50 kDa, but SOH-Al comprised protein or peptide having MW of less than 50 kDa. The lower FC of SOH-Pa was plausibly caused by the slower diffusion rate of larger proteins molecules into air-water interface (Table 7). It is known that the larger size proteins possessed higher FS but lower FC (Zayas, 1997).

Thus, SOH prepared using Alcalase with hydrolysis time of 30 min having DH of 1.78% (SOH-Al-30) could form the stable foam with high FC. SOH-Al-30 was subjected to freeze drying and the resulting powder was further studied comparatively with SOP.

4.5.3 Characterization of SOP and SOH-Al-30 powder

4.5.3.1 Solubility

SOH-Al-30 powder showed the higher solubility (~75%) as compared to SOP (~63%) (Table 8). Shorter chain peptides with higher ionizable groups as a result of hydrolysis more likely enhanced the hydrophilicity. Therefore, smaller proteins or peptides with exposed charged domains were able to interact with water, resulting in higher solubility. Severin and Xia (2006) also observed the increased solubility of whey protein from 75 to 85% when DH of Alcalase was increased from 5 to 20%. Chobert, Bertrand-Harb and Nicolas (1988) and Mutilangi, Panyam and Kilara (1995) reported that the decrease in MW of casein and whey protein isolates resulted in the higher solubility. Therefore, hydrolysis of squid ovary improved the solubility, which is a prerequisite for functional properties of proteins. The result reconfirmed the increase in FC of SO after Alcalase hydrolysis (Table 7).

Table 8. Solubility, surface hydrophobicity and particle size of squid ovary powder (SOP) and squid ovary hydrolysate (SOH) powder prepared using Alcalase for 30 min (SOH-Al-30).

	SOP	SOH-Al-30
Solubility (%)	63.27 ± 2.21^{b}	75.42 ± 0.96^{a}
Surface hydrophobicity (S_0ANS)	390.40 ± 10.7^{b}	495.7 ± 3.20^{a}
Mean particle size (µm)	21.33 ± 1.33^{b}	23.59 ± 0.66^{a}
Particle size (µm)		
<10%[A]	1.92 ± 0.45^{b}	7.03 ± 1.46^{a}
<50%[B]	14.57 ± 3.05^{b}	22.13 ± 1.37^{a}
<90%[C]	50.00 ± 6.00^{a}	43.97 ± 3.19^{b}

Values are mean ±SD (n=3). Different lowercase superscripts in the same row denote significant difference ($p<0.05$)

[A]Using Mie's theory it is determined that 10% particles have diameter less that stated.

[B]Using Mie's theory it is determined that 50% particles have diameter less that stated.

[C]Using Mie's theory it is determined that 90% particles have diameter less that stated.

4.5.3.2 Surface hydrophobicity

Surface hydrophobicity of SOP and SOH-Al-30 powder is shown in Table 8. Surface hydrophobicity of SOP and SOH-Al-30 powder was 390 and 496, respectively. The increase in surface hydrophobicity indicates the binding of ANS to aromatic amino acids in the reaction mixture (do Evangelho *et al.*, 2017). Hence, Alcalase hydrolysis favored the exposure of buried hydrophobic groups in proteins after cleavage of peptide bonds. Alcalase is an endopeptidase, which preferably cleaves peptide bonds containing the carboxylic sides with hydrophobic amino acids such as valine, tyrosine and leucine (Adamson and Reynolds, 1996). Similarly, surface hydrophobicity of black bean increased when treated with Alcalase and pepsin (do Evangelho *et al.*, 2017). Segura-Campos *et al.* (2012) observed the higher increase in surface hydrophobicity in hydrolysate produced by Flavourzyme (DH-7.27%) than Alcalase (DH-23.61%). Therefore, surface hydrophobicity depends on the type of hydrolytic enzyme and DH. After hydrolysis, solubility increased as indicated by shorter chain peptides or increased charged residue. Nevertheless, these peptides with exposed aromatic amino acids underwent the rapid orientation of protein molecules at the interfacial layer. Thus, high surface hydrophobicity resulted in lower surface activity, hence improving foaming property.

4.5.3.3 Particle size

In general, SOP had the slightly lower particle size than SOH-Al-30 powder (Table 8). This was most probably due to the higher surface hydrophobicity of SOH-Al-30 powder, which might enhance hydrophobic-hydrophobic interaction and the formation of aggregates during freeze-drying. During thermal inactivation of protease after hydrolysis, aggregation of proteins occurred to some extent during heating. Spellman *et al.* (2005) also reported the increase in particle size of whey protein hydrolysates treated with Alcalase for 5 h. Proteins with lower particle size are able to form stable foam due to the uniform distribution of smaller particles at the interface (Lazidis *et al.*, 2016). However, Morales *et al.* (2015) observed that rate of foam formation was not influenced by the particles when particle size was lower than 100 µm. In the present study, size of both SOP and SOH-Al-30 powder were below

100 µm. However, the varying distribution in size was observed between both samples (Table 8). This might determine the difference in foaming properties between both samples as shown in Table 7.

4.5.3.4 FTIR spectra

SOP and SOH-Al-30 powder showed similar FTIR spectra Figure 14. Amide A, amide B, amide I, amide II and amide III bands, representing proteins, were detected in both samples. The amide I band of SOP and SOH-Al-30 powder was found at wavenumbers of 1635 and 1628 cm^{-1}, respectively. This band is related to a C=O stretching vibration coupled to contributions from the CN stretch (Sinthusamran *et al.*, 2018). Amide I band of SOP was shifted to the lower wavenumber after the Alcalase hydrolysis. This was more likely due to exposure of the NH-group after hydrolysis, which might undergo the interactions with C=O groups of peptide backbone. Amide II bands of SOP and SOH-Al-30 powder were observed at wavenumber ranging from 1530 and 1532 cm^{-1}, respectively. Amide II band

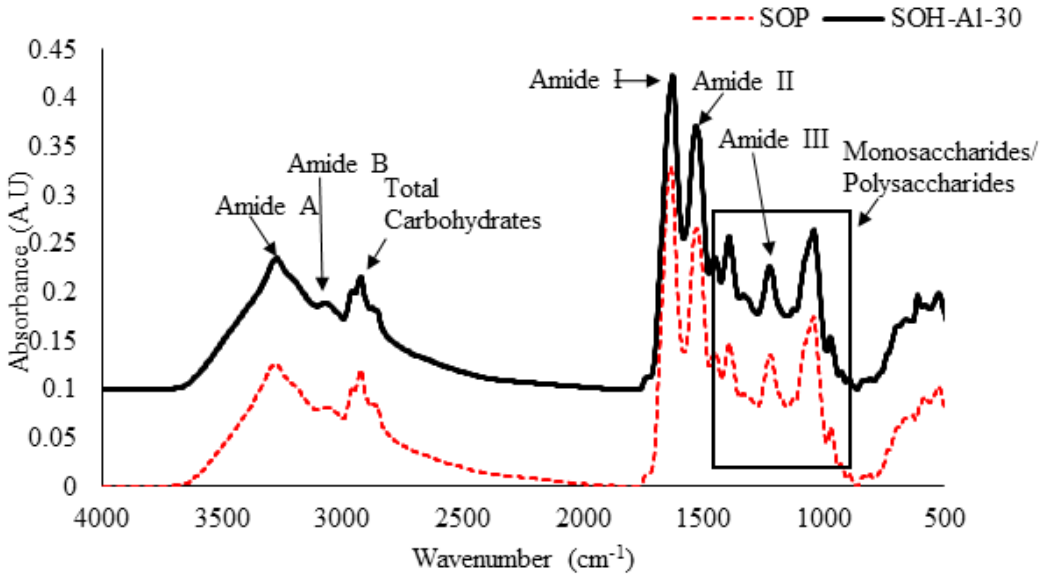

Figure 14. FTIR spectra of squid ovary powder (SOP) and preheated squid ovary hydrolysate (SOH) prepared using Alcalase for 30 min (SOH-Al-30).

represents the combination between bending vibration of N–H groups and stretching vibrations of C–N groups (Sinthusamran *et al.*, 2018). Moreover, the amide III bands of both samples were observed at wavenumbers in the range of 1223-1224 cm^{-1}. The amide III was more likely associated with C–N stretching vibrations and N–H deformation from the amide linkages as well as the absorptions arising from wagging vibration of CH_2 groups (Sinthusamran *et al.*, 2018). Amide A bands of both samples were shown at wavenumber in the range of 3274-3275 cm^{-1}. Amide B peak of SOP was found at 3060 cm^{-1}, whereas SOH-Al-30 showed Amide B at wavenumber of 3066 cm^{-1}. Amide A arises from the stretching vibrations of the NH group coupled with hydrogen bonding, whereas amide B represents CH stretching vibrations of the –CH_2 groups (Sinthusamran *et al.*, 2018). The result suggested that hydrolysis more likely exposed CH and -CH_2 of peptides as indicated by higher wavenumber of amide B band in SOH-Al-30 sample.

FTIR spectrum showed large number of peaks at wavenumbers in the range of 900-1400 cm^{-1} in both samples (Figure 14). Leopold *et al.* (2011) documented large number of peaks representing sucrose, fructose and glucose in the spectral range between 900 and 1400 cm^{-1}. Peaks in between the wavenumber of 900–1153 cm^{-1} are assigned to C–O and C–C stretching modes, whereas those in the 1400–1199 cm^{-1} region are due to O–C–H, C–C–H and C–O–H bending vibrational modes of carbohydrates. FTIR band at wavenumber of 2924 cm^{-1} is due to C-H and it was reported that band near wavenumber of 2930 cm^{-1} represents total carbohydrate (Guerrero, Kerry and de la Caba, 2014). This result reconfirmed the presence of carbohydrates (26.6%) in SO as reported by (Singh *et al.*, 2017). After hydrolysis, the whole sample was subjected to freeze-drying without any separation of carbohydrates. As a result, carbohydrates were still present in SOH-Al-30 powder. Amir *et al.* (2013) observed pyranoid and furanoid ring vibrations, which represent anomeric region of monosaccharides and polysaccharides at wavenumber in range of 950 to 750 cm^{-1}. This observation is frequently found for IR spectra in carbohydrate analysis. Presence of carbohydrates at high percentage might play a profound role in foam formation of both SOP and SOH-Al-30 powder. Viscosity of aqueous phase could be enhanced, thus increasing foam stability.

4.5.4 Effect of pre-heating and whipping time on foaming properties of SOP and SOH-Al-30 powder

4.5.4.1 Effect of heating on foaming properties of SOH-Al-30 powder

FC and FS of SOH-Al-30 solution (4%) heated at various temperatures (50, 60, 70 and 80 ºC) are shown in Figure 15A and B. SOH-Al-30 showed the highest FC (201%) when temperature was increased up to 60 ºC ($p<0.05$). However, FC decreased and showed the lowest value (173%) when heated at 80 ºC ($p<0.05$) (Figure 15A). FS was determined by calculating destruction of foam during the aging for 60 min and its was noted that SOH-Al-30 without heating showed the highest foam destruction (48%) (Figure 15B). However, foam collapse was lowered (30-32%) when SOH-Al-30 was heated at different temperatures. There was no significant difference in FS between sample heated at 50 and 60 ºC ($p>0.05$). When temperature was increased to 70-80 ºC, FS of SOH-Al-30 was decreased ($p<0.05$). Increases in FC and FS were more likely due to the unfolding of hydrophobic groups during heating, associated with the increased flexibility of protein molecules. Increased surface hydrophobicity and flexibility allows proteins molecules to orient rapidly at the interface to surround the air bubble. Heating at appropriate temperature also enhanced the solubility of proteins, in which the increasing number of protein molecules could migrate to the interface and enhance FC (Zayas, 1997). It was observed that SO contained a large amount of carbohydrates (Figure 14) (Singh *et al.*, 2017). Heating might help in dispersion of carbohydrates, which more likely play a role in the stabilization of foam by increasing the viscosity of mixture. It was noted that solution of SOH-Al-30 showed high FC (237%) and foam volume was reduced by 27% (Table 7). However, FC of SOH-Al-30 powder obtained after freeze-drying decreased to 184% and foam volume was decreased by 48%. This was more likely due to the aggregation during the drying process, which could decrease the solubility. Hence, the increased solubility more likely contributed to the increased FS and FC of SOH-Al-30 after heat treatment. Therefore, SOH-Al-30 solution pre-heated at 60 ºC was used for the further study.

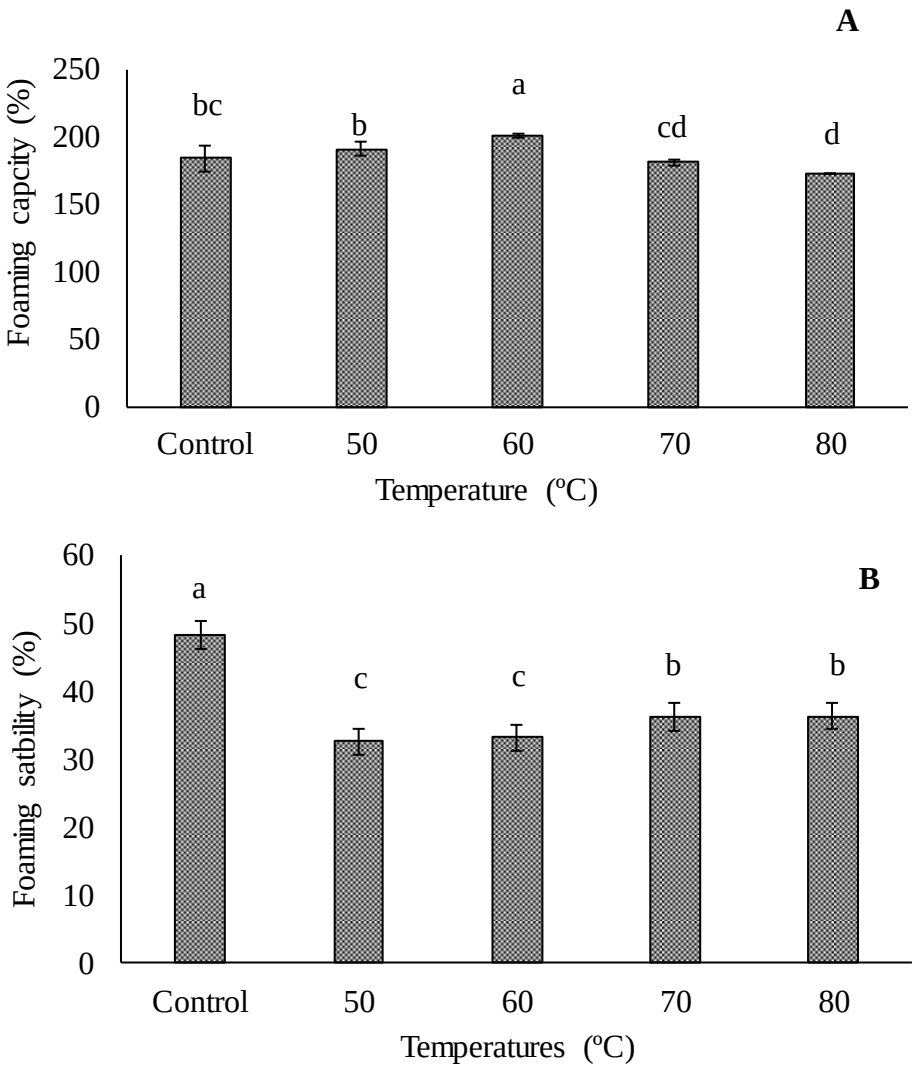

Figure 15. Effect pre-heating at different temperatures on foaming capacity (A) and foaming stability (B) of squid ovary hydrolysate (SOH) prepared using Alcalase for 30 min (SOH-Al-30). Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicates significant difference ($p<0.05$). Solutions of 4% were used for foam preparation and whipping time of 1 min was used.

4.5.4.2 Effect of whipping time on FC and FS of SOP and preheated SOH-Al-30 powder

FC of SOP and preheated SOH-Al-30 solutions (4%) as affected by various whipping times (1, 2, 3 and 4 min) is shown in Figure 16A. FC of SOP and preheated SOH-Al-30 solutions was increased with increasing whipping time

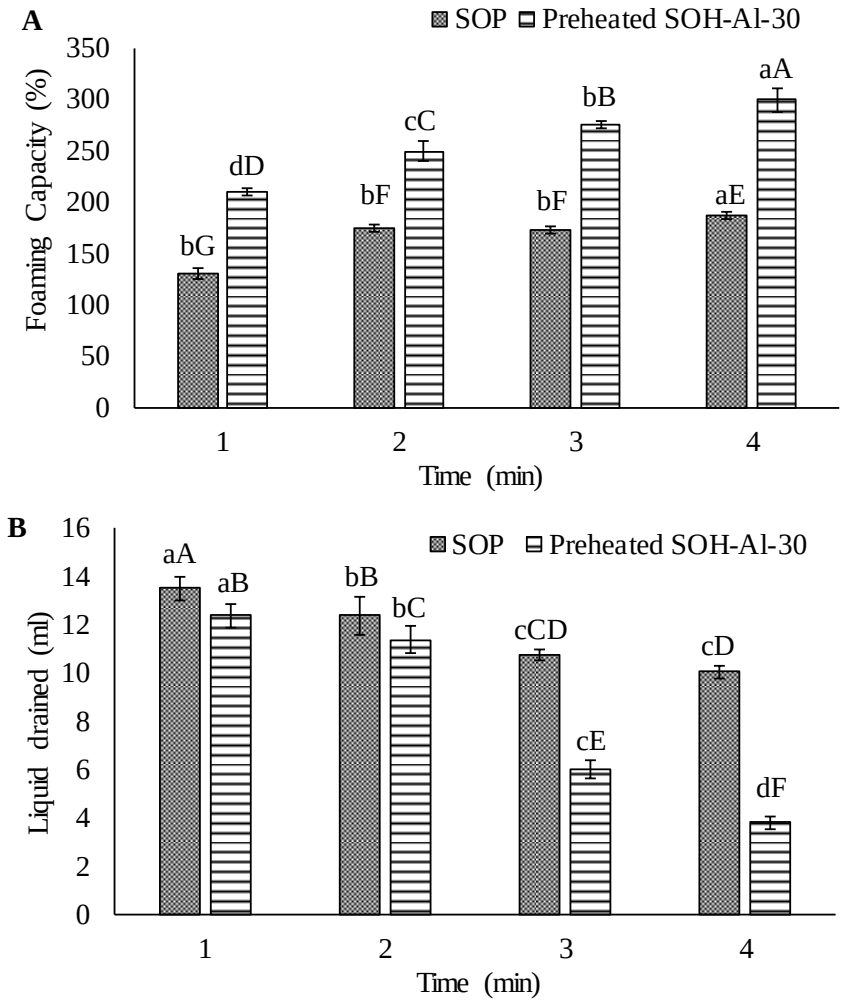

Figure 16. Effect of whipping time on foaming capacity (A) and liquid drainage (B) of foam from solutions of squid ovary powder (SOP) and squid ovary hydrolysate (SOH) prepared using Alcalase for 30 min, followed by preheating at 60 ºC (Preheated SOH-Al-30). Bars represent the standard deviation (n = 3). Different lowercase letters on the bars within the same sample indicates significant difference ($p < 0.05$). Different uppercase letters on the bars indicates significant difference between all the samples ($p < 0.05$). Solutions of 4% were used for foam preparation.

($p<0.05$). FC increased from 81 to 136% and from 210 to 300% for the former and the latter, respectively. Preheated SOH-Al-30 solution showed the higher FC at all whipping times than SOP ($p<0.05$). Hydrolysis increased solubility and surface hydrophobicity (Table 8). As a consequence, more flexible proteins could migrate rapidly to air-water interface. Another possible mechanism for increased FC could be the formation of lower MW protein molecules, which had the higher diffusion rate to the interface (Patino *et al.*, 2007). These mechanisms might be responsible for the enhanced FC of preheated SOH-Al-30 solution.

Liquid drainage is the loss of liquid from Plateau border of foam under the influence of gravity. Liquid drainage determines the stability of foam. Foams from SOP showed the higher loss of liquid than those of preheated SOH-Al-30 at all whipping times used (Figure 16B). Liquid drainage was decreased with increasing whipping time and the lowest liquid drainage was observed when whipping time of 4 min was used ($p<0.05$). This was more likely due to the exposure of hydrophobic groups by the hydrolysis and pre-heating of SOH-Al-30. During foam formation, more proteins migrated to the interfacial layer and formed thick layer, which help resist the flow of liquid from Plateau borders. Generally, liquid is drained under the influence of gravity or due to the collapse of air bubbles during aging (Narsimhan and Ruckenstein, 1986). Apart from pre-heating, whipping time was another prime factor determining foam properties of SOH.

Thus, SOH-Al-30 solution preheated at 60 ºC for 10 min was whipped for 4 min and the resulting foam was subjected to further analysis.

4.5.5 Characteristics of foams from SOP and preheated SOH-Al-30

4.5.5.1 Foam rheology

Foams generated from preheated SOH-Al-30 solution showed higher storage (G′) and loss (G′′) moduli values than those from SOP (Figure 17A and B). G′ and G′′ were recorded as function of frequency. G′ and G′′ indicate solid-elastic

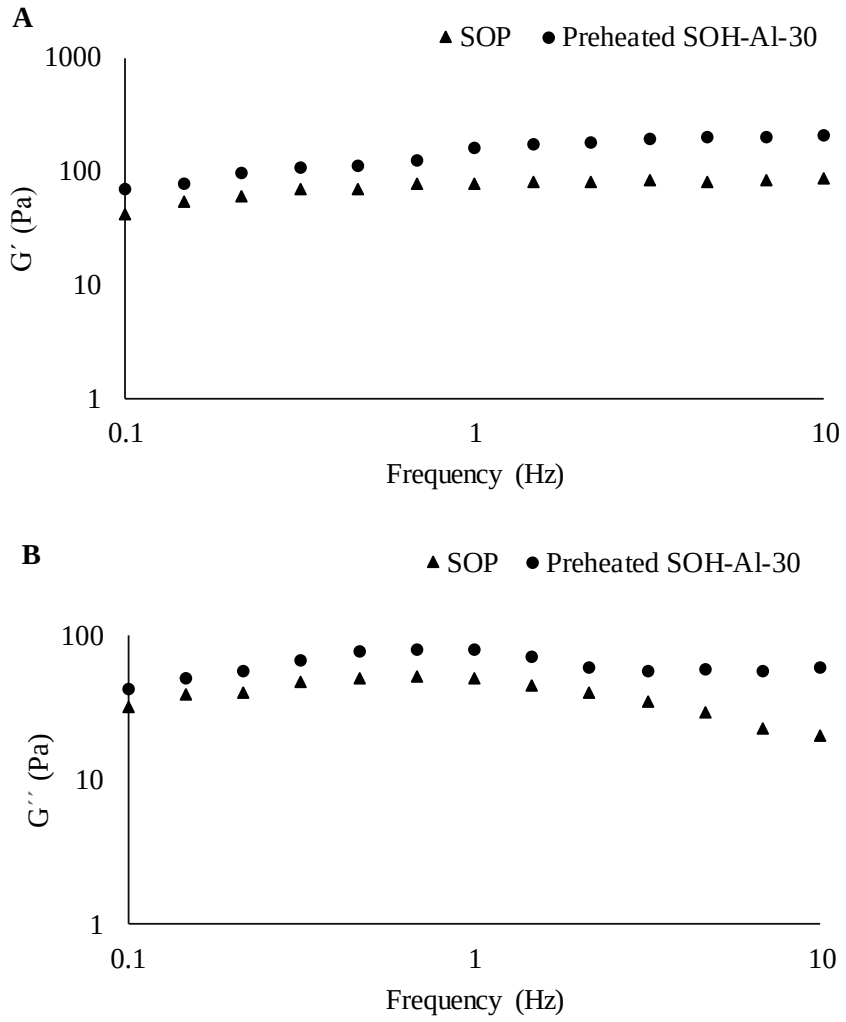

Figure 17. Storage modulus (A) and loss modulus (B) of foam from solutions of squid ovary powder (SOP) and squid ovary hydrolysate prepared using Alcalase for 30 min, followed by preheating at 60 ºC (Preheated SOH-Al-30). Solution of 4% and whipping time of 4 min were used for foam preparation.

and liquid-viscous nature of foam, respectively. Hydrolysis resulted in the conformational changes of protein molecules as well as increased solubility of SOH-Al-30 (Table 8). Alcalase hydrolysis along with subsequent heating resulted in the exposure of more hydrophobic polypeptide chains. High hydrophobic-hydrophobic interaction between protein or peptide brought about the strong and thick viscoelastic

film surrounding air bubbles. As previously mentioned, SOP contained high amount of carbohydrates, which more likely contributed to high viscosity of preheated SOH-Al-30 solution. These factors increased viscoelasticity of foam. This elastic behavior of interfacial layer more likely had the influence on foam stability.

4.5.5.2 Confocal laser scanning microscopy (CLSM)

Micrographs of foam whipped from SOP and preheated SOH-Al-30 solutions taken at different times (5 and 60 min) are shown in Figure 18. After 5 min of whipping, foam from SOP showed the larger bubble with thinner lamella as compared to foam prepared from preheated SOH-Al-30. Foam from preheated SOH-Al-30 solution showed thick lamella with smaller air bubbles. During whipping,

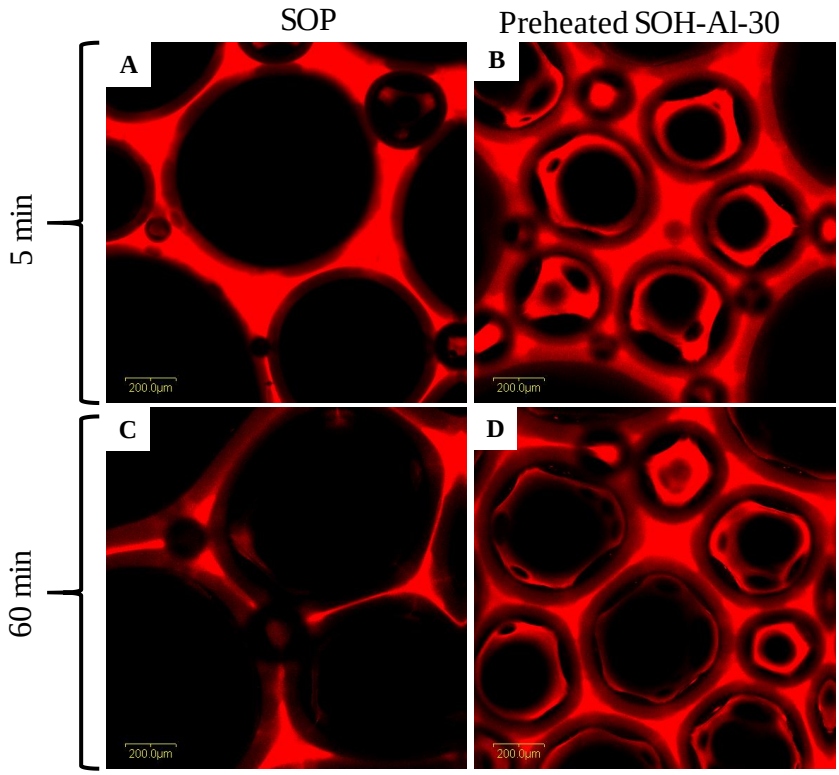

Figure 18. Confocal microscopy image of foam from solutions of squid ovary powder (SOP) and squid ovary hydrolysate prepared using Alcalase for 30 min, followed by preheating at 60 ºC (Preheated SOH-Al-30) at 5 min (A, B) and 60 min (C, D) after whipping. Solution of 4% and whipping time of 4 min were used for foam preparation.

proteins or peptides with smaller MW probably form the film easily, in which a large number of bubbles with smaller size were generated. Smaller bubbles with thicker lamella coincided with high FS of foam from preheated SOH-Al-30 solution (Figure 13). After incubation or aging of foam for 60 min, thinning of lamella and increased bubble size were observed in both samples (Figure 18C and D). This is mostly associated with the drainage of liquid from the Plateau border under gravity or due to collapse of air bubbles. However, foams from SOP showed more thinning of lamella than those of preheated SOH-Al-30. Generally, foams with smaller bubble size and uniformity provide desirable texture to the resultant products (Zayas, 1997). The results reconfirmed that enzymatic hydrolysis followed by pre-heating could improve the foaming capacity and stability of SO.

4.6 Conclusion

Squid ovary hydrolysate prepared by Alcalase (1%, w/w solid content) for 30 min with equivalent DH of 1.79% named 'SOH-Al-30', having the altered physicochemical properties showed the improved foaming property. Further pre-heating could enhance foaming capacity and foam stability of SOH-Al-30. Whipping time showed the profound impact on foam formation of preheated SOH-Al-30. Foam from preheated SOH-Al-30 solution showed smaller bubble size with thicker lamella with the improved viscoelastic properties as observed by the increased G′ and G′′. Thus, foaming property of squid ovary could be improved via hydrolysis and preheat treatment under the appropriate conditions.

4.7 References

Adamson, N. J. and Reynolds, E. C. 1996. Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. Enzyme and Microbial Technology. 19: 202-207.

Ali, A. M. M., Kishimura, H. and Benjakul, S. 2018. Extraction efficiency and characteristics of acid and pepsin soluble collagens from the skin of golden carp (*Probarbus Jullieni*) as affected by ultrasonication. Process Biochemistry. 66: 237-244.

Amir, R. M., Anjum, F. M., Khan, M. I., Khan, M. R., Pasha, I. and Nadeem, M. 2013. Application of Fourier transform infrared (FTIR) spectroscopy for the identification of wheat varieties. Journal of Food Science and Technology. 50: 1018-1023.

Benjakul, S., Karnjanapratum, S. and Visessanguan, W. 2017. Production and characterization of odorless antioxidative hydrolyzed collagen from seabass (*Lates calcarifer*) skin without descaling. Waste and Biomass Valorization. 9: 549–559.

Benjakul, S. and Morrissey, M. T. 1997. Protein hydrolysates from Pacific whiting solid wastes. Journal of Agricultural and Food Chemistry. 45: 3423-3430.

Chobert, J. M., Bertrand-Harb, C. and Nicolas, M. G. 1988. Solubility and emulsifying properties of caseins and whey proteins modified enzymically by trypsin. Journal of Agricultural and Food Chemistry. 36: 883-892.

Davis, J. P., Doucet, D. and Foegeding, E. A. 2005. Foaming and interfacial properties of hydrolyzed beta-lactoglobulin. Journal of Colloid and Interface Science. 288: 412-422.

do Evangelho, J. A., Vanier, N. L., Pinto, V. Z., De Berrios, J. J., Dias, A. R. G. and da Rosa Zavareze, E. 2017. Black bean (*Phaseolus vulgaris* L.) protein hydrolysates: physicochemical and functional properties. Food Chemistry. 214: 460-467.

Foegeding, E. A., Davis, J. P., Doucet, D. and McGuffey, M. K. 2002. Advances in modifying and understanding whey protein functionality. Trends in Food Science and Technology. 13: 151-159.

German, J. B., O'Neill, T. E. and Kinsella, J. E. 1985. Film forming and foaming behavior of food proteins. Journal of the American Oil Chemists' Society. 62: 1358.

Gornall, A. G., Bardawill, C. J. and David, M. M. 1949. Determination of serum proteins by means of the biuret reaction. Journal of Biological Chemistry. 177: 751-766.

Guerrero, P., Kerry, J. P. and de la Caba, K. 2014. FTIR characterization of protein–polysaccharide interactions in extruded blends. Carbohydrate Polymers. 111: 598-605.

Guo, X., Zhang, J., Ma, Y. and Tian, S. 2013. Optimization of limited hydrolysis of proteins in rice residue and characterization of the functional properties of the products. Journal of Food Processing and Preservation. 37: 245-253.

Hoque, M. S., Benjakul, S. and Prodpran, T. 2010. Effect of heat treatment of film-forming solution on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. Journal of Food Engineering. 96: 66-73.

Jeewanthi, R. K. C., Lee, N.-K. and Paik, H.-D. 2015. Improved functional characteristics of whey protein hydrolysates in food industry. Korean Journal for Food Science of Animal Resources. 35: 350-359.

Kilara, A. and Panyam, D. 2003. Peptides from milk proteins and their properties. Critical Reviews in Food Science and Nutrition. 43: 607-633.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2012. Effect of extraction temperature on functional properties and antioxidative activities of gelatin from shark skin. Food Bioprocess Technology 5: 2646-2654.

Klomklao, S. and Benjakul, S. 2018. Protein hydrolysates prepared from the viscera of skipjack tuna (*Katsuwonus pelmamis*): antioxidative activity and functional properties. Turkish Journal of Fisheries and Aquatic Sciences. 18: 69-79.

Kong, X., Zhou, H. and Qian, H. 2007. Enzymatic preparation and functional properties of wheat gluten hydrolysates. Food Chemistry. 101: 615-620.

Lazidis, A., Hancocks, R. D., Spyropoulos, F., Kreuß, M., Berrocal, R. and Norton, I. T. 2016. Whey protein fluid gels for the stabilisation of foams. Food Hydrocolloids. 53: 209-217.

Leopold, L. F., Leopold, N., Diehl, H.-A. and Socaciu, C. 2011. Quantification of carbohydrates in fruit juices using FTIR spectroscopy and multivariate analysis. Journal of Spectroscopy. 26: 93-104.

Mamboya, E. A. F. 2012. Papain, a plant enzyme of biological importance: a review. American Journal of Biochemistry and Biotechnology. 8: 99-104.

Morales, R., Martínez, K. D., Pizones Ruiz-Henestrosa, V. M. and Pilosof, A. M. R. 2015. Modification of foaming properties of soy protein isolate by high ultrasound intensity: particle size effect. Ultrasonics Sonochemistry. 26: 48-55.

Mune-Mune, M. A. 2015. Influence of degree of hydrolysis on the functional properties of cowpea protein hydrolysates. Journal of Food Processing and Preservation. 39: 2386-2392.

Mutilangi, W., Panyam, D. and Kilara, A. 1995. Hydrolysates from Proteolysis of Heat- denatured Whey Proteins. Journal of Food Science. 60: 1104-1109.

Narsimhan, G. and Ruckenstein, E. 1986. Hydrodynamics, enrichment, and collapse in foams. Langmuir. 2: 230-238.

Panyam, D. and Kilara, A. 1996. Enhancing the functionality of food proteins by enzymatic modification. Trends in Food Science and Technology. 7: 120-125.

Rodríguez Patino, J. M., Miñones Conde, J., Linares, H. M., Pedroche Jiménez, J. J., Carrera Sánchez, C., Pizones, V. and Rodríguez, F. M. 2007. Interfacial and foaming properties of enzyme-induced hydrolysis of sunflower protein isolate. Food Hydrocolloids. 21: 782-793.

Segura-Campos, M., Espinosa-García, L., Chel-Guerrero, L. and Betancur-Ancona, D. 2012. Effect of enzymatic hydrolysis on solubility, hydrophobicity, and in vivo digestibility in cowpea (*Vigna unguiculata*). International Journal of Food Properties. 15: 770-780.

Severin, S. and Xia, W. S. 2006. Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. Journal of Food Biochemistry. 30: 77-97.

Singh, A., Benjakul, S. and Kijroongrojana, K. 2017. Effect of ultrasonication on physicochemical and foaming properties of squid ovary powder. Food Hydrocolloids. 77: 286-296.

Singh, A., Benjakul, S. and Kishimura, H. 2017. Characteristics and functional properties of ovary from squid *Loligo formosana*. Journal of Aquatic Food Product Technology. 26: 1083-1092.

Sinthusamran, S., Benjakul, S., Hemar, Y. and Kishimura, H. 2018. Characteristics and properties of gelatin from seabass (*Lates calcarifer*) swim bladder : impact of extraction temperatures. Waste and Biomass Valorization. 9: 315-325.

Spellman, D., Kenny, P., O'Cuinn, G. and FitzGerald, R. J. 2005. Aggregation properties of whey protein hydrolysates generated with *Bacillus licheniformis*

proteinase activities. Journal of Agricultural and Food Chemistry. 53: 1258-1265.

Steel, R. G. D. and Torrie, J. H. 1986. Principles and procedures of statistics: a biometrical approach. McGraw-Hill.

van der Ven, C., Gruppen, H., de Bont, D. B. and Voragen, A. G. 2002. Correlations between biochemical characteristics and foam-forming and -stabilizing ability of whey and casein hydrolysates. Journal of Agricultural and Food Chemistry. 50: 2938-2946.

Ye, A. and Singh, H. 2006. Heat stability of oil-in-water emulsions formed with intact or hydrolysed whey proteins: influence of polysaccharides. Food Hydrocolloids. 20: 269-276.

Zayas, J. F. 1997. Foaming Properties of Proteins. *In* Functionality of Proteins in Food. p. 260-309. Springer Berlin Heidelberg, Heidelberg.

Zeng, M., Adhikari, B., He, Z., Qin, F., Huang, X. and Chen, J. 2013. Improving the foaming properties of soy protein isolate through partial enzymatic hydrolysis. Drying Technology. 31: 1545-1552.

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

CHAPTER 5
USE OF ULTRASONICATED SQUID OVARY POWDER AS A REPLACER OF EGG WHITE POWDER IN CAKE

5.1 Abstract

Ultrasonicated squid ovary powder (USOP) was used to replace egg white powder (EWP) at different substitution levels (12.5-100%) and its effects on properties of batter and cake were investigated. High elastic modulus (G´) and average bubble size of batter added with 100% USOP resulted in higher volume and lower baking loss, when compared to the control cake (100% EWP). For textural analysis, the lowest values of hardness, gumminess and chewiness were noticeable for cake containing 100% USOP ($p<0.05$). Crust showed the lower moisture content than crumb and bottom part of all the cakes ($p<0.05$). No difference in moisture content was observed for all parts of the cakes containing 25-100% USOP ($p>0.05$), while lower moisture content was obtained for cake added with 12.5% USOP and the control ($p<0.05$). The color difference (ΔE^*) between the control and cake added with USOP was increased with increasing USOP levels. When USOP was used to replace EWP at 100%, higher likeness score was obtained for firmness and overall likeness ($p<0.05$). Microstructure study of cake added with 100% USOP revealed that oil phase was distributed in gluten matrix more uniformly, when compared to the control. Thus, replacement of EWP with 100% USOP resulted in the production of cake with superior quality and increased overall acceptance by consumers.

5.2 Introduction

Foam is a two-phase system formed by trapping pockets of gas in liquid or solid structure via the lamellar phase (Singh, Benjakul and Kijroongrojana, 2018). In culinary, foam is a complex mixture of gases, solids, liquids, and surfactants. Proteins such as liquid egg white (LEW), egg white powder (EWP), casein, whey protein, etc. are widely used as foaming agents (Zayas, 1997; Cho *et al.*, 2004; Agyare, Addo and Xiong, 2009; Song *et al.*, 2009). However, some of them have limited applications due to the impaired functional properties such as solubility, etc. Therefore, proteins have been modified using chemical (enzymatic hydrolysis,

deamidation, Maillard reaction, etc.) and physical methods (ultrasonication and irradiation etc.). Owing to the adverse effects of chemical modification, particularly related with toxicity, physical methods have been employed more widely (Singh *et al.*, 2018).

Ultrasonication has lately gained substantial interest due to a wide range of applications including preservation, extraction and modification of proteins, carbohydrates, lipid, etc. High frequency ultrasonication is used for analytical assessment of physicochemical properties, whereas ultrasonication at low frequency has been employed for alteration of food structure by lowering the surface tension and increasing the rate of adsorption of proteins. Cavitational effect of ultrasonication causes the generation and collapse of the bubbles, which changes the protein conformation in the way that hydrophilic parts of amino acids from inside are opened and exposed to aqueous phase (Moulton and Wang, 1982; Jambrak *et al.*, 2008). This increases the solubility and other functional aspects of proteins such as foaming, emulsifying properties, etc. Ultrasonication also reduces molecular weight of proteins, indicating that larger area of protein is covered by water (Jambrak *et al.*, 2008).

Fish roe and squid ovary are considered as the alternative ingredients with bioactivity and high nutritive value for humans (Singh, Benjakul and Kishimura, 2017). Singh and Benjakul (2017) documented that squid ovary possessed the excellent foaming properties, especially high foaming stability. Foaming properties of squid ovary were enhanced with the aid of partial enzymatic hydrolysis and ultrasonication (Singh and Benjakul, 2018; Singh *et al.*, 2018). However, squid ovary subjected to ultrasonication showed superior foaming properties to partially hydrolyzed squid ovary. Therefore, ultrasonicated squid ovary could serve as a novel foaming agent, especially for food applications.

Generally, fresh egg white has been used for the production of cake. Owing to undesirable flavor and allergy of fresh egg white, various food proteins such as soy protein isolate, whey protein isolate, etc. have been used to replace egg white in various types of cakes (Jyotsna *et al.*, 2007; Majzoobi *et al.*, 2014; Lin *et al.*, 2017). Egg white powder is another form, which can be used in bakery product because of the long shelf-life and the ease of transportation. Song *et al.* (2009) observed that the

quality of angel cake was enhanced with the addition of irradiated EWP. No study has been reported on the utilization of squid ovary in the cake.

5.3 Objective

To use ultrasonicated squid ovary powder (USOP) to replace EWP at various substitution levels and to investigate the impact of replacement on properties of batter and resulting cake.

5.4 Materials and methods

5.4.1 Ingredient and egg white powder

Commercial whole wheat flour (Imperial, KCG Corporation Co., Ltd, Bangkok, Thailand), unsalted butter (Orchid, Indofood Sukses Makmur Tbk, Jawa Tengah, Indonesia), baking powder (Best Foods, Unilever Thai Holdings Limited, Chachoengsao, Thailand), cake flour (Ribbin, Thai Floor Mill Industry Co., LTD, Bangkok, Thailand), fresh milk, sugar and salt were obtained from a local market in Hat Yai, Songkhla, Thailand. Egg white powder (EWP) was purchased from Mathawach Flavour Limited Partnership, Samut Sakhon, Thailand.

5.4.2 Preparation of ultrasonicated squid ovary powder (USOP)

The frozen squid (*Loligo formosana*) viscera were gifted from Sea Wealth Frozen Food, Songkhla, Thailand. The ovary was manually separated and chopped manually, followed by freeze-drying using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The obtained powder was defined as ‘squid ovary powder’ or ‘SOP’. Dried ovary was ground and sieved using a screen (40 mm mesh). Sixteen grams of SOP was mixed with 400 mL of distilled water to obtain final concentration of 4% (w/v). The mixture was subjected to ultrasonication (Sonics, Model VC750, Sonic & Materials, Inc., Newtown, CT, USA) with operating frequency of 20 kHz ± 50 Hz and high intensity power of 750 W at an amplitude of 70% for 30 min (Singh *et al.*, 2018). The temperature of the mixture was maintained at 25-30 °C during the whole operation. The obtained mixture was freeze-dried and the resultant powder was named as ‘ultrasonicated squid ovary’ or ‘USOP’.

5.4.3 Batter preparation

Cake batter was firstly prepared using the method of Levy (1982) with slight modifications. Based on the total volume of ingredient, cake recipe contained 17.48% of all-purpose flour, 16.26% of cake flour, 20.32% of sugar, 19.27% of fresh milk, 18.37% of unsalted butter, 4.3% of water, 0.57% of baking powder, 0.5% of salt, and one tea-spoon of vanilla. EWP or USOP alone or the mixtures was added at 3.25%.

To study the replacement of EWP using USOP, EWP was substituted by USOP at levels of 0%, 12.5%, 25%, 50% and 100% (w/w). The batter was prepared in two steps; first, EWP or USOP or mixtures were whipped with water for 3 min along with the continuous addition of sugar. After foam was formed, the prepared mixture of melted butter, milk and vanilla flavor was added. The obtained mixture was manually mixed with spatula for 3 min. Finally, dry ingredients, including all-purpose and cake flours, baking powder and salt were mixed with the whipped batter for another 3 min with spatula. The obtained batter was subjected to following analysis.

5.4.3.1 Microstructure

Approximately 500 mg of batter was transferred on a microscope slide. Batter was pressed carefully with another slide to make a thin layer of batter without entrapment of air bubbles. Both slides were fixed with two paper clips to create a layer of batter of constant thickness between the slides. The samples were imaged using a fluorescence stereo microscope (Leica, DFC450 C, Wetzlar, Germany) at 40× magnification.

5.4.3.2 Rheological property

Viscoelastic properties of batters were determined using a rheometer (HAAKE RheoStress 1, Thermo Fisher Scientific, Karlsruhe, Germany) with cone and plate geometry (4° angle, 35 mm diameter) as per the method described by Hesso et al. (2015). The batter was carefully placed in the center of the lower plate. Afterward the upper geometry was descended to a gap of 2 mm. The excessive batter

was carefully removed using a plastic spatula. The linear viscoelastic region (LVR) was measured previously for an oscillation frequency of 1 Hz with 0.1% strain. Temperature sweep from 25 to 95 °C at a heating rate of 2.4 °C/min was performed at a frequency of 1 Hz and strain of 0.1%. Elastic (G´) and viscous (G´´) moduli were documented

5.4.4 Cake preparation

The prepared batter (150 mL) was poured into greased aluminum pans (250 mL) and baked at 150 ºC for 60 min in an oven. After baking, the cakes were taken out from the aluminum pan and cooled at room temperature (26-28 ºC) for 3 h. The cakes were placed in a zip lock bag until used for the analysis.

5.4.4.1 Baking loss

Baking loss of the control and cakes added with USOP at various EWP substitutions (12.5-100%, w/w) was determined as described by Barcenilla *et al.* (2016). It was calculated by weighing batter before baking (W_0) and after baking (W_f).

Baking loss (%) = (W_0 -W_f /W_0) x100

5.4.4.2 Cake volume

Cake volume was measured by the sesame seed displacement method (Sinthusamran and Benjakul, 2015). Cake volume was also examined visually from digital image of cross-sectioned cake samples.

5.4.4.3 Color measurement

The crust of cake was removed and cut into 0.5 × 2 × 2 cm^3 pieces and crumb was taken from the center of cake and cut into the pieces (2 × 2 × 2 cm^3). The *L**, *a**, and *b** of crust and crumb of cake were measured using a calibrated colorimeter (Hunter Lab, Color Flex, Hunter Associates Laboratory, Reston, VA, USA). The total difference in color (ΔE^*) was calculated as follows:

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

where ΔL^{*}, Δa^{*} and Δb^{*} are the difference between the color parameters of the samples and those of the control.

5.4.4.4 Textural profile

Textural profile analysis (TPA) of the cake samples was conducted using a texture analyzer (Model TA-XT2, Stable MicroSystems, Surrey, UK) as per the procedure of Sowmya *et al.* (2009).

5.4.4.5 Sensory property

Sensory property of cakes was evaluated by a panel of 80 panelists using 9-point hedonic scale for various quality parameters, namely appearance, color, odor, firmness, flavor and taste as per the procedure described by Sowmya *et al.* (2009).

5.4.4.6 Microstructure

The samples were cut into small blocks with a sharp blade followed by freeze-drying using a freeze drier. Cake samples were fixed on the sample holder using double sided scotch tape (O-BIZ, Samutprakarn, Thailand) and was exposed to gold sputtering (2 min, 2 mbar). Microstructures of cakes were examined using a scanning electron microscope (Quanta 400, FEI, Eindhoven, the Netherlands).

5.4.5 Statistical analysis

All experiments were performed in triplicate. Data were subjected to analysis of variance. Comparison of means was carried out by the Duncan's multiple range tests (Steel and Torrie, 1986). Analysis was performed using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL, USA).

5.5 Results and discussion

5.5.1 Impact of USOP replacement of EWP on properties of cake batter

5.5.1.1 Microscopy of batter

The micrographs of batter added without and with USOP at several EWP substitution levels (12.5-100%, w/w) are shown in Figure 19A(1-5). Micrograph of control batter (100% EWP), showed small air bubbles uniformly distributed in the matrix (Figure 19A1). However, with increasing concentration of USOP in batter, a number of air bubbles with slightly larger size were noticeable (Figure 19A2-5). Singh *et al.* (2018) reported that USOP foam had slightly larger sized air bubble surrounded by thick layer of proteins. Foam prepared from USOP had an excellent stability after 60 min at room temperature (Singh *et al.*, 2018). Uniform distribution of air bubbles in the matrix more likely resulted in the formation of stable matrix (Zayas, 1997). Generally, batter with small average air bubble size is required for good texture, volume, shape and grain of cake (Jia *et al.*, 2014). However, batter with larger stabilized air bubble could entrap more air, which might result in the formation of cake with high volume and softer texture.

5.5.1.2 Rheology of batter

Viscoelastic properties of batter added with and without USOP at various EWP substitution levels (12.5-100%, w/w) during heating from 25 to 95 °C are shown in Figure 20. Elastic modulus (G´) was varied with increasing temperatures for all batters. Firstly, G´ was decreased for all the samples when heated from 25 to 40 °C. This was more likely due to the melting of fat associated with the reduction in liquid phase viscosity (Hesso *et al.*, 2015). Carbon dioxide released during baking might be another possible reason to reduce the elasticity of batter (Meza *et al.*, 2011; Schirmer *et al.*, 2012). In general, batter added with 100% USOP showed the higher G´ when heated up to 78 °C. It was noted that G´ of the sample was quite constant during 45-80 °C. For the other samples, G´ was increased with increasing temperatures. Thus, both EWP and USOP has the different impact on viscoelastic

A
Cake batter
1
2
3
4
5
500µm
B
Top view of cake
C
Cross-section of cake
0
12.5
25
50
100
USOP (%)

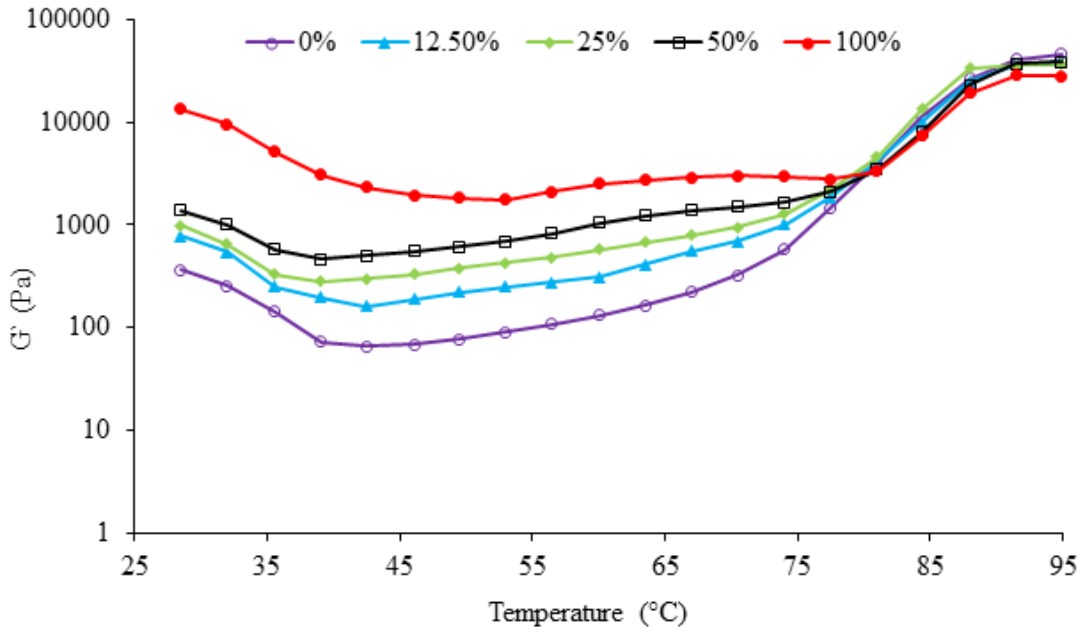

Figure 20. Elastic modulus (G`) of the control (100% EWP) and cakes added with USOP at various EWP substitution levels during heating from 25 to 95 ºC.

property of batter more likely due to various compositions. The higher value of G´ for batter containing USOP until 78 °C more likely related with the presence of high amount carbohydrate in USOP (Singh *et al.*, 2017; Singh *et al.*, 2018), which might increase the viscosity of batter. The higher viscosity might provide elastic structure to the batter during baking. In addition, ultrasonication resulted in the exposure of buried hydrophobic domains of USOP, which might increase hydrophobic-hydrophobic interaction. This interaction could formed thick viscoelastic film around the bubbles (Singh *et al.*, 2018). Generally, 55 to 78 °C region indicated fluidization of batter while heating (Hesso *et al.*, 2015). At the end, there was an increase in G´ in the region of 78 to 88 °C, which is characteristics of protein denaturation, starch gelatinization, and structure fixation (Lee, Inglett and Carriere, 2004). However, during gelatinization, structure fixation plausibly resulted in similar value of G´ for all batters. Thus, addition of USOP improved the viscoelastic properties of cake batter, which more likely strengthened the cake structure during baking.

5.5.2 Effect of USOP replacement of EWP on properties of cake

5.5.2.1 Baking loss and cake volume

Baking loss and volume of cake added without and with USOP at several EWP replacement levels (12.5-100%) are shown in Table 9. Gas formation during baking caused an increase in vapor pressure, which was likely due to the expansion of liquids when heat was applied to the batter. The loss of gas during baking is called ‘baking loss’ (Lee, 2015). Baking losses of all the samples were in the range of 8.41-8.88%. The highest baking loss was noted for cake added with 12.5% USOP and the control (100% EWP) ($p<0.05$), whereas, cake added with 50 and 100% USOP showed slightly lower baking loss ($p<0.05$). USOP addition could therefore strengthen the batter matrix via formation of strong and viscoelastic films surrounding the air bubbles. This might help entrap and hold more gas during the expansion of cake matrix throughout the baking process.

Volume of cake indicated the ability of batter to expand and incorporate gas during the baking process. The volume of cake was increased when the levels of USOP was increased from 25 to 100% EWP substitution ($p<0.05$), whereas the lowest volume was obtained for cake added with 12.5% USOP followed by the control ($p<0.05$). Generally, the presence of initial water generates the sufficient vapor, which increases volume of cake and provides a moist texture (Choi, Bae and Han, 2007). Figure 19C also depicts the increases in porosity and volume of crumb of cake, when USOP levels were increased from 12.5 to 100%. Disappearance of dome of cake was observed on the addition of USOP, particularly at high level. Presence of dome indicated inconsistency of batter, which resulted in the fast baking at surface of cake and a dome was formed (Figure 19B-1). Presence of carbohydrates in USOP might increase water holding capacity of cake and caused higher volume. Additionally, batter containing 100% EWP showed smaller bubbles as compared to that added with 100% USOP. This might cause higher volume of cake from the latter than the former. Furthermore, EWP had high foaming capacity but lower foaming stability, as compared to USOP (data not shown).

Table 9. Baking loss, cake volume and textural properties of the control (100% EWP) and cakes added with USOP at various EWP substitution levels.

USOP (%)	Baking loss (%)	Volume (cm^3)	Hardness (g)	Springiness (g)	Cohesiveness (g)	Gumminess (g)	Chewiness (g)
0	8.82±0.19^{a}	157.5±2.88^{d}	4605.30±57.10^{a}	0.94±0.01^{a}	0.62±0.01^{a}	4243.94±86.54^{a}	2221.06±70.29^{a}
12.5	8.88±0.20^{a}	153.75±2.5^{e}	4538.56±91.01^{b}	0.94±0.00^{a}	0.63±0.01^{a}	4129.39±71.93^{b}	2085.97±40.79^{b}
25	8.79±0.14^{a}	163.75±3.88^{c}	4070.40±46.53^{c}	0.93±0.01^{b}	0.62±0.01^{a}	3876.41±91.83^{c}	2909.28±47.75^{c}
50	8.55±0.17^{b}	167.00±2.74^{b}	3872.48±55.94^{d}	0.92±0.01^{b}	0.61±0.01^{a}	3641.89±68.82^{d}	2722.80±89.15^{d}
100	8.40±0.20^{b}	173.57±5.56^{a}	3779.80±47.00^{e}	0.90±0.00^{c}	0.61±0.01^{a}	3494.03±64.21^{e}	2408.04±87.39^{e}

Values are expressed as mean ± SD (n = 3). Different lowercase superscripts in the same column indicate significant differences ($p<0.05$).

In the complex system of cake, unable to form a stable foam in the presence of sugar, flour, lipid etc. This resulted in the baking loss and lower volume of cake added with EWP.

5.5.2.2 Textural profile

Textural profile of cake in the presence and absence of USOP at different EWP substitution levels (12.5-100%, w/w) is shown in Table 9. Hardness is the applied force to compress sample to attain a certain deformation. The highest hardness was obtained for the control cake and the cake added with 12.5% USOP, as compared to other cakes ($p<0.05$). When USOP level was increased up to 100%, hardness was continuously decreased ($p<0.05$). The result was in agreement with the higher volume of cake added with 100% USOP (Table 9). The higher volume indicated more entrapment of gas during baking, which provided softer texture and lower hardness. Cohesiveness, an ability to deform the internal structure of food, showed no difference at all the levels (0-100%) of USOP incorporated into cake. Springiness, elastic recovery after the compressive force is removed, was decreased to the minimum value when 100% USOP was added to the cake ($p<0.05$). No difference was observed in springiness of cake added with 25-50% USOP ($p>0.05$). Also, similar springiness was noted between cake added with 12.5% USOP and the control ($p>0.05$). Gumminess and chewiness reflect energy needed to breakdown and masticate the food to the point needed for swallowing, respectively. Both parameters were decreased with increasing levels of USOP ($p<0.05$). Thus, USOP could be used for the preparation of cake with acceptable textural properties. Song *et al.* (2009) reported that irradiation improved the foaming capacity and stability of EWP. This resulted in enhanced textural properties and quality of angel cake added with modified EWP as compared to the control. Therefore, inferior quality of cake added with EWP might be due to its impaired functional properties of EWP, as compared to USOP

5.5.2.3 Moisture content (MC)

MC of different parts of the control and cake added with USOP at various EWP substitution level is shown in Table 10. The lowest MC was observed at

the top of cake, especially crust as compared to the other parts (center and bottom) of the cake ($p<0.05$). No difference in MC of center and bottom parts was noted for the cake added with 25-100% USOP ($p>0.05$). The lowest MC of crust was more likely due to the substantial decrease in surface water content during baking process (Lostie *et al.*, 2002). For the control cake and cake added with 12.5% USOP, the lower MC was noticeable for all parts as compared to the remaining samples ($p<0.05$). With increasing USOP levels, the higher MC was obtained for all parts (crust, crumb and bottom) of the cake ($p<0.05$). However, the addition of USOP at 25-100% did not show any difference in MC of all parts of cake. The results suggested that USOP might help in holding water in the cake matrix. This result was in agreement with higher volume and lower hardness of cake containing USOP (Table 9). The higher MC was plausibly due to the interaction between of water molecules with hydrophilic amino acids of squid ovary exposed by ultrasonication. Additionally, the presence of large amount of carbohydrates might help hold water in cake matrix. MC play an important role in textural properties of cake. Sani *et al.* (2014) stated that typical cake has MC between 15 and 30%. In present study, the MC was in the recommended range. Thus, USOP had the potential to hold water during baking, which could increase the quality of cake.

5.5.2.4 Color

Color of crust and crumb of the control and cake added with USOP at various levels is presented in Table 10. The *L** and *b** values of crust were decreased with increasing levels of USOP, whereas *a** was increased with the addition USOP at all levels ($p<0.05$). The color of crust is mainly governed by the Maillard reaction and caramelization during baking (Lin *et al.*, 2017). The major constituents of USOP were protein and carbohydrates accounting for 67 and 27%, respectively (Singh *et al.*, 2017), which might enhance the Maillard reactions, especially during baking at high temperature. This caused more redness and lower yellowness of the crust. Figure 19B shows the increase in the redness of crust with increasing concentration of USOP. For crumb, the lower value of *L** was observed for the cake added with 100% USOP ($p<0.05$). The *a** of crumb was increased with increasing levels of USOP from 12.5

Table 10. Moisture content and color of different parts of the control (100% EWP) and cakes added with USOP at various EWP substitution levels.

USOP (%)		0	12.5	25	50	100
Moisture content	Top	16.86 ± 0.11^{bC}	17.09 ± 0.59^{bC}	$18.83\pm.94^{aB}$	18.74 ± 0.41^{aB}	19.03 ± 0.31^{aB}
	Centre	29.46 ± 0.36^{bA}	30.11 ± 0.57^{abA}	31.16 ± 0.82^{aA}	31.51 ± 0.20^{aA}	$31.39\pm.70^{aA}$
	Bottom	26.78 ± 0.42^{bB}	27.47 ± 0.52^{bB}	30.74 ± 0.33^{aA}	30.83 ± 0.98^{aA}	31.48 ± 0.50^{aA}
Crust color	*L**	68.68 ± 0.46^{a}	66.18 ± 0.71^{b}	64.71 ± 0.84^{c}	63.78 ± 2.58^{cd}	63.12 ± 0.24^{d}
	*a**	6.98 ± 0.49^{b}	11.39 ± 1.02^{a}	11.87 ± 0.70^{a}	11.69 ± 1.31^{a}	12.30 ± 0.95^{a}
	*b**	42.33 ± 0.43^{a}	42.53 ± 1.16^{a}	40.51 ± 1.33^{b}	40.90 ± 0.90^{b}	39.46 ± 1.16^{c}
	*ΔE**	-	0.77 ± 0.01^{d}	1.27 ± 0.03^{c}	1.96 ± 0.07^{b}	2.76 ± 0.19^{a}
Crumb color	*L**	76.54 ± 0.99^{a}	76.17 ± 0.47^{ab}	75.42 ± 0.21^{bc}	74.89 ± 0.36^{cd}	74.35 ± 0.54^{d}
	*a**	0.59 ± 0.01^{d}	0.73 ± 0.05^{c}	0.85 ± 0.02^{b}	0.90 ± 0.4^{b}	1.48 ± 0.12^{a}
	*b**	23.32 ± 0.92^{a}	23.24 ± 0.61^{a}	23.82 ± 0.27^{a}	23.95 ± 0.87^{a}	24.29 ± 1.28^{a}
	*ΔE**	-	0.77^{d}	1.27^{c}	1.96^{b}	2.76^{a}

Values are expressed as mean ± SD (n = 3). Different lowercase superscripts in the same row indicate significant differences ($p<0.05$). Different uppercase superscripts in the same column indicate significant differences ($p<0.05$).

to 100% ($p<0.05$). No difference in b^* value was observed for the control and cake added with USOP at all concentrations ($p>0.05$). ΔE^* indicated the color difference between the control (cake containing 100% EWP) and cake added with USOP at various concentrations. It was noted that color difference was increased with increasing levels of USOP for both crust and crumb of cakes ($p<0.05$). Generally, crumb color of the cakes is affected by the ingredient used and its formulation (Lin *et al.*, 2017). The difference in chemical composition of EWP and USOP might provide different colors of the cake.

5.5.2.5 Likeness score

Likeness scores of the control and cake incorporated with USOP at various EWP substitution levels (12.5-100%) are shown in Table 11. All the samples had no difference in likeness score for appearance, color, odor attributes ($p>0.05$). The highest likeness score for firmness was obtained for cake containing 100% USOP ($p<0.05$). Cake added without and with 12.5% USOP showed no difference in likeness score for firmness and the lowest value was obtained for the control ($p<0.05$). This result was in agreement with hardness of cake (Table 9). The higher likeness score was noted for flavor and taste of cake added with 50 or 100% USOP ($p<0.05$), however no difference in likeness score was obtained for other cakes ($p>0.05$). The maximum likeness score was generally obtained for cake containing 100% USOP, followed by the 50% USOP ($p<0.05$), but no difference was obtained among the other samples ($p<0.05$). The major factor contributing to the acceptance of cake was firmness. Other sensory attributes such as flavor, taste and odor might be masked or dominated by the presence of other ingredient such as butter, flavoring agent etc. Therefore, USOP could be incorporated to replace EWP completely into cake with consumer acceptability and without negative effect on sensory properties.

5.5.2.6 Microstructure of cake

Microstructures of the control cake (100% EWP) and cake added with 100% USOP at different magnifications (40 and 160x) are shown in Figure 21. Cake added with 100% USOP was more porous than the control cake (Figure 21A and B).

Table 11. Likeness score of the control (100% EWP) and cakes added with USOP at various EWP substitution levels.

USOP (%)	Appearance	Color	Odor	Firmness	Flavour	Taste	Overall
0	7.46 ± 0.88^{a}	7.54 ± 0.88^{a}	6.08 ± 0.52^{b}	5.92 ± 0.65^{c}	6.87 ± 0.76^{b}	6.69 ± 0.86^{b}	6.92 ± 0.36^{c}
12.5	7.68 ± 0.64^{a}	7.85 ± 1.14^{a}	6.55 ± 0.50^{b}	6.38 ± 0.66^{bc}	6.92 ± 0.85^{b}	6.92 ± 0.67^{b}	6.92 ± 0.25^{c}
25	7.62 ± 0.96^{a}	7.77 ± 0.83^{a}	7.31 ± 0.48^{a}	6.69 ± 0.55^{ab}	7.23 ± 0.83^{ab}	7.16 ± 0.77^{ab}	7.08 ± 0.23^{c}
50	7.69 ± 0.63^{a}	7.62 ± 0.51^{a}	7.31 ± 0.95^{a}	6.92 ± 0.34^{b}	7.62 ± 0.52^{a}	7.23 ± 0.83^{ab}	7.33 ± 0.23^{b}
100	7.58 ± 0.96^{a}	7.77 ± 0.83^{a}	7.53 ± 0.59^{a}	7.43 ± 0.43^{a}	7.63 ± 0.69^{a}	7.45 ± 0.73^{a}	7.63 ± 0.12^{a}

Values are expressed as mean ± SD (n = 3). Different lowercase superscripts in the same column indicate significant differences ($p<0.05$).

The pores of varying sizes were uniformly distributed throughout the gluten matrix of cake added with 100% USOP (Figure 21B). Whereas, control cake showed less pores with non-uniformity (Figure 21A). The result was in line with the higher number of air bubbles of batter containing 100% EWP (Figure 19A5). At the high magnification, micrograph of the control cake containing 100% EWP showed continuous gluten matrix with starch granules (SG) embedded in the matrix. The shape of the starch granules was changed due to the gelatinization during the baking. Sowmya et al. (2009) stated the proteinaceous components of flour used for baking might act as matrix covering the starch granules. Cake added with 100% USOP showed scattered gluten matrix embedded with starch granules. This was more likely due to the

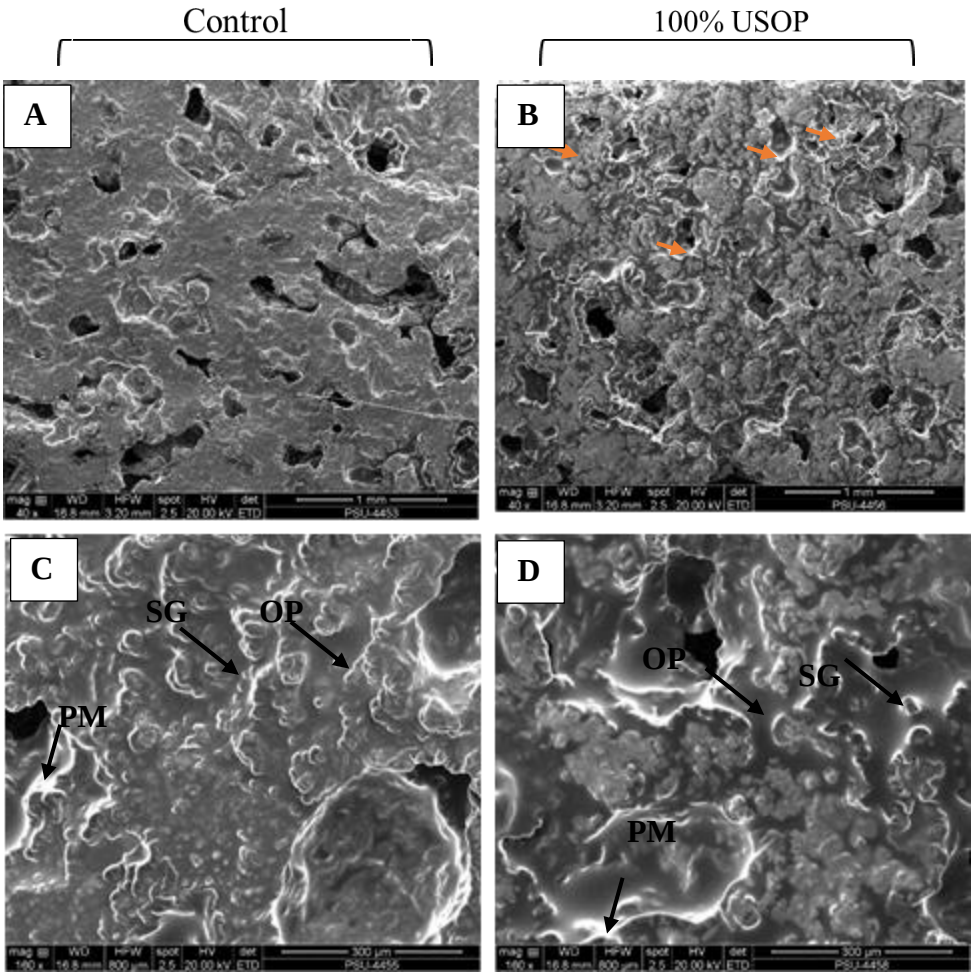

Figure 21. Scanning electron microscopic images of the control (100% EWP) (A and C) and cake added with 100% USOP (B and D). Magnification: 160x. SG: starch granules, PM: protein matrix, OP:

different chemical composition between both EWP and USOP. Micrograph of cake added with USOP showed more oil phase as indicated by smooth dark area in the matrix as compared to the control. USOP treated with ultrasound had the exposed buried hydrophobic domains. Hydrophobic amino acids might interact with the oil in the matrix, which might stabilize the oil phase in the cake. Oil is one of the important factors providing the softness to cake. The matrix of the cake is controlled by various factors associated with nature and properties of ingredients used. Therefore, USOP could help stabilize the cake via interaction with other ingredients of cake.

5.6 Conclusion

EWP was replaced with USOP in cake at different levels ranging from 12.5 to 100%. Cake batter added with USOP had better viscoelastic properties than that of cake containing EWP. USOP at 100% yielded the cake with slightly superior properties to EWP. The addition of USOP alone yielded the cake with lower baking loss, higher volume and softer texture. Moreover, USOP rendered the cake with slightly higher moisture and darker color. Overall, USOP had no negative impact on the sensory attributes. Thus, USOP could be used as a novel foaming agent in various types of cake.

5.7 References

Agyare, K. K., Addo, K. and Xiong, Y. L. 2009. Emulsifying and foaming properties of transglutaminase-treated wheat gluten hydrolysate as influenced by pH, temperature and salt. Food Hydrocolloids. 23: 72-81.

Barcenilla, B., Román, L., Martínez, C., Martínez, M. M. and Gómez, M. 2016. Effect of high pressure processing on batters and cakes properties. Innovative Food Science and Emerging Technologies. 33: 94-99.

Cho, S. M., Kwak, K. S., Park, D. C., Gu, Y. S., Ji, C. I., Jang, D. H., Lee, Y. B. and Kim, S. B. 2004. Processing optimization and functional properties of gelatin from shark (*Isurus oxyrinchus*) cartilage. Food Hydrocolloids. 18: 573-579.

Choi, G.-Y., Bae, J.-H. and Han, G.-J. 2007. The quality characteristics of sponge cake containing a functional and natural product (1. mulberry leaf powder). Journal of the East Asian Society of Dietary Life. 17.

Hesso, N., Garnier, C., Loisel, C., Chevallier, S., Bouchet, B. and Le-Bail, A. 2015. Formulation effect study on batter and cake microstructure: Correlation with rheology and texture. Food Structure. 5: 31-41.

Jambrak, A. R., Mason, T. J., Lelas, V., Herceg, Z. and Herceg, I. L. 2008. Effect of ultrasound treatment on solubility and foaming properties of whey protein suspensions. Journal of Food Engineering. 86: 281-287.

Jia, C., Huang, W., Ji, L., Zhang, L., Li, N. and Li, Y. 2014. Improvement of hydrocolloid characteristics added to angel food cake by modifying the thermal and physical properties of frozen batter. Food Hydrocolloids. 41: 227-232.

Jyotsna, R., Sai Manohar, R., Indrani, D. and Venkateswara Rao, G. 2007. Effect of whey protein concentrate on the rheological and baking properties of eggless cake. International Journal of Food Properties. 10: 599-606.

Lee, J. H. 2015. Physicochemical and sensory characteristics of sponge cakes with Rubus coreanus powder. Preventive Nutrition and Food Science. 20: 204.

Lee, S., Inglett, G. E. and Carriere, C. J. 2004. Effect of nutrim oat bran and flaxseed on rheological properties of cakes. Cereal Chemistry. 81: 637-642.

Lin, M., Tay, S. H., Yang, H., Yang, B. and Li, H. 2017. Replacement of eggs with soybean protein isolates and polysaccharides to prepare yellow cakes suitable for vegetarians. Food Chemistry. 229: 663-673.

Lostie, M., Peczalski, R., Andrieu, J. and Laurent, M. 2002. Study of sponge cake batter baking process. Part I: Experimental data. Journal of Food Engineering. 51: 131-137.

Majzoobi, M., Ghiasi, F., Habibi, M., Hedayati, S. and Farahnaky, A. 2014. Influence of soy protein isolate on the quality of batter and sponge cake. Journal of Food Processing and Preservation. 38: 1164-1170.

Meza, B. E., Chesterton, A. K. S., Verdini, R. A., Rubiolo, A. C., Sadd, P. A., Moggridge, G. D. and Wilson, D. I. 2011. Rheological characterisation of cake batters generated by planetary mixing: Comparison between untreated and heat-treated wheat flours. Journal of Food Engineering. 104: 592-602.

Moulton, K. J. and Wang, L. C. 1982. A pilot-plant study of continuous ultrasonic extraction of soybean protein. Journal of Food Science. 47: 1127-1129.

Sani, N. A., Taip, F. S., Kamal, S. and Aziz, N. 2014. Effects of temperature and airflow on volume development during baking and its influence on quality of cake. Journal of Engineering Science and Technology. 9: 303-313.

Schirmer, M., Jekle, M., Arendt, E. and Becker, T. 2012. Physicochemical interactions of polydextrose for sucrose replacement in pound cake. Food Research International. 48: 291-298.

Singh, A. and Benjakul, S. 2017. Chemical compositions and functional properties of ovary from squid *Loligo formosana*. Journal of Aquatic Food Product Technology. 26: 1083-1092.

Singh, A. and Benjakul, S. 2018. Effect of partial enzymatic hydrolysis on physicochemical and foaming properties of ovary from squid *Loligo formosana*. Waste and Biomass Valorization. doi: 10.1007/s12649-018-0348-0.

Singh, A., Benjakul, S. and Kijroongrojana, K. 2018. Effect of ultrasonication on physicochemical and foaming properties of squid ovary powder. Food Hydrocolloids. 77: 286-296.

Singh, A., Benjakul, S. and Kishimura, H. 2017. Characteristics and functional properties of ovary from squid *Loligo formosana*. Journal of Aquatic Food Product Technology. 26: 1083-1092.

Sinthusamran, S. and Benjakul, S. 2015. Effect of drying and frying conditions on physical and chemical characteristics of fish maw from swim bladder of seabass (*Lates calcarifer*). Journal of the Science of Food and Agriculture. 95: 3195-3203.

Song, H.-P., Kim, B., Choe, J.-H., Jung, S., Kim, K.-S., Kim, D.-H. and Jo, C. 2009. Improvement of foaming ability of egg white product by irradiation and its application. Radiation Physics and Chemistry. 78: 217-221.

Sowmya, M., Jeyarani, T., Jyotsna, R. and Indrani, D. 2009. Effect of replacement of fat with sesame oil and additives on rheological, microstructural, quality characteristics and fatty acid profile of cakes. Food Hydrocolloids. 23: 1827-1836.

Zayas, J. F. 1997. Foaming Properties of Proteins. *In* Functionality of Proteins in Food. p. 260-309. Springer Berlin Heidelberg, Heidelberg.

CHAPTER 6

SERINE PROTEASE INHIBITORS FROM SQUID OVARY: EXTRACTION AND ITS EFFECT ON PROTEOLYSIS AND GEL PROPERTIES OF SURIMI

6.1 Abstract

Serine protease inhibitors from squid ovary (SOSPI) were extracted using different extraction media and conditions. Optimal condition included 0.45 M NaCl for 1 h. After heat treatment at 70 °C for 10 min, the highest specific activity was obtained. Based on Sephadex G-75 gel filtration and activity staining, the major SOSPIs were present as monomer with molecular weight of 9.10 and 10.27 kDa. Impact of SOSPI on autolysis of bigeye snapper surimi was studied. Myosin heavy chain was more retained with coincidentally lower trichloroacetic acid-soluble peptide content as the level of SOSPI increased. When SOSPI at various levels (0.5-3%) was added into surimi, the highest breaking force of both kamaboko and modori gels containing 1% SOSPI was obtained ($p<0.05$). However, SOSPI had no effect on deformation of kamaboko gel ($p>0.05$) but slightly increased the deformation of modori gel. SOSPI increased water holding capacity of both kamaboko and modori gels in a dose dependent manner, as indicated by the lowered expressible moisture content. SOSPI had no effect on the whiteness of both gels. Thus, SOSPI can be used as the protein additive in surimi for increasing breaking force.

6.2 Introduction

Proteolysis mediated by indigenous proteases has been known as one of the major reasons for gel weakening of surimi. These proteases are activated by the heat, particularly temperature around 50-60 °C (Alvarez *et al.,* 1999). Severe cleavage of myofibrillar proteins at high temperature inhibits the development of three-dimensional network of surimi gel (Kudre and Benjakul, 2013). This phenomenon is called modori, which contributes to the loss in gel quality and the market value (Morrissey *et al.,* 1993). To lower the impact of modori phenomenon, several protease inhibitors have been widely used in surimi. Those include beef plasma

protein (BPP), porcine plasma protein (PPP), egg white (EW), potato powder and whey protein concentrate, etc. Due to religious obliges of porcine origin product and the outbreak of bovine or mad cow diseases, the inhibitors have been prohibited for use in surimi. High price of EW, egg like flavor and allergic reasons limit its use in surimi. Similarly, potato powder also leads to off-white color (Rawdkuen and Benjakul, 2008). Hence, alternative food grade protease inhibitors for surimi industry are still required.

Thailand and other Southeast Asian countries are the major exporters for the marine fisheries, specially squid and cuttlefish (Hoque *et al.,* 2010). In marine capture, 78% are used for human consumption and the rest is discarded or used for non-food purposes, especially for fishmeal production. Among the leftovers, roe and ovary can be utilized as nutritive proteinaceous food. Fish roe are considered as the important source for the bioactive compounds, which exhibit antioxidant, antibacterial and immunomodulatory activities. Intarasirisawat *et al.* (2011) reported that fish roe is rich in polyunsaturated fatty acids (PUFAs). Fish egg protein hydrolysates showed fat adsorption capacity, foam capacity and emulsifying capacity (Chalamaiah *et al.,* 2009). Furthermore, trypsin inhibitor from yellowfin tuna roe was recovered and used to prevent the modori in surimi (Klomklao *et al.,* 2016). Recently, fish ovarian fluid has been reported to possess proteases inhibitory activity (Minin and Ozerova, 2015). During evisceration of female squid, the ovary accounting about 10 to 15% of total weight is discarded by the squid processing industries. The ovary could be used as an alternative source of protease inhibitors for possible use in surimi.

6.3 Objective

To optimize the recovery of serine protease inhibitor from squid ovary (SOSPI) and to study the impact of SOSPI on autolysis and properties of gel from bigeye snapper surimi.

6.4 Materials and Methods

6.4.1 Chemicals

All chemicals used were of analytical grade. Na-Benzoyl-DL-arginine-p-nitroanilide (BAPNA), and high molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), and Coomassie Blue R-250 were procured from Merck (Darmstadt, Germany). Sephadex G75 gel and molecular weight marker (bovine serum albumin, albumin from chicken egg white and cytochrome C) for size exclusion chromatography were obtained from GE Healthcare Life Science (Chicago, IL, USA).

6.4.2 Extraction of serine protease inhibitor from squid ovary

6.4.2.1 Squid ovary and surimi

The frozen squid (*Loligo formosana*) viscera were obtained from Sea Wealth Frozen Food, Songkhla, Thailand. The ovary was manually separated. The obtained ovary was chopped using a blender to attain homogeneity, followed by freeze-drying using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The freeze-dried powder was transferred into polyethylene bag and stored at -40 °C until use.

Grade A frozen surimi from bigeye snapper (*Priacanthus macranthus*) was obtained from Man A Frozen Foods Co., Ltd. (Songkhla, Thailand) and kept at -20 °C until use but not longer than 2 months.

6.4.2.2 Effect of different extraction media and times on the recovery of serine protease inhibitor

Distilled water and NaCl solution at different concentrations (0.15, 0.3, 0.45 and 0.6 M) were used for the extraction of serine protease inhibitor from squid ovary powder. The squid ovary powder was mixed with extraction media at a powder/media ratio of 1:30 (w/v). The mixture was homogenized for 1 min at 5,000 rpm using a homogenizer (IKA, Labortechnik homogenizer Selangor, Malaysia). The

homogenate was stirred for 1 h at room temperature. The mixture was centrifuged at 10,000 xg for 30 min (Allegra 25R centrifuge, Beckman Coulter, Palo Alto, CA, USA). The supernatant or extract was collected and determined for trypsin inhibitory activity and specific activity.

To study the effect of extraction time on recovery of serine protease inhibitor from squid ovary powder, the medium yielding the extract with the highest total and specific activities was selected. The extraction was performed for different times (1, 2, 3, 4 and 5 h). The extracts were examined as previously described.

6.4.2.3 Effect of heat treatment on the recovery of serine protease inhibitor

The extract containing SOSPI was heated at different temperatures (60, 70, 80, 90 and 100 °C) for 10 min and suddenly cooled on ice. The extracts were then centrifuged at 8,000 xg for 10 min to remove the coagulated debris. Pre-heated extracts were subjected to analysis. The preheated extract showing the highest total and specific activities was chosen for the further study.

6.4.3 Serine protease inhibitory activity assay

Serine protease inhibitory activity was measured according to the method of Klomklao *et al.* (2010) with slight a modification. The sample solution (200 µL) was incubated with 200 µL (1 mg/mL) porcine pancreas trypsin at 37 °C for 15 min. Then, 1,000 µL of reaction buffer (50 mM Tris– HCl buffer, pH 8.0, containing 10 mM $CaCl_2$) were added. To initiate reaction, 200 µL of BAPNA (2 mg/mL) were added. After incubation 15 min, 200 µL of 30% acetic acid (v/v) were added to terminate the reaction. The residual activity of trypsin was determined by measuring the release of p-nitroaniline spectrophotometrically at 410 nm (UV-16001, Shimadzu, Kyoto, Japan). One unit of trypsin activity was defined as the enzyme causing an increase of 0.001 absorbance unit/min under the assay condition. One unit of protease inhibitory activity was defined as the amount of inhibitor, which reduced trypsin activity by one unit.

6.4.4 Gel filtration and activity staining

The selected preheated-extract containing SOSPI was separated by size exclusion chromatography using a Sephadex G-75 gel filtration column (GE Healthcare, Bio-Science AB, Uppsala, Sweden). One milliliter of extract (120 mg/mL) was loaded onto the column at room temperature (28-30 °C). After loading the sample, column was eluted with the double distilled milli-Q water and the fractions (3mL) were collected using a fraction collector (Model 2128, Bio-Rad Laboratories, Hercules, CA, USA) at the flow rate of 1 mL/min. The absorbance was recorded at 280 nm. Blue dextran (200 kDa) was use for void volume measurement. Molecular weight (MW) markers included albumin (66.5 kDa), albumin from chicken egg white (44.3 kDa), and cytochrome C (12.4 kDa). Serine protease inhibitory activity was determined for all fractions. Activity peaks were calculated for MW. Plot between available partition coefficient (Kav) and the logarithm of the MW of protein standards was prepared.

Inhibitory activity staining was conducted with SDS-polyacrylamide gel electrophoresis using casein as substrate according to the method of Gracia-Carreno *et al.* (1993) with a slight modification. The fractions with the highest trypsin inhibitory activity from the size exclusion chromatography were pooled and separated using a Mini-Protein III unit (Bio-Rad Laboratories, Hercules, CA, USA) under reducing and non-reducing conditions. One gel was fixed and stained for total proteins using Coomassie Blue R-250. Another gel was used for activity staining. The gel was washed with 2.5% Triton X-100 for 15 min twice to remove SDS. The trypsin was allowed to diffuse into the gel by incubating gel with 0.2 mg/mL trypsin for 60 min at 4 °C. The gel was incubated at 60 °C for 60 min. Thereafter, the gel was immersed in 10 mg/mL casein solution for 120 min at 60 °C. The gel was rinsed with distilled water after every treatment. Then the gel was stained with Coomassie blue R-250 to develop inhibitory zone as dark band on a clear background. The MW of inhibitor was calculated.

6.4.5 Effect of SOSPI on autolysis of bigeye snapper surimi

Autolytic activity assay was performed according to the method of Morrissey *et al.* (1993). SOSPI at various levels (0.5, 1, 2 and 3%, w/w) was mixed with surimi paste (3g) containing 2.5% NaCl (w/w). The mixture was thoroughly mixed and spread at the bottom of the beaker. Samples without and with SOSPI were incubated at 60 °C for 60 min. Then, the reaction was terminated by adding 27 mL of cold 5% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 8,000 xg for 10 min. TCA-soluble peptide content in the supernatant was determined by the Lowry method (Lowry *et al.*, 1951) and was expressed as µmole of tyrosine equivalent/g sample.

To determine the autolytic pattern of bigeye snapper surimi, autolysis was conducted in the same manner, except that 27 mL of 5% sodium dodecyl sulfate (SDS) (85 °C) were added instead of 5% TCA. The mixtures were homogenized and centrifuged. The supernatant was subjected to SDS-PAGE analysis (Laemmli, 1970) using 10% running gel and 4% stacking gel. Quantitative analysis of protein band intensity was performed by image analysis system using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4.

6.4.6 Effect of SOSPI on gel properties of bigeye snapper surimi

6.4.6.1 Preparation of surimi gel

Frozen surimi was partially thawed at 4 °C for 2–3 h, cut into small pieces with an approximate thickness of 1 cm and blended using a blender for 1 min. Salt (2.5%, w/w) was then added. SOSPI was subsequently added into surimi paste to obtain the final concentration of 0.5, 1, 2 and 3% (w/w). The final moisture content of surimi paste was adjusted to 80% (w/w). The mixture was chopped for 2 min. The temperature of surimi paste during chopping was maintained below 7 °C. The paste was stuffed into a polyvinylidine chloride casing with a diameter of 2.5 cm, and both ends were sealed tightly. Modori and kamaboko gels were prepared by incubating the paste at 60 °C and 40 °C for 30 min, respectively, followed by heating both gels at 90

°C for 20 min. All gels were cooled in iced water for 60 min and stored at 4 °C for 15-18 h prior to analysis.

6.4.7 Analyses

6.4.7.1 Textural analysis

Textural analysis of surimi gels was carried out using a texture analyzer (Model TA-XT2, Stable Micro Systems, Surrey, UK). Gels were equilibrated at room temperature (28–30 °C) before analysis for 2 h. Cylindrical samples of 2.5 cm in diameter and 2.5 cm in length were prepared. A spherical probe with a diameter of 5 mm was pressed into the cut surface of a gel specimen perpendicularly at a constant depression speed of 60 mm/min until puncture occurred as per the method of Benjakul *et al.* (2010). Breaking force and deformation were recorded.

6.4.7.2 Determination of whiteness

Gel samples were subjected to whiteness measurement using a colorimeter (Hunter Lab, Color Flex, Hunter Associates Laboratory, Reston, VA, USA). The instrument was calibrated using white and black standards plates. Illuminant C was used as the light source of measurement. L^*, a^* and b^* values were measured. Whiteness was calculated using the following equation (Park, 1994):

$$\text{Whiteness} = 100 - [\,(100 - L^*)^2 + a^{*2} + b^{*2}\,]^{1/2}$$

where L^* is the lightness; a^* is the redness/greenness and b^* is the yellowness/blueness

6.4.7.3 Determination of expressible moisture content

Expressible moisture content was measured according to the method of Rawdkuen and Benjakul (2008). Gel samples of 5 mm thickness were weighed (X) and placed between three pieces of filter papers (Whatman No.1, Whatman International, Ltd., Maidstone, England) at the bottom and two pieces of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (Y). Expressible

moisture content was calculated with the following equation and expressed as the percentage of sample weight:

$$\text{Expressible moisture content (\%)} = [X - Y / X] \times 100$$

6.4.8 Statistical Analysis

All experiments were run in triplicate. Data were subjected to analysis of variance. Comparison of means was carried out by the Duncan's multiple range tests (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL).

6.5 Results and discussion

6.5.1 Effect of extraction media and time on the recovery of serine protease inhibitor

6.5.1.1 Effect of extraction media and time on the recovery of serine protease inhibitor

Different media yielded the extracts with varying total inhibitor activity and specific activity as shown in the Table 12. The extraction with the NaCl at concentrations of 0.3 and 0.45 M rendered the extract with higher total inhibitor activity than others ($p<0.05$). This was more likely due to the increased solubility of inhibitor at the appropriate salt concentrations used. Chloride ions increased solubility of protease inhibitor by enhancing the electrostatic repulsions after binding to the positively charge groups of the protein. However, the decrease in total activity was found as NaCl solution at high concentration (0.6 M) was used ($p<0.05$). There was no difference in total inhibitor activity between water extract and that extracted using 0.6 M NaCl ($p>0.05$). At high level of NaCl, the water molecules could be tightly bound to salt, in which the competition between salt ions and protein for water molecules took place. Hence, solubility of protease inhibitor was lowered and some protease inhibitors might be denatured at high salt concentration as evidenced by the decrease in inhibitory activity. It was noted that lower protein content was obtained in

Table 12. Total inhibitor activity, protein concentration and specific activity of SOSPI as affected by different extraction media.

Extraction media	Concentration (M)	Total inhibitor activity (unit/mL)	Protein concentration (mg/mL)	Specific activity (unit/mg protein)
Water	-	392.39±16.24^{c}	16.95±0.63^{a}	23.17±1.40^{e}
NaCl	0.15	394.63±1.97^{c}	4.16±0.08^{b}	94.85±2.20^{d}
	0.3	426.85±8.96^{b}	3.55±0.02^{c}	120.33±2.75^{b}
	0.45	452.46±16.91^{a}	3.11±0.09^{c}	145.76±3.46^{a}
	0.6	394.21±1.77^{c}	3.60±0.08^{c}	109.64±2.16^{c}

Values are given as mean ± SD (n=3). Different lowercase superscripts in the same column denote the significant differences ($p<0.05$). The extraction time of 1 h was used.

the extract using NaCl solution with concentration of 0.3-0.6 M ($p<0.05$). NaCl might cause the precipitation of other proteins in squid ovary. As a result, proteins were retained at a lower extent in the extract. This was also evidenced by the highest protein content in water extract. When considering specific activity, the extract showed the increases in specific activity when the NaCl solutions with concentrations up to 0.45 M were used ($p<0.05$). Nevertheless, at high salt concentration (0.6 M), the decrease in the specific activity was noticeable ($p<0.05$). Specific activity of protease inhibitor from yellowfin tuna roe (Klomklao *et al.*, 2014) and Thai legume seeds (Benjakul *et al.*, 2000) decreased as the salt concentration increased. Based on the highest amount of inhibitors recovered and the highest specific activity, 0.45 M NaCl solution was considered as the most appropriate medium for extraction of serine protease inhibitor from squid ovary.

Total inhibitor activity was decreased when extraction time was longer than 1 h ($p<0.05$) (Table 13). On the other hand, protein content was increased with increasing extraction time ($p<0.05$). Very lower inhibitor activity was found in the extract when the extraction time was lower than 1 h. This was plausibly due to the

Table 13. Total inhibitor activity, protein content and specific activity of SOSPI as affected by different extraction time.

Time (h)	Total inhibitory activity (units/mL)	Protein concentration (mg/mL)	Specific activity (unit/mg protein)
1	485.40±0.92^{a}	3.31±0.17^{b}	146.55±0.28^{a}
2	451.73±4.25^{b}	3.92±0.27^{a}	115.36±1.08^{b}
3	447.01±4.33^{b}	3.97±0.18^{a}	114.22±1.11^{bc}
4	433.14±2.1^{c}	3.86±0.26^{a}	112.07±0.533^{d}
5	434.4±3.45^{c}	3.81±0.25^{a}	112.66±0.56^{cd}

Values are given as mean ± SD (n=3). Different lowercase superscripts in the same column denote the significant differences ($p<0.05$) NaCl solution (0.45 M) was used the extraction medium.

insufficient time for extraction of inhibitor from squid ovary into the extraction medium. Nevertheless, no difference in the protein content was observed as extraction time ranging from 2 to 5 h was used ($p>0.05$). For specific activity, the marked decrease was found when extraction time was longer than 1 h ($p<0.05$). Decrease was more pronounced with increasing extraction time ($p<0.05$). The denaturation of proteins was plausibly caused by the mechanical shearing and adsorption of proteins at liquid-air interface during extraction (Damodaran, 1996; Klomklao *et al.,* 2010).

The extraction time of 0.5 h was optimum for the isolation of protease inhibitor from the yellowfin tuna fish roe (Klomklao *et al.,* 2014) Extraction time of 0.5, 1 and 3 h was suggested for isolation of trypsin inhibitor from adzuki bean, cowpea and pigeon, respectively (Klomklao *et al.,* 2010; Benjakul *et al.,* 2000). Many factors are involved in the protein solubility and isolation of target protease inhibitors. Those include particle size and nature of tissue, ionic strength, type of concentration of extracting medium and hydration characteristics of protein (Benjakul *et al.,* 2000;

Klomklao *et al.*, 2011). Therefore, extraction time of 1 h was selected for the recovery of SOSPI from squid ovary powder.

6.5.1.2 Effect of heat treatment on the recovery of serine protease inhibitor

When the extract was subjected to heat treatment at different temperatures, the specific activity was increased when heated up to 70 °C ($p<0.05$) (Figure 22). When heating at temperature higher than 70 °C was applied, the continuous decrease in specific activity was noticeable ($p<0.05$). The denaturation of both inhibitors and proteins occurred simultaneously. However, the higher rate of denaturation of inhibitor was pertained in comparison with other proteins. Heating of

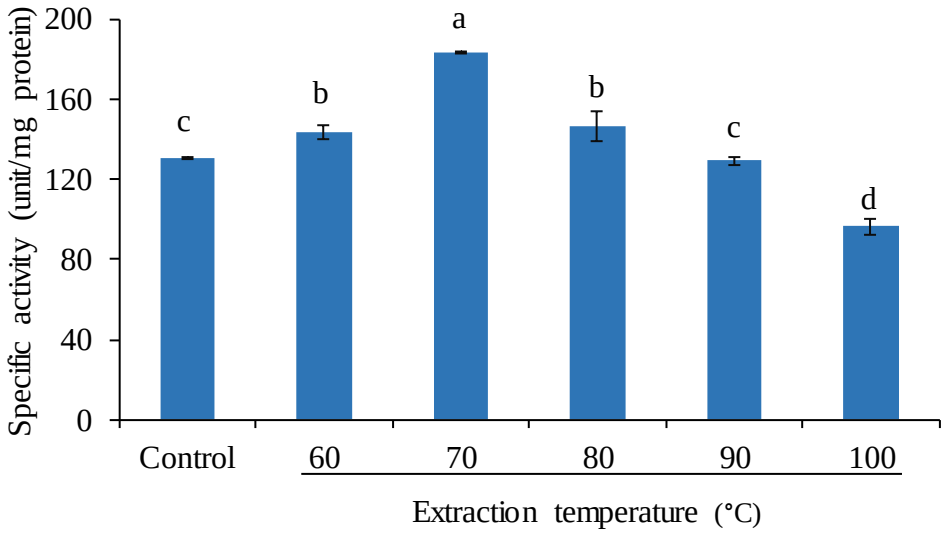

Figure 22. Specific activity of the extract from squid ovary preheated at different temperatures. Bars represent the standard deviation (±SD). Different lowercase letters on the bars denote the significant differences ($p<0.05$)

samples causes the looser structure of inhibitor from compact native form, which is stabilized by the several bonding (Benjakul *et al.*, 2000). The trypsin inhibitor from the egg of skipjack tuna and yellowfin tuna fish showed the decreases in specific activity at temperature above 40 °C and 70 °C, respectively (Choi *et al.*, 2002;

Klomklao *et al.*, 2014). The result revealed that heat treatment at 70 °C for 10 min could be used for partial purification of serine protease inhibitor from squid ovary.

6.5.1.3 Molecular weight distribution of serine protease inhibitors

Gel filtration chromatogram and inhibitor activity profile of SOSPI are shown in Figure. 23A. Two peaks (A280) with molecular weight of 35.09-70.60 and 3.32-16.83 kDa were obtained. Only one activity peak was observed. MW of Sephadex G-75 fraction showing the highest inhibitor activity was estimated to be in the range of 9.05- 11.47 kDa. MW of serine protease inhibitor from SOSPI was lower than those of trypsin inhibitor from chickpeas (28 kDa), soybean (19 kDa) and mustard seeds (20 kDa) (Kansal *et al.*, 2008). Trypsin inhibitor from yellow tuna fish roe was reported to have MW of 70 kDa based on size exclusion chromatography (Klomkalo *et al.*, 2015). Thus, serine protease inhibitor from SOSPI had the differences in size and the number of amino acids, in comparison with the other serine protease inhibitors.

Pooled active fractions containing inhibitor obtained from gel filtration were subjected to SDS-PAGE and inhibitory activity staining under both reducing and non-reducing conditions (Figure 23B and C). Based on SDS-PAGE, there were two

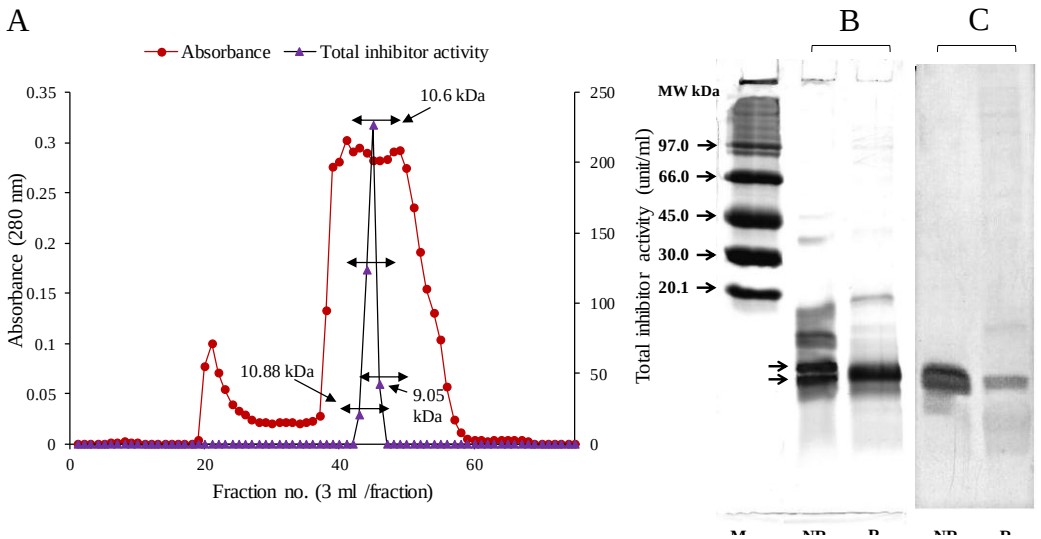

Figure 23. Sephadex G-75 elution profile and inhibitory activity (a), SDS-PAGE (b) and inhibitory activity staining (c) of active Sephadex G-75 fraction of squid ovary extract. M, Low molecular weight marker; NR, non-reducing condition; R, reducing condition. (↔ pooled active fractions).

major bands with MW 10.27 and 9.10 kDa when examined under non-reducing conditions. Additionally, proteins with MW of 14.10, 36.73 and 46.85 kDa were also found. Under reducing condition, those protein bands disappeared. The result suggested that those proteins were more likely stabilized by disulfide bond. However, protein of MW 9.10 kDa was still retained under the reducing condition (Figure 23B).

Inhibitor activity staining study reveals that two protein bands with apparent MW of 10.27 and 9.10 kDa under non-reducing conditions were serine protease inhibitors (Figure 23C). Apparent MW of inhibitor was supported by the results from size exclusion chromatography, which showed MW in the range of 9.05-11.47 kDa. The results also indicated that serine protease inhibitors were present as monomer. Under reducing conditions, inhibitor band with MW 10.27 kDa disappeared, indicating the loss of inhibitory activity in the presence of βME. Thus, the inhibitor with MW of 10.27 kDa more likely contained intramolecular disulfide bonds. Kunitz and Bowman inhibitor have seven and two disulfide bonds, respectively. When these disulfide bonds were reduced, the loss in inhibitor activity against trypsin was found (Benjakul *et al.,* 2000). Trypsin inhibitor from yellow tuna fish roe of MW 70 kDa contained two subunits with MW of 30 and 40 kDa, which are stabilized by disulfide bond. The results indicated that SOSPI had two inhibitors with MW of 10.27 and 9.10 kDa, in which the former was stabilized by disulfide bond.

6.5.2 Effect of SOSPI on autolysis of bigeye snapper surimi

Autolysis of bigeye snapper surimi in the absence and presence of SOSPI at various levels was expressed in term of TCA-soluble peptides content (Figure 24A). Heat activated proteases in surimi have been known to hydrolyze the muscle proteins (Kudre and Benjakul, 2013). TCA soluble peptides indicate the degradation of muscle proteins during heat induced gelation of surimi. The highest content of TCA soluble peptides was observed in the control (without SOSPI). The TCA-soluble peptide content was decreased when surimi paste was added with SOSPI in a dose-dependent manner. The result indicated the inhibition of proteolysis in surimi paste by SOSPI. The inhibitory activity of SOSPI was confirmed by SDS-PAGE results (Figure 24B). MHC and actin were found as the major proteins in

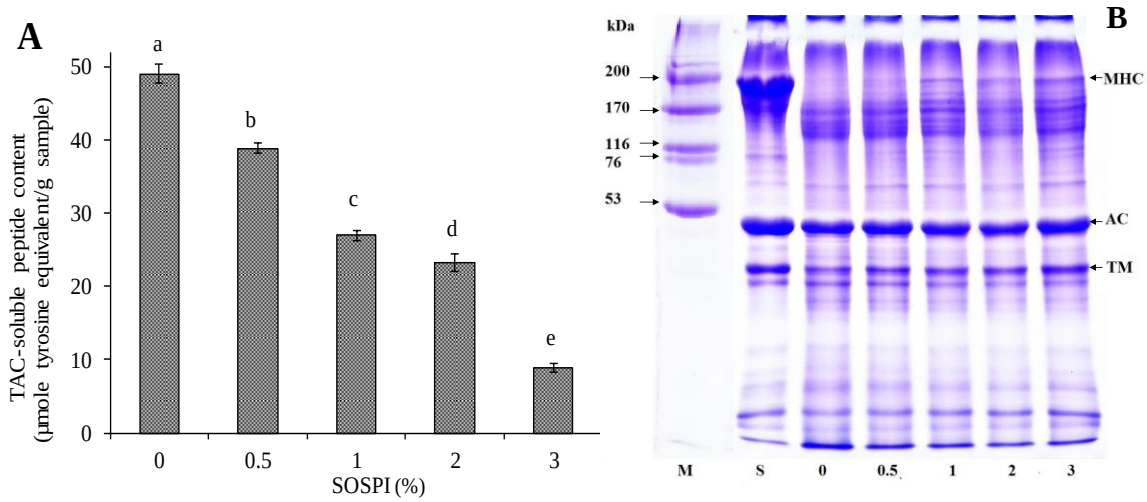

Figure 24. TCA-soluble peptide contents (A) and protein pattern of bigeye snapper surimi incubated at 60°C for 60 min (B) in the absence and presence of the extract from squid ovary. MHC: myosin heavy chain; AC: actin; TM: tropomyosin. M: high molecular weight; S: surimi; numbers are denoted as concentration of SOSPI (% w/w). Bars represent the standard deviation. Different lowercase letters on the bars denote the significant differences ($p<0.05$)

surimi paste. When the surimi paste was incubated at 60 °C for 60 min, the MHC band completely disappeared. Nevertheless, the addition of SOSPI was able to maintain MHC band to a higher extent as SOSPI levels increased. MHC band intensity increased by 5.8, 8.9, 9.4 and 31.3% as compared to control (without SOSPI) when the levels of SOSPI increased from 0.5 to 3%. Actin band density almost remained unchanged. Actin was resistant toward the proteolysis (Rawdkuen and Benjakul, 2008). MHC of sardine surimi was more retained when the protein isolate from mung bean, black bean and bambara groundnut were incorporated (Kudre and Benjakul, 2013). Furthermore, whey protein concentrate was also reported to exhibit inhibitory effect toward proteolysis of various tropical fish (Rawdkuen and Benjakul, 2008).

6.5.3 Effect of SOSPI on gel properties of bigeye snapper surimi

6.5.3.1 Effect of SOSPI on the textural properties of surimi gels

Breaking force and deformation of modori (60/90 °C) and kamaboko gel (40/90 °C) of bigeye snapper surimi added with SOSPI at different levels (0-3%) are shown Table 14. For modori gel, the lowest breaking force and deformation were observed in gels without SOSPI addition ($p<0.05$). Modori gel added with SOSPI up to 1% showed the increases in both breaking force and deformation ($p<0.05$). However, gel added with SOSPI at concentrations above 1% showed no marked difference in breaking force ($p>0.05$). There was no difference in deformation between samples added with SOSPI ranging from 0.5 to 3% ($p>0.05$). With addition of 1% SOSPI, breaking force and deformation of modori was increased by 40% and 40.7%, respectively, compared with the control (without SOSPI). Results suggested that SOSPI had the potential to enhance the gel strength of bigeye snapper surimi via inhibition of heat activated protease. When muscle proteins, especially myosin heavy chain, were more retained, those proteins were able to undergo gel formation more effectively. Long chain proteins could form the inter-junction or gel network more effectively. This was evidenced by the increases in breaking force when the SOSPI was added. The surimi from bigeye snapper contain serine proteases as the major indigenous enzyme associated with myofibrillar proteins (Benjakul *et al.*, 2003; Benjakul *et al.*, 2004), which are responsible for the degradation of myofibrillar proteins in surimi. Trypsin inhibitor from yellowfin tuna roe and protein isolate from bambara ground nut increased the breaking force and deformation in the modori surimi gel from bigeye snapper and threadfin bream, respectively (Klomklao *et al.*, 2015; Oujifard *et al.*, 2012).

For kamaboko gels, breaking force increased ($p<0.05$) as SOSPI amount increased up to 1%, in which the increase by 10.37% was attained, compared with the control. There was no difference in breaking force between kamaboko gels added with 1% and 2% SOSPI ($p>0.05$). Nevertheless, the decrease in breaking force was observed as SOSPI at 3% was incorporated ($p<0.05$). SOSPI generally showed higher inhibition toward protein degradation in modori gel than in kamaboko gel. This

Table 14. Breaking force, deformation, whiteness and expressible moisture content (EMC) of modori and kamaboko gels from bigeye snapper surimi added with SOSPI at different levels.

Gel samples	SOSPI (%)	Breaking Force (g)	Deformation (mm)	Whiteness	EMC (%)
Modori Gel	0	185.14±10.69^{c}	4.59±0.31^{b}	80.21±0.56^{a}	5.03±0.38^{a}
	0.5	230.28±11.41^{b}	5.94±0.11^{a}	79.96±0.52^{a}	4.12±0.51^{ab}
	1	259.91±16.09^{a}	6.46±0.42^{a}	80.03±0.23^{a}	4.01±0.8^{ab}
	2	283.15±4.03^{a}	6.27±0.11^{a}	79.07±0.12^{a}	3.27±1.06^{b}
	3	274.06±5.21^{a}	6.47±0.12^{a}	79.73±0.41^{a}	3.22±0.39^{b}
Kamaboko Gel	0	716.72±13.09^{c}	11.6±0.6^{a}	81.34±1.23^{a}	2.71±0.13^{a}
	0.5	757.52±25.1^{b}	10.86±0.27^{a}	81.44±0.24^{a}	2.32±0.06^{b}
	1	791.06±23.16^{a}	11.37±0.45^{a}	81.35±1.08^{a}	2.29±0.07^{b}
	2	811.58±10.65^{a}	11.76±0.19^{a}	82.18±0.54^{a}	2.23±0.04^{bc}
	3	665.51±12.19^{d}	11.25±0.2^{a}	81.85±0.27^{a}	2.12±0.049^{c}

Values are mean ± SD (n=3). Different lowercase superscripts in the same column under the same gel denote the significant differences ($p<0.05$).

confirmed the role of heat activated protease in bigeye snapper surimi. When those proteases, which were active at 60 ºC, were inactivated by SOSPI, the proteins were more retained and better gel could be formed. The result was supported by the highest increase in breaking force in modori gel than kamaboko gel, as compared to the controls from both gels (Table 14). In addition, the formation of non-disulfide covalent bonds such as ε-(γ-glutamyl) lysine linkages in kamaboko gel during setting at 40 ºC was resistant to protease activity (Kumazawa *et al.*, 1995). The setting phenomenon in kamaboko might proceed the degradation inhibition by SOSPI. This was indicated by the lower increase in breaking force of kamaboko gel when SOSPI was incorporated. Bambara groundnut protein isolate, porcine plasma protein and

casein at high concentration also alleviated the weakening of gel in threadfin bream and walleye pollack surimi mediated by indigenous proteases (Oujifard *et al.,* 2012; Benjakul *et al.,* 2004 and Yamashita *et al.,* 1996). Apart from the inhibitory activity against the proteolysis, SOSPI at a proper level might act as a filler, thereby increasing gel strength of kamaboko gel.

6.5.3.2 Effect of SOSPI on whiteness of surimi gels

Whiteness of modori and kamaboko added with SOSPI at different levels is shown in Table 14. No difference in whiteness was observed in both modori and kamaboko gels, regardless of SOSPI concentrations ($p>0.05$). Color characteristic of surimi gels were mainly dependent on the type and amount of additives added in surimi gels (Benjakul *et al.,* 2004). However, other protein additives including Thai legumes and whey protein concentrate resulted in the decreased whiteness of surimi gel from sardine and tropical fishes, respectively (Kudre and Benjakul, 2013; Rawdkuen and Benjakul, 2008). When comparing the whiteness of modori and kamaboko gels, kamaboko gels had a slightly higher whiteness, compared with modori gel ($p<0.05$). Incubation of modori gel at higher temperature (60 °C) might induce the browning reaction, particularly Maillard reaction to a higher extent, compared to kamaboko gel, with setting at lower temperature (40 °C). The overall results suggested that the SOSPI had no negative effect on the color of both kamaboko and modori surimi gels.

6.5.3.3 Effect of SOSPI on the expressible moisture content (EMC) of surimi gel

Modori gel showed the decrease in EMC ($p<0.05$), when SOSPI at level above 1% was added ($p<0.05$), suggesting the ability of SOSPI in enhancing water holding capacity (WHC) of modori gel (Table 14). For kamaboko gel, the continuous decrease in EMC was noticeable with increasing concentration. SOSPI` was suggested to assist in retaining more water in gel matrix which is the requirement for the better gel quality. Whey protein concentrate also decreased EMC in the kamaboko gel from the bigeye snapper (Rawdkuen and Benjakul, 2008). Protein isolates from black bean, mung bean and bambara groundnut were able to lower EMC

of kamaboko gels from sardine and threadfin bream surimi (Kudre and Benjakul, 2013; Kudre *et al.*, 2013; Oujifard *et al.*, 2012). In the present study, SOSPI rich in proteinaceous matters might exhibit high WHC, thus reducing water loss. In comparison between the controls (without SOSPI) of both gels, modori showed higher EMC than kamaboko gel, indicating the higher water holding capacity of the latter. The degradation of muscle proteins in modori gel caused the gel matrix poorer in holding the water. Furthermore, the addition of SOSPI directly prevented the degradation of surimi proteins, resulting in the strong gel network with capacity of water holding. The increased water holding capacity of gel was in accordance with the increased breaking force.

6.6 Conclusion

Serine protease inhibitors from the squid ovary were successfully isolated from squid ovary using 0.45 M of NaCl for 1 h, followed by heat treatment at 70 °C for 10 min. Protease inhibitors having apparent MW of 9.10 and 10.27 kDa were detected. It was able to inhibit the proteolysis of surimi paste from bigeye snapper and improved the gelling properties of both modori and kamaboko gel. Thus, serine protease inhibitor from squid ovary could be used as alternative additive for improving the gelling properties of surimi.

6.7 References

Alvarez, C. Couso, I., Tejada, M. 1999 Thermal gel degradation (modori) in sardine surimi gels. Journal of Food Science. 64: 633–637.

Benjakul, S. and Visessanguan, W. 2003. Transglutaminase-mediate setting in bigeye snapper surimi, Food Research International. 36: 253-266.

Benjakul, S. Visessanguan, W., and Thummaratwasik, P. 2000. Isolation and characterization of trypsin inhibitors from some Thai legume seeds. Journal of Food Biochemistry. 24: 107–127.

Benjakul, S., Visessanguan, W. and Chantarasuwan, C. 2004. Effect of porcine plasma protein and setting on gel properties of surimi produced from fish caught in Thailand, LWT- Food Science and Technology. 37: 177–185.

Benjakul, S., Visessanguan, W., Tueksuban, J. and Tanaka, M. (2004) Effect of some protein additives on proteolysis and gel-forming ability of lizardfish (*Saurida tumbil*). Food Hydocolloid. 18: 395-401.

Benjakul, S., Yarnpakdee, S., Visessanguan, W. and Phatcharat, S. 2010. Combination effects of whey protein concentrate and calcium chloride on the properties of goatfish surimi gel. Journal of Texture Studies. 41: 341-357.

Carreno, G. F., Dimes, L. E. and Haard, N. F. 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinase or proteinaceous proteinase inhibitors. Analytical Biochemistry. 214: 65-69.

Chalamaiah. M., Rao, G. N., Rao, D. G. and Jyothirmayi, T. 2010. Protein hydrolysates from meriga (*Cirrhinus mrigala*) egg and evaluation of their functional properties. Food Chemistry. 120:52–657.

Choi, J. H., Park, P. J. and Kim, S. W. 2002. Purification and characterization of a trypsin inhibitor from the egg of skipjack tuna *Katsuwonus pelamis*. Fisheries Science. 68: 1367–1373.

Damodaran, S. 1996. Amino acids, peptides, and proteins. In: Food Chemistry. (edited by Fennema, O.R.), p. 322–429. Marcel Dekker, New York.

Hoque, M. S., Benjakul, S. and Prodpran, T. 2010. Effect of heat treatment of film-forming solution on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. Journal of Food Engineering. 96: 66-73.

Intarasirisawat, R., Benjakul, S. and Visessanguan, W. 2011. Chemical compositions of the roes from skipjack, tongol and bonito. Food Chemistry. 124: 1328–1334.

Kansal, R., Kumar, M., Kuhar, K., *et al*. 2008. Trypsin protease inhibitor from *Cicer arietinum* L. and its efficacy against *Helicoverpa armigera*. Brazilian Journal of Plant Physiology. 20 :313–322.

Klomklao, S., Benjakul, S. and Kishimura, H. 2014. Optimum extraction and recovery of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe and its biochemical properties. International Journal of Food Science and Technology. 49: 168–173.

Klomklao, S., Benjakul, S., Kishimura, H. and Chaijan, M. 2011. Extraction, purification and properties of trypsin inhibitor from Thai mung bean (*Vigna radiata* (L.) R. Wilczek). Food Chemistry. 129: 1348–1354.

Klomklao, S., Benjakul, S., Kishimura, H., Osako, K. and Tanaka, M. 2010. A heat-stable trypsin inhibitor in adzuki bean (*Vigna angularis*): effect of extraction media, purification and biochemical characteristics. International Journal of Food Science and Technology. 45: 163–169.

Klomklao, S., Benjakul, S., Kishimura, H., Osako, K. and Simpson, B. K. 2015. Trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe: Effects on gel properties of surimi from bigeye snapper (*Priacanthus macracanthus*). LWT - Food Science and Technology. 65:122-127.

Klomklao, S., Benjakul, S., Kishimura, H., Osako, K., and Benjamin, K. S. 2016. Purification and characterization of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe. Jounal of Food Biochemistry. 40: 140–147.

Kudre, T., Benjakul, S. and Kishimura, H. 2013. Effects of protein isolates from black bean and mungbean on proteolysis and gel properties of surimi from sardine (*Sardinella albella*). LWT - Food Science and Technology. 50: 511-518.

Kudre, T. and Benjakul, S. 2013. Effect of legume seed protein isolates on autolysis and gel properties of surimi from sardine (*Sardinella albella*). International Journal of Chemical and Environmental Biological Sciences. 1: 2320–4087.

Kudre, T. G. and Benjakul, S. 2013. Combining effect of microbial transglutaminase and bambara groundnut protein isolate on gel properties of surimi from sardine (*Sardinella albella*). Food Biophysics. 8: 240–249.

Kumazawa, Y., Numazawa, N. and Seguro, M. 1995. Motoki suppression of surimi gel setting by transglutaminase inhibitors. Journal of Food Science. 60: 715–717.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature .227: 680-685.

Lowry, O. H., Rosebrough, N. J., Fan, A. L. and Randall, R. J. 1951. Protein measurement with folin phenol reagent. The Journal of Biological Chemistry. 193: 256–275.

Minin, A. and Ozerova, S. G. 2015. Fish ovarian fluid contains protease inhibitors. Russian Journal of Developmental Biology. 46: 33–37.

Morrissey, M. T., Wu, J. W., Lin, D. and An, H. 1993. Protease inhibitor effects on torsion measurements and autolysis of pacific whiting surimi. Journal of Food Science. 58: 1050-1054.

Oujifard, A., Benjakul, S., Ahmad, M. and Seyfabadi, J. 2012. Effect of bambara groundnut protein isolate on autolysis and gel properties of surimi from threadfin bream (*Nemipterus bleekeri*). LWT-Food Science and Technology. 47: 261-266.

Park, J. W. 2000. Ingredient technology and formulation development *In* Surimi and Surimi Seafood. (Park, J.W. ed.), p. 343-392. Marcel Dekker. New York.

Rawdkuen, S. and Benjakul, S. 2008. Whey protein concentrate: sutolysis inhibition and effects on the gel properties of surimi prepared from tropical fish. Food Chemisty. 106: 1077–108.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and Procedures of Statistics: A Biometrical Approach (2nd ed.) McGraw-Hill, New York.

Yamashita, T., Araki, H. and Seki, Y. 1995. Effects of casein on gelling properties of surimi paste. Fisheries Science. 62: 421-426.

CHAPTER 7

EFFECT OF SERINE PROTEASE INHIBITOR FROM SQUID OVARY ON GEL PROPERTIES OF SURIMI FROM INDIAN MACKEREL

7.1 Abstract

Effects of serine protease inhibitor from squid ovary (SOSPI) and egg white powder (EWP) on gel properties of surimi from Indian mackerel (*Rastrelliger kanagurta*) were investigated. Breaking force of gel increased when SOSPI levels increased up to 2%. However, EWP showed higher efficiency in increasing breaking force of resulting surimi gel. Nevertheless, SOSPI (0.5-3%) had no effect on deformation. TCA-soluble peptide content in surimi gel decreased when the levels of SOSPI and EWP increased ($p<0.05$). This was coincidental with more retained myosin heavy chain (MHC). Water holding capacity was increased with the addition of these protein additives, but resulted in lower whiteness. Microstructure study revealed that surimi gel added with 2% EWP was denser and had higher connectivity as compared to that containing 2% SOSPI. The SOSPI had no negative effect on sensory attributes and could serve as the alternative protein additive to improve gel strength of surimi.

7.2 Introduction

Surimi is washed fish mince rich in myofibrillar proteins, which are solubilized at high ionic strength. It has unique gelling properties and can be used for manufacturing of several products. Normally, lean fish are the major source for surimi production since they provide surimi with white color and better gel properties, compared with the dark fleshed fish. Owing to over-exploitation, surimi industries are moving towards the dark fleshed fish as an alternative raw material. These fish possess high lipid, myoglobin and sarcoplasmic protein contents. Additionally, heat activated proteases in meat of those species show adverse effects on gel properties. Although most enzymes are removed during the washing process of fish mince, some endogenous proteases are still retained. Amounts of proteases generally vary from species to species (Benjakul *et al.,* 2003). These proteases mainly cause degradation

of myofibrillar protein at temperatures between 50 and 70C, resulting in gel weakening termed ‘modori phenomenon’ (Park, 2000). To overcome this problem, various food grade proteases inhibitors, including bovine plasma protein (BPP), egg white, porcine plasma protein (PPP), soy protein isolate and whey protein concentrate etc. have been used. These inhibitors were able to inhibit the proteases and enhanced the gel strength (Benjakul *et al.*, 2004a; Rawdkuen and Benjakul, 2008; Kudre and Benjakul, 2013). With increasing concern on mad cow disease and religious constraint, BPP and PPP are prohibited to use in surimi. High cost and off-odor of egg white and off-white of plant protein isolates also limit their uses in surimi gel.

Thailand is in the list of major country for marine capture, estimated to be 1, 630, 047 tones (FAO, 2014). Approximately 78% capture is for human consumption and the rest (about 22%) is discarded or used for non-food purposes, especially for fishmeal production. Squid is one of marine product popular for consumption in Asian countries. During processing or degutting, internal organs are removed and can serve as the potential sources of beneficial compounds (Morrissey and Okada, 2007). In female squid, ovary has accountable percentage (10-15%) with respect to body weight and is generally discarded by squid processing industry. Recently, Singh and Benjakul (2016) reported that squid ovary contained serine protease inhibitor and the extract containing serine protease inhibitor could inhibit autolysis of bigeye snapper surimi in a dose dependent manner. However, there is no information regarding the impact of SOSPI on surimi gel properties has been reported.

7.3 Objective

To investigate the gel properties of Indian mackerel surimi as influenced by incorporation of SOSPI at various levels.

7.4 Materials and methods

7.4.1 Chemicals

All chemicals used were of analytical grade. Trypsin, Na-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) and high molecular weight protein marker were obtained from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), and Coomassie Blue R-250 were purchased from Merck (Darmstadt, Germany). Egg white powder was purchased from Mathawach Flavour Limited Partnership, Samut Sakhon, Thailand.

7.4.2 Preparation of squid ovary serine protease inhibitor (SOSPI)

Squid (*Loligo formosana*) viscera were obtained from Sea Wealth Frozen Food, Songkhla, Thailand. The ovary was manually separated from other internal organs. The obtained ovary was chopped using a blender to attain homogeneity, followed by freeze-drying using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The freeze-dried squid ovary powder was stored in a polyethylene bag at -40 ºC until use.

To extract protease inhibitors, the squid ovary powder was mixed with 0.45 M NaCl in Na-phosphate buffer (pH 7.0) at a ratio of 1:30 (w/v). The mixture was homogenized (IKA, Labortechnik homogenizer Selangor, Malaysia) for 1 min at 5,000 rpm, followed by stirring for 1 h at room temperature (25-30 ºC). The squid ovary extract was recovered by centrifugation (Allegra 25R centrifuge, Beckman Coulter, Palo Alto, CA, USA) at 10,000 xg for 30 min at 4 ºC. The obtained supernatant was heated for 10 min at 70 ºC. Heated extract was centrifuged at 8,000 xg for 10 min to remove coagulated debris. The supernatant containing protease inhibitor was freeze-dried using a freeze dryer. Squid ovary serine protease inhibitor (SOSPI) obtained was stored at -40 ºC in a polyethylene bag.

The inhibitory activities of SOSPI and EWP were 2.27 and 13.2 k unit/g, respectively, as determined by the method of Kudre and Benjakul (2013).

7.4.3 Surimi preparation

Fresh Indian mackerel (*Rastrelliger kanagurta*) with the size of 100-120 g/fish were obtained from a local fish market of Hat Yai, Songkhla, Thailand. Fish were transported in ice with fish/ice ratio 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand within 1 h. The fish were washed with cold water. Thereafter, fish were beheaded and eviscerated. Fish flesh was recovered manually. The fish flesh was minced using a mincer with a hole (diameter of 5 mm). Then the mince was washed with cold water (5-8 ºC) using a mince/water ratio of 1:3 (w/v). The mixture was stirred gently with a plastic spatula and then filtered through a layer of cheesecloth. The washing step is repeated for totally three times. After filtration, the mince was centrifuged using a basket centrifuge (Model CE 21 K, Grandiumpiant, Belluno, Italy) for 15 min at 700 xg. Washed minced was mixed thoroughly with 4% cryoptotectants (sorbitol/sucrose, 1:1), packed into polyethylene bag and stored at -20 ºC. The frozen surimi was used within two weeks.

7.4.4 Effect of SOSPI on gel properties of Indian mackerel surimi

7.4.4.1 Preparation of surimi gel

Kamaboko surimi gel from Indian mackerel was prepared as described in previous section 6.4.6.1.

7.4.4.2 Analyses

7.4.4.2.1 Breaking force and deformation

Breaking force and deformation of surimi gel were determined as described in section 6.4.7.1.

7.4.4.2.2 Determination of whiteness

Whiteness of surimi gel was determined as described in section 6.4.7.2.

7.4.4.2.3 Determination of expressible moisture content

Expressible moisture content was measured as described in previous section 6.4.7.3.

7.4.4.2.4 TCA-soluble peptide content

TCA-soluble peptide content was determined as described in section 6.4.5.

7.4.4.2.5 Textural profile

Gel samples were subjected to texture profile analysis (TPA). Hardness, springiness, cohesiveness, gumminess and chewiness were determined using a texture analyzer (Model TA-XT2, Stable MicroSystems, Surrey, UK) with a cylinder probe (a diameter of 35 mm) following the method of Buamard and Benjakul (2015).

7.4.4.2.6 Protein pattern

The gel samples were subjected to SDS-PAGE analysis as described in section 6.4.5.

7.4.5 Characterization of surimi paste and gel added with SOSPI and EWP at the selected level

7.4.5.1 Dynamic rheology of surimi paste

Surimi pastes added with SOSPI or EWP at a level of 2% were prepared as previously described and were subjected to dynamic rheological measurements following the method of Buamard and Benjakul (2015). Rheometer (HAAKE RheoStress1, ThemoFisher Scientific, Karlsruhe, Germany) with 35 mm 4° slope cone and plate geometry was used for monitoring the change in storage or elastic modulus (G'). An oscillation of 1 Hz with 1% deformation was used for measurements. This condition yielded a linear response in the viscoelastic region. The temperature sweep was recorded during heating up from 10 to 90 ºC with heating rate

of 1 ºC/min. Silicone oil was used to minimize water evaporation of surimi pastes during measurement.

7.4.5.2 Microstructure of surimi gel

Microstructures of surimi gel containing SOSPI or EWP at a level of 2% (w/w) were examined using a scanning electron microscope (SEM). The samples with a thickness of 2-3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 3 h at room temperature followed by rinsing with distilled water. Fixed specimens were dehydrated in ethanol with serial concentrations of 25, 50, 70, 80, 90 and 100%. Samples were critical point dried using CO_2 as transition fluid. The prepared samples were mounted on a bronze stub and sputter-coated with gold. The specimens were visualized using SEM (Quanta 400, FEI, Eindhoven, the Netherlands).

7.4.5.3 Sensory properties of surimi gel

Gel samples in the absence and presence of 2% SOSPI or 2% EWP, were cut into a bite-size (1 cm thick and 2.5 cm in diameter), equilibrated at room temperature (28-30 ºC) for 30 min and coded with 3-digit random numbers. Gel samples were served on the white paper dishes at room temperature under the fluorescent daylight-type illumination. Eighty non-trained panelists (aged between 20 and 45) were the students and staffs at the Department of Food Technology, who were acquainted with surimi products. The panelists were asked to evaluate for color, taste, texture and overall liking of gel samples using 9-point hedonic scale (1, extremely dislike; 2, very much dislike; 3, moderately dislike; 4, slightly dislike; 5, neither like nor dislike; 6, slightly like; 7, moderately like; 8, very much like; 9, extremely like) as per the method of Meilgaard *et al.* (1999). Between samples, the panelists were asked to rinse their mouth with distilled water.

7.4.6 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance. Comparison of means was carried out by the Duncan's multiple range

tests (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL).

7.5 Results and discussion

7.5.1 Effect of SOSPI and EW on textural properties of Indian mackerel surimi gel

7.5.1.1 Breaking force and deformation

Breaking force and deformation of surimi gel incorporated with SOSPI or EWP at different levels are shown in Figure 25A and B. Breaking force of surimi gel increased as the concentrations of SOSPI and EWP were increased up to 2% (w/w) ($p<0.05$) (Figure 25A). Breaking force was decreased when the level of SOSPI added was above 2%. The decrease by 21.44% was obtained when 3% SOSPI was added, in comparison with that of 2% SOSPI addition. For EWP added samples, there was no difference in breaking force between those containing 2 and 3% ($p>0.05$). It was noted that no difference in deformation between samples added with SOSPI in the range of 0.5-3% was observed ($p>0.05$). Nevertheless, higher deformation was found in gels added with SOSPI, regardless of levels used ($p<0.05$). For the sample added with EWP, the increased deformation was noticeable with increasing levels ($p<0.05$). However, similar deformation was found between those containing 2 and 3% EWP ($p>0.05$). When SOSPI or EWP at level of 2% was added, breaking force of surimi gel was increased by 207.2% and 417.24%, while deformation was increased by 61% and 125.5%, respectively, as compared to the control (without SOSPI and EWP). During setting at 40 ºC, endogenous transglutaminase induced the cross-linking of MHC via nondisulfide bond (Benjakul and Visessanguan, 2003). Simultaneously, endogenous heat-stable proteases with the optimum temperature of 50-60 ºC could induce the degradation of MHC during setting (Benjakul *et al.*, 2003). The results suggested that both SOSPI and EWP were effective in enhancing the gel strength, more likely via lowering the degradation by indigenous protease inhibitors. EWP contained several trypsin inhibitors and ovoinhibitor was the most active trypsin inhibitor in EWP (Weerasinghe *et al.*, 1996). Ovastatin with MW of 780 kDa, has a potential to inhibit trypsin and is homologous to α2-macroglobulin (α2M) in

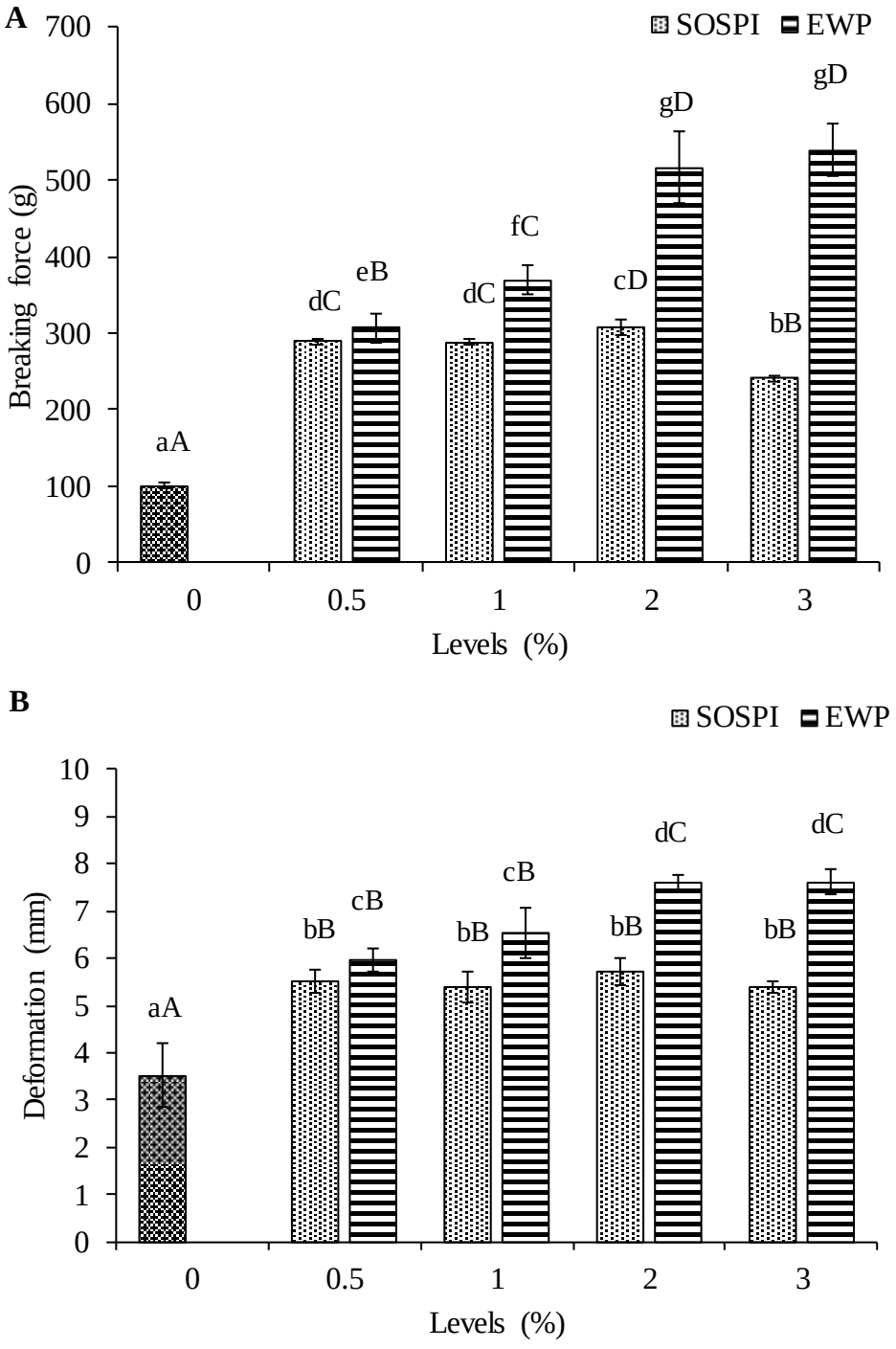

Figure 25. Breaking force and deformation of gels from Indian mackerel surimi in the absence and presence of SOSPI or EWP at different levels. Bars represent the standard deviation (n = 3). Different uppercase letters in the same sample including the control (without additives) on the bars indicate significant differences ($p<0.05$). Lowercase letters on the bars indicate significant difference ($p<0.05$).

molecular structure, function, and inhibition mechanism (Nagase and Harris, 1983). Cystatin, a cysteine proteinase inhibitor, was also found in egg a white (Anastasi *et al.*, 1983). Apart from their protease inhibitory activities, both SOSPI and EW were able to form gel, thus having the filler effect or binding ability to strengthen the surimi gel matrix. Lu and Chen (1999) reported that the EWP also functioned as a binder in meat. The result suggested that SOSPI was able to improve the gel properties of gel from Indian mackerel surimi to some degree. Nevertheless, the efficacy was lower than EWP as indicated by the lower increase in breaking force and deformation of surimi gel in comparison with EWP.

7.5.1.2 Whiteness

Whiteness of surimi gel added with SOSPI or EWP at different levels is shown in Table 15. Addition of SOSPI and EWP resulted in the decrease in whiteness ($p<0.05$). No difference in whiteness was observed for gel added with SOSPI or EWP in the range of 0.5-3% ($p>0.05$). At the same level, EWP yielded the gel with slightly higher whiteness, as compared to SOSPI ($p<0.05$). This might be due to the different indigenous pigments between EWP and SOSPI. Whiteness of surimi gel were affected by the amount and type of additives added (Benjakul *et al.*, 2004b). Thai legumes and whey protein concentrate also decreased the whiteness of surimi gel from sardine and tropical fishes (Kudre and Benjakul, 2013; Rawdkuen and Benjakul, 2008). Indian mackerel is a dark fleshed fish having high lipid content, and lipids could undergo lipid oxidation. Lipid oxidation products were able to participate in Maillard reaction during setting or heating (Arfat and Benjakul, 2012).

7.5.1.3 Expressible moisture content (EMC)

The marked decrease in EMC was observed when SOSPI or EWP at a level of 0.5% was incorporated ($p<0.05$) (Table 15). It indicated the ability of SOSPI and EWP to enhance water holding capacity of gel. SOSPI at high concentrations (2-3%, w/w) decreased EMC of resulting gels more efficiently than at lower level ($p<0.05$). The result suggested that SOSPI at sufficient amount could increase water holding capacity of gel more potentially. Increased water holding capacity of surimi

Table 15. TCA-soluble peptide content, whiteness and expressible moisture content (EMC) of gels from Indian mackerel surimi in the absence and presence SOSPI or EWP at different levels

Samples	Levels (%)	TCA-soluble peptide (µmole tyrosine release/g)	Whiteness	EMC (%)
	0	126.61 ± 1.84^{hE}	70.11 ± 0.24^{cB}	9.99 ± 0.43^{cCB}
SOSPI	0.5	67.16 ± 0.30^{gD}	68.30 ± 0.31^{aA}	3.80 ± 0.47^{bB}
	1	64.29 ± 0.52^{fC}	68.25 ± 0.28^{aA}	3.41 ± 0.46^{abAB}
	2	45.70 ± 0.57^{cB}	68.29 ± 0.25^{aA}	3.17 ± 0.19^{aA}
	3	38.34 ± 0.43^{bA}	68.22 ± 0.2^{aA}	3.02 ± 0.083^{aA}
EWP	0.5	56.10 ± 0.74^{eD}	69.72 ± 0.31^{bA}	3.36 ± 0.23^{aA}
	1	52.29 ± 0.75^{dC}	69.26 ± 0.44^{bA}	2.84 ± 0.61^{aA}
	2	39.05 ± 0.37^{bB}	69.44 ± 0.16^{bA}	2.70 ± 0.22^{aA}
	3	34.44 ± 0.35^{aA}	69.56 ± 0.08^{bA}	2.70 ± 0.49^{aA}

Values are mean ± SD (n=3). Different uppercase superscripts in same column under the same sample including control (without additives) indicate the significant differences ($p<0.05$). Different lowercase superscripts in same column indicate significant difference ($p<0.05$).

gel added with 2% SOSPI was coincidental with the increased breaking force (Figure 25). However, EMC of gels added with EWP at all levels (0.5-3%, w/w) had similar EMC ($p>0.05$). In general, there was no difference in EMC between gel added with SOSPI and EWP in the range of 1-3% ($p>0.05$). Rawdkuen and Benjakul (2008) reported that EMC of surimi from tropical fish was decreased with increasing level of whey protein concentrate up to 3%. Among all the samples, the control (without EWP or SOSPI) showed the highest EMC. This might be due to the higher degradation of myofibrillar proteins, in which poor gel network with lower ability in imbibing water was formed. In the presence of SOSPI or EWP, the degradation of myofibrillar

proteins could be retarded and the developed gel network could hold water more efficiently.

7.5.1.4 TCA-soluble peptide content

The highest TCA-soluble peptide content was observed in surimi gel without addition of SOSPI or EWP ($p<0.05$) (Table 15). TCA-soluble peptide content in gel decreased as the concentrations of SOSPI or EWP increased ($p<0.05$). This result suggested that SOSPI and EWP exhibited inhibitory activity towards degradation of muscle proteins in surimi during heat-induced gelation process. SOSPI and EWP showed the highest inhibition at the level of 3% (w/w). The decreases in TCA-soluble peptide content by 65.9% and 72.8%, respectively, were obtained as compared to the control. At all levels, TCA-soluble peptide content in surimi gels added with EWP was lower than that found in those containing SOSPI ($p<0.05$). Higher trypsin inhibitory activity of EWP (13.2 k unit/g) mostly contributed to the higher ability in inhibition of degradation of surimi proteins. As a consequence, properties of Indian mackerel surimi gel could be improved, mostly associated with the maintenance of muscle proteins for the development of strong gel network

7.5.1.5 Textural profile

TPA profile of surimi gel from Indian mackerel added with SOSPI or EWP at different levels is shown in Table 16. All TPA parameters, hardness, springiness, cohesiveness, gumminess and chewiness of surimi gel were drastically increased with the addition of EWP and SOSPI, compared to the control ($p<0.05$). Hardness, the force required to compress surimi gel to achieve a given deformation, was increased when SOSPI levels increased up to 2% ($p<0.05$). The increase in hardness was more likely due to formation of stronger gel network. This result was coincidental with the increased breaking force (Figure 25). The decrease in hardness was observed when 3% SOSPI was added ($p<0.05$). The dilution effect towards myofibrillar protein might lower the ability of gel formation of surimi. For surimi gel added with EWP the continuous increase in hardness was found with increasing EWP levels up to 3% ($p<0.05$). Higher inhibitory activity of EWP could conquer the dilution effect, leading to the increased hardness. Cohesiveness, capability in breaking

Table 16. Textural properties of gels from Indian mackerel surimi in the absence and presence of EWP or SOSPI at different levels.

Samples	Levels (%)	Hardness	Springiness	Cohesiveness	Gumminess	Chewiness
Control	0	25.46 ± 0.51^{aA}	0.48 ± 0.13^{aA}	0.20 ± 0.03^{aA}	5.65 ± 0.37^{aA}	3.15 ± 0.19^{aA}
SOSPI	0.5	101.58 ± 1.53^{dC}	0.89 ± 0.01^{bB}	0.62 ± 0.01^{dC}	62.66 ± 0.94^{eD}	55.63 ± 0.57^{dC}
	1	100.61 ± 0.69^{dC}	0.89 ± 0.02^{bB}	0.61 ± 0.02^{cdC}	61.38 ± 1.02^{edD}	54.45 ± 0.46^{dC}
	2	103.97 ± 0.83^{eD}	0.88 ± 0.01^{bB}	0.59 ± 0.02^{bcBC}	58.73 ± 0.78^{cC}	54.85 ± 0.70^{dC}
	3	89.20 ± 0.84^{bB}	0.87 ± 0.01^{bB}	0.57 ± 0.01^{bB}	49.92 ± 1.13^{bB}	43.71 ± 1.03^{bB}
EW	0.5	92.45 ± 0.49^{cB}	0.89 ± 0.02^{bB}	0.64 ± 0.01^{eB}	60.83 ± 1.69^{eB}	52.53 ± 0.57^{cB}
	1	116.82 ± 0.42^{fC}	0.88 ± 0.00^{bB}	0.66 ± 0.01^{efCB}	73.22 ± 1.10^{fC}	65.99 ± 1.34^{eC}
	2	128.75 ± 1.32^{gD}	0.89 ± 0.01^{bB}	0.67 ± 0.00^{fgCB}	86.81 ± 0.28^{gD}	76.09 ± 0.86^{fD}
	3	133.41 ± 1.27^{hE}	0.89 ± 0.02^{bB}	0.69 ± 0.00^{gC}	85.40 ± 0.59^{gD}	74.88 ± 0.96^{fD}

Values are mean ± SD (n=3). Different lowercase uppercase superscripts in same column under the same sample including the control (without and with additives) indicate the significant differences ($p<0.05$). Different lowercase superscripts in same column indicate significant difference ($p<0.05$).

down the internal structure of sample and gumminess, the energy required to breakdown a semi-solid food ready for swallowing, of surimi gels were decreased as the concentration of SOSPI increased ($p<0.05$). For chewiness, the required energy to chew the sample to the point required for swallowing it, no difference was observed when SOSPI was added up to 2% ($p>0.05$). The decrease was noticeable when SOSPI at the level of 3% (w/w) was used ($p<0.05$). Overall, hardness of surimi gel added with EWP increased as the concentration increased, whereas cohesiveness, gumminess and chewiness of resulting gel also increased with increasing EWP levels ($p<0.05$). This was in accordance with the continuous increase in breaking force and deformation of surimi gel added with increasing levels of EWP (Figure 25). Springiness, elastic recovery that occurs when the compressive force is removed, was unaffected by incorporation of both SOSPI and EWP. Therefore, SOSPI at an appropriate level (2%) could be used to improve the textural properties of Indian mackerel surimi gel.

7.5.1.6 Protein patterns of surimi gel

Protein patterns of surimi gel added with SOSPI and EWP at different levels are shown in Figure 26. Myosin heavy chain (MHC) was the major protein present in the surimi paste, followed by actin. Control gel (without SOSPI and EWP) showed the complete disappearance of myosin heavy chain (MHC), whereas actin was still retained. This indicated that MHC was susceptible to proteolysis. This was confirmed by the highest TCA-soluble peptide content in the control sample. Apart from the degradation, cross-linking of proteins mediated by indigenous transglutaminase also played a major role in the disappearance of MHC band (Benjakul *et al.,* 2003). The band intensity of MHC was increased with addition of SOSPI and EWP, suggesting the inhibitory activity of SOSPI and EWP against degradation of myofibrillar proteins. The result was supported by the decreased TCA-soluble peptide content of gel in the presence of SOSPI and EWP (Table 15). With addition of SOSPI or EWP at 2%, MHC band was retained by 49.04% and 60.38%, respectively, as compared to that of the control. When 3% SOSPI or 3% EWP was incorporated, band intensity of MHC was decreased by 10.19% and 4.9%, as compared to the samples added with 2% SOSPI or 2% EWP, respectively. The

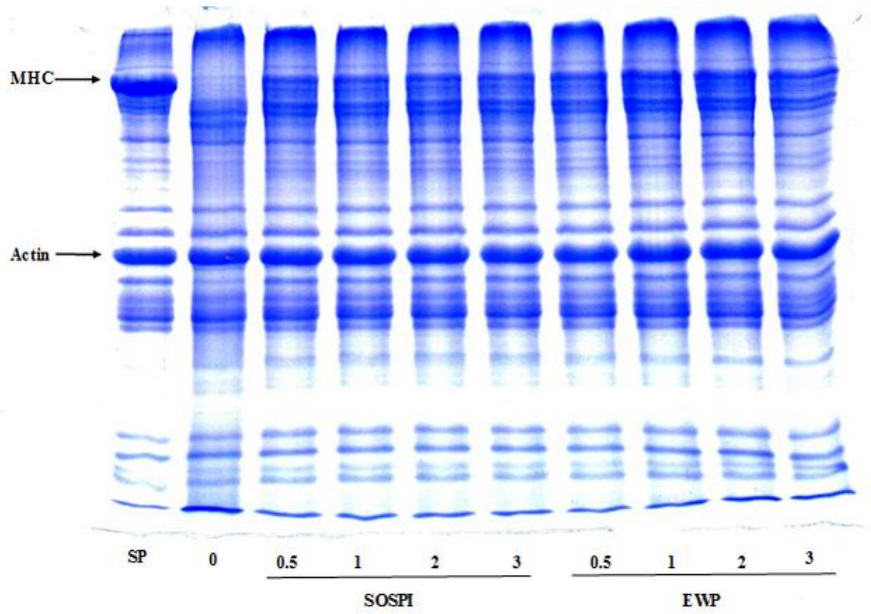

Figure 26. Protein patterns of gel from Indian mackerel surimi in the absence and presence of the SOSPI or EWP at different levels. MHC: myosin heavy chain, SP: surimi paste; numbers denote as the levels of SOSPI and EWP (% w/w).

decrease in band intensity was in accordance with the decreases in breaking force in sample added with 3% SOSPI. Nevertheless, slight decrease in band intensity of MHC in 3% EWP containing sample had no adverse effect on breaking force (Figure 25). In general, MHC band intensity was retained more slightly in gel added with EWP, compared with those added with SOSPI. MHC at higher level could form the stronger gel network during heat-induced gelation process. This was concomitant with higher breaking force of gel with addition of EWP or SOSPI.

7.5.2 Characteristics of surimi paste and gel added with SOSPI and EWP at the selected level

7.5.2.1 Dynamic rheology

Storage modulus (G´) of Indian mackerel surimi paste added without and with SOSPI and EWP at a level of 2% (w/w) during heating at different temperatures was monitored as shown in Figure 27. Storage modulus, is a measure of deformation energy stored in the sample during shear process, representing the elastic behavior of a sample (Vate and Benjakul, 2016). G´ was increased continuously and

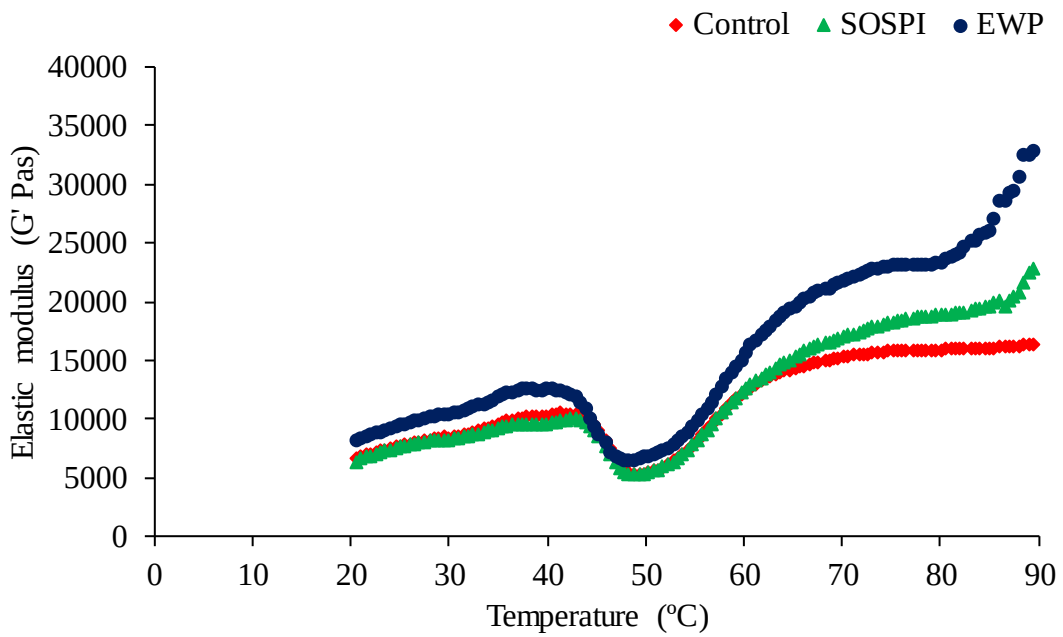

Figure 27. Elastic modulus (G′) of Indian mackerel surimi paste in the absence and presence of 2% SOSPI or 2% EWP during heating from 20 to 90 ºC.

reached the highest value at approximately 40 ºC in all samples. This indicated the formation of protein network via weak bondings between protein molecules (Buamard and Benjakul, 2015). At temperature lower than 40 ºC, myofibrillar components dissociate from its super helix structure (Rawdkuen *et al.*, 2008). Subsequently, G´ value decreased and the lowest value was obtained when heated at temperature around 50 ºC. This was more likely due to degradation of myofibrillar proteins by the action of endogenous proteases. The decrease in G´ value was also governed by the increased fluidity of surimi paste at this temperature due to the dissociation, unfolding and denaturation of actin-myosin complex, myosin and meromyosin, respectively. (Egelandsdal *et al.*, 1986). Thereafter, the continuous increase in G´ was observed up to 90 ºC. When temperature was increased, unfolded proteins entangled and formed gel network via the interaction of unfolded proteins. Surimi paste with EWP showed the highest G´ value at all temperatures treated. This was coincidental with the higher breaking force and deformation of gel added with EWP than that containing SOSPI at the same level (2%). G´ of sample added with SOSPI was similar to that of control at temperature lower than 35 ºC. Slightly higher G´ was observed in the former at temperature around 35-45 ºC. Nevertheless, the sample added with SOSPI showed the

higher G´ than the control after heating up to 90 ºC. The result implicated that SOSPI might enhance the entanglement or interaction of proteins in surimi, particularly at higher temperature. This was plausibly due to the inhibition of degradation of myofibrillar proteins by SOSPI or EWP. This resulted in the formation of stronger gel network. The increased G´ value of sample added with SOSPI was in accordance with the low TCA-soluble peptide content (Table 15) and higher breaking force (Figure 25). Apart from serving as protease inhibitor, SOSPI might act as filler or binder in gel network, which contributed to the higher G´ value.

7.5.2.2 Microstructure of surimi gel

Microstructures of gel from Indian mackerel surimi without and with 2% SOSPI or 2% EWP are illustrated in Figure 28. Control gel showed a coarser network with coagulated proteins distributed in the network. Finer network with higher connectivity was found in gel containing SOSPI or EWP. It was noted that gel containing EWP was more compact and had the smaller voids, compared to that with SOSPI. This reconfirmed the role of EWP as an effective protease inhibitor, which exhibited higher activity than SOSPI. Addition of whey protein concentrate, black bean protein isolate and mung bean protein isolate yielded more compact and ordered gel from bigeye snapper and sardine when added at the level of 3% (w/w) and 1% (w/w), respectively (Kudre *et al.*, 2013; Rawdkuen and Benjakul, 2008). Thus, addition of 2% SOSPI or 2% EWP rendered the finer gel network with enhanced gel strength and increased water holding capacity.

7.5.2.3 Likeness score of surimi gel

Likeness score of gel from Indian mackerel surimi in the absence and presence of 2% SOSPI or 2% EWP is shown in Table 17. Surimi gel without and with additives, showed no significant difference in score of color, appearance and taste likeness ($p>0.05$). Among all samples, the control showed the lowest texture and overall likeness score, compared to those added with SOSPI or EWP. This was coincidental with the increased breaking force and decreased expressible moisture content of gels added with SOSPI and EWP (Figure 25 and Table 15). However, no significant difference was found in texture and overall likeness score between sample

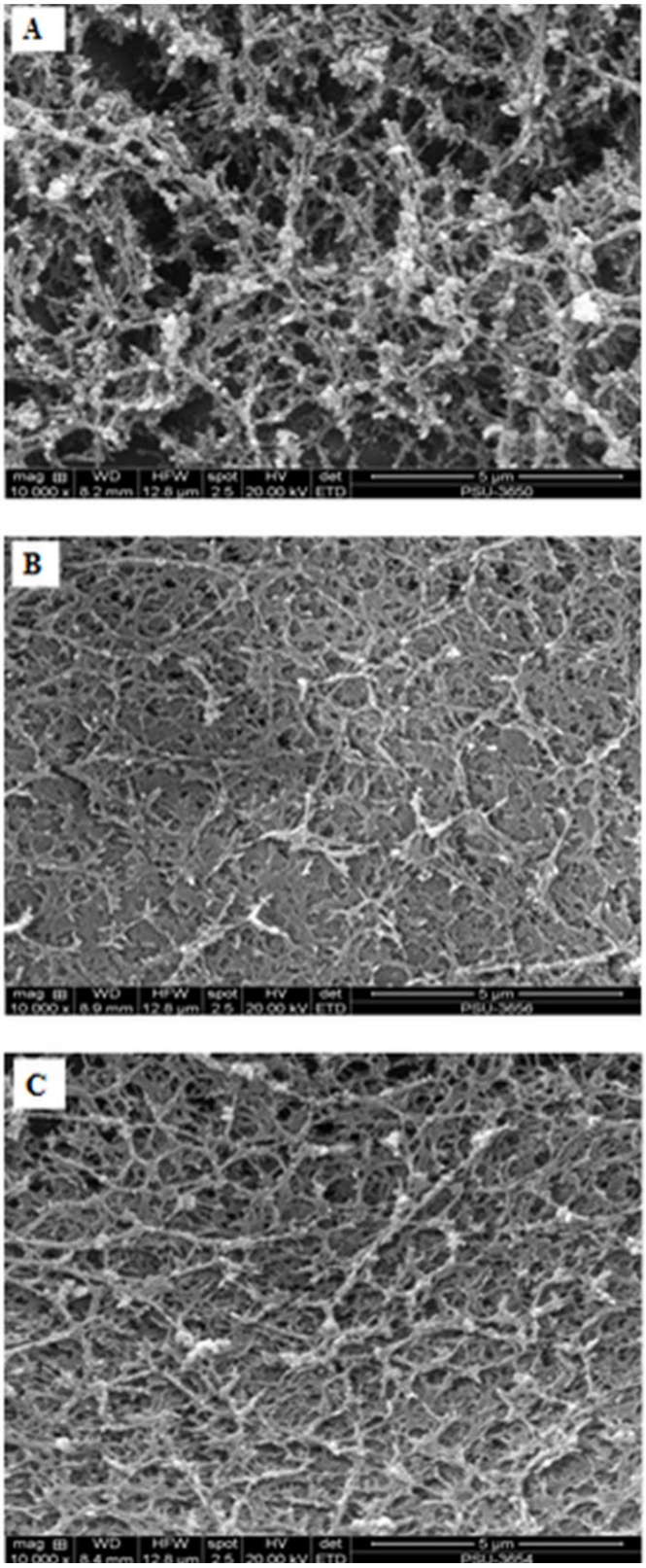

Figure 28. Scanning electron microscopic image of Indian mackerel surimi without (A) and with 2% of EWP (B) or 2% SOSP (C). Magnification: 10,000X.

added with SOSPI and EWP ($p>0.05$). The SOSPI added gel showed higher odor likeness score than that containing EWP ($p<0.05$). This was more likely due to strong typical smell of EWP (Pigott and Tucker, 1990). No detrimental effect on the acceptability of surimi gel from common carp was observed, when samples were incorporated with 10% soy protein isolate and 3% potato starch and egg white (Jafarpour *et al.,* 2012). Therefore, the addition of 2% SOSPI was able to enhance the sensory properties of surimi gel.

Table 17. Likeness score of gels from Indian mackerel surimi without and with 2% SOSPI or 2% EWP.

Samples	Appearance	Color	Odor	Texture	Taste	Overall
Control	7.67 ± 0.90^{a}	8.00 ± 0.76^{a}	6.87 ± 0.92^{ab}	6.47 ± 0.99^{a}	6.87 ± 0.92^{a}	6.67 ± 0.49^{a}
SOSPI	7.80 ± 0.86^{a}	8.00 ± 0.76^{a}	7.40 ± 0.83^{b}	7.13 ± 0.74^{b}	7.00 ± 0.70^{a}	7.40 ± 0.63^{b}
EWP	7.87 ± 0.64^{a}	8.07 ± 0.80^{a}	6.60 ± 0.99^{a}	7.47 ± 0.92^{b}	7.13 ± 0.92^{a}	7.53 ± 0.74^{b}

Values are mean ± SD (n=3). Different lowercase superscripts in the same column indicate the significant differences ($p<0.05$). Control: without SOSPI and EWP.

7.6 Conclusion

The addition of 2% SOSPI yielded the gel with increased breaking force along with improved water holding capacity and textural properties. Nevertheless, whiteness was slightly reduced with addition of SOSPI. SOSPI increased G´ and provided the compact three-dimensional structure. Although EWP had higher potential to improve the gel characteristics of Indian mackerel surimi, SOSPI could use as an alternative food grade additive for surimi industry without the negative effect on sensory property. Hence, the SOSPI could be used as the novel protease inhibitor to enhance the gel strength of surimi gel. Further study on the gelling property of SOPSI and its impact on surimi gel strengthening should be conducted.

7.7 References

Anastasi, A., Brown, M. A., Kembhavi, A. A., Nicklin, M. J. H., Sayers, C. A., Sunter, D. C. and Barrett, A. J. 1983. Cystatin, a protein inhibitor of cysteine proteinases: improved purification from egg white, characterization, and detection in chicken serum. Biochemical Journal. 211: 129-138.

Arfat, Y. A. and Benjakul, S. 2012. Gelling characteristics of surimi from yellow stripe trevally (*Selaroides leptolepis*). International Aquatic Research. 4: 1-13.

Benjakul, S. and Visessanguan, W. 2003. Transglutaminase-mediate setting in bigeye snapper surimi. Food Research International. 36: 253-266.

Benjakul, S., Visessanguan, W. and Chantarasuwan, C. 2004a. Effect of porcine plasma protein and setting on gel properties of surimi produced from fish caught in Thailand. LWT-Food Science and Technology. 37: 177–185.

Benjakul, S., Visessanguan, W., Tueksuban, J. and Tanaka, M. 2004b. Effect of some protein additives on proteolysis and gel-forming ability of lizardfish (*Saurida tumbil*). Food Hydrocolloids 18: 395-401.

Benjakul, S., Visessanguan, W. and Tueksuban, J. 2003. Heat-activated proteolysis in lizardfish (*Saurida tumbil*) muscle. Food Research International. 36: 1021-1028.

Buamard, N. and Benjakul, S. 2015. Improvement of gel properties of sardine (*Sardinella albella*) surimi using coconut husk extracts. Food Hydrocolloids 57: 146-155.

Egelandsdal, B., Fretheim, K. and Samejima, K. 1986. Dynamic rheological measurements on heat-induced myosin gels: effect of ionic strength, protein concentration and addition of adenosine triphosphate or pyrophosphate. Journal of the Science of Food and Agriculture. 37: 915–926.

FAO (2014). The state of world fisheries and aquaculture. Food and agriculture organization. United Nations, Rome.

Jafarpour, A., Hajiduon, H. A. and Rez Aie, M. 2012. A comparative study on effect of egg white, soy protein isolate and potato starch on functional properties of common carp (*Cyprinus carpio*) surimi gel. Food Processing Technology. 3: 1-6.

Kudre, T. and Benjakul, S. 2013. Effect of legume seed protein isolates on autolysis and gel properties of surimi from sardine (*Sardinella albella*). International Journal of Chemical, Environment and Biological Sciences. 1: 91-101.

Kudre, T., Benjakul, S. and Kishimura, H. 2013. Effects of protein isolates from black bean and mungbean on proteolysis and gel properties of surimi from sardine (*Sardinella albella*). LWT-Food Science and Technology. 50: 511-518.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature. 227: 680-685.

Lowry, O. H., Rosebrough, N. J., Fan, A. L. and Randall, R. J. 1951. Protein measurement with Folin phenol reagent. The Journal of Biological Chemistry. 193: 256–275.

Lu, G. H. and Chen, T. C. 1999. Application of egg white and plasma powders as muscle food binding agents. Journal of Food Engineering. 42: 147–51.

Meilgaard, M., Civille, G. V. and Carr, B. T. 1999. Sensory evaluation techniques (3rd ed.). Florida: CRC Press.

Morrissey, M. T. and Okada, T. 2007. Marine enzymes from seafood by-product. *In* Maximizing the value of marine by-product. (Shahid, F., ed). p. 374-389. Woodhead Publishing Ltd., CRC Press. Cambridge,

Morrissey, M. T., Wu, J. W., Lin, D. and An, H. 1993. Protease inhibitor effects on torsion measurements and autolysis of Pacific whiting surimi. Journal of Food Sceince. 58: 1050-1054.

Nagase, H. and Harris E. D. Jr. 1983. Ovostatin: a novel proteinase inhibitor from chicken egg white. II. Mechanism of inhibition studied with collagenase and thermolysin. The Journal of Biological Chemistry. 258: 7490-8.

Park, J. W. 2000. Ingredient technology and formulation development. *In* Surimi and Surimi Seafood. (Park, J.W. ed.) p. 343-392. Marcel Dekker. New York.

Pigott, G. M. and Tucker, B. W. 1990. Seafood effects of technology on nutrition. Marcel Dekker, Inc. New York.

Rawdkuen, S. and Benjakul, S. 2008. Whey protein concentrate: Autolysis inhibition and effects on the gel properties of surimi prepared from tropical fish. Food Chemistry. 106: 1077–1084.

Rawdkuen, S., Benjakul, S., Visessanguan, W. and Lanier, T. C. 2008. Rheological and textural properties of Pacific whiting surimi gels as influenced by chicken plasma. International Journal of Food Properties. 11: 820-832.

Singh, A. and Benjakul, S. 2016. Serine protease inhibitors from squid ovary: extraction and its effect on proteolysis and gel properties of surimi. Journal of Food Science and Technology 54: 267–275.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: A biometrical approach (2nd ed.). McGraw-Hill. New York.

Vate, N. K. and Benjakul, S. 2016. Effect of the mixtures of squid ink tyrosinase and tannic acid on properties of sardine surimi gel. Journal of Food Science and Technology. 53: 411–420.

Weerasinghe, V. C., Morrissey, M. T. and An, H. 1996. Characterization of active components in food-grade proteinase inhibitors for surimi manufacture. Journal of Agricultural and Food Chemistry. 44: 2584–90.

CHAPTER 8

ULTRASOUND-ASSISTED EXTRACTION OF CHITOSAN FROM SQUID PEN: MOLECULAR CHARACTERIZATION AND FAT BINDING CAPACITY

8.1 Abstract

Chitosan from squid (*Loligo formosana*) pens was prepared and characterized. Firstly, ultrasonication condition was optimized for deproteinization of squid pen using central composite design (CCD) of response surface methodology (RSM). Squid pen ultrasonicated at amplitude 69% for 41.46 min at the solid/solvent ratio of 1: 18 yielded 34% (w/w) chitin with the lowest remaining protein (3.31 mg/100 mg sample). Therefore, ultrasonication effectively reduced the extraction time for chitin production from squid pen as compared to traditional method. When the resultant chitin was subjected to deacetylation at different temperatures and times, yield and degree of deacetylation (DDA) of chitosan was in the range of 65-50% (w/w) and 78-90%. Intrinsic viscosity and molecular weight (MW) of chitosan was in the range of 3.2-6.52 dL/g and 1.2 x105-3.2 x105 Dalton, respectively. All the chitosans with different DDA were able to bind oil droplets under the mimicked pH condition of gastrointestinal tract. Chitosan produced by deacetylation at 130 °C for 2 h (CH130-2) showed the optimum yield (54%) and had medium MW (1.5 x105 Dalton). DDA of CH130-2 determined using 1H-NMR was 89%, which was similar to that (87%) obtained from FTIR. XRD results showed destruction of chitin structure and decreased crystallinity index from 55 to 27% after deacetylation. CH130-2 stabilized the emulsion under the simulated gastrointestinal conditions. Therefore, it could be used as dietary fiber to control the adsorption of fat/oil in the human digestive tract.

8.2 Introduction

Chitin is a natural polysaccharide mainly found in invertebrates, vertebrates as well as some species of plants (Croisier and Jérôme, 2013). Chitin

occurs as α and β-forms, depending on the sources. Moreover, γ-chitin, showing similar morphology to α and β-forms, has also been reported in fungi and yeast (Rudall, 1969; Blackwell, 1973; Rudall and Kenchington, 1973; Atkins, 1985). Crab, shrimp and krill shells mainly consist of α-chitin, whereas β-chitin is mainly present in squid pens (Elieh-Ali-Komi and Hamblin, 2016). α-chitin has anti-parallel structure with hydrogen bonds, which limits its reactivity with other solvents. On the other hand, due to parallel structure and absence of inter-hydrogen bonding in β-chitin, it has high reactivity and affinity toward solvents (Jung and Zhao, 2011). In general, chitin extraction involves demineralization and deproteinization, which are time consuming. Industrial processing condition vary widely in terms of acid or base type and concentration, time and temperature used. Quality of chitin can be improved by optimization of processing parameters. Additionally, the appropriate and potential technology can be implemented. Recently, ultrasonication has been widely used for the extraction of numerous polysaccharides including pectin, hemicellulose and starch from various sources (Kjartansson *et al.*, 2006). Ultrasonication has been documented to reduce the processing cost and time. Chitin has been extracted from Atlantic shrimp and freshwater prawn using ultrasonication (Kjartansson *et al.*, 2006; Kjartansson *et al.*, 2006). However, no study has been reported on the extraction of chitin with the help of ultrasonication from squid pen, containing different types of chitin from crustaceans.

Chitosan is deacetylated product of chitin, in which acetyl groups are removed with help of alkali treatment. Generally, chitosan is soluble in aqueous acidic medium when degree of deacetylation (DDA) reached up to 50% (Rinaudo, 2006). Chitosan is biodegradable, biocompatible and less toxic. Therefore, it has been widely utilized in various applications, especially in medical and pharmaceutical areas (Panith *et al.*, 2016). It has been used in different forms such as solution, gel, film and fiber (Rinaudo, 2006). Chitosan is a versatile substance, it has wide applications in various field such as, cancer diagnosis, drug delivery, antibacterial, tissue engineering, antiaging. It is also known for its ability to enhance the response of antibodies (Elieh-Ali-Komi and Hamblin, 2016). Chitosan has resistance towards digestive enzymes which allow it to use as a dietary fibre to improve human health.

Sugano *et al.* (1988) reported that chitosan has the potential to bind cholesterol and fat in the digestive tract. Its fat binding property can help protect fat/oil to be digested by lipase. Chitosan and its carboxymethylated derivative showed potential to bind to bile acid, implying they had cholesterol lowering ability (Zhao *et al.*, 2011; Huang *et al.*, 2012). As a consequence, an intake of chitosan is a means to lower health-related problems like obesity, high cholesterol, etc.

8.3 Objective

To optimize the ultrasonic condition for the extraction of chitin from squid pen using RSM and to study the impact of deacetylation conditions on chitosan production. Molecular properties and *in vitro* fat binding capacity of chitosan were also examined.

8.4 Materials and Methods

8.4.1 Chemicals

All chemicals were of analytical grade. Fluorescein isothiocyanate (FITC) was procured from Sigma Chemical Co. (St. Louis, MO, USA).

8.4.2 Squid pen procurement and preparation

Squid pens were gifted from the Sea Wealth Frozen Food, Songkhla, Thailand. Squid pens with the length of 14-16 cm were cleaned with tap water to remove all water-soluble material, followed by the hot air oven drying at 60 °C for 24 h. Dried squid pens were reduced to smaller size manually, followed by blending using a Panasonic blender (Model MX-898N, Malaysia). Blended squid pens were seived to obtain the size less than 1 cm. Squid pens had low ash content (0.02%, w/w) as analyzed by AOAC method (AOAC, 2000). Prepared squid pens were kept in polythene bag, sealed and stored under dried conditions.

8.4.3 Chitin extraction

Due to the low ash content, demineralization of squid pen was omitted (Cortizo *et al.*, 2008; Cuong *et al.*, 2016). Deproteinization was carried out using 1.0

M NaOH with the aid of ultrasonic treatment designed with the help of central composite design (CCD) of response surface methodology (RSM) (Table 18).

8.4.3.1 Optimization of ultrasonication conditions for deproteinization

Ultrasonication conditions were optimized with the help of RSM. Three independent variables, ultrasonication amplitude (X_1, %), sonication time (X_2, min) and solid: solvent (X_3, g/L) ratio at five code levels were implemented with CCD. Two dependent variables included yield and remaining protein content. The second-order polynomial model was implemented in RSM as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \beta_{ij} X_i X_j$$

where Y is the dependent variables (yield and protein content); X_i and X_j are the coded forms of the independent variables; β_0, β_i, β_{ii} and β_{ij} is the constant, linear coefficient, quadratic coefficient and interaction coefficient of the model, respectively.

The optimum conditions were determined using the Design-Expert Statistical package version 7 (Statease, Inc Minneapolis, MN, USA). The contour plots were used to show the relationship between dependent and independent variables.

8.4.3.2 Sample preparation

Ground squid pen (10 g) was mixed with one molar NaOH to obtain various solid:solvent ratios as shown in Table 18. The mixtures were subjected to ultrasonication using an ultrasonic equipment (Sonics, Model VC750, Sonic & Materials, Inc., Newtown, CT, USA) with high intensity power of 750 W and operating frequency of 20 kHz ± 50 Hz under different conditions (Table 18). The mixture temperature was maintained at 30-40 ºC throughout operation using the iced

Table 18. Central composite design for RSM and experimental data for protein content and yield of chitin extracted under different ultrasonication treatment.

Standard order	Coded levels			Actual levels			Yield (%)	Protein content (mg/100 mg sample)
	Amplitude (%)	Time (min)	Solid: Solvent ratio	Amplitude (%)	Time (min)	Solid: Solvent ratio		
1	-1	-1	-1	33.00	19.00	18.00	50.27	15
2	1	-1	-1	69.00	19.00	18.00	37.43	4.7
3	-1	1	-1	33.00	50.00	18.00	34.27	3.1
4	1	1	-1	69.00	50.00	18.00	35.42	4.0
5	-1	-1	1	33.00	19.00	42.00	50.6	16
6	1	-1	1	69.00	19.00	42.00	40.91	11
7	-1	1	1	33.00	50.00	42.00	37.79	5.4
8	1	1	1	69.00	50.00	42.00	33.83	2.6
9	-1.682	1	0	20.73	34.50	30.00	44.68	4.0
10	1.682	0	0	81.27	34.50	30.00	35.08	3.8
11	0	-1.682	0	51.00	8.43	30.00	45.00	18
12	0	1.682	0	51.00	60.57	30.00	36.23	6.1
13	0	0	-1.682	51.00	34.50	9.82	37.52	6.9
14	0	0	1.682	51.00	34.50	50.18	35.51	6.0
15	0	0	0	51.00	34.50	30.00	32.34	5.0
16	0	0	0	51.00	34.50	30.00	35.95	7.0
17	0	0	0	51.00	34.50	30.00	33.63	7.8
18	0	0	0	51.00	34.50	30.00	36.34	7.0
19	0	0	0	51.00	34.50	30.00	34.95	8.0
20	0	0	0	51.00	34.50	30.00	31.93	7.7

bath. All resultant squid pen chitin samples were brought to neutral pH by washing with water, followed by vacuum filtration.

Squid pen chitin was also extracted using traditional process as described by Chandumpai *et al.* (2004). In brief, deproteinization was carried out at 50 °C with constant stirring for 5 h using 1.0 M NaOH solution at a solid:solvent ratio of 1:20 (w/v). Chitin was washed as described above. All chitin samples were dried using a hot-air oven at 60 ºC for 12 h and further subjected to analyses.

8.4.3.3 Analyses

8.4.3.3.1 Protein content

One gram of sample was dissolved in 1.0 M NaOH to obtain the final concentration of 1% (w/v) followed by stirring over night at room temperature (25 °C). Solubilized proteins were filtered through a Whatman paper no. 4. Biuret method was used to determine protein content of filtrates (Gornall *et al.*, 1949). Protein content indicated amount of protein remained in the samples.

8.4.3.3.2 Yield

The yield of chitin was estimated as the percentage of initial dry squid pen as follows:

$$\text{Yield (\%)} = \frac{Weight\ of\ chitin\ (g)}{Weight\ of\ squid\ pen\ (g)} \times 100$$

The ultrasonic condition rendering the chitin with the highest yield and the lowest protein remained was selected for chitosan preparation.

8.4.4 Chitosan production

Chitosan was prepared from chitin using different deacetylation temperatures (110 and 130 °C) and times (2, 4 and 8 h). Chitin was slowly added into three-necked round bottom flask containing 50% (w/v) NaOH and the final chitin/solution ratio was 1:50 (w/v). After deacetylation processes designated, chitosan was rinsed with distilled water to obtain neutral pH of washed water.

Chitosan was filtered by vacuum-filtration, followed by drying using a hot-air oven at 60 °C for 12 h. Chitosan samples were placed in polyethylene bag and kept into a desiccator. Yield was determined based on weight of chitin as starting material. Chitosan produced at deacetylation temperature of 110 °C for 2, 4 and 8 h were defined as CH110-2, CH110-4 and CH110-8, respectively. Those produced at 130 °C for 2, 4 and 8 h were denoted as CH130-2, CH130-4 and CH130-8, respectively.

8.4.5 Characterization of chitosan

8.4.5.1 Determination of degree of deacetylation (DDA)

DDA was determined using an attenuated total reflection (ATR)-Fourier transform infrared (FTIR) spectroscopy with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) as described by Ali *et al.* (2018). DDA was calculated using following equation (He *et al.*, 2016):

$$\text{DDA (\%)} = (1\text{-}A_{1320}/A_{1420} \times 0.3192) \times 100$$

Selected chitosan sample was analyzed by ^{1}H-NMR as described by Lavertu *et al.* (2003). DDA was obtained from proton signal H2, H3, H4, H5, H6, H6′ (H2-6) of both acetylated and deacetylated monomers and the peak of acetyl group (H-Ac) as per the method proposed by Hirai *et al.* (1991).

$$\text{DDA (\%)} = \left\{1 - (1/3\ I_{CH_3}/1/6\ \ I_{H2-H6})\right\} \times 100$$

where I_{CH3} and $I_{H2\text{-}H6}$ are the integral intensities of CH_3 of N-acetyl and H2, H3, H4, H5, H6, H6′ protons.

8.4.5.2 Measurement of intrinsic viscosity

Intrinsic viscosity was determined using an Ubbelohde capillary type viscometer. Chitosan was dissolved in the solution containing 0.2 M acetic acid and 0.15 M ammonium acetate (solvent) to get the concentrations of 0.05, 0.1, 0.2 and 0.3 (g/dL). Chitosan solutions were stirred for 24 h for complete solubilization at room temperature. The efflux time was calculated for solvent and chitosan solutions.

Temperature of samples during the viscosity measurement was sustained at 25 ± 0.1°C using a cooling water bath. All measurements were done within 24 h.

The average efflux times of solvent and chitosan solutions were used to calculate relative and inherent viscosity using following equations:

$$\text{Relative viscosity } (\eta_{\text{rel}}) = \frac{\text{efflux time of sample}}{\text{efflux time of solvent}}$$

$$\text{Specific viscosity } (\eta_{\text{sp}}) = \frac{\text{efflux time of sample} - \text{efflux time of solvent}}{\text{efflux time of solvent}}$$

$$\text{Reduced viscosity } (\eta_{red}) = \frac{\text{Specifc viscosity}}{\text{concentration of sample solution}}$$

$$\text{Inherent viscosity } (\eta_{\text{inh}}) = \frac{\ln (\text{relative viscosity})}{\text{concentration of sample solution}}$$

The reduced viscosity or inherent viscosity were plotted against chitosan concentration (0.05-0.3 g/dL). Intrinsic viscosity was calculated from linear equation of either reduced viscosity or inherent viscosity at zero concentration. Similar value obtained from both reduced viscosity and inherent viscosity indicated the precision of measurements.

8.4.5.3 Viscosity-average molecular weight ($\bar{M}$v) determination

Viscosity-average molecular weight ($\bar{M}$v) was calculated using Mark-Houwink-Sakurada equation (Yacob *et al.*, 2013) as following equation:

$$[\eta] = K\bar{M}_v^{\propto}$$

where [η] = intrinsic viscosity, K= 9.66 x10^{-5} and α = 0.742 determined in the solvent at 25°C (Yacob *et al.*, 2013).

8.4.5.4 X-ray diffraction (XRD) analysis

XRD patterns of chitosan was obtained using an X-ray diffractometer (X'Pert MPD, Philips, Netherlands) with a diffracted bean monochromator Cu (λ = 0.154 nm) as per the method described by Jung and Zhao (2014).

The crystallinity index (Icr) was calculated by the following equation:

$$I_{cr} = [(I_{110} - I_{am})/I_{110}] \times 100$$

where I_{am} is the intensity of amorphous diffraction at 16°, and I_{110} is the maximum intensity at 20°.

8.4.5.5 Fat-binding ability of chitosan

8.4.5.5.1 Fluorescein isothiocyanate (FITC) labeling of chitosan

Chitosan was labelled with FITC dye as per the method of Panith *et al.* (2016) with a minor variation. Briefly, 1.0 mg FITC was solubilize in 10 mL of methanol followed by mixing with 10 mL of 1.0 g/dL chitosan in 0.1 M acetic acid solution. Mixture was stirred at 350 rpm in dark for 3 h at room temperature. The pH of mixture was raised to 12 with 0.5 M NaOH to precipitate FITC-labelled chitosan. Unbound FITC was removed using distilled water, followed by centrifugation (8000 xg for 20 min) until no fluorescence was detected in the supernatant.

8.4.5.5.2 Fat binding ability of FITC-labelled chitosan under the mimicked pH condition of gastrointestinal tract

For fat-binding determination, the *in vitro* digestive model focused on the ability of chitosan to bind and entrap fat molecules is based on the manipulation of chitosan's solubility under acidic condition (i.e. stomach pH) and chitosan's dissolubility under basic condition (i.e. duodenum pH) was performed by the method of Panith *et al.* (2016). Briefly, 0.1 g of FITC-labelled chitosan was added in 20 mL of 0.1 M HCl and incubated for 30 min at 37 °C with continuous shaking at 200 rpm.

FITC-labelled chitosan solution was subsequently mixed with soybean oil at a ratio of 1:40 (w/v) and stirred at 200 rpm for 30 min followed by adjusting pH to 6.8 with 1.5% (w/v) of $NaHCO_3$ to mimic the duodenum conditions. The resultant emulsion stabilized by chitosan was analyzed via confocal laser scanning microscopy (CLSM) at λ_{excit} = 492 nm and λ_{emis} = 518 nm.

CH130-2, showing the ability to bind oil under the different pH conditions of gastrointestinal tract, was selected for further study.

8.4.5.5.3 *In vitro* gastrointestinal digestion

The simulated *in vitro* gastrointestinal digestion system was prepared following the method of (Sae-Leaw *et al.*, 2016) with some minor modifications. Briefly, FITC-labeled CH130-2 stabilized emulsion was prepared as previously described and defined as the 'control emulsion'. To mimick the gastric phase of human digestion, freshly prepared pepsin (0.04 g/mL in 0.1 N HCl) was added into control emulsion. The pH of the mixture was adjusted to 2.0 using 0.1 N HCl followed by incubation at 37 °C for 1 h in an orbital shaking water bath (Grant OLS200, Grant Instruments, Cambridge, UK) at 95 rpm. For duodenal digestion, the pH was raised to 6.8 using 0.9 M sodium bicarbonate, followed by the addition of 300 µL of freshly prepared bile salts (0.04 g/mL glycodeoxycholate, 0.04 g/mL taurocholate and 0.025 g/mL taurodeoxycholate) and 300 µL of porcine pancreatin including lipase, trypsin, amylase, ribonuclease and protease (0.08 g/mL). Subsequently, the mixture was incubated for 2 h at 37 °C in an orbital shaking water bath. Control emulsion and emulsions obtained after gastric and duodenal phases were immediately subjected to CLSM analysis.

8.4.6 Statistical analysis

Optimization of deproteinization was conducted using CCD as mentioned above. All experiments were repeated three times. Data were subjected to analysis of variance. Comparison of means was carried out by the Duncan's multiple range tests (Steel and Torrie, 1986). Analysis was performed using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL, USA).

8.5 Results and discussion

8.5.1 Optimization of ultrasonication conditions for deproteinization of squid pen using RSM

Three independent variables including amplitude (X_1) ultrasonication time (X_2) and solid: solvent ratio (X_3) were used in CCD (Table 18). Twenty treatments were obtained from CCD to determine yield and remaining protein content of chitin samples. Valid model was obtained as indicated by significant F-value and p-value, based on ANOVA results. Additionally, p-values for yield and remaining protein content were 0.26 and 0.06, respectively, showing non-significant lack of fit. The results indicated the validity of the fitted model. R^2 for both yield and remaining protein content was 0.91 (Table 19). For a good fit model, the R^2 values have to be higher than 0.75 (Joglekar *et al.*, 1987). This result revealed that the data fit the statistical model very well.

8.5.1.1 Impact of independent variables on extracted yield of chitin

Amplitude, sonication time and solid:solvent ratio had significant effect on yield of chitin (Figure 29A-C). Yields of chitins were in the range of 31-50%. Among all the variables tested, yield was highly influenced by linear term of amplitude (X_1) and sonication time (X_2) ($p<0.0001$) and interactions between both variables (X_1X_2) were significant ($p<0.05$). Nevertheless, linear term of solid:solvent ratio (X_3), interactions between amplitude and solid:solvent ratio (X_1X_3) as well as interaction between sonication time and solid:solvent ratio (X_2X_3) had non-significant effect on yield ($p>0.05$). Quadratic term of amplitude (X_1^2) and sonication time (X_2^2) had significant effect on yield ($p<0.001$). However, quadratic term of solid: solvent ratio (X_3^2) did not exhibit significant effect on the yield ($p>0.05$). Effect of various independent variables on the yield of chitin as tested by regression is shown below:

$$\textbf{Yield} = 34.17 - 3.04\,X_1 - 3.86\,X_2 + 0.17\,X_3 + 2.47\,X_1X_2 - 0.24\,X_1X_3 - 0.23\,X_2X_3 + 2.17\,X_1^2 + 2.43\,X_2 + 0.98\,X_3^2$$

Table 19. Regression coefficients of the predicted second-order polynomial models for yield and remaining protein content squid pen chitin subjected to ultrasonication.

Regression coefficients (β)	Yield	Protein content
Intercept		
β_0	34.17	0.71
Linear		
β_1	-3.04	-0.25
β_2	-3.86	-0.39
β_3	2.47	0.054
Cross product		
β_{12}	-0.24	0.17
β_{13}	-0.23	0.31
β_{23}	2.17	-0.089
Quadratic		
β_1	2.17	0.036
β_2	2.43	0.15
β_2	0.98	-0.05
R^2	0.91	0.91
Lack of Fit	0.06	0.26
p-value	< 0.0001	< 0.0001

Extraction of chitin is generally time consuming and requires high temperature. This is associated with the increased production cost of chitin. Therefore, ultrasonication was implemented for chitin extraction in order to reduce extraction time and enhance efficacy. Cavitational effect of ultrasound results in breaking covalent bonds in polymeric chains, depolymerizing macromolecules and dispersion of aggregates (Kjartansson *et al.*, 2006). This effect might result in the increase in solubilization of protein associated with chitin in squid pen. Relationship between different independent variable is presented in the form of contour plot (Figure 29A-C). With increasing amplitude and sonication time, yield of chitin was decreased (Figure 29A). Figure 29B and 1C demonstrated that solid:solvent ratio had no

significant effect on the yield, while amplitude and sonication time had the profound effect. Ultrasonication at high amplitude and prolonged sonication time more likely caused dissociation and breakdown of chitin from the pen complex along with the proteins and other compounds. The degradation of chitin and other compounds plausibly led to the formation of small molecules, which could be lost during washing. This brought about the lower yield. Chitin from squid pen of *Loligo lessoniana* and *L. formosana,* was extracted at 50 °C using 1.0 M NaOH solution for 5 h. The yield in the range of 35–38% based on initial material was obtained (Chandumpai *et al.*, 2004). In the current study, chitin yield of 34% was achieved within 42 min, in which deproteinization was maximized. Therefore, ultrasonication could reduce processing time as compared to the traditional methods.

8.5.1.2 Impact of independent variables on remaining protein content

Remaining protein contents of chitins as affected by amplitude, sonication time and solid:solvent ratio are shown in Figure 29D-F. Remaining protein content was varied in the range of 2.6-18 mg/ 100 mg of sample (Table 18). Protein content was vastly affected by amplitude (X_1) and sonication time (X_2) ($p<0.0001$), whereas, solid:solvent ratio (X_3) did not show significant impact ($p>0.05$). Interaction between amplitude and sonication time (X_1X_2) and quadratic term of sonication time (X_3^2) had significant effect on remaining protein content ($p<0.05$), However, other interactions (X_1X_3; X_2X_3) and quadric terms (X_1^2; X_3^2) had no significant influence ($p>0.05$). Impact of independent variables on the remaining protein content as tested by regression is shown below:

Remaining protein content= $0.71 - 0.25\,X_1 - 0.39\,X_2 + 0.054\,X_3 + 0.17\,X_1X_2 + 0.31\,X_1\,X_3 - 0.089X_2X_3 + 0.036\,X_1^2 + 0.15\,X_2^2 - 0.050\,X_3^2$

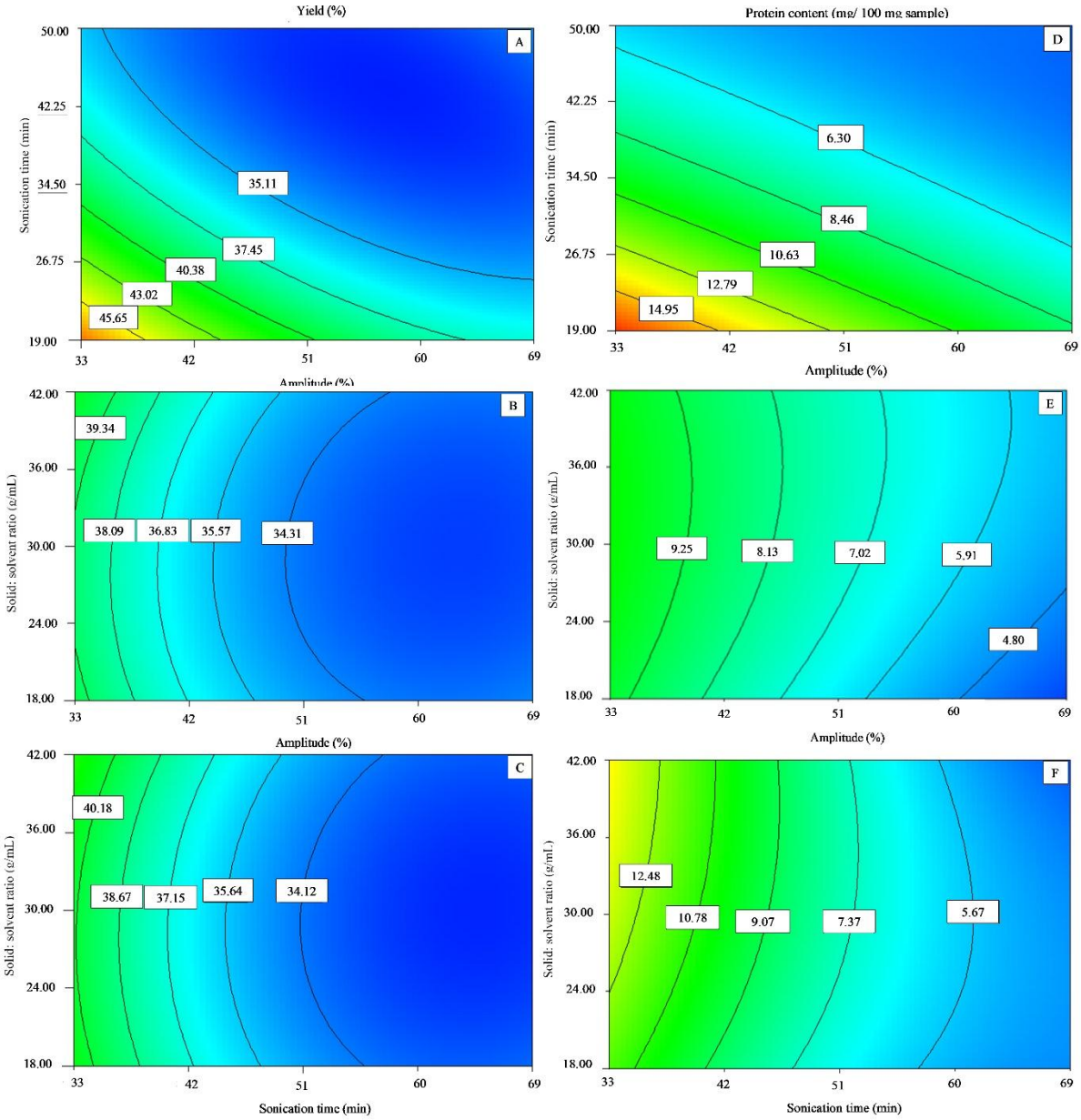

Figure 29. Contour plots of the yield (A-C) and remaining protein content (D-F) of chitin from squid pen using ultrasonic assisted process as affected by independent variable. Amplitude and sonication time (A, D), amplitude and solid: solvent ratio (B, C) and sonication time and solid: solvent ratio (C, F).

As amplitude and sonication time increased, remaining protein contents were decreased (Figure 29D). However, solid:solvent ratio did not have significant effect on the remaining protein content in comparison with other independent variables (Figure 29E-F). This was more likely due to the cavitational effect which induced breakage of hydrogen and other inter or intramolecular bonds. This caused the matrix of pen to be loosened, thus facilitating the leach out of proteins from the pens with increasing amplitude and sonication time. Kjartansson *et al.* (2006) reported that ultrasonication assisted chitin extraction process could lowered protein content of shrimp shell chitin. Protein content 44.01% was reduced to 12.55, 10.59, and 7.45% when prawn shell was treated with ultrasonic processor for 0, 1, and 4 h, respectively, in the presence of NaOH. Ultrasonication potentially enhanced protein extraction from shrimp shells. Nevertheless, it did not affect mineral extraction during the demineralization step (Kjartansson *et al.*, 2006).

Yield and remaining protein content of chitin extracted using traditional method (extraction time of 5 h) was 38% and 5.1 mg/100 mg sample, respectively. Yield of chitin was slightly higher than that extracted using ultrasonication. During ultrasonication, the degradation of chitin might be enhanced. Those degraded molecules could be washed away during washing process. Additionally, higher remaining protein content in chitin prepared by traditional method was also associated with the higher yield of chitin produced by traditional method. Therefore, ultrasonication could effectively reduce protein content of squid pen and decreased extraction time as compared to the traditional method

8.5.1.3 Model validation and optimization of ultrasonication

Based on CCD and contour plots, the optimized extraction conditions were: amplitude of 69% with sonication time of 41.46 min at the solid:solvent ratio of 1:18. Statistical model and regression equation of optimal condition were validated. The predicted values for chitin yield and remaining protein content were 34% and 3.31 mg/100mg sample, respectively. Similar observed values (yield: 34.65% and remaining protein content: 3.49 mg/ 100 mg sample) with small percentage of error, 1.97 and 6%, were obtained, respectively. The result reconfirmed the validity and

acceptability of the statistical model for the optimization of extraction condition of chitin (deproteinization) using ultrasonication.

8.5.2 Characterization of chitosan from squid pen with different deacetylation conditions

8.5.2.1 Yield of chitosan

Yields of chitosan from squid pen prepared by deacetylation using strong alkaline solution at different temperatures (110 and 130 °C) and times (2, 4 and 8 h) are shown in Table 20. When the deacetylation time of 2 h was used, the maximum yields of 65 and 54% were obtained for temperatures of 110 and 130 °C, respectively. However, yield was decreased when the extraction time was increased, regardless of temperatures ($p<0.05$). This was more likely due to depolymerization of chitosan molecules. Fragmentated molecules could be lost during thorough washing. It was observed that yield of chitosan was decreased from 65 to 51% with deacetylation temperature of 110 °C when deacetylation time was from 2 to 8 h. At 130 °C, yield was decreased from 54 to 50% as deacetylation time was increased from 2 to 8 h. In general, under alkaline and high temperature, hydrolysis of acetamido groups of acetylglucosamine in chitin occurred. This results in the formation of chitosan, a glucosamine polymer (Elieh-Ali-Komi and Hamblin, 2016). Kurita *et al.* (1977) produced chitosan from shells of *Penaeus japonicus* (DDA 48-55%) using concentrated NaOH solution (30g NaOH/45g H_2O) at 25 °C for 3 h. On the other hand, Aiba (1991) stated that deacetylation condition might give an uneven distribution of D-glucosamine and *N*-acetyl-D-glucosamine residues having some parts of acetyl group distribution along polymeric chains. This could affect yield and DDA of chitosan, thus influencing functional characteristics. The result suggested that deacetylation temperature and processing condition were crucial factors determining the yield of chitosan (Chang *et al.*, 1997). The higher temperature most likely induced depolymerization, which caused the loss of small MW molecules during washing.

Table 20. Yield, degree of deacetylation (DDA), intrinsic viscosity ([η]) and viscosity-average molecular weight ($M\bar{v}$) of chitosan from squid pen chitin deacetylated under different temperatures and times.

Temperature (°C)	Extraction time (h)	Yield (%)	DDA (%)	[η] (dL/g)	$\bar{M}$v (Dalton)
110	2	64.99±1.83^{aA}	78.21±1.28^{eC}	6.52±0.02^{aA}	3.2 x10^5
	4	54.12±1.67^{bB}	84.65±0.42^{dB}	4.19±0.04^{bB}	1.7x10^5
	8	50.54±0.80^{cC}	86.84±0.4^{cA}	3.47±0.02^{dC}	1.3 x10^5
130	2	53.51±1.22^{dA}	86.55±0.73^{cC}	3.79±0.02^{cA}	1.5 x10^5
	4	51.91±1.21^{bcB}	87.74±0.49^{bA}	3.39±0.08^{eB}	1.3 x10^5
	8	50.07±1.02^{cB}	89.72±0.37^{aB}	3.24±0.02^{fC}	1.2 x10^5

Values are mean ± SD (n = 3). Different uppercase superscripts within the same temperature in the same column indicated significant difference ($p < 0.05$). Different lowercase superscripts in the same column indicate significant difference ($p < 0.05$).

8.5.2.2 Characteristics of chitosan

8.5.2.2.1 FTIR analysis and DDA determination

FTIR spectra of chitosans prepared under different deacetylation temperatures and times are shown in Figure 30. FTIR spectra of all samples showed absorption pattern corresponding to the characteristic of chitosan (Osman and Arof, 2003). A prominent band in the region 3600 – 3250 cm^{-1} represents O-H and N-H stretching, as well as the inter- and intra-molecular hydrogen bonding. The bands around 2921 and 2872 cm^{-1} could be attributed to C-H asymmetric and symmetric stretching, respectively. The band around 1640 cm^{-1} (C=O stretching of amide-I) represent the residual *N*-acetyl groups was confirmed by the bands at around and 1320 cm^{-1} (C-N stretching of amide III). The band at around 1590 cm^{-1} representing the N-H bending of NH_2 in the primary amine was also observed. It was noted that the band at around 1540 cm^{-1} corresponding to N-H bending of amide (amide-II), another

characteristic band of typical *N*-acetyl groups, was not observed. Mohammed *et al.* (2013) reported that absence of bands around 1540 cm^{-1} indicated the removal of proteins. The result confirmed the effective removal of protein during the deproteinization process as indicated by the absence of characteristic band of protein.

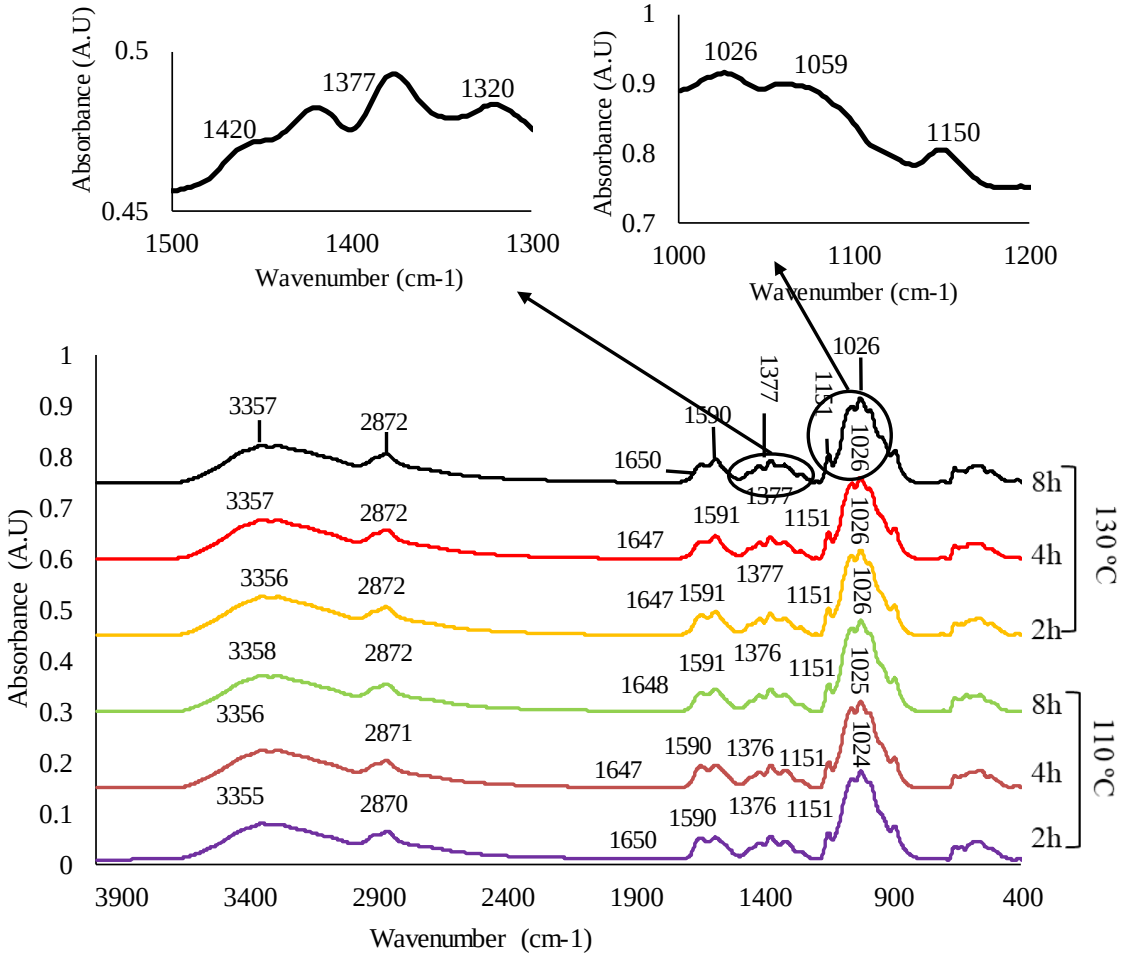

Figure 30. FTIR spectra of chitosan from squid pen chitin deacetylated under different temperatures and times.

he bands at around 1420 and 1377 cm^{-1} mostly represents CH_2 bending and CH_3 symmetrical deformation, respectively. Saccharide band around the wavenumber of 1150 cm^{-1} was due to anti-symmetric stretching of the C-O-C bridge and peaks representing C-O stretching were observed at 1026 and 1059 cm^{-1} (El Knidri *et al.*, 2016). From the IR spectra, it was noticed that the intensity of peaks around 1645 and 1320 cm^{-1} (characteristic of residue *N*-acetyl group) decreased, while that of peaks at 1420 and 1590 cm^{-1} (corresponding to CH_2 an NH_2 groups, respectively) increased,

when temperature and time of deacetylation increased. This result indicated the DDA of obtained chitosan increased when deacetylation temperature and time increased. Moreover, the samples with increasing deacetylation temperature and time had the shifts of peaks representing OH, NH, CH as well as amide I and II to the higher wavenumber. This was more likely owing to both free amine and acetamide groups of chitosan. Similarly, peak representing saccharides (1150 cm^{-1}) was also shifted to the higher wavenumber with increasing deacetylation temperature and time. Thus, chitosan prepared using varying temperatures and times of deacetylation possessed different functional groups, especially acetyl and amino groups. The DDA values, calculated from the absorption ratio of the 1320 to 1420 cm^{-1} peaks (A_{1320}/A_{1420}), of different chitosans obtained are shown in Table 20. Brugnerotto *et al.* (2001) reported that ratio A_{1320}/A_{1420} gives the lowest error and is sensitive to the chemical composition of chitin or chitosan, irrespective of technique, state and secondary structure. Characteristic band at 1320 cm^{-1} measures the amount of *N*-acetylation and 1420 cm^{-1} band is suitable for the comparison between D-glucosamine and *N*-acetylglucosamine. Therefore, the ratio A_{1320}/A_{1420} was suitable to calculate degree of acetylation and DDA (Brugnerotto *et al.*, 2001). It was observed that DDA was increased significantly with increasing deacetylation time from 2 to 8 for both temperatures used (Table 20) ($p<0.05$). The lowest DDA (78%) was observed when chitin was deacetylated at 110 °C for 2 h. However, it was increased up to 87% when deacetylation time of 8 h was used ($p<0.05$). Similar result was obtained when chitin was deacetylated at 130 °C. The highest DDA (90%) was obtained when deacetylation was performed at 130°C for 8 h ($p<0.05$). Result indicated that with increasing deacetylation temperature and time, DDA was increased. Chang *et al.* (1997) stated that temperature plays the dominant role in deacetylation. Moreover, alkaline concentration and solid-solvent ratio also had the impact on the DDA. The maximum DDA was obtained when chitin from pink shrimp was deacetylated at 107 °C using 60% NaOH (Chang *et al.*, 1997). However, high deacetylation temperature and time might result in the severe degradation of chitin, which led to the decreased yield and formation of low MW chitosan. Therefore, appropriate deacetylation temperature and time were required to obtain optimum yield and increased DDA.

8.5.2.2.2 Intrinsic viscosity and average MW

Intrinsic viscosity [η] and viscosity-average MW ($\overline{M}\,v$) of chitosan are shown in Table 20. Average MW of chitosans was determined by the intrinsic viscosity using Mark–Houwink–Sakurada equation. Intrinsic viscosity was decreased with increasing deacetylation temperature and time ($p<0.05$). Intrinsic viscosity of chitosan prepared at 110 °C was decreased from 6.52 to 3.47 dL/g when deacetylation time was increased from 2 to 8 h ($p<0.05$). Similarly, when chitin was deacetylated at 130 °C, intrinsic viscosity of resulting chitosan was decreased from 3.79 to 3.24 dL/g as deacetylation time increased from 2 to 8 h ($p<0.05$). Deacetylation at high temperature for extended time resulted in the more degradation of backbone of chitosan. This was associated with shorter chain, hence reducing viscosity. Reduced viscosity with increasing temperature and time was supported by the increased DDA (Table 20). Average MW is directly related to the intrinsic viscosity of chitosan (Yacob *et al.*, 2013). Deacetylation of chitosan at 110 °C for 2 h yielded chitosan (CH110-2) with the highest MW (3.2×10^5 Dalton) ($p<0.05$). Nevertheless, the lowest MW chitosan (CH130-8) (1.2×10^5 Dalton) was prepared by deacetylation at 130°C for 8 h ($p<0.05$). The result suggested that deacetylation condition directly determined MW and viscosity of resulting chitosan.

8.5.2.3 Structural characteristics of the selected chitosan

8.5.2.3.1 ^{1}H-NMR

^{1}H-NMR spectrum of chitosan extracted from chitin via deacetylation at 130 °C for 2 h (CH130-2) measured at 400 MHz is shown in Figure 31A. Four resonances were found, including H-1 (D), H-2/6, H-2(D), and H-Ac at chemical shift of 5.14, 4.17, 3.45 and 2.33 ppm, respectively. H-1 (D) and H-2(D) represent proton of H1 and H2 of deacetylated monomer, respectively, whereas H-(Ac) signifies proton of acetyl group. H-2/6 and other peaks between 3-4 ppm are known as proton of pyranose ring (Hirai *et al.*, 1991). There was disappearance of peak around 4.9 ppm (H1-A), which represents proton of H1 of acetylated monomers (Lavertu *et al.*, 2003). Generally, disappearance of H1-A peaks was related with high DDA (Lavertu *et al.*,

2003). DDA of CH130-2 chitosan was 89% using ^{1}H-NMR data, which was similar to DDA (87%) obtained from FTIR analysis (Table 20).

8.5.2.3.1 XRD analysis

XRD pattern of chitosan from squid pen is depicted in Figure 31B. Chitosan showed only one crystalline plane at 19.97°, chitin showed two crystalline planes at reflections of 8.1 and 19.66°. Similarly, Jaworska *et al.* (2003) also observed disappearance of peak after deacetylation of chitin from shrimp, squid and fungi. The disappearance of second crystalline plane more likely took place during the deacetylation. Jung and Zhao (2014) also observed two crystalline planes (020 and 110) at reflection of 8.9 and 19.7° in chitin from jumbo squid pen. In general, chitin consists of various types of crystal lattices. Five lattices were reported in α-chitin including 020, 110, 120, 101, or 130 planes. However, only two (020) and (110) planes were documented in the native and processed β-chitin (Jung and Zhao, 2014). This indicated that the β-chitin is less crystalline as compared to α-chitin. This is more likely due to compact parallel and anti-parallel arrangements of chain in α-chitin as compared to β-chitin, which has parallel arrangements of chain (Lavall *et al.*, 2007). The XRD pattern was supported by lower crystallinity index of chitosan (27%) than that of squid pen chitin (55%). Heating of chitin under strong alkaline conditions might distort the structure of chitin and arrangement of chain, resulting in the lower crystalline index. The d-spacing (distance between the different chains) of crystalline plane (110) of chitin (4.51 Å) was slightly decreased to 4.30 Å after deacetylation. Generally, α-chitosan exhibited two crystalline peaks at $2\theta \approx 10°$ and 21°. However, β-chitosan showed single characteristic crystalline peak at $2\theta \approx 21°$ (He *et al.*, 2016). This typical peak reflects the weak intermolecular bonding of β-chitosan (He *et al.*, 2016). Since the single crystalline peak (19.97°) was obtained for the chitosan from squid pen, it could be classified as β-form. The different XRD pattern of squid pen chitin and chitosan indicated different structural arrangements after deacetylation. The result was supported by those from FTIR and ^{1}H-NMR analysis.

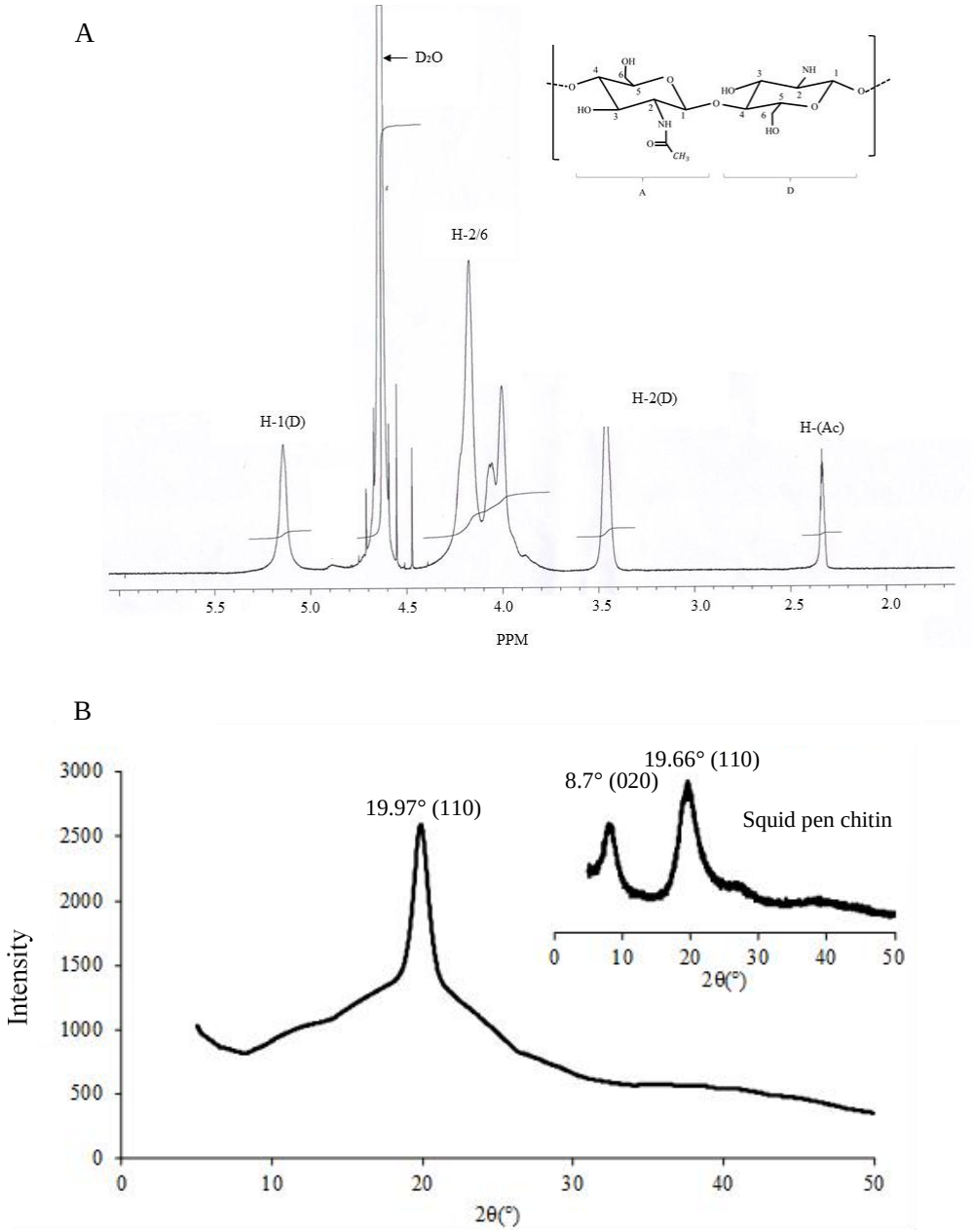

Figure 31. [1]HNMR spectrum (A) and XRD pattern (B) of chitosan (CH130-2) from squid pen.

8.5.2.4 Fat-binding capacity of FITC-labelled chitosan under the mimicked pH condition of gastrointestinal tract

Microstructure of emulsion solubilized by FITC-labelled chitosan extracted under different conditions is shown in Figure 32. It was observed that all chitosan samples with different DDA could form an emulsion under the mimicked pH conditions of gastrointestinal tract. Chitosan is a weak cationic polyelectrolyte (Panith *et al.*, 2016). Under acidic aqueous condition (*in vitro* human gastro-intestinal), it was hydrated and dispersed throughout the medium. Protonation of amino group in chitosan molecule provided strongly positive charge, which was able to attract negatively charged particle including oils, fat containing phospholipids, etc. (Panith *et al.*, 2016). This caused the formation of micelles and fat emulsion complex. Chitosan

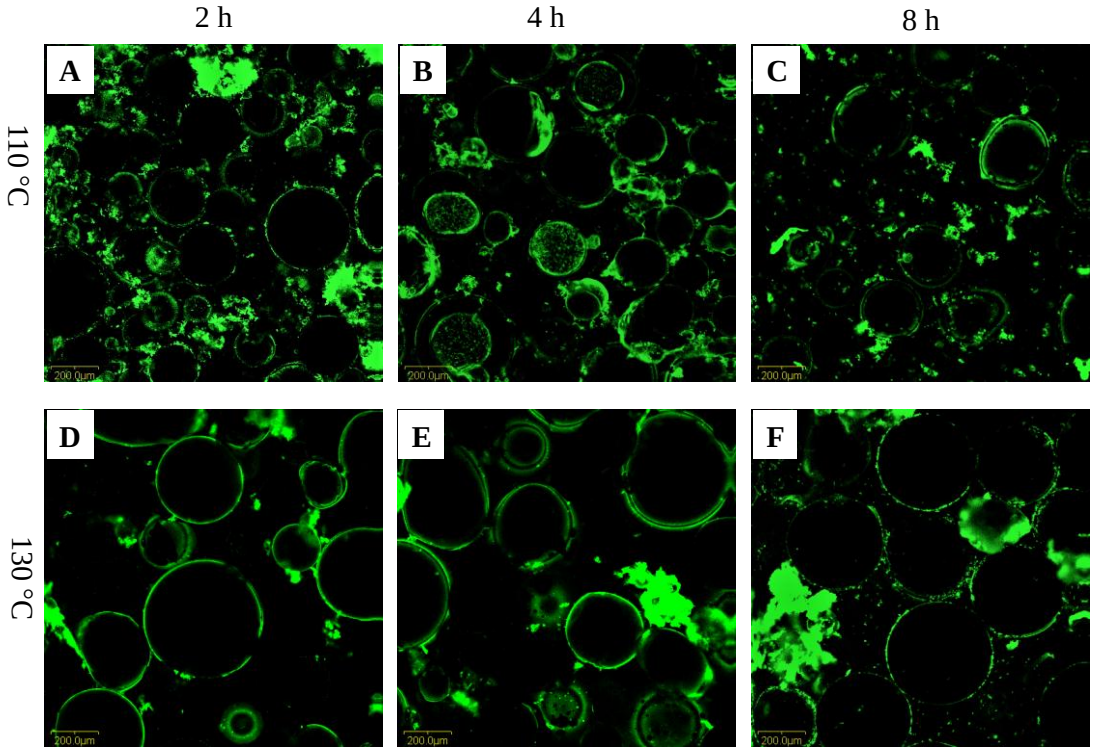

Figure 32. Microstructure of emulsion stabilized by FITC-labelled chitosan under the mimicked pH of gastrointestinal tract (pH 2.0 for 1 h, followed pH 6.8 for 2 h). CH110-2 (A), CH110-4 (B), CH110-8 (C), CH130-2 (D), CH130-4 (E) and (F) CH130-8.

with low DDA and high MW yielded emulsion with smaller droplet sizes (Figure 32A-B) as compared to other samples. High MW molecules, especially with low DDA, had high fat binding ability than the low MW counterpart, and could maintain the size of oil droplets effectively (Panith *et al.*, 2016). Chitosan with low DDA had less positive charge and more likely aligned at the soybean oil droplet surface used for fat binding capacity test. Additionally, long chain chitosan could form the thick and stable film surrounding oil droplets, thus stabilizing the emulsion. Hence, chitosan could be more likely used in the prevention of obesity and related problems. Although droplet size of emulsion obtained from CH130-2 was larger than those stabilized by CH110-2 and CH110-4, the uniform distribution was observed in system containing CH130-2. This was more likely due to the high solubility of chitosan with high DDA (Figure 32A-B). Considering cost-effective and less time-consuming process, along with medium MW and appropriate yield and DDA, chitosan produced at 130 °C for 2 h (CH130-2) was therefore selected for simulated *in vitro* gastrointestinal study.

8.5.2.5 *In vitro* gastrointestinal digestion

Microstructures of FTIC-labelled CH130-2 stabilized emulsion as affected by different gastrointestinal conditions are illustrated in Figure 33. Initial control emulsion showed uniformly distributed smaller oil droplets surrounded by chitosan (Figure 33A). After being subjected to gastric phase, smaller droplet size was obtained. Under the acidic condition, oil droplets might further undergo

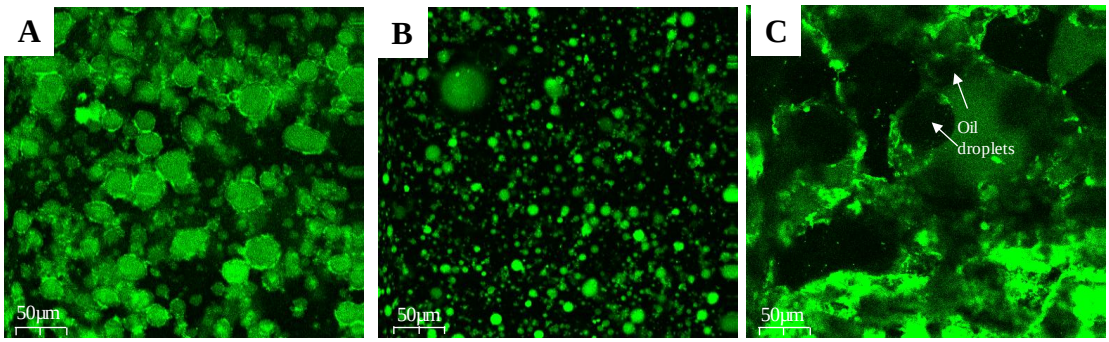

Figure 33. Microstructure of emulsion stabilized by FITC-labelled CH130-2 under the simulated gastrointestinal tract. Control emulsion (A), emulsion after gastric phase (B) and emulsion after duodenal phase (C).

emulsification with ease and soluble chitosan could localize at oil surface to a higher extent. It was suggested that acidic condition and long shaking (1 h) of mixture increased the solubility of chitosan and enhanced the interactions of chitosan with oil droplets. Additionally, pepsin might cause the partial hydrolysis of chitosan, resulting in the lower MW chitosan (Gohi *et al.*, 2016). Low MW chitosan could migrate into interface rapidly and stabilize emulsion. Nevertheless, oil droplet size was increased when the emulsion was brought to duodenal condition. At higher pH (6.8) of duodenum, the precipitation of chitosan more likely occurred, partially destabilization of emulsion (Patil and Benjakul, 2017). Result indicated that more precipitation of chitosan was observed in duodenum (pH 6.8) with addition of bile salt (Figure 32D and C). Binding of sulfite group of bile salts to the amine groups of chitosan via electrostatic attraction might occur with increasing hydrophobic forces. This resulted in the formation of micelle-like clusters of bile acids within the chitosan (Helgason *et al.*, 2008). When chitosan was precipitated, oil droplets were closely packed, leading to more coalescence (Figure 33C). Nevertheless, oil droplets or coalesced oils were stilled entrapped in chitosan gel formed under duodenal condition, as shown by the green background. It was documented that the increases in oil droplet size resulted in lower hydrolysis by lipase due to the decreased oil surface area (Helgason *et al.*, 2008). Moreover, the accessibility of digestive enzymes to hydrolyze oil entrapped in chitosan gel was minimized. As a result, the fat or oil was less hydrolyzed by lipase in the presence of chitosan during the duodenal tract (Mun *et al.*, 2006). Therefore, chitosan can be utilized as a dietary fiber, which could reduce fat digestion or absorption up to some extent.

8.6 Conclusion

Ultrasonication for 41.46 min at an amplitude 69% and 1:18 of solid: solvent ratio produced squid pen chitin with the maximum yield and minimum remaining protein content. DDA of chitosan produced from squid pen chitin under different temperatures and times was varied in the range 78-90%. Chitosans were able to stabilize the emulsion prepared under mimicked gastrointestinal conditions. High DDA chitosan with the ability to stabilize emulsion was produced under the

appropriate deacetylation conditions (deacetylation at 130 °C for 2 h). It could be used to fat absorption after intake of fatty foods.

8.7 References

Aiba, S.-i. 1991. Studies on chitosan: 3. Evidence for the presence of random and block copolymer structures in partially N-acetylated chitosans. International Journal of Biological Macromolecules. 13: 40-44.

Ali, A. M. M., Kishimura, H. and Benjakul, S. 2018. Extraction efficiency and characteristics of acid and pepsin soluble collagens from the skin of golden carp (*Probarbus Jullieni*) as affected by ultrasonication. Process Biochemistry. 66: 237-244.

AOAC. 2000. Official Methods of Analysis. Association of Official Analytical Chemists.

Atkins, E. 1985. Conformations in polysaccharides and complex carbohydrates. Journal of Biosciences. 8: 375-387.

Blackwell, J. 1973. Chitin. *In* Biopolymers. . (Blackwell, J. and Walton, A., eds.). p. 474–489. Academic Press. New York/London.

Brugnerotto, J., Lizardi, J., Goycoolea, F. M., Argüelles-Monal, W., Desbrières, J. and Rinaudo, M. 2001. An infrared investigation in relation with chitin and chitosan characterization. Polymer. 42: 3569-3580.

Chandumpai, A., Singhpibulporn, N., Faroongsarng, D. and Sornprasit, P. 2004. Preparation and physico-chemical characterization of chitin and chitosan from the pens of the squid species, *Loligo lessoniana* and *Loligo formosana*. Carbohydrate Polymers. 58: 467-474.

Chang, K. L. B., Tsai, G., Lee, J. and Fu, W.-R. 1997. Heterogeneous N-deacetylation of chitin in alkaline solution. Carbohydrate Research. 303: 327-332.

Cortizo, M. S., Berghoff, C. F. and Alessandrini, J. L. 2008. Characterization of chitin from *Illex argentinus* squid pen. Carbohydrate Polymers. 74: 10-15.

Croisier, F. and Jérôme, C. 2013. Chitosan-based biomaterials for tissue engineering. European Polymer Journal. 49: 780-792.

Cuong, H. N., Minh, N. C., Van Hoa, N. and Trung, T. S. 2016. Preparation and characterization of high purity β-chitin from squid pens (*Loligo chenisis*). International Journal of Biological Macromolecules. 93: 442-447.

El Knidri, H., El Khalfaouy, R., Laajeb, A., Addaou, A. and Lahsini, A. 2016. Eco-friendly extraction and characterization of chitin and chitosan from the shrimp shell waste via microwave irradiation. Process Safety and Environmental Protection. 104: 395-405.

Elieh-Ali-Komi, D. and Hamblin, M. R. 2016. Chitin and chitosan: production and application of versatile biomedical nanomaterials. International Journal of Advanced Research. 4: 411-427.

Gohi, B. F. C. A., Zeng, H.-Y. and Pan, A. D. 2016. Optimization and Characterization of Chitosan Enzymolysis by Pepsin. Bioengineering (Basel, Switzerland). 3: 17.

Gornall, A. G., Bardawill, C. J. and David, M. M. 1949. Determination of serum proteins by means of the biuret reaction. Journal of Biological Chemistry. 177: 751-766.

He, X., Li, K., Xing, R., Liu, S., Hu, L. and Li, P. 2016. The production of fully deacetylated chitosan by compression method. The Egyptian Journal of Aquatic Research. 42: 75-81.

He, X., Xing, R., Li, K., Qin, Y., Zou, P., Liu, S., Yu, H. and Li, P. 2016. Beta-chitosan extracted from *Loligo Japonica* for a potential use to inhibit Newcastle disease. International Journal of Biological Macromolecules. 82: 614-620.

Helgason, T., Weiss, J., McClements, D. J., Gislason, J., Einarsson, J. M., Thormodsson, F. R. and Kristbergsson, K. 2008. Examination of the interaction of chitosan and oil-in-water emulsions under conditions simulating the digestive system using confocal microscopy. Journal of Aquatic Food Product Technology. 17: 216-233.

Hirai, A., Odani, H. and Nakajima, A. 1991. Determination of degree of deacetylation of chitosan by 1 H NMR spectroscopy. Polymer Bulletin. 26: 87-94.

Huang, J., Zhao, D., Hu, S., Mao, J. and Mei, L. 2012. Biochemical activities of low molecular weight chitosans derived from squid pens. Carbohydrate Polymers. 87: 2231-2236.

Jaworska, M., Sakurai, K., Gaudon, P. and Guibal, E. 2003. Influence of chitosan characteristics on polymer properties. I: Crystallographic properties. Polymer International. 52: 198-205.

Joglekar, A. M., May, A. T., Graf, E. and Saguy, I. 1987. Product excellence through experimental design. *In* Food Product and Development: From Concept to the Marketplace. (Graf, E. and Saguy, I. S., eds.). p. 211-230. An Aspen Publication. Gaithersburg, Maryland.

Jung, J. and Zhao, Y. 2011. Characteristics of deacetylation and depolymerization of β-chitin from jumbo squid (*Dosidicus gigas*) pens. Carbohydrate Research. 346: 1876-1884.

Jung, J. and Zhao, Y. 2014. Alkali- or acid-induced changes in structure, moisture absorption ability and deacetylating reaction of beta-chitin extracted from jumbo squid (*Dosidicus gigas*) pens. Food Chemistry. 152: 355-362.

Kjartansson, G. T., Zivanovic, S., Kristbergsson, K. and Weiss, J. 2006. Sonication-assisted extraction of chitin from North Atlantic shrimps (*Pandalus borealis*). Journal of Agricultural and Food Chemistry. 54: 5894-5902.

Kjartansson, G. T., Zivanovic, S., Kristbergsson, K. and Weiss, J. 2006. Sonication-assisted extraction of chitin from shells of fresh water prawns (*Macrobrachium rosenbergii*). Journal of Agricultural and Food Chemistry. 54: 3317-3323.

Kurita, K., Sannan, T. and Iwakura, Y. 1977. Studies on chitin, 4. Evidence for formation of block and random copolymers of N- acetyl- D- glucosamine and D- glucosamine by hetero- and homogeneous hydrolyses. Macromolecular Chemistry and Physics. 178: 3197-3202.

Lavall, R. L., Assis, O. B. and Campana-Filho, S. P. 2007. β-Chitin from the pens of *Loligo* sp.: Extraction and characterization. Bioresource Technology. 98: 2465-2472.

Lavertu, M., Xia, Z., Serreqi, A., Berrada, M., Rodrigues, A., Wang, D., Buschmann, M. and Gupta, A. 2003. A validated 1H NMR method for the determination of

the degree of deacetylation of chitosan. Journal of Pharmaceutical and Biomedical Analysis. 32: 1149-1158.

Mohammed, M. H., Williams, P. A. and Tverezovskaya, O. 2013. Extraction of chitin from prawn shells and conversion to low molecular mass chitosan. Food Hydrocolloids. 31: 166-171.

Mun, S., Decker, E. A., Park, Y., Weiss, J. and McClements, D. J. 2006. Influence of interfacial composition on in vitro digestibility of emulsified lipids: potential mechanism for chitosan's ability to inhibit fat digestion. Food Biophysics. 1: 21-29.

Osman, Z. and Arof, A. K. 2003. FTIR studies of chitosan acetate based polymer electrolytes. Electrochimica Acta. 48: 993-999.

Panith, N., Wichaphon, J., Lertsiri, S. and Niamsiri, N. 2016. Effect of physical and physicochemical characteristics of chitosan on fat-binding capacities under in vitro gastrointestinal conditions. LWT - Food Science and Technology. 71: 25-32.

Patil, U. and Benjakul, S. 2017. Characteristics of albumin and globulin from coconut meat and their role in emulsion stability without and with proteolysis. Food Hydrocolloids. 69: 220-228.

Rinaudo, M. 2006. Chitin and chitosan: Properties and applications. Progress in Polymer Science. 31: 603-632.

Rudall, K. 1969. Chitin and its association with other molecules. Journal of Polymer Science: Polymer Symposia. 28: 83-102.

Rudall, K. and Kenchington, W. 1973. The chitin system. Biological Reviews. 48: 597-633.

Sae-Leaw, T., O'Callaghan, Y. C., Benjakul, S. and O'Brien, N. M. 2016. Antioxidant activities and selected characteristics of gelatin hydrolysates from seabass (*Lates calcarifer*) skin as affected by production processes. Journal of Food Science and Technology. 53: 197-208.

Steel, R. G. D. and Torrie, J. H. 1986. Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill.

Sugano, M., Watanabe, S., Kishi, A., Izume, M. and Ohtakara, A. 1988. Hypocholesterolemic action of chitosans with different viscosity in rats. Lipids. 23: 187-191.

Yacob, N., Talip, N., Mahmud, M., Sani, N., Samsuddin, N. and Fabillah, N. 2013. Determination of viscosity-average molecular weight of chitosan using intrinsic viscosity measurement. Journal of Nuclear and Related Technologies. 10: 39-44.

Zhao, D., Huang, J., Hu, S., Mao, J. and Mei, L. 2011. Biochemical activities of N, O-carboxymethyl chitosan from squid cartilage. Carbohydrate Polymers. 85: 832-837.

CHAPTER 9
CHITOOLIGOSACCHARIDES FROM SQUID PEN PREPARED USING DIFFERENT ENZYMES: CHARACTERISTICS AND THE EFFECT ON QUALITY OF SURIMI GEL DURING REFRIGERATED STORAGE

9.1 Abstract

Chitooligosaccharides (COS) from squid pen produced using amylase, lipase and pepsin were characterized. COS produced by 8% (w/w) lipase (COS-L) showed the maximum FRAP and ABTS radical scavenging activity than those prepared using other two enzymes. COS-L had the average MW of 79 kDa, intrinsic viscosity of 0.41 dL/g and water solubility of 49%. DPPH, ABTS radical scavenging activities, FRAP and ORAC of COS-L were 5.68, 322.68, 5.66 and 42.20 μmol TE/g sample, respectively. Metal chelating activity was 2.58 μmol EE/g sample. For antibacterial activity, MIC and MBC of COS-L against the targeted bacteria were in the range of 0.31-4.91 mg/mL and 0.62-4.91 mg/mL, respectively. Sardine surimi gel added with 1% (w/w) COS-L showed the lower PV, TBARS and microbial growth in surimi gel during 10 days of storage at 4 °C. COS-L from squid pen could inhibit lipid oxidation and extend the shelf-life of refrigerated sardine surimi gel.

9.2 Introduction

Chitosan is a non-toxic deacetylated chitin, with a variety of applications such as antibacterial and antiaging agents, tissue engineering, cancer diagnosis and drug delivery. It also has ability to enhance the response of antibodies (Elieh-Ali-Komi and Hamblin, 2016). Due to low water solubility, its applications are limited. In general, low molecular weight (MW) chitosan (2.8–87.7 kDa) possessed much improved solubility and functional activities than high MW chitosan (604–931 kDa) (Laokuldilok *et al.*, 2017). Therefore, physical, chemical or enzymatic methods have been employed to hydrolyze chitosan, in which short chain chitooligosaccharide (COS) can be produced (Il'ina and Varlamov, 2004). Physical methods such as irradiation and ultrasonication, etc. produced partially depolymerized chitosan but the reduction in MW is restricted (Lodhi *et al.*, 2014). In contrast, chemical hydrolysis is harsh and is uncontrolled to produce the desired (COS) (Lodhi

et al., 2014). Enzymatic hydrolysis has been implemented to manufacture low MW (COS) with controlled degree of depolymerization (Lee *et al.*, 2008; Lodhi *et al.*, 2014). However, enzymes specific for chitosan degradation such as chitosanase and chitinase are costly and less available. Therefore, non-specific enzymes such as amylase, pepsin, pectinase, papain, cellulase and lipase have been used to hydrolyze chitosan for production of COS (Lodhi *et al.*, 2014).

Surimi is concentrated myofibrillar protein obtained from fish mince. It has exceptional textural properties and high nutritional value and can be used for the productions of various products (Singh and Benjakul, 2017). Owing to the overexploitation of lean fish, surimi industry is showing great interest to utilize dark fleshed fish for example sardine, mackerel etc. (Singh and Benjakul, 2017). Dark flesh of small pelagic fish species is mainly due to high contents of myoglobin and lipid (Chaijan *et al.*, 2010). This leads to the susceptibility of surimi toward lipid oxidation. This can shorten shelf-life caused by off-flavor of resulting gel products stored for an extended time (Chaijan *et al.*, 2010). A wide range of antioxidants has been implemented into surimi to tackle this problem. Owing to unhealthy impact of synthetic antioxidants on human body, natural antioxidants have been utilized in foods widely (Sae-Leaw *et al.*, 2018).

Chitosan can be also produced from squid pen and it is classified as β-form (Elieh-Ali-Komi and Hamblin, 2016). Squid pen chitosan can be transformed to COS with antioxidant and antimicrobial activities, which could be used as preservative in some food products, particularly those prone to lipid oxidation or spoilage. Fernandes *et al.* (2008) documented COS from crab shell (DDA, 80-85%) as an antimicrobial agent against common food borne pathogens including *Staphylococcus aureus* and *Escherichia coli* in milk and apple juice. Chitosan was fortified into surimi gel to enhance textural properties as well as storage stability of gel kept at refrigerated temperature (Mao and Wu, 2007; Amiza and Kang, 2013). However, no information is available on utilization of COS from squid pen into surimi gel.

9.3 Objective

To find the appropriate enzyme for the production of COS and to investigate its impact on storage stability of sardine surimi gel stored at 4 °C.

9.4 Material and methods

9.4.1 Chemicals, surimi and bacterial strains

All chemicals were of analytical grade. Amylase, lipase, pepsin, ethylenediaminetetraacetic acid (EDTA), 2,4,6- tripyridyltriazine (TPTZ), 2,2diphenyl-1-picrylhydrazyl (DPPH), fluorescein, 2,2′- azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2carboxylic acid (Trolox) and 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were procured from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Tryptic soy broth and tryptic soy agar were obtained from Difco, Le Port de claix, France.

Frozen sardine (*Sardinella albella*) surimi (AA grade) was bought from Chaichareon Marine Co., Ltd. (Pattani, Thailand). Surimi was stored at −20°C and used within two months.

Pseudomonas aeruginosa PSU.SCB.16S.11, *Listeria monocytogenes* F2365, *Vibrio parahaemolyticus* PSU.SCB.16S.14, *Staphylococcus aureus* DMST 4745, *Salmonella enterica* serovar Enteritidis S5-371 were collected from Food Safety Laboratory, Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand

9.4.2 Preparation of chitooligosaccharide (COS)

Chitosan was firstly extracted from squid pen as described in previous chapter 7. Degree of deacetylation (DDA) was 87%. To prepare COS, chitosan (1 g) was dissolved in 1% (v/v) acetic acid to get a final concentration of 1% (w/v). Final pH of prepared solution was adjusted to 5 with 6 M NaOH. Amylase, lipase or papain were added to chitosan solution at the concentration of 8%. Amylase and lipase assisted hydrolysis was carried out at 50 °C and hydrolysis using papain was done at 37 °C for different times (0, 1, 3, 6, 12, 24, 36, 48, 60 and 72 h). The hydrolysis was

terminated by heating the solution at 95 °C for 10 min. All the hydrolysates were determined for degree of depolymerization (DDP) by measuring reducing sugar using dinitrosalicylic acid (DNS) method (Miller, 1959) in comparison with total sugar content analyzed by the Dubois method (Dubois *et al.*, 1956). In addition, hydrolysates were subjected to determination of ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) as described by Tongnuanchan *et al.* (2012).

9.4.3 Characterization of the selected COS

COS produced by lipase exhibiting the highest antioxidative activity was adjusted to pH 7 using 6 M NaOH followed by vacuum filtration. The obtained filtrate was subsequently freeze-dried using a freeze-dry (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The resultant powder named as 'COS-L' was used for characterization.

9.4.3.1 Water solubility

Percent water solubility of COS-L was measured using the method of Laokuldilok *et al.* (2017).

9.4.3.2 Intrinsic viscosity and viscosity-average molecular weight ($\bar{M}_v$)

Intrinsic viscosity and viscosity-average molecular weight were determined as described in sections 8.4.5.2 and 8.4.5.3, respectively.

9.4.4 Antioxidative activities

FRAP and ABTS radical scavenging activity of COS-L sample was determined as previously described. In addition, DPPH radical scavenging activity was measured using the method of Benjakul *et al.* (2014) and was expressed as μmol Trolox equivalent (TE)/g sample. Chelating activity against ferrous ion (Fe^{2+}) was examined by the method of Benjakul *et al.* (2014) and was expressed as μmol EDTA equivalent (EE)/g sample. Oxygen radical absorbance capacity (ORAC) was

measured as tailored by Buamard and Benjakul (2017) and expressed as μmol Trolox equivalent (TE)/g sample.

9.4.5 Bacterial cell suspension and culturing condition

Cultures were grown separately for 18 h in tryptic soy broth (TSB) until the absorbance of wavelength 600 nm was 0.8. Afterward, the cultures were mixed with 15% glycerol and stored at −80°C until use. Before use, firstly cultures were thawed and sub-cultured on tryptic soy agar at 37°C for 24 h. Cells were resuspended in TSB and incubated for 4 h at 37°C. Then serial dilutions were performed to dilute the cell concentration to 10^6 CFU/mL.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the COS-L were determined against *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella enterica* as per the method of Olatunde *et al.* (2018).

9.4.6 Impact of the COS-L on storage stability of sardine surimi gel during refrigerated storage

9.4.6.1 Surimi gel preparation

Surimi gel added without and with 1% COS-L was prepared as per method described in section 6.4.6.1. For storage study, gels added without and with COS-L were sealed in a zip lock bag and kept for 10 days at 4 °C. Surimi gels were randomly taken at 0, 5, and 10 days for analyses.

9.4.6.2 Analyses

9.4.6.2.1 Lipid oxidation

Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) of sardine surimi gel incorporated without and with COS-L (1%) were measured by the method described by Sae-Leaw *et al.* (2018).

9.4.6.2.2 Microbial analysis

Spread plate method was conducted for microbiological analyses (Sallam, 2007). Ten gram of gel samples were added into 90 mL of 0.85% (w/v) sterile saline solution (supplemented with 0.1% peptone) followed by homogenization for 2 min at 220 rpm using a Stomacher blender (Mode l400, Seward Ltd. West Sussex, England). Obtained homogenate was serially diluted ten-fold in 0.85% sterile saline solution contained peptone. Total viable count (TVC) and psychrophilic bacterial count (PBC), total fungal count (TFC), spoilage bacteria count including *Pseudomonas,* and *Enterobacteriaceae* were determined (Vanderzant and Splittstoesser, 1995)

9.4.7 Statistical analysis

All experiments were performed in triplicate. Data were applied to analysis of variance. Comparison of means was done by the Duncan's multiple range tests (Steel and Torrie, 1986). Analysis was achieved using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL, USA).

9.5 Results and discussion

9.5.1 Degree of depolymerization and antioxidative activities of COS produced using various enzymes for different hydrolysis times

Degrees of depolymerization (DDP) of chitosan treated with 8% amylase or 8% lipase or 8% pepsin are shown in Figure 34. Chitosan (time 0) had DDP of around 7%, indicating that depolymerization occurred to some degree, particularly during deacetylation at high temperature. For each enzyme used, an increase in DDP was observed with increasing hydrolysis time up to 36 h ($p<0.05$). Afterward, no further changes in DDP were attained ($p>0.05$). This was plausibly due to lowered substrate or decreased enzyme activity due to the prolonged hydrolysis (Rokhati *et al.*, 2013). The highest DDP was observed for COS produced by amylase (12 to 31%), followed those produced by lipase (15 to 26%) and pepsin (12 to 23%), respectively. DDP of COS produced by amylase was quite high, probably due to the presence of β-1, 4 bond between glucosamine, which was similar to that found in

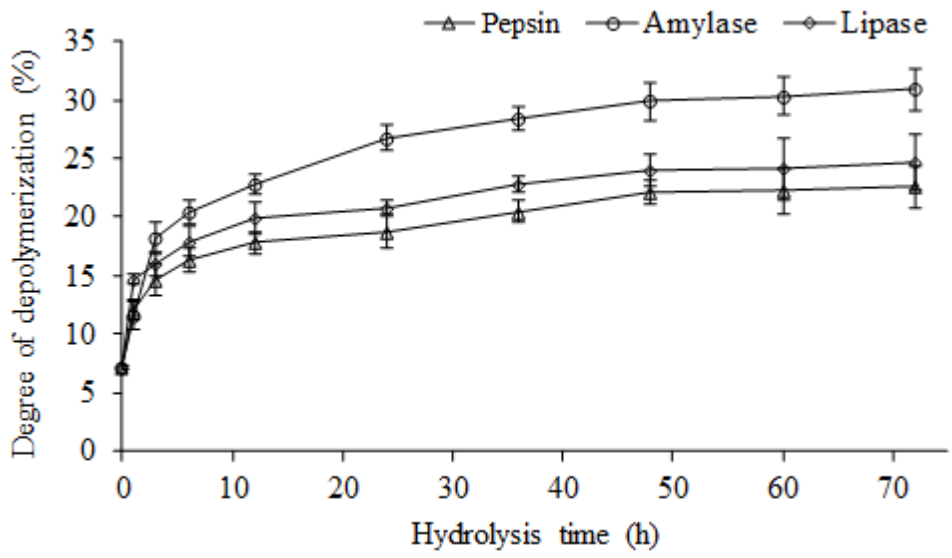

Figure 34. Degree of depolymerization of chitooligosaccharides (COSs) from squid pen prepared using pepsin, amylase or lipase for different hydrolysis times. Bars represent the standard deviation (n=3). Enzymes at 8% (w/w) were used.

amylose (Rokhati *et al.*, 2013). Pan and Wu (2011) documented the optimum condition for hydrolysis of chitosan from shrimp shell using glucoamylase at pH 4.5 and 55 °C for 24 h. Optimum hydrolysis of chitosan from shrimp shells was conducted using α-amylase (pH 5; temperature 50 °C) for 6 h (Wu, 2011). Chitosan have 4 different kinds of glycosidic linkages (Roncal *et al.*, 2007). Enzymes can cleave more than one type of these linkages owing to different affinities of enzyme towards different bonding. This led to varying cleavage rates by different enzymes (Roncal *et al.*, 2007). Therefore, DDP of chitosan generally depends on the type and source of enzyme.

FRAP or ABTS radical scavenging activity of COS produced by various enzymes for different hydrolysis time are presented in Figure 35A and B, respectively. All COS samples showed the higher FRAP or ABTS radical scavenging activity than chitosan (hydrolysis time: 0) ($p<0.05$). Overall, COS produced by lipase revealed the greater FRAP and ABTS radical scavenging activity, compared to those produced by pepsin and amylase, regardless of hydrolysis time. The highest FRAP activity was observed in COS produced using lipase at the hydrolysis time of 12 h ($p<0.05$). Nevertheless, the maximum ABTS radical scavenging was obtained for

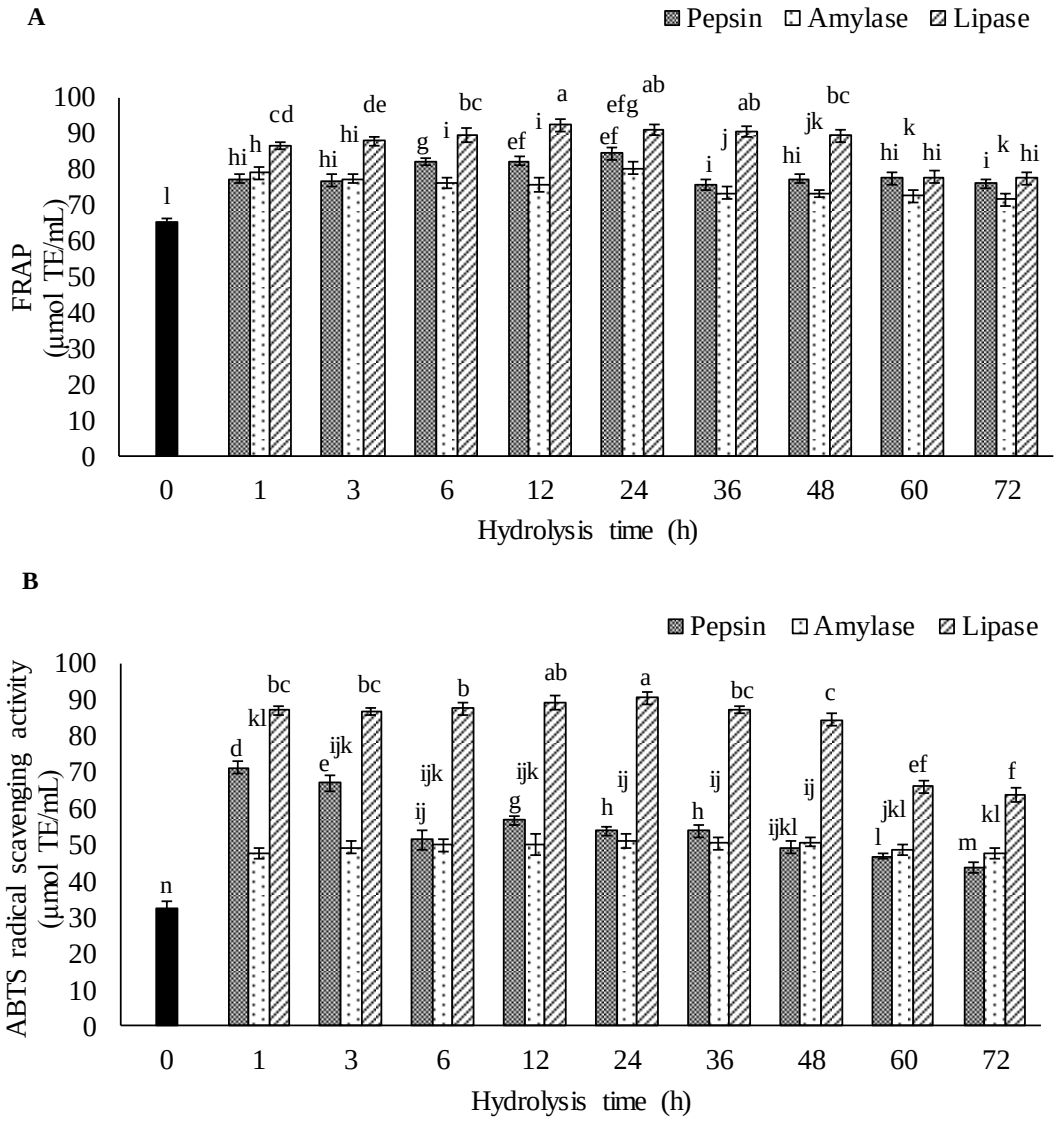

Figure 35. FRAP (A) and ABTS radical scavenging activity (B) of chitooligosaccharides (COSs) from squid pen chitosan prepared using pepsin or amylase or lipase for different hydrolysis times. Bars represent the standard deviation (n=3). Enzymes at 8% (w/w) were used. Different lowercase letters on the bars indicate significant difference ($p<0.05$).

COS when lipase was utilized for hydrolysis for 12 or 24 h ($p<0.05$). Considering time effectiveness, hydrolysis time of 12 h was selected for the production of COS using lipase, which yielded the resulting COS with high antioxidative activity. Antioxidative activity of chitosan or COS were generally governed by the amino and hydroxyl groups present in pyranose ring. Antioxidative activity of COS could vary with the degree of deacetylation of chitosan and MW of COS. Lee *et al.* (2008) reported that lipase could produce both oligomers and monomers of chitosan due to its endo and exo-type specificity towards chitosan. Hence, high ABTS radical scavenging activity and FRAP of COS prepared by lipase might be due to the appropriate size and conformation of COS produced by lipase, in which amino or hydroxyl groups were available and acted as H-donor or radical scavenger.

Since COS produced by lipase at 12 h of hydrolysis showed the highest antioxidative potential as indicated by FRAP and ABTS radical scavenging assays, it was further freeze-dried and the resultant powder was defined as COS-L.

9.5.2 Characteristics and properties of COS-L

9.5.2.1 Intrinsic viscosity, average MW and water solubility

Intrinsic viscosity, average MW and water solubility of COS-L are shown in Table 21. Initial intrinsic viscosity and average MW of chitosan (DDA of 87%) were 3.79 dL/g and 1.5 $x10^5$ Da, respectively (Singh et al., unpublished results). Both intrinsic viscosity and average MW were decreased to 0.41 dL/g and 79 kDa, respectively after 12 h hydrolysis by lipase. Reduction in MW and viscosity of chitosan was documented to be governed by the type and source of enzyme used (Il'ina and Varlamov, 2004). Lipase showed the strong affinity and higher activity to chitosan having the moderate DDA (Lee *et al.*, 2008). Lee *et al.* (2008) reported that lipase cleaved chitosan with the exo-type mode as well as in end-splitting manner. This resulted in higher reduction in viscosity and MW. COS-L showed 49% solubility in water. Generally, chitosan is not soluble in water. With the lower MW after hydrolysis by lipase, hydrogen bonding of hydroxyl group in COS produced underwent interaction or binding with water. Laokuldilok *et al.* (2017) also observed

increased water solubility after 16 h hydrolysis of chitosan (DDA 80 and 90%) using lysozyme, papain, or cellulase. However, chitosan of DDA 90% showed higher water solubility. Higher accessibility of enzyme to substrate was presumed, resulting in the formation of COS with lower MW.

Table 21. Water solubility, viscosity, average molecular weight (MW) and antioxidative activity of COS-L from squid pen.

Water Solubility (%)	49.14±0.89
Viscosity (dL/g)	0.41±0.00
MW (kDa)	78.82±2.55
Antioxidative activity	
DPPH (μmol TE/g sample)	5.68±0.36
ABTS (μmol TE/g sample)	322.68±8.46
FRAP (μmol TE/g sample)	5.66±0.15
MCA (μmol EE/g sample)	2.58±0.11
ORAC (μmol TE/g sample)	42.20±4.85

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

9.5.2.2 Antioxidative activities

DPPH, ABTS radical scavenging activities, FRAP, metal chelating activity and ORAC of COS-L are depicted in Table 21. COS-L had high ABTS radical scavenging activity (323 μmol TE/g sample). ORAC (42 μmol TE/g sample), FRAP (5.7 μmol TE/g sample), DPPH radical scavenging activity (5.7 μmol TE/g sample) were also noted. COS-L showed metal chelating activity of 2.6 μmol EE/g sample. Free amino group of glucosamine can form ammonium group by taking a hydrogen ion from the system, which later can react with radicals (Senphan and Benjakul, 2014; Laokuldilok *et al.*, 2017). Additionally, more hydroxyl groups generated after hydrolysis could function as hydrogen donor to radicals. Chitosan, which has strong intra-molecular hydrogen bonding, showed lower antioxidant activity (Tomida *et al.*, 2009). The results confirmed that COS-L with lower MW possessed higher radical scavenging activity than chitosan. Kim and Thomas (2007)

stated that chitosan with MW 30 kDa had the higher DPPH radical scavenging activity as compared to those of 90 and 120 kDa chitosan. In present study, COS from squid pen chitosan showed both ABTS and DPPH radical scavenging activities ($p<0.05$) (Table 21). ABTS radical scavenging activity assay can be used for both hydrophilic and lipophilic substances, whereas DPPH radical scavenging activity assay is applicable in the lipophilic system (Senphan and Benjakul, 2014). ORAC assay determined the oxidative degradation of the fluorescent molecule azo-initiator compounds. Azo-initiators produce the peroxyl radical by heating, which damages the fluorescent molecule, resulting in the loss of fluorescence. In the presence of COS-L, the oxidation of fluorescent molecule was lowered or terminated. Hence, COS-L had the potential to scavenge radicals, thus having an ability to prevent lipid oxidation by chain termination reaction.

FRAP assay generally determines the reducing power of antioxidant, showing the potential of substance to reduce TPTZ–Fe (III) complex to TPTZ–Fe (II) complex. Reducing power demonstrated the potential of COS-L to reduce Fe^{3+} to Fe^{2+} by donating electron and prevent or retard propagation step. Chitosan and COS has been known to remove various toxic metals from the aqueous environment due to the presence of reactive hydroxyl group at C-3 and C-6 with amino group at C-2 (Zhang *et al.*, 2016). COS can remove metal ions through different mechanisms such as chelation via lone electron pairs of amino or ion exchange of protonated amino group (Guzman *et al.*, 2003). Huang *et al.* (2006) modified COS at amino position with carboxyl and quaternized amino groups to alter the amount of hydrogen atoms. After modification, the reduction in DPPH radical scavenging activity was observed. The results suggested the crucial role of amino group and hydroxyl groups in COS-L for its antioxidative activities.

9.5.2.3 Antibacterial activity

MIC and MBC of COS-L against targeted bacteria are presented in Table 22. MIC were 4.91, 0.62, 0.31, 4.91 and 1.23 mg/mL for *Listeria monocytogenes, Vibrio parahaemolyticus, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella enterica,* respectively. The lowest MIC value (0.31 mg/mL)

was observed for *P. aeruginosa*, whereas the highest MIC value (4.91 mg/mL) was found for *L. monocytogenes* and *S. aureus*. Gram-negative bacteria were sensitive to COS-L as indicated by lower MIC and MBC than those of Gram-positive. This was more likely due to thinner cell wall of the former (Olatunde *et al.*, 2018). Chitosan and COS are known to possess antibacterial activity, which is influenced by the MW (No *et al.*, 2002). Moreover, susceptibility of microorganism towards COS could be varied. *Bacillus subtilis, Pseudomonas aureofaciens, Bifidobacterium bifidum 791* and *Enterobacter agglomerans* were resistant to chitosan with an average MW of 5 to 27 kDa (DDA-85%), while *Escherichia coli* was more sensitive to the 5 kDa chitosan (Gerasimenko *et al.*, 2004). Gerasimenko *et al.* (2004) also reported the sensitivity of *Candida krusei, Staphylococcus aureus* and *Bifidobacterium bifidum* ATCC 14893 against chitosan with different MW, which were varied between 5 and 27 kDa. Park *et al.* (2004) studied the effect chitosan having DDA 90%, 75% and 50% and their COS of varying MWs on 32 strains of *V. parahaemolyticus.* MIC were 0.5 and 1.0 mg/mL for 14 and 18 strains, respectively. In addition, MIC of most hetero-chitosan oligosaccharides was 8.0 mg/mL. Hence, antimicrobial potential of COS-L with low MW could inhibit the number of bacteria, both pathogenic and spoilage bacteria.

Table 22. Minimum inhibitory concentration (MIC) and minimum bacterial concentration of COS-L from squid pen.

Targeted microbes	MIC (mg/mL)	MBC (mg/mL)
Listeria monocytogenes	4.91±0.2	4.91±0.21
Vibrio parahaemolyticus	0.62±0.01	2.46±0.2
Pseudomonas aeruginosa	0.31±0.00	0.62±0.01
Staphylococcus aureus	4.91±0.3	4.91±0.5
Salmonella enterica	1.23±0.96	2.46±1.8

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

MBC were 4.91, 2.46, 0.62, 4.91, 2.46 mg/mL for *Listeria monocytogenes, Vibrio parahaemolyticus, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella enterica*, respectively. Antibacterial effect of any substance can be assured by comparing the MBC and MIC values (Olatunde *et al.*, 2018). The ratio of MBC/MIC determined the bacteriostatic and bactericidal effect of the tested substances. Generally, the ratio of ≤2 indicated 'bacteriostatic' effect, where ratio >2 reflect bactericidal impact. MBC/MIC ratio of *L. monocytogenes, V. parahaemolyticus, P. aeruginosa, S. aureus* and *S. enterica* were 1, 3.97, 2, 1 and 2, respectively. Therefore, COS-L could act as bactericidal agent for *V. parahaemolyticus, P. aeruginosa* and *S. enterica* and possessed bacteriostatic effect towards *L. monocytogenes* and *S. aureus*.

9.5.3 Storage stability of sardine surimi gel added without and with COS-L

9.5.3.1 Lipid oxidation

TBARS values of sardine surimi gel in the absence and presence of 1% COS-L from squid pen chitosan are presented in Figure 36A. The increases in TBARS value were observed with increasing storage time from day 0 to 10 in both samples ($p<0.05$). However, surimi gel without COS-L showed higher TBARS value than that of gel added with COS-L at the same storage time ($p<0.05$). TBARS represent the secondary oxidation products in the oxidized lipid (Sae-Leaw *et al.*, 2018). Similarly, PV of sardine surimi gel with and without COS-L was also increased as the storage time increased from 0 to 10 days as shown in Figure 36B. However, higher PV was observed for gel without COS-L than that of gel added with COS-L at the same storage time ($p<0.05$). This was more likely due to antioxidative capacity of COS-L (Table 22). The result was in line with that for TBARS (Figure 36A). Lipid oxidation resulted off-flavor and off-odor of fish muscles due to oxidative deterioration of polyunsaturated fatty acid, thereby shortening shelf-life of food (Mao and Wu, 2007). TBARS and PV value are both well-known methods to determine lipid oxidation in the food system. Mao and Wu (2007) observed the lower PV and TBARS values in the gel from grass carp surimi incorporated with commercial

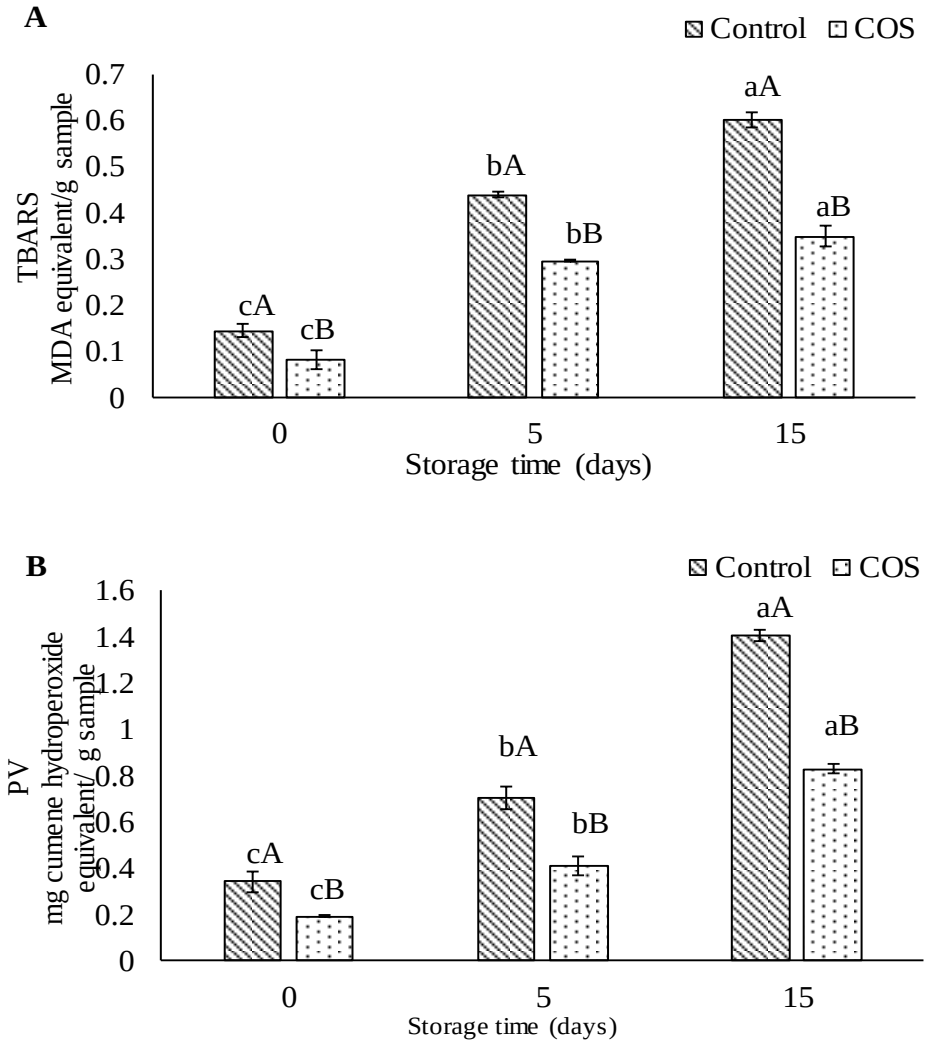

Figure 36. Thiobarbituric acid reactive substances (TBARS) (A) and peroxide value (PV) (B) of sardine surimi gels without and with 1% COS-L from squid pen during refrigerated storage at 4°C for 10 days. CON: gel without addition of COS-L; COS-L: gel added with 1% COS-L. Bars represent the standard deviation (n = 3). Different lowercase letters on the bars within the same sample indicate significant differences ($p<0.05$). Different uppercase letters on the bars within the same storage time indicate significant differences ($p<0.05$).

chitosan with different MW. Low MW chitosan (10 kDa) exhibited higher capacity to inhibit lipid oxidation as compared to high MW chitosan (300 kDa). Similarly, Carmen Gómez-Guillén *et al.* (2005) observed lower increases in TBARS and PV of gel from horse mackerel surimi when 1% chitosan from shrimp shells (DDA-97%) was incorporated. Antioxidative activities of COS-L was more likely due to its radical scavenging and metal binding capacities (Table 21). Fish tissue, particularly dark fleshed fish, e.g. sardine etc., contains iron bound to the protein, which could be released during heat induced gelation. Free iron could act as prooxidant and accelerated lipid oxidation (St. Angelo *et al.*, 1996). COS-L might chelate ferrous ions from the system, thus retarding lipid oxidation. Furthermore, amino group at C-2 might participate in metal chelation (Mao and Wu, 2007). Therefore, the addition of COS-L into sardine surimi gel could retard lipid oxidation during the extended storage at 4 °C.

9.5.3.2 Microbial load

TVC, PBC, TFC, *Enterobacteriaceae* and *Pseudomonas* counts of sardine surimi gel added without and with 1% COS-L during storage at 4 °C are depicted in Table 23. All initial counts of gel without and with COS-L addition were in the range of 2.28-3.24 and 1.07-1.51 log CFU/g, respectively. Higher counts of all microorganism tested were observed in the control gel as compared to the gel added with COS-L at the same storage time ($p<0.05$). Psychrophilic bacteria and *Pseudomonas* were not found in gel added with COS-L (<1 log CFU/g sample) at day 0. At day 10, all counts were in the range of 5.49-6.57 and 3.18-4.77 CFU/g sample for gels added without and with COS-L, respectively. The result showed significant reduction in counts of surimi gel in the presence of COS-L during 10 days of storage. This was more likely due to the antimicrobial capacity of COS-L (Table 22). Amiza and Kang (2013) studied the effect of food grade chitosan (MW-10 kDa, DDA-95%) on gel from catfish surimi. Gels added with 2% chitosan showed lower microbial counts than control gel ($p<0.05$) during storage at 4 °C. In general, antimicrobial activity of COS was more likely due the inhibition of mRNA and protein translation. Chelation of essential nutrients and metals are the known mechanisms advocating antimicrobial activity of COS. No *et al.* (2002) reported that COS from crab shell with

MW of 1 kDa showed the higher antimicrobial activity against Gram-negative bacteria, while those having MW of 4 and 2 kDa exhibited high activity towards Gram-positive bacteria. COS from exoskeletons of marine crustaceans (1%) inactivated 4.5 and 0.5 log CFU/g of *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*, respectively after 120 min of exposure (Choi *et al.*, 2001). For antifungal activity, COS might penetrate to fungal cell and caused structural and molecular changes in the cells (Younes *et al.*, 2014). Younes *et al.* (2014) reported that commercially available chitosan with varying DDA and MW retarded the growth of food spoilage microbes such as *Aspergillus niger*, *Fusarium oxysporum* and *Alternaria solani.* Hence, COS-L successfully inhibited the growth of microorganisms in sardine surimi gel during the refrigerated storage of 10 days.

Table 23. Microbial count of sardine surimi gel added without and with 1% COS-L from squid pen.

Samples	Storage time (days)	Microbial load (log CFU/g)				
		TVC	PBC	Total fungal count	*Enterobacteriaceae* count	*Pseudomonas* count
CON	0	3.24±0.01^{c}	2.28±0.02^{c}	2.96±0.01^{c}	2.69±0.03^{c}	2.98±0.06^{c}
	5	4.62±0.04^{b}	5.20±0.02^{b}	5.41±0.03^{b}	4.30±0.03^{b}	4.58±0.06^{b}
	10	5.94±0.06^{a}	6.57±0.03^{a}	5.55±0.02^{a}	5.49±0.04^{a}	5.88±0.01^{a}
COS-L	0	1.32±0.03^{c}	ND	1.51±0.01^{c}	ND	1.07±0.03^{c}
	5	2.48±0.04^{b}	2.30±0.04^{b}	2.39±0.03^{b}	1.90±0.09^{b}	2.20±0.04^{b}
	10	3.75±0.02^{a}	4.77±0.03^{a}	3.79±0.05^{a}	3.18±0.06^{a}	3.62±0.04^{a}

Values are expressed as mean ± SD (n = 3). CON: gel without addition of COS-L; COS-L: gel added with 1% (w/w) COS-L. Different lowercase superscripts within the same sample in the same column indicate significant differences ($p<0.05$).

ND: Not detected

9.6 Conclusion

Chitooligosaccharide (COS) produced from squid pen chitosan using lipase named COS-L showed the highest antioxidative activity as compared to those

prepared using amylase and pepsin. COS-L had medium MW and showed improved solubility in water. Addition of 1% COS-L could lower lipid oxidation in sardine surimi gel during refrigerated storage shown by the decreased PV and TBARS values. COS-L also suppressed the growth of spoilage bacteria *Pseudomonas* and *Enterobacteriaceae* as well as fungus in surimi gel during the refrigerated storage. Thus, COS-L from squid pen could increase the shelf-life of sardine surimi gel kept at refrigerated temperature.

9.7 References

Amiza, M. and Kang, W. 2013. Effect of chitosan on gelling properties, lipid oxidation, and microbial load of surimi gel made from African catfish (*Clarias gariepinus*). International Food Research Journal. 20: 1585.

Benjakul, S., Kittiphattanabawon, P., Sumpavapol, P. and Maqsood, S. 2014. Antioxidant activities of lead (*Leucaena leucocephala*) seed as affected by extraction solvent, prior dechlorophyllisation and drying methods. Journal of Food Science and Technology. 51: 3026-3037.

Buamard, N. and Benjakul, S. 2017. Ethanolic coconut husk extract: In vitro antioxidative activity and effect on oxidative stability of shrimp oil emulsion. European Journal of Lipid Science and Technology. 119: 1700131.

Carmen Gómez-Guillén, M., Montero, P., Teresa Solas, M. and Pérez-Mateos, M. 2005. Effect of chitosan and microbial transglutaminase on the gel forming ability of horse mackerel (*Trachurus* spp.) muscle under high pressure. Food Research International. 38: 103-110.

Chaijan, M., Panpipat, W. and Benjakul, S. 2010. Physicochemical properties and gel-forming ability of surimi from three species of mackerel caught in Southern Thailand. Food Chemistry. 121: 85-92.

Choi, B.-K., Kim, K.-Y., Yoo, Y.-J., Oh, S.-J., Choi, J.-H. and Kim, C.-Y. 2001. In vitro antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. International Journal of Antimicrobial Agents. 18: 553-557.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. t. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry. 28: 350-356.

Elieh-Ali-Komi, D. and Hamblin, M. R. 2016. Chitin and chitosan: production and application of versatile biomedical nanomaterials. International Journal Of Advanced Research. 4: 411-427.

Fernandes, J. C., Tavaria, F. K., Soares, J. C., Ramos, Ó. S., Monteiro, M. J., Pintado, M. E. and Malcata, F. X. 2008. Antimicrobial effects of chitosans and chitooligosaccharides, upon *Staphylococcus aureus* and *Escherichia coli*, in food model systems. Food Microbiology. 25: 922-928.

Gerasimenko, D. V., Avdienko, I. D., Bannikova, G. E., Zueva, O. and Varlamov, V. P. 2004. [Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms]. Prikladnaya Biokhimiya i Mikrobiologiya. 40: 301-306.

Guzman, J., Saucedo, I., Revilla, J., Navarro, R. and Guibal, E. 2003. Copper sorption by chitosan in the presence of citrate ions: influence of metal speciation on sorption mechanism and uptake capacities. International Journal of Biological Macromolecules. 33: 57-65.

Huang, R., Rajapakse, N. and Kim, S.-K. 2006. Structural factors affecting radical scavenging activity of chitooligosaccharides (COS) and its derivatives. Carbohydrate Polymers. 63: 122-129.

Il'ina, A. V. and Varlamov, V. P. 2004. Hydrolysis of chitosan in lactic acid. Applied Biochemistry and Microbiology. 40: 300-303.

Kim, K. W. and Thomas, R. L. 2007. Antioxidative activity of chitosans with varying molecular weights. Food Chemistry. 101: 308-313.

Laokuldilok, T., Potivas, T., Kanha, N., Surawang, S., Seesuriyachan, P., Wangtueai, S., Phimolsiripol, Y. and Regenstein, J. M. 2017. Physicochemical, antioxidant, and antimicrobial properties of chitooligosaccharides produced using three different enzyme treatments. Food Bioscience. 18: 28-33.

Lee, D.-X., Xia, W.-S. and Zhang, J.-L. 2008. Enzymatic preparation of chitooligosaccharides by commercial lipase. Food Chemistry. 111: 291-295.

Lodhi, G., Kim, Y.-S., Hwang, J.-W., Kim, S.-K., Jeon, Y.-J., Je, J.-Y., Ahn, C.-B., Moon, S.-H., Jeon, B.-T. and Park, P.-J. 2014. Chitooligosaccharide and its derivatives: preparation and biological applications. BioMed Research International. 2014: 1-13.

Mao, L. and Wu, T. 2007. Gelling properties and lipid oxidation of kamaboko gels from grass carp (*Ctenopharyngodon idellus*) influenced by chitosan. Journal of Food Engineering. 82: 128-134.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31: 426-428.

No, H. K., Young Park, N., Ho Lee, S. and Meyers, S. P. 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. International Journal of Food Microbiology. 74: 65-72.

Olatunde, O. O., Benjakul, S. and Vongkamjan, K. 2018. Antioxidant and antibacterial properties of guava leaf extracts as affected by solvents used for prior dechlorophyllization. Journal of Food Biochemistry. https://doi.org/10.1111/jfbc.12600

Pan, S. and Wu, S. 2011. Preparation of water-soluble chitosan by hydrolysis with commercial glucoamylase containing chitosanase activity. European Food Research and Technology. 233: 325-329.

Park, P.-J., Lee, H.-K. and Kim, S.-K. 2004. Preparation of hetero-chitooligosaccharides and their antimicrobial activity on Vibrio parahaemolyticus. Journal of Microbiology and Biotechnology. 14: 41-47.

Rokhati, N., Widjajanti, P., Pramudono, B. and Susanto, H. 2013. Performance comparison of α-and β-amylases on chitosan hydrolysis. ISRN Chemical Engineering. 2013: 0-5.

Roncal, T., Oviedo, A., de Armentia, I. L., Fernández, L. and Villarán, M. C. 2007. High yield production of monomer-free chitosan oligosaccharides by pepsin catalyzed hydrolysis of a high deacetylation degree chitosan. Carbohydrate Research. 342: 2750-2756.

Sae-Leaw, T., Buamard, N., Vate, N. K. and Benjakul, S. 2018. Effect of squid melanin-free ink and pre-emulsification on properties and stability of surimi

gel fortified with seabass oil during refrigerated storage. Journal of Aquatic Food Product Technology. 1-15.

Sallam, K. I. 2007. Chemical, sensory and shelf life evaluation of sliced salmon treated with salts of organic acids. Food Chemistry. 101: 592-600.

Senphan, T. and Benjakul, S. 2014. Antioxidative activities of hydrolysates from seabass skin prepared using protease from hepatopancreas of Pacific white shrimp. Journal of Functional Foods. 6: 147-156.

Singh, A. and Benjakul, S. 2017. Effect of serine protease inhibitor from squid ovary on gel properties of surimi from Indian mackerel. Journal of Texture Studies. 48: 541-549.

Singh, A. and Benjakul, S. 2017. Serine protease inhibitors from squid ovary: extraction and its effect on proteolysis and gel properties of surimi. Journal of Food Science and Technology. 54: 267-275.

St. Angelo, A. J., Vercellotti, J., Jacks, T. and Legendre, M. 1996. Lipid oxidation in foods. Critical Reviews in Food Science and Nutrition. 36: 175-224.

Steel, R. G. D. and Torrie, J. H. 1986. Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill.

Tomida, H., Fujii, T., Furutani, N., Michihara, A., Yasufuku, T., Akasaki, K., Maruyama, T., Otagiri, M., Gebicki, J. M. and Anraku, M. 2009. Antioxidant properties of some different molecular weight chitosans. Carbohydrate Research. 344: 1690-1696.

Tongnuanchan, P., Benjakul, S. and Prodpran, T. 2012. Properties and antioxidant activity of fish skin gelatin film incorporated with citrus essential oils. Food Chemistry. 134: 1571-1579.

Wu, S. 2011. Preparation of water soluble chitosan by hydrolysis with commercial α-amylase containing chitosanase activity. Food Chemistry. 128: 769-772.

Younes, I., Sellimi, S., Rinaudo, M., Jellouli, K. and Nasri, M. 2014. Influence of acetylation degree and molecular weight of homogeneous chitosans on antibacterial and antifungal activities. International Journal of Food Microbiology. 185: 57-63.

Zhang, L., Zeng, Y. and Cheng, Z. 2016. Removal of heavy metal ions using chitosan and modified chitosan: A review. Journal of Molecular Liquids. 214: 175-191.

CHAPTER 10

SARDINE SURIMI GEL ADDED WITH SQUID PEN CHITOOLIGOSACCHARIDE: PROPERTIES AND THEIR CHANGES DURING REFRIGERATED STORAGE

10.1 Abstract

Effect of chitooligosaccharide from squid pen prepared using lipase (COS-L) at various concentrations (0-3%) on gel properties of sardine surimi gel was investigated. Breaking force and deformation of gel were increased, when COS-L level was increased up to 1% ($p<0.05$). However, both parameters were decreased when COS-L of 1.5-3% was added. Water holding capacity of gel was increased as COS-L was added up to 2% ($p<0.05$). Incorporation of COS-L slightly increased whiteness of gel. Gel containing 1% COS-L had higher hardness, cohesiveness gumminess, springiness, and chewiness, compared with the control gel. Gel added with 1% COS-L had the denser network with higher connectivity than the control. COS-L increased likeness score for all sensory attributes of gel as compared to control. When gel incorporated with 1% COS-L was stored at 4 °C, breaking force, deformation and whiteness were maintained during 10 days of storage. Based on textural profile analysis, properties of surimi gel added with COS-L were shown to be higher than those of control throughout the storage. Thus, incorporation of 1% COS-L could improve gel properties of sardine surimi gel and retarded the deterioration of gel properties during refrigerated storage.

10.2 Introduction

Chitooligosaccharide (COS) has been gaining interest in pharmaceutical, medicinal and food applications due to their non-toxicity and high solubility (Lodhi *et al.,* 2014). COS is the hydrolyzed product of chitosan with varying molecular weight (MW). COS is generally produced by physical, chemical or enzymatic methods, however physical and chemical methods impair physicochemical and functional properties of COS (Lodhi *et al.,* 2014; Liang *et al.,* 2018). Therefore,

enzymatic method has been used more widely to produce low MW COS with the controlled degree of depolymerization (Lee *et al.*, 2008; Lodhi *et al.*, 2014). Since chitosanase and chitinase are less available and have high price, chitosan has been hydrolyzed using non-specific enzymes such as pectinase, lipase, proteases, amylase and cellulase (Mourya *et al.*, 2011).

Squid pen, a byproduct generated from squid processing industry, contains β-form of chitin. β-chitin has high reactivity toward various solvent due to looser confirmations than α-chitin (Elieh-Ali-Komi and Hamblin, 2016). β-chitin can be transformed to chitosan or COS with appropriate processes (Elieh-Ali-Komi and Hamblin, 2016). Owing to the high solubility of COS in comparison with chitosan, COS could be applied as food additives more efficiently. COS has been known to possess antibacterial activity (Olatunde and Benjakul, 2018) and antioxidant activity (Lodhi *et al.*, 2014; Laokuldilok *et al.*, 2017).

Surimi is washed fish mince rich in myofibrillar proteins and has unique gelling properties. It is used to produce several products (Singh and Benjakul, 2017). Generally, white fleshed fish such as bigeye snapper etc. are main source for surimi manufacturing, because of their superior properties than those made from dark fleshed fish. However, some surimi especially dark fleshed fish possessed lower gel ability, thus lowering their acceptability and market value. To enhance the gel properties, a variety of additives with different functions have been incorporated into surimi. Some of them inhibit proteolysis (Quan and Benjakul, 2017; Singh and Benjakul, 2018), or induce protein cross-linking (Vate *et al.*, 2015; Sae-Leaw *et al.*, 2018). Phenolic compounds or hydrocolloids such as gellan have been used to enhance gel properties surimi (Buamard *et al.*, 2017; Petcharat and Benjakul, 2018). Nevertheless, some additives impose the adverse effect on gel properties and religious constraints limits their applications (Singh and Benjakul, 2017). Therefore, surimi industry still required the alternative food grade additives. Chitosan was incorporated to enhance gel properties of surimi (Mao and Wu, 2007; Amiza and Kang, 2013). Nevertheless, its solubility in acidic condition limits its use in surimi gel. On the other

hand, COS is water soluble and possesses bioactivities (Lodhi *et al.*, 2014). Thus, it can be used as the novel additive for surimi gel without limitation.

10.3 Objective

To determine the impact of COS from squid pen on gel properties of sardine surimi gel and to study the changes in properties of surimi gel added with COS at 4 °C for 10 days.

10.4 Materials and Methods

10.4.1 Chemicals and surimi

All chemicals were of analytical grade. Lipase (300 unit/mg protein) was acquired from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

Frozen sardine (*Sardinella albella*) surimi (AA grade) was procured from Chaichareon Marine Co., Ltd. (Pattani, Thailand). Surimi was stored at −20°C and used within two months.

10.4.2 Preparation of chitooligosaccharide

Twenty grams of chitosan from squid pen (degree of deacetylation of 87%) was mixed with 2 L of 1% acetic acid and stirred for overnight. The pH of chitosan solution was adjusted to 5 with 6 M NaOH. Lipase (8%) was added to chitosan solution followed by incubation at 50 °C for 12 h with constant stirring. The hydrolysis was terminated by heating the solution at 95 °C for 10 min. The pH of hydrolysate was adjusted to neutral with 6 M NaOH, followed by vacuum filtration. The resultant filtrate was freeze-dried using a freeze drier (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The obtained powder was named as 'COS-L'. The intrinsic viscosity and average MW of COS-L were 0.41 dL/g and 79 kDa, respectively, as measured using the method of Yacob *et al.* (2013). Water solubility was 49% as calculated following the procedure of Laokuldilok *et al.* (2017).

10.4.3 Impact of COS-L at different levels on gel properties of sardine surimi gel

10.4.3.1 Surimi gel preparation

Surimi gel added without and with COS-L at different concentrations (0.5-3%, w/w) was prepared as described in section 6.4.6.1.

10.4.3.2 Analysis

10.4.3.2.1 Textural analysis

Breaking force and deformation of surimi gel were determined as described in section 6.4.7.1.

10.4.3.2.2 Determination of whiteness and expressible moisture content

Gel samples were subjected to whiteness and expressible moisture content determination as previously described in sections 6.4.7.2 and 6.4.7.3, respectively.

10.4.3.2.3 Textural profile analysis (TPA)

TPA of surimi gel was done using a texture analyzer as described in section 7.4.4.2.5.

10.4.3.2.4 Microstructure

Microstructures of gel without and with 1% COS-L were examined using a SEM as described in section 7.4.5.2.

10.4.3.2.5 Sensory analysis

Gel samples incorporated without and with of 1% COS-L were assessed for the sensory attributes as previously described in section 7.4.5.3.

10.4.4 Effect of COS-L addition on properties of sardine surimi gel during refrigerated storage

Surimi gel was prepared without and with 1% COS-L as described previously. All the gels were placed in a zip lock bag and kept for 10 days at 4 °C. Gels were randomly taken at 0, 5, and 10 days and subjected to textural analysis, whiteness and TPA determination as described previous sections.

10.4.5 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance. Comparison of means was carried out by the Duncan's multiple range tests. T-test was used for pair comparison (Steel and Torrie, 1986). Analysis was performed using a SPSS package (SPSS 22 for Windows, SPSS Inc., Chicago, IL, USA)

10.5 Results and discussion

10.5.1 Effect of COS-L on textural properties of sardine surimi gel

10.5.1.1 Breaking force and deformation

Breaking force and deformation of sardine surimi gel added with COS-L at various levels (0-3%, w/w) are presented in Figure 37A and B, respectively. The lowest values of Breaking force and deformation were noted for the control (without COS-L addition) ($p<0.05$). Breaking force and deformation were increased with increasing level of COS-L up to 1% ($p<0.05$). Nevertheless, both values were decreased, when COS-L levels were further increased ($p<0.05$). Breaking force and deformation of gel containing 1% COS-L were increased by 52 and 43%, as compared to those of the control gel, respectively. It was inferred that COS-L at an appropriate level had a potential to strengthen the surimi gel. This was more likely due to the increased cross-linkage between myofibrils bridged by amino groups at carbon number 2 of glucosamine present in COS-L. With increasing bridges or connections, the three-dimensional gel network was strengthened. Furthermore, setting (40 °C) activated endogenous transglutaminase (TGase), which resulted in the

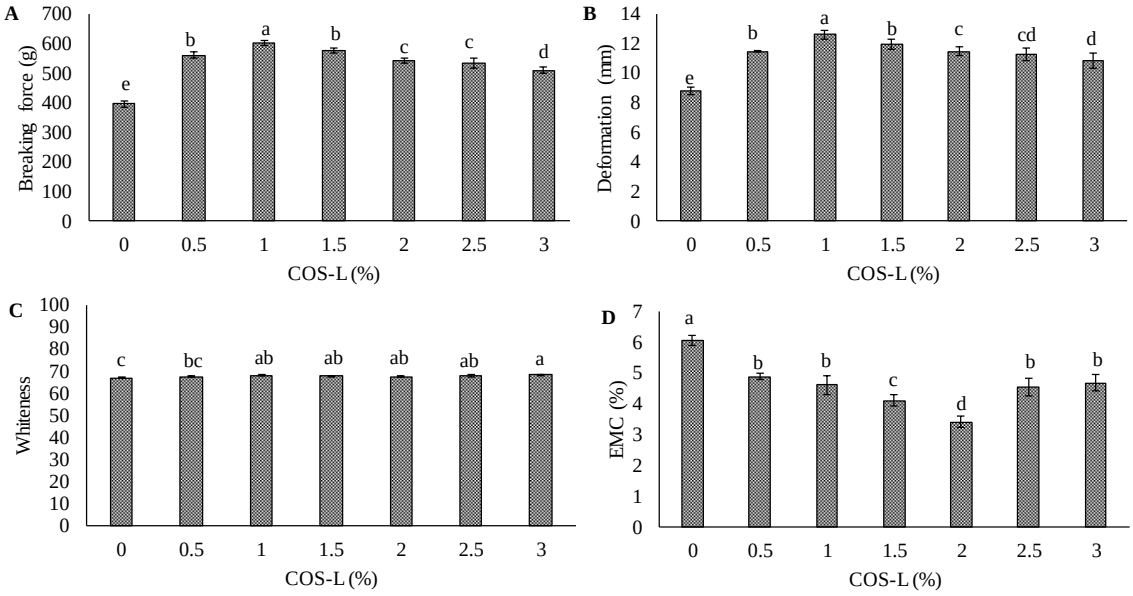

Figure 37. Breaking force (A) deformation (B) whiteness (C) and expressible moisture content (D) of gels from sardine surimi in the absence and presence of COS-L from squid pen at different levels. Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicate significant difference ($p<0.05$)

formation of ε-(γ-glutamyl) lysine cross-linking, thus strengthening the gel network (Singh and Benjakul, 2017). Additionally, amino group of glucosamine could act as acyl acceptor for glutaminyl residue (acyl donor) resulting in the formation of protein-COS-L conjugates mediated by TGase (Benjakul *et al.*, 2001). Nevertheless, excessive amount of COS-L plausibly disrupted the aggregation and polymerization of myofibrils, a major contributor for gelation, during either setting or heating. Additionally, COS-L at high concentration might dilute myofibrillar proteins, thus reducing the aggregation of proteins. This resulted in a less ordered and weaker gel network (Petcharat and Benjakul, 2017). Amiza and Kang (2013) reported that gel of surimi from African catfish added with 1.5% commercial food grade chitosan (MW 10 kDa) had the increase in gel strength by 58.92%. Thus, COS-L at a suitable level could enhance gel properties of sardine surimi gel.

10.5.1.2 Whiteness

Whiteness of gel as affected by various levels of COS-L is revealed in Figure 37C. No difference was observed in whiteness between gel added without and with 0.5% COS-L ($p>0.05$). Whiteness was increased with addition of COS-L at 10 g/kg or higher levels ($p<0.05$). On the other hand, no difference in whiteness among gels containing COS-L at concentration ranging from 1 to 3% was noted ($P>0.05$). Generally, whiteness is governed by amount, type and presence of indigenous pigments or additives (Singh and Benjakul, 2017). Improved whiteness of surimi gel with the addition of COS-L was more likely due to interaction of water molecules with protein and COS-L. Denser gel network caused by protein-COS-L interaction plausibly resulted in the increased light scattering, which produced more lustrous and transparent gel. Additionally, white color of COS-L partially contributed to the higher whiteness of gel sample. Mao and Wu (2007) observed the increased whiteness of gel from grass carp surimi, when commercial chitosan of high MW (300 kDa) or low MW (10 kDa) was added at the level of 0.5 or 1%. Similarly, whiteness of surimi gel from African catfish was increased by 13% with addition of 1.5% low MW (10 kDa) food grade chitosan.

10.5.1.3 Expressible moisture content (EMC)

The decreased EMC of sardine surimi gel was observed at all levels (0.5-3%, w/w) of COS-L added ($p<0.05$) (Figure 37D). Lower EMC was observed for gel added with 2% COS-L ($p<0.05$), whereas the maximum EMC was detected for the control ($p<0.05$). EMC of gel was decreased by 19-43%, when COS-L was incorporated into gel, as compared to that of control. Result indicated the capacity of COS-L to augment water holding ability of gel. During gelation, three-dimensional gel network formed by myofibrillar protein imbibe water more efficiently (Rawdkuen *et al.*, 2004). COS-L rich in OH- and NH_2 groups more likely enhanced water binding ability of resulting gels. COS-L had potential to bind water via hydrogen bonding due to the hydroxyl group at carbon 3 and 6 of glucosamine (Lodhi *et al.*, 2014). Ability

of COS-L in improving water holding capacity synergistically improved the property of sardine surimi gel.

10.5.1.4 Textural profile

TPA parameters of gel added with COS-L at different levels are shown in Table 24. Hardness, cohesiveness, springiness, chewiness and gumminess of gel containing COS-L were higher as compared to those of the control gel (without COS-L) ($p<0.05$). Hardness is the applied force to compress gel to attain a certain deformation. It was increased when COS-L was added to gel up to the level of 1% ($p<0.05$), however the lower value of hardness was obtained when higher levels of COS-L (1.5-3%) were used ($p<0.05$). The maximum value of hardness was plausibly owing to formation of firmer gel network. The improved hardness was coincidental with enhanced breaking force of gel incorporated with COS-L (Figure 37A). The decrease in hardness, especially with the addition of COS-L at higher levels, was more likely due to dilution effect of COS-L towards myofibrillar proteins. Cohesiveness is ability to deform the internal gel structure and gumminess reflects the energy needed to breakdown a semi- solid gel ready for swallowing. Both parameters showed the similar trend with hardness. Cohesiveness was increased with addition of COS-L up to 1%. Thereafter cohesiveness was decreased when higher levels of COS-L was incorporated ($p<0.05$). No difference in cohesiveness was obtained for gel added with COS-L in the range of 1.5-2.5% ($p>0.05$). Among all the gels added with COS-L, the lowest value was obtained for the sample containing 3% COS-L ($p<0.05$). For gumminess, the maximum value was observed for gel added with 1%COS-L ($p<0.05$). However, gumminess was decreased with further increase in COS-L. The lowest value was obtained for gel containing 3% COS-L, when compared with the gel added with the lower levels of COS-L ($p<0.05$). Similar result was obtained for chewiness, energy needed to masticate the gel to the point needed for swallowing. The maximum value was obtained for gel containing 1% COS-L ($p<0.05$). The decrease was detected when 3% COS-L was added to surimi gel ($p<0.05$). Springiness, elastic recovery after the compressive force is removed, was increased as COS-L was added up to 1%. With further increasing levels of COS-L, lower

springiness was noticeable ($p<0.05$). COS-L at a proper level could form protein-COS-L conjugates. Therefore, bondings between COS-L and myofibrillar protein might strengthen the network of surimi gel.

Accordingly, COS-L at 1% (w/w) showing the highest efficacy in enhancement of gel properties of sardine surimi gel was selected for the further study.

Table 24. Textural properties of sardine surimi gel added without and with COS-L from squid pen at different levels.

Levels (%)	Hardness (g)	Springiness (g)	Cohesiveness (g)	Gumminess (g)	Chewiness (g)
0	6957.96±93.78^{e}	0.68±0.1^{e}	0.52±0.01^{d}	4711.79±135.23^{d}	3984.859±34.76^{e}
0.5	7558.06±18.11^{b}	0.81±0.01^{b}	0.71±0.00^{b}	5802.78±107.22^{b}	5280.35±51.40^{b}
1.0	7936.33±96.98^{a}	0.91±0.02^{a}	0.74±0.01^{a}	6773.90±42.59^{a}	6140.88±41.86^{a}
1.5	7424.68±75.75^{b}	0.80±0.02^{b}	0.72±0.01^{b}	5712.29±108.62^{b}	5273.59±66.02^{b}
2.0	7218.95±51.26^{c}	0.80±0.00^{b}	0.72±0.02^{b}	5473.98±104.22^{bc}	5119.42±34.61b^{c}
2.5	7223.79±57.59^{c}	0.79±0.02^{bc}	0.71±0.00^{b}	5459.50±183.45^{bc}	5080.98±47.62^{c}
3.0	7125.96±49.23^{d}	0.78±0.1^{d}	0.70±0.00^{c}	5247.09±110.05^{c}	4893.28±95.52^{d}

Values are expressed as mean ± SD (n = 3). Different lowercase superscripts in the same column indicate significant differences ($p<0.05$).

10.5.1.5 Microstructure

Microstructure of gels added without and with 1% COS-L was visualized by SEM as shown in Figure 38. Control gel (without COS-L) had a coarser network with coagulated proteins distributed in three-dimensional network, whereas gel containing COS-L had higher connectivity between protein strands, resulting in the formation of finer gel network. Gel added with COS-L showed smaller voids as compared to the control. COS-L might be associated with myofibrillar proteins via COS-L-protein conjugate, or isopeptide induced by indigenous TGase. Ionic interaction between amino groups of glucosamine and the anionic groups of surimi protein might not be a major factor contributing to gel network(Benjakul *et al.*, 2001).

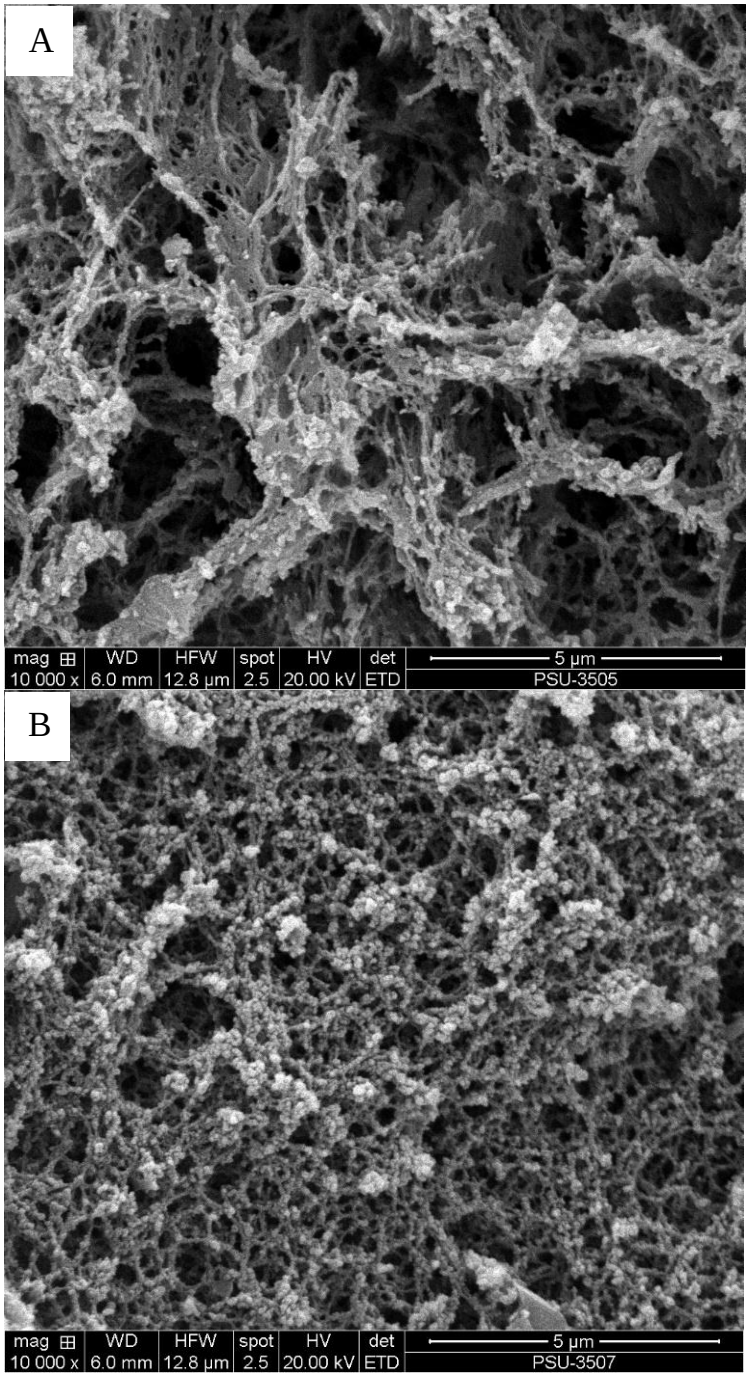

Figure 38. Scanning electron microscopic image of sardine surimi without (A) and with 1% COS-L from squid pen (B). magnification: 10,000X

This was probably due the lower difference in pk_a value of amino group (6.3) of glucosamine and pH of surimi paste (7-8). However, uncharged amino groups of COS-L plausibly act as acyl acceptors in the acyl transfer reaction induced by TGase and contributed to gel network strengthening via protein-COS-L conjugation. As a result, addition of COS-L effectively improved textural properties and water holding capacity of surimi gel.

10.5.1.6 Likeness score

Likeness score of sardine surimi gel incorporated without and with 1% COS-L is shown in Table 25. Higher likeness score was obtained for appearance, color, texture, and overall attribute for gel containing 1% COS-L, as compared to the control ($p<0.05$). The result was in line with the improved texture, whiteness and lower EMC of gels added with COS-L (Figure 37A-D). Gel containing COS-L had higher odor and taste likeness score than the control ($p<0.05$). Generally, sardine surimi is dark fleshed fish and has high susceptibility to lipid oxidation, which causes off-flavor and off-odor of resulting products during processing, particularly during off-flavor and off-odor of resulting products during processing, particularly during heat-induced gelation. Singh *et al.* (2018) reported that COS-L possessed excellent antioxidant activity and have potential to inhibit lipid oxidation of sardine surimi gel during storage at 4 °C for 10 days. Therefore, COS-L might inhibit

Table 25. Likeness score of sardine surimi gel without and with 1% COS-L from squid pen.

Sample	Appearance	Color	Odor	Texture	Taste	Overall
Control	7.65 ± 0.26^{b}	7.52 ± 0.21^{b}	7.33 ± 0.27^{a}	7.59 ± 0.31^{b}	7.45 ± 0.23^{a}	7.21 ± 0.52^{b}
COS	7.94 ± 0.37^{a}	8.00 ± 0.45^{a}	7.71 ± 0.14^{a}	8.06 ± 0.20^{a}	7.71 ± 0.15^{a}	7.99 ± 0.47^{a}

Values are expressed as mean ± SD (n = 80). Different lowercase superscripts in the same column indicate significant differences ($p<0.05$).

the lipid oxidation during surimi gel preparation. Hence, the incorporation of 1% COS-L was able to improve the sensory properties and consumer acceptability of surimi gel.

10.5.2 Effect of COS-L on properties of sardine surimi gel during refrigerated storage

10.5.2.1 Breaking force and deformation

Breaking force and deformation of gel added without and with 1% COS-L during 10 days storage at 4 °C are depicted in Figure 39A and B, respectively. Higher breaking force and deformation were observed for gel containing 1% COS-L than the control gel (without COS-L) during the storage of 10 days ($p<0.05$). No difference was observed in breaking of gel containing 1% COS-L at day 0 and 5 ($p>0.05$), however breaking force was decreased by 5% at day 10 ($p<0.05$). There was no difference in deformation of gel containing COS-L during 10 days of storage ($p>0.05$). Breaking force and deformation of control gel were increased by 10 and 14% at day 5 of storage, respectively ($p<0.05$). However, breaking force and deformation were decreased by 19 and 18%, after 10 days of storage, compared to that obtained at day 0. Increased breaking force of control gel at day 5 was more likely owing to water loss, thus increasing the solid content of control gel. Additionally, lipid oxidation products might lead to cross-linking of proteins, which strengthened gel network. Sae-Leaw *et al.* (2018) reported that sardine surimi fortified with seabass oil showed the increased breaking force with increasing storage days, mainly mediated by lipid oxidation products. No increase in breaking force of gel added with COS-L was observed at day 5 which is more likely due to antioxidative activity of COS-L. At day 10, the decrease in breaking force of control gel (19%) was greater than that of gel containing COS-L (5%). Degradation of myofibrillar proteins in surimi gel caused by psychrophilic bacteria during refrigerated storage might lead to the lowered breaking force (Benjakul *et al.*, 2005). Proteases from microflora might result in proteolysis of myofibrillar protein and produced gel with lower breaking force during storage for 10 days. Singh *et al*. (2018) documented the

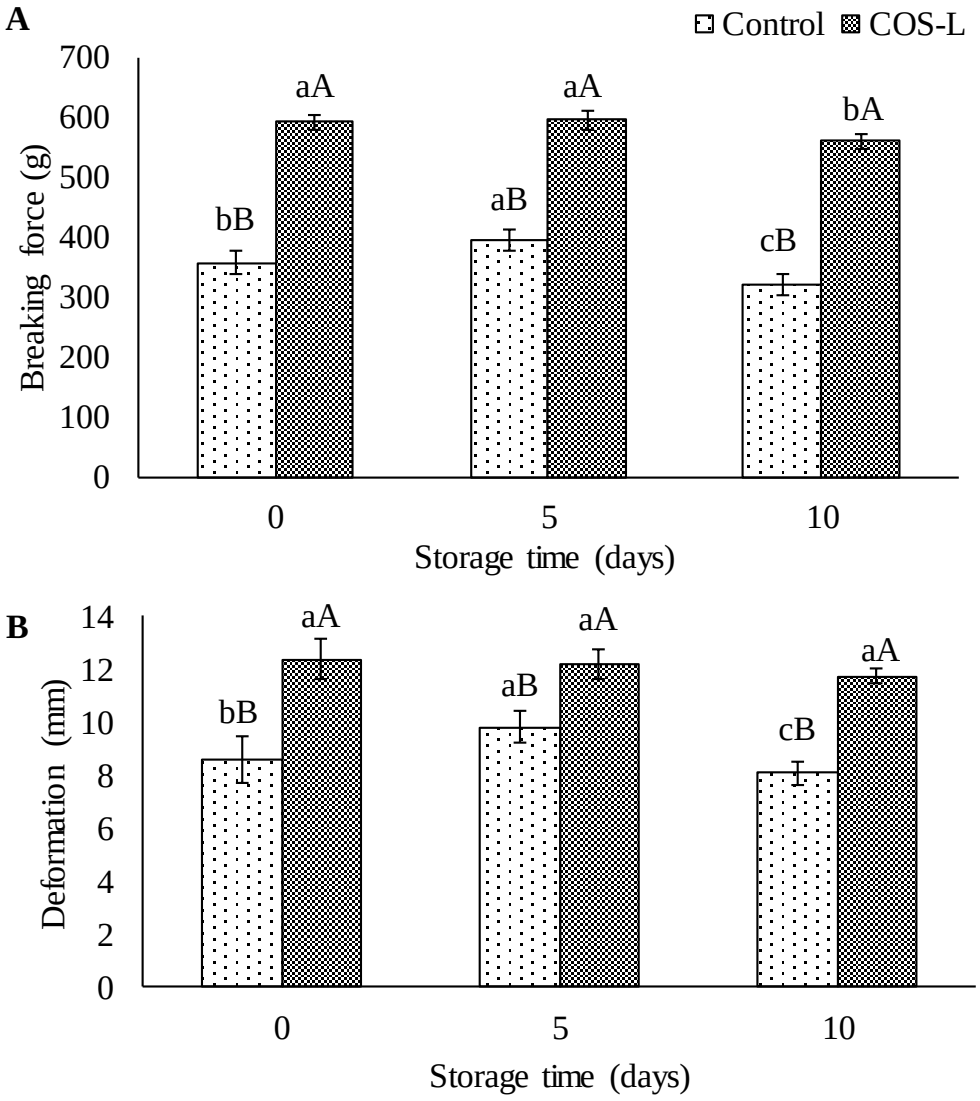

Figure 39. Breaking force (A) and deformation (B) of gels from sardine surimi in the absence and presence of 1% COS-L from squid pen during refrigerated storage at 4 °C for 10 days. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same sample indicate significant difference ($p<0.05$). Different uppercase letters on the bars within the same storage time indicated significance different ($p<0.05$).

retarded microbial growth of sardine surimi gel containing COS-L during 10 days of storage. COS-L with antibacterial activity might inhibit spoilage bacteria, thus preventing proteolysis and maintaining the strong network. Therefore, addition of COS-L efficiently enhanced and maintained the textural characteristics of sardine surimi gel throughout the storage at 4 °C of 10 days.

10.5.2.2 Whiteness

Whiteness of gel incorporated without and with 1% COS-L during the refrigerated storage is depicted in Table 26. Higher whiteness was detected for gel added with COS-L than the control at all storage time ($p<0.05$). No difference in whiteness of gel without COS-L was noticeable between day 0 and 5 ($P>0.05$), however whiteness was reduced with further increase in storage time (day 10) ($p<0.05$). Non-enzymatic browning due to reaction between lipid oxidation products and amino groups of surimi protein mediated by spoilage microorganisms (Sae-Leaw *et al.*, 2018) more likely contributed to the discoloration of surimi gel. Gel containing COS-L had no difference in whiteness during the storage of 10 days ($p>0.05$). The result suggested that COS-L might inhibit lipid oxidation and spoilage of gel during extended storage. Therefore, addition of COS-L could retain whiteness of surimi gel during storage at 4 °C.

10.5.2.3 Textural profile

TPA parameters of gel added without and with 1% COS-L as influenced by storage time are shown in Table 26. All TPA parameter tested showed higher value for gel containing COS-L as compared to the control gel at the same day of storage ($p<0.05$). There was no difference in hardness of gel at day 0 and 5 ($p>0.05$), but the decrease in hardness was noted at day 10 ($p<0.05$) The results were in agreement with breaking force of surimi gel during storage (Figure 39A). The lowest springiness, cohesiveness gumminess and chewiness of gel containing COS-L were observed at day 10 ($p<0.05$), but no difference was noticeable between day 0 and 5 ($p>0.05$). All TPA parameters for gel without COS-L were increased with increasing storage time up to day 5. Nevertheless, the decreased values were obtained

Table 26. Whiteness and textural properties of gels from sardine surimi gel added without and with 1% COS-L from squid pen during refrigerated storage at 4 °C for 10 days.

Samples	Storage time (days)	Whiteness (%)	Hardness (g)	Springiness (g)	Cohesiveness (g)	Gumminess (g)	Chewiness (g)
Control	0	67.90 ± 0.76^{aB}	6923.42 ± 33.33^{bB}	0.69 ± 0.1^{bB}	0.51 ± 0.01^{bB}	4730.52 ± 27.34^{bB}	3819.98 ± 69.38^{bB}
	5	67.45 ± 0.57^{aB}	7042.84 ± 39.80^{aB}	0.70 ± 0.01^{aB}	0.52 ± 0.00^{aB}	4998.80 ± 61.12^{aB}	4014.98 ± 44.78^{aB}
	10	66.84 ± 0.19^{bB}	6846.43 ± 52.94^{cB}	0.67 ± 0.1^{cB}	0.50 ± 0.00^{cB}	4619.74 ± 17.18^{cB}	3620.11 ± 53.65^{cB}
COS-L	0	68.72 ± 0.47^{aA}	7983.54 ± 35.55^{aA}	0.93 ± 0.01^{aA}	0.72 ± 0.00^{aA}	6850.39 ± 43.73^{aA}	6227.36 ± 49.97^{aA}
	5	68.89 ± 0.89^{aA}	8015.21 ± 54.33^{aA}	0.92 ± 0.01^{aA}	0.73 ± 0.01^{aA}	6859.63 ± 45.51^{aA}	6236.46 ± 47.65^{aA}
	10	67.70 ± 0.47^{aA}	7859.19 ± 27.91^{bA}	0.91 ± 0.00^{bA}	0.71 ± 0.00^{bA}	6735.90 ± 61.56^{bA}	6152.30 ± 43.80^{bA}

Values are expressed as mean ± SD (n=3). Different lowercase superscripts within the same sample in the same column indicate significant differences ($p<0.05$). Different uppercase superscripts within the same storage time in the same column indicate significant differences ($p<0.05$).

after 10 days of storage ($p<0.05$). Within the first 5 days, the increased values of hardness, springiness, gumminess, cohesiveness and chewiness were more probably due to moisture loss and cross-linking of proteins induced by secondary products generated during lipid oxidation. The decreases in textural properties were plausibly owing to gel weakening mediated by microbial proteases. The result was in line with the decrease in breaking force of gel without COS-L (Figure 39A). Therefore, the improved textural properties of gel added with COS-L throughout storage were more likely governed by antioxidative and antibacterial activity of COS-L (Singh *et al.*, 2018).

10.6 Conclusion

Surimi gel added with COS-L at 1% had the increased gel strength. Whiteness and water holding capacity were also improved. Addition of 1% COS-L could lower deterioration of gel properties during extended storage at 4 °C for 10 days. Therefore, COS-L could be used as a potential alternative food grade additive for surimi without the adverse effect on sensory characteristics.

10.7 References

Amiza, M. and Kang, W. 2013. Effect of chitosan on gelling properties, lipid oxidation, and microbial load of surimi gel made from African catfish (*Clarias gariepinus*). International Food Research Journal. 20: 1585.

Benjakul, S., Visessanguan, W., Tanaka, M., Ishizaki, S., Suthidham, R. and Sungpech, O. 2001. Effect of chitin and chitosan on gelling properties of surimi from barred garfish (*Hemiramphus far*). Journal of the Science of Food and Agriculture. 81: 102-108.

Benjakul, S., Visessanguan, W., Thongkaew, C. and Tanaka, M. 2005. Effect of frozen storage on chemical and gel-forming properties of fish commonly used for surimi production in Thailand. Food Hydrocolloids. 19: 197-207.

Buamard, N., Benjakul, S. and Konno, K. 2017. Improvement of gel quality of sardine surimi with low setting phenomenon by ethanolic coconut husk extract. Journal of Texture Studies. 48: 47-56.

Elieh-Ali-Komi, D. and Hamblin, M. R. 2016. Chitin and chitosan: production and application of versatile biomedical nanomaterials. International Journal of Advanced Research. 4: 411-427.

Laokuldilok, T., Potivas, T., Kanha, N., Surawang, S., Seesuriyachan, P., Wangtueai, S., Phimolsiripol, Y. and Regenstein, J. M. 2017. Physicochemical, antioxidant, and antimicrobial properties of chitooligosaccharides produced using three different enzyme treatments. Food Bioscience. 18: 28-33.

Lee, D.-X., Xia, W.-S. and Zhang, J.-L. 2008. Enzymatic preparation of chitooligosaccharides by commercial lipase. Food Chemistry. 111: 291-295.

Liang, S., Sun, Y. and Dai, X. 2018. A review of the preparation, analysis and biological functions of chitooligosaccharide. International Journal of Molecular Sciences. 19:

Lodhi, G., Kim, Y.-S., Hwang, J.-W., Kim, S.-K., Jeon, Y.-J., Je, J.-Y., Ahn, C.-B., Moon, S.-H., Jeon, B.-T. and Park, P.-J. 2014. Chitooligosaccharide and its derivatives: preparation and biological applications. BioMed Research International. 2014:

Mao, L. and Wu, T. 2007. Gelling properties and lipid oxidation of kamaboko gels from grass carp (*Ctenopharyngodon idellus*) influenced by chitosan. Journal of Food Engineering. 82: 128-134.

Mourya, V. K., Inamdar, N. N. and Choudhari, Y. M. 2011. Chitooligosaccharides: synthesis, characterization and applications. Polymer Science Series A. 53: 583-612.

Olatunde, O. O. and Benjakul, S. 2018. Natural preservatives for extending the shelf-life of seafood: a revisit. Comprehensive Reviews in Food Science and Food Safety. 17: 1595-1612.

Petcharat, T. and Benjakul, S. 2017. Effect of gellan and calcium chloride on properties of surimi gel with low and high setting phenomena. RSC Advances. 7: 52423-52434.

Petcharat, T. and Benjakul, S. 2018. Effect of gellan incorporation on gel properties of bigeye snapper surimi. Food Hydrocolloids. 77: 746-753.

Quan, T. H. and Benjakul, S. 2017. Comparative study on the effect of duck and hen egg albumens on proteolysis and gel property of sardine surimi. International Journal of Food Properties. 20: S2786-S2797.

Rawdkuen, S., Benjakul, S., Visessanguan, W. and Lanier, T. C. 2004. Chicken plasma protein affects gelation of surimi from bigeye snapper (*Priacanthus tayenus*). Food Hydrocolloids. 18: 259-270.

Sae-Leaw, T., Buamard, N., Vate, N. K. and Benjakul, S. 2018. Effect of squid melanin-free ink and pre-emulsification on properties and stability of surimi gel fortified with seabass oil during refrigerated storage. Journal of Aquatic Food Product Technology. 1-15.

Singh, A. and Benjakul, S. 2017. Effect of serine protease inhibitor from squid ovary on gel properties of surimi from Indian mackerel. Journal of Texture Studies. 48: 541-549

Singh, A. and Benjakul, S. 2017. Serine protease inhibitors from squid ovary: extraction and its effect on proteolysis and gel properties of surimi. Journal of Food Science and Technology. 54: 267-275.

Singh, A. and Benjakul, S. 2018. Proteolysis and its control using protease inhibitors in fish and fish products: a review. Comprehensive Reviews in Food Science and Food Safety. 17: 496-509.

Singh, A., Benjakul, S. and Prodpran, T. (2018). Chitooligosaccharides from squid pen prepared using different enzymes: characteristics and the effect on quality of surimi gel during refrigerated storage. Food Chemistry (submitted).

Steel, R. G. D. and Torrie, J. H. 1986. Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill.

Vate, N. K., Benjakul, S. and Agustini, T. W. 2015. Application of melanin- free ink as a new antioxidative gel enhancer in sardine surimi gel. Journal of the Science of Food and Agriculture. 95: 2201-2207.

Yacob, N., Talip, N., Mahmud, M., Sani, N., Samsuddin, N. and Fabillah, N. 2013. Determination of viscosity-average molecular weight of chitosan using

intrinsic viscosity measurement. Journal of Nuclear and Related Technologies. 10: 39-44.

CHAPTER 11

CONCLUSION AND SUGGESTION

11.1 Conclusions

1. Squid ovary (SO) had high amount of protein followed by carbohydrate. SO was rich in non-essential amino acid and n-3 PUFA. SO powder (SOP) has excellent foaming properties, especially foaming stability.
2. Squid ovary hydrolysate (SOH) produced using 1% Alcalase for 30 min (SOH-Al-30) showed the improved foaming properties associated with high solubility and hydrophobicity. Further pre-heating could enhance foaming capacity and foam stability of SOH-Al-30.
3. Ultrasonication at amplitude 70% for 30 min increased the hydrophobicity and foaming capacity of SOP. Highest foaming stability of ultrasonicated SOP (USOP) was also obtained.
4. Addition of USOP yielded a cake with strong and porous matrix. Cake batter added with USOP had better viscoelastic properties than that of cake containing EWP. Cake added with USOP had higher moisture and darker color with increased overall consumer acceptance.
5. Serine protease inhibitor from squid ovary (SOSPI) could be extracted using 0.45 M NaCl for 1 h followed by heating at 70 °C for 10 min. Protease inhibitors with apparent MW of 9.10 and 10.27 kDa inhibited the proteolysis of surimi paste from bigeye snapper.
6. SOSPI at 2% rendered Indian mackerel surimi gel with highest breaking force and improved water holding capacity and textural properties. Although EWP showed higher capacity to improve gel properties of surimi than the SOSPI,

SOSPI could be used as an alternative food grade additive for surimi industry without negative impact on sensory properties.

7. Chitin from squid pen could be prepared with the aid of ultrasonication (41.46 min at an amplitude 69% and 1:18 of solid: solvent ratio). DDA of chitosan produced from chitin under different temperatures and times was varied in the range 78-90%. Chitosan produced at 130 °C for 2 h could be use as dietary fibre for human consumption to inhibit fat absorption.
8. Chitooligosaccharide (COS) produced from squid pen chitosan using lipase named COS-L showed the highest antioxidative activity as compared to those prepared using amylase and pepsin. Addition of 1% COS-L increased gel strength, whiteness and water holding capacity of gel from sardine surimi.
9. COS-L at 1% could lower deterioration of gel properties and lipid oxidation as well as growth of spoilage bacteria and fungus in sardine surimi gel during refrigerated storage.

11.2 Suggestions

1. Hydrocolloids such xanthan, gellan, etc. are known to enhance foaming stability of various foaming agent. Therefore, effect of hydrocolloids in conjugation with SOP on foaming properties could be studied.
2. Purification and kinetic study of SOSPI should be carried out to determine kinetic mechanism of inhibitor.
3. Effect of chitosan on fat and bile absorption during *in vitro* gastrointestinal tract should be further studied.
4. Chitosan along with COS should be used to prepare edible coating for the preservation of various kinds of foods.

VITAE

Name Mr. Avtar Singh

Student ID 5811030003

Education Attainment

Degree	Name of Institution	Year of Graduation
Master of Science (Biotechnology)	Punjabi University, Patiala	2012
Bachelor of Science	Punjab University, Chandigarh	2010

Scholarship Awards during Enrolment

- Thailand's Education Hub for Southern Region of ASEAN Countries (TEH-AC) Scholarship 2014.
- Graduate School Research Support Funding for Thesis.

List of Publication and Proceedings

Publications

1. Singh, A. and Benjakul, S. 2017. Serine protease inhibitors from squid ovary: extraction and its effect on proteolysis and gel properties of surimi, Journal of Food Science and Technology, 54: 267.
2. Singh, A. and Benjakul, S. 2017. Effect of serine protease inhibitor from squid ovary on gel properties of surimi from Indian mackerel. Journal of Texture Study, 48: 541-549.
3. Singh, A. and Benjakul, S. and Kishimura, H. 2017. Characteristics and functional Properties of ovary from squid *Loligo Formosana*, Journal of Aquatic Food Product Technology, 26: 1083-1092.
4. Singh, A., Benjakul, S. and Kijroongrojana, K. 2018. Effect of ultrasonication on physicochemical and foaming properties of squid ovary powder. Food Hydrocolloids, 77: 286-296.

5. Singh, A. and Benjakul, S. 2018. Effect of partial enzymatic hydrolysis on physicochemical and foaming properties of ovary from squid *Loligo formosana*, Waste and Biomass Valorization. 10.1007/s12649-018-0348-0.
6. Singh, A., Benjakul, S., and Prodpran, T. 2018. Ultrasound-assisted extraction of chitosan from squid pen: molecular characterization and fat binding capacity. Journal of Food Science (accepted).
7. Singh, A., Benjakul, S. and Prodpran, T. 2018. Chitooligosaccharides from squid pen prepared using different enzymes: characteristics and the effect on quality of surimi gel during refrigerated storage. Food Chemistry (under review).
8. Singh, A., Benjakul, S. and Prodpran, T. 2018. Sardine surimi gel added with squid pen chitooligosaccharide: properties and their changes during refrigerated storage. LWT-Food Science and Technology (submitted).
9. Singh, A., and Benjakul S. and Karnjanapratum, S 2018. Use of ultrasonicated squid ovary powder as a replacer of egg white powder in cake. Journal of Food Science and Technology (submitted).

Review article

1. Singh, A. and Benjakul, S. 2017. Proteolysis and its control using protease inhibitors in fish and shellfish: Comprehensive Reviews in Food Science and Food Safety. 17: 496-509.