Development of Virgin Coconut Oil Emulsified Surimi Gel with Nutraceutical Properties

Asir Gani

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

Prince of Songkla University

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Author	Mr. Asir Gani
Major Program	Food Science and Technology

Major Advisor

……....
(Prof. Dr. Soottawat Benjakul)

Examining Committee:

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

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

…….....Committee
(Assoc. Prof. Dr. Saroat Rawdkuen)

……....Committee
(Dr. Thanasak Sae-leaw)

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. Asir Gani)
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. Asir Gani)

Candidate

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ABSTRACT

Effects of virgin coconut oil (VCO) at various levels (0-25%) on the properties of croaker surimi gels were studied. As the levels of VCO increased up to 15%, breaking force continuously decreased ($p < 0.05$). No differences in breaking force, deformation and fracture constant were noticeable when VCO of 15-25% was incorporated ($p > 0.05$). Based on texture profile analysis (TPA), hardness and chewiness decreased as the level of added VCO increased up to 10% ($p < 0.05$), while no marked changes were observed with the addition of 10-25% VCO ($p > 0.05$). Addition of VCO had no profound impact on springiness and cohesiveness. No remarkable change in protein pattern among all surimi gel samples was noticed, regardless of VCO levels. Lower elastic (G') as well as loss moduli (G'') of surimi paste were attained when VCO was added, compared to the control. Nevertheless, there was no marked difference in the moduli among samples containing VCO at all levels. Whiteness of surimi gel increased, whereas expressible moisture content decreased as VCO levels increased ($p < 0.05$). Microstructure study revealed that VCO droplets were distributed uniformly in gel network. Overall likeness of surimi gel was also increased for gel added with VCO.

To minimize the interfering effect of VCO on gel properties, pre-emulsified VCO was incorporated in the form of VCO nanoemulsion. Nanoemulsion based on 5% virgin coconut oil (VCO) was prepared with the aid of ultrasound using sodium caseinate (2 and 3%) as emulsifier for various ultrasonication times (5-20 min). The smallest average particle size of resulting nanoemulsion (3.88 nm) was obtained when 3% sodium caseinate and ultrasonication of 5 min were used.

Nevertheless, the nano droplets were flocculated when visualized using confocal laser scanning microscope. The effects of VCO and nanoemulsion at a level of 5% on properties of croaker surimi gel with the mixing time of 3 and 5 min were studied. No differences in breaking force, deformation and fracture constant were observed between the control gel and that added with nanoemulsion, regardless of mixing time ($p > 0.05$). No difference in TPA parameters was found between the control gel and that added with nanoemulsion, however mixing time had a significant impact on the hardness and chewiness ($p < 0.05$). No change in protein pattern of all the surimi gel samples was found for both mixing times, irrespective of VCO or nanoemulsion added. No remarkable effect on elastic (G') as well as loss (G") moduli of surimi paste was noticeable with the addition of either VCO or nanoemulsion, but higher G' was generally attained for the control. Whiteness increased, while the expressible moisture decreased with the addition of either VCO or nanoemulsion. VCO droplets from nanoemulsion were embedded in the gel matrix and distributed uniformly. In general overall likeness was increased for the gels added with VCO nanoemulsion, compared to the control, most likely owing to the increased whiteness.

To elucidate the effects of β-glucan on surimi gel, VCO nanoemulsion stabilized by β-glucan at levels of 5-20% (based on solid weight of surimi) on croaker surimi gel were comparatively studied. Increases in breaking force, deformation and fracture constant were found in surimi gel containing VCO nanoemulsion stabilized by 5% β-glucan, whereas the decrease in all properties were observed as level of β-glucan in nanoemulsion increased. The addition of β-glucan solutions led to continuous decreases in breaking force, deformation and fracture constant, compared to the control. Addition of both β-glucan stabilized VCO nanoemulsion (βG-V-N) and β-glucan solutions resulted in the decreases in viscoelastic moduli. Power law model represented viscoelastic behavior of all the gels. Expressible moisture content decreased, while whiteness increased with the addition of βG-V-N, compared with gel containing VCO. However, both expressible moisture content and whiteness increased with the addition of β-glucan solutions, at all levels used. Addition of βG-V-N resulted in finer gels, than the control gel added with only VCO. Generally,

overall likeness score was increased for gel containing VCO nanoemulsion stabilized by 5% β-glucan.

To improve the storage stability and enhance value, addition βG-V-N containing epigallocatechin gallate (EGCG) and α-tocopherol at levels of (0-0.3%) on properties and storage stability of surimi gel were investigated. Augmented breaking force, deformation and fracture constant were obtained in gels containing 0.2% EGCG or 0.1% α-tocopherol ($p < 0.05$). Expressible moisture content increased as EGCG levels were higher than 0.2%. Smoother microstructure was observed in gels containing 0.2% EGCG. No change in protein pattern of gels was observed, regardless of antioxidant incorporation. The incorporation of 0.2% EGCG or 0.1% α-tocopherol lowered the decrease in G'. Gels containing EGCG and α-tocopherol at selected levels had the improved oxidative stability and lowered microbial loads.

To investigate digestibility and bioactivity of surimi gels containing βG-V-N, gel samples were subjected to simulated gastrointestinal digestion system and the resulting digest was analyzed for nutraceutical properties. βG-V-N remarkably improved antioxidant activities of the surimi digest. When EGCG was added in nanoemulsion, the surimi digest showed the highest antioxidant activities. Antidiabetic activity of the digest was also improved by the addition of βG-V-N comprising EGCG. Nevertheless, the addition of βG-V-N lowered ACE inhibitory activity of surimi digest. The surimi digest from the gel added with βG-V-N possessed an inhibitory effect on five cancer cell lines including HEK (human embryonic kidney 293 cells), MCF-7 (breast cancer cell line), U87 (human glioma), HeLa (human cervical cancer), and IMR-32 (human neuroblastoma), regardless of EGCG or α-tocopherol incorporated. This study demonstrated that surimi gel supplemented with βG-V-N in the presence of EGCG exhibited nutraceutical potential and could be used as a functional food.

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I dedicate this thesis to my beloved mother who has left for heavenly abode and her absence has shattered me. She is always there in my thoughts and prayers.

Asir Gani

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Surimi is a stabilized myofibrillar protein obtained by washing fish mince with water. Surimi is the intermediate foodstuff for various surimi-based seafood products. Development of surimi-based products manifests the most suitable food application for underutilized fish species (Panpipat *et al.,* 2010). The increasing popularity of surimi-based products is due to its unique textural properties and high nutritive value (Jia *et al.,* 2019). Common fish used in south-east Asia for surimi production are threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.), croaker (*Pennahia* and *Johnius* spp.) and lizardfish (*Saurida* spp.) (Morrissey and Tan, 2000). However, dark fleshed fish have been paid more attention as an alternative raw material for surimi production due to the depleting amount of lean fish, due to their overexploitation in the Gulf of Thailand (Morrissey and Tan, 2000). Nevertheless, the poor gel forming ability of surimi produced from dark fleshed fish has limited the utilization of those species (Arfat and Benjakul, 2013). Physical and textural properties of surimi gels have been improved with the addition of different ingredients or additives. Addition of some oils resulted in whiter gel but they might cause deleterious effect on gel properties (Benjakul *et al.,* 2004d, Shi *et al.,* 2014). Incorporation of camellia tea oil (0-8 g/100 g) augmented the hardness, shear force, whiteness, water holding capacity, overall acceptability and elastic modulus (G') of surimi gel (Zhou *et al.,* 2017). In emulsion gel products, lipids are found to be essential for the textural and rheological properties of comminuted meat products in addition to contributing towards unique flavor (Jiao *et al.,* 2019). Absence of lipids results in a rubbery gel with unpleasant texture and mouth feel (Choi *et al.,* 2010). Therefore, emulsion-type surimi-based products such as fish tofu, fish sausage, etc. are always incorporated with cooking oil (Jiao *et al.,* 2019). Owing to the health concerns associated with animal fat, which possesses high saturated acid and cholesterol contents, most consumers prefer food products containing no animal fat. Thus, different vegetable oils such as peanut, rapeseed, soybean, and corn oils have been incorporated in surimi-based products to modify gel properties (Shi *et al.,* 2014).

Virgin coconut oil (VCO) is a rich source of medium-chain fatty acids (MCFAs), notably lauric acid (Marina *et al.*, 2009a), which are also found in human breast milk and are easily digestible. Lauric acid has antiviral, antibacterial, anticaries, antiplaque and antiprotozoal functions (German and Dillard, 2004). VCO has high content of polyphenols, which are primarily hydroxycinnamic acids such as ferulic, *p*-coumaric, and caffeic acids (Robinson *et al.*, 2019). Trilaurin and tripalmitin fractions of coconut oil were reported to inhibit the promotion stage of carcinogenesis (Nolasco *et al.*, 1994). The anticancer properties of VCO have been documented by Narayanankutty *et al.* 2018. VCO finds its application in medicines, baby foods and has been known as weight loss aid. Emulsion based products such as sausage, etc. has been popular due to the desirable smooth-in-mouth texture. However, distribution of oil droplets is crucial factor determining the quality of emulsion product. Pre-emulsification of oil can be carried out to ensure uniform distribution of oil or fat in the muscle food products. The process can improve fat binding ability, enhance physical stability, and is usually easier to disperse into a water based system such as a meat batter (Jimenez-Colmenero, 2007). When oil/fat is pre-emulsified with a non-meat protein, gel formation of meat proteins becomes better, compared to those containing oil without pre-emulsification (Hoogenkamp, 1987). Gao *et al.* 2018 reported that emulsification of fish oil into surimi gels enhanced gel properties such as water holding capacity and gel-forming ability in addition to providing health promoting ingredients. It was also suggested that the improving effect on gel properties by emulsification of surimi is related to the formation of a protein membrane surrounding the oil particles emulsified within the surimi gel.

Ultrasonication has been implemented as an efficient technique for the preparation of nanoemulsions that has better control over the characteristics of emulsions (Ramisetty *et al.*, 2015). Ultrasonication is a high energy disruptive technique, which mechanically breaks larger droplets into smaller ones (Mehmood *et al.*, 2019). Nanoemulsions are kinetically stable systems with higher bioavailability and mean diameter is generally less than 200 nm (McClements and Rao, 2011). Abbasi *et al.* (2019a) reported the production of flaxeed oil nanoemulsion coated by a combination of whey protein-sodium alginate (WP/SA) having particle size of less than

1000 nm with uniform size distribution. When ultrasound waves are transferred through the liquid medium, they create cavitation phenomena (Sivakumar *et al.,* 2014). Such intense cavitation conditions can initiate the desired physical transformation during emulsification. Ultrasound is capable of producing the nanoemulsion of droplet sizes below 100 nm (Ramisetty *et al.,* 2015). Abbasi *et al.* 2019b prepared flaxeed oil based nanoemulsion having mean droplet diameter of 464 nm with the aid of ultrasound. The nanoemulsion showed the potential for targeted delivery and to enrich broiler meat with ω-3 fatty acids. The emulsion having lower droplet size possesses long term stability. Therefore, ultrasound can effectively control the particle size distribution as well as improve stability of the emulsions, thereby governing the final quality of emulsion products.

β-Glucan has gained increasing interest for consumers and is regarded as an important functional ingredient to lower serum cholesterol, promote weight management, reduce glycemic response, enhance immune system, besides having a prebiotic effect (Shah *et al.,* 2016; Zhu *et al.,* 2016). The health benefits of β-glucan are fundamentally due to its fermentability and formation of high viscosity solutions in the intestines (Bozbulut and Sanlier, 2019). β-glucan is regarded as biological response modifier due to their ability to modulate immune system (Shah *et al.,* 2015b). β-glucan from barley and oat was found to reduce cardiovascular disease risk including reduction in blood glucose. In order to meet the demands for low glycemic index (GI) diets and antioxidant property, β-glucan can be used as an ingredient in the products to develop new functional foods (Lee *et al.,* 2016). β-glucan as a hydrocolloid can be used to manipulate the rheological and textural properties of many food products (Shah *et al.,* 2017). It can be used as the stabilizer for emulsion due to its high viscosity, particularly when the ultrasonication is applied properly. As a result, the product can have the functional ingredient to help stabilize the emulsified oil incorporated into the product.

Plant polyphenolics are the compounds holding one or more phenolic rings and are derived from the secondary metabolism of plants (Parr and Bolwell, 2000). In addition to being present in many foods naturally, phenolic compounds can be extracted from their respective sources and thereafter can be added to some foods for their antioxidant effects (Maqsood *et al.,* 2013). To prevent quality loss due to lipid

oxidation, synthetic antioxidants have been widely utilized in the past to retard lipid oxidation in seafood (Boyd *et al.*, 1993). However, the uses of synthetic antioxidants have raised concerns about possible toxicity and food safety (Maqsood *et al.*, 2013). In addition, those antioxidants can serve as nutraceutical, which can scavenge radicals generated in human body. As a consequence, it can be of health promotion, especially after digestion, in which bioactive compounds can be released and absorbed. Thus, the development of surimi gel containing VCO stabilized by β-glucan with high oxidative stability via the incorporation of selected natural additives can be of choice for the consumers who are of health concern. Moreover, oxidized phenolic compounds were demonstrated to act as the alternative protein cross linking agents (Balange and Benjakul, 2009c).

Due to high content of proteins in surimi, peptides or free amino acids after digestion have been documented to have a variety of bioactivities. These peptides have shown broad spectrum of bioactivities including immunomodulatory (Chalamaiah *et al.*, 2018). As an ideal food matrix for supporting high quality protein, the gastrointestinal digestion of surimi gel is imperative for development of surimi based products with enhanced nutraceutical value and regulated release of nutrients in the gastric tract (Fang *et al.*, 2019). Anticancer activity has been documented in hydrolysates as well as peptides derived from a variety of food proteins (Chalamaiah *et al.*, 2018). Flounder surimi digest (FSD) exhibited ACE inhibitory activity after an *in vitro* gastric model (Oh *et al.*, 2019). Similarly, Wang *et al.* (2014) found an increase in degree of hydrolysis in gelatin from 0.17% to 26.08%, while the DPPH radical scavenging rate increased from 1.20% to 44.76% under simulated gastrointestinal digestion. Recently, Sinthusamran and Benjakul (2018) reported the increased antioxidant activity of fish gelatin gels incorporated with β-glucan in the simulated gastrointestinal tract model. Although surimi gel containing β-glucan stabilized VCO was developed, no information on nutraceutical properties has been reported, particularly in the conjunction of antioxidant incorporation.

The outcome of this research can be of great benefit to the surimi industry with the development of emulsified surimi gel having augmented gel properties as well as enhanced nutraceutical potential.

1.2 Literature Review

1.2.1 Surimi

Surimi is a Japanese word describing the resulting material from fish mince that has been deboned, washed with water and mixed with cryoprotectants. The surimi process involves heading, gutting, deboning, and then washing minced flesh with water to concentrate the fish myofibrillar proteins. The washing and dewatering is continued until water soluble impurities such as heme and enzymes are removed and the meat becomes odorless and colorless (Hunt *et al.*, 2009). Surimi consists of salt-soluble myofibrillar protein and has unique gelling properties that make it useful as a food base in seafood analogues e.g shrimp, scallop, lobster, elver and crab leg analogues (Benjakul *et al.*, 2003). The unique textural properties and high nutritional value contribute to the increasing popularity of surimi based products (Shi *et al.*, 2014). The underutilized fish species could also be processed into surimi based products for better exploitation (Shimizu *et al.*, 1992). Surimi can be produced from any fish, however the rheological properties of the surimi gel depend on the properties of myofibrillar proteins, which are affected by the species and freshness of the fish as well as on the processing parameters such as protein concentration, pH, ionic strength and temperature (Niwa, 1992; Shimizu *et al.*, 1992). In general, lean fish have been commonly used for surimi production. For surimi production in south-east Asia, threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.), croaker (*Pennahia* and *Johnius* spp.) and lizardfish (*Saurida* spp.) are the major raw material (Morrissey and Tan, 2000). Alaska pollock (*Therma chalcogramma*) and Pacific Whiting (*Merluccius productus*) are the two most widely used fish species for the production of surimi and surimi seafood in the USA (Yoon *et al.*, 2004).

With increasing demand for nutritional and health foods, the use of surimi based products have grown substantially (Moosavi-Nasab *et al.*, 2005). These products are prepared by incorporating ingredients such as starch, egg white proteins, salt and vegetable oils into a continuous protein matrix, resulting in a gel-based protein composite foods. Physico-chemical properties, distribution and volume fraction of the added ingredients and their interaction with the protein gel matrix affect the textural properties of surimi based products (Lee, 2002).

1.2.2 Surimi Gelation

Gelation is the most critical step in the development of surimi based products to achieve a desirable texture. The functionality of myofibrillar protein has a direct impact on gelation process (Cando *et al.,* 2016). Prior to gelation, solubilization is required. Salt addition during chopping solubilizes the myofibrillar proteins and an optimum gel strength is obtained when 2.5-3% NaCl is added to the surimi (Lanier *et al.,* 1985; Cando *et al.,* 2016). Salt addition to the surimi paste unfolds the myofibrillar proteins and exposes the reactive sites buried in the interior of the protein, which gels rapidly upon heating at 80-90 °C. Salt addition enhances protein gelation which results in good texture and flavor as well as imparts microbial safety (Tahergorabi *et al.,* 2012). Addition of salt and salt substitute at 0.17 and 0.34 mol/L enhanced ($p < 0.05$) gel texture in Alaska pollock surimi gel (Tahergorabi *et al.,* 2012). Unfolding allows the proteins to interact and form a three-dimensional protein network (Lanier *et al.,* 2005; Omana *et al.,* 2011). Hydrogen bonds and hydrophobic interactions are involved with retaining water in the gel network (Niwa, 1971). Nunez-Flores *et al.* (2018) studied the effect of salt and temperature in myosin polymerization during two step surimi gelation (5 ºC/24 h and 30 ºC/30 min, followed by heating at 90 ºC/30 min). In the absence of salt, there was no myosin heavy chain (MHC) polymerization during setting, and surimi gels were characterized by highly aggregated proteins, reduced elasticity and a poorly stabilized protein network. Gel was of low quality. On the contrary, the disruption and dissociation of the actomyosin complex led to myosin unfolding, resulting in greater exposure of reactive groups. This facilitates endogenous transglutaminase activity, thus inducing MHC polymerization prior to heat induced aggregation. Salted surimi gels were thus characterized by the formation of a well-stabilized protein network, resulting in the better rheological properties due to MHC polymerization, especially after setting at 30 ºC. Therefore, the degree of protein unfolding prior to gelation is an important factor to improve gel qualities. The inclusion of 2% salt into fish protein isolate (FPI) improved gel strength, but addition of 3% salt into FPI decreased G' as well as gel strength (Kobayashi and Park, 2017).

Gelation is an aggregation of proteins, forming a three dimensional network, which entraps water (Pomeranz, 1991; Arafat and Benjakul, 2013). Myofibrillar

proteins, mainly myosin and actin, are involved in gelation. The gel network is developed when sufficient intermolecular bonding takes place. This gel network is stabilized by ionic linkages, hydrophobic interactions, covalent bonds including disulfide linkages and hydrogen bonds (Lanier, 2005). The intrinsic protein properties and environmental conditions determine the proportion of various bonds involved in forming the gel network. Environmental factors that can influence protein gelation include protein concentration, pH, temperature, and ionic strength (Smith, 1994). Heat-induced gelation unfolds the protein, exposing the hydrophobic sites from the interior of the protein and results in protein aggregation (Nakai, 1983). Benjakul *et al.* (2001a) also reported that disulfide bonds were found in bigeye snapper actomyosin during thermal gelation process. Sano *et al.* (1988) reported that the gelation of carp actomyosin takes place in two stages: at temperature ranges of 30-41 °C and 51-80 °C. The first stage of gel formation was attributed to the interactions among tail portions of myosin molecule, while the second stage governed by hydrophobic interactions taking place among the head portions of myosin (Sano *et al.*, 1990). However, heat induced gelation of myosin via two reactions was proposed by Samejima *et al.*, (1981). Those include (1) aggregation of the globular head segments of the myosin molecules and (2) network formation, resulting from unfolding of helical tail segment. Protein unfolding takes place during heating of surimi paste, thus exposing the reactive surfaces to the neighboring protein molecules, which interact to form intermolecular bonds (Lanier, 2000).

Heating process has been known to affect the properties of and is crucial to obtain the desired surimi gel with good elasticity (Jiao *et al.* 2019). Traditionally surimi products are heated is two-steps, the first step involves endogenous transglutaminase (TGase) meditated cross-linking of myofibrillar proteins below 40 °C (Benjakul and Visessanguan, 2003), followed by protein aggregation at a higher temperature. Ohmic heating was reported to minimize proteolysis of surimi from lizardfish and goatfish, compared to 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

(Tadpitchayangkoon *et al.*, 2012). Additionally, rapid heating method with shorter heating time improved water holding capacity and whiteness of tropical surimi gels when compared to water bath heating (Tadpitchayangkoon *et al.*, 2012). Treatment of *Collichthys lucidus* surimi with 5 kGy dose of electron irradiation produced gels having significantly higher gel strength, improved whiteness and increased water holding capacity, compared to non-irradiated gels (Deng *et al.*, 2017). During the heat-induced gel formation, the α-helix content decreased, whilst the β-sheet and β-turn content increased in samples. Irradiation treatments similarly decreased the α-helix content and increased the β-sheet content. This transformation is beneficial for protein denaturation and gel formation. During dense phase carbon dioxide gelation (DPCD) of myosin, the α-helices of myosin became unfolded and were gradually converted to β-sheet, β-turn and random coil structures during the gelation process. Due to α-helix unfolding, active groups such as sulfhydryl and hydrophobic groups were exposed, leading to protein-protein interactions and chemical cross-linking induced by DPCD. This in turn led to the formation of a three-dimensional stable network retaining water, resulting in enhanced gel strength (Guo *et al.*, 2017).

Some additives or chemicals have been employed to improve properties of gel or to lower salt required for solubilization of proteins. Cando *et al.* (2016) reported that addition of cystine (0.1%), tetra-sodium pyrophosphate (0.05%) and lysine (0.1%) enhance gelation of Alaska pollock surimi in the presence of only 0.3% NaCl. The action of these additives is mainly based on inducing primary protein denaturation or unfolding of myofibrillar proteins, thus facilitating the formation of different types of bonds.

1.2.2.1 Setting phenomenon (Swari)

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 *et al.*, 2000). It involves gel network formation of muscle proteins initiated by protein unfolding. Setting temperature depends 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 low temperature setting is time consuming, it is avoided at the industrial scale (Benjakul *et al.*, 2003). High temperature settings are the most common practice due to shorter time, but this can cause protein degradation, induced by moduri-inducing proteases, which are active at 50-60 °C (Benjakul *et al.*, 2003). Hence, medium temperature setting can be an alternative for the manufacturer to obtain a better gel quality without severe proteolytic degradation. Setting of Alaska pollock surimi was done either at 5 °C (low temperature) or 25 °C (medium temperature) (Zhang *et al.*, 2013; Zhu *et al.*, 2014). High temperature (40 °C) setting has been implemented for surimi from tropical or warm water fish species such as threadfin bream, Indian mackerel, sardine, yellow stripe travelly, goatfish and lizardfish (Arfat and Benjakul, 2012; Chanarat *et al.*, 2012; Tadpitchayangkoon *et al.*, 2012). Heating process induces protein aggregation, stabilized by hydrophobic interactions and disulfide bonds (Benjakul *et al.*, 2003). Formation of large aggregates is presumably a prerequisite for formation of a good elastic gel (Chan *et al.*, 1992). During the heating process, the alpha helix unfolds due to instability of hydrogen bonds, thereby exposing hydrophobic amino acids, which further undergo hydrophobic-hydrophobic interaction (Benjakul *et al.*, 2003). The high-quality of mackerel surimi with the highest gel strength and whiteness was obtained when it was produced from short-bodied mackerel with the setting at 40 °C prior to heating at 90 °C (Chaijan *et al.*, 2010).

Transglutaminase (TGase) has been reported to be involved in polymerization of myosin during setting (Kamath *et al.*, 1992). It helps in the formation of cross-links between MHC via the formation of ε-(γ-glutamyl) lysine isopeptides (Benjakul *et al.*, 2004a; Fang *et al.*, 2019). The subsequent setting of surimi at appropriate temperature aims to improve the gel strength by activating TGase (Benjakul *et al.*, 2004a; Moreno *et al.*, 2008). Calcium ions (Ca^{2+}) is required for the catalytic activity of indigenous TGase (Yongsawatdigul *et al.*, 2002) and plays an important role in gel strengthening via cross-linking of MHC (Kumazawa *et al.*, 1995). Addition of calcium ions in surimi resulted the increased gel strength of surimi (Benjakul *et al.*, 2004b; Ding *et al.*, 2011; Yin and Park, 2014). 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). Due to the insufficient TGase in surimi from some fish species, microbial transglutaminase (MTGase) has been widely employed in surimi. Breaking force of red tilapia surimi gel was enhanced by 240% fold with addition of 2g/kg MTGas/g surimi as reported by Duangmal and Taluengphol (2010). Cando *et al.* (2016) used MTGase and the combination of MTGase and/or lysine (0.1%) or cystine (0.1%) to improve physicochemical properties of low salt (0.3%) surimi gels. Chanarat and Benjakul, (2013) reported that MTGase (0.4 units/g surimi) in the absence of formaldehyde was able to increase the gel strength of surimi from lizardfish. High pressure processing (HPP) at 300 MPa also improved physicochemical properties of surimi gel, especially when MTGase was combined with cystine. HPP induced primary protein denaturation or unfolding of myofibrillar proteins, facilitating the further formation of different types of bonds. These bonds improved the conformational stability of the protein network as evidenced by the lower MHC band density and δ value (phase angle) and the predominance of β-sheet structures when the additives were added and/or HPP was applied (Cando *et al.,* 2016). 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). Fish gelatin up to 10% was used in combination with 1.2 units MTGase/g surimi to obtain surimi with grade AA (Kaewudom *et al.,* 2013). 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). MTGase was able to increase the gel strength of surimi from lizardfish. However, formaldehyde (FA) present in surimi had a negative impact on gel improvement and cross-linking ability induced by MTGase. Therefore, it was recommended to prepare surimi from fresh lizardfish to avoid formaldehyde formation to maximize the gel strengthening effect of MTGase (Chanarat and Benjakul, 2013).

1.2.2.2 Gel weakening (modori)

Degradation of proteins in surimi occurred due to the endogenous heat-activated proteinases (Benjakul *et al.,* 1997), which results in the formation of weak or soft gel. Gel softening or gel weakening is termed "modori" (Benjakul *et al.,* 2004c;

Morrissey *et al.,* 1993). This phenomenon causes the negative effects on the quality of surimi products and reduces their commercial value (Hu *et al.,* 2010). Modori is mainly due to the autolysis caused by sarcoplasmic proteases, especially serine- and cysteine-type proteases, which are activated at postmortem pH (Hu *et al.,* 2012). Although sarcoplasmic proteins including proteases are water-soluble, some of them are still retained after washing process (Hossain *et al.,* 2004). 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 *et al.,* 1993) during spawning migration (Yamashita and Konagaya, 1990). Higher proteolytic activity, caused by cathepsin L, is found at temperature above 50 ^{o}C and leads to the rapid and severe degradation of myofibrillar proteins, especially MHC (Kudre *et al.,* 2013). Among the numerous proteases present in muscle, cysteine endoproteases cause the most adverse effects on texture, owing to their thermostability and ability to cleave internal peptide bonds. Softening of threadfin bream is due to a cysteine protease, which has the maximum autolytic activity at 50-60 ^{o}C (Oujifard *et al.,* 2012). When Pacific whiting surimi was incubated at 55 ^{o}C, most MHC was degraded when the incubation time increased, which was in accordance with the lower breaking force or gel strength of the resulting surimi gel (Rawdkuen *et al.,* 2007).

Numerous food grade protease inhibitors capable of limiting modori have been used in surimi (Rawdkuen *et al.,* 2007; Campo-Deaño and Tovar, 2009; Fowler and Park, 2015a). The most frequently used protease inhibitors are egg white, whey protein concentrate and soy protein isolate (Campo-Deaño and Tovar, 2009; Oujifard *et al.,* 2012; Kudre *et al.,* 2013). However, some undesirable odor, flavor and color have been reported in surimi gel with the addition of those inhibitors. Fowler and Park (2015a) reported that the addition of salmon plasma protein significantly improved gel strength of Pacific whiting surimi subjected to the two step ohmic heating, ohmically heating to 60 ^{o}C and holding for 30 min, followed by ohmically heating to 90 ^{o}C. Similarly, salmon plasma (SP) from Chinook salmon was shown as an effective inhibitor of proteolysis in both Pacific whiting surimi and salmon mince. SP was found

to contain both cysteine and serine protease inhibitors. It was proposed that SP may be effectively used at a concentration of 0.25% in surimi or be injected into salmon fillets to inhibit protease mediated texture softening (Fowler and Park, 2015b). Furthermore, Klomklao *et al.* (2015) reported that protease inhibitor from tuna roe effectively improved the gel strength of surimi from bigeye snapper via inhibiting proteolysis. Protease inhibitor purified from adzuki bean seed (0.5-3%) was also reported as a potential trypsin inhibitor as indicated by the lowered TCA soluble peptide content in threadfin bream muscle and increased breaking force of threadfin bream surimi gel (Klomklao and Benjakul, 2015). Singh and Benjakul (2017a) used serine protease inhibitors isolated from squid ovary (0.5-3%) to inhibit the proteolysis of surimi paste from bigeye snapper and improved the gelling properties of both modori and kamaboko gel. Similarly, the addition of 2% serine protease inhibitor from squid ovary (SOSPI) increased breaking force of surimi gel from Indian mackerel by 207.2%, compared to control sample (Singh and Benjakul, 2017b).

1.2.3 Use of oils in surimi gel

Different biopolymers have been added into surimi gel to improve its various physicochemical properties as well as to improve the nutritional status of the product. Oil has been used in surimi products as texture modifier, color enhancer, or processing aid. Vegetable oil can replace water up to 6% without changing shear stress and shear strain values (Park, 2005). The whiteness of surimi-based products with vegetable oil is often higher than those without vegetable oil (Benjakul, *et al.*, 2004d; Hsu and Chiang, 2002), which is attributed to the light scattering effect of oil droplets. Additionally, vegetable oil has no cholesterol but higher ratio of unsaturated fatty acids than animal fat (Liu *et al.*, 1991). Benjakul *et al.* (2004d) used soybean oil (1-5%) in the mixture of bigeye snapper and mackerel surimi. Sample containing bigeye snapper and mackerel at ratio of 7:3 (w/w) and added with 5% soyabean oil showed 9% increase in whiteness, compared to the control, whereas the sample containing bigeye snapper SA grade and mackerel in ratio of 5:5 (w/w) and added with 5% soyabean oil showed an increase in whiteness by 11%, compared to the control. However, breaking force and deformation were reduced by oil addition. Pietrowski *et al.* (2011) added the ω-3 polyunsaturated fatty acids (PUFAs)-rich oils (flaxseed, algae, menhaden, krill and

blend) at level of 9 g/100 g to nutritionally enhanced surimi-based seafood products but the texture was not affected. Color properties of ω-3 PUFAs nutrified surimi seafood were generally improved except when krill oil or blend was added. In another study, Pietrowski *et al.* (2012) added ω-3 PUFAs-rich oils (flaxseed, algae, menhaden, krill, and blend) to the Alaska pollock surimi. Differential scanning calorimetry showed that oil addition enhanced thermal transition of actin and did not compromise the transition of myosin. The addition of oil improved heat-induced protein gelation as demonstrated with dynamic rheology. Elastic modulus increased when oil was added. There were no differences ($p > 0.05$) in shear stress between surimi gels with and without oil, indicating that nutrification with ω-3 PUFAs rich oils within the range tested (0-9 g/100 g) did not alter gel strength. Chang *et al.* (2015) reported that addition of soybean oil (0-5%) increased whiteness of the gel of surimi from frozen silver carp (Grade AA) but reduced the breaking force, hardness and chewiness. Vegetable oils (soybean, peanut, corn, and rap oils) were added (0-50 g/kg surimi) to improve the properties of surimi gel from frozen silver carp. As the vegetable oil concentration increased in surimi gels, breaking force of gels was decreased ($p < 0.05$), while expressible water and whiteness values were increased ($p < 0.05$). Surimi gels with peanut oil had higher breaking force values, compared to those with other vegetable oils. Storage modulus and loss modulus decreased along with increasing vegetable oil concentrations. Results demonstrated that vegetable oils could be used potentially to modify the qualities of surimi-based products, such as color and taste (Shi *et al.,* 2014). Zhou *et al.* (2017) determined the effects of different concentrations of camellia tea oil on physicochemical properties and protein secondary structure of surimi gel from white croaker (Grade A). With the increasing camellia tea oil concentration (0-8 g/100 g of surimi), hardness, whiteness, WHC, overall acceptability, storage modulus and the indexes of ionic bonds and hydrophobic interactions of surimi gel were increased ($p < 0.05$). The presence of the oil could change the micro-environment and molecular structure of surimi proteins and further affected the physicochemical properties of surimi gels. In general, when the concentration of camellia tea oil was 8 g/100 g of surimi, the surimi gel showed the most favorable properties. Jiao *et al.* 2019 reported that incorporation of fish oil (FO) into surimi gel disrupted the protein matrix and decreased gel strength. However, the application of microwave heating at the protein aggregation stage improved the gel

strength and whiteness, yielded surimi gel with a soft texture and compact network and reduced expressible moisture content.

1.2.3.1 Effect of pre-emulsified oil on muscle protein gel

The effects of fat reduction (25.0%, 17.5%, and 10.0%) and substituting beef fat with canola oil or pre-emulsified canola oil (using soy protein isolate, sodium caseinate or whey protein isolate as emulsifiers) on cooking loss, texture and color of comminuted meat products were investigated by Youssef and Barbut (2011). Reducing fat from 25 to 10% increased cooking loss and decreased hardness. The use of pre-emulsified oil using sodium caseinate as an emulsifier resulted in the highest hardness value. Both canola oil and pre-emulsified oil resulted in a significant reduction in redness. Pre-emulsification can offset some changes in reduced fat meat products when more water is used to substitute for the fat and that pre-emulsification can also help to produce a more stable meat matrix. Zhao *et al.* (2014) used pre-emulsified plant lipids prepared using pulsed ultrasound to replace animal fat. Chicken breast myofibrillar protein (MP)–soybean oil emulsion composite gels (SMG) were prepared from 3% chicken breast MP with 27.5% soybean oil pre-emulsified with 0.5% sodium caseinate (0.6 M NaCl and 50 mM phosphate solution, pH 6.25). The ultrasound treatment time had a profound influence on the functional properties of SMG. The addition of soybean oil to the MP sols resulted in shear-thinning flow behavior. When SMG was prepared using an ultrasound-treated pre-emulsion, a more viscoelastic network was formed. Hardness, chewiness, and other textural properties were significantly improved by the addition of ultrasound-treated pre-emulsion. A substantial increase in water and fat binding (WFB) capacity of SMG was achieved by selecting an appropriate ultrasound treatment time. When the emulsion was prepared from a 6 min-ultrasound treatment, smaller oil droplets and a more ordered, higher density microstructure were obtained. Pulsed ultrasound treatment of pre-formed emulsion is therefore an efficient method, and has potential for use in improving the gel characteristics of low-saturated fat meat products.

Cheetangdee (2017) partially replaced porcine fat with soybean oil (SBO) to modify the characteristics of sausages. Pre-emulsified SBO with fish protein isolate (FPI) as the emulsifier successfully improved stability of the meat matrix, as indicated

by reduced cooking loss relative to the control. Moreover, improved homogeneity of the microstructure was found in these sausage samples. Partial replacement of porcine fat with pre-emulsified SBO appears to be a promising way to produce meat products with desirable stability, and the FPI could be a potent non-meat protein that enhances the stability of comminuted meat products. Kang *et al.* (2017) investigated the physical-chemical and rheological properties of pork batters as affected by replacing pork back-fat with pre-emulsified sesame oil. Replacement of pork back-fat with pre-emulsified sesame oil improved L* value, moisture and protein content, hardness, cohesiveness, and chewiness, but decreased a* value, fat content and energy. When pre-emulsified sesame oil was used to replace pork back-fat at 50%, the sample had the highest L* value. According to the results of dynamic rheology, replacement of pork back-fat by pre-emulsified sesame oil increased the storage modulus (G') values at 80 °C, and resulted in firm gel. Batters with pre-emulsified sesame oil had higher water holding capacity than the control. Overall, the batters with pre-emulsified sesame oil enabled the lowering of fat and energy contents and yielded the pork batter with the better texture. The results showed that by adding pre-emulsified sesame oil it was possible to produce low fat meat batter and having desired qualities. Heat-induced composite gels with 20 g/kg (2%) myofibrillar protein (MP) sol and 100 g/kg (10%) olive oil pre-emulsified by MP or non-meat proteins in 0.6 mol/L NaCl, at pH 6.2 were produced by Wu *et al.* (2017). The effect of different non-meat protein (soy protein isolate, egg-white protein isolate and sodium caseinate) pre-emulsions on the rheological properties and microstructure of MP gel was evaluated. Addition of olive oil emulsified by non-meat proteins could enhance MP gel strength (except the SPI emulsion group) and increased the water-holding capacity. The emulsion substantially improved the dynamic rheological properties of MP composites during the transformation from sol to gel. When the emulsions containing non-meat proteins were added into MP sol, some of these non-meat proteins including 7S globulin, 11S globulin in SPI, ovotransferrin, lysozyme in EPI, and β-casein in SC would participate in the formation of gels to some extent. However, gels with non-meat proteins as emulsifier exhibited structures that were weaker than those of gels containing emulsion emulsified by MP. The results indicate the potential and feasibility to modify the textural properties of comminuted meat products by means of the non-meat protein as emulsifier to substitute meat protein.

Buamard *et al.* 2019 reported that pre-emulsification of seabass oil in the presence of ECHE (0.20–0.25 g ECHE/100 g) yielded the surimi gel with improved textural properties and oxidative stability. Liu et al. 2019 investigated the effects of peanut oil pre-emulsification by three food-grade emulsifiers (soy protein isolate (SPI), konjac glucomannan (KGM), and acetylated distarch phosphate (ADSP)) on the properties of emulsified surimi sausage. The water holding capacity, emulsification stability, and whiteness properties improved significantly after adding the pre-emulsified peanut oil. Uniform distributions of oil droplets in the SPI, KGM, and ADSP groups were observed by optical microscopy. Overall, KGM was found as the ideal candidate for the pre-emulsification of peanut oil for emulsified surimi sausages.

1.2.4 Virgin coconut oil

Based on the definition of virgin oil, as long as the oil does not go through the refining/bleaching/deodorization (RBD) process and has no alteration of the nature of the oil, the oil can be deemed as virgin oil (Marina et al., 2009a). Virgin coconut oil (VCO) is obtained from fresh, mature kernel of the coconut by mechanical or natural means, with or without the use of heat but chemical refining is omitted (Villarino *et al.*, 2007). Chemical refining, bleaching or de-odorizing processes are not required. Therefore the intrinsic nature of oil is maintained. VCO is regarded as the healthiest oil on the earth (Gupta *et al.*, 2010). Purest form of VCO is visualized as water clear or colorless. It contains natural vitamin E and has not undergone any hydrolytic and atmospheric oxidation as demonstrated by very low FFA content and peroxide value (Marina et al., 2009a). It has a fresh coconut aroma that can be mild to intense, depending on the oil extraction process used. VCO is generally used as frying and cooking oil due to its excellent resistance to rancidity development.

Various extraction methods have been used to extract VCO. Wet extraction method which eliminates the use of solvent was used by Wen (2010). Physical methods have been used by Seow and Gwee (1977). Supercritical carbon dioxide extraction of VCO was carried out at pressure and temperature ranges of 20.7–34.5 MPa and 40–80 C, respectively by Norulaini *et al.*, (2009). Fermentation method was used by Satheesh and Prasad (2014). Enzymatic method, in which proteases were employed to hydrolyze proteins stabilizing emulsions of coconut milk, was used by (Raghavendra and

Raghavarao, 2010; Senphan and Benjakul, 2015; Senphan and Benjakul, 2016; Patil and Benjakul, 2017; Patil and Benjakul, 2019).

1.2.4.1 Advantages of VCO

VCO serves as an important source of energy in the diet. It acts as a lubricating agent and a flavor enhancer. VCO also acts as carrier and protective agent for fat-soluble vitamins. VCO is a rich source of medium-chain fatty acids (MCFAs), notably lauric acid (C12) (Marina et al., 2009a), which are also found in human breast milk and are easily digestible and provides immunity to babies. There are also similar beneficial effects in adults. VCO possesses anti-inflammatory, antimicrobial and antioxidative properties and boosts immune system (Shilling *et al.*, 2013; Marina et al., 2009b; Jose *et al.*, 2017). VCO is digested easily without the need for bile and goes directly to liver for conversion into energy (Dayrit, 2003). Shilling *el al.* (2013) evaluated the VCO and its most active individual fatty acids for the control of *C. difficile*, which is the major cause of hospital-acquired antibiotic-associated diarrhea. Lauric acid was most effective for growth inhibition, followed by capric acid (C10) and caprylic acid (C8). However, VCO did not inhibit the growth of *C. difficile*, but the growth was inhibited when bacterial cells were exposed to 0.15–1.2% lipolyzed coconut oil. Narayanankutty *et al.* 2018 documented that VCO, by virtue of their MCFA content and high amounts of phenolic antioxidants, exerts potential preventive and curative efficacy in several conditions such as hyperlipidemia, fatty liver, diabetes, and cancers.

1.2.5 Ultrasonication

1.2.5.1 Principle

Ultrasound is an inaudible high frequency (>20 kHz) sound wave. Acoustic waves propagate as longitudinal waves exhibiting compression and rarefaction (O'Sullivan et al., 2017). Ultrasound application in foods can be classified into two categories based on the frequency range. High frequency ultrasound (100 kHz-1 MHz) is mainly used for non-destructive quality evaluation of foods including measurement of food properties, flow rates and inspect food packages (Malik and Saini, 2018). Low energy ultrasound used for non-destructive quality evaluation does not influence the

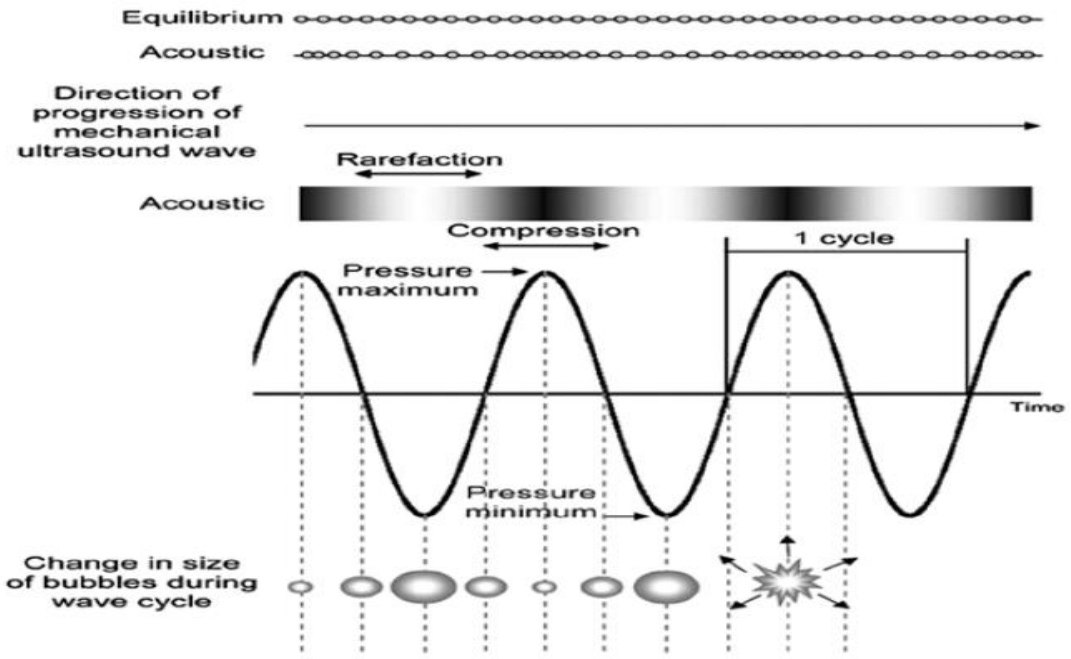

Figure 1. Cavitation caused by ultrasonication

Source: Soria and Villamiel (2010).

physical properties of materials, but in turn is influenced by the material properties (Goulserene and Coupland, 2007). Low frequency (20-100 kHz) high intensity (10-1000 W cm^{-2}) ultrasound is used for modification of physicochemical properties of foods. High intensity ultrasound (HIUS) causes physical disruption of food material and promotes crosslinking between the denatured protein molecules and oxidation by the hydroxyl radicals generated during ultrasound treatment (Mason, Paniwnyk, & Lorimer, 1996). HIUS treatment can also break the water molecules generating highly reactive radicals that may react with other molecules (Riesz and Kondo, 1992). This broad range of mechanisms involved in HIUS may induce physical and chemical effects with potential applications in food processing (Soria and Villamiel, 2010). Ultrasonication involves the conversion of mechanical or electrical energy into acoustic energy with the aid of transducers. When ultrasound traverses through a liquid medium, the minute gas bubbles are continuously formed and imploded. The phenomenon is known as cavitation (Figure 1), resulting in tremendous increase in pressure (up to 500 MPa) and temperature (over 1000 K) at the micro-scale level (Ercan and Soysal, 2011). Cavitation bubbles are produced when the pressure produced by rarefaction exceeds the

inter-molecular force of the liquid medium. If the pressure is sufficient, the cavitation bubbles will enlarge radially and implodes upon exceeding the critical size. This phenomenon is known as transient cavitation, which is accompanied by release of enormous amounts of energy and extremely high temperature and pressure (up to 5000 K and 1000 atm) (Fu et al., 2020). Therefore, ultrasound technique has gradually grown to be considered as a promising, novel and green technology that can replace or assist traditional food-processing methods.

1.2.5.2 Application of ultrasound in food emulsions

Emulsification is the process of homogeneous mixing of two immiscible phases (e.g., oil and water) with the aid of an emulsifier into a homogeneous emulsion. An energy input by mechanical agitation or ultrasonication is required to facilitate the formation of small droplets. The release of high energy and generation of intense shear force during cavitation facilitates the emulsification process by ultrasonication (Thompson & Doraiswamy, 1999). Ultrasound has been employed to disperse oil phases into aqueous phases in a controlled manner, resulting in stable emulsified products (Kentish et al., 2008). Ultrasound produces emulsions involving two mechanisms. The interfacial waves produced by ultrasound result in dispersion of oil phase into water phase as mid-to large-sized droplets. Subsequent breakdown of these droplets of dispersed oil into sub-micron size droplets is due to cavitation effect (Thompson and Doraiswamy, 1999). Ultrasonically induced implosions due to cavitation disrupts the micron-sized oil droplets (>50 mm) and results in the formation of nano-sized emulsion droplets (~200 nm) (O'Sullivan et al., 2017).

The mouth feel, visual appearance and stability of food emulsions is influenced by the size of the emulsified droplets (Kentish et al., 2008). The emulsions with droplet size smaller than ~100 nm appear translucent and almost clear, however large sized emulsion droplets give a 'milky' opaque appearance (Leong et al., 2009). The thermodynamic unstability of emulsions is overcome by kinetic stability when the droplet size is less than ~100 nm (Jafari et al., 2007). The tendency of droplets to cream is overcome by the Brownian motion in nanoemulsions. However, instability of nanoemulsion is dependent on colloidal forces such as Ostwald Ripening and droplet–droplet collisions that leads to coalescence and eventual phase separation (Solans et al.,

2005). These processes proceed slowly and therefore nanoemulsions are stable for many months (Nakabayashi et al., 2011). Strong shear forces delivered by low frequency (20–100 kHz) and high power (>10 W/cm2) ultrasound are involved to break the liquid droplets and formation of nano-sized droplets. The emulsified droplets are then stabilized by the surfactant to prevent phase separation by coalescence (Leong et al., 2009).

Ultrasound has been used for effective mixing of different ingredients especially those which are not miscible. Shanmugam and Ashokkumar (2014) reported incorporation of 7% flax seed oil in homogenized skim milk with the aid of ultrasound at 20 kHz for 3 min at acoustic power of 176 W. The emulsion droplets with an average mean volume diameter of 0.64 mm were produced and were stable for at least 9 days at 4 ± 2 °C. It was also documented that less than 20% of partially denatured whey proteins provided stability to the emulsion droplets. Cucheval and Chow (2008) investigated the effect of ultrasonic power, time and duty cycle of commercial ultrasonic probe on the production of oil-in-water emulsion (5 wt% soybean oil stabilised with 1 wt% Tween 80). Stable droplet size of 0.7 μm was gained in 5 min regardless of the power and duty cycle used. Alzorqi et al. (2016) incorporated β-glucan isolated from *Ganoderma lucidum* in palm olein based nanoemulsions generated by ultrasonication. The physical properties of nanoemulsion were enhanced by achieving lower droplet diameter (263 nm), narrower polydispersity index (0.244) and lower viscosity (1.85 cP) when optimal formulation (water: 85%, oil/surfactant ratio: 3:1) as well as the ultrasonic emulsification conditions (power: 700 W, treatment time: 300 s) were employed. The antioxidant activity of β-glucan was enhanced by ultrasonic emulsification in palm olein-based nanoemulsions. Ramisetty et al. (2015) used different sonochemical reactor designs for preparation of coconut oil in water nanoemulsion as a model system. The increase in emulsification time, emulsifying agent volume fraction and power density led to the decrease in droplet size of emulsion. High power ultrasonic processor can therefore be effectively used for improved emulsification process. Carpenter and Saharan (2017) reported the preparation of mustard oil in water nanoemulsion stabilized by Span 80 and Tween 80 with the aid of ultrasound. Ultrasonication time of 30 min, HLB value of 10, surfactant volume fraction

of 0.08 (8%, v/v), oil volume fraction of 0.1 (10%, v/ v) and ultrasonic power amplitude of 40% produced the minimum droplet size of 87.38 nm. The produced nanoemulsion was found to be stable at least for 3 months at ambient conditions without any phase separation and creaming. Leong et al. (2009) produced nanoemulsions of sunflower oil in water with mean particle sizes of 40 nm by altering the oil content of a triglyceride/Tween/Span combination or by altering the PEG content in a triglyceride/SDS/PEG system. However, the minimum droplet size was found to be independent of equipment configuration. O'Sullivan et al. (2015) evaluated ultrasonic emulsification processes (batch and continuous) using Tween 80 or milk protein isolate as emulsifiers. Ultrasonic treatment yielded emulsion droplets of ~200 nm in both batch and continuous processes. However, the timescale of emulsification for continuous process and batch process is milliseconds and seconds, respectively. Ultrasonic treatments carried out at ultrasonic powers of 150, 300 and 450 W for 12 and 24 min on emulsion system stabilized by soybean protein isolate and lecithin was explored by Sui et al. (2017). Ultrasonic treatment improved the emulsifying activity index and emulsion stability index of the resulting emulsion. Nevertheless, the continuous increases of ultrasonic power and duration had undesirable effect on the stability of emulsion. The high energy input excessively exposed the hydrophobic domains of soybean protein molecules to the aqueous environment, leading to protein aggregation and consequently reduced the stability of soybean protein isolate and lecithin stabilized emulsion. Ghosh et al. (2013) formulated antibacterial basil oil nanoemulsion with droplet diameter of 29.3 nm using non-ionic surfactant Tween 80 and ultrasonic emulsification for 15 min. Stability and droplet diameter of nanoemulsion were significantly influenced by emulsification time, ratio of oil and surfactant and concentration of surfactant. Mehmood et al. (2017) used ultrasonication to prepare olive-oil based alpha-tocopherol containing nanoemulsions using Tween 80 as surfactant. The optimized emulsification conditions chosen were 4% olive oil, 2.08% surfactant concentration and ultrasonication treatment of 3 min. The resulting nanoemulsion had particle size of 151.68 nm, *p*-anisidine and antioxidant activity of 7.17% and 88.64%, respectively. Páez-Hernández et al. (2019) used two ultrasonic processors (130 W and 750 W) operated at different amplitudes (40% and 90%) for 30 min to produce curcumin nanoemulsions. The increase in time, amplitude and power of

ultrasonication caused the decreases in droplet size and polydispersity index of the nanoemulsions. Ultrasonic approach was used to produce vitamin D containing canola oil nanoemulsion using soya lecithin and Tween 80 as emulsifiers. Ultrasonication time of 4.32 min, surfactant/oil ratio of 0.62 and disperse phase volume of 7% produced nanoemulsion had droplet size of 115.47 nm and vitamin D retention of 73.44%. Abbasi et al. (2019) employed ultrasonication for the production of flaxseed oil nanoemulsions (mean droplet diameter of 464 nm) stabilized by combination of whey protein-sodium alginate to protect bioactive α-linolenic acid against gastric digestion and to deliver the bioactive at the posterior parts of chicken gastrointestinal tract. Therefore, ultrasound can be effectively used to generate nanoemulsions, which could in turn be used in food systems as carriers of bioactive components as well as to modify the properties of the food systems.

1.2.6 β-glucan

1.2.6.1 Source and structure

β-glucan is a non-starch polysaccharide consisting of repeating β-D-glucopyranose units arranged either in linear chains or branched structures, depending upon the source (Lam and Cheung, 2013). β-Glucan from baker's yeast consists of β-(1→3) and (1→6) linkages (Fig. 2A). However, cereal β-glucans have β-(1→3) and β-(1→4) linkages between glucose monomers. (Fig. 2B) (Zhu *et al.*, 2016). β-glucan is quite diversified and main sources include cereals, mushrooms, yeast, and some bacteria. Among cereals, oat and barley are reported as richest sources of β-glucan ranging between 4–7% (Lazaridou and Biliaderis, 2007; Bhatty, 1992). β-glucan from different sources vary in their molecular structure, chain conformation, solubility, number of linkage, and thus different biological activities (Descroix *et al.*, 2006). Generally cereal β-glucans are linear polysaccharides consisting of (1→3) and (1→4) glycosidic bonds, in which (1→4)-β-linkages occur mostly in groups of two or three and are interrupted by a single (1→3)-linkage (Wood, 1993; Wood *et al.*, 1994a). So

the β-glucans structure constitutes predominantly of β-(1→3)-linked

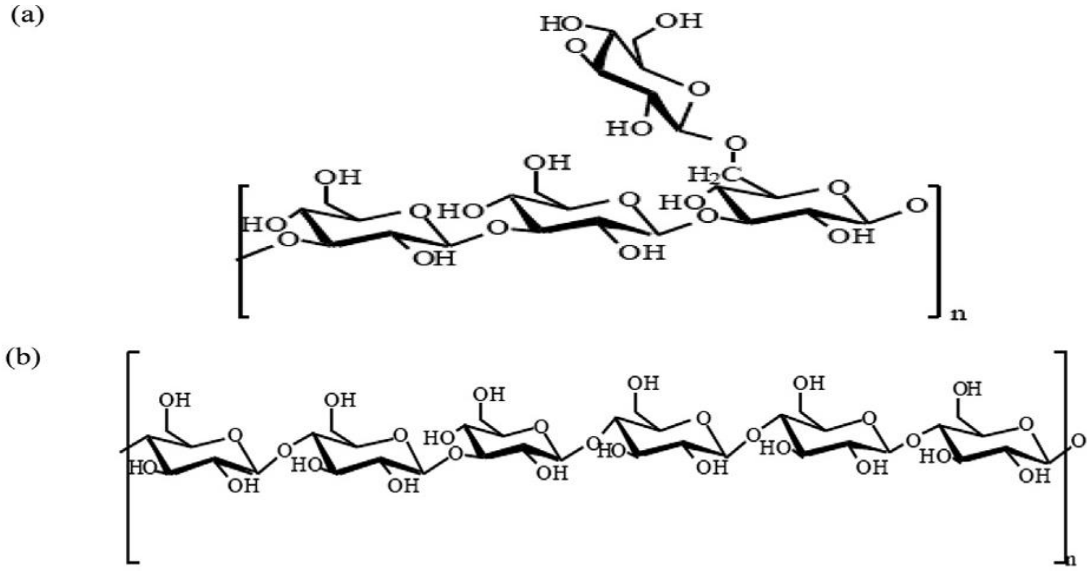

Figure 2. The structure of β-glucans. (a) (1→3) β-glucans with ramifications β (1→6); (b) (1→3) β-glucans with ramifications β (1→4)

Source: Zhu et al. (2016)

cellotriosyl and cellotetraosyl units (~90%) and lesser amount of long cellulosic oligosaccharides in (~5 to 10%), with a degree of polymerization (DP) between 5 and 20 (Ebringerova *et al.*, 2005; Woodward *et al.*, 1983). The β-(1→4)-linkage is capable of interchain aggregation, giving close packing to crystalline structures, whereas the β-(1→3) linkages interrupt the β-(1→4) linkage sequence and gives rise to kinks in the chain, making it soluble (Woodward *et al.*, 1983; Liu *et al.*, 2015; Shah *et al.*, 2017). Aman and Graham (1987) reported 20% and 46% of β-glucan was insoluble in oat and barley, respectively. β-glucan is hydrolyzed by lichenase, which specifically cleaves the (1→4)-linkage next to a (1→3)-linkage at its reducing end, yielding oligosaccharides with different degree of polymerization (Lazaridou and Biliaderis, 2007). Based on cereal β-glucan hydrolysis, DP3 (3-O-β-cellobiosyl-D-glucose) and DP4 (3-O-β-cellotriosyl-D-glucose) are the major products, which constitute about 90% of the total β-glucan content. Also a small amount of cellodextrin-like oligosaccharides containing more than three consecutive (1→4)-linked glucose residues terminated by a (1→3)-linkage at the reducing end are released. These constitute only 5–10% of the total β-glucan content with degree of polymerization in

the range of 5–20 (Wood *et al.*, 1994a; Wood *et al.*, 1994b; Izydorczyk *et al.*, 1998). The relative amount of DP3 in the β-glucan was found in the range of 67 –72% in wheat, 52 – 69% in barley, and 53 – 61% in oat, whereas DP4 constituted in the range of 25– 33% in barley, 34 – 41% in oat, and 21–24% in wheat. An often used indicator of structural difference among β-glucans is the ratio of cellotriose to cellotetraose units i.e, DP3:DP4 (Wood *et al.*, 1991; Izydorczyk *et al.*, 1998). DP3/DP4 is considered as a fingerprint of the structure of cereal β-glucans. Wood (2011) reported values of DP3 to DP4 ratio in the range of 2.1–2.4 for oat β-glucan, 3.0–3.8 for wheat β-glucan, and 2.8 – 3.4 for barley β-glucans.

1.2.6.2 Hydration properties of β-glucan

Hydration properties of fibre depend on the chemical structure, hydrophobic/ hydrophilic properties, surface area and particle size (Thibault and Ralet, 2001). The water binding capacity (WBC) measures the amount of water retained by the fiber when subjected to external stresses. Ahmad *et al.* (2008) reported that WBC of β-glucan extracted from barley flour using four different types of extraction methods was in the range of 2.91–3.79 g/g. The water holding capacity was reported in the range of 2.46 –13.94 g/g for oat β-glucan samples (Liu *et al.*, 2015) and 6.14 –6.74 g/g for native barley β-glucan samples (Lee *et al.*, 2016). Swelling power (SP) is defined as the volume occupied by a known weight of fiber when hydrated with water without any external stress except gravity. Swelling power of 3.45 (g/g) was reported for β-glucan from *Agaricus bisporus* (Khan *et al.*, 2015) and 14.05 (g/g) for native oat β-glucan (Moura *et al.*, 2011), 3.45, 3.74 and 4.49 (g/g) respectively for *Agaricus*, *Pleurotus* and *Coprinus* β-glucan (Khan *et al.*, 2017).

1.2.6.3 Emulsifying properties of β-glucan

The ability of biopolymer to adsorb on the interface reflects the emulsion activity and its ability to resist changes in its properties over time reflects its stability. Emulsifying activity (EA) and emulsion stability of β-glucan extracted from baker's yeast and button mushroom was found in the range of 64.26-79.24 % and 94.64-99.28 % respectively. The increase in γ-irradiation dose from 0-50 KGy for treatment of β-glucan could increase EA of resulting β-glucan (Khan *et al.*, 2015; Khan *et al.*, 2016). The potential

application of barley β-glucan as stabilizer in foams and emulsion type food products has been demonstrated by Burkus and Temelli (2000). Barley β-glucan increased stability of foams and emulsion using whey protein concentrate as emulsifier and foaming agent. Kontogiorgos et al. (2004) investigated the effects of barley and oat β-glucans on the rheological and creaming behavior of egg-yolk-stabilized model emulsions. Emulsion (o/w) was stabilized by high molecular weight β-glucans (Mw ~110 × 10^3) by increasing the viscosity of the continuous phase. However, the low molecular weight β-glucans (MW ~40 × 10^3) stabilized emulsion through network formation in the continuous phase. Salgado *et al.* (2017) developed antifungal formulation against *B. cinerea* by encapsulating resveratrol using barley and yeast β-glucans by emulsification-evaporation technique. It was suggested that the formulations could be developed with other polyphenols in the future works. Álvarez and Barbut (2013) evaluated the effects of fat level (20.0, 12.5 and 5.0%), inulin and β-glucan on emulsion stability, color, textural characteristics and microstructure of cooked meat batters. β-glucan, due to its ability to form dense matrices and hold large amounts of water, could act as a fat-replacer and also augment textural parameters of reduced-fat products. Santipanichwong and Suphantharika (2009) studied the influence of different β-glucan preparations including curdlan, barley, oat, and yeast β-glucans on egg yolk stabilized oil-in-water emulsions. Increase in oil droplet size and flocculation was noticed in the emulsion with the addition of β-glucan. However, viscoelastic properties, flow behaviour and emulsion stability were enhanced.

1.2.6.4 Health benefits of β-glucan

The change in life style and food habits have made people vulnerable to diseases like cancer, cardiovascular disorders, obesity, osteoporosis, and irritable bowel syndrome. These degenerative diseases are mostly associated with the free radical formation in human body, present externally, as well as produced internally. Therefore, people are looking for the kind of food that can limit this damage by directly scavenging reactive oxygen species or by stimulating endogenous defense systems (Shah *et al.*, 2017).

β-glucan is a polysaccharide that has received much attention from past few years due to its several health beneficial properties, including the ability to remove free

radicals in a way identical to antioxidants (Gardiner, 2000; Kofuji *et al.,* 2012). β-glucan is regarded as an important functional ingredient to lower serum cholesterol, promote weight management, reduce glycemic response, enhance immune system, besides having a prebiotic effect (Shah *et al.,* 2016; Zhu *et al.,* 2016). β-glucan from barley and oat at a 3 g/day dosage as recommended by FDA would reduce cardiovascular disease risk including reduction in blood glucose and also has satiety effect. In order to meet the demands of people related to diets that has low glycemic index (GI) and antioxidant property, nonstarch polysaccharides like β-glucan can be used as an ingredient in the products to develop new functional foods (Lee *et al.,* 2016). β-glucan from barley and oat has got excellent antioxidant and antiproliferative activities which might be because of its degree of branching, chain conformation and functional groups. These properties will help regulate carcinogenesis at the initiation as well as progressive stages (Shah *et al.,* 2017). Ahmad *et al.* 2016 concluded that germination of barley can be a good approach for enhancing the antioxidant potential of β-D-glucan. Gamma irradiation (0-10 KGy) of β-glucan extracted from oats and barley showed enhanced antioxidant and antiproliferative activities against three human cancer cell lines including Colo-205, T47D and MCF7 using MTT assay. The increased antioxidant and antiproliferative activities were attributed to easy access of small β-glucan into the target. The irradiated β- glucan can be used as natural antioxidant (Shah *et al.,* 2015a; Shah *et al.,* 2015b). The anti-inflammatory effect of fungal β-glucan is mediated through the regulation of various inflammatory cytokines, such as nitric oxide (NO), interleukins (ILs), tumor necrosis factor alpha (TNF)-α, interferon gamma (INF)-γ as well as non-cytokine mediator, prostaglandin E_2 (PGE_2) (Du *et al.,* 2015). Arena *et al.* (2016) reported that mixtures of probiotic microorganisms and barley β-glucans exhibited synergistic effects in modulating the transcriptional level of several immune-related genes, leading to an overall enhanced anti-inflammatory effect. These findings suggest a promising application of probiotic bacteria and β-glucans in the preparation of dietary mixtures presenting health-promoting features such as immunomodulatory properties.

1.2.6.5 Application of β-glucan in food products

Apart from its biological activities including anti-cancer, antiinflammatory, and immune-modulating properties, β-glucan possesses physical properties such as water solubility, viscosity, and gelation. Thus, it has been increasingly used by the food and other industries (Zhu *et al.*, 2016).

β-Glucan had noticeable effect on physical and sensory properties of sausage. Therefore, by the combination of β-glucan and resistant starch, production of prebiotic sausage was possible (Sarteshnizia *et al.*, 2015). Gluten free bread based on hydroxypropylmethylcellulose, yeast β-glucan, whey protein isolate and rice starch were developed by Kittisuban *et al.* (2014). The optimized rice starch bread formulated with yeast β-glucan was sensorially acceptable. Sharafbafi *et al.* (2014) incorporated high molecular weight oat β-glucan into milk to obtain calorie-reduced and cholesterol-lowering dairy products. β-glucan at low level (less than 0.2%) a concentration which would be insufficient to be nutritionally significant in food products, caused no phase separation. At higher concentrations, different microstructures were formed upon mixing. These structures are responsible for the changes in the bulk rheological behaviour for the mixes. Casein micelles are responsible for the thermodynamic incompatibility as whey proteins partitioned equally in both separated phases. Brennan *et al.* (2013) used β-glucan rich fractions from barley and mushroom in the production of extruded ready to eat snacks. Inclusion of barley β- glucan rich fractions and mushroom β-glucan fractions at 10% levels increased total dietary fibre content of extrudates, compared to the control. Substantial reductions in the *in vitro* glycamic response (up to 25% compared to the control product) illustrated the possibility of utilizing both barley and mushroom fractions to reduce the energy content of snack foods and help modulate the overall glycaemic response. Apostu *et al.* (2017) used yeast derived ingredients containing β-glucans (GOLDCELL® IY B and GOLDCELL® BETA GLUCAN) to improve the emulsifying capacity (up to 5 increments), the water holding capacity (up to 8 increments) as well as the emulsion stability of meat batters. A decrease in total fluid release up to 4.30% and 3.99%, respectively, was obtained when GOLDCELL® IY B and GOLDCELL® BETA GLUCAN at 1.5% addition level was incorporated, respectively. A significant decrease in hardness and fracturability

values was also observed, while maintaining the structural cohesiveness of the samples. Adding yeast β-glucan to meat batters can allow food to decrease NaCl and polyphosphate content in meat products. Omana *et al.* (2011) used β-glucan as a partial salt replacer in high pressure processed chicken breast meat. Addition of β-glucan with reduced NaCl (1%) and in the absence of sodium tripolyphosphate could produce gels with similar properties to those with 2.5% NaCl addition. Hence it was proposed that β-glucan could be used to reduce NaCl content of chicken products. The effect of β-glucan (BG) and phytosterols (PS) as fat replacers on textural, microstructural, and lubrication properties of reduced-fat cream cheese was investigated (Ningtyas *et al.*, 2017). The effect of β-glucan appeared to be more pronounced, imparting increased viscosity and firmness to reduced-fat cream cheese, similar to commercial high-fat cream cheese sample. The particle size distribution of cream cheese formulation containing β-glucan showed a monomodal curves with small globule size. Cream cheese with β-glucan as carbohydrate-based fat replacer showed a relatively high moisture content, firmness, and adhesiveness due to the ability to bind water and form a viscous solution. The presence of β-glucan in cream cheese matrix act as a filler and break the casein–casein interaction, resulting in a weak structure. The only drawback of adding β-glucan is the lack of lubrication properties with less spreadability. Wu *et al.* (2015) investigated the effect of curdlan at different levels as well as the method of addition, on the viscoelastic characteristics of ribbonfish meat gel. Due to the interaction of the RCs (thermal reversible curdlan gel) or the IRCs (thermal irreversible curdlan gel) with the ribbonfish meat protein gel matrix during the thermal gelation, the simple filled fish meat gels turned into complex filled gels, resulting in various changes in water holding capacity (WHC) and textural and rheological properties of the final reconstructed ribbonfish meat gel products. Compared with the control sample (fish meat gels without curdlan) and IRC samples, the addition of higher levels of the RC in the fish meat gels significantly increased the cross-linking density of the complex fish meat gel networks, leading to a more stable and ordered three-dimensional network complex gel structure with improved gel properties. The addition of the RC at an appropriate level (in the range of 4–6% for ribbonfish) effectively strengthens the gelation properties and sensory property of restructured fish meat gel. Therefore, application of the RC as a gel binder and as a dietary fiber source in fish meat gel-based

products appears feasible. Addition of oat β-glucan (OβG) deteriorated the gluten network structure and decreased dough stability. These effects can be explained by the dilution effect of OβG, competition for water and interaction between OβG and gluten proteins. Rheological evaluation revealed an increasing tendency to solid-like behavior with increasing addition of OβG (Wang *et al.,* 2017). Sinthusamran and Benjakul, 2018 reported that incorporation of β-glucan (0-20%) affected the rheological and gelling properties of fish gelatin (FG) gel. Moreover, the incorporation of β-glucan showed the effectiveness in increasing antioxidative activity of FG gel in the simulated gastrointestinal tract. Therefore, the incorporation of β-glucan at an appropriate level could improve fish gelatin gel product with increased antioxidative activities. Zhang *et al.* 2019 also documented the pronounced improvement in water-holding capacity (WHC), hardness, springiness, chewiness as well as gel strength of surimi gel fortified with 2% yeast β-glucan (YG). YG strengthened the protein network as a semi-rigid filler to fill the interspaces and enhance its structure resulting in the manufacture of the multifunctional surimi products fortified with YG having health benefits.

1.2.7 Polyphenols

Plant phenolic compounds, also denoted polyphenols, are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s). Phenolics are derived from the secondary metabolism of plants (Parr and Bolwell, 2000). They are mostly present in various types of foods of plant origin. Phenolic compounds can be broadly divided into 2 categories, flavonoids and nonflavonoid polyphenols. Nonflavonoids and phenolic acids are abundant in foods. Flavonoids, the target class of polyphenols, can be divided into different subclasses according to the degree of oxidation of the heterocyclic ring: anthocyanins, flavonols, flavans, flavanol, flavones, and isoflavones (Scalbert and Williamson, 2000). The most common polyphenols are hydroxylated cinnamic acids such as caffeic acid (3, 4-dihydroxycinnamic acid), chlorogenic acid (its quinic acid ester), caftaric acid (its tartaric acid ester), and flavonols such as quercetin and rutin (its rutinoside) (Spanos and Wrolstad, 1992). These compounds have an ortho-diphenol (or a 1-hydroxy-2-methoxy) structure (Strauss and Gibson, 2004).

1.2.7.1 Antioxidant activity

Phenolic compounds have been used as antioxidants in food and beverages (Ghasemzadeh and Ghasemzadeh, 2011; Jiang *et al.,* 2013; Utrera and Estévez, 2013). Polyphenol including catechins, stilbenes, flavonoids, proanthocyanidins, ellagitannins and anthocyanins, the natural occurrence of which in plants contribute to health benefits through diets (Hu *et al.,* 2017). Several phenolic compounds such as catechin, caffeic acid, ferulic acid, and tannic acid showed preventive effect on lipid oxidation of fish mince and fish oil-in-water emulsion (Maqsood and Benjakul, 2010). These natural antioxidants from plants, in the form of extracts, have been obtained from different sources such as fruits (grapes, pomegranate, date, kinnow), vegetables, (broccoli, potato, drumstick, pumpkin, curry, nettle), herbs and spices (tea, rosemary, oregano, cinnamon, sage, thyme, mint, ginger, clove) and could prevent the lipid oxidation (Shah *et al.*, 2014). Therefore, natural antioxidants are widely employed to control lipid oxidation and limit the deleteriousness during processing as well as storage of fish products (Kulawik *et al.,* 2013). Addition of tea catechins significantly reduced TBARS value of cooked white muscle of mackerel stored at 4 °C for 7 days (He and Shahidi, 1997). Lipid oxidation and fishy odor development were retarded in fish emulsion sausages by the use of 0.02% or 0.04% tannic acid and ethanolic kiam wood extract (0.08%) during 20 days of the refrigerated storage (Maqsood *et al.,* 2012). Sardine surimi gels containing ethanolic coconut husk extract (ECHE) showed lower PV and TBARS, with increasing levels of ECHE (0-0.25 g/100 g) (Buamard and Benjakul, 2019). Caffeic acid (100 mg/kg) was successfully used to inhibit lipid oxidation in wheat dietary fibre minced fish restructured products after 10 days of chilled storage (Sánchez-Alonso *et al.,* 2011). Lipid oxidation in mackerel mince was retarded by the use of numerous phenolic compounds (100 ppm) during 15 days of iced storage (Maqsood and Benjakul, 2010). Tannic acid and catechin were effectively used at 200 ppm to retard lipid oxidation and microbial growth in ground camel meat throughout 9 days of refrigerated storage (Maqsood *et al.,* 2015). The addition of young apple polyphenols (YAP) at 0.1% into grass carp surimi (GCS) effectively delayed lipid oxidation during refrigerated storage of 7 days (Sun *et al.*, 2017). Sun *et al.* (2017) reported that young apple polyphenols (YAP) showed the strong antioxidant activities

in vitro and preservative effects on grass carp surimi (GCS) during cold storage. The treatment of GCS with YAP retarded the increase of PV, TBARs, TVB-N and b* values; inhibited the degradation of soluble myofibrillar protein; and extended the sensory shelf-life of GCS. Chlorogenic acid was found to be the primary component showing preservative effects in YAP. Besides, YAP was shown to protect the physicochemical quality of GCS during cold storage in terms of protein functional properties (emulsifying activity, emulsifying stability and surface hydrophobicity), gel strength and textural properties (hardness, springiness, cohesiveness, gumminess and chewiness). YAP, as one kind of polyhydroxyl extract therefore had the protective effects on the quality GCS during cold storage.

1.2.7.2 Protein cross-linking activity

Diphenol moiety of a phenolic acid or other polyphenol is readily oxidized to an ortho-quinone, either enzymatically as in plant tissues, or by molecular oxygen. The quinone forms a dimer in a side reaction, or reacts with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds with the phenolic ring, with regeneration of hydroquinone. The latter can be reoxidized and bind a second polypeptide, resulting in a cross-link. Alternatively, two quinones, each carrying one chain, can dimerize, also producing a cross-link (Strauss and Gibson, 2004). Rawel *et al.* (2002) reported that the phenolic compounds react with proteins, resulting in the formation of cross-links.

During the course of acting as antioxidant through donation of protons (hydrogen) and electrons, a phenolic compound is converted to a quinone derivative(s) via its radical intermediate, which can subsequently react with a nucleophilic group in proteins to form a covalent adduct (Jongberg *et al.* 2011; Kroll *et al.*, 2003). Protein functional properties in food products could be improved by the treatment of phenolic acids or flavonoids, for example, foaming capacity of β-lactoglobulin (Sarker *et al.*, 1995), emulsifying activity of α-lactalbumin (Wang *et al.*, 2014), and gel strength of bigeye snapper surimi (Balange and Benjakul, 2009a). The efficacy of phenolics appeared to be determined by the oxidative status of the phenolic compounds. Balange and Benjakul (2009a) reported that treatment of bigeye snapper surimi with ferulic acid

without oxygenation at alkaline pH had no effect on surimi gelation; however, the treatment became evident upon oxygenation, in which oxidation took place.

Two types of complexation mechanisms can be distinguished: a monodentate and a multidentate mechanism (Haslam, 1989). Both complexation mechanisms lead to aggregation and precipitation of proteins (Haslam, 1989). Lower amounts of tannic acid and catachin (0.05%) were required to increase breaking force and deformation of surimi gel, compared with ferulic acid and caffeic acid. The multidentate mechanism generally requires a much lower phenolic compound/protein molar ratio and thus a lower phenolic compound concentration (Haslam, 1989). On the contrary, ‘‘Monodentate” means that a phenolic compound interacts with only one protein site at a higher phenolic compound concentration (Haslam, 1989). Both oxidized ferulic acid (OFA) and oxidized caffeic acid (OCA) having 1 and 2 hydroxyl groups, respectively, at higher levels (0.20 and 0.15%) were required to increase breaking force and deformation of surimi gel. Balange and Benjakul (2009b) reported that oxidized tannic acid showed the synergistic effect with alkaline washing process in improving the gel properties of mackerel surimi without any adverse effect on sensory properties. Alkaline-saline washing process could remove most interfering components including myoglobin, lipid and other impurities more effectively than conventional washing process. This possibly facilitated the better interaction of OTA with MHC.

Nevertheless, sarcoplasmic protein (SP) exhibited the interfering effect on crosslinking activity of OTA towards NAM, a major contributor for gel formation. OTA might preferably interact with SP instead of NAM, in which a small aggregate or cluster was formed and impede the aggregation of NAM. Jongberg *et al.* (2013) reported that increased thiol loss and a distinct loss of myosin heavy chain and actin due to polymerization were caused by the addition of green tea extract in Bologna type sausages produced from oxidatively stressed pork. The enhanced protein polymerization was ascribed to the reaction between quinone compounds from the plant extracts and protein thiol groups to yield phenol-mediated protein polymerization.

In addition to the improvement of gel strength, phenolics could be used to improve properties of films based on proteins. Under alkaline and heating condition,

the incorporation of tannins (tannic acid and apple procyanidin) resulted in the polymerization of myofibrillar protein and produced a film with higher tensile strength and lower water vapor permeability (Nie *et al.*, 2017). Therefore, tannins especially condensed tannins can be used as a good crosslinker of myofibrillar protein. The structure and molecular weight of polyphenols play an important role in protein–polyphenol interactions. High molecular weight polyphenols (tannins) are able to bond more strongly or preferentially to proteins (Frazier *et al.*, 2010). The flexibility of the polyphenol molecule appears to be important. Structurally flexible polyphenols have shown equal binding strength to different proteins (gelatin and bovine serum albumin), whereas less flexible polyphenols (ellagitannins) exhibited stronger binding to some proteins (gelatin) and weaker to others (bovine serum albumin) (Frazier *et al.*, 2010).

1.2.7.3 Nutritional impact

Phenolic compounds have both positive and negative effect under the light of health effect. Some studies suggested the blocking of some essential amino acids by polyphenols when they are bonded to proteins, which could affect the availability of some amino acids. Rawel *et al.* (2002b) suggested that phenolic acids and flavonoids, which reacted with soy proteins, might affect the blocking of lysine, tryptophan and cysteine residues in protein molecules, and hence decrease the availability of the essential amino acids, lysine and tryptophan.

Some studies suggested positive effects of polyphenol–protein interactions. Polyphenols could be delivered to lower parts of the gastrointestinal tract due to polyphenol–protein interactions. Certain proteins carrying polyphenols were even called nanovehicles (Shpigelman *et al.*, 2010). Stojadinovic *et al.* (2013) also suggested proteins as good carriers of polyphenols in the gastrointestinal tract. When bound to proteins, polyphenols such as those from green tea could preserve their anti-proliferative activity (Von Staszewski *et al.*, 2012).

1.2.7.4 Tea polyphenols

Tea polyphenols are popular plant extracts that have been widely used in various foods (Perumalla and Hettiarachchy, 2011). Tea polyphenols have attracted attention in recent years due to their enzyme inhibition, antibacterial and antioxidant

activity (Erol *et al.,* 2009; Gondoin *et al.,* 2010). Catechins are monomeric flavanols abundantly present in unfermented tea. The principal catechins are catechin, catechin gallate, epicatechin (EC), epi-gallocatechin (EGC), epi-catechin gallate (ECG), epigallocatechin gallate (EGCG), gallocatechin, and gallocatechin gallate. The catechins have the general structure of C6–C3–C6 with two aromatic rings and several hydroxyl groups (Sharma and Rao, 2009; Hara, 2001). The scavenging ability of tea catechins on superoxide anions ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), the free radicals generated from 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was reported by Guo *et al.,* (1999).

Green tea catechins are stable under acidic conditions. With the increase of pH, oxygen concentration or temperature, they degrade faster (Shpigelman *et al.,* 2012). Stabilization of EGCG by binding to native or heated betalactoglobulin (BLG) has been demonstrated (Lestringant *et al.,* 2014). It has also been shown that dietary proteins can be good vehicles for transport and preservation of antioxidant capacity of bioactive compounds. Thermally treated beta-lactoglobulin was described as a promising nanovehicle which was able to bind EGCG with a higher affinity than native protein and protect the polyphenol from degradation (Shpigelman *et al.,* 2010). Rashidinejad *et al.,* (2014) reported that encapsulation of catechin and EGCG in liposomes is a promising technique to protect and deliver antioxidants to the gut. Goh *et al.* (2015) reported a significant reduction in glucose release from the green tea extract-fortified steamed and baked bread during the first 90 min of pancreatic digestion phase, particularly at concentrations of 2% GTE. Thus the fortification of GTE into bread products may show a significant impact on reducing the glycaemic response. Jia *et al.* (2017) reported that catechin at 200 μmol/g protein led to the greatest increase in the surface hydrophobicity and significant loss of thiols (SH) content of pork myofibrillar protein (MP). The low concentration of catechin of 10 μmol/g caused slight changes in the MP gel strength and water-holding capacity. However, catechin at higher concentrations (50, 100 and 200 μmol/g protein) resulted in the severe deterioration of MP gelation. Therefore, catechin caused the changes in MP conformational and gel properties, which may be due to the covalent interactions between catechin and the exposed hydrophobic domains induced by catechin.

1.2.8 Tocopherols

Tocopherol or Vitamin E occurs in the form of tocopherols (α, β, γ and δ), which are composed of a chromanol ring with a different number and positions of methyl groups and phytyl tail. Alpha-tocopherol is the most active form of this fat-soluble vitamin. Alpha-tocopherol is often used as the antioxidant in many pharmaceutical, cosmetic and food products. Its antioxidative activity results from the phenolic hydroxyl group present in the molecule (Cieśla *et al.,* 2017). The radical-scavenging properties of tocopherols and related compounds is determined by the ease of donation of the phenolic hydrogen, or its bond dissociation energy (BDE), which is enhanced by methyl substituents in the two ortho positions, in addition to the alkoxy substitution in the para position (Kamal-Eldin and Appelqvist, 1996). Thus, α-tocopherol (with two ortho methyl substituents) is a stronger hydrogen donor than either β- or γ-tocopherols (with only one ortho methyl substituent). Those are more potent than δ-tocopherol with no ortho methyl substituent. An antioxidant potency in the order of α > β > γ > δ would be expected, but this order is not consistent and is affected by the concentrations of tocopherols (Chotimarkorn *et al.,* 2008; Dolde, 2009; Kamal-Eldin and Appelqvist, 1996). Hwang and Moser (2017) reported antioxidant activity of amino acids largely relied on the synergism with tocopherols in soybean oil at frying temperature, although amino acids themselves could act as primary antioxidants in the absence of tocopherols. Similarly, Dai *et al.* (2008) reported that the mixture of the green tea polyphenol, vitamin E and vitamin C could act synergistically to protect lipid peroxidation. Kinetic and mechanistic studies on the antioxidation process revealed that this antioxidant synergism was due to the regeneration of vitamin E by the green tea polyphenol and the regeneration of the latter by vitamin C. Alpha-Tocopherol can also inhibit oxidation of protein. Est´evez and Heinonen (2010) demonstrated that α-tocopherol reduced formation of α-aminoadipic and γ -glutamic semialdehydes from oxidized myofibrillar proteins.

1.2.9 Simulated gastrointestinal tract digestion

Food proteins may act as sources of bioactive peptides. These peptides are encrypted in the proteins and can be released *in vitro* during the food processing or *in vivo* by the gastrointestinal digestion. Bioactive peptides can be absorbed in the

gastrointestinal system and exert their activity after absorption (Delgado *et al.*, 2011). The application of *in vitro* digestion models is a necessary step to study the release of potentially bioactive and functional peptides from food proteins (Bax *et al.*, 2013; Bordoni *et al.*, 2014). Paolella *et al.* (2015) reported that simulated gastrointestinal digestion of Parma ham using a physiological digestion model gave rise to complex mixtures of peptides derived mainly from myofibrillar and sarcoplasmic proteins, with molecular weight ranging from 200 to 1700 Da. The peptide pattern was strongly influenced by the proteolysis process occurring in ham during ageing, leading to a different profile in 18 and 24 months aged products. Among the identified peptide sequences, many are known to be bioactive or precursors of potential bioactive sequences. Capriotti *et al.* (2015) characterized potential bioactive peptides in soybean seeds and milk. The identified peptides were 1173 in soybean seed samples, 1364 in untreated soy milk samples and 1422 in soy milk samples. Soybean proteins underwent an extensive degradation process during gastrointestinal digestion and generated a large number of bioactive peptides, some with established activity and some with predicted antimicrobial activity. Sangsawad *et al.* (2017) reported that *in vitro* GI digestion of cooked chicken breast generated a number of potent angiotensin converting enzyme (ACE) inhibitory peptides identified as KPLLCS, ELFTT and KPLL. Pepe *et al.* (2016) identified two abundant hexapeptides (EAMAPK and AVPYPQ) from Stracchino cheese gastrointestinal digestion. The tested peptide fractions showed significant effects in reducing the oxidative cellular stress, both inhibiting reactive oxygen species (ROS) and increasing an antioxidant response. Phongthai *et al.* (2018) reported that hydrolysis of rice bran protein concentrate under *in vitro* gastrointestinal digestion (pepsin-trypsin system) greatly improved the antioxidant properties. Only digestion by pepsin enhanced the DPPH radical scavenging activity, while further digestion with trypsin improved ferric reducing antioxidant power by 13.9%. Liu and Pischetsrieder (2017) reported that physiological digestive processes may promote bioactive peptide formation from proteins and oligopeptides in kefir.

Lipids or fats have been digested differently after ingestion. The bioavailability depends on the structure and composition of lipid as well as emulsifier, etc. Gumus *et al.* (2017) created a simulated gastrointestinal tract (mouth, stomach,

small intestine stages) to compare gastrointestinal fate of lipid droplets coated by plant proteins (lintil, pea, faba bean proteins) with those coated with animal protein (whey protein). Extensive droplet aggregation occurred in mouth and stomach but oil droplets were mostly digested in the small intestime. Ban *et al.* (2018) studied the colloidal stabilities and gastrointestinal tract (GIT) digestions of tristearin nanoparticles stabilized with various polyethylene glycol (PEGylated emulsifiers). Particularly, the solid lipid nanoparticle (SLN) lipolysis induced by the action of bile salts, colipase, and pancreatic lipase in the small intestinal tract was minutely studied *in vitro* under the simulated conditions. SLNs coated with small-chained PEGylated emulsifiers were more significantly hydrolyzed than those covered with large-chained PEGylated emulsifiers. It was demonstrated that the digestion fate of the orally administered SLNs could be controlled by rational design in terms of choosing the type and concentration of PEGylated emulsifiers.

For emulsion stabilizing by protein, the localization and susceptibility to hydrolysis in gastrointestinal tract have the impact on bioavailability. Mun *et al.* (2017) used fluorescence imaging to visualize special location of lipids and proteins during simulated gastrointestinal tract digestion of protein-stabilized oil-in-water emulsion. It was revealed from the study that proteins remained attached to the lipid droplet surfaces in the mouth and stomach, but formed large aggregates in the small intestine. The lipid droplets were highly flocculated in the mouth, highly coalesced in the stomach, and fully digested in the small intestine. It was remarked that the simultaneous study of protein-labeled and lipid-labeled emulsions can provide valuable information into the physicochemical events occurring within the gastrointestinal tract. This knowledge may prove useful for designing functional foods for increasing the bioavailability of encapsulated bioactive agents, for delivering bioactives to specific regions within the GIT, or for controlling satiety responses. Lamothe *et al.* (2017) studied the influence of dairy matrices on nutrient release in a simulated gastrointestinal environment. Intense heat treatment of milk induced faster digestion of proteins in the gastric environment. Cheeses were more resistant to protein and lipid digestion than liquid or semi-solid matrices. Fatty acid release in the intestinal phase was much faster when matrices were produced from homogenized milk. Calcium soaps were produced in the intestinal

environment, and their concentration was higher during the digestion of cheeses in comparison with milks and yogurts. Processing-induced modifications to the composition, microstructure, and rheological properties of dairy matrices could be used to control nutrient delivery.

Plant extracts show the varying bioactivity in gastrointestinal tract. Sun *et al.* (2015) reported that the antioxidant activity and cytoprotective effect of purple rice anthocyanins were not changed during simulated gastric digestion, but dropped sharply when subjected to intestinal digestion. Synergies between gastric and intestinal digested anthocyanins were observed when evaluated by antioxidant and cytoprotective effects. Murugan *et al.* (2016) reported that most of the polyphenolic compounds (gallic acid, catechin, rutin and quercetin) are easily bioaccessible in the fruit than peduncle of *Phoenix loureirii* fruit during *in vitro* digestion. The stability of antioxidants and acetylcholinesterase inhibition activities were found maximum after gastric digestion than pancreatic digestion. Thus polyphenolic compounds were not completely degraded and made easily accessible to exert their biological effects in the gastro-intestinal tract. Yen *et al.* (2017) reported that oral digestive product of propolis ethanol extract showed the best antioxidant and cyclooxygenase-2 (COX-2) inhibitory activity than gastric and gastrointestinal products. It was recommended that propolis could be used along with standard chemotherapeutic agents to attenuate the progression of various cancer. Rueda *et al.* (2017) evaluated the bioaccessibility of individual phenolic compounds in extra virgin argan oil using an *in vitro* digestion method. Hydroxyphenylacetic acid (4,245 mg/kg), ferulic acid (2,478 mg/kg), vanillin (2,429 mg/ kg) and 3,4-dihydroxybenzoic acid (2247 mg/kg) were the major compounds identified in argan oil extracts. After the digestion process, values of bioaccessibility of the different compounds varied from a minimum of 2% (hydroxyphenylacetic acid) to a maximum of 84% (coumaric acid), but some such as caffeic acid became detectable, as a result from digestive transformations. Therefore, the phenolic compounds of the argan oil were strongly affected during the digestive process. Fang *et al.* (2019) reported that silver carp surimi gels added with MTGase prohibited the hydrolysis during *in vitro* pepsin digestion at the beginning because the enhanced cross-linking reduced the accessibility of pepsin to the hydrolytic site and the hydrolysis rate increased more rapidly once the network was

destroyed during the first 30 min of digestion. These findings were claimed to help explore the impact of structure of surimi gel, which has a delayed digestion that potentially enhances the feeling of satiety and control caloric intake without affecting nutritional value. Li *et al.* (2019) built a theoretical foundation of surimi gels gastric digestion using Logistic model. The relationship between content of free amino acids and digestion time of surimi gels showed an S-shaped curve and could be represented by Logistic equation. The tight and regular gel structure developed by TGase was difficult to degrade in acid enviromment, resulting in decreased initial free amino acids in digestion juice. As digestion proceeded, the compact structure was destroyed and hydrophobic amino acids were exposed and consequently the gastric digestibility increased until reaching the maximum digestibility. The results suggested that the addition of proper amount TGase to surimi could sustain the release of nutrients.

1.3 Objectives

1. To study the effect of virgin coconut oil (VCO) on properties of surimi gel.
2. To evaluate the effect of pre-emulsified virgin coconut oil (VCO) on properties of surimi gel.
3. To elucidate the effect of ultrasonicated β-glucan on properties of VCO based surimi gel.
4. To investigate the effect of selected antioxidants on properties and storage stability of VCO based surimi gel.
5. To study the nutraceutical potential of the developed surimi gel generated during simulated gastrointestinal (SGI) tract digestion.

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CHAPTER 2

EFFECT OF VIRGIN COCONUT OIL ON PROPERTIES OF SURIMI GEL

2.1 Abstract

Effects of virgin coconut oil (VCO) at various levels (0-25%) on the properties of croaker surimi gels were studied. As the levels of VCO increased up to 15%, breaking force continuously decreased ($p < 0.05$). No differences in breaking force, deformation and fracture constant were noticeable when VCO of 15-25% was incorporated ($p > 0.05$). Based on texture profile analysis, hardness and chewiness decreased as the level of added VCO increased up to 10% ($p < 0.05$), while no marked changes were observed with the addition of 10-25% VCO ($p > 0.05$). Addition of VCO had no profound impact on springiness and cohesiveness. No remarkable change in protein pattern among all surimi gel samples was noticed, regardless of VCO levels. Lower elastic (G') as well as loss moduli (G'') of surimi paste were observed when VCO was added, compared to the control. Nevertheless, there was no marked difference in the moduli among samples containing VCO at all levels. Whiteness of surimi gel increased, whereas expressible moisture content decreased as VCO levels increased ($p < 0.05$). Microstructure study revealed that VCO droplets were distributed uniformly in gel network. Overall likeness of surimi gel was also increased for gel added with VCO. Therefore, VCO addition directly affected textural properties and improved the whiteness as well as sensory property of surimi gel.

2.2 Introduction

Surimi is a concentrated myofibrillar proteins obtained by washing fish mince with water. Surimi is the functional ingredient for various surimi-based seafood products. The increasing popularity of surimi-based products is due to its unique textural properties and high nutritive value. Owing to the health concerns associated with animal fat, which possesses high saturated acid and cholesterol contents, most consumers prefer food products containing no animal fat. Thus, different vegetable oils such as peanut, rapeseed, soybean, and corn oils have been incorporated in surimi-based products to modify gel properties (Shi *et al.*, 2014). Also the vegetable oil

supplemented surimi products have the increased whiteness caused by light scattering effect of oil droplets in the matrix (Benjakul *et al.*, 2004; Hsu and Chiang, 2002). Pietrowski *et al.* (2011) reported that supplementation of surimi with flaxseed, algae, menhaden, krill oils and blend resulted in the improved nutritional value and colour without affecting the texture. 'Fish tofu' is the emulsified surimi gel, which is subsequently fried to obtain the yellow husk/surface. Gel is very white in color and has the smooth and soft texture associated with oil droplets distributed throughout the gel. This product has been popular in Thailand and other countries in Asia.

Virgin coconut oil (VCO) is a rich source of medium-chain fatty acids (MCFAs), notably lauric acid (Marina *et al.*, 2009), which are also found in human breast milk and are easily digestible. Lauric acid has antiviral, antibacterial, anticaries, antiplaque and antiprotozoal functions (German and Dillard, 2004). Trilaurin and tripalmitin fractions of coconut oil were reported to inhibit the promotion stage of carcinogenesis (Nolasco *et al.*, 1994). Short chain fatty acids such as capric, caproic and caprylic acids, which are present in considerable amounts in VCO also had antimicrobial and antiviral effects (Bergsson *et al.*, 1998; German and Dillard, 2004). In general, VCO finds its application in medicines, baby foods and has been known as weight loss aid. Functional food industry is growing at a faster pace and the consumer demand for health foods has been increasing. Due to its health benefits, the incorporation of VCO instead of other vegetable oils could be a means to prepare a functional surimi gels. However, no information regarding the impact of VCO on the properties of surimi gel exists. The present work was undertaken to incorporate VCO at different levels into surimi gels. Properties of resulting surimi gels were examined.

2.3 Objective

To study the effect of virgin coconut oil (VCO) on properties of surimi gel.

2.4 Materials and Methods

2.4.1 Materials and chemicals

Frozen croaker surimi (AA grade) and virgin coconut oil (VCO) with cold press process were procured from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand) and Posture Trading, Ltd (Pathumthani, Thailand) respectively. Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, *N*,*N*,*N'*,*N'*–tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.4.2 Preparation of surimi gel

After thawing for 3-4 hr using a running water, surimi with the temperature of 0-2 °C was cut into small pieces and minced in Moulinex Masterchef 350 mixer (Paris, France) for 1 min. Salt (2.5% w/w) dissolved in iced water was added to minced surimi. The mixture was blended for 1 min and the moisture content of paste was adjusted to 80%. Thereafter, VCO at various levels (5, 10, 15, 20 and 25%) was added into surimi paste, followed by mixing for 1 min. The pastes were then stuffed in polyvinylidine casing with a diameter of 2.5 cm and sealed at both ends. The gels were set at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water baths (Memmert, Schwabach, Germany). All gels were then cooled in iced water. Subsequently gels were stored at 4 °C for 24 h prior to analysis.

2.4.3 Analysis

2.4.3.1 Textural properties

A penetration test was performed using a Model TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England) equipped with a spherical plunger (diameter of 5 mm, depression speed of 60 mm/min). Cylinder-shaped gel samples of 2.5 cm in length were equilibrated to room temperature (25 °C) for 1 h before testing. Breaking force and deformation were determined (Arfat and Benjakul, 2013). The breaking force was defined as the force required in grams to break the gel, and the breaking deformation represented the distance in millimeters travelled by probe from surface of the gel at the point of breakage. Fracture constant (K_f) was calculated as the ratio

of breaking force/deformation. K_f provided a measure of relative rigidity or firmness of gels at the failure point (Herranz *et al.*, 2012).

Texture profile analysis (TPA) of the gels was carried out according to Cheret *et al.* (2005). Cylindrical gels (height of 2.50 cm, diameter of 2.5 cm) were used for the TPA measurement. Gel samples were subjected to two-cycle compression at 50% compression using the texture analyzer with a 70-mm TPA compression plate attachment moving at a speed of 127 mm/min. From the resulting force–time curves, hardness, springiness, cohesiveness, chewiness and resilience were determined.

2.4.3.2 Expressible moisture content

Expressible moisture content was estimated by the method of Benjakul *et al.* (2001) with some modifications. A cylindrical gel sample with a thickness of 0.5 cm was weighed (X g) and sandwiched between two Whatman filter papers No. 1 (Whatman International Ltd., Maidstone, England) at the top and three pieces of the same type of filter paper at the bottom. The sample was pressed by a standard weight of 5 kg for 2 min and weighed again (Y g). Expressible moisture content was expressed as percentage of sample weight as follows:

$$\text{Expressible moisture (\%)} = \left[\frac{(X-Y)}{X}\right] x100$$

2.4.3.3 Whiteness

Whiteness of surimi gels was measured using Hunterlab (ColorFlex, Hunter Associates Laboratory, Reston, VA, USA). L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) were firstly determined. Whiteness was then calculated as described by NFI (1991) as follows:

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

2.4.3.4 Protein patterns

Protein patterns of the surimi gels were determined by Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The protein sample was prepared by adding 3 g of sample to 27 mL

of 5% (w/v) SDS solution heated at 85 °C for 15 min. The mixture was then homogenized for 2 min at a speed of 11,000 rpm using a homogenizer (IKA Labortechnik, Selangor, Malaysia). The homogenate was incubated at 85 °C for 1 h. The samples were centrifuged at 8000 × g for 20 min at room temperature (26–28 °C) using a centrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Tuttlingen, Germany). Protein concentration in the supernatant was determined as per the method of Lowry *et al.* (1951). Solubilized samples were mixed with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% β-ME) at a ratio of 1:1 (v/v) and boiled for 3 min. Samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 10% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, staining of the gel was carried out with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid. The protein standards (Bio-Rad Laboratories, Inc., Richmond, CA, USA) containing myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa) and ovalbumin (45 kDa) were used to estimate the molecular weight of the proteins.

2.4.3.5 Microstructure

Microstructure of gel samples was analyzed using a scanning electron microscope (Quanta 400, FEI, Brno, Czech Republic). Operation was performed at 20 kV with spot size of 4 under an Environmental SEM mode. Microstructure was obtained using Secondary Electron Image (SEI) with Gaseous Secondary Electron Detector (GSED). A standard working distance of 10 mm and pressure of 600 Torr were used for optimal image quality. The samples were visualized at magnification of 300×.

2.4.3.6 Acceptability test

Surimi gel samples were cut into bite size (1 cm in thickness and 2.5 cm in diameter) and equilibrated at room temperature for 30 min. Each sample was assigned a random 3-digit code and served on white paper dish at room temperature

under the fluorescent day-light type illumination. Thirty non-trained panelists (aged between 20 and 32) were the students at the Department of Food Technology, who were acquainted with surimi products. The panelists were asked to evaluate color, texture, odor, flavor and overall likeness of surimi gels 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). Panelists were asked to rinse their mouth after evaluating each sample.

2.4.4 Dynamic rheological properties of surimi paste containing VCO

Dynamic rheological test of surimi pastes containing VCO at various levels was conducted following the method of Shi *et al.* (2014) with a minor modification. The pastes added with VCO at different levels (5-25%) were subjected to oscillatory testing using a rheometer (HAAKE RheoStress1, ThermoFisher Scientific, Karlsruhe, Germany) equipped with a 40 mm parallel steel plate. A gap of 1 mm was set and silicon oil was used to prevent water evaporation. Stress and frequency sweep tests were conducted to obtain the linear viscoelastic region. Stress of 12.69 Pa and frequency of 1 Hz were used to conduct temperature sweep tests, in which the sample was gradually heated from 20 to 90 °C at a heating rate of 1 °C/min. Storage modulus (G') loss modulus (G'') and loss tangent (tan δ) values were recorded as a function of temperature.

2.4.5 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

2.5 Results and Discussion

2.5.1 Properties of surimi gels incorporated with VCO at various levels

2.5.1.1 Textural properties

Breaking force and deformation of surimi gels incorporated with VCO at different levels are shown in Fig 3A and B, respectively. Breaking force decreased continuously as VCO was added up to 15% ($p < 0.05$), in which the decrease by 29% was obtained in gel containing 15%, compared with that of control (without oil). It was noted that no difference in breaking force was found ($p > 0.05$) when VCO at a level of 5% was added. Similar breaking force was attained in gels containing VCO at 15-25% ($p > 0.05$). Deformation of all gels was in the range of 12.03-14.31 mm. A decrease in deformation was noticed when VCO level was incorporated at level up to 15% ($p < 0.05$). No marked change in deformation was found when VCO in the range of 15-25% was added ($p > 0.05$). The result was in agreement with Benjakul *et al.* (2004) who found that addition of soybean oil significantly decreased the breaking force but showed no impact on deformation of surimi gel from bigeye snapper *(Priacanthus tayenus)* and mackerel *(Trachurus japonicus)*. Hsu and Chiang (2002) also reported significant influence of oil concentration on breaking force of golden threadfin bream *(Nemipterus virgatus)* surimi gel, however the deformation was less affected by oil incorporation. With addition of 4% oil, 50% reduction in the breaking force was obtained, however, only 10% reduction in deformation was found.

In general, protein content of surimi gels decreased as VCO was present, especially at high level. VCO droplets also showed the interfering effect on protein-protein interaction in the gel matrix, thus lowering the strength as evidenced by the decreased breaking force. Breaking force and breaking distance of surimi gels from Alaska pollock, common carp, grass carp, and silver carp were well correlated with the protein concentrations of surimi (Luo *et al.*, 2001). When oil was added, oil-protein interaction could occur in gel network. Oil might act as filler in the voids of gel matrix in meat products, thus limiting the movement of the matrix (Dickinson and Chen, 1999; Wu *et al.*, 2009; Yost and Kinsella, 1992). Generally, the reduced surimi protein content in the network structure could not be compensated by the increased

oil content (Shi *et al.*, 2014). Therefore, the decrease in breaking force and deformation were found with increasing VCO level, except in the range of 15-25%, where VCO had no profound effect on reduction of those two parameters. The fracture constant (K_f) is the ratio of breaking force and deformation and it provides a measure of relative rigidity or firmness of gels at the failure point (Herranz *et al.*, 2012). The fracture constant slightly decreased with increasing VCO and ranged from 37.55 to 49.22 (Fig 3C). The result indicated the less rigidity in texture of gel added with VCO. Although the decrease in fracture constant was noticed between the control sample and those added with VCO at levels above 10%, no difference in fracture constant was found in gel added with VCO in the range of 15-25%. Therefore, level of VCO added could significantly influence the textural properties of surimi gel.

Texture profile analysis (TPA) is a rheological method to imitate the mastication process (Figura and Teixeira, 2007). The test involves two complete cycles of compression and decompression of a food material (Lu and Abbott, 2004). Table 1 shows the TPA parameters of surimi gel incorporated with VCO at various levels. The hardness, representing the force required to compress the sample to attain a given deformation, displayed a gradual decrease with increasing VCO levels, and ranged from 52 to 60 N. The decreases in hardness were noticed only after addition of VCO at the levels of 10% or above. The result was concomitant with the breaking force. Chang *et al.* (2015) reported that the surimi gels containing higher soybean oil concentration showed lower hardness and chewiness values. Hardness and chewiness were probably related to the fish protein content. The increasing soybean oil concentration directly resulted in the lower content of fish protein. Pietrowski *et al.* (2011) also found that corn oil addition decreased the hardness and chewiness of Alaska pollock surimi gel.

There were no differences in springiness, the elastic recovery that occurs when the compressive force is removed, among all gels tested, regardless of VCO level used ($p > 0.05$). Chang *et al.* (2015) also noticed insignificant difference in the springiness values of gels with different soybean oil concentrations. The cohesiveness, the capability of breaking down the internal structure, ranged from 0.80

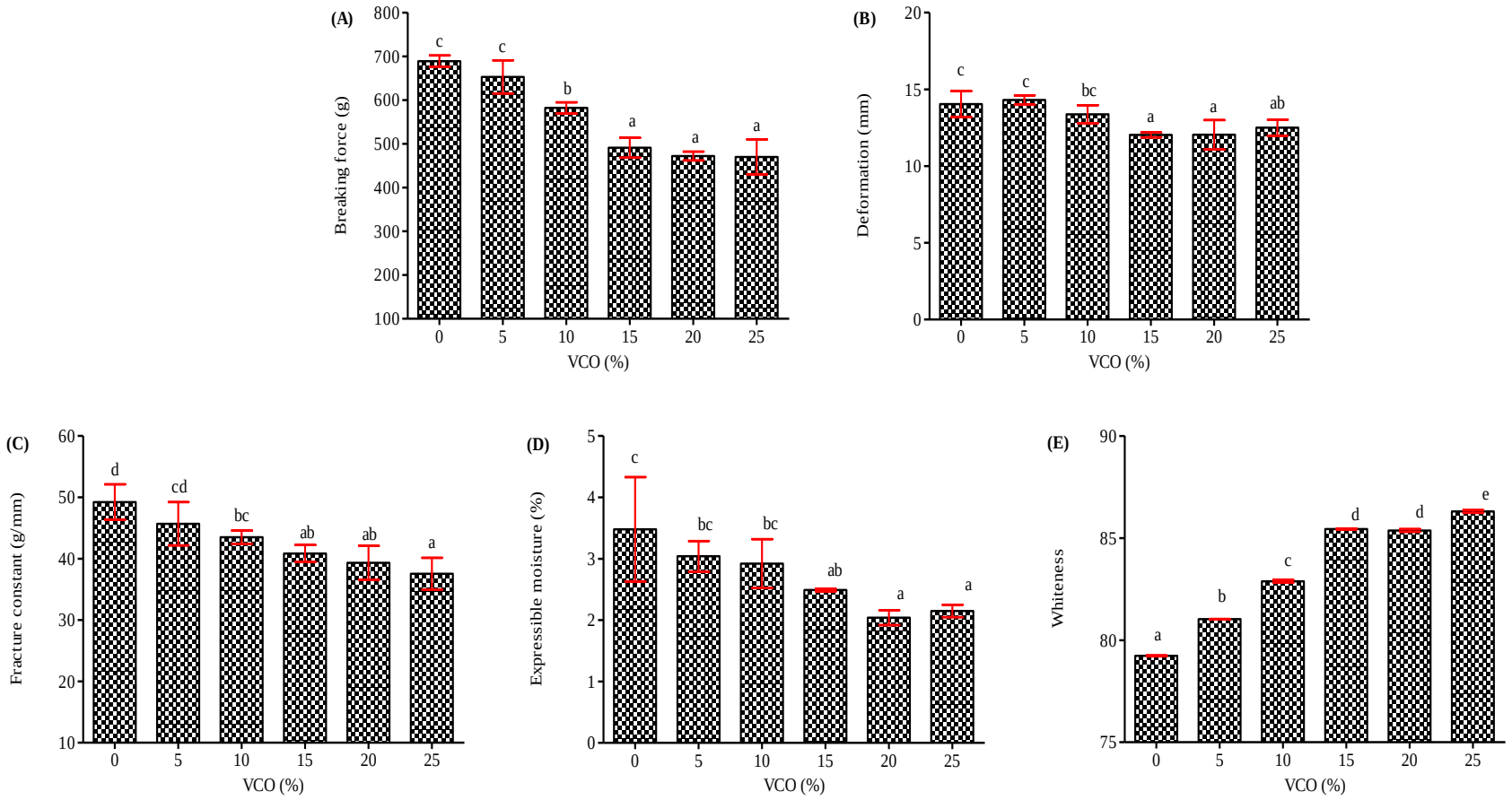

Figure 3. Breaking force (A), deformation (B), fracture constant (k_f) (C), expressible moisture (D) and whiteness (E) of croaker surimi gels added with VCO at different levels. Bars represent the standard deviation (n = 3). Different letters on the bars indicate significant differences ($p < 0.05$)

Table 1. Texture profile analysis of croaker surimi gel added with VCO at different levels

VCO (%)	Hardness (N)	Springiness	Cohesivenss	Chewiness (N)
0	60±3.89*^{c}	0.89±0.03^{a}	0.82±0.01^{bc}	44±4.24^{c}
5	58±0.51^{bc}	0.91±0.02^{a}	0.82±0.00^{bc}	43±0.86^{bc}
10	54±0.44^{ab}	0.90±0.01^{a}	0.83±0.00^{c}	40±0.08^{ab}
15	55±1.02^{ab}	0.90±0.01^{a}	0.80±0.01^{a}	39±0.72^{a}
20	55±1.05^{ab}	0.92±0.01^{a}	0.82±0.00^{bc}	42±1.23^{c}
25	52±0.36^{a}	0.92±0.01^{a}	0.82±0.00^{b}	39±0.71^{b}

* Mean ± S.D (n=3). Different superscripts in the same column indicate significant differences ($p < 0.05$).

to 0.83 and was rarely changed with increasing VCO levels. However, slight decrease in cohesiveness was observed when VCO of 15% was added ($p < 0.05$). Chewiness, representing the energy required for chewing a solid food until it is ready for swallowing, ranged from 39 to 44 N. Chewiness displayed the gradual decrease with increasing VCO levels up to 10%. Nevertheless, no difference was observed among gels containing VCO ranging from 10 to 25% ($p > 0.05$).

2.5.1.2 Expressible moisture content

As shown in Fig 3D, expressible moisture content of surimi gel was decreased with increasing VCO level up to 15% ($p < 0.05$). No further changes were noticeable when VCO was added at the range of 15-25% ($p > 0.05$). The decrease in expressible moisture was observed when VCO was incorporated, more likely associated with the lower water in gel. This was caused by dilution effect of VCO in the gel system. Furthermore, oil droplets might act as water migration barrier from gel network, in which the water could be trapped. However, Shi *et al.* (2014) reported that increasing vegetable oil concentration increased expressible water content of surimi gel.

2.5.1.3 Whiteness

Figure 3E displays the whiteness of surimi gels incorporated with VCO at various levels. Whiteness of surimi gels increased as VCO levels progressively increased and ranged from 79.23 to 86.30. The highest whiteness was observed when VCO at a level of 25% was added ($p < 0.05$). Benjakul *et al.* (2004) found the increased whiteness for mixed surimi from bigeye snapper (SSA and SA grade) and mackerel with the addition of soybean oil. The increased whiteness of surimi with added vegetable oil is attributed to the light scattering effect of the emulsion that is created when oil is comminuted with surimi and water (Park, 2000). Shi *et al.* (2014) also found the increased whiteness of silver carp surimi gel with increasing oil content. Corn oil added Alaska pollock surimi gels had higher whiteness, compared to those without oil (Pietrowski *et al.*, 2011). Therefore, VCO addition directly improved the whiteness of emulsified surimi gels.

2.5.1.4 Protein pattern

Protein patterns of surimi gels without and with the addition of VCO at different levels are depicted in Fig. 4. Surimi paste contained myosin heavy chain (MHC) as the dominant protein, followed by actin and tropomyosin. Disappearance of MHC band was found in the control as well as all VCO containing samples, compared to that observed in surimi paste. The decrease in band intensity of MHC indicated the formation of ε-(γ-glutamyl) lysine intra- and inter-molecular crosslinks of proteins induced by endogenous transglutaminase (TGase) (Kaewudom *et al.*, 2013). However, no marked changes in actin and tropomyosin bands were observed. Endogenous TGase plays a role in setting of surimi by forming non-disulfide covalent bonds (Benjakul and Visessanguan, 2003). Crosslinks were not dissociated by the mixture of SDS and β-mercaptoethanol used for electrophoresis (DeJong and Koppelman, 2006). Therefore, VCO incorporation in the range of 5-25% had no remarkable effect on the protein crosslinking of the surimi gel, particularly by covalent bonds mediated by endogenous TGase.

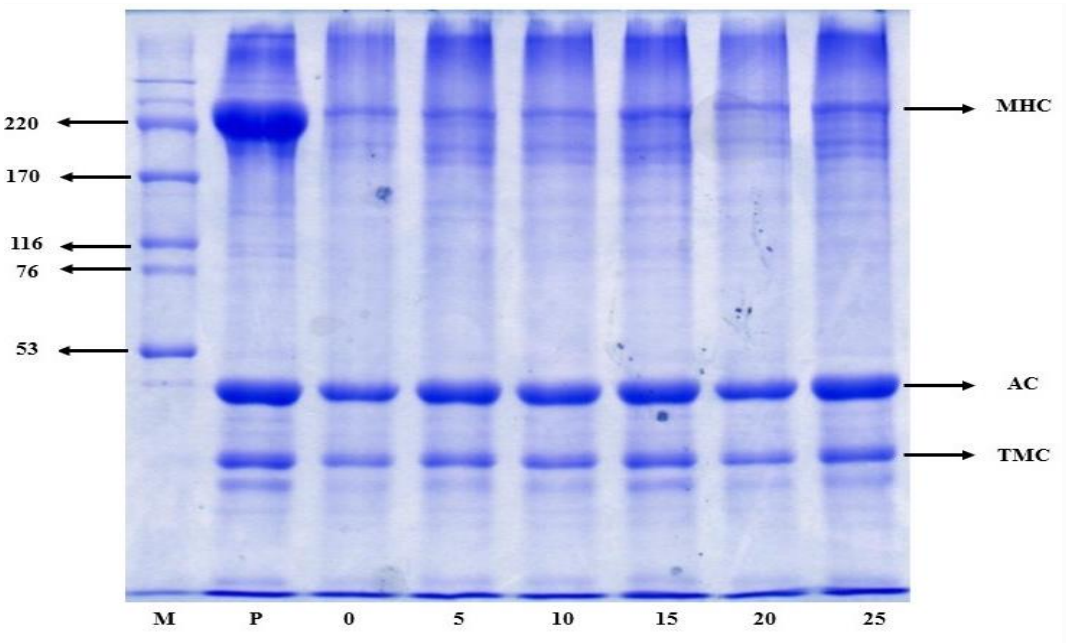

Figure 4. SDS-page patterns of croaker surimi gels added with VCO at different levels. M: marker; P: surimi paste; numbers (0-25) denote the level of VCO added (%); MHC: myosin heavy chain; AC: actin; TMC: tropomyosin

2.5.1.5 Microstructure

The microstructure of croaker surimi gel without and with VCO added at different levels (5–25%) is shown in Fig. 5. A typical fibrous structure was observed in the control sample, however VCO droplets were embedded in the gel matrix of VCO containing samples. It was noted that the number of oil droplets was increased with increasing amounts of VCO added. Benjakul *et al.* (2004) reported the spherical soybean oil droplets dispersed in the matrix of the mixed snapper and mackerel surimi gels. At higher VCO levels, the larger oil globules were also observed. Lower proportion of myofibrillar proteins in the paste containing higher amount of VCO more likely resulted in their reduced emulsifying capacity. This was evidenced by the coalescence, in which the larger oil droplets were formed. The shape of VCO droplets varied from spherical to oval. Some irregular shaped droplets were also embedded in the matrix. The light scattering by the VCO droplets in the gel matrix was responsible for whiter color of VCO added gels, compared to the control gel (Fig. 3E). Moreover, oil droplets showed the higher interfering effect on protein–protein interaction in the gel matrix by increasing the intermolecular distances between protein chains, thus lowering the strength as evidenced by the decreased breaking force (Fig. 3A) and hardness (Table 1). Such a phenomenon was more pronounced as VCO levels increased. However, Zhou *et al.* (2017) reported that camellia tea oil could occupy the void spaces of surimi protein matrix and formed a firmer gel.

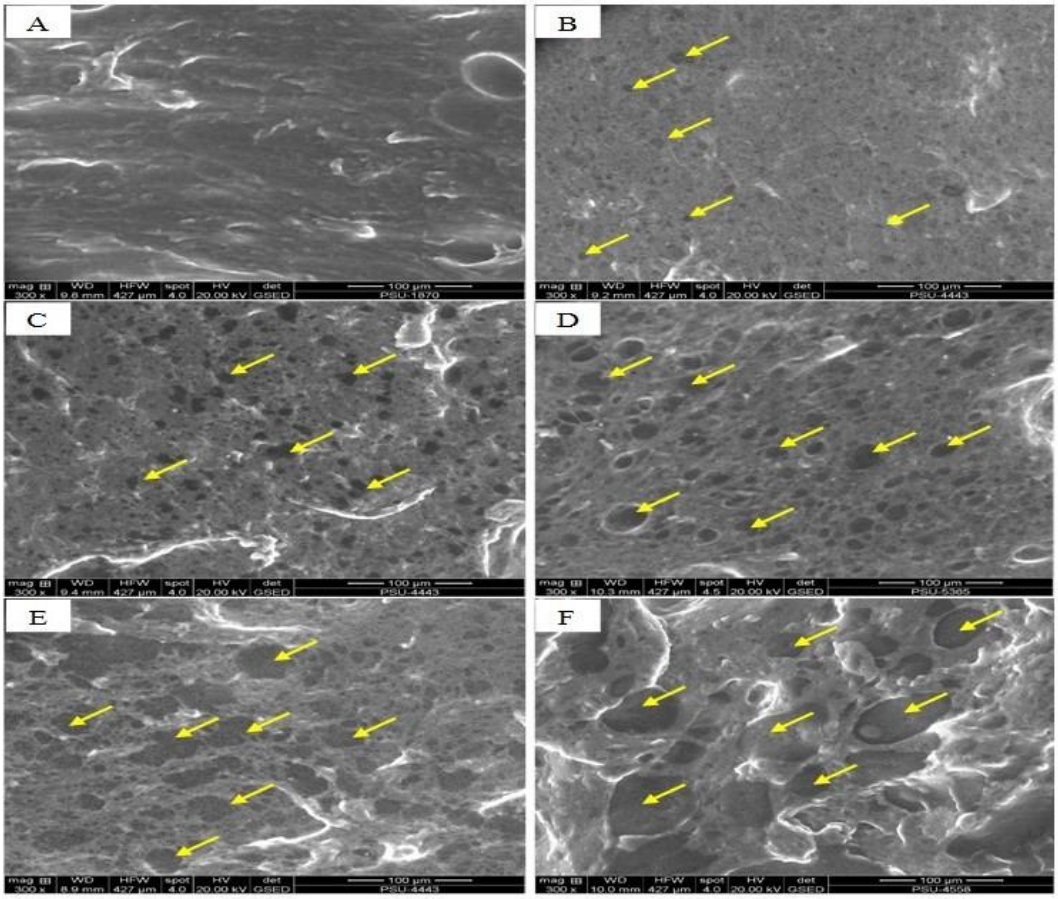

Figure 5. Electron microscopic images of croaker surimi gel added without (A) and with 5% VCO (B), 10% VCO (C), 15% VCO (D), 20% VCO (E) and 25% VCO (F) The arrows show the embedded VCO droplets. Magnification: 300×

2.5.1.6 Likeness score

Likeness score of surimi gel supplemented with VCO at various concentrations is presented in Table 2. VCO incorporation increased the color likeneness of surimi gels but there was no difference in score between samples added with VCO at 10-25% ($p > 0.05$). With increasing VCO levels, the higher whiteness was attained (Fig 3E). Gel with whiter color is more desirable and VCO droplets played a role in improved whiteness. For texture likeness, no difference in score was found among all samples ($p > 0.05$). The likeness score for odor significantly increased with the addition of VCO ($p < 0.05$), compared to the control. For flavor, higher score was gained for gels added with VCO at all concentrations used,

compared to the control. The overall likeness also increased with increasing VCO concentrations. Nonetheless, there was no difference between the control and 5% VCO added sample ($p > 0.05$). Also similar scores were found among samples incorporated with VCO ranging from 10 to 25% ($p > 0.05$). Therefore, the addition of VCO was able to improve sensory property of surimi gel, particularly when VCO at higher amount was added.

2.5.2 Dynamic rheological properties of surimi paste incorporated with VCO at various levels

Rheological behavior of surimi paste containing VCO at various levels in comparison with the control during thermal treatment from 20 to 90 °C is illustrated in Fig. 6. For the control (surimi paste without VCO), G' steadily increased and reached the peak value at around 40 °C. The initial increase of G' at low temperature could be related to actomyosin molecular interactions and formation of protein network structure via hydrogen bonds between protein molecules, which formed a weaker three-dimensional gel network (Lefevre *et al.*, 1998; Zhang *et al.*, 2013). The lower values of G' for the samples incorporated with VCO at temperature above 25 °C, compared to the control, could be due to the plasticizing effect of liquefied fat globules distributed in three dimensional protein network. This led to the reduction of the attractive forces between protein chains, thus making it more flexible (Shima and Tanimoto, 2016).

Subsequently, G' decreased at 40 °C and then dropped to the minimum abruptly at around 50 °C for all the samples. At the higher temperature, a large number of hydrogen bonds were destroyed, thus destabilizing the protein aggregate previously formed (Liu *et al.*, 2007). Additionally, the breakdown of gel structure might take place by degradation of myosin mediated by endogenous proteolytic enzymes in this temperature range termed 'Modori' (Wasson, 1992). Increased protein mobility and breakdown of the gel network structure also occurred due to the dissociation of actin-myosin complex and the denaturation of myosin tail (Shi *et al.*, 2014). Upon further heating, G' increased until reaching the temperature of 65 °C and then remained

Table 2 Likeness score of croaker surimi gel added with VCO at different levels

VCO (%)	Color	Texture	Odor	Flavor	Overall likeness
0	5.9±1.4*[a]	6.9±1.7[a]	5.7±1.5[a]	6.4±0.8[a]	6.0±1.1[a]
5	6.5±0.9[ab]	6.8±1.0 [a]	6.6±1.1[b]	6.8±0.7 [a]	6.4±0.9[ab]
10	6.9±0.6[bc]	6.6±1.3 [a]	6.9±0.8[b]	6.6±1.0 [a]	6.8±0.7[bc]
15	7.2±0.9[bc]	7.2±1.0 [a]	7.0±1.2[b]	6.7±1.0 [a]	7.1±0.9[bc]
20	7.2±1.2[bc]	6.8±1.1 [a]	7.2±0.9[b]	6.8±0.9 [a]	6.9±0.8[bc]
25	7.8±0.7[c]	7.5±1.0 [a]	7.2±1.1[b]	7.0±1.0 [a]	7.2±0.7[c]

* Mean ± S.D (n=3). Different superscripts in the same column indicate significant differences ($P < 0.05$).

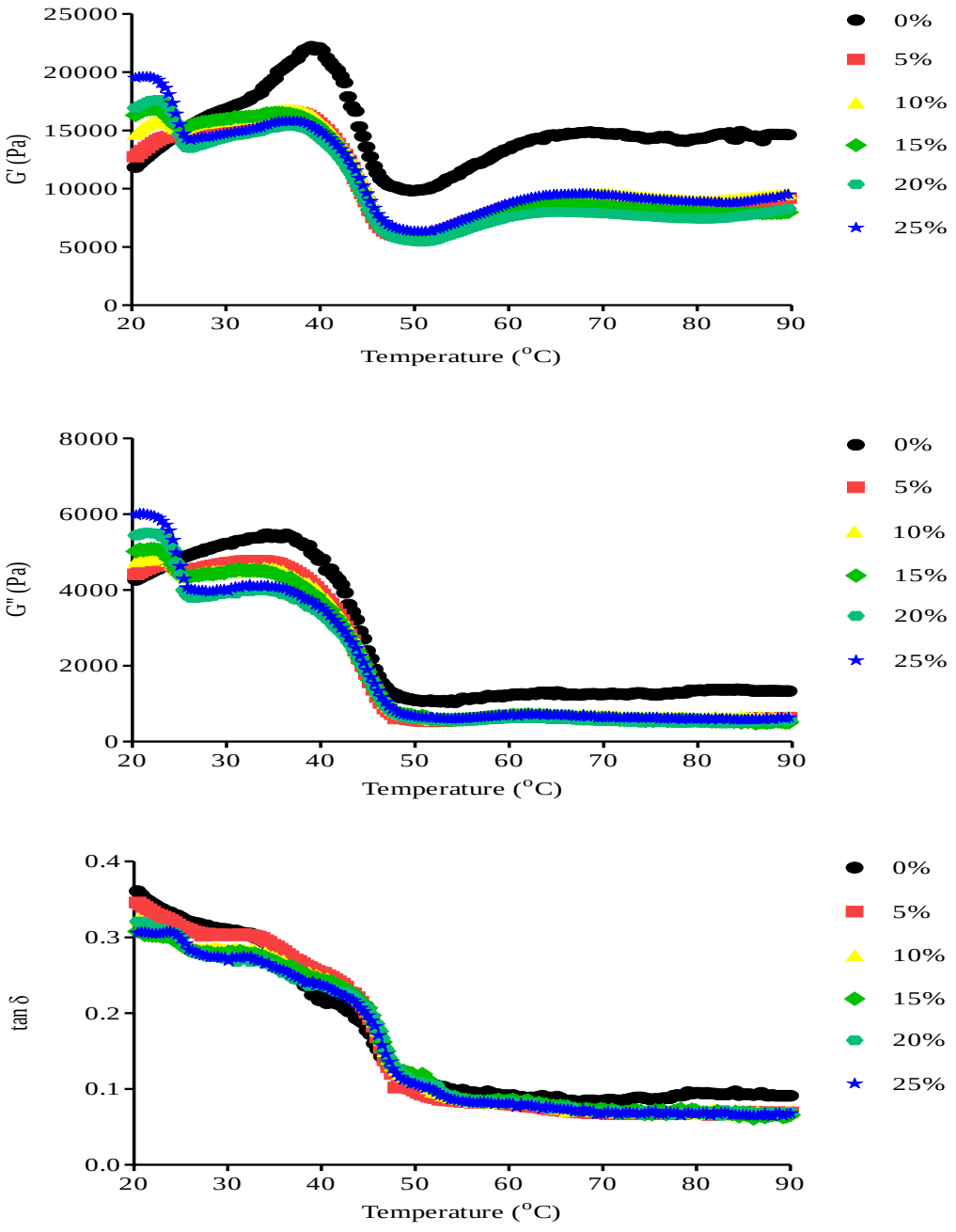

Figure 6. Viscoelastic property of croaker surimi paste added with VCO at different levels as a function of temperature. G': storage modulus G'': loss modulus and tan δ: loss tangent

almost constant up to 90 °C. The phenomenon might be caused by the formation of a stable gel structure associated with enhanced protein aggregation (Liu *et al.*, 2007). The G' values correlated well with the springiness values of the samples. The progress and patterns of the G' as a function of heating temperature were similar, regardless of VCR levels added. All the VCO added samples displayed higher values of moduli (G' and G") in the temperature range of 20-24 °C, compared to the control. Since the melting point of VCO was in the range of 22-27 °C (Srivastava *et al.*, 2017), VCO was solidified at temperature lower than 25 °C. VCO in solid state might function as a filler. Consequently, it could increase viscoelasticity of paste. When heat was applied, the phase change from solid to liquid state took place in the VCO and resulted in an abrupt decline in G' of VCO added samples in the temperature range of 23-24 °C.

In general, the G" curves were similar to those of G', but G" values were much lower than G' values over the entire temperature range tested (Fig. 6). VCO containing pastes had the sharp decrease in G" at around 24 °C, which could be due to phase transition of VCO from solid to liquid state. On the other hand, G" value for the control increased gradually until 35 °C and then rapidly decreased and reached the minimum value at 50 °C. Upon further heating, G" remained almost constant over the remaining temperature up to 90 °C.

With increasing temperature, the value of tan δ was decreased, indicating the formation of stronger gel network. Smaller value of tan δ reflects stronger gel structure and more elastic behavior (Malik and Saini, 2017). However, a sharp decrease in tan δ was noticed in the temperature range of 50-60 °C, implying that this temperature range was crucial for a stronger gel network formation. Overall, the final G', G" and tan δ of VCO added gels were lower than those of control. This was consistent with the lower breaking force of surimi gel added with VCO

2.6 Conclusion

Addition of VCO significantly influenced the texture and whiteness of surimi gel. Breaking force and deformation decreased as VCO level increased up to 15%. However whiteness of the gel increased with increasing VCO levels.

Viscoelastic study revealed that VCO addition affected elastic (G') as well as loss moduli (G'') of surimi paste. Nevertheless, addition of VCO had no impact on protein pattern of all gels. Uniform distribution and anchoring of VCO droplets in the protein matrix were noticed. VCO incorporation increased the likeness scores for color, odor and overall likeness but had no impact on texture and flavor likeness. Thus, VCO at an appropriate level could be used to replace other vegetable oils, in which health benefits could be claimed, especially as a source of medium chain fatty acids.

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CHAPTER 3

IMPACT OF VIRGIN COCONUT OIL NANOEMULSION ON PROPERTIES OF CROAKER SURIMI GEL

3.1 Abstract

Nanoemulsion based on 5% virgin coconut oil (VCO) was prepared with the aid of ultrasound using sodium caseinate (2 and 3%) as emulsifier for various times (5-20 min). The smallest average particle size of resulting nanoemulsion (3.88 nm) was obtained when 3% sodium caseinate and ultrasonication of 5 min were used. Nevertheless, the nano droplets were flocculated when visualized using confocal laser scanning microscope. The effects of VCO and nanoemulsion at a level of 5% on properties of croaker surimi gel with the mixing time of 3 and 5 min were studied. No differences in breaking force, deformation and fracture constant were observed between the control gel and that added with nanoemulsion, regardless of mixing time ($p > 0.05$). No difference in TPA parameters was observed between the control gel and that added with nanoemulsion, however mixing time had a significant impact on the hardness and chewiness ($p < 0.05$). No change in protein pattern of all the surimi gel samples was found for both mixing times, irrespective of VCO or nanoemulsion added. No remarkable effect on elastic (G') as well as loss (G") moduli of surimi paste was found with the addition of either VCO or nanoemulsion, but higher G' was generally attained for the control sample. Whiteness increased, while the expressible moisture decreased with the addition of either VCO or nanoemulsion. VCO droplets from nanoemulsion were embedded in the gel matrix and distributed uniformly. In general overall likeness was increased for the gels added with VCO nanoemulsion, compared to the control, most likely owing to the increased whiteness.

3.2 Introduction

Emulsions are fine dispersant of one liquid in another immiscible liquid. Because of their large interfacial area, emulsions are thermodynamically unstable structures and small molecule or polymeric surfactants are required to act at the interface (Elwell *et al.,* 2004). The formation of an emulsion includes the mechanical

mixing of the immiscible liquids and the arrangement of surfactant molecules at the interface of the two phases. The mechanical energy can be achieved by Rotor-stator systems or high-pressure homogenizers (Cucheval and Chow, 2008). Ultrasound can be used as an alternative powerful method to produce an emulsion. The advantages of ultrasound include lower energy consumption, use of less surfactant and production of more homogeneous emulsion, compared to a typically mechanical process (Abismail *et al.,* 1999). Ultrasonic homogenisation is considered as a 'green technology' due to its high efficiency, economic performance and low instrumental requirements (Abbas *et al.,* 2015). It is well known that ultrasonics can be used to produce very small emulsion droplets that are exceptionally stable (Leong *et al.,* 2009).

Ultrasound can be delineated into low and high intensity ultrasound according to its frequency range (Awad *et al.,* 2012). Low intensity ultrasound is used to ensure safety and quality of food unlike high intensity ultrasound, which is used to modify the functional properties including improving the stability of emulsions (Chemat and Khan, 2011). In an emulsion system containing a combination of whey protein isolate and xanthan, either increasing ultrasonic power or duration of ultrasonic treatment resulted in significant reduction in the droplet size of oil phase (Kaltsa *et al.,* 2014). Similarly, an emulsion system based on coconut milk protein had a reduced mean droplet size with improved stability after ultrasonic treatment (Lad and Murthy, 2012). Nanoemulsions are metastable dispersions of nanoscale droplets less than of 100 nm made by application of high shear such as ultrasonication, etc. (Augustin and Hemar, 2009). Ultrasound has been proposed as a potential technique to produce nanoemulsions (10-100 nm radius). It can impart a sufficiently high energy input to reduce the droplet dimensions at nano-level of oil-in-water mixtures (Canselier *et al.,* 2002; Abbas *et al.,* 2013; Silva *et al.,* 2012). Kentish *et al.* (2008) prepared nanoemulsions of flexseed oil using ultrasonic batch process at 20 kHz with flow-through process at 24 kHz and Tween-40 was used as a surfactant. The produced nanoemulsions possessed good stability against creaming, sedimentation, flocculation and coalescence because of the small droplet size.

The delivery of bioactive components in a complex/real food matrix has been considered as a vast area to be explored. Some particular oils have been incorporated in food products. Shanmugam and Ashokkumar (2014) incorporated flax seed oil in pasteurized homogenized skim milk using high intensity ultrasound to produce emulsion droplets of 0.64 µm size, which was stable for at least 9 days. Additionally, Anandan *et al.* (2017) incorporated 7% of black seed oil in pasteurized homogenized skim milk (PHSM) with the aid of 20 kHz high intensity ultrasound.

Pre-emulsified oil can be prepared before addition into meat or muscle food product to ensure the uniform distribution of oil or fat in finished products. Pre-emulsification is a process of preparing an emulsion (oil in water in this case) stabilized with an emulsifier, which is typically a protein of a non-meat origin (Cáceres *et al.*, 2008). The process can improve fat binding ability, enhance physical stability, and is usually easier to disperse into a water-based system such as a meat batter (Jiménez-Colmenero, 2007). When oil/fat is pre-emulsified with a non-meat protein, gel formation of meat proteins become better, compared to those added with oil without pre-emulsification (Hoogenkamp, 1987).

Recently, virgin coconut oil (VCO) has been added into surimi gel as the additional source of medium chain fatty acids. However, oil droplets showed the interfering impact on textural property of surimi gel to some degree (Gani *et al.*, 2017). The use of pre-emulsified oil, especially in form of 'nanoemulsion', could be a means to ensure the stability of emulsion and avoid the collapse of oil droplets in surimi gel matrix. The light scattering effect of nanoemulsion might improve whiteness of surimi gel, while providing health benefits via supplying medium chain fatty acids to consumers. The objective of this study was to investigate the impact of nanoemulsion based on VCO on properties of surimi gel.

3.3 Objective

To evaluate the effect of pre-emulsified virgin coconut oil (VCO) on properties of surimi gel.

3.4 Materials and Methods

3.4.1 Materials and chemicals

Frozen croaker surimi (AA grade) and virgin coconut oil (VCO) produced by cold press process were procured from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand) and Posture Trading, Ltd (Pathumthani, Thailand), respectively. Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, *N*,*N*,*N'*,*N'*–tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

3.4.2 Effect of emulsification conditions on characteristics of VCO nanoemulsion

Nanoemulsion based on VCO was prepared with the aid of ultrasound. Emulsions were prepared by mixing VCO and sodium caseinate solution (2 and 3%, w/w) at a ratio of 1:3.5 (w/w). The mixture was homogenized at 11,000 rpm for 5 min using a homogenizer (IKA, Labortechnik homogenizer Selangor, Malaysia). The obtained coarse emulsions were then ultrasonicated using the ultrasonic processor (Sonics, Model VC750, Sonica & Materials, Inc., Newtown, CT, USA) equipped with a titanium alloy (Ti-6Al-4V) probe (13 mm diameter) with an on-time and off-time of 5 sec at an amplitude of 60% with a constant frequency of 20 kHz ± 50 Hz and high intensity power of 750W for different times (5, 10, 15 and 20 min). The emulsion samples were subjected to analysis.

A laser diffraction method was used to measure the size distribution of emulsion droplets using a ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). The emulsion samples were analyzed without further dilution during the size measurement. The particle sizes were reported as the Z-average mean diameter. The refractive index for water (25 °C) was set at 1.33. The broadness of the droplet size distribution was indicated by the polydispersity index (PDI).

The microstructure of the selected emulsion sample, which had the smallest particle size, was observed with confocal laser scanning microscope (CLSM)

(Model FV300; Olympus, Tokyo, Japan). The sample was dissolved in Nile blue A solution (1:10) and mixed well to obtain uniformity. Fifty microliters of sample were smeared on the microscope slide. The CLSM was operated in the fluorescence mode at the excitation wavelength of 533 nm and the emission wavelength of 630 nm using a Helium Neon Red laser (HeNe-R) for lipid analysis. A magnification of 400× was used.

3.4.3 Effect of VCO nanoemulsion on properties of surimi gel

After thawing for 3–4 h using running water, surimi with the core temperature of 0–2 °C was cut into small pieces and minced in a Moulinex Masterchef 350 mixer (Paris, France) for 1 min. Salt (2.5% w/w) dissolved in iced water was added to minced surimi. The mixture was blended for 1 min and the moisture content of paste was adjusted to 80%. Thereafter, VCO at a level of 5% was added into surimi paste, followed by mixing for 1 min. For the sample containing VCO nanoemulsion, the prepared nanoemulsion showing the smallest particle size was added into surimi paste to obtain the final VCO content of 5% (based on surimi paste). A control sample was prepared without VCO or nanoemulsion. The pastes were then stuffed in polyvinylidene casing with a diameter of 2.5 cm and sealed at both ends. The gels were set at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). All gels were then cooled in iced water. Subsequently gels were stored at 4 °C for 24 h prior to analysis.

3.4.4 Analysis

3.4.4.1 Textural properties

A penetration test was conducted following the method of Buamard and Benjakul (2015) using a Model TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England) equipped with a spherical plunger (diameter of 5 mm, depression speed of 60 mm/min). Cylindrical gel samples of 2.5 cm in length were equilibrated to room temperature (25 °C) for 1 h before testing. A spherical plunger was pressed into the cut surface of a gel sample perpendicularly at a constant depression speed. The force to puncture into the gel (breaking force) and the distance at which the

plunger punctured into the gel (deformation) were both recorded. Fracture constant (K_f), which provides a measure of relative rigidity of gels at the failure point, was estimated as the ratio of breaking force/deformation (Herranz *et al.*, 2012).

Texture profile analysis (TPA) of the gels was also carried out according to Kaewudom *et al.* (2013) with a slight modification. Cylindrical gels (height of 2.50 cm, diameter of 2.5 cm) were used for TPA. Gel samples were subjected to two cycle compression at 50% compression using the texture analyzer with a 70-mm TPA compression plate attachment moving at a speed of 127 mm/min. From the resulting force–time curves, hardness, springiness, cohesiveness, chewiness and resilience were determined.

3.4.4.2 Expressible moisture content

Expressible moisture content was determined following the method of Benjakul *et al.* (2007). Cylindrical gel samples were cut into a thickness of 5 mm, weighed accurately (X) and placed between three pieces of Whatman filter paper (Whatman International Ltd., Maidstone, UK) at the bottom and two pieces on the top of the sample. After being placed with a standard weight of 5 kg on the top for 2 min, the sample was then removed from the papers and weighed again (Y). Expressible moisture content was calculated with the following equation and expressed as percentage of sample weight:

$$\text{Expressible moisture content (\%)} = \left[\frac{(X-Y)}{X}\right] x100$$

3.4.4.3 Whiteness

Whiteness of gel samples was determined using a colorimeter (HunterLab, Colorflex, Hunter Associates Laboratory, VA, USA). CIE L*, a* and b* values were measured and whiteness was then calculated using the following equation (Yang *et al.*, 2014):

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

3.4.4.4 Protein patterns

Protein patterns of the surimi gels were determined by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) as modified by Buamard and Benjakul (2015). The protein sample was prepared by adding 3 g of sample to 27 mL of 5% (w/v) SDS solution heated at 85 °C for 15 min. The mixture was then homogenized for 2 min at a speed of 11,000 rpm using a homogenizer (IKA Labortechnik, Selangor, Malaysia). The homogenate was incubated at 85 °C for 1 h. The samples were centrifuged at 8,000 × g for 20 min at room temperature (26–28 °C) using a centrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Tuttlingen, Germany). Protein concentration of the supernatant was determined by the Biuret method (Robinson and Hogden, 1940) using bovine serum albumin as standard. Solubilized samples were mixed with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% β-ME) at a ratio of 1:1 (v/v) and boiled for 3 min. Samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 10% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gels were stained with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid. The protein standards (Bio-Rad Laboratories, Inc., Richmond, CA, USA) containing myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa) and ovalbumin (45 kDa) were used to estimate the molecular weight of the proteins.

3.4.4.5 Microstructure

Microstructure analysis of gel was performed at 20 kV with spot size of 4 under an Environmental SEM mode using a scanning electron microscope (Quanta 400, FEI, Brno, Czech Republic). Microstructure was obtained using SEI (Secondary Electron Image) with Gaseous Secondary Electron Detector (GSED). A standard working distance of 10 mm and pressure of 600 Torr were used for optimal image quality. The samples were visualized at a magnification of 300×.

3.4.4.6 Acceptability test

Surimi gel samples were cut into bite size (thickness of 1 cm and diameter of 2.5 cm) and equilibrated at room temperature for 30 min. Each sample was assigned a random 3-digit code and served on white paper dish at room temperature under the fluorescent day-light type illumination. Thirty non-trained panelists (aged between 20 and 32) were the students at the Department of Food Technology, who were acquainted with surimi products. The panelists were asked to evaluate for color, texture, odor, flavor and overall likeness of surimi gels 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.* (2007). Panelists were asked to rinse their mouths after evaluating each sample.

3.4.5. Dynamic rheological properties of surimi paste without and with VCO or VCO nanoemulsion

Dynamic rheological test of surimi pastes containing VCO, VCO nanoemulsion and the control sample was conducted following the method of Petcharat and Benjakul (2017) with a minor modification. The pastes were subjected to oscillatory testing using a rheometer (HAAKE RheoStress1, ThermoFisher Scientific, Karlsruhe, Germany) equipped with a 40 mm parallel steel plate. A gap of 1 mm was set and silicon oil was used to prevent water evaporation. Stress and frequency sweep test was conducted to obtain the linear viscoelastic region. Stress of 12.69 Pa and frequency of 1 Hz were used to conduct temperature sweep test, in which the sample was gradually heated from 20 to 90 °C at a heating rate of 1 °C/min. Storage modulus (G'), loss modulus (G'') and loss tangent (tan δ) values were recorded as a function of temperature.

3.4.6 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test (Steel and Torrie, 1980).

For pair comparison, *t*-test was used. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3.5 Results and discussion

3.5.1 Characteristics of VCO nanoemulsion as affected by sodium caseinate (SCN) levels and ultrasonication time

The particle size distribution of nanoemulsion samples prepared under various conditions is shown in Fig 7. Based on the preliminary studies, ultrasonic amplitude of 60% yielded emulsion with the lowest droplet size (data not shown). All the nanoemulsions exhibited unimodal particle size distribution with polydispersity index (PDI) ranging from 0.24 to 0.28. PDI measures the broadness of size distribution and it indicates the deviation from mean particle size (Tadros *et al.*, 2004). A value of >0.5 indicates a broad particle size distribution (Gibis, Rahn, & Weiss, 2013). The average particle sizes of droplets in emulsion containing 2 and 3% SCN were 4.20-5.88 nm and 3.88-4.84 nm, respectively. At the same ultrasonication time, the decrease in particle size was found as SCN level was increased from 2 to 3% (Fig 7). The results indicated that SCN level had the pronounced effect on the droplet size of nanoemulsions and SCN acted as emulsifier, which stabilized emulsion formed.

Droplet coalescence was enhanced when insufficient emulsifier was used. The result suggested that SCN at 3% was able to align and occupy fully at the oil-water interface, thus preventing the coalescence. A moderate increase in emulsifier concentration causes a considerable decrease in particle size because the extra emulsifier is able to stabilize more interfacial area (Periasamy *et al.*, 2016). Rebolleda *et al.* (2015) successfully incorporated wheat bran oil into water systems by the formation of nanoemulsions with droplet size of 40 nm using a combination of high speed blender (29,000 rpm–5 min) and ultrasonic processor (50 s). The mixture of 1% of wheat bran oil (WBO) and 7.3% of a surfactant mixture Span 80 (37.4%) and Tween 80 (62.6%) was required to stabilize emulsion. It was noted that ultrasonication time longer than 5 min resulted in the larger droplet. The increase in

particle size with increasing sonication time indicated that droplets coalesced during sonication (Anandan *et al.*, 2017). Ultrasound cavitation has been employed for the formation of oil-in-water nanoemulsions using different edible oils such as coconut oil (Ramisetty *et al.*, 2015), sunflower oil (Leong *et al.*, 2009), flaxseed oil (Shanmugam and Ashokkumar, 2014; Shanmugam and Ashokkumar, 2015) and basil oil (Ghosh *et al.*, 2013). Furtado *et al.* (2017) produced the stable emulsions containing sunflower oil in the presence of sodium caseinate used as emulsifier by ultrasonication with fixed power and frequency of 300 W and 20 kHz, respectively with sonication time of 2-6 min. Sonication time of 6 min resulted in the smallest mean droplet size (1.89 µm).

The high shear and temperatures generated during acoustic cavitation can also partially unfold and denature proteins (Shanmugam *et al.*, 2012). This might promote the alignment of proteins at the interface. Ultrasonication was reported to facilitate cross-linking of proteins to form aggregates (Cavalieri *et al.*, 2008). The protein cross-linking can be reversible, for instance through hydrophobic interactions and hydrogen bonding, or irreversible if covalent links via disulphide bond are produced, mainly induced by free radicals generated through ultrasonic cavitation (Cavalieri *et al.*, 2011). However, long time of ultrasonication could enhance the destabilization of protein films surrounding the oil droplets. This led to the collapse of emulsion as indicated by the increase in droplet size. Thus, the sonication time of 5 min and SCN at 3% were found as the optimal condition, yielding the VCO nanoemulsion with the smallest droplet size of 3.88 nm. It was found that sonication time less than 5 min was not sufficient to provide the stable nanoemulsion (data not shown).

Fig. 8 shows CLSM image of nanoemulsion emulsified with 3% SCN using ultrasonication at 60% amplitude for 5 min. Minute particulates were distributed uniformly under CLSM. Based on the particle size analysis, the droplets were in the nanoscale (Fig. 7). The image indicated that nano droplets were flocculated, in which the large flocs were noticeable by CLSM. However, the nanoemulsion prepared were stable when stored at room temperature for more than 2 days (data not shown).

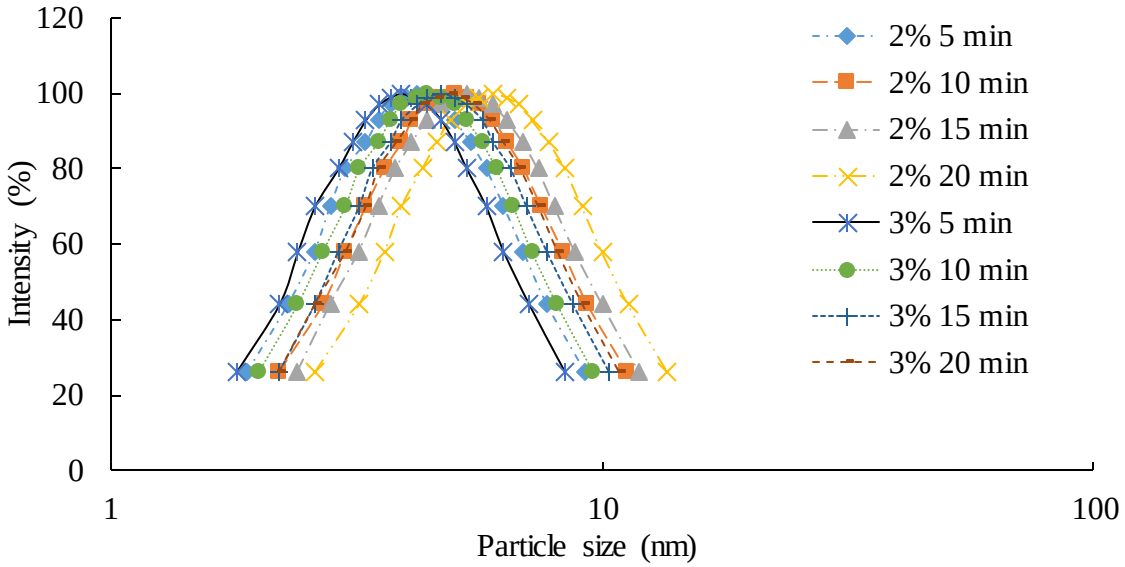

Figure 7. Particle size distribution of nanoemulsion stabilized by 2% and 3% SCN and ultrasonicated at 60% amplitude for 5-20 min

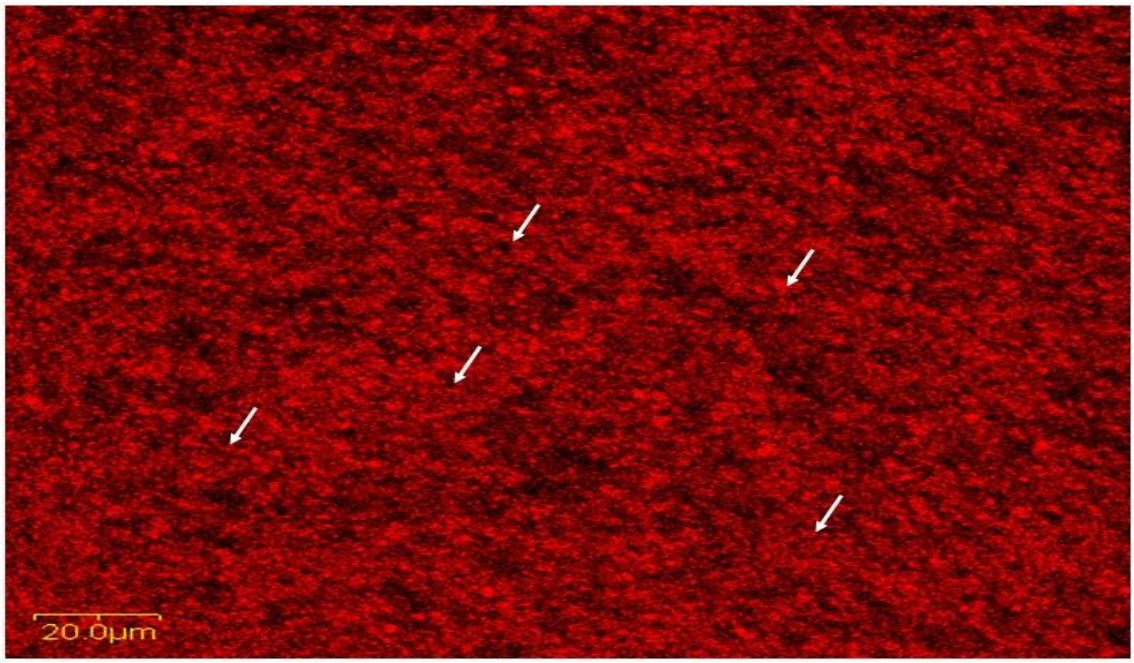

Figure 8. Confocal laser scanning image of 5% VCO nanoemulsion stabilized by 3% SCN and produced at ultrasonic amplitude of 60% and time of 5 min. The arrows point to the flocculated nano droplets.

3.5.2 Properties of surimi gels incorporated with VCO nanoemulsion

3.5.2.1 Textural properties

Breaking force and deformation of croaker surimi gels incorporated without and with 5% VCO or 5% VCO nanoemulsion, in which mixing times of 3 and 5 min were employed, are shown in Fig. 9 (A) and (B), respectively. For the gels containing VCO, breaking force decreased significantly, compared to the control ($p < 0.05$), irrespective of the mixing time. However, no change in breaking force was found in the gels added with VCO nanoemulsion, compared to the control ($p > 0.05$). Similar result was observed when mixing times of 3 and 5 min were implemented. Therefore, mixing time had no impact on breaking force of surimi gel, regardless of VCO or VCO nanoemulsion incorporated. Generally, the protein content of surimi gels decreased as VCO or VCO nanoemulsion was added. VCO droplets more likely showed the interfering effect on protein-protein interaction in the gel matrix, thus lowering the strength as shown by decreased breaking force. Nevertheless, the addition of VCO nanoemulsion had no effect on breaking force of surimi gel. Thus, droplets of nanoemulsion with very small size did not disrupt the interaction of muscle proteins in gelation process.

Deformations of all the gels were in the range of 11.74-12.80 mm. No change in deformation of gels was noticeable when VCO was incorporated, compared to the control (without VCO or nanoemulsion) and that added with VCO nanoemulsion, when the mixing time of 3 min was used ($p > 0.05$). However, VCO added gel showed higher deformation, compared to the control, when the mixing time was 5 min ($p < 0.05$). Also, mixing time showed no impact on deformation of all the gels tested. Nevertheless, Shi *et al.* (2014) reported significant decrease in breaking force of silver carp surimi gel when oil concentration was increased, whereas deformation was slightly decreased. Benjakul *et al.* (2004) reported that addition of soybean oil significantly decreased the breaking force but did not cause much change in the deformation of surimi gel. Hsu and Chang (2002) reported 50% reduction in the breaking force of golden threadfin bream *(Nemipterus virgatus)* surimi gel with the addition of 4% oil. The gel showed 10% decrease in deformation. Furthermore,

Zhou *et al.* (2017) suggested that camellia tea oil could occupy the void spaces of surimi protein matrix and formed a firmer gel. It was noted that increasing trend in deformation of gel added with VCO was plausibly attributed to the lower protein-protein interaction by VCO droplets, thus increasing extensibility of gel.

The fracture constant (K_f) is the ratio of breaking force and deformation and it provides a measure of relative rigidity or firmness of gels at the failure point (Herranz *et al.*, 2012). The fracture constant was decreased with the addition of VCO for both mixing times. Similar fracture constant was found between the control and gel added with VCO nanoemulsion, for both mixing times used ($p > 0.05$). Fracture constants were 50-57 g/mm and 49-60 g/mm for the gels with mixing time of 3 and 5 min, respectively. The result indicated that nanoemulsion had no influence on fracture constant of surimi, irrespective of the mixing time. Therefore, the addition of VCO nanoemulsion had no influence on textural properties of surimi gel. VCO could be added as a source of medium chain fatty acids as the preemulsified form with droplets of nanoscale (nanoemulsion).

Texture profile analysis (TPA) is a testing method to simulate the mastication process (Figura and Teixeira, 2007). The test involves two complete cycles of compression and decompression of a food material (Lu and Abbott, 2004). Textural properties of surimi gels added without and with 5% VCO or 5% VCO nanoemulsion are presented in Table 3. Hardness, representing the force required to compress the sample to attain a given deformation, was lower for VCO added samples, compared to the control ($p < 0.05$). This was in accordance with the decrease in breaking force of gel incorporated with VCO (Fig. 9A). However, no difference in hardness was found between the control and VCO nanoemulsion added samples for both mixing times ($p > 0.05$). Increasing mixing time had effect on hardness for all the samples tested ($p > 0.05$). Longer mixing time (5 min) yielded the gels with higher hardness than the shorter time (3 min) ($p < 0.05$). With longer mixing, proteins were allowed to be more solubilized and entanglement or protein-protein interaction could take place to a higher extent. Simultaneously, VCO or nanoemulsion could be more dispersed and uniformity was achieved. Chang *et al.* (2015) reported that the surimi

gels containing higher soybean oil concentration showed lower hardness. Hardness and chewiness of surimi gel were directly related to the fish protein content. The increasing soybean oil concentration directly lowered the content of fish protein. Pietrowski *et al.* (2011) also found that corn oil addition decreased the hardness of Alaska pollock surimi gel.

There were no differences in springiness, the elastic recovery that occurs when the compressive force is removed, among all samples, irrespective of mixing time ($p > 0.05$). Moreover, all gel samples showed similar cohesiveness, capability in breaking down the internal structure. Zhou *et al.* (2017) reported no change in cohesiveness of surimi gel prepared from white croaker as camellia tea oil was added up to 4%. However, there was slight difference in the cohesiveness of the gels containing VCO nanoemulsion between mixing times of 3 and 5 min ($p < 0.05$). The springiness and cohesiveness values were 0.88-0.91 and 0.79-0.83 for all the gel samples, respectively. Chang *et al.* (2015) also noticed insignificant difference in springiness values of gels from silver carp surimi (grade AA) with different soybean oil concentrations ranging from 0 to 2%. In our previous study, no significant change in springiness was found in gel samples added with VCO ranging from 5 to 25%, compared to the control (Gani *et al.*, 2017).

Chewiness, representing the energy required for chewing a solid food until it is ready for swallowing, ranged from 43 to 48 N and from 47 to 54 N for the samples with mixing time of 3 and 5 min, respectively. Chewiness displayed the decrease with addition of 5% VCO ($p < 0.05$). Nevertheless, the addition of VCO nanoemulsion did not affect the chewiness of gel for both mixing times ($p > 0.05$). It was noted that the gels added with VCO nanoemulsion had the similar chewiness to that of controls ($p > 0.05$). Different mixing times had the effects on the chewiness of all the gels ($p < 0.05$). Similar to hardness, the increase in chewiness was gained with increasing mixing time from 3 to 5 min ($p < 0.05$).

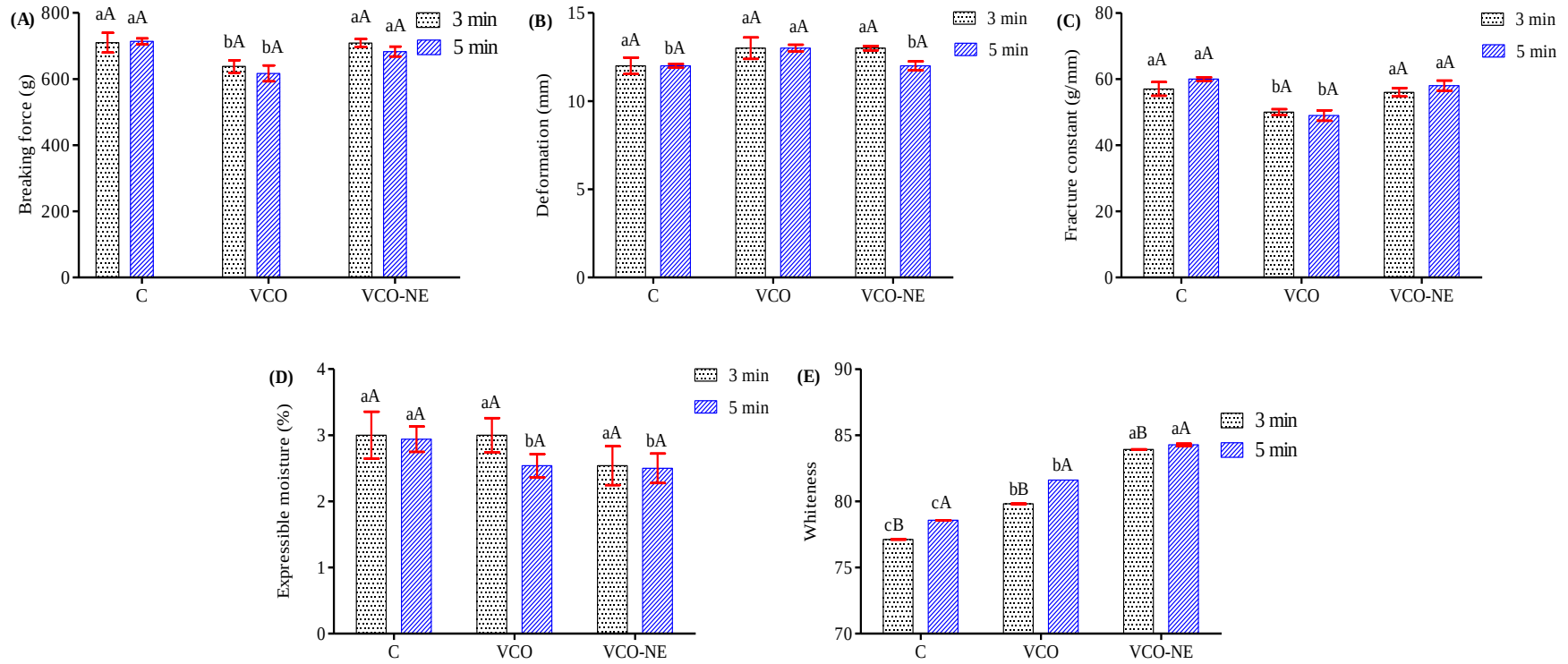

Figure 9. Breaking force (A), deformation (B), fracture constant (C), expressible moisture (D) and whiteness (E) of croaker surimi gel added without and with 5% VCO or VCO nanoemulsion prepared with different mixing times. Different lowercase letters on the bars with the same mixing time indicate significant difference ($p < 0.05$). Different uppercase letters within the same sample indicate significant difference ($p < 0.05$). C: Control (without VCO or VCO nanoemulsion); VCO: Surimi gel added with 5% VCO; VCO-NE: Surimi gel added with 5% VCO nanoemulsion.

Table 3. Texture profile analysis of croaker surimi gel added without and with 5% VCO or 5% VCO nanoemulsion prepared with different mixing times

Mixing time	Samples	Hardness (N)	Springiness	Cohesiveness	Chewiness (N)
3 min	C	65 ± 0.22^{aB}	0.90 ± 0.03^{aA}	0.80 ± 0.02^{aA}	46 ± 0.30^{aB}
	VCO	58 ± 0.84^{bB}	0.91 ± 0.01^{aA}	0.82 ± 0.01^{aA}	43 ± 1.32^{bB}
	VCO-NE	64 ± 0.82^{aB}	0.90 ± 0.01^{aA}	0.83 ± 0.00^{aA}	48 ± 0.30^{aB}
5 min	C	76 ± 3.35^{aA}	0.89 ± 0.01^{aA}	0.81 ± 0.01^{aA}	54 ± 1.47^{aA}
	VCO	65 ± 0.33^{bA}	0.90 ± 0.01^{aA}	0.81 ± 0.01^{aA}	47 ± 0.52^{bA}
	VCO-NE	73 ± 0.08^{aA}	0.88 ± 0.02^{aA}	0.79 ± 0.00^{aB}	54 ± 0.74^{aA}

C: Control (without VCO or VCO nanoemulsion); VCO: Surimi gel added with 5% VCO; VCO-NE: Surimi gel added with 5% VCO nanoemulsion. Different lowercase superscripts within the same column under the same mixing time indicate significant difference ($p < 0.05$). Different uppercase superscripts within the same column under the same sample indicate significant difference ($p < 0.05$).

3.5.2.2 Expressible moisture content

Expressible moisture content of gel added without and with 5% VCO or 5% VCO nanoemulsion is shown in Fig. 9D. Expressible moisture content decreased in both surimi gels containing VCO and VCO nanoemulsion, compared to the control gel ($p < 0.05$), when mixing time of 5 min was employed. Nevertheless, no difference in expressible moisture content was found for either VCO or VCO nanoemulsion added samples compared to control when mixing time of 3 min was used ($p > 0.05$). There was no difference in expressible moisture content between gels with mixing time of 3 and 5 min, for all samples tested ($p > 0.05$). The expressible moisture content of the gels produced by mixing times of 3 and 5 min was 2.54-3.32 and 2.5-2.94%, respectively. The decrease in expressible moisture was observed when VCO or VCO nanoemulsion was incorporated, more likely associated with the lower water in gel. This was caused by dilution effect of VCO or VCO nanoemulsions in the gel system. Furthermore, oil droplets might act as water migration barrier from gel network, in which the water could be trapped. However, Shi *et al.* (2014) reported that increasing vegetable oil concentration increased expressible water content of surimi gel. Our previous study also demonstrated the decreased expressible moisture content with increasing level of VCO from 5% to 25% (Gani *et al.*, 2017).

3.5.2.3 Whiteness

Whiteness of all the gels increased ($p < 0.05$) with the addition of 5% VCO or 5% VCO nanoemulsion, as shown in Fig. 9E. For the same gel, the higher whiteness was found for gels prepared with the longer mixing time ($p < 0.05$). Among all the samples, the highest whiteness was observed with the samples added with VCO nanoemulsion with the mixing of 5 min ($p < 0.05$). Benjakul *et al.* (2004) found the increased whiteness for mixed surimi from bigeye snapper (SSA and SA grade) and mackerel with the addition of soybean oil. The increased whiteness of surimi added with vegetable oil is attributed to the light scattering effect of the emulsion, created when oil is comminuted with surimi and water (Park, 2000). Shi *et al.* (2014) also reported the increased whiteness of silver carp surimi gel with increasing oil content. Corn oil added Alaska pollock surimi gels had higher whiteness, compared to those

without oil (Pietrowski *et al.*, 2011). VCO nanoemulsion contained smaller droplets of oil, which were stabilized by Na caseinate. It was milky or creamy in appearance. The incorporation of nanoemulsion with sufficient mixing time (5 min) allowed small oil droplet to disseminate uniformly in gel matrix, leading to the increased light scattering. Therefore, VCO addition, especially in the form of nanoemulsion, directly improved the whiteness of surimi gel.

3.5.2.4 Protein pattern

Protein patterns of surimi gels added without and with 5% VCO or 5% VCO nanoemulsion, in which mixing time of 3 and 5 min was used, are shown in Fig. 10. Surimi paste contained myosin heavy chain (MHC) as the dominant protein, followed by actin and tropomyosin. Marked decrease in MHC band was found in the control, 5% VCO added sample and VCO nanoemulsion added sample, compared to that observed in surimi paste. The decrease in band intensity of MHC indicated the formation of ε-(γ-glutamyl) lysine intra- and inter-molecular crosslinks of proteins induced by endogenous transglutaminase (TGase) (Kaewudom *et al.*, 2013). However, no marked changes in actin and tropomyosin bands were observed. Endogenous TGase plays a role in setting of surimi by forming non-disulfide covalent bonds (Benjakul and Visessanguan, 2003). Crosslinks were not dissociated by the mixture of SDS and β-mercaptoethanol used for electrophoresis (DeJong and Koppelman, 2006). The results suggested that the incorporation of VCO or emulsified VCO nanoemulsion had no remarkable effect on the protein cross-linking of the surimi gels. Additionally, mixing times showed the negligible impact on protein cross linking of surimi gel, regardless of VCO or VCO nanoemulsion addition. In our previous study, the addition of VCO up to 25% had no effect on the protein pattern of croaker surimi gel (Gani *et al.*, 2017).

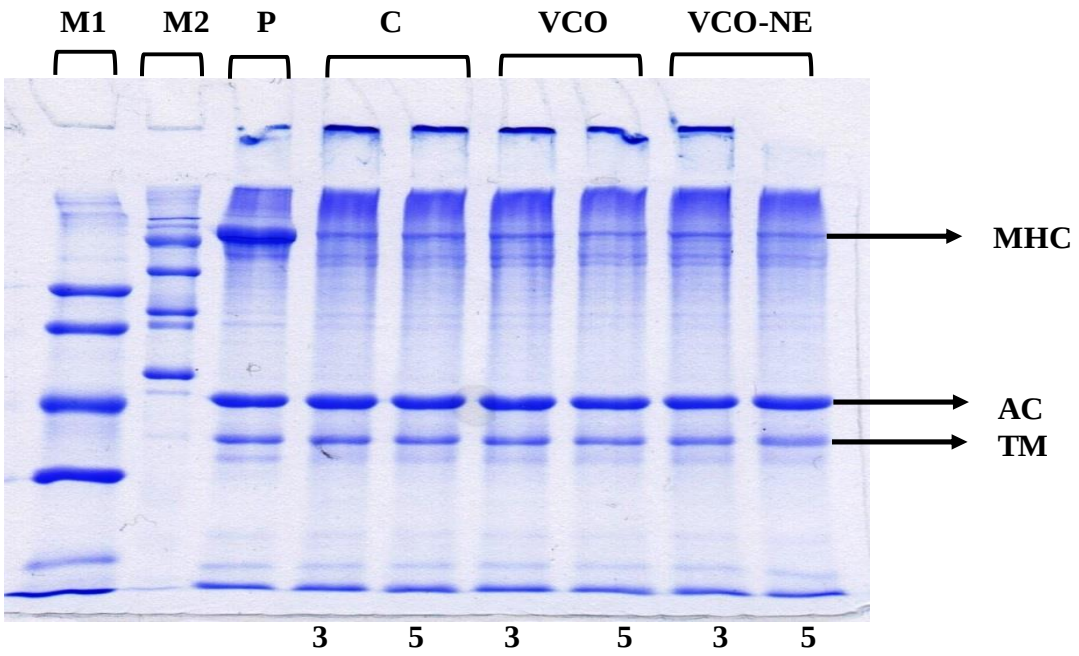

Figure 10. SDS PAGE pattern of croaker surimi gel added without and with 5% VCO or 5% VCO nanoemulsion prepared with different mixing times. M1: Low MW markers, M2: High MW markers, P: surimi paste, MHC: myosin heavy chain, TM: tropomyosin, AC: actin, C: Control (without VCO or VCO nanoemulsion); VCO: Surimi gel added with 5% VCO; VCO-NE: Surimi gel added with 5% VCO nanoemulsion. Numbers (3, 5) represent mixing time (min)

3.5.2.5 Microstructure

The microstructures of croaker surimi gel in the absence and presence of 5% VCO or 5% VCO nanoemulsion having the mixing times of 3 and 5 min are illustrated in Fig. 11. A typical fibrous structure was observed in the control samples. The sample with 5 min mixing time showed slightly finer structure, compared to that mixed for 3 min. This was in agreement with the higher hardness and chewiness of gel with 5 min mixing time, compared to 3 min (Table 3). VCO droplets were found anchored in the gel matrix of VCO containing samples. However, more uniform distribution can be noticed in the gel mixed for 5 min, compared to 3 min. The shape of VCO droplets varied from spherical to oval. Benjakul *et al.* (2004) reported the spherical soybean oil droplets dispersed in the matrix of the mixed snapper and

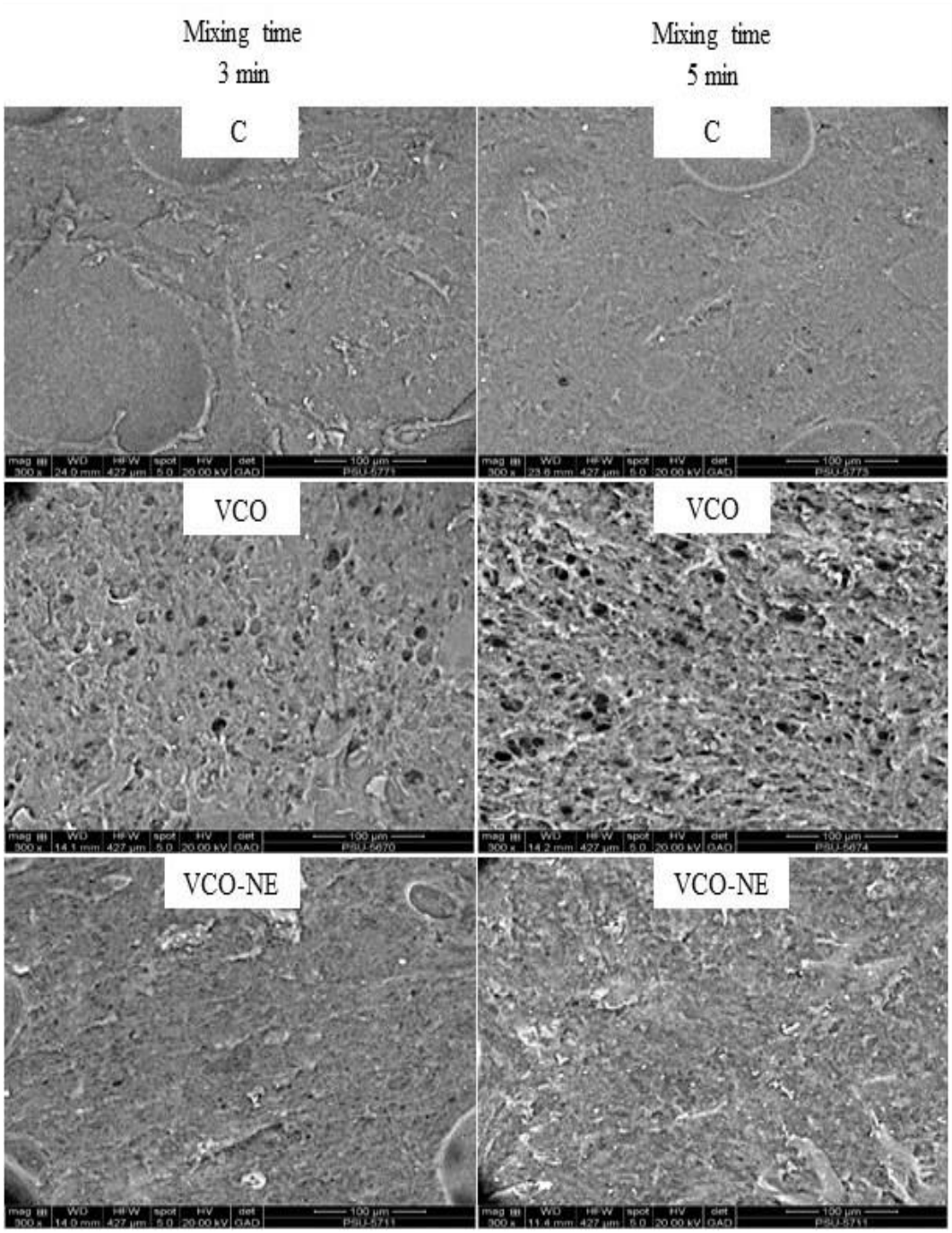

Figure 11. Electron microscopic images of croaker surimi gel added without and with 5% VCO or 5% VCO nanoemulsion prepared with mixing time of 3 min and 5 min.Magnification: 300× C: Control (without VCO or VCO nanoemulsion); VCO: Surimi gel added with 5% VCO; VCO-NE: Surimi gel added with 5% VCO nanoemulsion.

mackerel surimi gels. The results were consistent with our previous study, in which VCO droplets were embedded in the gel network (Gani *et al.*, 2017). For the gels containing VCO nanoemulsions, no visible oil droplets could be found, owing to their size in the nanometer range (Fig. 7). However, slightly coarser network was observed in comparison with that of control gel. The light scattering by the nanoemulsion in the gel matrix was responsible for whiter color of VCO nanoemulsion added gels, compared to the control gel (Fig 9E). Moreover, large oil droplets showed the higher interfering effect on protein-protein interaction in the gel matrix by increasing the intermolecular distances between protein chains as indicated by the larger voids, thus lowering the strength as evidenced by the decreased breaking force in 5% VCO added gels. On the other hand, the addition of VCO nanoemulsion did not affect breaking force, irrespective of mixing times (Fig. 9A).

3.5.2.6 Likeness score

Likeness score of surimi gel in comparison with gels with 5% VCO and 5% VCO nanoemulsion with different mixing times (3 and 5 min) is presented in Table 4. VCO or VCO nanoemulsion incorporation increased the color likeness of surimi gels for both 3 and 5 min mixing time ($p < 0.05$). However, mixing time had no impact on the color likeness for all the gels tested ($p > 0.05$). Addition of either 5% VCO or 5% VCO nanoemulsion increased the whiteness of resulting gel (Fig 9E). The results clearly demonstrated that gels with whiter color were more desirable. VCO droplets and VCO nanoemulsion directly improved the whiteness of the gels. For texture likeness, sample added with VCO nanoemulsion with mixing time of 3 min had a higher score than the control ($P < 0.05$). However, no difference in score of texture likeness was found among samples when the mixing time of 5 min was used ($p > 0.05$). There was an increase in the odor likeness score when VCO or VCO nanoemulsion was incorporated, compared to the controls ($p < 0.05$), irrespective of mixing times. Nontheless, no difference in flavor likeness score among all the samples ($p > 0.05$) was found. For overall likeness, sample added with nanoemulsion prepared by both mixing times had higher scores than control and VCO added sample ($p < 0.05$). This was most likely due to the increase in whiteness of gel added with VCO

nanoemulsion (Fig. 9E). Color likeness score was generally in accordance with overall likeness.

3.5.3 Dynamic rheological properties of surimi paste incorporated with VCO nanoemulsion

Rheological behavior of surimi paste added without and with 5% VCO and 5% VCO nanoemulsion using the mixing time of 3 and 5 min is shown in Fig. 12. G' steadily increased and reached the peak value at around 40 °C. The initial increase of G' at low temperature could be related to actomyosin molecular interactions and formation of protein network structure via hydrogen bonds between protein molecules, which formed a weaker three-dimensional gel network (Lefevre *et al.*, 1998; Zhang *et al.*, 2013).

Subsequently, G' decreased at 40 °C and then dropped to the minimum abruptly at around 50 °C for all the samples. At the higher temperature, a large number of hydrogen bonds were destroyed, thus destabilizing the protein aggregate previously formed (Liu *et al.*, 2007). Additionally, the breakdown of gel structure might take place by degradation of myosin mediated by endogenous proteolytic enzymes in this temperature range, which is known as 'Modori' (Buamard and Benjakul, 2015; Singh and Benjakul, 2017). Increased protein mobility and breakdown of the gel network structure also occurred due to the dissociation of actin-myosin complex and the denaturation of myosin tail (Shi *et al.*, 2014). Upon further heating, G' increased until reaching the temperature of 65 °C and then remained almost constant up to 90 °C. The phenomenon might be caused by the formation of a stable gel structure associated with enhanced protein aggregation (Liu *et al.*, 2007; Buamard and Benjakul, 2015). The increased G' values correlated well with the springiness values of the samples. The progress and patterns of the G' as a function of heating temperature were generally similar, regardless of the sample. Overall, G' value was higher in the control sample mixed for 3 min, indicating the formation of a firmer gel network. Mixing time of 5 min could disrupt the muscle proteins filaments, in which the entanglement during gelation could be retarded. This was evidenced by the lower G' when the mixing time was longer (5 min). This result was opposite to TPA, in which hardness

Table 4. Likeness score of croaker surimi gel added without and with 5% VCO or 5% VCO nanoemulsion prepared with different mixing times

Mixing time	Samples	Color	Texture	Odor	Flavor	Overall
3 min	C	6.9 ± 0.8^{cA}	6.7 ± 1.5^{bA}	6.3 ± 1.4^{bA}	6.5 ± 1.3^{aA}	6.3 ± 1.0^{cA}
	VCO	7.5 ± 0.8^{bA}	7.2 ± 1.1^{abA}	7.1 ± 1.4^{aA}	6.8 ± 1.4^{aA}	7.0 ± 1.2^{bA}
	VCO-NE	8.1 ± 0.8^{aA}	7.4 ± 1.1^{aA}	7.1 ± 1.4^{aA}	7.2 ± 1.4^{aA}	8.0 ± 0.9^{aA}
5 min	C	7.0 ± 0.9^{bA}	6.7 ± 1.6^{aA}	6.6 ± 1.3^{bA}	6.7 ± 0.9^{aA}	6.4 ± 0.8^{cA}
	VCO	7.6 ± 1.0^{aA}	7.2 ± 1.3^{aA}	7.4 ± 1.3^{aA}	7.1 ± 1.1^{aA}	7.1 ± 1.0^{bA}
	VCO-NE	8.0 ± 1.0^{aA}	7.3 ± 1.3^{aA}	7.4 ± 1.0^{aA}	7.3 ± 1.2^{aA}	7.8 ± 0.9^{aA}

C: Control (without VCO or VCO nanoemulsion); VCO: Surimi gel added with 5% VCO; VCO-NE: Surimi gel added with 5% VCO nanoemulsion. Different lowercase superscripts within the same column under the same mixing time indicate significant difference ($p < 0.05$). Different uppercase superscripts within the same column under the same sample indicate significant difference ($p < 0.05$).

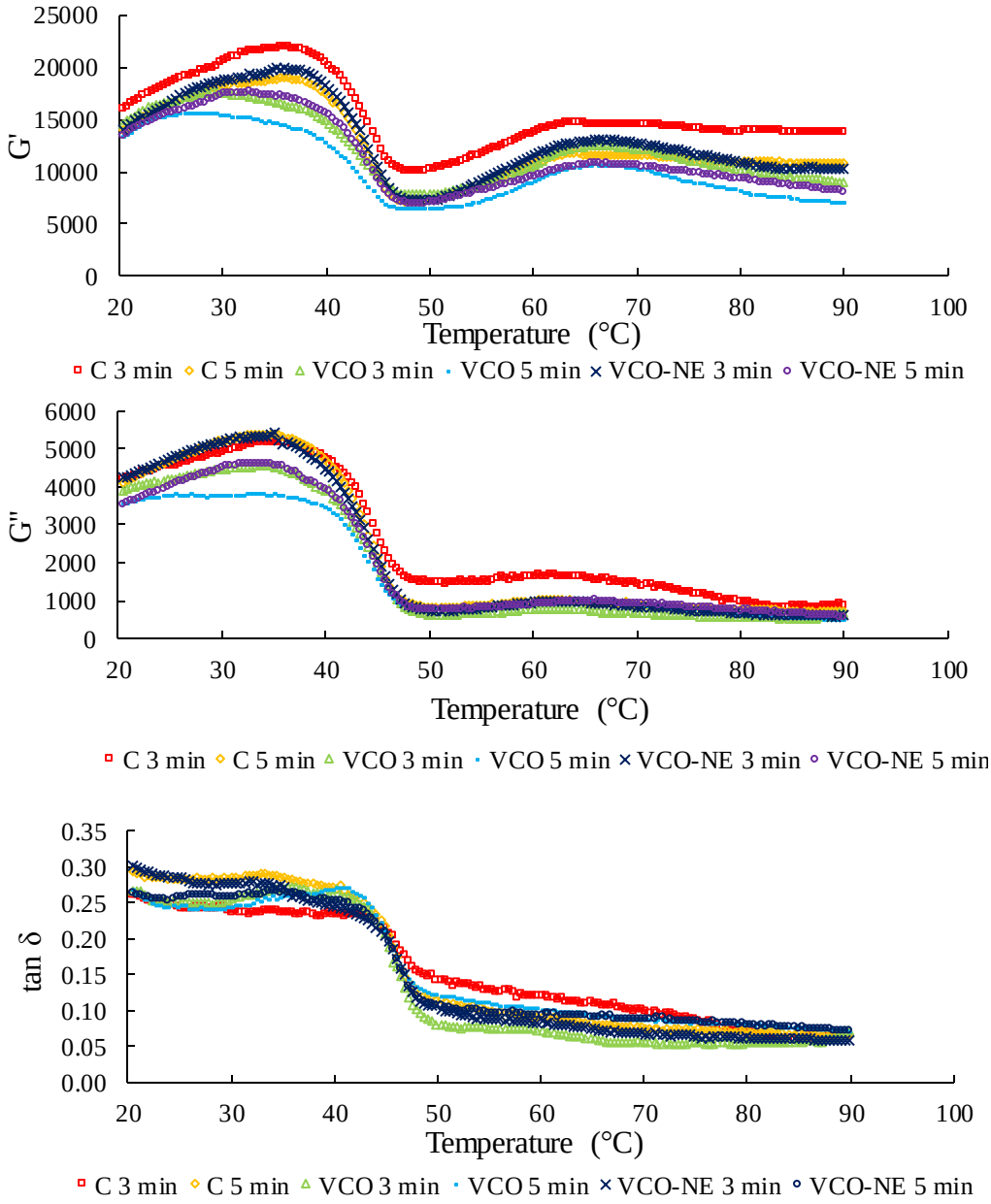

Figure 12. Viscoelastic property of croaker surimi paste added without and with 5% VCO or 5% VCO nanoemulsion as a function of heating temperature. C: Paste without VCO or VCO nanoemulsion; VCO: Paste added with 5% VCO; VCO-NE: Paste added with 5% VCO nanoemulsion; 3 min and 5 min represent mixing time

was increased with increasing mixing time. Dynamic rheology measures the dynamic change in viscoelastic behavior, while the system is undergoing transition from sol to gel state. Hardness indicates the force required to compress the sample to a given deformation when it is in the static gel state. For the samples mixed for 5 min, disaggregation of actin-myosin network structure more likely enhanced mobility of protein, resulting in a decreased G' (Zhang *et al.*, 2015). When considering for gel, the degree of unfolding protein structure in surimi paste prepared became higher by increasing chopping time, which provided more sites for protein-protein interaction in the evolved gel network. (Kobayashi *et al.*, 2017). As a consequence, enhanced protein-protein interaction in the evolved gel structure could increase hardness for the gel samples mixed for 5 min. For samples added with VCO or VCO nanoemulsion, the lower G' was noticeable. Oil droplets could interfere the interaction or association of protein chains or filament. Comparing VCO and VCO nanoemulsion added sample, the former showed a lower value of G' than the later.

In general, the G'' curves were similar to those of G', but G'' values were much lower than G' values over the entire temperature range tested (Fig. 12). However, no remarkable effect of 5% VCO or 5% VCO nanoemulsion could be noticed on the loss modulus.

With increasing temperature, the value of tan δ was decreased, indicating the formation of stronger gel network. Smaller value of tan δ reflects stronger gel structure and more elastic behavior (Malik and Saini, 2017). However, a sharp decrease in tan δ was noticed in the temperature range of 40-50 °C, implying that this temperature range was crucial for a stronger gel network formation. Overall, the final G' and G'' of VCO and VCO nanoemulsion added surimi paste were lower than those of control. When comparing the mixing time between 3 and 5 min, the mixing time of 5 min rendered the lower values of parameters tested.

3.6 Conclusion

VCO nanoemulsion was successfully produced by ultrasonication. Addition of VCO nanoemulsion had no remarkable effect on textural properties of surimi gel, while VCO showed some impact, mainly by lowering breaking force. However, the gel whiteness showed the profound increase with the addition of nanoemulsion and led to the increase in likeness. Addition of VCO nanoemulsion had no impact on the protein pattern and microstructure. Therefore, VCO nanoemulsion was the appropriate form to be incorporated in the surimi without deteriorating its textural properties and significantly increased whiteness and likeness of surimi gel.

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CHAPTER 4

EFFECT OF β-GLUCAN STABILIZED VIRGIN COCONUT OIL NANOEMULSION ON PROPERTIES OF CROAKER SURIMI GEL

4.1 Abstract

Effects of VCO nanoemulsion stabilized by β-glucan and β-glucan solution at final β-glucan levels of 5-20% (based on solid weight of surimi) on croaker surimi gel were comparatively studied. Increases in breaking force, deformation and fracture constant were found in surimi gel containing VCO nanoemulsion stabilized by 5% β-glucan, whereas the decrease in all properties were observed as level of β-glucan in nanoemulsion increased. The addition of β-glucan solutions led to continuous decreases in breaking force, deformation and fracture constant, compared to the control. Addition of both β-glucan stabilized nanoemulsion and β-glucan solutions resulted in the decreases in viscoelastic moduli. Power law model represented viscoelastic behavior of all the gels. Expressible moisture content decreased, while whiteness increased with the addition of β-glucan stabilized nanoemulsion, compared to gel containing VCO. However, both expressible moisture content and whiteness increased with the addition of β-glucan solutions, at all levels used. Addition of β-glucan stabilized nanoemulsion resulted in finer gels, than the control gel added with only VCO. Generally, overall likeness score was increased for gel containing VCO nanoemulsion stabilized by 5% β-glucan. The surimi gel containing both medium chain fatty acids and β-glucan, functional ingredients, could be developed as a new product.

4.2 Introduction

β-Glucan is a polysaccharide of D-glucose molecules joined by β(1→3) and β(1→4) linkages. It is present in barley and oats as cell wall polysaccharide and is present in meagre amount in some other cereals (Bohm and Kulicke, 1999). β-glucan shows diversity in both the chain conformation as well as the number of β-(1→3) or β(1→4) linkage, which greatly influences its bioactivity (Ghavami *et al.*, 2014). β-glucan has various health beneficial effects such as serum cholesterol

reduction, blood sugar regulation, antioxidant activity and immunomodulatory activity (Kontogiorgos *et al.*, 2006; Shah *et al.*, 2015). The addition of some polysaccharide or dietary fiber in food products as antioxidants could enhance the bioactivity in the final products (Alakhrash *et al.*, 2016). Application of β-glucan in the treatment of cancer and other diseases, e.g. microbial infections, diabetes and hypercholesterolaemia, has been documented (Chen and Seviour, 2007). The molecular structure of polysaccharide including molecular mass, branch length and degree of branching determine its biological activity (Shah *et al.*, 2015). β-glucan has received considerable attention in recent years due to its health beneficial properties, notably its ability to scavenge free radicals similar to antioxidants (Gardiner, 2000; Kofuji *et al.*, 2012).

Barley and oat β-glucan at a level of 3 g/day as per FDA recommendation would lessen the risk of cardiovascular disease and reduce blood glucose in addition to providing satiety effect. β-glucan can be used for development of functional foods having low glycemic index and antioxidant property to meet the consumer demand (Lee *et al.*, 2016). However, reduced solubility of β-glucan due to its high molecular weight is a challenge for food industries. To reduce the molecular mass of β-glucan, various degradation processes have been employed such as enzymatic degradation (Ilyina *et al.*, 2000), oxidative degradation (De Moura *et al.*, 2011), acid hydrolysis (Jeon and Kim, 2000), and radiation degradation (Khan *et al.*, 2015) to improve the solubility and decrease the viscosity.

Ultrasound was effectively used to produce nanoemulsions based on sodium caseinate and virgin coconut oil (VCO) (Gani and Benjakul, 2018). High efficiency, economic performance and low instrument requirements make ultrasonic operation a green technology (Abbas *et al.*, 2015). It also results in the production of more homogenous emulsions with smaller emulsion droplets that are highly stable (Leong *et al.*, 2009). To enhance emulsion stability, several natural emulsifiers (e.g., enzymatically modified lecithin and sodium caseinate) have been used (Gani and Benjakul, 2018; Khalid *et al.*, 2017). Additionally, some polysaccharides have been used to stabilize emulsion. The cavitation bubbles formed during ultrasonication

produce implosions and create highly localized regions of hydrodynamic shear. These ultrasonically induced implosions generate powerful shock waves, which result in breakdown of micron sized droplets and formation of nano-sized emulsion droplets (O'Sullivan *et al.*, 2017).

The addition of cereal β-glucan offers several beneficial effects by altering the textural properties as well as improving the stability of emulsions during storage (Santipanichwong and Suphantharika, 2009). It is due to their ability to form stable gels and increase the viscosity of aqueous solutions. VCO as a supplementary source of fatty acids was added into surimi gel, however, it had an intrusive effect on the gel texture to some extent (Gani *et al.*, 2018). Using β-glucan as stabilizing agent for VCO-based nanoemulsion could result in good quality surimi gel containing health-promoting ingredients. β-glucan has been employed increasingly in the food industry due to its specific physical properties such as water solubility, viscosity, and gelation in addition to health-promoting and prebiotic properties (Zhu *et al.*, 2016). Therefore, β-glucan could be exploited as a stabilizing agent for the development of stable VCO nanoemulsion. The purpose of this study was to demonstrate the effect of β-glucan-stabilized VCO nanoemulsion on the properties of surimi gel.

4.3 Objective

To elucidate the effect of ultrasonicated β-glucan on properties of VCO based surimi gel.

4.4 Materials and Methods

4.4.1 Materials and chemicals

Frozen croaker surimi (AA grade) was acquired from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand). Virgin coconut oil (VCO) produced by cold press process was purchased from Posture Trading, Ltd (Pathumthani, Thailand) and β-glucan (70% purity) was obtained from Hangzhou Asure Biotech Co, Ltd. (Hangzhou, China). The chemicals used for electrophoresis were acquired from Bio-Rad Laboratories (Hercules, CA, USA).

4.4.2 Preparation of β-glucan solution

To prepare β-glucan solution, β-glucan powder was firstly dissolved in sufficient volume of distilled water to obtain various β-glucan levels (5, 10, 15 and 20% based on surimi dry weight) in the finished gel. The resulting β-glucan solutions were left overnight to fully hydrate the β-glucan powder and used either directly or emulsified with VCO prior to addition into the paste.

4.4.2.1 Characteristics of β-glucan stabilized VCO nanoemulsion as affected by emulsification conditions

Ultrasound assisted VCO nanoemulsion was prepared using β-glucan as a stabilizer. Emulsions were produced by mixing 5% VCO (based on total surimi paste) and β-glucan solutions at various levels (based on surimi dry weight) as mentioned above. Homogenization of the mixture was carried out for 5 min at 11,000 rpm using a homogenizer (IKA, Labortechnik homogenizer Selangor, Malaysia), followed by ultrasonication of the coarse emulsions using the ultrasonic processor (Sonics, Model VC750, Sonica & Materials, Inc., Newtown, CT, USA) with an on-time and off-time of 5 s at an amplitude of 20-80% and constant frequency of 20 kHz ± 50 Hz for 5 and 10 min.

The obtained emulsion samples stabilized by 20% β-glucan were subjected to analysis of particle size and distribution using a ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). The emulsion samples were not diluted during the size measurement and the refractive index of water (25 °C) was set at 1.33. The particle sizes were presented as the Z-average mean diameter and the broadness of the size distribution was represented by the polydispersity index (PDI).

4.4.3 Influence of β-glucan stabilized VCO nanoemulsion on properties of surimi gel

Frozen surimi was thawed to temperature of 0-2 °C, subsequently cut and chopped for 1 min in a Moulinex Masterchef 350 mixer (Paris, France). Salt (2.5% w/w) was added to the chopped surimi and blended again for 1 min. For the sample

containing β-glucan stabilized nanoemulsion, the prepared nanoemulsion showing the lowest particle size was added into surimi paste to obtain the final VCO content of 5% (based on surimi paste) and β-glucan contents of 5-20% based on surimi solid content. The moisture content of the paste was adjusted to 80% using distilled water. A control sample was also prepared without β-glucan but added with 5% VCO.

Another set of samples was prepared by addition of β-glucan (5-20%) based on surimi solid content, without VCO addition. All the paste samples were then filled in 2.5 cm diameter polyvinylidene casing and sealed at the ends. The setting of the gels was carried out at 40 °C for 30 min, immediately followed by a heating step at 90 °C for 20 min in a temperature-controlled water bath (Memmert, Schwabach, Germany). All the gels were then submerged in iced water for immediate cooling. The prepared gels were kept at 4 °C for 24 h before analysis.

4.4.4 Analysis

4.4.4.1 Textural properties

Surimi gels were subjected to penetration test using texture analyser (Model TA-XT2i, Stable Micro Systems, Surrey, England) following the method of Buamard and Benjakul (2015). Both breaking force and deformation were noted. Fracture constant (K_f) was measured as the ratio of breaking force/deformation to access the relative rigidity of gels at the failure point (Gani and Benjakul, 2018).

4.4.4.2 Expressible moisture content

Expressible moisture content of surimi gel was estimated following the method of Kaewudom *et al.* (2013). Cylindrical gel samples, 5 mm thick, were weighed (X g) and placed between Whatman filter paper No. 1, three at the bottom and two pieces on the top of the gel. The standard weight of 5 kg was placed on top of the sample for 2 min and weighed again (Y). Expressible moisture content was estimated as follows:

$$\text{Expressible moisture content (\%)} = \left[\frac{(X-Y)}{X}\right] x100$$

4.4.4.3 Whiteness

Whiteness of gel samples was estimated following the method of Yang *et al.* (2014). CIE L*, a* and b* values were measured using a colorimeter (HunterLab, Colorflex, Hunter Associates Laboratory, VA, USA), and whiteness was then estimated using the following equation.

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

4.4.4.4 Protein patterns

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was used to examine the protein patterns of surimi gel using the method of Laemmli (1970) as modified by Petcharat and Benjakul (2018). Protein separation was carried out after loading the samples (15 µg protein) onto the SDS-PAGE gel. After electrophoresis, gels were stained with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid.

4.4.4.5 Microstructure

Microstructure of surimi gels were analyzed using a scanning electron microscope (Quanta 400, FEI, Eindhoven, the Netherlands) as tailored by Buamard and Benjakul (2018). Briefly, 2–3mm thick gel samples were fixed with 2.5% v/v glutaraldehyde solution in 0.2 M phosphate buffer (pH 7.2) for 3 h. After washing with distilled water, dehydration of fixed specimens was carried out in ethanol with serial concentrations of 25, 50, 70, 80, 90 and 100%. Critical point drying of samples was done using CO_2 as a transition fluid. The samples were gold coated prior to mounting on a bronze stub and visualized using SEM.

4.4.4.6 Acceptability test

Sensory evaluation of gel samples was done using 9-point hedonic scale (Meilgaard *et al.*, 2007) following the method of Petcharat and Benjakul (2018) with minor modification. Briefly, bite-size surimi gel samples equilibrated at room temperature for 30 min were served in plastic cups under the fluorescent daylight-

type illumination and coded with 3-digit random numbers. Sensory attributes such as color, texture, odor, flavor and overall likeness were evaluated by fifty non-trained panelists, familiar with surimi products. Panelists rinsed their mouth between samples with drinking water.

4.4.5 Rheological behavior of surimi paste as affected by β-glucan stabilized VCO nanoemulsion or β-glucan solution

The surimi pastes containing β-glucan solution, β-glucan stabilized nanoemulsion and the control sample were analyzed for dynamic rheological properties using the method of Gani and Benjakul (2018) with a minor modification. A rheometer (HAAKE RheoStress1, ThermoFisher Scientific, Karlsruhe, Germany) attached with a 40 mm parallel steel plate and set with gap of 1 mm was used for testing. The circumference of the plate was coated with silicon oil to avoid the evaporation. The linear viscoelastic region (LVR) was obtained by conducting stress and frequency sweep tests. The temperature sweep test was conducted by gradually heating the paste from 20 to 90 °C at a heating rate of 1 °C/min, stress of 10 Pa and frequency of 1 Hz. Storage (G') and loss moduli (G") values were plotted against temperature. Frequency sweeps were performed from 0.01 to 10 Hz with 1% strain. Power-law model was fitted to the angular frequency (ω) dependence of G' and G" as tailored by Ji *et al.* (2017).

$$G' = G_0'.\omega^{n'}$$

4.4.6 Statistical analysis

Completely randomized design was used for all the experiments. Three different sample lots were used to carry out the experiments, which were performed in triplicate. Data were subjected to analysis of variance (ANOVA), and *t*-test was used for pair comparison. Mean comparisons were determined using the Duncan's multiple range test (Steel and Torrie, 1980). The data was subject to statistical analysis using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

4.5 Results and discussion

4.5.1 Characteristics of VCO based nanoemulsion as influenced by β-glucan and ultrasonication condition

Particle size distribution of nanoemulsion samples containing 20% β-glucan as stabilizer prepared under varying ultrasonic amplitudes and times is shown in Fig. 13. The mean particle size of emulsion droplets containing 20% β-glucan and ultrasonicated at 5 and 10 min at 20−80% amplitude were 2.97±0.06−21.17±0.06 nm and 2.97±0.06−3.60±0.1 nm, respectively. At the same ultrasonication time, decreased particle size was found as amplitude was increased from 20 to 80%. Considering the short time and less energy consumed, ultrasonic operation carried out for 5 min at 60% amplitude resulted in the small average droplet size (2.97±0.06) (Table 5). All the nanoemulsions showed unimodal particle size distribution with polydispersity index (PDI) ranging from 0.212 to 0.232 and from 0.208 to 0.213 when ultrasonic treatment of 5 min and 10 min was implemented, respectively (Table 5). PDI depicts the deviation from mean particle size and is a measure of broadness of the size distribution (Tadros *et al.*, 2004). A value of >0.5 indicates a broad particle size distribution (Gibis *et al.*, 2013). In the present study, PDI was lower than 0.5, indicating the narrow size distribution of oil droplets in emulsion.

Size reduction increased with increasing amplitudes of ultrasonication. This was more likely due to the increased energy imparted for breakdown of oil droplets and β-glucan molecules. In the presence of small oil droplets, β-glucan with the reduced size or low molecular weight could migrate and cover the interface more effectively. Ultrasonic treatment could also shorten β-glucan, making it more surface active. Chen *et al.* (2015) reported partial depolymerization of β-glucan extracted from the sclerotium of a medicinal mushroom, *Poria cocos* by the application of ultrasound. Ultrasonication might be a potent technique for improving solution properties of β-glucan without modifying its primary chemical structure (Wang *et al.*, 2010). β-glucan with low MW at sufficient amount (20%) could localize surrounding oil droplets, thus stabilizing the emulsion effectively.

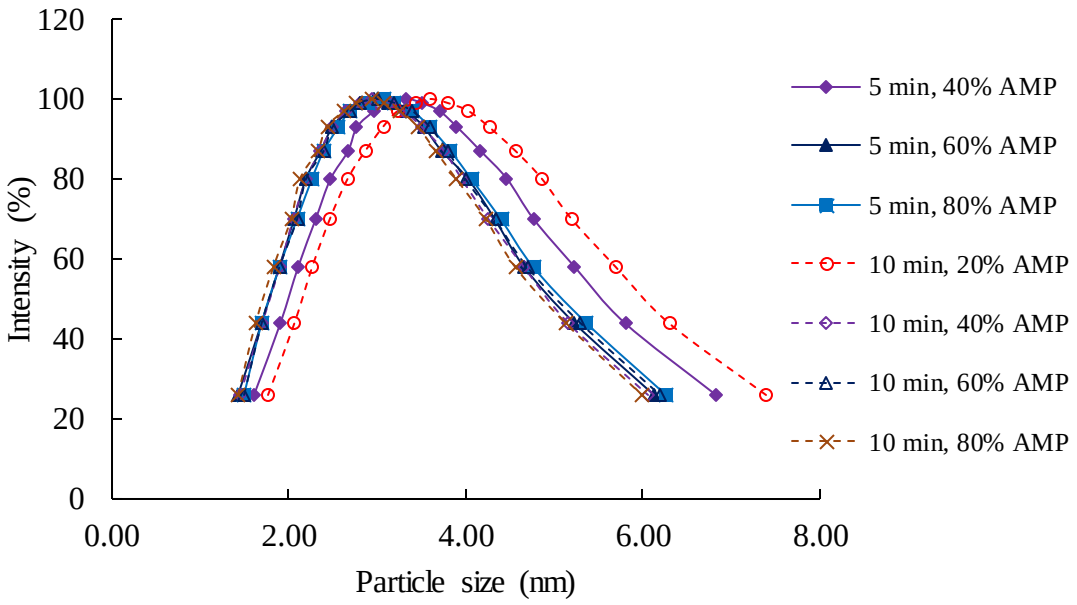

Figure 13. Particle size distribution of nanoemulsion stabilized by 20% β-glucan subjected to ultrasonication at 20-60% amplitude (AMP) for 5 and 10 min. Trend line for 5 min, 20% amplitude has been ignored as it falls outside the range.

Table 5. Effect of ultrasonic time and amplitude on particle size and polydistribution index of VCO-nanoemulsion stabilized by 20% β-glucan

	Z-average (nm)		Polydispersity index	
Amplitude (%)	5 min	10 min	5 min	10 min
20	21.17±0.06^{aA}	3.60±0.1^{aB}	0.232±0.00^{aA}	0.212±0.002^{aB}
40	3.50±0.00^{bA}	2.97±0.06^{bB}	0.214±0.00^{bA}	0.208±0.002^{bB}
60	2.97±0.06^{dA}	3.00±0.00^{bA}	0.212±0.00^{bA}	0.213±0.001^{aA}
80	3.07±0.06^{cA}	2.93±0.06^{bB}	0.212±0.00^{bA}	0.213±0.002^{aA}

Values are presented as mean±SD (n = 3). Different lowercase superscripts within the same column under the same ultrasonication time denote significant difference ($p < 0.05$). Different uppercase superscripts within the same row under the same parameter tested denote significant difference ($p < 0.05$).

4.5.2 Properties of surimi gels added with β-glucan VCO nanoemulsion and β-glucan solution

4.5.2.1 Textural properties

Breaking force and deformation of croaker surimi gels incorporated without and with β-glucan stabilized nanoemulsions or β-glucan solutions having the final β-glucan levels of 0-20%, are shown in Fig. 14A and B, respectively. For control surimi gel, the lower breaking force was observed when VCO was added. VCO droplets likely disrupted the aggregation and inter-connection between myofibrillar proteins. This confirmed the result reported by Gani and Benjakul (2018) who documented that VCO addition led to a decrease in breaking force of surimi gel. For the gels containing β-glucan stabilized nanoemulsion, breaking force decreased as the level of β-glucan increased ($p < 0.05$). Although β-glucan was located at oil-water interface, the rest was distributed in the aqueous phase. Aforementioned β-glucan might cause the hinderence in protein-protein interaction. This was evidenced by the decreased breaking force in the presence of higher level of β-glucan. Generally, the protein content of surimi gels diminished as β-glucan nanoemulsion or β-glucan solution was added. This mainly led to the dilution of myofibrillar proteins to some degrees, thus lowering the strength as manifested by the decreased breaking force. Addition of 5% β-glucan in the nanoemulsion had a significant influence on increasing the breaking force, compared to the control (added with VCO without β-glucan) ($p < 0.05$). At the same level of β-glucan, gel added with VCO nanoemulsion displayed the lower breaking force than that without VCO ($p < 0.05$). VCO addition resulted in the diluted protein concentration. VCO shows an interfering effect on protein-protein interaction, hence decreasing the breaking force, irrespective of β-glucan incorporated (Gani and Benjakul, 2018).

A decrease in deformation was found in all the samples, as the levels of β-glucan added increased, regardless of VCO incorporated ($p < 0.05$). However, the sample added with 5% β-glucan nanoemulsion had higher deformation than the control containing VCO ($p < 0.05$). The control without β-glucan solution also had lower deformation when VCO was added ($p < 0.05$). The deformation of gels added

with nanoemulsion increased when 5% β-glucan was present. Nevertheless, deformation continuously decreased as the levels of β-glucan increased ($p < 0.05$). Sánchez-Alonso *et al.* (2007) reported decrease in breaking force ($p < 0.05$) of giant squid surimi gel when wheat dietary fibre at levels of 3% and 6% were added, however, no change in deformation was noticed ($p > 0.05$). At the same level of β-glucan, higher deformation was found in gel containing nanoemulsion stabilized by 5 and 10% β-glucan, compared to those without VCO but contained β-glucan at the same level. Nonetheless, there was no difference in deformation between gel without and with VCO nanoemulsion when β-glucan at levels of 15 and 20% was present ($p > 0.05$).

The measure of firmness or relative rigidity of a gel at the failure point known as the fracture constant (K_f) is provided by the ratio of its breaking force and deformation (Herranz *et al.*, 2012). Increase in fracture constant was found in β-glucan nanoemulsion added sample when 5% β-glucan was used as a stabilizer ($p < 0.05$) compared to the control (added with only VCO). For the samples added with β-glucan solutions (without VCO), significant decrease in fracture constant was found only when β-glucan higher than 10% was added, compared to the control ($p < 0.05$). When comparing the samples added with β-glucan nanoemulsion and β-glucan solution, significant difference was found only when 20% β-glucan was used ($p < 0.05$). Fracture constants were 46-52 g/mm and 40-55 g/mm for the gels containing β-glucan nanoemulsions and β-glucan solutions, respectively. The results showed that nanoemulsion had impact on fracture constant at all β-glucan levels used. Therefore, the addition of nanoemulsion using 5% β-glucan as a stabilizer could improve the textural properties of surimi gel added with VCO in form of nanoemulsion. Preemulsified VCO in the form of nanoemulsion could be incorporated as a source of medium chain fatty acids. β-glucan has numerous health benefits such as antioxidant, anti-cancerous, anti-diabetic, reducing blood serum cholesterol and a source of soluble dietary fibre (Ahmad *et al.*, 2012; Sinthusamran and Benjakul, 2018). The result indicated that the use of 5% β-glucan could stabilize VCO nanoemulsion, in which VCO droplets could be entrapped and stabilized. Those small size droplets had less interfering effect on protein-protein interaction or aggregation.

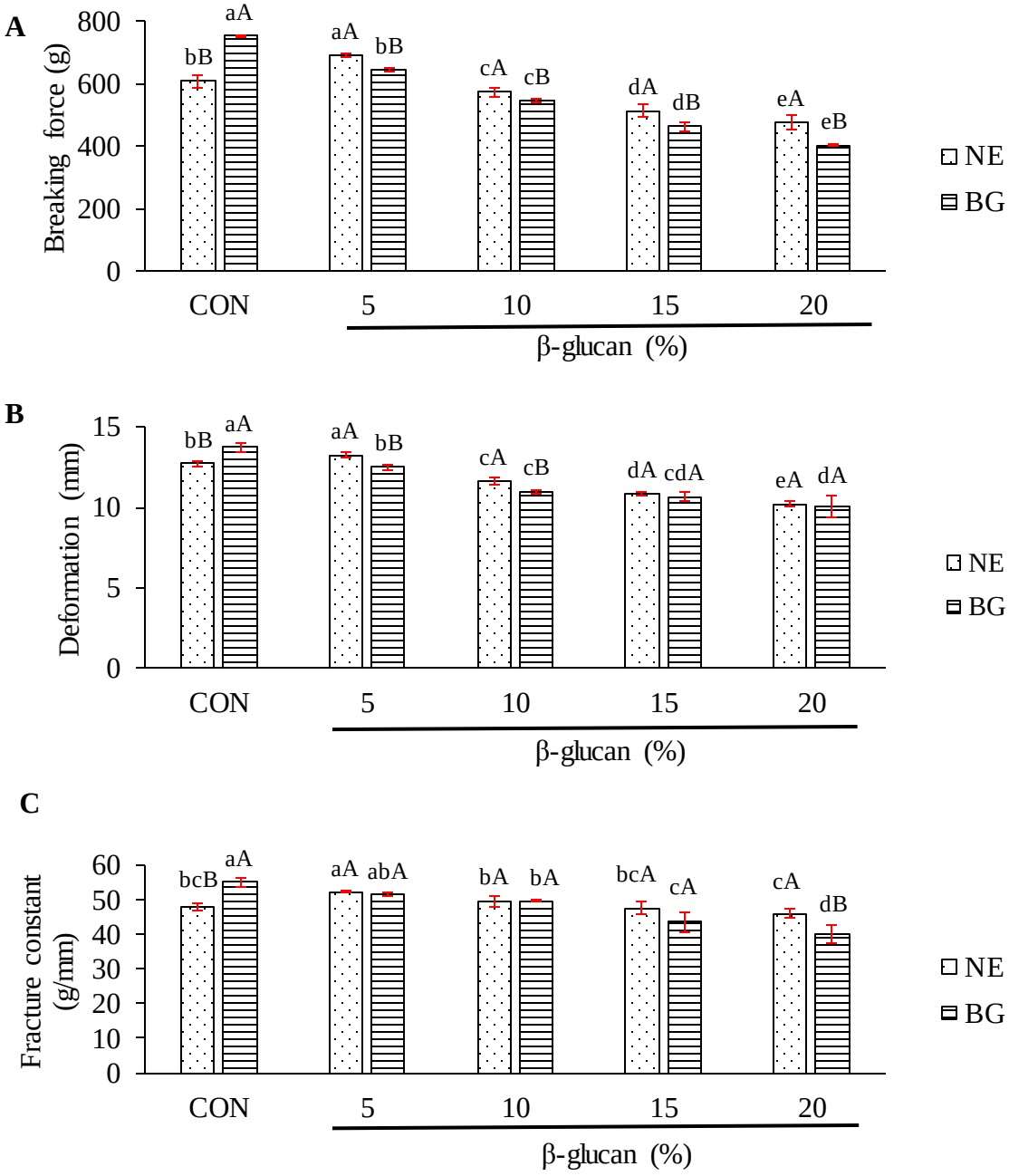

Figure 14. Breaking force (A), deformation (B) and fracture constant (C) of croaker surimi gel added without and with VCO in the presence of β-glucan at different levels. NE: gel with VCO nanoemulsion except for control, in which VCO was added directly; BG: gel without VCO or VCO nanoemulsion. Bars represent standard deviation (n=3). Different uppercase superscripts on the bars with the same level of β-glucan incorporated indicate significant difference ($p < 0.05$). Different lowercase superscripts within within same level of VCO incorporated indicate significant difference ($p < 0.05$).

4.5.2.2 Expressible moisture content

Expressible moisture content of gel added without and with β-glucan stabilized nanoemulsion or β-glucan solution is shown in Fig. 15A. The expressible moisture contents of β-glucan nanoemulsion and β-glucan solution added samples ranged from 2.73−3.63% and 2.41−2.98%, respectively. For the gels added with β-glucan stabilized nanoemulsion, the decrease in expressible moisture content was found for gels added with nanoemulsion when β-glucan at levels higher than 10% was present ($p < 0.05$). Ultrasonic treatment used to produce the nanoemulsion might depolymerize the large molecular weight β-glucan, and a concomitant surge in the proportion of shorter polymeric chains was found. Ultrasonication causes breakdown of glycosidic linkages or depolymerization (Li *et al.*, 2005). This could favor transition from compact random coil conformation to more flexible and even shorter extended chains. As a result, the smaller new β-glucan could have the increased hydration capacity, in which gels could imbibe more water. It was noted that the control gels containing VCO (without β-glucan) had higher expressible moisture content, whereas the gel added with β-glucan (without VCO) had the drastic decrease in expressible moisture content, indicating the superior water holding capacity of the latter, associated with the excellent water entrapement of β-glucan.

For the gels added with β-glucan solution, the increase in expressible moisture content was found at all levels ($p < 0.05$), up to 15%, compared to the control. However, at a level of 20% β-glucan, no change in expressible moisture was found, in comparison with the corresponding control ($p > 0.05$). Since the control sample had no β-glucan, no dilution was attained for myofibrillar protein. As a consequence, a finer network with high ability in water entrapment was developed. Chaijan *et al.* (2006) reported that the ability of gel to hold water under pressure is a measure of strength of the gel network. However, when β-glucan was added, the interaction between myofibrillar proteins was disrupted to some degree, resulting in reduced ability to hold water. Similar results were obtained by Tudorica *et al.* (2002), who reported that competitive hydration of dietary fiber led to disruption of gel matrix. Under the competitive condition that was intensified by β-glucan addition,

protein-water and β-glucan-water interactions might not be balanced. This contributed to poor water holding capacity of gel. However, with high level of β-glucan (20%), water was more entrapped by β-glucan, as indicated by the decrease in expressible moisture content. There was no difference in the expressible moisture between β-glucan nanoemulsion and β-glucan solution added samples at all β-glucan levels used ($p > 0.05$). Thus, β-glucan at higher level more likely improved water holding capacity of surimi gel added with VCO nanoemulsion.

4.5.2.3 Whiteness

Increase in whiteness of all the gels was found with the addition of β-glucan nanoemulsion or β-glucan solution at all the levels used, compared to the controls ($p < 0.05$), as shown in Fig. 15B. However, the addition of nanoemulsion stabilized by 5% β-glucan yielded whiter gels, compared to other samples, when β-glucan at levels higher than 5% was used as stabilizer ($p < 0.05$). In our previous study, increased whiteness was found in the croaker surimi gel with the addition of 5% VCO nanoemulsion (Gani and Benjakul, 2018). The increased whiteness may be attributed to the light scattering effect caused by the VCO based nanoemulsion. However, the higher amounts of β-glucan plausibly resulted in the offset of light scattering by nanoemulsion. This led to the decreased whiteness at higher levels of β-glucan used as the stabilizer in nanoemulsion ($p < 0.05$). However, for the gels added with β-glucan solutions, significant increase ($p < 0.05$) in whiteness was noticed when β-glucan was added up to 15%. Nevertheless, no difference in whiteness was found between gels added with 15% and 20% β-glucan ($p > 0.05$). In general, β-glucan nanoemulsion added gels were whiter, compared to the β-glucan solution added gels at all the levels of β-glucan used ($p < 0.05$). Alipour *et al.* (2018) reported that gel whiteness of silver carp surimi was not adversely affected by the addition of sulfated polysaccharides from green alga *Ulva intestinalis* up to 0.75%. Debusca *et al.* (2014) reported whitening effect of long chain powdered cellulose on Alaska pollock surimi gel up to 6%. However, Alakhrash *et al.* (2016) reported the decreased whiteness of Alaska pollock surimi gel fortified with oat bran up to 6% ($p < 0.05$). It was reported that the color of mixed gel was influenced by the color and the method of dispersion

and interaction of hydrocolloid (Sinthusamran *et al.*, 2017). This study indicated that the incorporation of β-glucan in surimi gel without or with VCO nanoemulsion remarkably influenced the whiteness of the resulting gel.

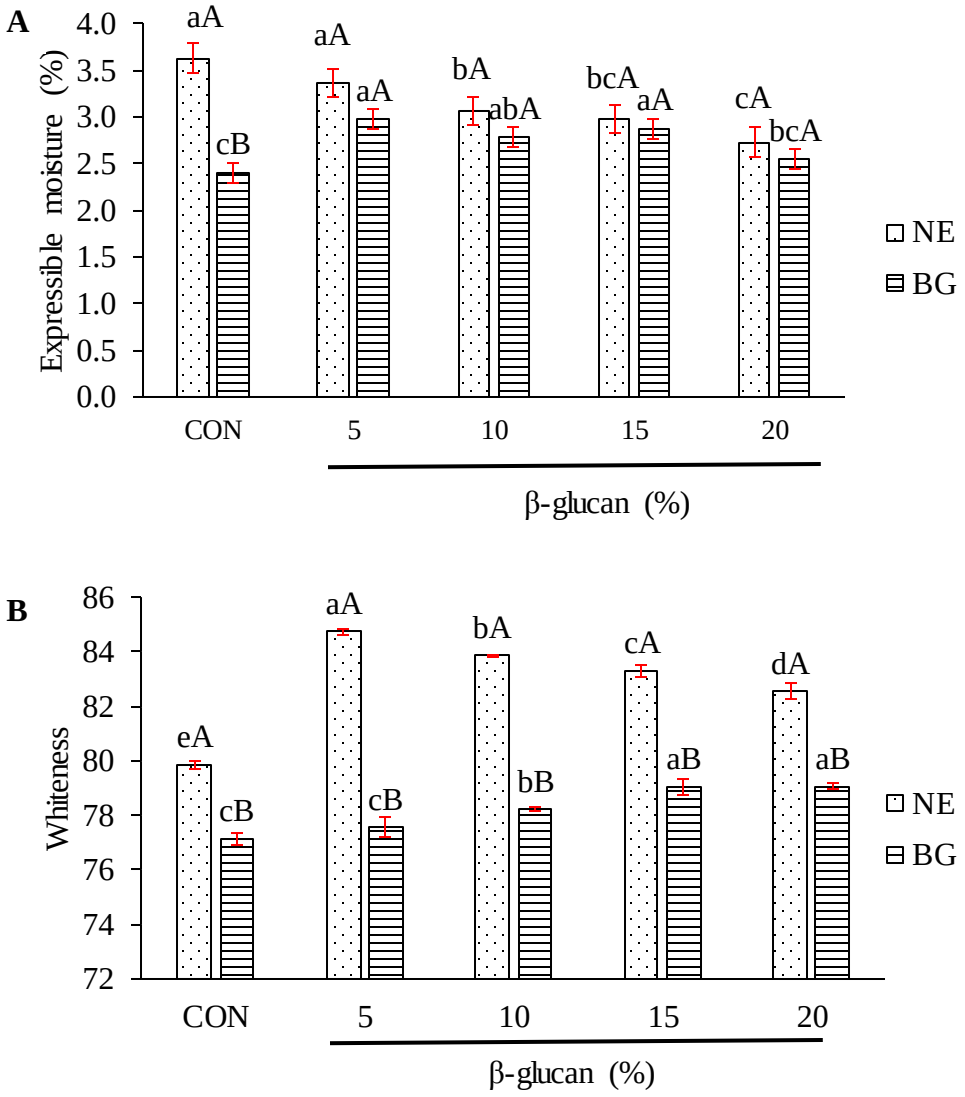

Figure 15. Expressible moisture (A) and whiteness (B) of croaker surimi gel added without and with VCO in the presence of β-glucan at different levels. NE: gel with VCO nanoemulsion except for control, in which VCO was added directly; BG: gel without VCO or VCO nanoemulsion. Bars represent standard deviation (n=3). Different uppercase superscripts on the bars with the same level of β-glucan incorporated indicate significant difference ($p < 0.05$). Different lowercase superscripts within within same level of VCO incorporated indicate significant difference ($p < 0.05$).

4.5.2.4 Protein pattern

Protein patterns of surimi gels without and with the addition of β-glucan nanoemulsion or β-glucan solution at various β-glucan levels are shown in Fig. 16. Myosin heavy chain (MHC) and actin were the major proteins in surimi paste. MHC completely disappeared in the control gels. Indigenous transglutaminase-mediated cross-linking of proteins had a vital role in polymerization of MHC (Buamard and Benjakul, 2018). When β-glucan nanoemulsion or β-glucan solution was added, MHC band was observed to some extent at all the levels of β-glucan used. This indicated that β-glucan retarded the cross-linking of MHC to some degree, regardless of the VCO incorporated. Transglutaminase-induced formation of ε-(γ-glutamyl) lysine isopeptide was reported by Benjakul and Visessanguan (2003). It was noted that cross-linking of myofibrillar proteins was not drastically affected by the levels of β-glucan, as indicated by similar protein pattern. However, breaking force and deformation decreased with increasing β-glucan. This confirmed the dilution effect on myofibrillar protein, as β-glucan level increased. Petcharat and Benjakul (2018) reported that gellan incorporation up to 8% (based on surimi solid weight) had no effect on myosin heavy chain polymerization. No alterations in intensity of actin band were found for all samples, irrespective of the amount of β-glucan or the presence of VCO. Therefore, actin did not serve as the desirable substrate for transglutaminase and was resistant to proteolysis (Buamard and Benjakul, 2015).

4.5.2.5 Microstructure

Microstructures of control croaker surimi gels without and with VCO nanoemulsion visualized by SEM are shown in Fig. 17. The gel containg 5% VCO (control gel without β-glucan) had a coarser network with a larger void or cavities, while the gel containing 5% β-glucan nanoemulsion had a smoother and finer network. Ultrasonic treatment uniformly distributed β-glucan in the gel, resulting in filling of crevices and channel in the surimi gel matrix. β-glucan stabilized nanoemulsion could be distributed throughout the surimi gel matrix, which resulted in decreased interconnection of proteins in the resulting gel. This was in agreement

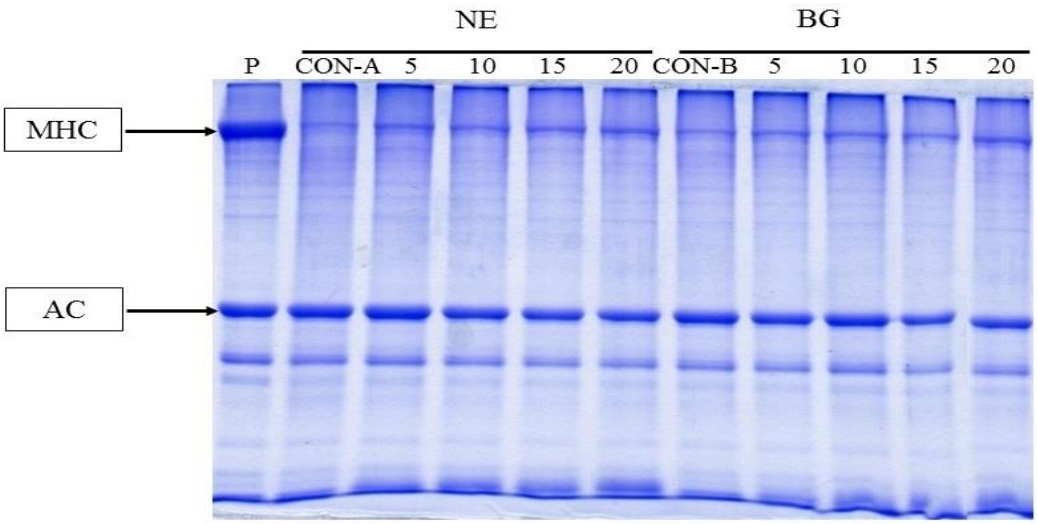

Figure 16. SDS PAGE pattern of croaker surimi gel added without and with VCO in the presence of β-glucan at different levels. NE: gel with VCO nanoemulsion except for control, in which VCO was added directly; BG: gel without VCO or VCO nanoemulsion; CON-A: control with VCO; CON-B: control without VCO; P: surimi paste, MHC: myosin heavy chain, AC: actin.

with the lowered breaking force (Fig. 14A). However, the network of surimi gel became denser and finer with the addition of 5% β-glucan nanoemulsion, as compared to the control gel (added with only 5% VCO). Fine and ordered arrangement of gel network probably imbibed water (Buamard *et al.*, 2017). This might result in insignificantly increased water holding capacity of gels added with β-glucan nanoemulsion compared with that containing only VCO. Moreover, ultrasonication treatment could depolymerize the β-glucan chains, thus increasing water holding capacity.

The gels containing 5% β-glucan solution had a slightly coarser microstructure with larger voids compared with the control (without β-glucan and VCO). A decrease in protein aggregation as interrupted by β-glucan more likely caused the decreased breaking force (Fig. 14A). Repulsive force between protein and polysaccharide reduced cross-linking between these two biopolymers (Zhang *et al.*, 2015).

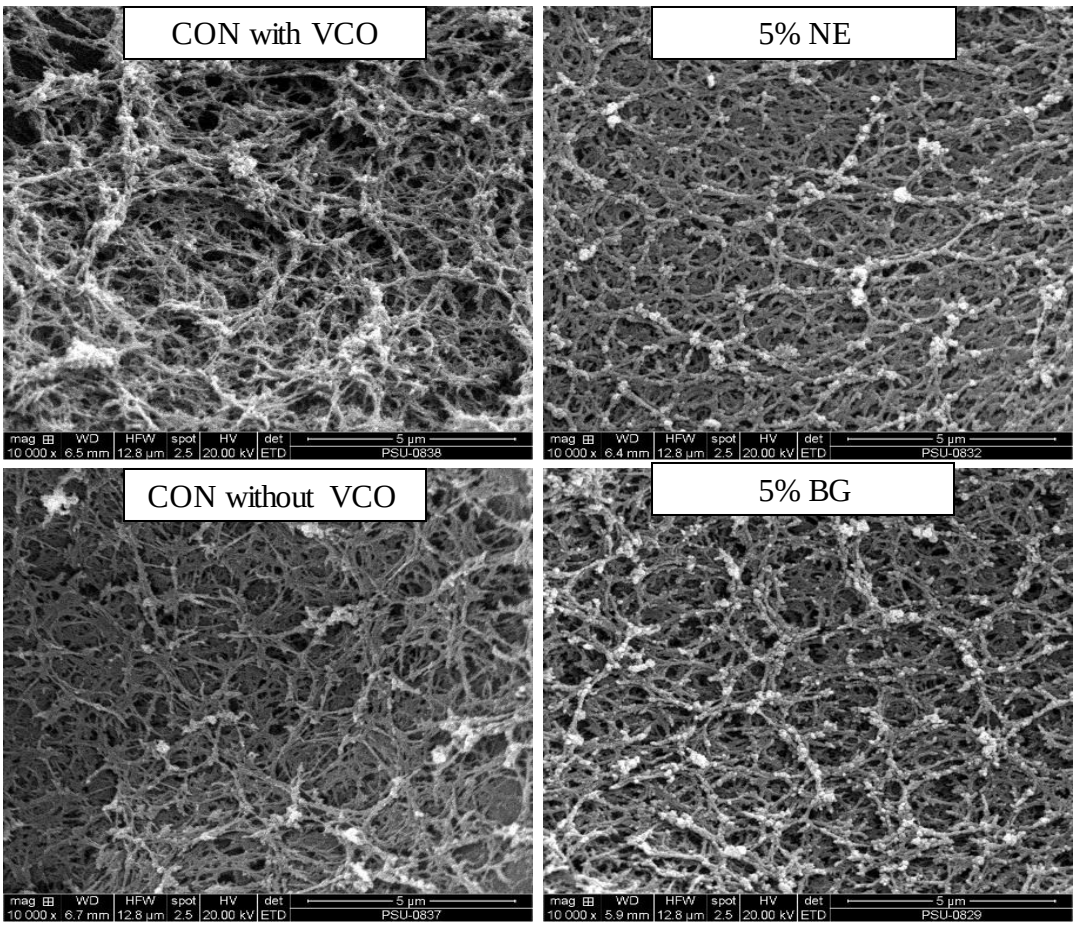

Figure 17. Electron microscopic images of croaker surimi gel added without and with VCO in the presence of 5% β-glucan; NE: gel with VCO nanoemulsion containing 5% β-glucan; BG: gel without VCO or VCO nanoemulsion containing 5% β-glucan. Magnification: 10,000×

4.5.2.6 Likeness score

Likeness score of surimi gels added with β-glucan stabilized nanoemulsion and β-glucan solution at various β-glucan levels is presented in Table 6. Addition of β-glucan nanoemulsion increased the color likeness, especially when 5% β-glucan was used as a stabilizer ($p < 0.05$). However, addition of β-glucan in the solution form did not have any remarkable impact on color likeness ($p > 0.05$). Addition of VCO nanoemulsion containing 5% β-glucan resulted in whiter gels compared to other samples including controls ($p < 0.05$). The results clearly demonstrate that whiter gels were more desirable. β-glucan nanoemulsion directly enhanced the whiteness of the

gel compared to those added with β-glucan, especially when β-glucan at levels of 5 and 10% was incorporated. No change in texture likeness was perceived by the addition of β-glucan or VCO nanoemulsion stabilized by β-glucan ($p > 0.05$). Panelists rated similarly odor and flavor likeness score for the samples added with β-glucan nanoemulsion or β-glucan solution, regardless of level of β-glucan used ($p > 0.05$). However, an increase in odor and flavor likeness score was found when nanoemulsion containing 5% β-glucan was incorporated compared to their respective counterparts added with β-glucan solution at the same level ($p < 0.05$). For overall likeness, the sample containing VCO nanoemulsion stabilized by 5% β-glucan had the higher score than the control (added with VCO) ($p < 0.05$). This was most likely due to increased whiteness of the gel added with β-glucan stabilized nanoemulsion. No differences in overall likeness score were found for the samples added with nanoemulsion containing other levels of β-glucan (Table 6). For the samples added with β-glucan solution, no change in overall likeness was attained in all the samples compared with the control, irrespective of the level of addition. In general, color likeness score was largely in agreement with overall likeness.

Table 6. Likeness score of croaker surimi gel added without and with VCO in the presence of β-glucan at different levels.

β-glucan form	β-glucan level (%, based on surimi solid weight)	Color	Texture	Odor	Flavor	Overall
CON with VCO	0	7.0 ± 1.0^{bA}	6.6 ± 1.3^{aA}	6.8 ± 1.2^{aA}	6.7 ± 1.0^{aA}	6.9 ± 0.8^{bA}
NE	5	8.0 ± 0.7^{aA}	7.1 ± 1.2^{aA}	7.2 ± 0.9^{aA}	6.9 ± 0.8^{aA}	7.5 ± 0.6^{aA}
	10	7.6 ± 0.7^{abA}	7.2 ± 1.0^{aA}	6.8 ± 1.1^{aA}	6.5 ± 1.2^{aA}	7.2 ± 0.6^{abA}
	15	7.5 ± 1.3^{abA}	6.7 ± 1.5^{aA}	6.7 ± 1.6^{aA}	6.4 ± 1.3^{aA}	6.8 ± 1.0^{bA}
	20	7.5 ± 1.2^{abA}	7.0 ± 1.2^{aA}	6.4 ± 1.6^{aA}	6.4 ± 1.2^{aA}	7.0 ± 0.8^{bA}
CON without VCO	0	6.4 ± 1.4^{aA}	6.0 ± 1.5^{aA}	5.8 ± 1.6^{aB}	6.0 ± 1.2^{aA}	6.2 ± 1.1^{aB}
BG	5	6.8 ± 1.3^{aB}	6.4 ± 1.5^{aA}	6.3 ± 1.5^{aB}	6.2 ± 1.4^{aB}	6.4 ± 1.0^{aB}
	10	6.9 ± 1.3^{aB}	6.9 ± 1.2^{aA}	6.2 ± 1.7^{aA}	6.2 ± 1.4^{aA}	6.4 ± 1.2^{aB}
	15	6.9±1.2aA	6.6 ± 1.4^{aA}	6.3 ± 1.5^{aA}	6.5 ± 1.3^{aA}	6.4 ± 1.1^{aA}
	20	6.8 ± 1.1^{aA}	6.5 ± 1.6^{aA}	6.3 ± 1.4^{aA}	6.6 ± 1.6^{aA}	6.7 ± 1.6^{aA}

Values are presented as mean±SD (n = 50). NE: gel with VCO nanoemulsion; BG: get without VCO or VCO nanoemulsion. Different uppercase superscripts within the same column under the same level of β-glucan denote significant difference ($p < 0.05$). Different lowercase superscripts within the same column under the same VCO incorporated denote significant difference ($p < 0.05$)

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4.5.3 Dynamic rheological properties of surimi paste incorporated with β-glucan stabilized nanoemulsion and β-glucan solution

Rheological behavior of surimi pastes added without and with β-glucan stabilized nanoemulsion or β-glucan solution at various levels of β-glucan is shown in Fig. 18. The elastic modulus curves showed similar trends for surimi pastes incorporated without and with β-glucan stabilized nanoemulsion or β-glucan solution. In general, G' is a measure of stored mechanical energy after the sample is subjected to a deforming force and is related to the change in elasticity of the sample during gelation (Hunt and Park, 2013). A continuous increase in G' of all the samples was found, and the highest value was reached at about 35 °C. This was associated with the development of protein network through weak hydrogen bonds between protein molecules (Buamard *et al.*, 2017). Subsequently, a rapid decrease in G' was found and reached the lowest value at approximately 50 °C. Endogenous proteolytic enzyme mediated degradation took place in the temperature range of 50-60 °C (Buamard and Benjakul, 2015) and enhanced protein mobility as a consequence of disaggregation of actin-myosin network structure (Zhang *et al.*, 2015). This resulted in the decreased G'. Subsequent heating up to approximately 65 °C led to further increase in G', due to the enhanced cross-linking between dissociated or denatured MHC. As a consequence, the formation of a thermo-irreversible gel network was developed (Mleko and Foegeding, 2000). Unfolded proteins might undergo aggregation via reactive groups or domains. Hydrophobic domains likely interacted via hydrophobic-hydrophobic interaction and disulfide bonds were formed due to oxidation of sulfhydryl groups (Buamard and Benjakul, 2015). Thereafter, slight decrease in G' was again noticed till temperature was 90 °C. High temperature might destroy the weak bonds, such as hydrogen bonds.

Paste added with β-glucan stabilized VCO nanoemulsion showed the higher G' than the control (with 5% VCO only). Among all samples containing VCO, those added with nanoemulsion stabilized by 5% β-glucan had the highest G'. The results were in agreement with breaking force and fracture constant, in which the addition of nanoemulsion stabilized by 5% β-glucan resulted in the highest value of

both parameters. This might be due to the hydrogen bond or ionic interaction between β-glucan and myofibrils in surimi. Ziegler and Foegeding (1990) reported that emulsified omega-3 polyunsaturated fatty acid (ω-3 PUFA)-rich oil possibly acted as a co-polymer, filling the voids in the network of myofibrillar protein gel matrix thus enhancing its elasticity. For the gels added with β-glucan solution, lower value of G' was found, compared to the control, at all the levels used. This could be due to dilution effect of β-glucan towards myofibrillar proteins in the paste and might be associated with the less inter-connected network. The results were in agreement with protein patterns, in which the addition of β-glucan interfered the polymerization of MHC to some extent (Fig. 16). Similar behavior was observed for loss moduli, but G'' values were much lower than G', indicating the dominance of elastic component. All the gels exhibited slight frequency dependence in the tested frequency range (Fig. 18C and F). The frequency independence of G' is an indication of strong gel formation with no disruption of junction zones within the experimental time scale. Generally, G' of weak gels show high frequency dependence, indicating less interaction between the molecules (Doublier, 1992).

Frequency sweep test was conducted at 25 °C to determine the viscoelastic properties and interactions between β-glucan chains and protein in the mixed gel. The values of the viscoelastic moduli of all samples exhibited very similar trends. All the samples displayed a remarkably higher value of elastic modulus (G') than viscous component (G''), with no crossover along the frequency range (Fig. 18C and 18F), indicating the formation of a gel network (Bertrand and Turgeon, 2007; Neiser *et al.*, 2000). All the surimi gels showed a small frequency dependence for G' and G'', indicating the formation of strong gels (Wang *et al.*, 2016). The frequency independence of G' and G'' indicates strong gel network with strong junction zones, which were resistant to rupture within the time-scale of experiment (Doublier, 1992).

The power law model adequately represented the frequency dependence of G' for mixed gels (Table 7). As shown in Table 7, the value n' represents a positive number, indicating that the mixed gels can be classified as physical gel with inter-connected structures (Ross-Murphy, 1984). Physical gels are characterized by weak

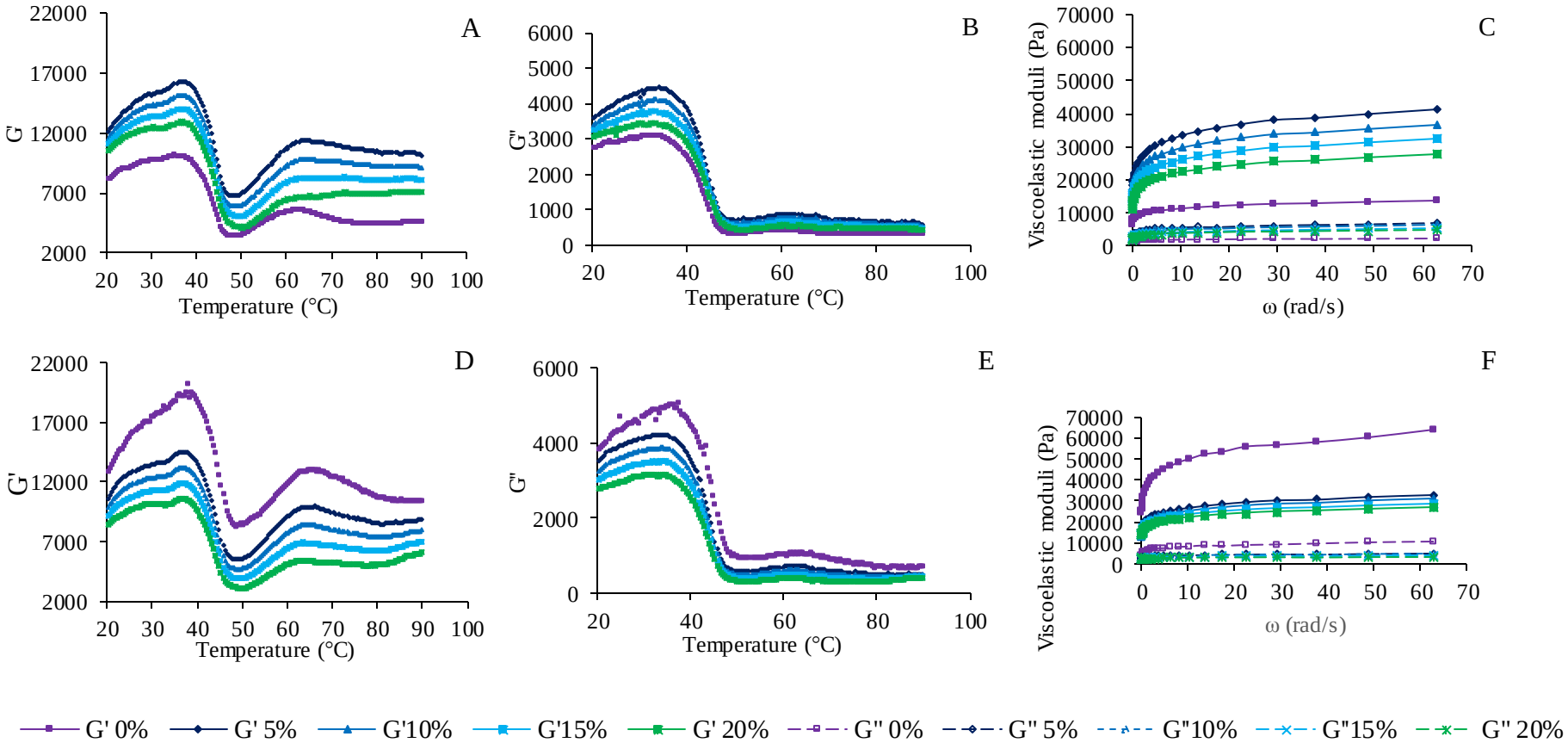

Figure 18. Viscoelastic property of croaker surimi gel added without (D, E, F) and with (A, B, C) VCO in the presence of β-glucan at different levels.

Table 7. Power law parameters of croaker surimi gel added without and with VCO in the presence of β-glucan at different levels

β-glucan form	β-glucan level (%, based on surimi solid weight)	G_0' (kPa)	n'	r^2
CON with VCO	0	8.972 ± 0.3^{eB}	0.100 ± 0.00^{bB}	0.986
NE	5	24.043 ± 1.1^{aA}	0.139 ± 0.00^{aA}	0.990
	10	21.617 ± 0.7^{bA}	0.136 ± 0.0^{aA}	0.993
	15	19.189 ± 0.4^{cA}	0.132 ± 0.00^{aA}	0.995
	20	16.758 ± 0.3^{dB}	$0.127\pm.005^{aA}$	0.996
CON without VCO	0	35.656 ± 0.5^{aA}	0.143 ± 0.00^{aA}	0.997
BG	5	20.254 ± 0.7^{bB}	0.126 ± 0.00^{bB}	0.987
	10	19.505 ± 0.7^{bcB}	0.116 ± 0.00^{cB}	0.994
	1	18.741 ± 0.6^{cdA}	$0.105\pm.002^{dB}$	0.999
	20	17.962 ± 0.2^{dA}	0.093 ± 0.00^{eB}	0.998

Values are presented as mean ± SD (n = 3). G_0': storage modulus at 1 rad/s; n': Power law exponent; NE: gel with VCO nanoemulsion; BG: gel without VCO or VCO nanoemulsion. Different uppercase superscripts within the same column under the same level of β-glucan denote significant difference ($p < 0.05$). Different lowercase superscripts within the same column under the same VCO incorporated denote significant difference ($p < 0.05$).

physical interactions among their polymer chains at the microscopic level (An *et al.*, 2010).

4.6 Conclusion

β-glucan stabilized nanoemulsion was produced by ultrasonication under the appropriate condition. Addition of β-glucan stabilized nanoemulsion and β-glucan solutions had remarkable effect on textural properties. However, addition of VCO nanoemulsion stabilized by 5% β-glucan increased the breaking force and whiteness of surimi gel significantly. This was associated with the smoother and finer microstructure, compared to gels directly added with VCO. Therefore, VCO nanoemulsion stabilized by 5% β-glucan was the appropriate form to be incorporiated as a source of functional ingredients in the surimi gel and also could improve textural properties, whiteness and likeness of surimi gel.

4.7 References

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CHAPTER 5

EFFECT OF ANTIOXIDANTS IN COMBINATION WITH VCO NANOEMULSION ON GEL PROPERTIES AND STORAGE STABILITY OF REFRIGERATED SARDINE SURIMI GEL

5.1 Abstract

Impacts of β-glucan–VCO nanoemulsion containing epigallocatechin gallate (EGCG) and α-tocopherol at levels of 0-0.3% on properties and storage stability of surimi gel were investigated. Augmented breaking force, deformation and fracture constant were obtained in gels containing 0.2% EGCG or 0.1% α-tocopherol ($p < 0.05$). Expressible moisture content (EMC) increased as EGCG levels were more than 0.2%. Smoother microstructure was observed in gels containing 0.2% EGCG. Whiter gels were obtained when β-glucan–VCO nanoemulsion was incorporated. No change in protein pattern of gels was observed regardless of antioxidant incorporation. Viscoelastic moduli decreased as β-glucan–VCO nanoemulsion was added, however incorporation of 0.2% EGCG or 0.1% α-tocopherol lowered the decrease in G'. β-glucan–VCO nanoemulsion containing gels had higher likeness scores than the control ($p < 0.05$). Gels containing EGCG and α-tocopherol at selected levels had the improved oxidative stability and lowered microbial loads.

5.2 Introduction

Surimi rich in myofibrillar proteins can form gel when subjected to hydrothermal processing. It is highly nutritious with delicacy, mainly due to the elastic texture. Physical and textural properties of surimi gels have been enhanced using a variety of ingredients or additives. Addition of β-glucan-VCO nanoemulsion stabilized by 5% β-glucan yielded whiter gel without any deleterious effect on gel properties (Gani and Benjakul, 2019). Seabass oil pre-emulsified in presence of soy protein isolate (SPI) and incorporated with ECHE improved the oxidative stability and also strengthened sardine surimi gels (Buamard and Benjakul, 2019). The addition of cereal β-glucan is used to alter the textural properties and improve the emulsion stability during storage (Santipanichwong and Suphantharika, 2009). β-

glucan has been shown to stabilize nanoemulsion, which was further added into surimi gel to obtain superior health effects (Gani and Benjakul, 2019). The increasing use of β-glucan in the food industry is because of its functional properties such as high water solubility, viscosity as well as gelation along with its health-promoting and prebiotic properties (Zhu *et al.*, 2016).

VCO contains natural vitamin E and lauric acid, and resists to oxidation and has tremendous health benifits (Senphan and Benjakul, 2017). VCO is used in medicines and in baby food formulation. VCO has several claimed medicinal values, including anti-hypercholesterol, antimicrobial, antioxidant, anti-viral and other health promoting properties (Ng *et al.*, 2014). Vegetable oil is normally used in surimi products to modify texture and enhance color (Shi *et al.*, 2014). The texture of surimi gels is mainly a consequence of heat induced gelation of actin and myosin. The lipid content can affect the protein gelation and final texture. Emulsified lipids acted as fillers or co-polymers, which can fill the voids distributed in the gel network, resulting in tailoring of overall properties of composite gels (Pietrowski *et al.*, 2012). Recently, β-glucan stabilized VCO nanoemulsion (βG-V-N) has been added as a functional ingredient and could augment the textural properties, whiteness and acceptability of surimi gel (Gani and Benjakul, 2019). Addition of nanoemulsion yielded the gel with better properties than the direct incorporation of VCO.

Phenolic compounds are the chemical species with one or several aromatic rings containing at least one hydroxyl group. Oxidized phenolic compounds were demonstrated to act as the alternative protein cross linking agents (Balange and Benjakul, 2009a). Seafood is prone to lipid oxidation and microbiological spoilage. Natural antioxidants are widely employed to control lipid oxidation and limit the deleteriousness during processing as well as storage of fish products (Kulawik *et al.*, 2013). Addition of tea catechins significantly reduced TBARS value of cooked white muscle of mackerel stored at 4 °C for 7 days (He and Shahidi, 1997). Lipid oxidation and fishy odor development were retarded in fish emulsion sausages by the use of 0.02 or 0.04% tannic acid and ethanolic kiam wood extract (0.08%) during 20 days of the refrigerated storage (Maqsood *et al.*, 2012). Sardine surimi gels containing

ethanolic coconut husk extract (ECHE) showed lower PV and TBARS, with increasing levels of ECHE (0-0.25%) (Buamard and Benjakul, 2019). Caffeic acid (100 mg/kg) was successfully used to inhibit lipid oxidation in wheat dietary fibre minced fish restructured products after 10 days of chilled storage (Sánchez-Alonso *et al.*, 2011). Lipid oxidation in mackerel mince was retarded by the use of numerous phenolic compounds (100 ppm) during 15 days of iced storage (Maqsood and Benjakul, 2010). Tannic acid and catechin were effectively used at 200 ppm to retard lipid oxidation and microbial growth in ground camel meat throughout 9 days of refrigerated storage (Maqsood *et al.*, 2015). The addition of young apple polyphenols (YAP) at 0.1% into grass carp surimi (GCS) effectively delayed lipid oxidation during refrigerated storage of 7 days (Sun *et al.*, 2017).

This study aimed to determine the influence of antioxidant loaded β-glucan-stabilized VCO nanoemulsion (βG-V-N) on properties of sardine surimi gel as well as its inhibitory potential on lipid oxidation and microbial growth of gels during prolonged storage at refrigerated temperature.

5.3 Objective

To investigate the effect of selected antioxidants on properties and storage stability of VCO based surimi gel.

5.4 Materials and Methods

5.4.1 Materials and chemicals

Frozen sardine surimi (AA) grade, VCO and β-glucan (70% purity) were purchased from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand), Posture Trading, Ltd. (Pathumthani, Thailand) and Hangzhou Asure Biotech Co, Ltd. (Hangzhou, China), respectively. All other chemicals used for analyses were procured from Sigma Chemical Co. (St. Louis, MO, USA) and Bio-Rad Laboratories (Hercules, CA, USA).

5.4.2 Preparation of β-glucan-stabilized VCO nanoemulsion (βG-V-N) containing antioxidants

βG-V-N was prepared as detailed by Gani and Benjakul (2019). Emulsions were firstly prepared by mixing 5% VCO (based on total surimi paste) and 5% β-glucan solutions (based on surimi dry weight). EGCG or α-tocopherol were solubilized in distilled water and VCO respectively, to attain the level of 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3% each, based on protein content in surimi. All the mixtures were homogenized for 5 min at 11,000 rpm. This was followed by ultrasonication for 5 min at 60% amplitude as tailored by Gani and Benjakul (2019).

5.4.3 Preparation of surimi gel containing VCO nanoemulsion

Frozen surimi was tempered to reach of 0-2 °C at the core. After thawing, surimi block was reduced to pieces or flakes, followed by mincing in a Moulinex Masterchef 350 mixer (Paris, France) for 1 min. To surimi paste, 2.5% salt was added and mixed for another 1 min. For the samples containing βG-V-N without or with antioxidants, the prepared nanoemulsions were incorporated into paste to possess the final VCO, β-glucan and antioxidant levels of 5% (based on surimi paste), 5% (based on dry matter) and 0.05-0.3% (based on protein content), respectively. The paste was mixed again for 1 min. The control was also prepared without addition of βG-V-N and was referred to as 'C-gel'. The sample added with βG-V-N was referred as 'βG-V-N-gel' while those added with βG-V-N along with EGCG or α-tocopherol at different levels were termed as 'βG-V-N-EG-gel' and 'βG-V-N-TC-gel', respectively.

The pastes were filled and sealed in polyvinylidene casing (25 mm in diameter). Gel setting was done at 40 °C and 90 °C for 30 min and 20 min, respectively using a water bath (Memmert, Schwabach, Germany). Subsequently gels were subjected to cooling using iced water. The gel samples were kept for 24 h at 4 °C before analysis.

5.4.4 Analysis

5.4.4.1 Textural properties

Breaking force (B-F), deformation (D-M) and fracture constant (F-C) were measured following the method as tailored by Gani *et al.* (2018). Texture analyzer (Model TA-XT2, Stable Micro Systems, Surrey, UK) attached with a spherical probe (5 mm diameter) was used for examining B-F and D-M. The ratio of B-F/D-M was derived as F-C (K_f)

5.4.4.2 Expressible moisture content (EMC)

The procedure of Gani *et al.* (2018) was adopted for EMC determination. Cylindrical gels (5 mm diameter) were weighed (X) and kept between filter papers (Whatman International Ltd., Maidstone, UK). A 5 kg standard weight was used to press the sample for 2 min and the samples were subsequently weighed (Y) after removing the weight. EMC was expressed as follows:

$$\text{EMC (\%)} = \left[\frac{(X-Y)}{X}\right] x100$$

5.4.4.3 Whiteness

L*, a* and b* values were determined by a colorimeter (HunterLab, Colorflex, Hunter Associates Laboratory, VA, USA). Whiteness was calculated as follows:

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

5.4.4.4 Protein patterns

The protein pattern of surimi gel was examined under reducing conditions using the method of Laemmli (1970) as modified by Gani *et al.* (2018). Sample (15 µg protein) was loaded onto SDS-PAGE gel (4% stacking gel, 10% running gel) to carry out protein separation. Subsequently, gels were stained with 0.02% (w/v) Coomassie Blue R-250 and destained with 50 % (v/v) methanol and 7.5% (v/v) acetic acid as described by Gani *et al.* (2018).

5.4.4.5 Microstructure

The microstructural details of surimi gels was analyzed as detailed by Gani and Benjakul (2019) using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, the Netherlands). After fixing the gel samples with glutaraldehyde solution (2.5% v/v) for 3 h dehydration was done using serial concentrations of ethanol ranging from 25 to 100%, followed by critical point drying (CPD) using carbon dioxide. Gold-coating of the samples was done and finally examined under SEM.

5.4.4.6 Sensory property

Sensory analysis of surimi gel was carried out using 9-point hedonic scale (Meilgaard *et al.*, 2007) as detailed elsewhere by Gani *et al.* (2018). Fifty non-trained panellists were requested to assess the surimi gel samples for color, texture, odor, flavor, and overall likeness.

5.4.4.7 Rheological behavior of surimi paste as affected by βG-V-N containing antioxidants

Dynamic rheological analysis of surimi pastes added without and with βG-V-N in the absence or presence of EGCG or α-tocopherol at 0.1 and 0.2%, respectively, was adopted from the procedure of Gani *et al.* (2018). Testing was done using a rheometer (HAAKE RheoStress1, ThermoFisher Scientific, Karlsruhe, Germany). LVR region was determined and temperature sweep of the paste was performed from 20 to 90 °C, in which stress of 10 Pa, and frequency of 1 Hz along with heating rate of 1 °C/min were used. Both storage (G′) and loss moduli (G″) were measured.

5.4.5 Storage stability of surimi gels containing βG-V-N added with antioxidants

Surimi gels added with βG-V-N in the absence or presence of EGCG or α-tocopherol at the levels rendering the highest B-F were selected for further analysis.

5.4.5.1 Peroxide value

PV of surimi gel was estimated as tailored by Buamard and Benjakul (2019). Cumene hydroperoxide (0.05–2 ppm) was used as standard and PV was reported as mg hydroperoxide equivalents/kg sample.

5.4.5.2 Thiobarbituric acid reactive substances (TBARS)

TBARS of gel samples were estimated by distillation method as tailored by Bumard and Benjakul (2019). Distillate (5 mL) was mixed with TBA reagent (5 mL) and heated at 100 °C for 35 min. Blank was also prepared in the same fashion but distilled water was used instead of sample. A standard curve of 1,1,3,3-tetramethoxypropane (0–6 ppm) was prepared and TBARS were expressed as mg malonaldehyde/kg sample.

5.4.6 Microbiological analyses

Total viable count (TVC) and psychrophilic bacteria count (PBC) were estimated by spread plate method using plate count agar (PCA) incubated at 35 °C for 2 days and 4 °C for 12 days, respectively (Buamard and Benjakul, 2019).

5.4.7 Statistical analysis

Three different sample lots were employed to run the experiments in triplicate. The data was subjected to Analysis of Variance and the Duncan's multiple range was used for mean comparison (Steel & Torrie, 1980). Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA) software was adopted for analysis of the data.

5.5 Results and discussion

5.5.1 Properties of surimi gels added with βG-V-N containing antioxidants

5.5.1.1 Textural properties

Addition of βG-V-N did not remarkably reduce the B-F, however 5% reduction in B-F was noticed, compared to that of C-gel (Fig. 19A). VCO nanoemulsion at 5% level did not affect B-F of the croaker surimi gel

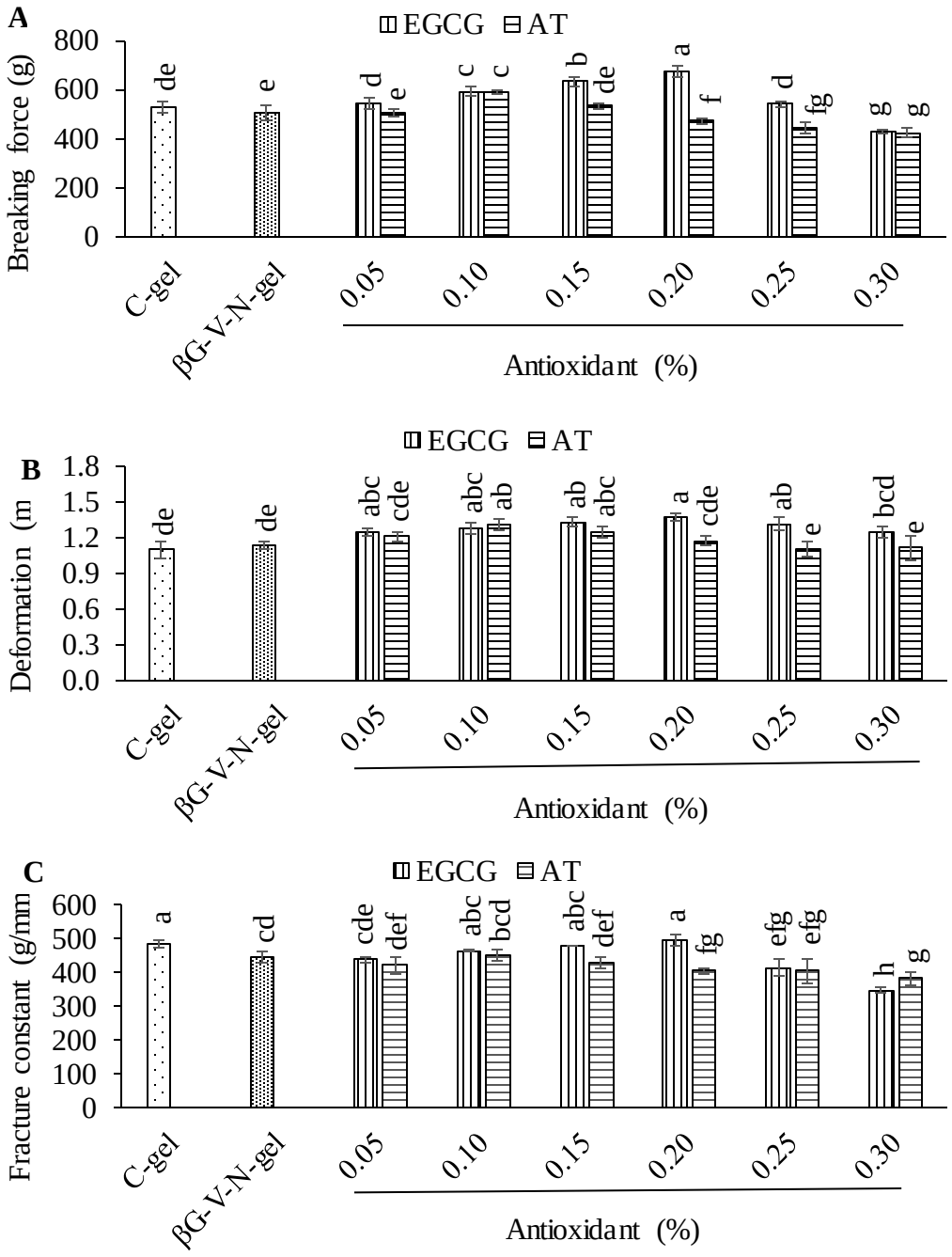

Figure 19. B-F (A), D-M (B) and F-C (C) of sardine surimi gel added without and with βG-V-N in the presence and absence of EGCG or α-tocopherol at various levels. C-gel: control, βG-V-N-gel: Sample added with 5% β-glucan stabilized nanoemulsion, 0.05-0.3: gels containing βG-V-N added with EGCG or α-tocopherol at various levels ranging from 0.05 to 0.3% of protein. Bars represent standard deviation (n=3). Different letters on the bars indicate significant differences ($p < 0.05$).

(Gani and Benjakul, 2018). Nonetheless, a gradual increase in B-F was attained by the addition of antioxidants, which might be due to formation of hydrogen bonding between C=O group of proteins with (−OH) groups of antioxidants via H-bond. The samples containing 0.2% EGCG (βG-V-N-0.2EG-gel) had the highest B-F ($p < 0.05$), while further addition of EGCG caused the reduction in B-F. Similarly, addition of α-tocopherol at the level of 0.1% displayed the highest B-F ($p < 0.05$), while further incorporation led to the decrease in B-F. This might be due to coalescence of VCO nanoemulsion driven by hydrophobic α-tocopherol when added at higher levels, resulting in creation of pockets of VCO in the gel. These VCO pockets were related with larger crevices in the gel network and yielded the little resistance for the penetrating probe as evidenced by looser microstructure of the gel added with 0.2% α-tocopherol (Figure 22). Similar result was reported by Cao *et al.* (2018), in which higher EGCG levels (500 and 1000 ppm) hindered gel formation and emulsifying property of myofibrillar protein, but the lower levels (50-200 ppm) did not jeopardize textural property. Due to large number of hydroxyl groups in EGCG, it might intensely interact with polypeptide chains, to a higher extent compared to α-tocopherol. Moreover, the hydrophobic nature of α-tocopherol may prevent its interaction with the peptide chains. Therefore, pronounced effect of EGCG on gel strength was attained. Balange and Benjakul (2009b) documented that oxidized polyphenols had the enhancing effect on gel formation when used at an optimum level. Self-aggregation of phenolic compounds at higher levels might result in reduced protein cross-linking and consequently decreased breaking force (Balange and Benjakul, 2009a). Phenolic compound with larger size can restrict its conformational flexibility, which is important for protein–phenolic interactions (Frazier *et al.*, 2003).

Addition of βG-V-N had no impact on the deformation of the C ($p > 0.05$), however with the incorporation of EGCG up to level of 0.2% and α-tocopherol up to 0.1% increased the deformation of resulting gels ($p < 0.05$) (Fig. 19B). D-M of surimi gel was augmented where oxidized ferulic acid and oxidized caffeic acid were added up to levels of 0.2 and 0.15%, respectively (Balange and Benjakul, 2009b). The addition of βG-V-N led to decrease in F-C of gel ($p < 0.05$), however with the addition

of EGCG up to 0.2% and α-tocopherol up to 0.1% increased F-C of βG-V-N-gel (Fig. 19C).

5.5.1.2 Expressible moisture content (EMC)

Incorporation of βG-V-N did not remarkably alter the EMC of the C-gel ($p > 0.05$) (Fig. 20A). Surimi gels exhibited the decreased in EMC as the EGCG levels were increased up to 0.1%, compared to the C-gel ($p < 0.05$). The discernible increase in EMC was found when EGCG at 0.25% was incorporated ($p < 0.05$). This was concomitant with the reduction in B-F when levels of EGCG incorporation was more than 0.2%. Nevertheless, the EMC of gel added with 0.2% EGCG was similar to that of C-gel ($p > 0.05$). Addition of phenolic compound at an optimum level enhanced the protein crosslinking and development of stronger gel network with higher WHC (Balange and Benjakul, 2009b). Gani and Benjakul (2019) also reported that addition of βG-V-N at 5% level did not affect the EMC, however when the levels of β-glucan were increased up to 20%, the gradual decrease in EMC was found when compared to the control. For gel added with α-tocopherol at all the levels used, no marked differences in EMC were found ($p > 0.05$). The high concentration of the extracts or phenolic addition led to lower WHC of surimi gel (Balange and Benjakul, 2009a; Buamard and Benjakul, 2015).

5.5.1.3 Whiteness

Addition of βG-V-N remarkably increased the whiteness of the C-gel ($p < 0.05$) by 12% (Fig. 20B). However, the incorporation of EGCG at all levels decreased the whiteness of gels when compared to βG-V-N-gel ($p < 0.05$). Addition of α-tocopherol up to 0.2% showed no impact on whiteness of resulting gels ($p > 0.05$). The addition of nanoemulsion remarkably enhanced the whiteness of the gels, which might be due to light scattering effect of the nanoemulsion distributed uniformly inside the gel. Incorporation of VCO nanoemulsions increased whiteness of the croaker surimi gel (Gani and Benjakul, 2018). Incorporation of soybean oil up to 5% (w/w) increased the whiteness of mixed surimi gels produced from bigeye snapper and mackrel (Benjakul *et al.*, 2004). Progressive increase in whiteness of silver carp surimi gel was observed when vegetable oil concentration was augmented from 0 to

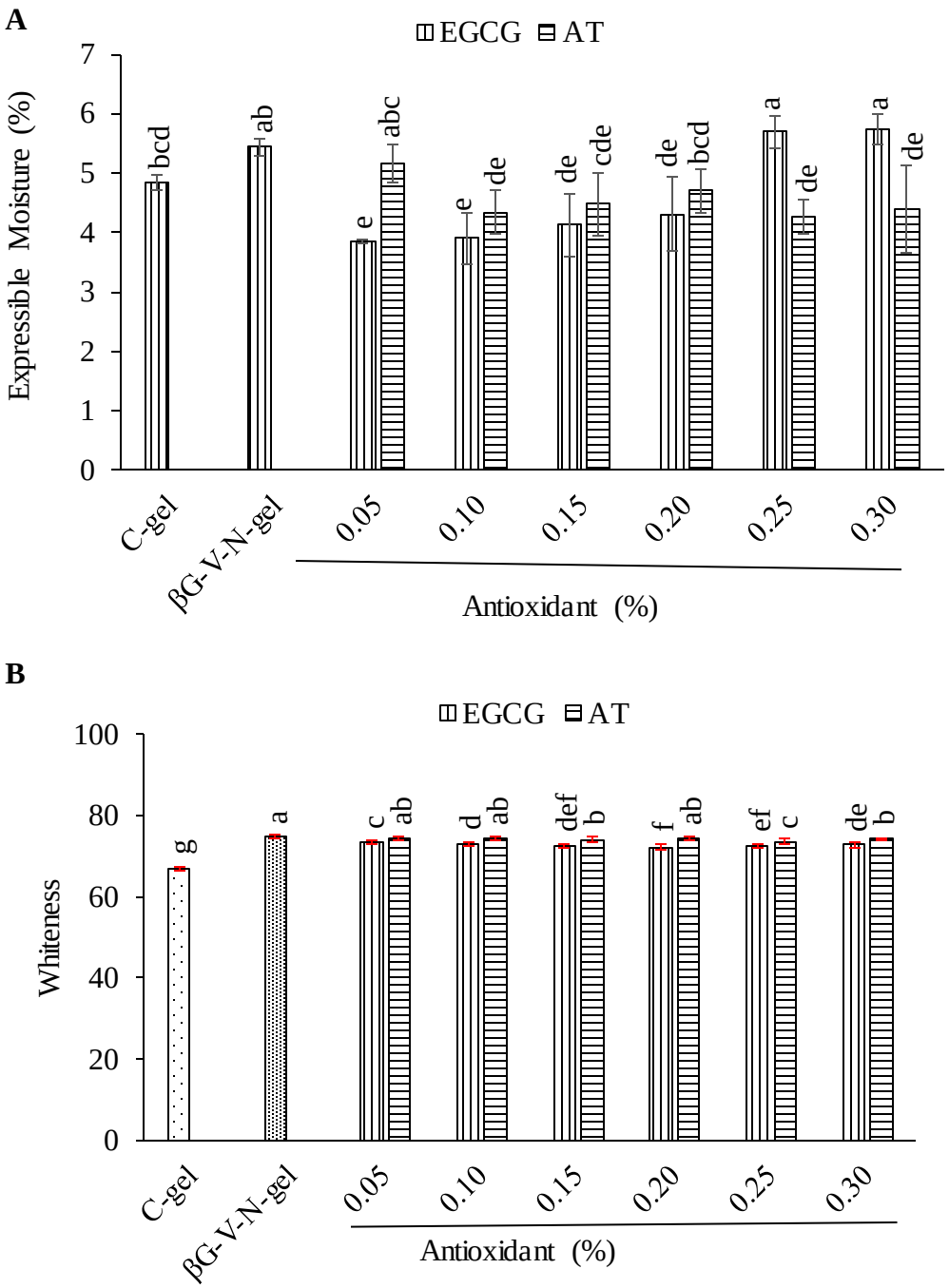

Figure 20. EMC (A) and whiteness (B) of sardine surimi gel added without and with βG-V-N in the presence and absence of EGCG or α-tocopherol at various levels. C-gel: control, βG-V-N-gel: Sample added with 5% β-glucan stabilized nanoemulsion, 0.05-0.3: gels containing βG-V-N added with EGCG or α-tocopherol at various levels ranging from 0.05 to 0.3% of protein. Bars represent standard deviation (n=3). Different letters on the bars indicate significant differences ($p < 0.05$).

5% (w/w) (Shi *et al.*, 2014). Therefore, addition of βG-V-N containing EGCG or α-tocopherol plausibly enhanced the whiteness of gels.

5.5.1.4 Protein pattern

Surimi paste (P) had myosin heavy chain (MHC) and actin as the major proteins. MHC was not retained in the control gel (Fig. 21). Protein cross-linking via indigenous transglutaminase showed the profound role in polymerization of MHC and gel enhancement of surimi (Benjakul and Visessanguan, 2003; Buamard and Benjakul, 2015). Incorporation of βG-V-N with or without EGCG or α-tocopherol at various levels could not interfere with the cross linking of MHC as evidenced by disappearance of MHC in all the samples. This indicated that inter- and intra-molecular cross-linking of MHC mediated by non-disulfide covalent bonds occurred without interfering impact by EGCG or α-tocopherol (Petcharat and Benjakul, 2018). Benjakul and Visessanguan (2003) reported that the formation of ε-(γ-glutamyl) lysine isopeptide was induced by transglutaminase in surimi gel. However, B-F and D-M increased with incorporation of EGCG and α-tocopherol up to an optimum level, which might be due to enhanced non-covalent interactions with the addition of EGCG and α-tocopherol, particularly via H-bond and hydrophobic-hydrophobic interaction. Moreover, quinones, obtained from oxidation of phenolic compounds are reported to induce polymerization of proteins via the promotion of S-S bonds (Coa and Xiong, 2015). Actin band remained unchanged for all samples, regardless of the levels and type of antioxidants. Therefore, actin was not the required substrate for transglutaminase and was more likely tolerant towards proteolysis (Benjakul and Visessanguan, 2003; Buamard and Benjakul, 2015).

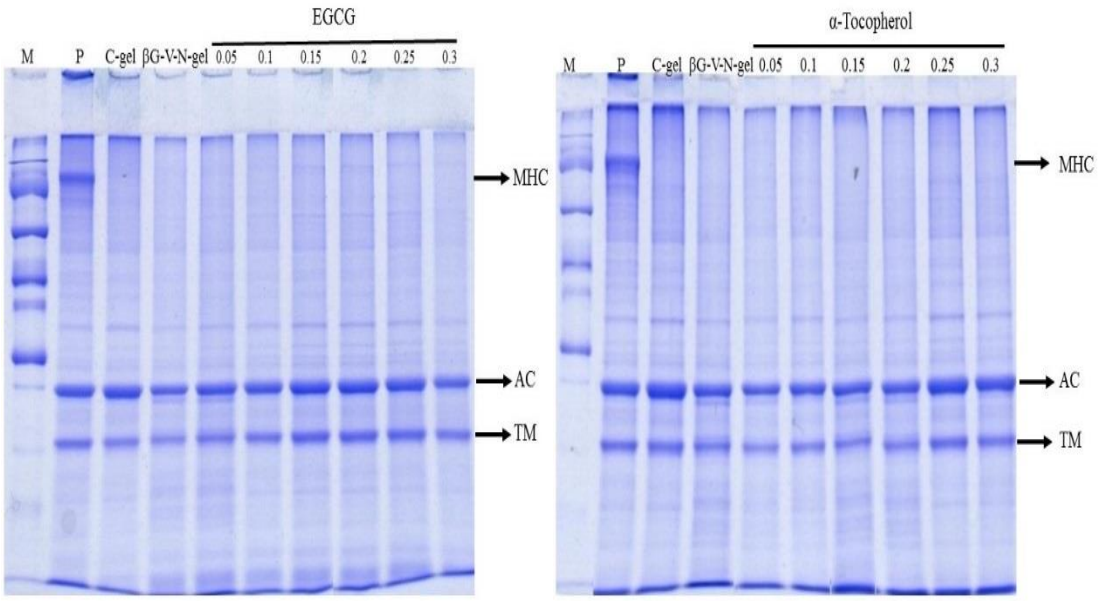

Figure 21. SDS PAGE pattern of sardine surimi gel added without and with βG-V-N. M: marker; P: paste; C-gel: control, βG-V-N-gel: gel containing 5% β-glucan stabilized VCO nanoemulsion, 0.05-0.3: gel containing βG-V-N added with EGCG or α-tocopherol at various levels ranging from 0.05 to 0.3% of protein.

5.5.1.5 Microstructure

The control gel had a finer compact network with smaller voids (Fig. 22). Addition of βG-V-N did not interfere the fine structural connectivity of the gel as shown in Fig. 22. Addition of 0.2% EGCG further strengthened the gel network as shown by the finer structure with development of more interconnected network. However, the addition of α-tocopherol at 0.2% resulted in the coarser network with larger voids or cavities. Balange and Benjakul (2009) documented that self-aggregation of phenolic compounds when used at high level caused the loss in their ability to cross-link proteins.

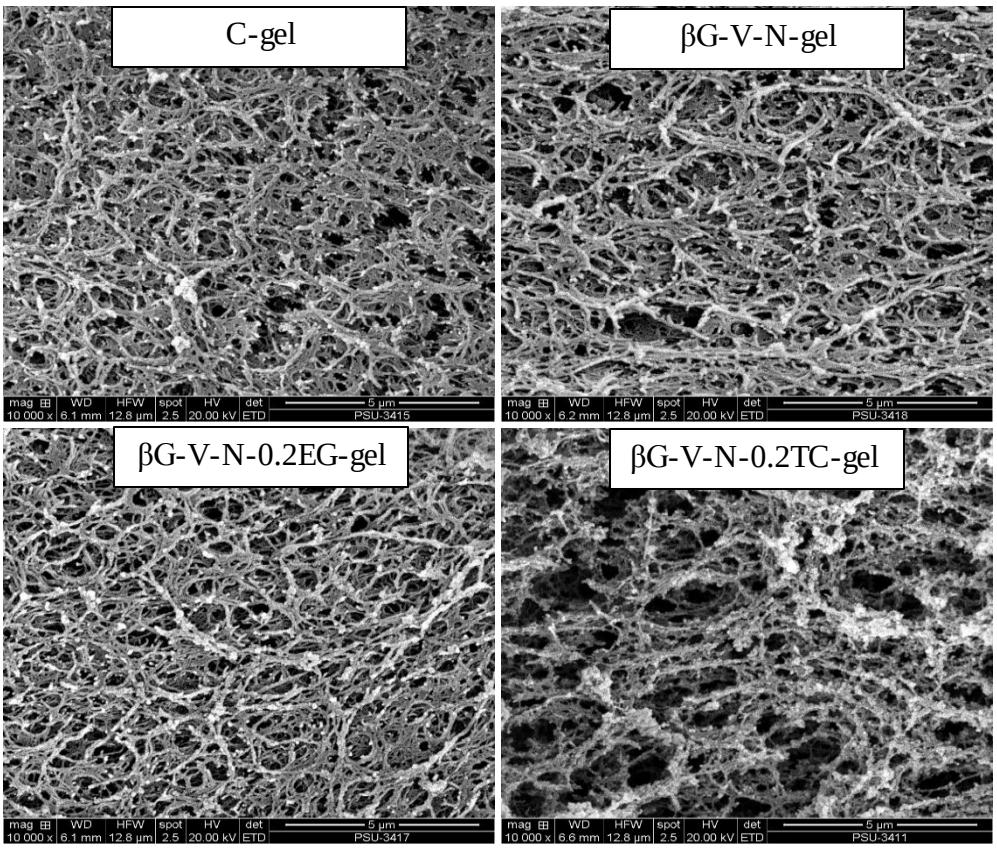

Figure 22. SEM of sardine surimi gel added without and with βG-V-N. Caption: C-gel: control, βG-V-N-gel: gel added with 5% β-glucan stabilized VCO nanoemulsion, βG-V-N-0.2EG: gel added with βG-V-N along with 0.2% EGCG (protein basis); βG-V-N-0.2TC: gel added with βG-V-N along with 0.2% α-tocopherol (protein basis). Magnification: 10,000×.

5.5.1.6 Likeness score

Addition of βG-V-N increased the color likeness of resulting gel, irrespective of the type or level of antioxidant added ($p < 0.05$) (Table 8). This might be due to whiter appearance of nanoemulsion added in surimi gels. However, the addition of EGCG or α-tocopherol had no impact on color likeness, compared to βG-V-N-gel. The results indicated that whiter gels were more preferred. Likeness for texture was not altered by the addition of βG-V-N without or with antioxidants, compared to the C-gel ($p > 0.05$). For odor and flavor likeness, βG-V-N added samples were preferred, irrespective of the antioxidant added, compared to C-gel (p

< 0.05). This might be due to the masking effect towards fishy odor by VCO. For overall likeness, the βG-V-N containing samples were preferred over the C-gel, irrespective of the antioxidants added ($p < 0.05$). Thus antioxidants added had no influence on the likeness, however the samples containing VCO in the form of βG-V-N were generally preferred, most likely owing to pleasant coconut flavor.

5.5.1.7 Rheological behavior of surimi paste as affected by βG-V-N containing antioxidants

Rheological patterns of surimi paste containing βG-V-N without or with added antioxidants are shown in Fig. 23. The control sample (C) showed a characteristic G' curve with a transition peak at about 40 °C, plausibly owing to the association of denatured myosin globular head (Coa *et al.,* 2018). Temperature dependent increase in G′ was found, irrespective of the sample and the peak value was arrived at about 40 °C. Protein network formation through the weak H-bonds between the protein molecules led to the increase in G′ (Buamard *et al.,* 2017). This was followed by a swift decline in G′. At 50 °C the lowest value was gained. Endogenous proteases have optimum temperature in the range of 50-60 °C (Klomklao *et al.,* 2008). Further heating up to approximately 65 °C caused the development of thermo-irreversible gel network, in which aggrigation between dissociated or denatured MHC occurred (Mleko and Foegeding, 2000). Aggregation could take place via hydrophobic–hydrophobic interaction and disulphide bonds formation by oxidation of sulfhydryl groups (Buamard and Benjakul, 2015). Thereafter, the reduction in G′ was attained up to 90 °C. Heating at high temperature destroyed the weak bonds, e.g. hydrogen bonds, etc.

The addition of βG-V-N decreased the G' and G" over the entire temperature range, compared to the C-gel (Fig. 23). Decrease in protein concentration with the addition of βG-V-N in the gels was presumed. However, the addition of EGCG and α-tocopherol at the appropriate levels augmented the interfering effect of βG-V-N on G′. Sample added with 0.2% EGCG showed higher values for storage and loss moduli. The addition of EGCG up to 200 ppm did not modify general rheological profile but diminished the transition peak of myofibrillar protein (MP)-soybean oil

Table 8: Likeness score of sardine surimi gel added without and with βG-V-N in the presence or absence of EGCG or α-tocopherol at various level

Antioxidant	Level (% protein)	Colour	Texture	Odour	Flavour	Overall
C-gel	–	6.20±1.01[b]	7.26±1.36abc	6.24±1.00c	6.0±0.89b	6.05±0.86b
βG-V-N-gel	–	8.14±1.03[a]	7.58±1.12a	7.95±1.07a	7.33±1.20a	7.62±1.16a
βG-V-N-0.1EG-gel	0.1	7.74±1.14[a]	7.44±1.15ab	7.62±1.20ab	7.19±1.25a	7.43±1.21a
βG-V-N-0.2EG-gel	0.2	7.61±1.24[a]	7.70±1.09a	7.38±1.07ab	7.43±1.08a	7.52±1.08a
βG-V-N-0.1TC-gel	0.1	7.95±1.30[a]	6.78±1.08bc	7.10±1.18b	7.33±1.11a	7.10±1.0a
βG-V-N-0.2TC-gel	0.2	7.80±1.21[a]	6.63±1.15c	7.19±1.03b	7.10±1.14a	6.95±1.02a

Different superscripts in the same column denote the significant differences ($p < 0.05$). C-gel: control, βG-V-N-gel: gel containing 5% β-glucan stabilized VCO nanoemulsion, βG-V-N-0.1EG-gel, βG-V-N-0.2EG-gel: gels containing βG-V-N along with EGCG at 0.1 and 0.2% of protein, respectively; βG-V-N-0.1TC-gel, βG-V-N-0.2TC-gel: gels containing βG-V-N along with α-tocopherol at 0.1 and 0.2% of protein, respectively.

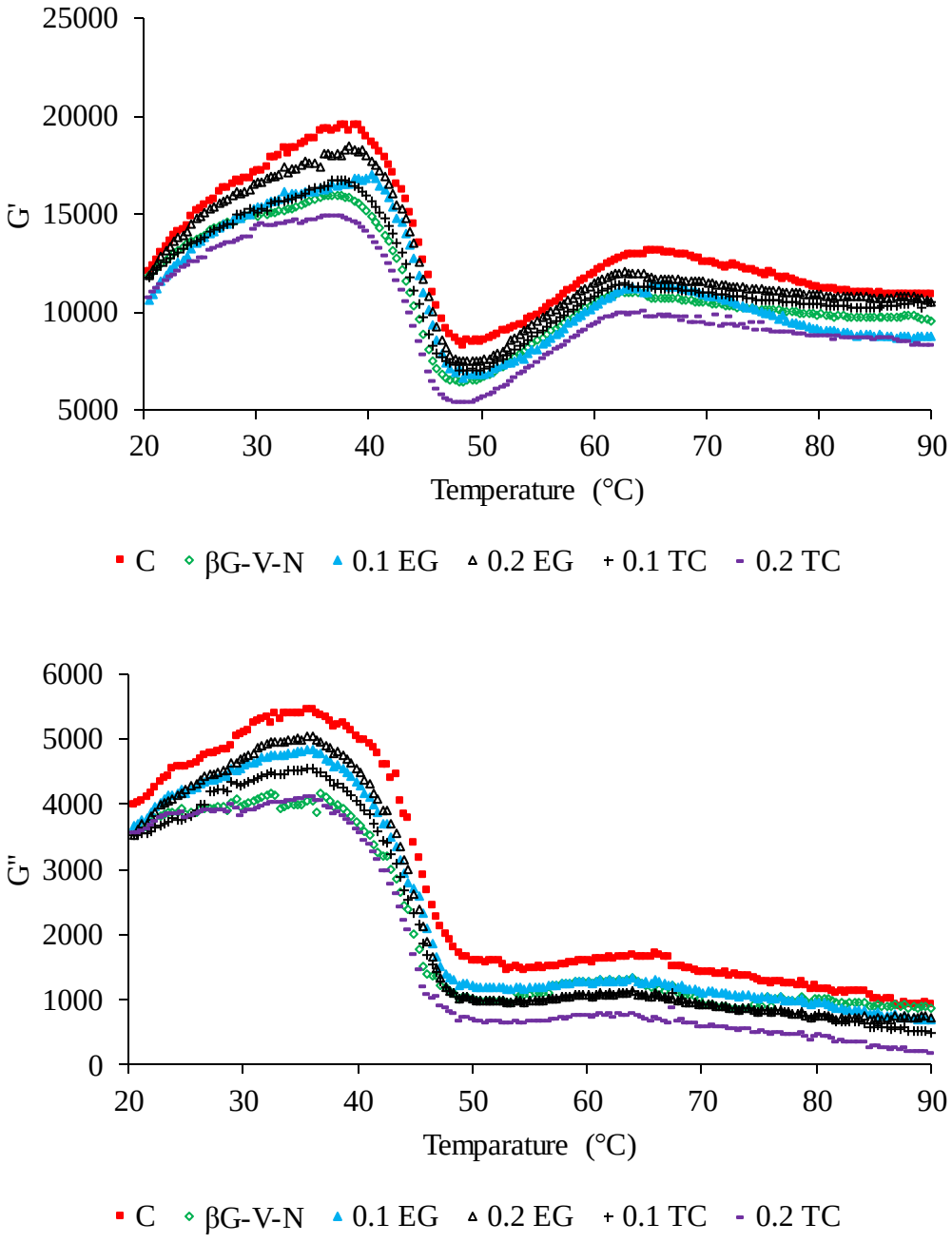

Figure 23. Viscoelastic properties of sardine surimi gel without and with βG-V-N. C: control paste, βG-V-N: surimi paste added with 5% β-glucan stabilized VCO nanoemulsion, 0.1 EG, 0.2 EG: surimi paste added with βG-V-N along with 0.1 and 0.2% EGCG (protein basis); 0.1 TC, 0.2 TC: surimi paste added with βG-V-N along with 0.1 and 0.2% α-tocopherol (protein basis).

emulsion systems (Cao *et al.*, 2018). Denser and interconnected strands were linked to higher G' and breaking force of surimi gels except 2% α-tocopherol containing gels which showed poor gel strength and G'. Buamard and Benjakul (2015) documented that addition of coconut husk ethanolic extracts increased the G' of surimi gel, most likely owing to the increased cross-linking of proteins during temperature sweep.

5.5.2 Oxidative stability of surimi gel as affected by antioxidants

No difference in PV among the samples was observed at day 0, irrespective of antioxidant added ($p > 0.05$) (Fig 24A). PV of βG-V-N-gels with or without added antioxidants was lower than that of the C-gel during the storage ($p < 0.05$). However, PV was constant after day 9 for C- and βG-V-N-gels ($P > 0.05$). For the gels containing EGCG at 0.1% and 0.2%, the increase in PV was noticed at day 6 and day 12 respectively ($p < 0.05$). Similarly, the gels containing α-tocopherol at 0.1 and 0.2% had the increase in PV at day 3 and day 6, respectively ($p < 0.05$). Generally for the gels containing βG-V-N in the presence of antioxidants, the lower PV was found throughout 12 days of storage ($p < 0.05$) (Fig. 24A). Since antioxidants were added into pre-emulsified oil, they could localize adjacent to oil droplets and acted as antioxidants efficiently (Buamard and Benjakul, 2019). The result indicated that βG-V-N-gel added with EGCG or α-tocopherol could suppress the formation of primary oxidation products during the refrigerated storage. Nonetheless, at day 12, PV of C-gel was decreased ($p < 0.05$). Hydroperoxides could be decomposed to the low molecular weight secondary lipid oxidation products, especially aldehydes etc (Bumard and Benjakul, 2019).

At the day 0, no variation in TBARS between the samples were found, irrespective of antioxidants added ($p > 0.05$) (Fig 24B). TBARS of all the samples augmented continuously within the first 6 days of storage ($p < 0.05$). However, the sample containing 0.2% EGCG showed an increase in TBARS at day 9 ($p < 0.05$). During the last 6 days, TBARS of C-gel was continuously decreased, possibly due to the loss in volatile products (Bumard and Benjakul, 2019). The C-gel and βG-V-N-gel had the discernible augmentation in TBARS up to 6 day of storage, possibly

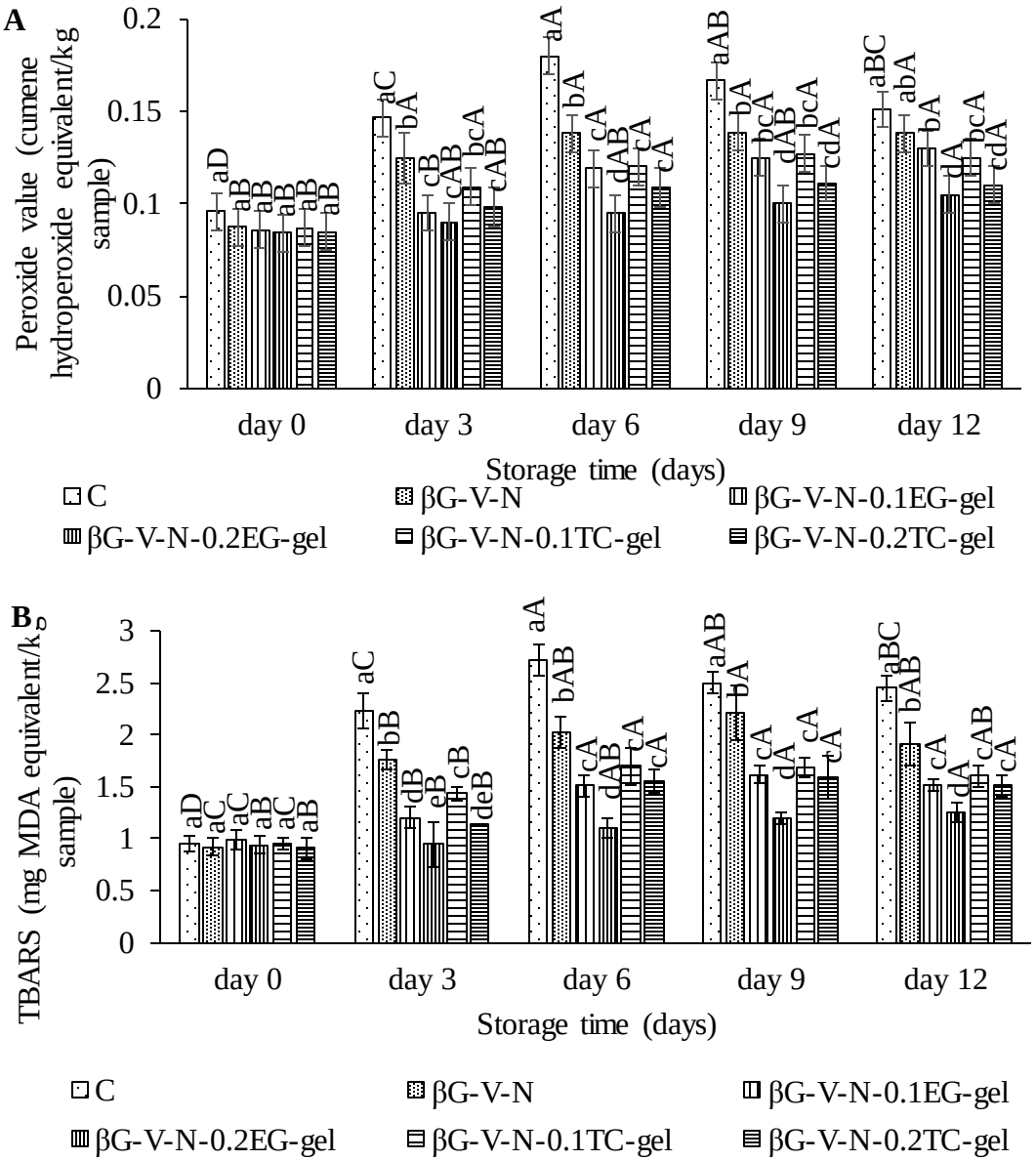

Figure 24. PV (A) and TBARS (B) of sardine surimi gel without and with βG-V-N during storage at 4 °C for 12 days. C-gel: control gel, βG-V-N-gel: gel added with 5% β-glucan stabilized nanoemulsion, βG-V-N-0.1EG-gel, βG-V-N-0.2EG-gel: gels added with EGCG at levels of 0.1 and 0.2% protein basis; βG-V-N-0.1TC, βG-V-N-0.2TC : gel added with α-tocopherol at levels of 0.1 and 0.2% protein basis. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same storage time indicate significant differences ($p < 0.05$). Different uppercase letters on the bars within the same sample indicate significant differences ($p < 0.05$).

because of the decomposition of hydroperoxides into the secondary oxidation products. Until the end of storage, decrease in TBARS values were observed. TBARS of samples added with antioxidants was less than that of C-gel at all storage time. For sample added with 0.2% EGCG, the lower TBARS values were observed ($p < 0.05$).

The addition of βG-V-N caused the enhanced oxidative stability in emulsion surimi gel, owing to antioxidant activity of VCO (Marina *et al.*, 2009) and β-glucan. β-glucan was documented to have antioxidant property (Shah *et al.*, 2015; Khan *et al.*, 2017). Moreover, ultrasonication might help in the localization of antioxidants at the interface. In addition, the shorter β-glucan chains with enhanced antioxidant activity might be produced by ultraonication.

Thus, the preparation of βG-V-N in the presence of EGCG or α-tocopherol before the addition into surimi gel was shown as a potential means to augment oxidative stability of emulsion surimi gel.

5.5.3 Microbiological quality

At day 0, total viable count (TVC) of control (without any treatment) and other surimi samples ranged from 2.17 to 2.26 log CFU/g during storage at 4 °C (Table 9). After 6 days, TVC increased to the range of 3.27-4.12 log CFU/g. At day 12, TVC count ranged from 5.13 to 5.66 log CFU/g. Maximum allowable colony count of 6 log CFU/g for seafood was reported by Sanjee and Karim (2016). Psychrophilic bacterial count (PBC) of all the samples was enumerated in the order of1.91 to 2.21 log CFU/g at day 0. PBC increased to the range of 2.85-3.72 log CFU/g at day 6. At the end of storage (day 12), PBC count ranged from 4.26 to 4.86 log CFU log CFU/g. At day 0, all the samples had similar TBC and PBC ($p > 0.05$). TVC counts were reduced significantly in the βG-V-N-gel sample irrespective of added antioxidants ($p < 0.05$) at day 6. However PBC counts were reduced throughout the storage, compared to that of C sample ($p < 0.05$). Generally, reduction in microbial counts was evidenced in the βG-V-N containing samples, regardless of the antioxidants. This might be attributed to the antimicrobial effect exerted by VCO and β-glucan. Khan *et al.* (2016) demonstrated antimicrobial effect of yeast β-glucan

Table 9. Total viable count (TVC) and psychrophilic bacterial count (PBC) of surimi gels containing β-glucan-VCO nanoemusion in the presence of EGCG or α- tocopherol at various levels during storage at 4 °C for 12 days.

				EGCG (% protein)		AT (% protein)	
Microbial load	Storage time (days)	C-gel	BG-V-N-gel	0.1	0.2	0.1	0.2
TVC	0	2.17 ± 0.12^{aC}	2.21 ± 0.11^{aC}	2.25 ± 0.20^{aC}	2.24 ± 0.01^{aC}	2.26 ± 0.12^{aC}	2.18 ± 0.10^{aC}
	6	4.12 ± 0.20^{aB}	3.36 ± 0.22^{bB}	3.28 ± 0.21^{bB}	3.40 ± 0.20^{bB}	3.27 ± 0.20^{bB}	3.31 ± 0.30^{bB}
	12	5.66 ± 0.32^{aA}	5.27 ± 0.22^{aA}	5.14 ± 0.35^{aA}	5.16 ± 0.40^{aA}	5.18 ± 0.30^{aA}	5.13 ± 0.34^{aA}
PBC	0	1.97 ± 0.10^{abC}	2.01 ± 0.12^{abC}	2.08 ± 0.17^{abC}	2.1 ± 0.08^{abC}	2.21 ± 0.20^{aC}	1.91 ± 0.11^{aC}
	6	3.72 ± 0.10^{aB}	2.86 ± 0.10^{bB}	2.85 ± 0.17^{bB}	2.91 ± 0.22^{bB}	2.86 ± 0.13^{bB}	2.93 ± 0.20^{bB}
	12	4.86 ± 0.20^{aA}	4.45 ± 0.30^{bA}	4.34 ± 0.20^{bA}	4.26 ± 0.22^{bA}	4.28 ± 0.20^{bA}	4.31 ± 0.20^{bA}

Different lowercase superscripts in the same row indicate significant differences ($P < 0.05$). Different uppercase superscripts in the same column within the same count tested indicate significant differences ($P < 0.05$). Key: For caption see Figure 24.

against *E. coli.* via zone of inhibition. Ultrasonic treatment might have produced low molecular weight β-glucan, which were reported to penetrate into microbial cell, causing cell lysis (Zheng and Zhou, 2003).

5.6 Conclusion

Antioxidant loaded nanoemulsions were produced with the aid of ultrasound and β-glucan. Antioxidant loaded nanoemulsions had profound impact on gel properties and oxidative stability of sardine surimi gel during refrigerated storage of 12 days. The gel containing EGCG at 0.2% had higher breaking force and G'. Generally, nanoemulsion containing gels were whiter in color, which increased their sensory overall likeness score. Antioxidant loaded nanoemulsions effectively prevented lipid oxidation of surimi gel during storage. TVC and PBC were restrained within the acceptable limits in nanoemulsion containing gels. Therefore, surimi fortified with β-glucan containing VCO nanoemulsion with improved gel properties and storage stability could be prepared in combination with 0.2% EGCG.

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CHAPTER 6

NUTRACEUTICAL PROFILING OF SURIMI GEL CONTAINING β-GLUCAN STABILIZED VIRGIN COCONUT OIL WITH AND WITHOUT ANTIOXIDANTS AFTER SIMULATED GASTRO-INTESTINAL DIGESTION

6.1 Abstract

Surimi gels containing β-glucan stabilized virgin coconut oil (VCO) were subjected to simulated gastrointestinal digestion and the resulting digest was analyzed for nutraceutical properties. β-glucan stabilized VCO nanoemulsion (βG-V-N) remarkably improved antioxidant activities of the surimi digest. When EGCG was added in nanoemulsion, the surimi digest showed the highest antioxidant activities. Antidiabetic activity of the digest was also improved by the addition of βG-V-N comprising EGCG. Nevertheless, the addition of βG-V-N lowered ACE inhibitory activity of surimi digest. The surimi digest from the gel added with βG-V-N possessed an inhibitory effect on five cancer cell lines including HEK (Human embryonic kidney 293 cells), MCF-7 (breast cancer cell line), U87 (human glioma), HeLa (human cervical cancer), and IMR-32 (human neuroblastoma), regardless of EGCG or α-tocopherol incorporated. This study demonstrated that surimi gel supplemented with βG-V-N in the presence of EGCG exhibited nutraceutical potential and could be used as a functional food.

6.2 Introduction

Surimi rich in myofibrillar proteins can form gel when subjected to hydrothermal processing. It is highly nutritious with delicacy, mainly due to the elastic texture. Physical and textural properties of surimi gels have been improved with the addition of different ingredients or additives. Addition of virgin coconut oil (VCO) at 5 % level has resulted in whiter gel without any deleterious effect on gel properties (Gani and Benjakul, 2019). Incorporation of gellan in both powder and suspension forms increased water holding capacity, hardness, whiteness and breaking

force of surimi gel when the gellan amounts were augmented (Petcharat and Benjakul, 2018).

VCO is generally obtained from coconut milk, meat or residue in which any chemical refining, bleaching or deodorization is omitted. Production can be done at low temperature. VCO contains natural vitamin E and lauric acid, which resists to oxidation and has incredible health benifits (Senphan and Benjakul, 2017). VCO is considered as a functional oil due to its several biological activities involving antiviral and antimicrobial (Rohman and Che Man, 2011). Recently, β-glucan stabilized VCO nanoemulsion (βG-V-N) has been added as a functional ingredient and was able to augment the textural properties, whiteness and acceptability of surimi gel (Gani and Benjakul, 2019). Nanoemulsion addition yielded the gel with better properties than the direct incorporation of VCO. β-glucan is reported to help treatment and management of diabetes, cardiovascular diseases, obesity, hyperlipidemia and cancer. The health benefits of β-glucan are fundamentally due to its fermentability and formation of high viscosity solutions in the intestines (Bozbulut and Sanlier, 2019). β-glucan is regarded as biological response modifier due to their ability to modulate immune system (Shah *et al.,* 2015). Recently, Sinthusamran and Benjakul (2018) reported the increased antioxidant activity of fish gelatin gels incorporated with β-glucan in the simulated gastrointestinal tract model.

Due to high content of proteins in surimi, peptides or free amino acids after digestion have been documented to have a variety of bioactivities. These peptides have shown broad spectrum of bioactivities including immunomodulatory (Chalamaiah *et al.,* 2018). As an ideal food matrix for supporting high quality protein, the gastrointestinal digestion of surimi gel is imperative for development of surimi based products with enhanced nutraceutical value and regulated release of nutrients in the gastric tract (Fang *et al.,* 2019). Anticancer activity has been documented in hydrolysates as well as peptides derived from a variety of food proteins (Chalamaiah *et al.,* 2018). Flounder surimi digest (FSD) exhibited ACE inhibitory activity after an *in vitro* gastric model (Oh *et al.,* 2019). Similarly, Wang *et al.* (2014) found an increase in degree of hydrolysis in gelatin from 0.17 to 26.08%, while the 2,2-

diphenyl-1-picrylhydrazyl (DPPH) radical scavenging rate increased from 1.20 to 44.76% under simulated gastrointestinal digestion. Although surimi gel containing β-glucan stabilized VCO was developed, no information on nutraceutical properties has been reported, particularly in the conjunction of antioxidant incorporation.

The *in vitro* antioxidant, antidiabetic, hypertensive and anticancer properties of the digest surimi gels added with βG-V-N in the absence or presence of selected antioxidants were evaluated.

6.3 Objective

To study the nutraceutical potential of content generated during simulated gastrointestinal (SGI) tract digestion of developed VCO emulsified surimi gel

6.4 Materials and Methods

6.4.1 Materials chemicals and cell lines

Frozen sardine surimi (AA) grade, VCO and β-glucan (70% purity) were purchased from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand), Posture Trading, Ltd. (Pathumthani, Thailand) and Hangzhou Asure Biotech Co, Ltd. (Hangzhou, China), respectively. All other chemicals used for analysis were procured from Sigma Chemical Co. (St. Louis, MO, USA). Five cancer cell lines HEK (Human embryonic kidney 293 cells), MCF-7 (breast cancer cell line), U87 (human glioma), HeLa (human cervical cancer), and IMR-32 (human neuroblastoma) were obtained from National Center for Cell Science (NCCS), Pune, India.

6.4.2 Preparation of β-glucan-stabilized VCO nanoemulsion (βG-V-N) containing antioxidants

βG-V-N was prepared with the aid of ultrasonication as detailed by Gani *et al.* (2019). Firstly 5% VCO (based on total surimi paste) and 5% β-glucan solutions (based on surimi dry weight) were prepared. EGCG or α-tocopherol were dissolved in water and VCO respectively, to obtain the level of 0.2% each, based on protein content in surimi. All the mixtures were homogenized for 5 min at 11,000 rpm. This was followed by ultrasonication using ultrasonic processor (Sonics, Model VC750,

Sonica & Materials, Inc., Newtown, CT, USA) for 5 min at 60% amplitude as tailored by Gani and Benjakul (2019).

6.4.3 Preparation of surimi gel containing VCO nanoemulsion

Frozen surimi was thawed until core temperature reached 0-2 °C. After thawing, surimi block was reduced to small pieces and it was minced in a Moulinex Masterchef 350 mixer (Paris, France) for 1 min. To surimi paste, 2.5% salt was added and mixed for another 1 min. For the samples containing βG-V-N without or with antioxidants, the prepared nanoemulsions were incorporated into paste to possess the final VCO, β-glucan and antioxidant levels of 5, 5 and 0.2 % based on surimi paste, dry matter and protein content of surimi, respectively. The paste was mixed again for 1 min. The control was also prepared without addition of βG-V-N.

The pastes were filled and sealed in polyvinylidene casing (25 mm in diameter). Gel setting was done at 40 °C and 90 °C for 30 min and 20 min, respectively using a water bath (Memmert, Schwabach, Germany). Subsequently gels were subjected to cooling using iced water. The gel samples were kept at 4 °C for 24 h before analysis.

6.4.4 Simulated gastrointestinal tract digestion

Simulated gastrointestinal tract model system (GIMS) was adopted as detailed by Sinthusamran and Benjakul (2018) with some modifications. Surimi gels were immersed in liquid nitrogen followed by pulverizing in a mixer to produce a fine powder. The sample (10 g) was taken and homogenized with 100 mL of phosphate buffered saline, pH 7.2 (0.8 g/100 mL NaCl, 0.191 g/100 mL Na_2HPO_4, 0.038 g/100 mL KH_2PO_4). To mimic the oral conditions, 200 mg of α-amylase was added into the solution (pH=7.2). The mixture was preheated for 5 min to 37 °C with continuous shaking (shaking water bath). Subsequently pH of solution was adjusted to 2 using 1 M HCl, followed by addition of 400 mg pepsin into the solution. The mixture was further incubated at 37 °C for 1 h with continuous shaking. Thereafter, 1 M NaOH was added to raise pH to 5.3 followed by addition of 200 mg pancreatin and 67 mg bile salt. Subsequently, pH of the mixture was raised to 7.5 using 1 M NaOH and the

mixture was incubated at the same temperature for 3 h with continuous shaking. The enzyme reaction was terminated by placing the sample in boiling water at 100 °C for total 10 min. The digest was centrifuged at 12,000×g for 10 min and the supernatant was obtained and kept at -40 °C for analysis. The digest from control surimi gel was referred to as 'C'. The digest from surimi gel added with βG-V-N was named 'Dig-N' while those added with βG-V-N with EGCG and α-tocopherol were termed 'Dig-N-EG' and 'Dig-N-TC', respectively.

6.4.5 Determination of antioxidant activities

6.4.5.1 DPPH radical scavenging activity (DRSA)

The procedure of Maqsood and Benjakul (2010) was followed for DRSA. The calibration curve of Trolox (0–600 μM) was used for estimation.

6.4.5.2 ABTS radical-scavenging activity (ARSA)

The protocol of Sinthusamran and Benjakul (2018) was adopted for ARSA. The calibration curve of Trolox (0 to 600 μM) was used for estimation.

6.4.5.3 Ferric reducing antioxidant power (FRAP)

FRAP was determined as tailored by Maqsood and Benjakul (2010) with minor modification. The calibration curve of Trolox (0 to 60 μM) was prepared.

6.4.5.4 Reducing Power (RP)

RP was determined by the procedure of Sinthusamran and Benjakul (2018). Trolox (0 to 60 μM) was used for standard curve.

6.4.5.5 Ferrous chelating activity (FCA)

FCA was estimated as tailored by Khantaphant *et al.* (2011). The standard curve of EDTA ranging from 0 to 60 μM was used.

Activities of DRSA, ARSA, FRAP and RP were expressed as μmol Trolox equivalent (TE)/g sample. Activity of FCA was reported as μmol EDTA equivalents (EE)/g sample.

6.4.6 Antidiabetic activity

α-Glucosidase inhibition assay (GIA)

GIA was estimated as detailed by Ahmad *et al.* (2019) with minor modification. Sample (50 µL) was added to 4 mM 4-nitrophenyl-α-D-glucopyranoside (pNPG) (25 µL), prepared in 0.1 M phosphate buffer, pH 6.8 and 50 µL of α-glucosidase enzyme solution (0.2U/mL) from yeast and mixed in 96 well plate. The plates were incubated at 37 °C for 30 min and the absorbance was read at 405 nm. The inhibition percentage was calculated as shown below:

$$\% \text{ Inhibition } = \left(1 - \left(\frac{C-D}{A-B}\right)\right) \times 100$$

Where,

A : Control, B :Control Blank, C :Reaction, D :Reaction blank

6.4.7 Antihypertensive activity

ACE inhibitory activity (ACE-IA)

ACE-IA was examined as tailored by Chalamaiah *et al.* (2015) with a slight modification. Sample (50 µL) was mixed with ACE solution (50 µL, 50 mU/mL). This mixture was preincubated at 37 °C for 10 min. Subsequently 100 µL of substrate (8.3 mM Hip-His-Leu in 50 mM sodium borate buffer containing 0.3 M NaCl, pH 8.3) was added. The mixture was incubated at 37 °C for 30 min. For the control, distilled water was used instead of sample. The enzymatic reaction was terminated using 500 µL of 1 M HCl. Ethyl acetate (1.5 mL) was used to extract hippuric acid. This was followed by centrifugation (3,000×g for 10 min) and evaporation of 1 mL upper layer to dryness at 80 °C. Hippuric acid was dissolved with 1 mL of distilled water and absorbance was read at 228 nm. The ACE-IA was calculated using the following formula:

$$\text{ACE-IA (\%)} = (Abs_{control} - Abs_{sample}/Abs_{control}) \times 100$$

6.4.8 Antiproliferative assay

Anticancer activity was examined by the method detailed by Chalamaiah *et al.* (2015) with some modifications. Dulbecco's modified Eagle's medium (DMEM) added with 10% (v/v) foetal bovine serum (FBS) and 1% antibiotic was used to maintain the cells. Humidified incubator (37 °C and 5% CO_2) level was used to grow the cells.

After achieving desired confluency, 200 µL of cell suspension with cell density of 2×10^4 cells/mL was seeded in 96-well plate, followed by incubation for 24 h at 37 °C in a humidified CO_2 incubator. The media were carefully removed. Wells were then replenished with fresh media (150 µL) and sample (150 µL). Incubation was done at 37 °C for another 24 h in a humidified CO_2 incubator. For control wells, no sample was added. After incubation, the contents of the wells were carefully removed and 20 µL of tetrazolium dye [MTT 3- (4,5-dimethylthiazol- 2-yl)−2,5-diphenyltetrazolium bromide] (5 mg/mL DMSO) was added to each well in dark. The 96-well plate was incubated at 37 °C for 3 h in CO_2 incubator. The precipitates formed were dissolved by adding DMSO (150 µl) and shaking for 15 min. The absorbance of 96-well plate was read at 590 nm and % inhibition was estimated as follows:

$$\% \text{ Inhibition} = (A_{control} - A_{sample}/A_{control}) \times 100$$

6.4.9 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.5 Results and discussion

Simulated gastrointestinal digestion is used as it is rapid, safe, inexpensive and without ethical constraints unlike *in vivo* methods. During recent times, it has been used to evaluate the bioavailability as well as bioactivity of proteins (Wang *et al.,* 2014). Simulated digestion of surimi gel is expected to release the bioactive peptides with nutraceutical properties.

6.5.1 Antioxidative activities of the digest of surimi gels added without and with βG-V-N and antioxidants from GIMs

6.5.1.1 DRSA

DRSA of the digests of all the samples were in the range of 401-600 μM Trolox equivalent/g sample (Fig 25A). Surimi gels containing βG-V-N without or with added antioxidants (α-tocopherol or EGCG) exhibited higher scavenging ability, than the control ($p < 0.05$). The highest activity was attained for Dig-N-EG sample ($p < 0.05$). Therefore, in addition to the peptides generated during digestion in GIMs, βG-V-N as well as EGCG remarkably contribute towards DPPH scavenging ability of the digest. The result revealed that the surimi digests most likely contained peptides, which were electron donors and terminated the radical chain reaction. Ultrasonic treatment during the preparation of βG-V-N plausibly degraded the β-glucan to expose functional groups, especially –COOH, >C=O, resulting in the increase in the activity. Hussain *et al.* (2018) found that increase in DRSA of γ-irradiated β-glucan due to the creation of new functional groups as a consequence of radiation degradation. García-Moreno *et al.* (2014) reported DPPH scavenging activity of sardine and horse mackerel protein hydrolysates with varying EC_{50} values (0.91 to 1.78 mg protein/mL). Similarly, sardinelle *(Sardinelle aurita)* hydrolysate had 53.76% scavenging activity towards DPPH at 2 mg/mL (Bougatef *et al.,* 2010). β-glucan itself also had antioxidant activity. Shah *et al.* (2017) documented that oat β-glucan showed higher DRSA than barley β-glucan. The radical scavenging ability of β-glucan was linked to the hydrogen atoms in its structure, which could be donated to free radicals (Shah *et al.,* 2015). EGCG was reported to show DRSA in duck egg hydrolysate (Quan and Benjakul, 2019), while α-tocopherol has been known to be a

lipid soluble antioxidant (Palozza and Krinsky, 1992). The higher degree of hydroxylation in EGCG was most likely linked to its capability of scavenging free radicals (Maqsood and Benjakul, 2010). Therefore, the combined effect of peptides, β-glucan and added antioxidants, either EGCG or α-tocopherol contributed to the increased DRSA of the digest.

6.5.1.2 ABTS radical-scavenging activity (ARSA)

ARTS of the digest from surimi gel incorporated without and with βG-V-N without and with antioxidants is shown in Fig 25B. ARTS was increased in Dig-N sample, compared with C sample ($p < 0.05$). Nevertheless, Dig-N-EG sample had the highest ARSA ($p < 0.05$). No difference in ARSA was found between Dig-N sample and Dig-N-TC sample. The activity ranged from 190 to 245 μM Trolox equivalent/g sample. The tendency of antioxidants to donate hydrogen atom to ABTS and convert it to a non-radical measures its ARSA (Sinthusamran and Benjakul, 2018). The seabass skin gelatin hydrolysates prepared using protease showed the increase in ARSA when subjected to simulated gastrointestinal digestion (Senphan and Benjakul, 2014). The antioxidative activity of surimi gel was augmented in the gastrointestinal tract by the addition of βG-V-N containing antioxidants. Pancreatin (mixture of amylase, lipase and protease) might cleave β-glucan (Sinthusamran and Benjakul, 2018). However, the role of ultrasonication in cleavage and production of small molecular weight β-glucan fragments could not be ruled out. Low molecular weight products produced from β-glucan could also be partially responsible for the increased ARSA (Hussain *et al.,* 2018). Both EGCG and α-tocopherol also contributed to ARSA. Among all the samples, Dig-N-EG sample showed the highest ARSA after digestion.

6.5.1.3 Ferric reducing antioxidant power (FRAP)

FRAP of digest from surimi gel containing βG-V-N in the absence or presence of antioxidants as depicted in Fig. 25C. FRAP measures the ability of sample to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Senphan and Benjakul, 2014). Dig-N sample had higher FRAP, compared to C sample (without nanoemulsion) ($p < 0.05$). However, the FRAP of both Dig-N-EG and Dig-N-TC were

higher, compared with Dig-N sample ($p < 0.05$). Generally the FRAP value of surimi digests gradually augmented as βG-V-N was added ($p < 0.05$) and ranged from 9.8 to 32.5 μM Trolox equivalent/g sample (Fig. 25C). The reducing ability is attributed to the presence of reducing sugars and hydrogen donating ability of β-glucan fragment induced by ultrasound (Hussain *et al.*, 2018). Sinthusamran and Benjakul, (2018) also reported that the addition of β-glucan up to 20% level augmented FRAP of the resulting digest in simulated gastrointestinal model. Among all samples, Dig-N-EG sample had the highest FRAP ($p < 0.05$). The increase in RP indicated that the surimi digest added with nanoemulsion and antioxidant, particularly EGCG was able to render an electron to stabilize free radicals, leading to prevention or suppression of propagation (Senphan and Benjakul, 2014).

6.5.1.4 Reducing Power (RP)

The reduction of Fe(III) complex to Fe(II) complex was used to evaluate the reductive ability of digest from surimi gel added without or with βG-V-N in the presence and absence of antioxidants (Fig. 25D). Generally, Dig-N sample had the increased reducing power of the surimi digests ($p < 0.05$), compared to the C sample. However, the antioxidant added samples showed stronger RP of digest, than the Dig-N ($p < 0.05$). No difference was found between Dig-N-EG and Dig-N-TC samples ($p > 0.05$). The tendency of an antioxidant to provide an electron or hydrogen to free radicals is assessed by its reducing power (Bougatef *et al.*, 2010). Sinthusamran and Benjakul (2018) reported the increase in RP of fish gelatin-β-glucan gels subjected to GIMs with increasing levels of β-glucan in the gel. Gradual increase in RP was found for the digests with progression in simulated digestion from mouth phase to duodenum phase (Sinthusamran and Benjakul, 2018). In addition to the peptides generated during simulated gastrointestinal digestion having antioxidative properties (Senphan and Benjakul, 2014), oligosaccharides and/or small molecular weight components from β-glucan resulted in the antioxidative property of surimi digests (Sinthusamran and Benjakul, 2018). Shah *et al.* (2017) found a dose dependent increase in RP of barley and oats β-glucan ($p < 0.05$) up to 100 mg/mL concentration. However, RP of oats β-glucan was higher than that of barley at all the levels tested (p

< 0.05). Ovissipour *et al.* (2013) reported RP in the range of 3.3-7.6 (μM TE/g protein) for anchovy sprat hydrolysates produced by various enzymes. Both EGCG and α-tocopherol drastically contributed to the enhanced RP of the digest. Thus, the combination of βG-V-N with antioxidant resulted the digest with enhanced RP. Similar results were found for FRAP and RP (Fig 25C, D). Both of these assays use different Fe-binding ligands, however the ability of antioxidant to reduce Fe^{3+} complex to Fe^{2+} complex can be examined (Sinthusamran and Benjakul, 2018).

6.5.1.5 Metal-chelating activity (MCA)

MCA of digest from surimi gel added without and with βG-V-N alone or in combination with antioxidants is depicted in Fig. 25E. Dig-N-EG sample exhibited the highest MCA ($p < 0.05$). However, no difference was attained in MCA between Dig-N and Dig-N-TC samples ($p > 0.05$). The control sample (without nanoemulsion) showed the lowest ability to chelate metal ions. MCA ranged from 2.69 ± 0.17 to 3.70± 0.23 (μM EE/g sample). Senphan and Benjakul (2014) found the increased MCA of hydrolysate (40% DH) produced from seabass skin, in the duodenal condition after being ingested in GIMs. Khantaphant *et al.* (2011) found that enhanced antioxidative activity of flavourzyme protein hydrolysate produced from brownstripe red snapper, after GIMs. Transition metals ions like Cu^{2+} and Fe^{2+} catalyze the generation of reactive oxygen species which induces lipid peroxidation. Therefore, the capacity of peptides to bind the transition metals could inhibit oxidation (Stohs and Bagchi, 1995). García-Moreno *et al.* (2014) reported EC_{50} values of 0.32 mg protein/mL for ferrous-chelating activity of hydrolysates from sardine and small-spotted catshark. The availability of effective metal chelating sites in hydrolysates more likely corresponds to its metal binding capacity (Ovissipour *et al.*, 2013).

β-glucan–Fe^{2+}complexes, might also increase MCA of the digest. Hussain *et al.* (2018) and Shah *et al.* (2015) also found an increase in MCA of γ-irradiated (15 kGy) oat ß-D glucan by 20% when tested at 1,000 μg/mL and 45% increase in chelating ability of barley β-glucan upon irradiation treatment of 8 kGy, respectively. EGCG has been known to be able to chelate metal ions due to its adjacent trihydroxy structure, in which electrons are donated by oxygen atoms to form bonds with the

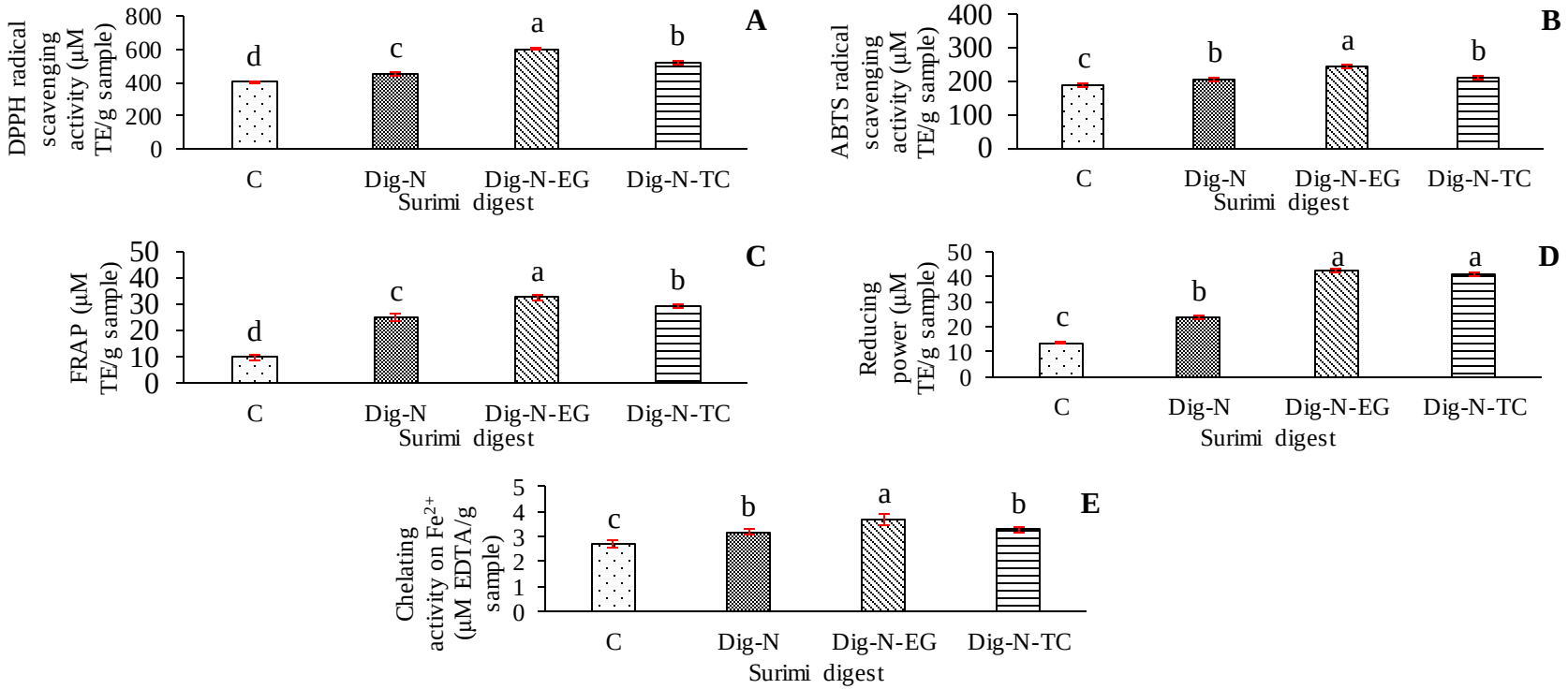

Figure 25. DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), metal chelating activity (C), reducing power (D) and FRAP (E) of digest of surimi gel added without and with β-glucan stabilized VCO nanoemulsion in the presence and absence of EGCG or α-tocopherol. C : control surimi gel; Dig-N : surimi gel added with β-glucan stabilized VCO nanoemulsion; Dig-N-EG : surimi gel added with β-glucan stabilized VCO nanoemulsion containing epigallocatechin gallate; Dig-N-TC : surimi gel added with β-glucan stabilized VCO nanoemulsion containing α-tocopherol. Bars represent standard deviation (n = 3). Different letters on the bars indicate significant differences ($p < 0.05$).

metal ions (Zhong *et al.*, 2012). EGCG has been reported to chelate metal ions like zinc, copper, iron, which are reported to be linked with various biological activities (Liu *et al.*, 2017). Therefore, addition of βC-V-N with EGCG remarkably improved MCA of the digest.

6.5.2 α-Glucosidase inhibition assay (GIA)

GIA of different surimi digests is depicted in Fig. 26. C sample had the lowest GIA, compared to others ($p < 0.05$). C sample showed less than 10% inhibition towards α-glucosidase, however Dig-N sample showed remarkable α-glucosidase inhibition (up to 35%). Peptides generated after digestion might contribute to inhibition of α-glucosidase. Matsui *et al.* (1999) isolated two α-glucosidase inhibitory peptides from sardine muscle hydrolysate prepared using alkaline protease from Bacillus licheniformis. The peptides were identified as Try-Tyr-Pro-Leu (IC_{50} = 3.7 mM) and Val-Trp (IC_{50} = 22.6 mM). The possible mechanism of inhibition was that peptides possibly interacted via hydrophobic bonds at the active site of enzyme.

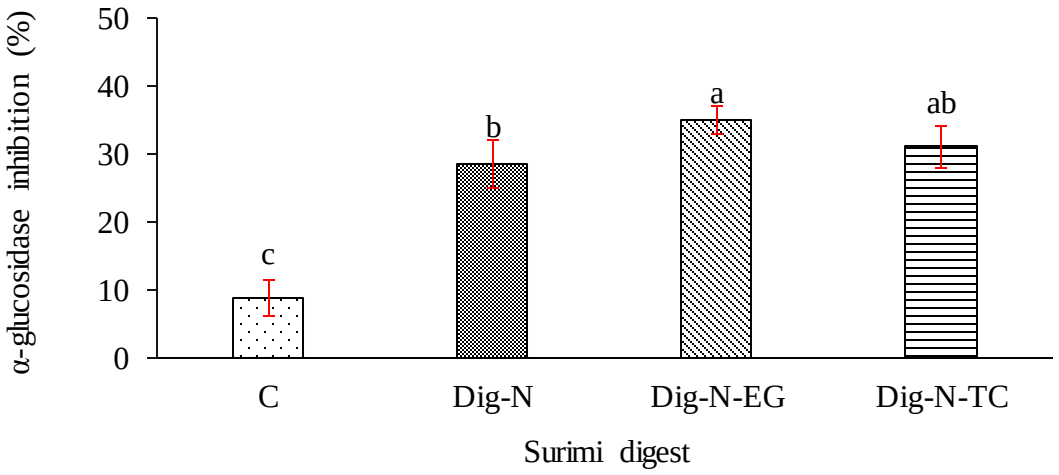

Figure 26. α-Glucosidase inhibitory activity of digest of surimi gel added without and with β-glucan stabilized VCO nanoemulsion in the presence and absence of EGCG or α-tocopherol. Bars represent standard deviation (n = 3). Different letters on the bars indicate significant differences ($p < 0.05$). Key: For caption see Figure 25.

Apart from peptides, Hussain *et al.* (2018) reported GIA of oats β-glucan which was increased by gamma irradiation. Kardono *et al.* (2013) isolated crude β-

glucan from silver ear mushroom at 50 ppm having GIA with IC_{50} value of 41.61%. Dig-N-EC and Dig-N-TC showed high inhibition towards α-glucosidase. Xu *et al.* (2019) demonstrated that the GIA of EGCG (IC_{50} = 19.5 µM) was higher than that of acarbose (IC_{50} = 278.7 µM). The inhibition was reversible and non-competitive. EGCG binding sites were in proximity to the active site of α-glucosidase as revealed by molecular docking. The inhibitory activity of EGCG possibly attributed to galloyl group bonding at the 3 position of catechins and was governed by the number of hydroxyl group on the B ring (Liu *et al.*, 2016). Therefore, the surimi gels added with βG-V-N with antioxidants can generate functional bioactives with promising antidiabetic properties.

6.5.3 ACE inhibitory activity (ACE-IA)

The digests surimi added without and with antioxidant loaded with βG-V-N were analyzed for ACE-IA as shown in Fig. 27. ACE-IA was in the range of 25 to 40%. C sample had the highest ACE-IA ($p < 0.05$), compared to other samples. Dig-N-EG sample showed higher ACE-IA activity than Dig-N-TC sample ($p < 0.05$). ACE is allosterically inhibited by EGCG, probably through conversion into an electrophilic quinone and subsequent binding to ACE (Liu *et al.*, 2017). EGCG is reported to chelate Zn at the active site of ACE, thus contributing towards ACE-IA (Persson *et al.*, 2006). Generally, βC-V-N decreased the ACE-IA of the surimi digest (Fig. 27). Flounder surimi digest had ACE-IA in a dose dependent manner ranging from 40 to 77% using an *in vitro* gastric model (Oh *et al.*, 2019). Bioactive peptides from flounder surimi effectively bound to ACE with negative binding energy and negative CDOCKER interaction energy in an *in silico* docking analysis. ACE converts angiotensin I into angiotensin II (vasoconstrictor) in the renin-angiotensin system (RAS) and inactivates bradykinin (a potent vasodilator), in the kinin nitric oxide system (KNOS). ACE inhibition leads to lowering blood pressure by reducing vasoconstriction (Neves *et al.*, 2017). The digest from surimi added with βG-V-N had lower activity. β-glucan might interact with ACE inhibitory peptides, thus lowering activity. Therefore, surimi digest had a potential to inhibit ACE, resulting in lowering of high blood pressure.

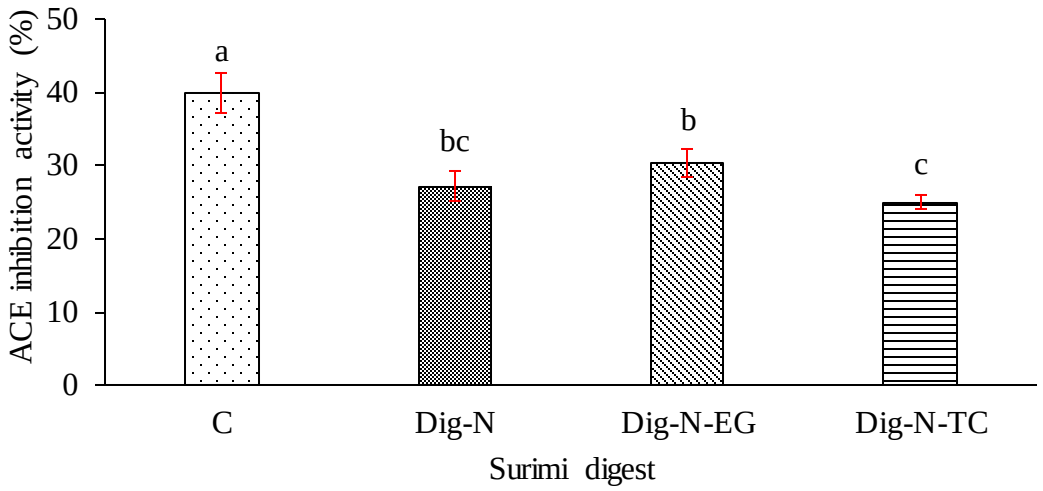

Figure 27. ACE inhibitory activity of digest of surimi gel added without and with β-glucan stabilized VCO nanoemulsion in the presence and absence of EGCG or α-tocopherol. Bars represent standard deviation (n = 3). Different letters on the bars indicate significant differences ($p < 0.05$). Key: For caption see Figure 25.

6.5.4 Antiproliferative activity

The *in vitro* gastrointestinal digests from surimi added without and with antioxidant loaded with βG-V-N were examined for antiproliferative activity against five cancer cell lines including HEK, MCF-7, U87, HeLa, and IMR-32. The samples remarkably inhibited the proliferation of cancer cells, however the C sample showed lower inhibition, than other samples ($p < 0.05$) for all the cell lines tested. The results indicated that % inhibition ranged from 31 to 54% in all the cell lines (Fig. 28). However, no difference was found in the inhibition ability of surimi digests containing βG-V-N with or without added antioxidants ($p > 0.05$). MCF-7 cancer cell line was more sensitive to surimi GI digest, resulting in higher % inhibition (41-54%), while U87 was the least sensitive as shown by the lower inhibition (31-38%). This indicated that the sensitivity of cancer cell lines towards surimi digests depended on the cell types. Picot *et al.* (2006) found that antiproliferative potential of hydrolysates from fish protein was dependent on the cell line investigated. Breast cancer cell line (MDA-MB-231), which is considered highly invasive, was less sensitive to fish protein hydrolysate treatment, compared to MCF-7/6.

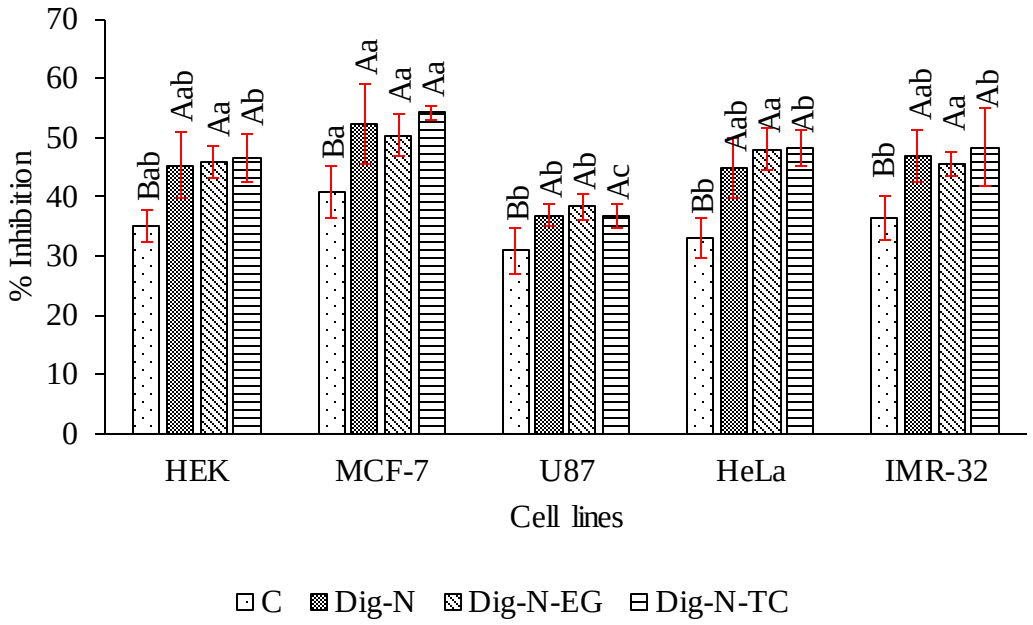

Figure 28. Antiproliferative activity of digest of surimi gel added without and with β-glucan stabilized VCO nanoemulsion in the presence and absence of EGCG or α-tocopherol. HEK: Human embryonic kidney 293 cells; MCF-7: breast cancer cell line; U87: human glioma; HeLa: human cervical cancer; and IMR-32: human neuroblastoma. Bars represent standard deviation (n=3). Different uppercase letters on the bars within same cell line indicate significant differences ($p < 0.05$). Different lowercase letters on the bars within same digest indicate significant differences ($p < 0.05$). Key: For caption see Figure 25.

The structural characteristics of peptides derived from food including amino acid sequence, composition, overall charge or hydrophobicity, length regulate their anticancer activity. The interaction between anticancer peptides and the outer leaflets of tumor cell membrane bilayers are enhanced by the presence of hydrophobic amino acids (Chi *et al.*, 2015). The shorter peptides are reported to show stronger anticancer activity due to their higher molecular mobility and diffusivity, thus enhancing interaction with the cancer cell components (Chalamaiah *et al.*, 2018). Chalamaiah *et al.* (2015) reported that pepsin hydrolysate obtained from rohu egg (roe) showed 65% inhibition of Caco-2 (human colon cancer cell line). Additionally β-glucan also showed antiproliferation of cancer cells. Shah *et al.* (2015) documented

that γ-irradiation treatment of β-glucan resulted in the formation of low molecular weight β-glucan, which had the increased antiproliferative activities against human cancer cell lines (Colo-205, T47D and MCF7). Choromanska *et al.* (2015) investigated the antitumor activities of low molecular weight β-glucan from oats in two cancer cell lines Me45 and A431. Cancer cell viability was markedly reduced by treatment with low molecular weight β-glucan. With the increasing incubation time and the β-glucan concentration, the cancer cells viability significantly deceased (Choromanska *et al.*, 2015). The ultrasonic treatment used in the preparation of βG-V-N most likely extended the β-glucan chains. Extended β-glucan chains had more chances to bind cell receptors than the compact conformation. Chen *at al.* (2009) reported that extended conformation of phosphorylated (1→3)-β-D-glucan from *Poria cocos* could enhance the anti-tumor activities as a result of the increasing the chance of binding with receptors on the immune cells.

Coconut oil contains medium chain saturated fatty acids including lauric acid, capric acid and caprylic acid. Lauric acid is reported to have anticancer activity. Lauric acid-induced dose-dependent cytotoxicity towards Raw 264.7 (murine macrophages), HCT-15 (human colon cancer), and HepG2 (human hepatocellular carcinoma) cells showed morphological characteristics of apoptosis (Sheela *et al.*, 2019). Lauric acid treatment at 30 and 50 µg/mL was found to downregulate the expression of epidermal growth factor receptor (EGFR) by 1.33 and 1.58 fold in HCT-15 cells. Downregulation of EGFR might be related to anticancer activity of lauric acid (LA), which resulted in decreased cell viability (Sheela *et al.*, 2019). Medium chain saturated fatty acids are also reported to interact with thymidine synthase in the folate pathway which plays significant roles in the DNA synthesis and cell division. The inhibition of thymidine synthase activity is reported to prevent the abnormal synthesis of DNA, thereby reducing the severity of cancers (Sheela *et al.*, 2019). Lappano *et al.* (2017) reported that LA promotes formation of stress fibre and upregulate the p21Cip1/WAF1 expression. The former has a crucial role in morphological changes related with apoptotic cell death, while the latter results in apoptosis of breast and endometrial cancer cells.

The surimi gel added with βG-V-N provided an effect on antiproliferative potential of the digest and resulted in higher inhibition compared to the control gels. However, the addition of antioxidants had no profound influence on the antiproliferative potential. Therefore, it was postulated that the simulated gastrointestinal digestion of surimi gel resulted in production of bioactive peptides with potential antiproliferative activity, while β-glucan or its fragments also showed the combined impact on antiproliferation activity of digest from surimi gel.

6.6 Conclusion

The incorporation of βG-V-N significantly improved the nutraceutical profile of surimi gel when subjected to simulated gastrointestinal digestion. Remarkable improvements in antioxidant, antidiabetic and anticancer activities of the digests were found with the addition of βG-V-N, especially in combination of EGCG. However, ACE-IA was not improved by the addition of βG-V-N. Therefore, incorporation of βG-V-N in conjunction with antioxidant could improve the nutraceutical profile of the surimi gel, in which the functional surimi with improved health benefits could be produced.

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CHAPTER 7

SUMMARY AND SUGGESTIONS

7.1 Summary

1. Addition of VCO significantly influenced the texture and whiteness of surimi gel. VCO addition affected elastic (G') as well as loss moduli (G'') of surimi paste, but had no impact on protein pattern of all gels. Uniform distribution and anchoring of VCO droplets in the protein matrix were noticed. Thus, VCO at an appropriate level could be used to replace other vegetable oils, in which health benefits could be claimed, especially as a source of medium chain fatty acids.

2. VCO nanoemulsion was successfully produced by ultrasonication. Addition of VCO nanoemulsion had no remarkable effect on textural properties of surimi gel, but increased gel whiteness. Addition of VCO nanoemulsion had no impact on the protein pattern and microstructure. Therefore, VCO nanoemulsion was the appropriate form to be incorporated in the surimi without deterioration of textural properties.

3. β-glucan stabilized VCO nanoemulsion (βG-V-N) was produced by ultrasonication under the appropriate condition. Addition of βG-V-N containing 5% β-glucan increased breaking force and whiteness of surimi gel and resulted in smoother and finer microstructure. Gel had the improved textural properties, whiteness and likeness.

4. Antioxidant loaded βG-V-N had profound impact on gel properties and oxidative stability of sardine surimi gel during refrigerated storage of 12 days. TVC and PBC were restrained within the acceptable limits in all the gels. Surimi fortified with βG-V-N in combination with 0.2% EGCG had the improved gel properties and storage stability.

5. The incorporation of βG-V-N significantly improved the nutraceutical profile of surimi gel when subjected to simulated gastrointestinal digestion. Remarkable improvements in antioxidant, antidiabetic and anticancer activities of the

digests were found with the addition of βG-V-N, especially in combination of EGCG. However, ACE-IA was not improved by the addition of βG-V-N. Therefore, incorporation of βG-V-N in conjunction with antioxidant, especially EGCG, could improve the nutraceutical profile of the surimi gel.

7.2 Suggestions

1. VCO nanoemulsification loaded with other bioactive compounds should be carried out with the aid of ultrasound and used as functional ingredients.

2. The bioactive molecules from gastrointestinal digest of VCO emulsified surimi gel should be further identified and characterized.

VITAE

Name Mr. Asir Gani

Student ID 5811030002

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Technology (Food Technology)	IUST, J & K, India	2010
Master in Technology (Food Engineering and Technology)	SLIET Longowal, Punjab	2013

Scholarship Awards during Enrollment

- Thailand's Education Hub for Southern Resion of ASEAN Countries (THE-AC) Scholarship 2015.

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