Characteristics and Applications of Active Components from Squid Melanin-Free Ink

Naveen Kumar Vate

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

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ABSTRACT

Antioxidative activities of melanin-free ink (MFI) from splendid squid (*Loligo formosana*) were investigated using different *in vitro* assays. MFI had DPPH, ABTS radical scavenging activities, and ferric reducing antioxidant power (FRAP) of 179.6±2.1, 957.8±89.3, and 171.2±7.3 µmol TE/g protein, respectively. It also possessed metal chelating activity of 4.0±1.2 µmol EE/g protein. MFI at 500 mg/L could prevent the oxidation in *β*-carotene-linoleic acid and lecithin liposome systems but its efficacy was lower than 200 mg/L butylated hydroxyl anisole (BHA) ($P<0.05$). MFI retarded the lipid oxidation in mackerel mince during iced storage of 15 days as evidenced by the lower peroxide value and thiobarbituric acid reactive substances, compared with the control. When MFI was subjected to heat treatment at 90°C, for up to 30 min, its DPPH and ABTS radical scavenging activities remained constant. The MFI fraction with MW less than 3 KDa showed the highest antioxidative activities ($P<0.05$).

Addition of MFI (0-0.1 g kg^{-1} surimi) increased breaking force and deformation of sardine surimi gel in a dose-dependent manner ($P<0.05$). The addition of MFI had no effect on whiteness ($P>0.05$) but decreased the expressible moisture content of gels ($P<0.05$). Based on microstructure study, gel added with MFI at a level of 0.08 g kg^{-1} surimi was denser and finer than that of the control (without MFI). Additionally, it was able to prevent lipid oxidation in surimi gels during refrigerated storage as evidenced by lower peroxide values (PV), thiobarbituric acid reactive substances (TBARS), nonanal and 2-decenal.

Effect of the mixture of squid ink tyrosinase (SIT) at 300 and 500 U/g protein and tannic acid (TA) at 0.5 and 1% (based on protein) with different reaction times (90 and 180 min) on gel properties of sardine surimi was investigated. Surimi gel incorporated with the mixture of SIT (500 U/g protein) and 1% TA with a reaction time

of 90 min had the highest breaking force and deformation (P<0.05), in which the increases by 29.3% and 11.9% were observed, in comparison with the control. However, gels added with SIT/TA mixture had the lower whiteness, compared to the control (P<0.05). Gel added with SIT/TA mixture showed more compact and finer network with higher connectivity of strands, compared to the control. This was coincidental with decreased expressible moisture content. Based on sensory evaluation, the highest overall likeness score was found in gel added with the mixture of SIT (500 U/g protein) and 1% TA (P<0.05). Physicochemical changes of muscle proteins induced by the mixture of SIT at 300 and 500 U/g protein and TA at 0.5 and 1% (based on protein), were studied. Turbidity and surface hydrophobicity of natural actomyosin (NAM) from sardine (*Sardinella albella)* were increased in a dose dependent manner. Total sulfydryl group content decreased in the NAM solutions with coincidental increase in disulfide bond content, when added with SIT/TA mixture. Ca^{2+}-ATPase activity was also lower in NAM solutions added with SIT/TA mixture. Higher aggregation of protein filaments was noticeable in NAM added with SIT at 500 U/g protein and 1% TA, which also had the highest storage modulus (G') and largest particle size. Negative charge of NAM was decreased when SIT/TA mixture was incorporated.

The use of SIT (500 KU kg^{-1} protein) in combination with ethanolic extract of coconut husk (EEC) (1.25 g kg^{-1} protein) yielded the gel with the highest breaking force and deformation (P<0.05). Nevertheless, the expressible moisture content and whiteness scores were markedly decreased. Protein cross-linking of surimi gels increased as evidenced by the decreased band intensity of myosin heavy chain (MHC). This coincided with the increased storage modulus (G'). Based on microstructure study, finer and more compact structure of surimi gels added with both SIT and EEC were obtained, compared to the control.

The effect of the MFI at different concentrations (0, 0.1, 0.2, 0.3 and 0.4 g L^{-1} of film forming solution (FFS)) on the properties and yellow discoloration of films from washed sardine mince was studied. Tensile strength (TS) of the film increased with increasing MFI concentration (P<0.05). Conversely water vapor permeability (WVP) of films decreased as the concentration of MFI increased (P<0.05). Fourier transform infrared (FTIR) spectra of films showed a slight shift to lower wavenumber of amide-B band of film added with MFI, indicating protein cross-linking. The

microstructure showed slightly rough surface of the films when amount of MFI increased. However, cracks in the films were much decreased when MFI at higher levels was incorporated. Films added with MFI had lower TBARS value, indicating the lowered lipid oxidation. Yellow discoloration decreased but the transparency of film increased when MFI concentration increased.

The film made from FFS added with SIT (5 k units L^{-1}) and TA (1 g L^{-1}) had the highest TS but lowest EAB ($P < 0.05$). The addition of mixture of SIT and TA reduced the water vapor permeability (WVP) of films ($P<0.05$). Fourier transform infrared (FTIR) spectra of films containing both SIT and TA showed a shift in the amide-III band to the lower wavenumber. The decrease in myosin heavy chain (MHC) band intensity in the film added with the mixture of SIT and TA was observed, indicating the increased cross-linking. The films added with the mixture of SIT and TA were less transparent, compared to the control film. The surface of films added with only SIT or the mixture of SIT and TA became slightly rougher.

MFI had antioxidant activity and hence could be used to inhibit the lipid oxidation in surimi gel and myofibrillar protein films. SIT in combination with the phenolic compounds could be utilized as protein cross-linker. Therefore the squid ink, a by-product from the squid processing industry, could be valorized as additive for the improvement of food properties.

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

About 50–60% of total marine catch is used for direct human consumption and about 25-30 million metric tons of fish and shellfish caught each year are discarded. Those discards can be the potential sources of beneficial compounds (Morrissey and Okada, 2007). Among the catch, squid and cuttlefish are one of the important fishery products in Thailand as well as other Southeast Asian countries, and are mainly exported worldwide (Hoque *et al.,* 2010). During processing, viscera along with the ink sac is generated as a by-product with the low market value and it can create serious ecological problems and environmental pollution without appropriate management. These by-products can be a promising source of bioactive compounds. Natural bioactive substances have the least quantum of side effects when compared with the synthetic counterparts. Due to the huge diversity of marine bioactive compounds with respect to their chemical structure, mode of action and applicability, the extreme concern has been increased towards the screening of marine natural products for their biomedical potential (McConnell *et al.,* 1994). Squid ink is known to possess many bioactivities such as antimicrobial, anticancer, antioxidant activities etc. (Girija *et al.,* 2011; Zhong *et al.,* 2009) and it has been used in some food products like pasta, etc. (Dowling, 2006).

Lipid oxidation is one of the major causes for deterioration of many food products. It leads to changes in texture, flavor, odor, thereby causing the loss in food quality. Lipid oxidation also causes some health hazards in human beings such as cardiovascular disease, cancer and neurological disorders as well as aging process (Gulcin, 2011, 2012). To prevent or retard lipid oxidation, several antioxidants including synthetic and natural antioxidants have been widely used. However, synthetic antioxidants are suspected of being toxic upon long term exposure and are banned in many countries (Madhavi and Salunkhe, 1996). As a consequence, natural and safe antioxidants have gained increasing attention. Natural antioxidants from

marine resources, especially from by-products of seafood processing, can be an alternative for food applications (Shahidi, 2006).

Surimi is minced fish flesh, washed to remove most of lipids, blood, enzymes and sarcoplasmic proteins and stabilized for frozen storage by cryoprotectants. In general, lean fish have been used for surimi production owing to the superior gelling property and whiteness to those from dark fleshed fish. Due to the limited fish resources, especially lean fish, pelagic dark-fleshed fish have been gained increasing attention as a potential alternative raw material for surimi production (Arfat and Benjakul, 2012). Apart from the dark color, surimi from dark fleshed fish has poorer gel properties than those produced from lean fish (Kudre *et al.,* 2013). To increase gel forming ability of surimi from dark fleshed fish, the uses of many natural and cheap additives capable of increasing gel strength have been implemented. Various food grade ingredients such as whey protein concentrate, plasma proteins, egg white etc. have been used to increase the gel strength of surimi (Rawdkuen *et al.,* 2007). However, some protein additives have been banned in surimi. Plasma proteins have been prohibited for the use in surimi due to the religious constraint as well as possible disease transmission (Rawdkuen and Benjakul, 2008). Egg white is expensive and associated with allergy problems (Balange and Benjakul, 2009; Kudre *et al.,* 2013). Protein cross-linking enzyme, especially transglutaminase, has been used to enhance gel strength of surimi (Benjakul and Visessanguan, 2003). Tyrosinase from squid ink can be an alternative cross-linking enzyme, which can improve gelling properties of surimi from dark fleshed fish. Simultaneously, antioxidant in squid ink can prevent lipid oxidation in the surimi gel.

Furthermore, tyrosinase in squid ink can be used to improve the strength of film based on surimi or surimi blend. Due to its antioxidant activity, the yellow discoloration of film from surimi can be retarded. Therefore, ink can be used as the alternative source of natural antioxidant and protein cross-linkers, which can be further used as processing aid in quality improvement of protein gel and film. As a consequence, the ink can be better exploited and new value-added product from squid processing discards can be gained.

1.2 Literature review

1.2.1 Squid

Squid is a cephalopod which is one of three groups of mollusks: (1) univalves having a single shell; (2) bivalves having two shells; and (3) cephalopods. Molluscs such as cockles and whelks have shells, but in the squid the shell is modified and consists of a strip of cartilage, known as the pen, buried in the flesh. There are almost 1000 species of cephalopods, however the species commercially caught are squid, cuttlefish, and octopus. Cephalopods are marketed in various forms including fresh, frozen, canned, dried, salted, and smoked (Suklim *et al.*, 2003). The most commercially important group of all cephalopods is squid. Squid has a distinct head, bilateral symmetry, a mantle, and arms. Squid, like cuttlefish, has eight arms arranged in pairs and two, usually longer, tentacles. Squid is strong swimmer and certain species can "fly" for short distances out of the water. Movement is by jet propulsion. Water is taken into the mantle cavity and forcibly expelled through a siphon, which can be rotated. Thus the animal can move forwards or backwards with great rapidity. Cephalopods have well developed eyes and many are rapacious predators. All have ink sac or reservoir containing a brown or black viscous fluid, which is ejected through the siphon when animal is alarmed (Hanlon and Messenger, 1996). The resulting cloud of ink forms an effective screen behind when animal can escape. In addition, it is believed that the alkaloids present in the ink can further confuse a potential predator by paralysing its olfactory sense (Jereb and Roper, 2010).

1.2.2 Squid ink

Squid ink produced at the end of the maturation process is a suspension of melanin granules in a viscous colorless medium. Ink gland cells of the digestive tract in the mantle cavity degenerate and shed their contents into the ink sac, acting as a reservoir of the exhausted material (Russo *et al.*, 2003). Ejection of dark ink from the sac is a defensive means squid employed to avoid dangers and risks. Squid ink is an intermixture. Besides large amounts of melanin, the ink contains proteins, lipids, glycosaminoglycans and various minerals, etc. (Derby *et al.*, 2007). The main

components are melanin and protein-polysaccharides complex. Double-layer structural melanin granule consists of inner high density melanin and external low density organic compound. Shakthi *et al.,* (2016) reported that ink from *Sepia prabahari* was composed of protein (1.16 – 1.49 mg/ml), lipid (0.25 – 1.42 mg/ml), carbohydrates (0.01 – 1.14 mg/ml), ash (0.4 & 0.1%) and moisture (93.33%). It is well documented that squid ink is a multifunctional marine bioactive-material. It not only promotes thromboxane and kills cancer cells, but also possesses leukocyte-number elevating, anti-oxidant, anti-radiation, anti-retrovirus and anti-bacterial properties (Zhong *et al.,* 2009).

The squid ink has been proved to be an alternative medicine and has the wide range of therapeutic applications (McConnell *et al.,* 1994). Growth performance, antioxidant functions and immunity in growing broiler chickens were affected by squid ink (Liu *et al.,* 2011). Melanin free ink from *Sepia prabahari* showed antioxidant effect and retarded the initiation of lipid oxidation process (Shakthi *et al.,* 2016). Antibacterial activity of the ink from *Sepioteuthis lessoniana* and *Sepia pharaonis* against biofilm bacteria was reported (Ramasamy and Murugan 2005). A protein extracted from cuttlefish (*Sepioteuthis lessoniana*) ink could inhibit the growth of *Staphylococcus aureus* (Mochizuki, 1979). Tyrosinase present in squid ink is known to play a key role in the defence against microbes (Takai *et al.,* 1992). Ink from *Sepia officinalis* (Lei *et al.,* 2007) and *Sepiella inermis* (Rajaganapathy *et al.,* 2000) showed antioxidative and antiretroviral activities, respectively. Raw extract of ink from *Loligo edulis* showed anti-vibriosis effect in grouper juveniles infected by *Vibrio alginolyticus* (Fadjar *et al.,* 2016). Melanin-free extract from the squid ink prolonged the shelf-life of yellowfin sea bream during cold storage by suppressing the putrefying bacteria (Shi *et al.,* 2015). Fresh ink from *Loligo duvauceli* showed antimicrobial effect on bacterial species including *Escherichia coli, Staphylococcus aereus, Bacillus subtilis* and *Pseudomonas aeruginosa* (Nicomrat and Tharajak, 2015).

1.2.3 Lipid oxidation and natural antioxidants

1.2.3.1 Lipid oxidation

Lipid oxidation or autoxidation is a chain reaction by which unsaturated fatty acids react with the molecular oxygen. The reaction can be influenced by both intrinsic factors (fatty acid composition, concentration of prooxidants, endogenous ferrous iron, heme proteins (myoglobin and hemoglobin) and enzymes) and extrinsic factors (pH, temperature and oxygen consumption) (Chan *et al.*, 1997; Grunwald and Richards, 2006; Nawar, 1996). The autoxidation includes several steps of reactions (Michael, 2001).

Initiation

At this step, a fatty acid radical is produced. The most notable initiators in living cells are reactive oxygen species (ROS), such as $OH^{\bullet}$ and H_2O_2, which combines with a hydrogen atom to make water and a fatty acid radical.

Propagation

The fatty acid radical is not a very stable molecule and reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This is an unstable species that reacts with another free fatty acid, producing different fatty acid radicals and lipid peroxide, or cyclic peroxide if it reacts with itself. This cycle continues, as the new fatty acid radical reacts in the same way.

Termination

This step is the final step of reaction. When a radical reacts with a nonradical, it produces another radical. This process is related with a "chain reaction mechanism". The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough for collision of two radicals.

Generally, fish muscle contains high levels of polyunsaturated fatty acids (PUFAs) and potential activators, such as heme pigments and trace metals

(Richards *et al.,* 2002). Oxidation process of fatty acids leads to the formation of free radicals and lipid hydroperoxides, primary products of oxidation, which break down to the secondary lipid oxidation compounds such as alcohols, aldehydes and ketones (Hultin, 1994). During storage at room temperature (28-30 °C), the increase in lipid oxidation was observed in dried fish powder from round scad (Artharn *et al.,* 2009) and even in frozen storage (below -18 °C) of horse mackerel patties (Giménez *et al.,* 2011). Due to high content of polyunsaturated fatty acid in fish muscle, lipid oxidation more likely takes place. This is associated with the increases in yellowness of muscle. Thanonkaew *et al.* (2006) reported the increase in *b**-value (yellowness) of squid muscle during frozen storage. Phospholipid has been involved in Maillard reaction by providing amine group in reaction with aldehydic oxidation products (Thanonkaew *et al.,* 2006).

1.2.3.2 Natural antioxidants and their uses

An antioxidant is a chemical compound capable of inhibiting the oxidation of lipids. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent (Fatemi and Gharehchahi, 2012). Oxidation can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions (Frankel, 1998; Sies, 1997). Antioxidants present in foods and other biological materials have attracted considerable interest because of their safety and potential nutritional and therapeutic effects. Synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, tertiary butylhydroquinone (TBHQ), trihydroxybutyrophenone, nordihydroguairetic acid and ethoxyquin have been reported to be effective in reduction of lipid oxidation (Shahidi *et al.,* 1987). However, the use of these types of antioxidants is controlled because of their carcinogenic potential and toxicity (Chen *et al.,* 1992; Sherwin, 1990; Whysner and Williams, 1996). The interest in natural replacements for synthetic antioxidants has been increasing.

1.2.3.3 Antioxidative peptides

Marine organisms have been reported to be the rich sources of structurally diverse bioactive compounds with valuable neutraceutical and pharmaceutical potential (Barrow and Shahidi, 2008). Bioactive peptides have been defined as specific protein fragments that have positive impact on body function of condition which ultimately may influence health (Tranter and Board, 1982). These peptides are in the size of 2 to 20 amino acids and molecular masses of less than 6000 Da (Meisel and FitzGerald, 2003; Sun *et al*., 2004). Marine fish species (finfish and shellfish) represent one of the richest sources of bioactive peptides which can be easily utilized for producing neutraceutical and functional food ingredients with antioxidant and antibacterial property (Ngo *et al*., 2011). Bioactive peptides are breakdown products of proteins by proteases present in gastrointestinal tract, which has specific biofunction only after release from parent protein source (Kitts and Weiler, 2003). Based on the sequence and size of the peptides, their biological activities such as antioxidant, antihypertension, immunomodulatory, anticancer, antimicrobial activity, etc. vary (Clare and Swaisgood, 2000). Food processing industry has also started to exploit the potential of bioactive peptides particularly antioxidant and antimicrobial peptides as alternative preservatives.

Peptides derived from fish proteins have shown the ability of exerting potent antioxidative activities in different oxidative systems (Jun *et al.*, 2004; Kim *et al*., 2000; Rajapakse *et al.*, 2005). Many peptides from fish have been reported to have antioxidant activities against peroxidation of lipids or fatty acids (Chan and Decker, 1994). Antioxidant activities of peptides from fish gelatins have been reported (Venugopal, 2009). Peptides derived from tryptic hydrolysate of hoki (*Johnius belengerii*) skin gelatin exhibited significant scavenging activities on superoxide, carbon-centered DPPH (Diphenyl- 2- picryl hydrazyl) radical as assessed by electron spin resonance spectroscopy (Mendis *et al*., 2005). This peptide could also function as an antioxidant against linoleic acid peroxidation and the activity was closer to that of butylated hydroxy toulene (BHT).

Peptide isolated from Alcalase-hydrolyzed protein from skipjack roe was effective in metal chelating activity, ABTS radical and singlet oxygen scavenging activities (Intarasirisawat *et al.,* 2013). Two peptides purified from oyster hydrolysate had high antioxidative activities based on their hydroxyl and DPPH radical scavenging activities (Wang *et al.,* 2014). Kittiphattanabawon *et al.* (2012) reported that peptides with shorter chain length trap or bind with singlet oxygen to a higher extent. A tetrapeptide isolated from Amur sturgeon skin gelatin exhibited scavenging activity against DPPH, ABTS and hydroxyl radicals and also effectively prevented lipid oxidation in minced fish (Nikoo *et al.,* 2014). Peptide from croaker (*Otolithes ruber*) muscle protein hydrolysate efficiently quenched DPPH and hydroxyl radicals and successfully inhibited the lipid peroxidation (Nazeer *et al.,* 2012). Ko *et al.* (2013) isolated two peptides from α-chymotrypsin hydrolysis of flounder fish muscle having good scavenging activity against DPPH free radical and total reactive oxygen species and high cryoprotective activity against 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH) without cytotoxicity. Peptide fractions obtained from cod protein hydrolysates showed DPPH radical scavenging and metal chelating activities (Farvin *et al.,* 2016). A list of antioxidative peptides with their amino acid sequences from marine organisms is given in Table 1.

The antioxidative properties of peptides are generally related to their amino acid composition, structure and hydrophobicity (Jung *et al.,* 2006). In addition, the presence of proper amino acids and their correct positioning in the peptide sequence are also important to display the antioxidant activity. Peptide conformation has also been claimed to influence antioxidant capacity, showing both synergistic and antagonistic effects (Hernandez-Ledesma *et al.,* 2005). Amino acids having aromatic residues can donate protons to electron deficient radicals (Rajapakse *et al.,* 2005). Tyr, Trp, Met, Lys, Cys and His are some examples of amino acids which exhibit antioxidant activity.

Table 1: Antioxidative peptides from marine organisms

Amino acid sequence of the peptides	**Sources**	**References**
Arg-Pro-Asp-Phe-Pro-Leu-Glu-Pro-Pro-Tyr	Yellowfin sole	Jun et al., 2004
Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu	Jumbo squid	Mendis *et al*., 2005
Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn	Conger eel	Ranathunga et al., 2006
Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Glu-Glu-Leu-Ser	Tuna	Je et al., 2007
Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	Microalga	Sheih et al., 2009
Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asn-His-Ala	Rotifer	Byun *et al.*, 2009
Ala-Cys-Phe-Leu	Horse mackerel	Sampath et al., 2011
Lys-Thr-Phe-Cys-Gly-Arg-His	Croaker	Nazeer et al., 2012
Tyr-Pro-Pro-Ala-Lys	Blue mussel	Wang et al., 2013
Val-Cys-Ser-Val and Cys-Ala-Ala-Pro	Flounder fish	Ko et al., 2013
Asp-Leu-Asp-Leu-Arg-Lys-Asp-Leu-Tyr-Ala-Asn2	Skipjack roe	Intarasirisawat et al., 2013
Phe-Tyr-Tyr Asp-Thr	Lanternfish	Chai et al., 2013
Pro-Ala-Gly-Tyr	Amur sturgeon	Nikoo et al., 2014
His-Asp-His-Pro-Val-Cys His-Glu-Lys-Val-Cys	Round scad	Jiang et al., 2014
Glu-Ser-Thr-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn	Hoki	Ali et al., 2014
Phe-Leu-Gln-Glu-Phe-Leu-His-Val	Salmon	Ahn et al., 2014
Leu-Ala-Asn-Ala-Lys	Oyster	Umayaparvathi et al., 2015
Trp-Pro-Pro	Blood Clam	Chi et al., 2015

1.2.3.4 Carnosine and anserine

Carnosine (*β*-alanylhistidine) is a dipeptide of the amino acids *β*-alanine and histidine whereas anserine is *β*-alanylmethylhistidine. Carnosine and anserine are highly concentrated in skeletal muscle and nervous tissues of many animals and humans. Carnosine and anserine have been shown to be efficient copper-chelating agents, and they may play a role in copper metabolism *in vivo* (Brown, 1981). They also have enzyme like activity and free radical scavenging activity (Kohen *et al.,* 1991; Chan *et al.,* 1994). In beef, treatment with carnosine decreased the non heme iron release by cooking. Also it inhibited the formation of metmyoglobin in raw beef which causes brown color and also off odor in the meat because of lipid peroxidation (Nerin *et al.,* 2006). Carnosine inhibits the formation of TBARS in various model systems such as fatty acid alcohol/aqueous solution (Yamashoji and Kajimoto 1980; Kohen *et al.,* 1988), liposomes (Decker and Faraji, 1990), sarcoplasmic reticulum membranes (Boldyrev *et al.,* 1988; 1989) and microsomes (Decker *et al.,* 1992). Carnosine inhibited oxidation of *β*-carotene and low density lipoprotein cholesterol (Ambigaipalan and Shahidi, 2015). Several mechanisms have been proposed to explain the inhibition of lipid oxidation by carnosine. One of the hypothesis is related to the buffering capacity of the peptide. Carnosine helps to keep the pH of the system at physiological values (Kohen *et al.,* 1988), at which lipid peroxidation is reduced (Misik *et al.,* 1991). Carnosine is more effective in quenching trans 2-hexanal than its constituent amino acids, histidine and *β*-alanine. Carnosine is capable of inhibiting iron-catalyzed lipid oxidation during the processing and storge of muscle foods (Decker and Faraji, 1990). Carnosine inhibited Cu^{2+} promoted low density lipoprotein oxidation (Decker *et al.,* 2001). Carnosine and anserine showed inhibition of linoleic acid autoxidation, scavenging effect on α, α-diphenyl-*β*-picrylhydrazyl free radical, reducing power, and chelating ability of Cu^{2+} (Wu *et al.,* 2003).

1.2.3.5 Plant phenols

Many plants and herbs contain a wide variety of phenolic compounds such as flavonoids, which act potentially as antioxidants to scavenge free radicals and

inhibit lipid peroxidation (Kumawat *et al.*, 2012). The phenolics in Greek medicinal and aromatic plant extracts showed DPPH and ABTS radical scavenging activities (Skotti *et al.,* 2014). The high polyphenolic potential and significant antioxidant and antimicrobial activities of leaf extracts of grape varieties have been reported (Katalinic *et al.,* 2013). Methanolic extracts from thyme, sage and marjoram had DPPH radical scavenging activity. Extracts contained rosmarenic acid, methyl rosmarenate, caffeic acid, cinnamic acid, chlorogenic acid and quinic acid as phenolic acids, besides some flavonoids such as ferulic acid, apigenin, luteolin and quercetin. The antioxidative activity of those extracts was higher than those of α-tocopherol and BHA (Roby *et al.,* 2013). Ethanol extracts of spice *Limnophila aromatica* showed the high total antioxidant activity, reducing power and DPPH radical scavenging activity. This activity was attributed to the highest phenolic contents and flavonoids present in the extract (Do *et al.,* 2014). The phenolics extracted from grape and papya seeds showed antioxidant activities and extended the shelf life of Indian mackerel during iced storage (Sofi *et al.,* 2016). Different solvent extracts of plant *Merremia borneensis* had DPPH radical scavenging activity, inhibition of oxidation in *β*-Carotene model system and also had reducing power. Positive correlation was observed between the antioxidant activity potential and total falvonoid levels of the extracts (Hossain and Shah, 2011). Catechins are natural phenols and have antioxidant activity. They belong to the group flavan-3-ols (flavanols) (Zheng *et al.,* 2008). Catechins in wine showed *in vitro* antioxidant acivities (Muselik *et al.,* 2007). Catechins in tea extracts showed radical scavenging acivities (Kerio *et al.,* 2013). The skin and seed extracts of grapes containing catechin with antiradical capacity (Iacopini *et al.,* 2008). Gallic acid and catechin have been extracted from spent flower and leaf of oil-bearing-rose (*Rosa damascena* Mill), respectively (Baydar and Baydar, 2013). These phenolic compounds exhibited more antiradical activity even better than synthetic antioxidants such as Trolox, BHA and BHT.

Ferulic acid is a hydroxycinnamic acid, a type of organic compound. It is a derivative of cinnamic acid with molecular formula $C_{10}H_{10}O_4$. It is an abundant phenolic phytochemical found in cell wall components such as arabinoxylans as covalent side chains. Ferulic acid is one of the ubiquitous compounds in nature,

especially as an ester form in rice bran pitch, which is yielded when rice oil is produced (Kikuzaki *et al.*, 2002). Ferulic acid together with dihydroferulic acid is a component of lingo celluloses, conferring cell wall rigidity by crosslinking lignin and polysaccharides. It is commonly found in seeds of plant such as rice, wheat and oats (Gelinas and McKinnon, 2006). As a component of lignin, ferulic acid is a precursor in the manufacture of other aromatic compounds. Ferulic acid, like many phenols, exhibits antioxidant effect in response to free radicals by donating hydrogen from its phenolic hydroxyl group. It has been revealed that the antioxidant capacity of phenolic acid is equivalent to lecithin on inhibition of time dependent peroxide value (Gupta *et al*., 1979). In addition, the reactive oxygen species scavenging effect of ferulic acid has been reported to be similar to that of superoxide dismutase (Toda *et al*., 1991). Antioxidant activity of ferulic acid alkyl esters was studied in a heterophasic system (Anselmi *et al.,* 2004). Ferulic acid was shown to inhibit the photoperoxidation of linoleic acid (Zheng and Wang, 2001). Ferulic acid was preventive against photo-oxidation of lutein and astaxanthin in Red Sea Bream (Maoka *et al.*, 2008).

Caffeic acid is an organic compound that is classified as hydroxycinnamic acid (Boerjan *et al.,* 2003). Caffeic acid consists of both phenolic and acrylic functional groups. It is found in all plants because it is a key intermediate in the biosynthesis of lignin, one of the principal components of plant biomass and its residues (Boerjan *et al.*, 2003). It has also been identified as one of the active antioxidant (Gulcin, 2006). Caffeic acid is naturally-occurring phenolic antioxidant containing a catechol ring, which largely accounts for its free radical scavenging capacity (Wang *et al.,* 2010) and other antioxidant activity (Sato *et al.,* 2011). Caffeic acid inhibited the lipid oxidation in chicken soup and preserved color and flavor and thus improved the shelf-life (Stojkovic *et al.,* 2013). The chitosan films grafted with caffeic acid had higher antioxidant activity. Caffeic acid also improved the mechanical properties of the chitosan film (Nunes *et al.,* 2013).

1.2.3.6 Coconut husk extract

Coconuts form a regular part of the diets of many people in the tropics and subtropics and are found abundantly in coastal areas of tropical countries. The husk of coconut is removed during processing and the millions of tons of husk are generated each year (Panyakaew and Fotios, 2011). For mature coconut, the white meat (28 wt.%) is surrounded by a hard protective shell (12 wt.%) and a thick husk (35 wt.%) (Thampan, 1991). The husk is mainly composed of lignin and cellulose, which are dietary fibre and recommended as GRAS (Woods and Gorbach, 2001). Additionally, husk is rich in phenolic compounds, namely 4-hydroxybenzoic acid (4-HBA), ferulic acid, tannic acid as well as lignin phenols such as vanillic acid, *ρ*-coumaric acid and syringic acid (Lobbes *et al.,* 1999; Rodrigues and Pinto, 2007). Phenolic compounds have been known to possess antioxidant (Maqsood *et al.*, 2012), antimicrobial and antiviral (Carvalho *et al.,* 2013; Hossain *et al.,* 2014) and anticancer activities (GawlikDziki *et al.,* 2012). Antibiofilm activity of coconut husk extract have also been reported (Viju *et al.,* 2013). Furthermore, the use of phenolic compounds has been extended as a protein cross-linking agent, particularly in the oxidized form (Balange and Benjakul, 2009a).

Ethanolic extract of coconut husk found to have tannic acid. Phenolic compounds present in coconut husk extract improved the gel properties of sardine surimi (Buamard and Benjakul, 2015). The ethanolic extracts of coconut husk increased the turbidity, surface hydrophobicity and disulfide bond content in heat induced aggregation natural actomyosin (Buamard and Benjakul, 2016). Extracts from coconut husk have been reported to enhance the properties of nanocomposite films prepared from tilapia skin gelatin (Nagarajan *et al.,* 2015). The use of coconut husk extract at an appropriate level improved the gel properties of gelatin from yellowfin tuna swim bladder (Kaewdang *et al.,* 2015). Coconut husk extract prepared using 60 % ethanol increased the gel strength of surimi prepared from mackerel (Mugale and Bhosale, 2016).

1.2.4 Surimi

Surimi has been known as the concentrated myofibrillar protein, mainly extracted from fish flesh via washing. The main steps in surimi preparation include heading, gutting and deboning, mincing, washing and dewatering, refining, screw press, stabilizing surimi with cryoprotectants; and at the end freezing and frozen storage (Flick *et al.,* 1990). During washing with cold water, fat and other water-soluble components are removed, whereas myofibrillar proteins become concentrated (Benjakul *et al.,* 1996). Surimi possesses the functionalities including gelling, water and fat binding and emulsifying properties and can be used as a functional protein ingredient in several products (Lanier, 1986). Myofibrillar proteins in surimi are mainly involved in the gel-forming ability (Benjakul *et al.,* 2003). Surimi is an intermediate product used in a variety of products ranging from the traditional "kamaboko" products of Japan to surimi seafood, especially imitated products due to their unique textural properties as well as high nutritional value (Park and Morrissey, 2000).

Fish muscle consists of two main types, dark and white, depending on the life-cycle of the species. Conventionally, the fish protein for surimi is obtained from white-muscle marine species such as Alaskan pollock, hake, and cod, etc (Chanarat and Benjakul, 2013). Strong-swimming species, such as tuna and mackerel, have a larger proportion of dark muscle than relatively sluggish fish such as cod, haddock and flat fish (Chaijan *et al.*, 2004). The two muscle types are essentially similar in composition but the dark muscle has a higher content of haem pigments, such as myoglobin, for oxygen transport and more non-structural lipids, to provide energy; this reflects its role in active strong-swimming (Hall and Ahmad, 1997). The higher levels of lipids and metabolites in dark muscle will affect the flavor and color of surimi. The actual amino-acid composition of dark/white muscle is roughly the same as in terrestrial species such as cattle, although the proportions of different protein types vary, reflecting the environment in which these creatures live (Hall, 1997). These groups of protein can be differentiated and separated by solubility in salt solutions of increasing concentration. The sarcoplsmic proteins are water soluble and normally found in the cell plasma where they act as enzymes and oxygen carriers.

They constitute ranging from 18 to 20% of total muscle protein. The largest proportion of muscle proteins, 65-80% of total protein, is myofibrillar protein, which gives the muscle its fibre-like structure and muscular activity. The major components are myosin, actin, tropomyosin and troponin (Park, 2005). These proteins can be extracted by the use of salt solutions up to 0.3M. The final groups of proteins are those making up the connective tissues, surrounding the muscle fibres and in skin; they include collagen and elastin. These proteins, known as the stroma, comprise about 3-5% of the total protein (which is much less than in terrestrial animals) and are easily solubilized by cooking. They are, however, resistant to solubilization except in strong salt solutions (Lanier *et al.,* 2005).

The demand and high utilization of surimi for the preparation of variety of food products has resulted in overfishing of marine white-muscle fishes, leading to the depletion of conventional sources of surimi (Buamard and Benjakul, 2015; Moreno *et al.,* 2016). This leads to the search of alternative sources for surimi production. Surimi has been prepared from various fishes such as yellow stripe trevally (Arfat and Benjakul, 2013), Alaska pollock (Yin and Park, 2014), myctophids (Shaviklo and Rafipour, 2013), threadfin bream (Wiriyaphan *et al.,* 2013), flying fish (Herranz *et al.,* 2013), lizardfish (Chanarat and Benjakul, 2013a), pacific whiting (Poowakanjana and Park, 2013), Indian mackerel (Chanarat and Benjakul, 2013b), Frigate mackerel (*Auxis thazard*) (Chaijan *et al.,* 2013), etc. and their properties have been studied. Surimi was also prepared from many fresh water fish such as common carp (*Cyprinus carpio*) (Liu *et al.,* 2014), bighead carp (*Aristichthys nobilis*) (Wang *et al.,* 2014), yellow drum (*Nibea albiflora*) (Zhao *et al.,* 2013), silver carp (*Hypophthalmichthys molitrix*) (Qiu *et al.,* 2013), catfish (*Clarias macrocephalus*) (Chaijan *et al.,* 2013) etc. Many additives and treatments have been employed to improve the quality of surimi from these sources (Kudre *et al.,* 2013; Arfat and Benjakul, 2013; Balange and Benjakul, 2009).

1.2.4.1 Surimi gelation

Gelation is an aggregation of proteins, forming a three-dimensional network in which water is entrapped (Pomeranz, 1991). Myofibrillar proteins

(myosin and actin) are generally solubilized by salt to obtain the paste prior to gelatin. Upon heating, the network is formed when sufficient intermolecular bonds occur and it is stabilized by ionic linkages, hydrophobic interactions, covalent bonds (disulfide bonds and covalent cross-linking), and hydrogen bonds (Lanier *et al.,* 2005). Myosin is the most important component for adequate gel formation in fish gel products (Sun and Holley, 2010). Gels prepared from myosin alone have higher gel strength and elasticity than those prepared from natural actomyosin (Sano *et al.,* 1988). Observations of dynamic viscoelastic behavior and differential scanning calorimetric analysis revealed that the gelation of carp actomyosin occurred in two stages: at temperature ranges of 30-41 ℃ and 51-80 ℃ (Sano *et al.,* 1988). Sano *et al.* (1990) proposed that the first stage of gel development was due to interactions among the tail portions of myosin molecules. The second stage was attributed to hydrophobic interactions among the head portions of myosin. However, Samejima *et al.* (1981) proposed that the heat-induced gelation of myosin consist of two reactions: (1) aggregation of the globular head segments of the myosin molecules, which is closely associated with the oxidation of sulfhydryl groups and (2) network formation resulting from the unfolding of the helical tail segment. Protein conformation changes during heating and the hydrophobic amino acids, which are found mainly in the head portion, become more exposed (Sano *et al.,* 1990). Actin has a synergistic or antagonistic effect on myosin gelation, depending upon the myosin/actin ratio in the gelling system (Matsumoto, 1980). The gel strength of myofibrillar proteins can be influenced by factors that affect myosin structure. The gelling property of myosin is highly related to the length of the double-stranded α-helical tail (Xiong, 1997). Proteolysis of myosin has been shown to lower surimi gel strength (An *et al.*, 1996; Morressy *et al.,* 1993) (Figure 1). The native conformation of myosin is of primary importance for proper gelation. Differences in cross-linking of MHC contribute to the differences in gel forming ability among the muscles of various fish (Benjakul *et al.*, 2001a; Yarnpakdee *et al.*, 2009).

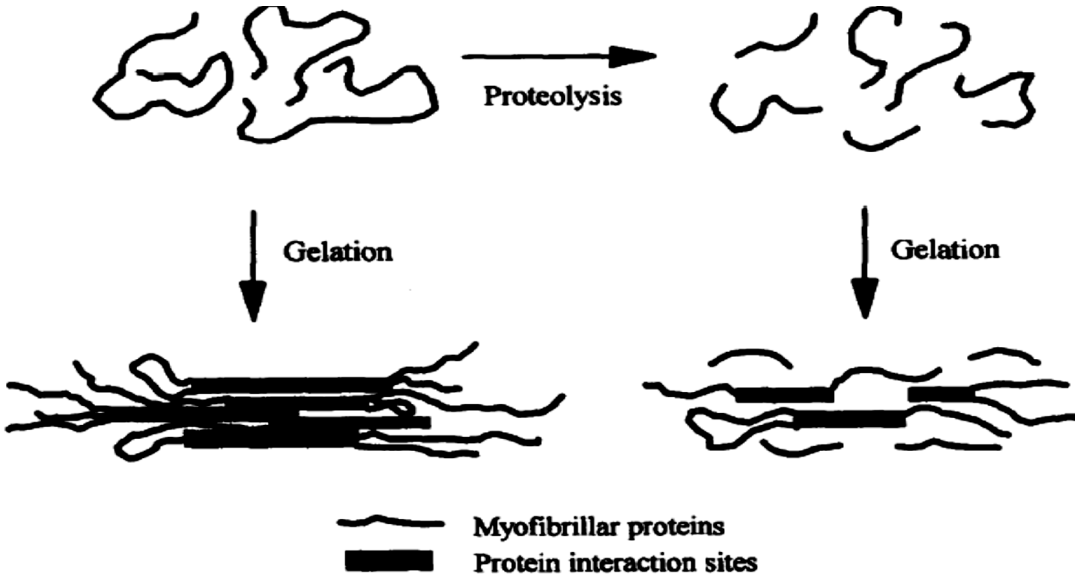

Figure 1. Surimi gelation and proteolysis of myofibrillar proteins.

Source: An *et al.* (1996)

1.2.4.2 Setting (Suwari)

Setting is a phenomenon explaining the increased textural properties of surimi gels after pre-incubation at a certain temperature below 40 °C for a specific period of time prior to cooking (Lanier *et al.*, 2000). A gel with higher rigidity and elasticity is obtained when setting is performed, followed by cooking (Roussel and Cheftel, 1990). This phenomenon involves gel network formation of muscle proteins triggered by protein unfolding. Setting temperature can be varied, depending on fish species, and setting phenomenon is related to habitat temperature of fish species. The different optimum temperature for setting among species may be determined by the heat stability of myosin (Morales *et al.*, 2001). Generally, setting can be carried out at low (0-4 °C), medium (25 °C), and high (40 °C) temperatures (Lanier, 1992). Low and medium temperatures are applied for setting of surimi from cold and temperate habitats. Setting of Alaska pollock surimi from Bering sea was achieved at 4-5 °C (Kim *et al.*, 1993). Nevertheless, Park *et al.* (1994) reported that optimum setting temperature for Pacific whiting surimi was 25 °C instead of 5 °C. High temperature setting at 40 °C has been applied for surimi from tropical or warm water fish species such as Atlantic croaker, Mexican flounder, Northern kingfish, threadfin bream,

bigeye snapper, barracuda, and white croaker (Lee and Park, 1998; Morales *et al.*, 2001; Yongsawatdigul *et al.*, 2002; Benjakul *et al.*, 2004a; VanPhu *et al.*, 2010).

Indigenous transglutaminase (TGase) has been reported to be the major force involving polymerization of myosin during setting (Kamath *et al.*, 1992). Tissue or indigenous TGases require Ca^{2+} for catalytic reaction (Yongsawatdigul *et al.*, 2002). Cross-linking of MHC was also the Ca^{2+}-dependent reaction in the setting and such cross-links play a crucial role in gel strengthening (Kumazawa *et al.*, 1995). The effect of Ca^{2+} on setting at 25 ℃ (3 h) or 5 ℃ (20 h) in surimi from Pacific whiting and Alaska pollock was reported (Lee and Park, 1998). Addition of Ca^{2+} significantly improved textural properties of surimi from threadfin bream after setting at either low or high temperatures but breaking force value of gels set at 40 ℃ was higher than at 25 ℃ (Yongsawatdigul *et al.*, 2002). The breaking force and deformation of suwari gels from Alaska pollock increase when added with nano-scaled fish bone (Yin and Park, 2014). Tsukamasa *et al.* (1993) found that the isopeptide bond formation of sardine sol was found at temperature below 30 ℃. An increase in gel strength was correlated with the amount of isopeptide bond. Setting phenomenon, gel strength, cross-linking of MHC, and the content of isopeptide bond increased with setting time (Kumazawa *et al.*, 1995). Takeda and Seki (1996) reported that complete suppression of myosin cross-linking of walleye pollock surimi gel was associated with the inhibition of indigenous TGase. Kumazawa *et al.* (1995) suggested that setting phenomenon was suppressed in the presence of TGase inhibitors, such as NH_4Cl and EDTA. The setting and gelling ability of fish mince increased when treated with transglutaminase (Binsi and Shamasuindar, 2012). The microwave heating of suwari gels prepared from silver carp surimi had increased gel strength (Fu *et al.*, 2012).

1.2.4.3 Gel weakening (modori)

Gel softening or gel weakening termed "modori" is a problem found in surimi, especially from some fish species. This is associated with degradation of muscle proteins caused by the endogenous heat-activated proteinase (An *et al.*, 1996; Benjakul *et al.*, 1997; Jiang *et al.*, 2000a). Proteolytic activity in muscle is high at temperature above 50°C and causes the rapid and severe degradation of myofibrillar

proteins, particularly myosin (Wasson *et al.,* 1992). Such proteolytic degradation of myofibrillar proteins inhibits the development of three-dimensional gel network leading to gel weakening of surimi-based products (Kudre and Benjakul, 2013). This has a detrimental effect on surimi quality, and substantially lowers the gel strength (Morrissey *et al.,* 1993; Benjakul *et al.,* 2004). Proteases associated with gel weakening can be categorized into two major groups: cathepsin (Seymour *et al.,* 1994; Toyohara *et al.,* 1993) and heat-stable alkaline protease (Wasson *et al.,* 1992). High level of cysteine protease activity mediated by cathepsin B, H, and L was found in Pacific whiting and arrowtooth flounder (An *et al.,* 1994; Wasson *et al.,* 1992), chum salmon and mackerel (Lee *et al.,* 1993) during spawning migration (Yamashita and Konagaya, 1990). Softening of arrowtooth flounder gel is due to a cysteine protease that has the maximum autolytic activity at 50-60°C (Greene and Babbitt, 1990). When Pacific whiting muscle was incubated at 60°C for 30 min before cooking at 90°C, most of MHC was degraded; the resultant surimi gel did not have measurable gel strength (Morrissey *et al.,* 1993). Klomklao *et al.* (2008) reported the presence of proteases in sardine (*Sardinops melanosticus*). Cathepsins B and H are easily washed off during surimi processing, whereas cathepsin L remains in the muscle tissue (An *et al.,* 1994; Park and Morrissey, 2000). Cathepsin L has an optimum temperature of 55°C and causes textural deterioration when the Pacific whiting surimi paste is slowly heated (An *et al.,* 1994). Benjakul *et al.* (2003c) compared the autolysis from two species of bigeye snapper and found that *P. macracanthus* had higher degradation of myosin heavy chain in both mince and washed mince than those from *P. tayenus*, especially when the incubation time was increased. Autolysis of washed mince from both species was inhibited by soybean trypsin inhibitor, suggesting that myofibril-associated proteases were serine proteases, while *P. tayenus* contained various proteases. The protease extracted from bigeye snapper muscle had an optimum pH and temperature of 8.5 and 60 ºC and was capable of hydrolyzing MHC effectively (Benjakul *et al.,* 2003c).

During setting, proteolysis takes place to some degree and may affect the properties of resulting gels. The modori gels from sardine surimi added with protein isolates from black bean and mungbean had the increased gel strength as the

protein isolates inhibited the protease activity in the surimi (Kudre *et al.,* 2013). Benjakul *et al.* (2004a) reported that suwari gel from some tropical fish, prepared by setting at 25°C showed lower degradation although the setting time increased up to 8 h, compared with setting at 40°C. Kamath *et al.* (1992) found that proteolysis in croaker paste increased with increasing temperature of setting, especially in the temperature range of 40-50°C. Proteolytic degradation of surimi gels is increased at temperatures above 50°C with the rapid and severe degradation of myofibrillar proteins, particularly myosin (Jiang *et al.,* 2000b). Arfat and Benjakul (2013) reported that the modori gels prepared from yellow stripe trevally surimi added with zinc salts had the increased gel strength. Bambara groundnut protein isolate with trypsin inhibitory activity increased the breaking force and deformation of gels prepared from threadfin bream surimi (Oujifard *et al.,* 2012).

1.2.4.4 Cross-linking enzymes

1.2.4.4.1 Microbial transglutaminase (MTGase)

Transglutaminases (TGs; EC 2.3.2.13, γ-glutamyl-peptide, amine-γ-glutamyl transferase) belong to the group of acyltransferases, which catalyze acyl-transfer reactions between a γ-carboxyamine group of a peptide- or protein-bound glutamyl residue and a primary amino group of various substrates including the ε-amino group of lysine or lysyl residues in proteins. This results in polymerization or amine incorporation (Figure 2). The crosslink formed is called an ε-(γ-glutamyl) lysine isopeptide bond (Folk and Finlayson, 1977; Griffin *et al.,* 2002). During the reaction, one molecule of ammonia is generated per crosslink. If the amine substrates are not available as acyl acceptors, TG can catalyze deamination of glutamyl residues using water as an acyl acceptor (Yokoyama *et al.,* 2004) (Figure 2). Cross-linking enzymes such as a non-calcium-dependent microbial TGase (MTGase) can be added to improve the mechanical properties of surimi. MTGase is more stable at higher temperatures than the endogenous TGase (Lee *et al.,* 1997; Gómez-Guillén *et al.,* 2005) and shows a greater activity than fish TGase (Hemung *et al.,* 2008). MTGase from *Streptoverticillium mobaraense* (Nonaka *et al.,* 1997) or from *Streptoverticillium ladakanum* (Tsai *et al.,* 1996) were shown to increase the gel

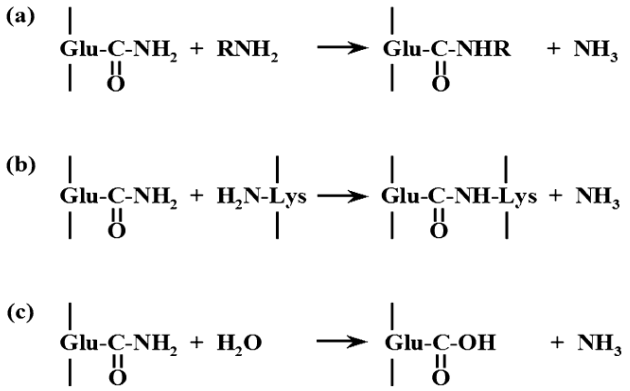

Figure 2: Reactions catalyzed by TGase (a) Acyl transfer (b) Crosslinking of Gln and Lys residues in proteins or peptides. The resulting bridge is called an ε-(γ-glutamyl) lysine (GL) bond. (c) Deamidation.

Source: Yokoyama *et al.* (2004)

strength of surimi (Benjakul and Visessanguan, 2003). Addition of MTGase to surimi significantly increases its gel strength, particularly when the surimi has lower natural setting ability, presumably due to lower indigenous TGase activity (Kumazawa *et al.*, 1995; Lee and Park, 1998). Furthermore, MTGase has been demonstrated to enhance the gel quality of lizardfish surimi, which was produced from fish stored even after 10 days in ice (Benjakul *et al.*, 2008), and was also effective in improving gel quality of surimi from dark and fatty fish, such as sardines (*Sardina pilchardus*) (Karayannakidis *et al.*, 2008). An increase in non-disulfide polymerization and formation of ε-(γ-glutamyl) lysine isopeptides was found with increasing setting time and MTGase concentration (Tsukamasa *et al.*, 1993). Jiang *et al.* (2000b) investigated the properties of surimi gels from threadfin bream and pollack surimi set at 30 or 45 °C with MTGase from *Streptoverticillium mobaraense*. The optimal amounts of MTGase and setting conditions were 0.3 unit/g surimi either at 30 °C for 90 min or at 45 °C for 20 min for threadfin bream surimi. For pollack surimi, MTGase at a level of 0.2 unit/g surimi with setting at 30 °C for 60 min was found to be the optimum condition. MTGase catalyzed MHC cross-linking of both pollack and threadfin bream surimi and increased the gel forming ability of surimi. Benjakul *et al.* (2008) studied the effect of MTGase at different levels (0 to 0.8 units/g samples) on the

properties of gels from lizardfish (*Saurida undosquamis*) mince set at 25 °C for 2 h or 40 °C for 30 min prior to heating at 90 °C for 20 min. MTGase showed the gel strengthening effect on lizardfish mince, particularly when high amounts of MTGase were used. For the gels added with MTGase at 0.8 units/g and set at 25 and 40 °C, the highest breaking force 93.1% and 90.7% was obtained, respectively. Karayannakidis *et al.* (2008) studied the effect of MTGase and Ca^{2+} ions on the textural characteristics of heat induced surimi gels from sardines (*Sardina pilchardus*). Incorporation of 2% MTGase (w/w) and 0.2 % Ca^{2+} ions (w/w) in surimi significantly affected the textural characteristics of heat-induced surimi gels. Fish gels with MTGase and $CaCl_2$ added were firmer and more cohesive, compared with the untreated gels. The former catalyzes the cross-linking reaction of myosin, while the latter activates indigenous TGase, which also leads to the formation of covalent non-disulfide cross-links. However, MTGase-containing fish gels exhibited a more elastic texture, compared with the untreated fish pastes and those containing $CaCl_2$.

Microbial transglutaminase was able to increase the gel strength of surimi from lizard fish (Chanarat and Benjakul, 2013a). MTGase improved the mechanical properties of blue crab protein gels (Martinez *et al.*, 2014). Addition of MTGase produced stronger and more rigid networks in flying fish surimi gel, mainly due to the formation of non-disulfide bonds between adjacent proteins (Herranz *et al.*, 2013). The gels prepared from protein isolate of Indian mackerel had improved properties with the addition of MTGase (Chanarat and Benjakul, 2013b). The incorporation of MTGase improved the properties of threadfin bream surimi gel added with 0-15% fish gelatin (Kaewdom *et al.*, 2013). Hong and Xiong (2012) reported that transglutaminase reaction was governed by pH of substrate protein and the reaction intensity was related to the solubility of protein. Transglutaminase has been extensively used in order to change the biological properties of different proteins *in vitro* (Porta *et al.*, 2013; Sorrentino *et al.*, 2012) and also to improve several features of hydrocolloid edible films (Giancone *et al.*, 2011; Mariniello *et al.*, 2008; Porta *et al.*, 2012). Preincubation of the proteins with the transglutaminase greatly improved the gelation of soy protein isolate stabilized emulsion gels (Tang *et al.*, 2013). The goat's milk yoghurt treated with transglutaminase had higher sensory

scores, improved textural property and lower syneresis (Domagala *et al.,* 2013). The addition of MTGase enhanced the gelling property and water binding capacity of surimi from Alaska pollock (Cando *et al.,* 2016).

1.2.4.4.2 Tyrosinase

Tyrosinase (monophenol, L-DOPA: oxygen oxidoreductase) is one of the copper- containing phenoloxidases that are widely distributed in nature (Claus and Decker, 2006; Faccio *et al.,* 2012; Halaouli *et al.,* 2006). The enzyme is known to be a key enzyme in the melanogenic pathway that catalyzes the oxygenation of mono-phenol to *o*-diphenols (monophenolase activity), as well as the oxidation of *o*-diphenols to corresponding *o*-quinones (*o*-diphenolase activity) (Mason, 1965; Rodriguez-Lopez *et al.,* 1992). Type 3 copper proteins, including tyrosinases, arthropod phenoloxidases and hemocyanins, have been isolated from many organisms. The evolutional relationships of the structures have also been elucidated on the basis of the amino acid sequences conserved around two copper-binding sites that form an oxygen-binding active center (Cuff *et al.,* 1998; Klabunde *et al.,* 1998; Morrison *et al.,* 1994; Decker and Tuczek, 2000). Arthropod phenoloxidases are known to be involved in host defence system termed the prophenoloxidase cascade as a terminally active molecule in the system (Satoh *et al.,* 1999). In ink of squid and cuttlefish, melanin constitutes as the major component (1g each ink sac). Ink contains about 1 U/ml of tyrosinase (Prota *et al.,* 1981) as well as a dopachrome rearranging activity (Palumbo *et al.,* 1994).

The application of tyrosinases to different fields, ranging from food to materials, relies on the ability of these enzymes to oxidize phenolic groups from both small molecules such as tyrosine to polymeric substrates such as proteins, thus enabling polymer cross-linking (Aytar and Bakir, 2008). Tyrosinases oxidize phenolic hydroxyl groups of small molecules or large polymeric substrates such as proteins. Tyrosinases catalyze firstly the ortho-hydroxylation of the phenolic substrate and subsequently induce oxidation to quinone (Figure 3) with the concomitant reduction of oxygen to water. The reaction is chromogenic as the quinones produced can undergo further non-enzymatic polymerization to form black eu-melanins. When

reacting with thiol groups, brownish pheo-melanins are formed (Eisenman and Casadevall, 2012). Tyrosinase catalyzed oxidation of the phenolic ring of tyrosine side chain is assumed to proceed via L-dopa (3,4-dihydroxy-Lphenylalanine) intermediate to diquinone as in the case of single tyrosine (Kim and Uyama 2005). These diquinones are extremely reactive and can further react with various amino acid side chains such as sulfhydryls, amines, amides, indoles, and other tyrosines commonly present in proteins, resulting in the formation of inter- and intramolecular crosslinks (Bittner, 2006; Mattinen *et al.,* 2008). In addition to many low molecular weight mono- and diphenolic molecules, surface-exposed tyrosyl side chains of proteins may also serve as substrates for tyrosinases that convert them to the respective *o*-quinones (Heck *et al.,* 2013). These are proposed to react spontaneously mainly via 1,4-additions with the side chains of lysine, tyrosine, histidine, and cysteine residues, depending on their abundance and accessibility on the target protein, to form covalent protein–protein crosslinks (Bittner, 2006). Proteins with weakly defined three-dimensional structure and unfolded proteins are the preferred targets for crosslinking by tyrosinases, whereas globular proteins are poorly converted by the enzyme (Hellman *et al.,* 2011; Selinheimo *et al.,* 2007). However, it has been shown that crosslink formation between proteins that are not accessible to tyrosinases can be induced by the addition of small-molecule phenolic compounds (Fairhead and Thöny-Meyer, 2010; Jus *et al.,* 2012; Thalmann and Lötzbeyer, 2002). Tyrosinase along with phenolic compound could stabilize protein nanoparticles by cross-linking via quinone formation (Xu *et al.,* 2016). These molecules likely function as crosslinking mediators to overcome the absence of surface-exposed tyrosine residues on the target proteins.

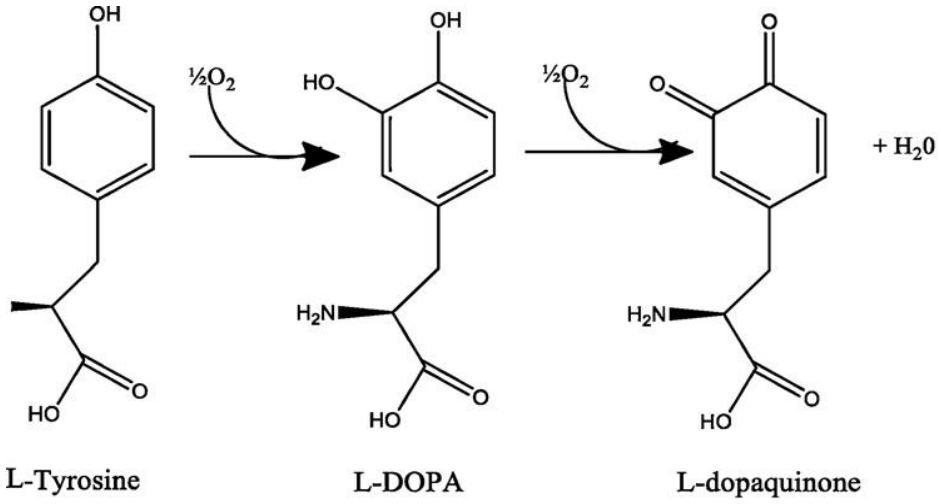

Figure 3. Oxidation of L-tyrosine to L-dopaquinone by tyrosinase.

Source: Faccio *et al.* (2012)

1.2.4.5 Phenolic compounds as protein cross-linker

Phenolic compounds have been extracted mainly from plant sources including fruits, vegetables, leaves, seeds, barks etc. (Maqsood *et al.,* 2013). Plant phenolic compounds are also known as polyphenols. They are defined as compounds containing one or more aromatic rings bearing hydroxyl substituents. Polyphenols are derived from the secondary metabolism of plants (Parr and Bolwell, 2000; Robards *et al.,* 1999). Phenolic compounds play an important role in plants, in processes including plant growth, reactions to stress and pathogen attack (Parr and Bolwell, 2000). Photochemical reactions of phenolic compounds lead to yellowing of paper over a long period of time (Zhu and Gray, 1995). Phenolic compounds are present in many drinks from plant origin, e.g. coffee (Clifford, 1999), tea (Lakenbrink *et al.,* 2000), beer, wine and chocolate (Arts *et al.,* 1999). Red wine has phenolic compounds at levels of 1-4 g/L (Shahidi and Naczk, 1995). Wine consumption therefore shows beneficial impact on cardiovascular diseases (Wallerath *et al.,* 2005). Dark chocolate has 1.6 g/kg of oligomeric phenolic compounds, called proanthocyanidins (USDA database, 2004). Procyanidins, which is a member of the proanthocyanidin sub-class, constitutes at high concentrations in apples and cider (2-3 g/L) (Shahidi and Naczk, 1995). Light and temperature affects the phenolic content in foods during storage

(Friedman, 1997). Considering as additives or ingredients, phenolic compounds are also used in some foods as coloring agent and antioxidant (O'Connell and Fox, 2001; Richelle *et al.,* 2001).

Phenolic extracts are usually prepared using different solvents including water, ethanol, methanol, isopropanol, acetone etc. according to the solubility of the major phenolic compounds in the materials (Li *et al.,* 2014). Agourram *et al.* (2013) found that 80% (v/v) aqueous acetone was more effective than 80% (v/v) aqueous methanol and 80% (v/v) aqueous ethanol in preparing phenolic extracts from fruit and vegetable byproducts. Ethanol was more efficient in producing phenolic extracts from *Smilax china* L. leaf with higher antioxidant activity and total phenolic content than methanol, acetone, or water (Sea *et al.,* 2012). Katalinić *et al.* (2010) reported that 80% (v/v) aqueous ethanol was efficient in the extraction of phenolic compounds from many plant materials.

The presence of chromophoric groups in phenolic compounds such as the red-purple anthocyanins (Bakowska *et al.,* 2003), or the brown and green reaction products of phenolic compounds with themselves or with proteins (Montavon *et al.,* 2003; Yabuta *et al.,* 2001) makes the phenolic compounds easy to identify. Taste of food is also affected by the presence of phenolic compounds. Phenolic compounds at low concentration may be responsible for desirable sweet, smoky or caramel flavors in foods e.g. dairy products (O'Connell and Fox, 2001). The astringent sensation in tea and wine is mainly caused by the presence of high concentrations of phenolic compounds (Baxter *et al.,* 1997; Charlton *et al.,* 2002). Milk proteins is able to bind with the phenolic compounds present in tea. The interactions between phenolic compounds and proteins may result in a decrease in protein digestibility, by inhibiting certain proteases or by blocking the substrate (Kroll *et al.,* 2003). Phenolic compounds are also known to improve the textural properties of certain foods either in reduced or oxidized form. Phenolics present in coconut husk ethanolic extract improved the gel strength of the sardine surimi (Buamard and Benjakul, 2015).

1.2.4.6 Oxidized phenolic compounds

Naturally derived plant phenolic compounds, especially in the oxidized form, have been shown to be the potential protein crosslinker (Rawel *et al.,* 2002). Delcour *et al.* (1984) found the formation of a haze in beer due to protein–phenolic compound interactions. Interactions of different phenolic acids and flavonoids with soy proteins were reported by Rawel *et al.* (2002). Plant phenols at pH 8 increased the bloom strength of gelatin gel (Strauss and Gibson, 2004). Addition of phenolic compound in combination with 0.1 M NaCl at pH 8.5 resulted in the improved gel properties of canola protein (Rubino *et al.,* 1996). *Ortho*-diphenolic compounds can be oxidized into *ortho*quinones. These quinones can be produced enzymatically or nonenzymatically. Non-enzymatic oxidation can occur readily under alkaline pH condition (Yabuta *et al.,* 2001).

1.2.4.6.1 Enzymatic oxidation

Polyphenol oxidases and peroxidases lead to the formation of quinones. Peroxidases produce the quinones via the formation of radicals. Peroxidases have a limited role in foods, since they require the presence of hydrogen peroxide (Matheis and Whitaker, 1984). Polyphenol oxidases (EC 1.14.18.1) can be classified into catechol oxidases and laccases. Both oxidases are able to oxidize phenolic substrates with the aid of molecular oxygen (Mayer and Harel, 1979; Osuga *et al.,* 1994). Catechol oxidases possess the catecholase activity which can lead to the oxidation of *ortho*-diphenols to *ortho*-quinones (Mayer and Harel, 1979) (Figure 4). In addition, cresolase activity shown by catechol oxidases may result in conversion of monophenols to *ortho* -diphenols (Mayer and Harel, 1979; Rodriguez-Lopez *et al.,* 2001) (Figure 4). Laccases are known to oxidize a wider range of substrates than catechol oxidases, which include *ρ*-diphenols (Mayer and Harel, 1979; Mayer and Staples, 2002) and non-phenolic compounds such as phosphorothiolates (Amitai *et al.,* 1998). They can also catalyze reactions other than oxidation, particularly demethylation and depolymerization of phenolic compounds (Mayer, 1987).

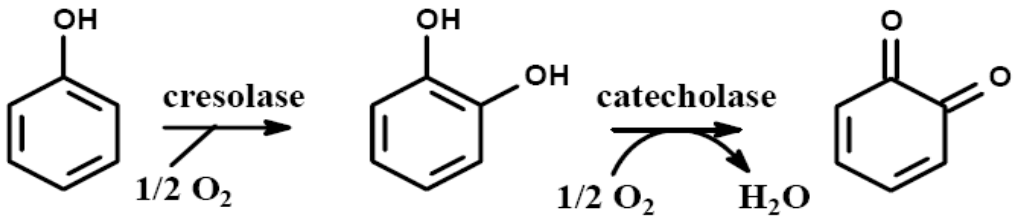

Figure 4. Cresolase and catecholase mechanism

Source: Matheis and Whitaker (1984)

Catechol oxidase, commonly known as tyrosinase in mammals and mushrooms. Catechol oxidases from fungi and higher plants have activity toward a wider range of mono- and *o*-diphenols, whereas tyrosinases from mammals are specific for tyrosine and DOPA (dihydroxyphenylalanine) (Mayer and Harel, 1979). The optimum pH of most catechol oxidases is 5-7. The maximal activity of mushroom tyrosinase was found at pH 7.0 and the lowest activity was obtained at pH 4.0 (McCord and Kilara, 1983). The enzyme is inhibited by an inhibitor, e.g. EDTA, in which copper in the active site is bound (Mayer and Harel, 1979).

1.2.4.6.2 Non-enzymatic oxidation

Quinones can also be formed in the absence of an enzyme. Deprotonation of hydroxyl groups in the phenolic compounds induced by alkaline pH eventually lead to the formation of quinones. Black olives have been produced by treating olives with diluted NaOH to oxidize the caffeic acid and hydroxytyrosol in olives (Garcia *et al.,* 1996). Use of an oxidizing reagent such as periodate can be an option to form quinones (Harrison and Hodge, 1982). Quinones themselves are unstable compounds, which tend to react by an oxido-reduction mechanism with other molecules, by oligomerization with other phenolic compounds, or by covalent reactions with other molecules such as proteins (Balange and Benjakul, 2010).

1.2.4.7 Protein cross-linking by oxidized phenolics

There are two different ways by which the phenolic compounds can interact with proteins. One is via non-covalent interactions which is a reversible

reaction and the another is via covalent interactions, which mostly are irreversible. The two kinds of distinguishable complexation mechanisms are monodentate and multidentate mechanisms (Haslam, 1989). Monodentate mechanism involves a phenolic compound interacting with only one protein site. When the phenolic compound to protein ratio is increased, the phenolics can form a layer surrounding a protein molecule, covering most of its surface, via a monodentate mechanism (Haslam, 1989). This may lead to aggregation of protein since the layer at the surface of the protein makes it less hydrophilic. In multidentate mechanism, the protein cross-linking is brought about by the interaction of phenolic compounds of sufficient size with more than one site in the protein (Haslam, 1989). Both complexation mechanisms can induce aggregation and precipitation (Charlton *et al.,* 2002; Haslam, 1989). Much lower phenolic compound / protein molar ratio is required in multidentate mechanism and a lower phenolic compound concentration is needed.

The non-covalent and covalent interactions between phenolic compounds and proteins also depend on several factors such as steric hindrance and the polarity of both protein and phenolic compound involved apart from the phenolic compound / protein ratio (Prigent, 2005). Therefore, the nature and the sequence of amino acids residues in the protein chain are the prime factors for deciding the nature of interaction (Prigent, 2005). The protein extraction, during the preparation of plant protein ingredients can be affected by phenolic compounds which interact with proteins. Removal of phenolic compounds is essential for the production of protein products from sunflower (Gonzalez-Perez *et al.*, 2002). Low solubility of protein caused by phenolic compounds affect the potato protein preparations (van Koningsveld *et al.,* 2002). Most of the protein functional properties depend on the protein solubility.

1.2.5 Edible film

Increasing use of synthetic packaging films has led to serious ecological problems due to their non-biodegradability. During the last decade, there has been growing interest in edible or biodegradable films based on biopolymers (Tongnuanchan *et al.,* 2013). Considerable efforts have been employed worldwide to

develop new biodegradable packaging materials from natural polymers. Edible films and coatings are of interest since they have potential to improve shelf-life, maintain quality and prevent microbial deterioration and physical damage of foods (Ahmad *et al.*, 2012). An edible film is defined as a thin layer, which can be consumed, coated on a food or placed as barrier between the food and the surrounding environment. Research on edible films and coatings in foods is mainly due to the high demand of consumers for longer shelf–life and better quality of fresh foods as well as of environmentally friendly packaging (Debeaufort *et al.,* 1998). Films can mechanically protect foods, prevent the contamination from microorganisms, prevent quality loss of foods due to mass transfer (e.g. moisture, gases, flavors, etc.) (Ahmad *et al.,* 2012). Edible films and coatings can be used as a vehicle for incorporating natural or chemical antimicrobial agents, antioxidants, enzymes or functional ingredients such as probiotics, minerals and vitamins (Vargas *et al.,* 2008). The edible films are classified into three categories taking into account the nature of their components: hydrocolloids (containing proteins, polysaccharides or alginates), lipids (constituted by fatty acids, acylglycerols or waxes) and composites (made by combining substances from the two categories) (Donhowe and Fennema, 1994).

The main biopolymers used in the edible film preparation are derived from polysaccharides (Nisperos-Carriedo, 1994) and proteins (Gennadios *et al.,* 1994). Edible films prepared from protein materials have been paid more attention for the use in the food protection and preservation, owing to their biodegradable, environmental characteristics (Tanaka *et al.,* 2001) and their ability to form films with satisfactory mechanical and gas barrier properties (Cuq *et al.,* 1998). Physical and chemical characteristics of the biopolymers greatly influence the properties of resulting films and coatings (Sothornvit and Krochta, 2000). In general, plasticizers are required to increase the flexibility of film by lowering the extension between polymers. Films can be incorporated with other additive for different purposes (Table 2).

Table 2. Materials used for edible films and coatings

Functional compositions	Materials
Film-forming materials	Proteins: myofibrillar protein, whey protein, casein, wheat gluten, soy protein, collagen, gelatin, corn zein, egg protein, pea protein, rice bran, sunflower, cottonseed protein, peanut protein, serum albumin, keratin, porcine plasma protein. Polysaccharides: starch, modified starch, modified cellulose (CMC, MC, HPC, HPMC), alginate, carrageenan, pectin, pullulan, chitosan, gellan gum, xanthan gum. Lipids: waxes (beeswax, paraffin, carnauba wax, candelilla wax, rice bran wax), resins (shellac, terpene), acetoglycerides.
Plasticizers	Glycerin, propylene glycol, sorbitol, sucrose, polyethylene glycol, corn syrup, water.
Functional additives	Antioxidants, antimicrobials, nutrients, nutraceuticals, pharmaceuticals, flavors, colors.
Other additives	Emulsifiers (lecithin, Tweens, Spans), lipid emulsions (edible waxes, fatty acids), cross-linkers (aldehyde, phenolic compounds).

*CMC, carboxy methylcellulose; MC, methylcellulose; HPC, hydroxypropyl cellulose; PMC, hydroxypropyl methylcellulose.

Source: Adapted from Han *et al.* (2005)

1.2.5.1 Myofibrillar protein films

Apart from gelation, film formation is another functionality of fish protein, which has been paid increasing attention (Shiku *et al.,* 2003; Cuq *et al.,* 1995; Paschoalick *et al.,* 2003). Fish proteins, including myofibrillar and sarcoplasmic proteins, have been used as film-forming material (Iwata *et al.,* 2000; Paschoalick *et al.,* 2003; Shiku *et al.,* 2003; Shiku *et al.,* 2004). The formation of edible packaging films from sardine meats and their properties were also investigated (Cuq *et al.,* 1995). Myofibrillar protein films have been produced from Atlantic sardine (Cuq *et al.,* 1996), round scad (Artharn *et al.,* 2009), red tilapia (Tongnuanchan *et al.,* 2011;

Nuanmano *et al.,* 2015). and silver carp mince (Nie *et al.,* 2015). Edible packaging films have also been produced from shrimp and squid myofibrillar proteins (Gómez-Estaca *et al.,* 2014; Leerahawong *et al.,* 2012; Blanco-Pascual *et al.,* 2014). Myofibrillar proteins are heteropolymers containing both polar and non-polar amino acids, which are able to form numerous intermolecular linkages. Generally, globular proteins must be denatured by heat, acid, base and/ or solvent to form more extended structures that are required for film formation (Krochta, 1997). Procedure and factors of film solubilization affected the film formation and its properties. The film properties were governed by many factors including pH, plasticizers, etc. Shiku *et al.* (2003) reported that pH was shown to influence the mechanical and physical properties of myofibrillar protein film. Transparent and flexible edible/biodegradable films were made from frozen threadfin bream surimi (Prodpran and Benjakul, 2005). The properties of films from surimi produced from tropical fish were affected by the pH used to solubilize the proteins, which directly influenced the proteolysis of muscle proteins (Prodpran and Benjakul, 2005; Chinabhark *et al.,* 2007).

The important functional characteristic of an edible film and coating is to retard the migration of moisture, oxygen, carbon dioxide, microbes or solutes, as well as to prevent collapse of products (Greener and Fennema, 1994). Protein concentration affects the properties of fish myofibrillar protein films. The highest mechanical properties of myofibrillar protein films from tilapia was obtained when the protein concentration of 2 % was used (Kaewprachu *et al.,* 2016). Edible films were prepared from silver carp surimi incorporated with ε-polylysine and their mechanical, barrier, optical properties and antimicrobial activity were studied (Weng *et al.,* 2014). The biodegradable films prepared from muscle proteins of whitemouth croaker (*Micropogonias furnieri*) had higher tensile strength and barrier properties with increasing protein concentrations (Zavareze *et al.,* 2014).

1.2.5.2 Film formation

There are two categories of film formation processes; dry and wet (Guilbert *et al.,* 1997) (Figure 5). The dry process of edible film production does not use liquid solvents, such as water or alcohol. Molten casting, extrusion and heat

pressing are good examples of dry processes. For the dry process, heat is applied to the film-forming materials to increase the temperature to above the melting point of the film-forming materials, to cause them to flow. Therefore, the thermoplastic properties of the film-forming materials should be identified in order to design film manufacturing processes. It is necessary to determine the effects of plasticizers and any other additives on the thermoplasticity of the film-forming materials (Guilbert *et al.,* 1997; Krochta, 2002). The wet process uses solvents for the dispersion of film forming materials, followed by drying to remove the solvent and form a film structure. For the wet process, the selection of solvents is one of the most important factors. Since the film-forming solution should be edible and biodegradable, only water, ethanol and their mixtures are appropriate as solvents (Krochta, 2002). All the ingredients of film-forming materials should be dissolved or homogeneously dispersed in the solvents to produce film-forming solutions (Cuq *et al.,* 1995; Gennadios *et al.,* 1994b; Guilbert and Gontard, 1995; Han and Floros, 1997; Han *et al.,* 2005). The film-forming solution should be applied to flat surfaces using a sprayer, spreader or dipping roller and dried to eliminate the solvent, forming a film structure. Phase separation of incompatible ingredients from the film-forming solution is not generally desirable unless the phase separation is intentionally designed for the formation of a bi-layer film structure (McHugh and Krochta, 1994). To produce a homogeneous film structure avoiding phase separation, various emulsifiers can be added to the film-forming solution (Krochta, 2002). The solvent compatibility of ingredients is very important to develop homogeneous edible film and coating systems carrying active agents. All ingredients, including active agents as well as biopolymers and plasticizers, should be homogeneously dissolved in the solvent to produce film-forming solutions. Most film-forming solutions possess much higher surface tension than the surface energy of dried films, since they contain excessive amounts of water or ethanol (Han *et al.,* 2005). During the solvent drying process, the film-forming solution is concentrated and its surface energy is decreased due to the loss of solvent (Skurtys *et al.,* 2010).

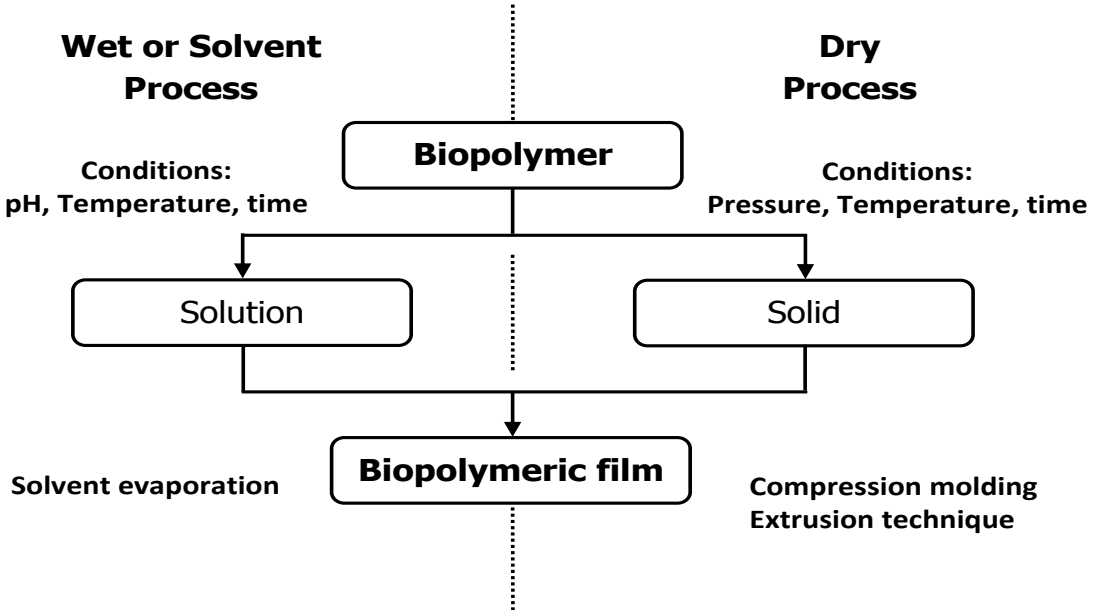

Figure 5. Processing methods of film formation: wet (or solvent) and dry process

Source: Guerrero *et al.* (2010)

1.2.5.3 Discoloration

Generally, myofibrillar fish protein-based film becomes yellowish when being stored for a long time (Artharn *et al.*, 2009; Cuq *et al.*, 1996a). Yellow discoloration of fish muscle protein-based film was mainly caused by non-enzymatic browning reaction or Maillard reaction (Artharn *et al.*, 2009; Limpan *et al.*, 2010; Tongnuanchan *et al.*, 2011b). Tongnuanchan *et al.* (2011b) reported that lipid oxidation played an essential role in yellow discoloration of fish muscle protein film by providing the carbonyl groups involved in Maillard reaction. Somanathan *et al.* (1992) reported that triethanolamine-treated casein films became brown in color and were considerably less resistant after 1 year of storage at 25 °C and 65% relative humidity (RH). The browning rate (*b**-value/week) of myofibrillar protein-based film from Atlantic sardine increased with increasing relative humidity and temperature (Cuq *et al.*, 1996b). Artharn *et al.* (2009) reported the increase in *b**- value of film from round scad muscle during storage at 25 °C for 8 weeks. Additionally, Chinabhark *et al.* (2007) also found the increase in *b**-value of protein based film from bigeye snapper surimi during storage of 60 days. Cuq *et al.* (1996a) reported that

fish myofibrillar protein-based films from Atlantic sardines showed yellow discoloration during storage for 8 weeks at 58.7% RH and 20 °C, which could be associated with the non-enzymatic browning reactions between protein and reducing sugars produced by partial hydrolysis of saccharose (as a plasticizing agent) introduced in the formulation of film. Discoloration of film directly limits the application of fish protein-based film.

Lipid oxidation under low pH system could be related with the enhanced autoxidation of hemoglobin at reduced pH (Tsuruga *et al.,* 1998). Low pH lowers oxygenation of hemoglobins and deoxy-form could be generated, thereby enhancing the lipid oxidation (Richards and Hultin, 2000). Fish muscle contained pro-oxidants such as hemoglobin, myoglobin and free irons (Richards and Hultin, 2000). Chaijan *et al.* (2006) found that the oxidation of myoglobin of sardine and mackerel muscles became intense with increasing storage time.

1.2.5.4 Improvement of film property

Proteins are promising biomaterials since films made with them are gas barriers. However, the main limitations of protein films, similar to other biopolymers, are their lack of mechanical strength and poor water vapor barrier because of their hydrophilic nature (Tongnuanchan *et al.,* 2013). Cross-linking of proteins in films is a very powerful method to improve their mechanical strength, water resistance, cohesion, rigidity and the barrier properties. Protein networks have the ability to interact with a wide range of active compounds. Modification via chemical, physical or enzymatic cross-linking take place and enhance the functional properties of the films.

1.2.5.4.1 Cross-linking chemicals

Chemical agents especially aldehydes including glutaraldehyde, glyoxal, formaldehyde etc. are used for the covalent cross-linking of protein (Orliac *et al.,* 2002; Hernandez-Munoz *et al.,* 2004). Formaldehyde is the simplest cross-linking agents, and has the widest reaction specificity. It mainly interacts with amine group of lysine and the side chains of cysteine, tyrosine, histidine, tryptophan, and arginine.

Formaldehyde can react bi-functionally and result in crosslinking activity, although it contains a single functional group (Bourtoom, 2009). Glutaraldehyde is more specific than formaldehyde and can react with lysine, cysteine, histidine and tyrosine (Tae, 1983). Glyoxyl leads to protein cross-linking via interaction between lysine and arginine side chain groups (Marquie, 2001) at alkaline pH. Hernandez-Munoz *et al.* (2004) reported that the incorporation of cross-linking agents such as formaldehyde, glutaraldehyde and glyoxal in glutenin rich films decreased their water vapor permeability by around 30%. The addition of formaldehyde resulted in highest tensile strength values followed by glutaraldehyde and glyoxal. Formaldehyde was better cross-linker compared to glutaraldehyde and gossypol. The enhanced cross-linking ability of formaldehyde can be attributed to the lack of specificity with respect to the different amino acid side chain groups (Bourtoom, 2009). In addition to amines, formaldehyde reacts with sulfydryl, phenolic, imidazolyl, indolyl and guanidinyl groups. The formation of methylene bridges occurred between lysine and tyrosine when protein was treated with formaldehyde (Hernandez-Munoz *et al.*, 2004). However, the major disadvantage of aldehydes is their toxicity which must be taken into account when synthesizing biodegradable materials.

Furthermore, plant phenolics have gained attention as protein cross-linker, which can improve the properties of protein based films. Phenolic compounds such as ferulic acid and tannic acid act as natural cross-linking agents and have been used to improve the mechanical properties of gelatin films (Cao *et al.*, 2007). The mechanical strength of gelatin film was maximum when ferulic acid was used as the cross-linking agent in the film-forming solution with pH value 7 or when tannic acid was used with pH value of film-forming solution being 9. The mechanical property of myofibrillar protein-based films from washed mince of silver carp was improved by the addition of grape seed procyanidine and polyphenols (Nie *et al.*, 2015). Tannic acid, caffeic acid, and ferulic acid at a level of 3% increased the tensile strength of porcine plasma protein-based film by 123.3, 194.3, and 19.5% and elongation at break (EAB) by 71.1, 86.3, and 10.2%, respectively, compared with the control film (Nuthong *et al.*, 2009). Tannic acid and caffeic acid showed a superior enhancing effect on the film strength, compared to ferulic acid, most likely due to their

multidentate mechanism (Nuthong *et al.*, 2009). Incorporation of tannic acid at 5% improved the mechanical properties of myofibrillar films from bigeye snapper (Prodpran *et al.*, 2012).

1.2.5.4.2 Cross-linking enzymes

Polymerization using cross-linking enzyme, e.g. transglutaminase has been investigated with various protein sources including casein, soy proteins and gelatin. The changes in gel strength were dependent on the reaction conditions and on the different protein sources (Sakamoto *et al.*, 1994). Larre *et al.* (2000) reported that transglutaminase was effective in introducing covalent bonds into films obtained from slightly deamidated gluten. The introduction of covalent bonds resulted in the formation of polymers of high molecular weight leading to the lowered solubility of the treated films but also reduced surface hydrophobicity. The film integrity and the capacity to stretch increased with the addition of covalent bonds by the use of transglutaminase (Larre *et al.*, 2000). Jiang *et al.* (2007) reported that the addition of transglutaminase can modify the properties of soy protein films, especially the tensile strength and the hydrophobicity. However, the modification of properties of soy protein films by transglutaminase was, to a various extent, dependent upon many processing parameters such as the concentration of enzyme, the condition of the film-forming solutions and the airdrying temperature. The influence of these processing parameters could account for the aggregation of soy protein films induced by transglutaminase. Thus, the improvement of the tensile strength of soy protein films by transglutaminase could be achieved by inhibiting or delaying the occurrence of this kind of aggregation. Generally, the cross-linkage by transglutaminase improves the tensile strength of protein films, while it decreases the elongation at break and solubility properties. In some cases, such as with isolated soy protein and deamidated gluten films, the transglutaminase treatment also significantly increased the surface hydrophobicity of films (Tang *et al.*, 2005). Microbial transglutaminase improved the mechanical properties of myofibrillar protein films from silver carp (*Hypophthalmichthys molitrix*) (Rostamzad *et al.*, 2016). Mechanical properties of gelatin films from tilapia scales were improved by addition of transglutaminase (Weng and Zheng, 2015). Gelatin and casein blend film added with transglutaminase

had the improved water barrier properties (Chambi and Grosso, 2006). The enzymes such as lipoxygenase, lysyl oxidase, polyphenol oxidase and peroxidase could also be utilized for improving the properties of protein film (Wittaya, 2012). Han and Zhao (2016) reported that improved properties of film from bovine gelatin were observed when prior cross-linking induced by horseradish peroxidase, glucose oxidase in the presence of glucose was implemented. The improvement in the properties of protein based edible films by using enzyme seems to be dependent upon the types of substrate protein and some processing parameters, such as the amount of enzyme applied. Thus, it is expected that the improvement in the properties of protein-based edible films by enzyme treatment is also affected by the enzyme concentration (Wittaya, 2012).

1.3 Objectives:

1.3.1 To study antioxidant capacity of squid melanin-free ink (MFI).

1.3.2 To investigate the effect of MFI on properties and oxidative stability of sardine surimi gel.

1.3.3 To evaluate the effect of mixture of squid ink tyrosinase and tannic acid on gelling property and quality of sardine surimi gel.

1.3.4 To study the effect of mixture of squid ink tyrosinase and coconut husk extract on the gel properties of sardine surimi.

1.3.5 To elucidate the effect of MFI on the properties and yellow discoloration of protein film from washed sardine mince.

1.3.6 To investigate the effect mixture of squid ink tyrosinase and tannic acid on the properties of protein film from washed sardine mice.

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CHAPTER 2
ANTIOXIDANTIVE ACTIVITY OF MELANIN-FREE INK FROM SPLENDID SQUID (*Loligo formosana*)

2.1 Abstract

Antioxidative activities of melanin-free ink (MFI) from splendid squid were investigated using different *in vitro* assays. MFI had DPPH, ABTS radical scavenging activities, and ferric reducing antioxidant power (FRAP) of 179.6±2.1, 957.8±89.3, and 171.2±7.3 μmol TE/g protein, respectively. It also possessed metal chelating activity of 4.0±1.2 μmol EE/g protein. MFI at 500 mg/L could prevent the oxidation of *β*-carotene-linoleic acid system but its efficacy was lower than 200 mg/L butylated hydroxyl anisole (BHA) ($P<0.05$). MFI (100-500 mg/L) showed the preventive effect on lipid peroxidation of lecithin liposome system in dose-dependent manner. When mackerel mince was added with MFI at levels of 100 and 200 mg/kg, lipid oxidation was retarded during ice storage of 15 days as evidenced by the lower peroxide value and thiobarbituric acid reactive substances, compared with the control. However, its effectiveness was lower than 200 mg/kg BHA. When MFI was subjected to heat treatment at 90 °C, for up to 30 min, its DPPH and ABTS radical scavenging activities remained constant, but FRAP decreased within the first 5 min without subsequent changes. When the MFI was separated using ultrafiltration into different fractions (<3 KDa, 3-10 KDa, and >10 KDa), the fractions with MW less than 3 KDa showed the highest antioxidative activities ($P<0.05$). Therefore, the squid ink could show the antioxidant activity after melanin removal.

2.2 Introduction

Lipid oxidation is one of the major causes for deterioration of many food products. It leads to changes in texture, flavor, odor and quality of foods. Lipid oxidation also causes some health hazards in human beings such as cardiovascular disease, cancer, and neurological disorders as well as aging process (Gulcin, 2011; Gulcin, 2012). To prevent or slow down lipid oxidation, several antioxidants including synthetic and natural antioxidants have been widely used. However,

synthetic antioxidants are suspected of being toxic upon long term exposure and are banned in many countries (Madhavi and Salunkhe, 1996). As a consequence, natural and safe antioxidants have gained increasing attention. Natural antioxidants from marine resources, especially from by-products of seafood processing, can be another alternative antioxidant for food applications.

Squid and cuttlefish have become an important fishery product of Thailand as well as other Southeast Asian countries, and are mainly exported worldwide (Hoque *et al.,* 2010). During processing, viscera along with the ink sac are generated as by-products with the low market value and it can create serious ecological problems and environmental pollution without appropriate management. These by-products can be a promising source of bioactive compounds. The squid ink has been proved to be an alternative medicine and has the wide range of therapeutic applications (McConnell *et al.,* 1994). Growth performance, antioxidant functions and immunity in growing broiler chickens were affected by squid ink (Liu *et al.,* 2011). Antibacterial activity of the ink from *Sepioteuthis lessoniana* and *Sepia pharaonis* against biofilm bacteria was reported (Ramasamy and Murugan, 2005). A protein extracted from cuttlefish (*Sepioteuthis lessoniana*) ink could inhibit the growth of *Staphylococcus aureus* (Mochizuki, 1979). Tyrosinase present in squid ink is known to play a key role in the defence against microbes (Takai *et al.,* 1992). Ink from *Sepia officinalis* (Lei *et al.,* 2007) and *Sepiella inermis* (Rajaganapathy *et al.,* 2000) showed antioxidative and antiretroviral activities, respectively. Due to a plenty of ink generated during squid processing, it can be used as the source of active compounds, particularly antioxidants. However, ink has black color, which may limit its application. The removal of melanin, a black pigment, prior to utilization should widen the application of ink. Therefore, the present study aimed to investigate antioxidative activity and properties of melanin-free ink from splendid squid (*Loligo formosana*), the most common squid used for processing in Thailand.

2.3 Materials and methods

2.3.1 Chemicals

2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), 1,1,3,3-tetramethoxypropane and Tween 40 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Linoleic acid, BHA, 2-thiobarbituric acid and cumene hydroperoxide were procured from Fluka (Buchs, Switzerland). Methanol, ethanol, acetone, chloroform, hydrochloric acid and ammonium thiocyanate were obtained from Lab-Scan (Bangkok, Thailand). All chemicals were of analytical grade.

2.3.2 Preparation of melanin-free ink

Fresh squids (24 h after capture) were purchased from a local market in Hat Yai, Songkhla, Thailand and transported in ice using squid/ ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 30 min. Upon arrival, ink sac was separated from the squid by cutting the ink duct and ink was squeezed out from the ink sac. The squid ink was diluted ten-fold using cold deionized water (4 °C). Thereafter, it was subjected to centrifugation at 18,000×g for 30 min at 4 °C using a refrigerated centrifuge (Allegra 25 R centrifuge, Beckman Coulter, Palo Alto, CA, USA). The supernatant obtained was referred to as “melanin-free ink; MFI”. MFI was subjected to analyses.

2.3.3 Determination of protein content

Protein content was determined using the Lowry method (1951). Bovine serum albumin (BSA) was used as a standard.

2.3.4 Determination of *in vitro* antioxidative activities

2.3.4.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Blois (1958) as modified by Binsan *et al.* (2008). To the sample (1.5 mL), 1.5 mL of

0.15 mM DPPH in 95% (v/v) ethanol was added. The mixture was mixed vigorously using a vortex mixer (model G-560E, Scientific Industries, Inc., Bohemia, NY, USA) and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that deionized water was used instead of sample. A standard curve was prepared using Trolox in the range of 0-50 µM. The activity was expressed as µmol Trolox equivalents (TE)/g protein.

2.3.4.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Blois (1958) as modified by Binsan *et al.* (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 mL of ABTS solution with 50 mL of methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared daily. Sample (150 µL) was mixed with 2850 µL of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm. A standard curve of Trolox ranging from 0 to 500 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/g protein.

2.3.4.3 Ferric reducing antioxidant power (FRAP)

FRAP was assayed according to the method of Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. A working solution was prepared freshly by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3.6H_2O$ solution. The mixed solution was incubated at 37°C for 30 min and was referred to as FRAP solution. A sample (150 µL) was mixed with 2850 µL of FRAP solution and kept for 30 min in dark. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. The control

was prepared in the same manner, except that deionized water was used instead of the sample. The standard curve was prepared using Trolox ranging from 0 to 500 µM. The activity was expressed as µmol Trolox equivalents (TE)/g protein.

2.3.4.4 Chelating activity toward Fe^{2+}

Chelating activity toward Fe^{2+} was measured by the method of Thiansilakul *et al.* (2007). The sample (4.7 mL) was mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature (26-28 °C). The absorbance was then read at 562 nm. The control was prepared in the same manner except that deionized water was used instead of the sample. A standard curve (0-50 µM EDTA) was prepared. Fe^{2+} chelating activity was expressed as EDTA equivalents (µmol EDTA equivalents (EE)/g protein).

2.3.5 Antioxidative effect of MFI in different model systems

2.3.5.1 *β*-Carotene-linoleate model system

Antioxidative activity of MFI in β-carotene linoleic acid emulsion model system was determined as described by Binsan *et al.* (2008). β-carotene (10 mg) was dissolved in 10 mL of chloroform. Thereafter, the solution (0.2 mL) was added to 20 mg linoleic acid and 200 mg Tween 40. Chloroform was then removed by purging with nitrogen. Fifty milliliters of oxygenated deionized water was added to β-carotene emulsion and mixed well. MFI (500 µL) was then mixed with 4.5 mL of oxygenated β-carotene emulsion to obtain the final concentrations of 100, 200 and 500 mg/L. The oxidation of β-carotene emulsion was monitored spectrophotometrically at 470 nm after 0, 10, 20, 30 40, 60, 90 and 120 min of incubation at 50 °C in dark. BHA at levels of 200 mg/L was also used. The control was prepared by using distilled water instead of MFI in the assay system.

2.3.5.2 Lecithin liposome system

Antioxidative activity of MFI in lecithin liposome system was determined according to the method of Frankel *et al.* (1997). Lecithin was suspended

in deionized water at a concentration of 8 mg/mL. The mixture was stirred with glass rod, followed by sonicating for 30 min using a sonicating bath (Elmasonic S 30 H, Elma, Germany). Sample (3 mL) was mixed with lecithin liposome (15 mL) to obtain the final concentrations of 100, 200 and 500 mg/L. The liposome suspension was then sonicated for 2 min. To initiate the assay, 20 μL of 0.15 M cupric acetate were added. The mixture was shaken at 120 rpm using a shaker (WNB 14 and SV 1422, Memmert, Germany) at 37°C in dark for 0, 6, 12, 24, 36 and 48 h. Liposome oxidation was monitored by determining thiobarbituric acid-reactive substances (TBARS). TBARS values were calculated from the standard curve (0 – 3 mg/L malonaldehyde (MDA)) and expressed as mg MDA/mL liposome.

2.3.6 Fish mince model system

2.3.6.1 Preparation of fish mince

Fish mince was prepared according to the method of Kamil *et al.* (2002) with a slight modification. Mackerel (*Rastrelliger kanagurta*) with an average weight of 100–150 g off-loaded 24 h after capture, were purchased from the local market in Hat Yai, Thailand. The fish were kept in ice during the transportation. Upon arrival, the fish were washed, dressed and meat was separated manually. The fish mince obtained was divided into four portions (100 g each). One portion, without the addition of MFI, was used as the control and 10 mL of deionized water was added instead. Two portions were mixed with 10 mL of MFI to obtain the final concentrations of 100 and 200 mg/kg mince. Another portion was added with BHA to obtain the final concentration of 200 mg/kg mince. The mince was then thoroughly mixed in order to ensure the homogeneous distribution of MFI and BHA in the mince. Different mince samples were placed in polyethylene bag and kept in ice using a mince/ice ratio of 1:2 (w/w). Molten ice was removed every day and the same quantity of ice was replaced. After the designated storage time (0, 3, 6, 9, 12 and 15 days), the samples were taken for analyses of TBARS and peroxide value (PV).

2.3.6.2 Determination of Peroxide Value (PV)

PV was determined according to the method of Richards and Hultin (2000) with a slight modification. Ground sample (1 g) was homogenized at a speed of 13,500 rpm for 2 min in 11 mL of chloroform/methanol (2:1, v/v). The homogenate was then filtered using a Whatman No.1 filter paper. Two milliliters of 0.5% NaCl was then added to 7 mL of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3,000 × *g* for 3 min at 4 °C using a refrigerated centrifuge to separate the sample into two phases. The lower phase (3 mL) was carefully pipetted out and 2 mL of cold chloroform: methanol (2:1) mixture were added. Then twenty-five microliters of 30% (w/v) ammonium thiocyanate and 25 μL of 20 mM iron (II) chloride were added to the mixture. The reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except deionized water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5 to 2 mg/L. PV was expressed as mg cumene hydroperoxide/kg sample.

2.3.6.3 Determination of TBARS

TBARS values of sample were determined as described by Buege and Aust (1978). Sample (0.5 g) was mixed with 2.5 mL of TBA solution containing 0.375 % thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture was heated in boiling water for 10 min to develop a pink color, cooled with running tap water and then sonicated for 30 min, followed by centrifugation at 5000×g at 25°C for 10 min. The absorbance of the supernatant was measured at 532 nm. Standard curve was prepared using 1,1,3,3- tetramethoxypropane (malonaldehyde; MAD) at the concentrations ranging from 0 to 10 ppm and TBARS were expressed as mg MAD equivalents/kg sample.

2.3.7 Thermal stability of MFI

Two milliliters of MFI solution (2.8 mg protein/ mL) were transferred to a screw-capped test tube. The tube was capped tightly and placed in a water bath

(90°C) for 0, 5, 10, 15, 20, 25 and 30 min. After designated heating times, the treated samples were immediately cooled in iced water. The samples were analyzed for DPPH, ABTS radical scavenging activities and FRAP as previously described. The remaining activities were expressed, relative to that of untreated sample.

2.3.8 Ultrafiltration of MFI

Ultrafiltration of MFI was carried out using a stirred ultrafiltration cell (Model 8050, Amicon Bioseparations, Millipore Corporation, Bedford, MA, USA). Initially, the filtration of MFI was done using the membrane with MW cut-off of 10 KDa. Then the permeate was filtered through the membrane with MW cut-off of 3 KDa. During filtration, the pressure was maintained at 60 psi with continuous stirring at 150 rpm. The fractions obtained were subjected to determination of antioxidative activities as described above.

2.3.9 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

2.4 Results and discussion

2.4.1 Antioxidative activities of MFI

MFI showed antioxidative activities when tested using several assays as shown in Table 3. MFI was effective in scavenging DPPH and ABTS radicals, in which DPPH and ABTS radical scavenging activities of 179.6±2.1 and 957.8±89.3 µmol TE/mg protein were observed, respectively. DPPH and ABTS radical scavenging activities are based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals, by converting it to the non-radical species (Binsan *et al.*, 2008; Chandrasekara and Shahidi, 2011). DPPH is a radical having an odd electron, and reacts with hydrogen donated from antioxidant. In case of ABTS

radical scavenging activity, the effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific functional groups (Hagerman *et al.*, 1998). Squid ink was reported to contain L-dopa and dopamine at concentrations of 1.15 mM and 0.19 mM, respectively (Lucero *et al.*, 1994). Hydroxyl groups of those compounds more likely donated hydrogen atom to radicals tested. FRAP of MFI was 171.1±07.3 µmol TE/mg protein and had metal chelating activity of 4.0±1.2 µmol EE/mg protein. FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Benzie and Strain, 1996; Binsan *et al.*, 2008). The result indicated that MFI was able to act as reducing agent which provided electron for stabilization. Additionally, some compounds in MFI could chelate prooxidative metals, thereby lowering or retarding the initiation of lipid oxidation process. The capacity of antioxidant for chelating metals is strongly dependent on the number of hydroxylic groups in ortho-position (Maqsood and Benjakul, 2010). Squid ink was reported to function as antioxidant in hyperlipidemia rats and broil chicken (Lei *et al.*, 2007; Liu *et al.*, 2011).

Table 3. Antioxidative activities of MFI from splendid squid

Assays	Activities
DPPH radical scavenging activity (µmol TE/g protein)	179.6 ± 02.1
ABTS radical scavenging activity (µmol TE/g protein)	957.8 ± 89.3
Ferric reducing antioxidant power (µmol TE/g protein)	171.2 ± 07.3
Metal chelating activity (µmol EE/g protein)	4.0 ± 01.2

Mean ± S.D (n=3)

2.4.2 Antiooxidative effect of MFI in different model systems

2.4.2.1 *β*-carotene-linoleate model system

MFI showed preventive effect toward oxidation of β-carotene-linoleate model system in a dose-dependent manner (Figure 6A). MFI at the level of 500 mg/L showed the highest activity, followed by 200 mg/L and 100 mg/L, respectively. The decrease in A_{470} indicates the oxidation of *β*-carotene in the system caused by free radicals from oxidation of linoleic acid (Chandrasekara and Shahidi, 2010). When the oxidation of linoleic acid occurs, free radicals formed are able to attack highly unsaturated *β*-carotene molecules. As a result, *β*-carotene losts in chromophore and characteristic orange color (Binsan *et al.,* 2008). In the presence of MFI, *β*-carotene bleaching was retarded, mainly due to the chain-breaking inhibition of lipid peroxidation by neutralizing linoleic free radical formed. When comparing antioxidative effect of MFI with BHA (200 mg/L), higher antioxidative of activity in the system was found for BHA. The ability to prevent the bleaching of *β*-carotene was more likely governed by their amphiphilic properties of amino acid compositions of peptides in MFI. When antioxidative compounds were oriented at linoleic acid/ water interface, antioxidative effect could be maximized (Binsan *et al.*, 2008). Thus, MFI could prevent oxidation of lipids in emulsion system.

2.4.2.2 Lecithin liposome model system

The ability of MFI to retard oxidation in the lecithin liposome system at various concentrations is depicted in Figure 6B. The formation of secondary lipid oxidation products in the lecithin liposome was evaluated by TBARS. MFI effectively retarded the oxidation of lecithin liposome system during incubation of 48 h. However, its preventive effect was less than that of BHA (200 mg/L) as indicated by lower TBARS values of system containing BHA. It was noted that the control sample (without MFI or BHA) had the sharp increase in TBARS after 24 h of incubation, suggesting that oxidation took place to a higher extent. Liposomes are appropriate lipid models to evaluate antioxidative activity in lipid food or lipoprotein particles containing phospholipids (Frankel *et al.,* 1997). The sample added with MFI at a level

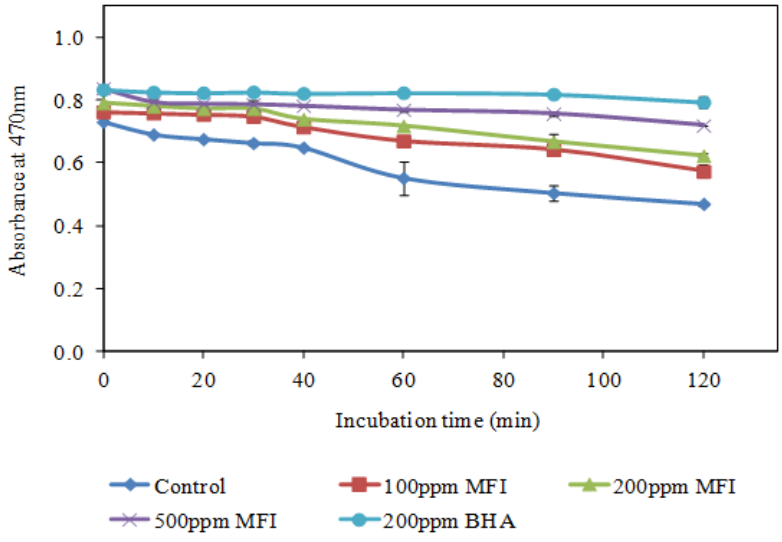
of 500 mg/L showed the lower TBARS value, compared to those containing MFI at levels of 200 and 100 mg/L. The result suggested that antioxidant activity of MFI in lecithin liposome system was in dose dependent manner. Furthermore, hydrophilic domains of peptides or proteins in MFI more likely migrated and localized at head portion of lecithin in liposome, thereby inhibiting oxidation of liposome via radical scavenging mechanism (Table 3). Due to metal chelating ability, MFI could chelate Cu^{2+}, a prooxidant in the system. As a result, the oxidation of liposome could be impeded with addition of MFI.

2.4.3 Fish model system

The formation of PV in the mackerel mince treated without and with MFI during ice storage is shown in Figure 7A. PV increased up to day 6 of storage in all samples (P<0.05), except for the control sample, in which PV still increased until the end of storage (day 15). The sample added with MFI at the level of 100 mg/kg had slight decrease in PV at day 9 but PV increased thereafter. It was noted that similar PV values were observed between samples added with 100, 200 mg/kg MFI and 200 mg/kg BHA at day 15 of storage (P<0.05). PV values of mince treated with MFI or BHA were lower than that of control. This indicated that MFI acted as an antioxidant and lowered the formation of primary lipid oxidation products.

Changes in TBARS of mackerel mince without and with MFI at the levels of 100 and 200 mg/kg during ice storage are shown in Figure 7B. TBARS values remained unchanged up to 3 days of storage (P<0.05). The marked increases in TBARS were observed in all samples up to day 12, except for the sample added with 200 mg/kg BHA, which had lower increase in TBARS. There was a slight decrease at day 15. When comparing TBARS values among the samples, the control contained higher TBARS value than other samples throughout 15 days of iced storage (P<0.05). The TBARS values of mince treated with MFI at the level of 200 mg/kg was less than that treated with 100 mg/kg (P<0.05). Nevertheless, TBARS values of mince added with MFI at the levels of 100 and 200 mg/kg were higher than those of sample added with BHA at a level of 200 mg/kg. At day 15 of storage, the decreases in TBARS values were obtained. This was plausibly due to the loss of volatile lipid

(A)



(B)

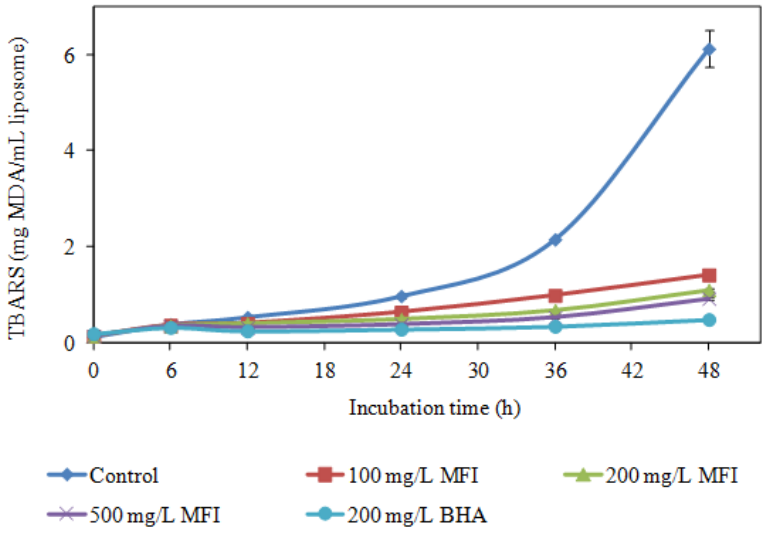


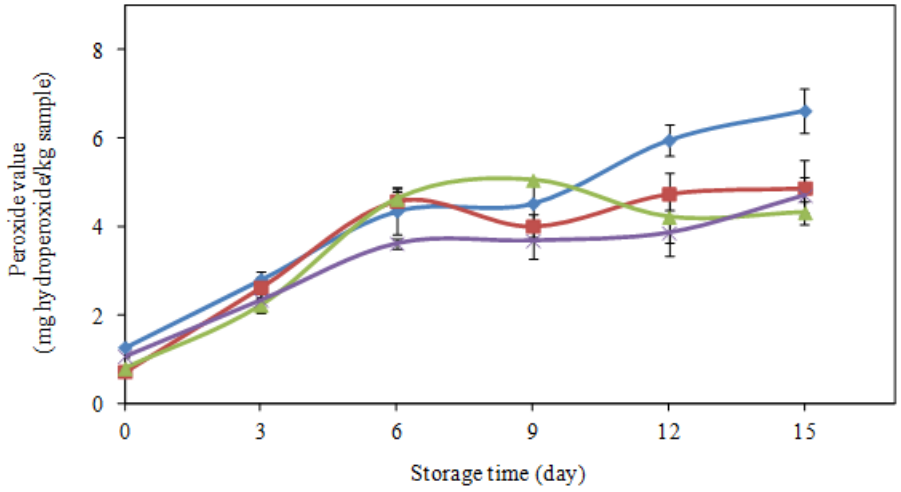
Figure 6. Antioxidative effects of MFI from splendid squid in *β*-Carotene-linoleate model system (A) and lecithin liposome model system (B). Bars represent the standard deviation (n=3).

oxidation products. Since MFI exhibited radical scavenging activity and capability of metal chelation (Table 3), it was able to retard the oxidation in fish mince. Mackerel meat contained high fat content (4.54±0.28 g/ 100g) (Osman *et al.*, 2001). It was reported that fish meat was rich in polyunsaturated fatty acid (PUFA) (Chantachum *et al.*, 2000). PUFAs are susceptible to oxidation, which is associated with rancidity. Additionally, mackerel meat contained a high amount of haem. The haem protein or other iron-containing proteins are denatured with coincidental release of iron as the storage time increased (Benjakul and Bauer, 2001). Degradation of protein during extended storage was also associated with the increase in non-haem iron. Denaturation and degradation might favour destruction of haem, thereby enhancing the release of iron. Chaijan *et al.* (2005) reported that the non-haem iron content increased in mackerel mince during the iced storage. Those non-haem iron could act as prooxidant in fish flesh or mince (Benjakul and Bauer, 2001). Owing to chelating ability of MFI (Table 3), it could scavenge Fe^{2+} or Fe^{3+} in the mince. As a result, the lipid oxidation could be retarded. Thus, MFI retarded the lipid oxidation in the mince as revealed by the lower TBARS values than that of control.

2.4.4 Thermal stability of MFI

MFI was subjected to heat treatment at 90 °C for 30 min and the remaining DPPH, ABTS radical scavenging activities and FRAP were monitored (Figure 8). DPPH and ABTS radical scavenging activities remained unchanged after heating up to 30 min (P<0.05). FRAP was decreased when heated for 5 min (P<0.05). Thereafter, no changes in FRAP were observed (P<0.05). Some peptides or compounds with FRAP might undergo some aggregation after being heated, while the heat stable counterparts were resistant to alteration. Zayas (1997) reported that smaller size peptides were more stable to aggregation at high temperatures. The smaller peptides or compounds in MFI could survive during heating and they more likely contributed to prevent lipid oxidation in thermally processed foods.

(A)



(B)

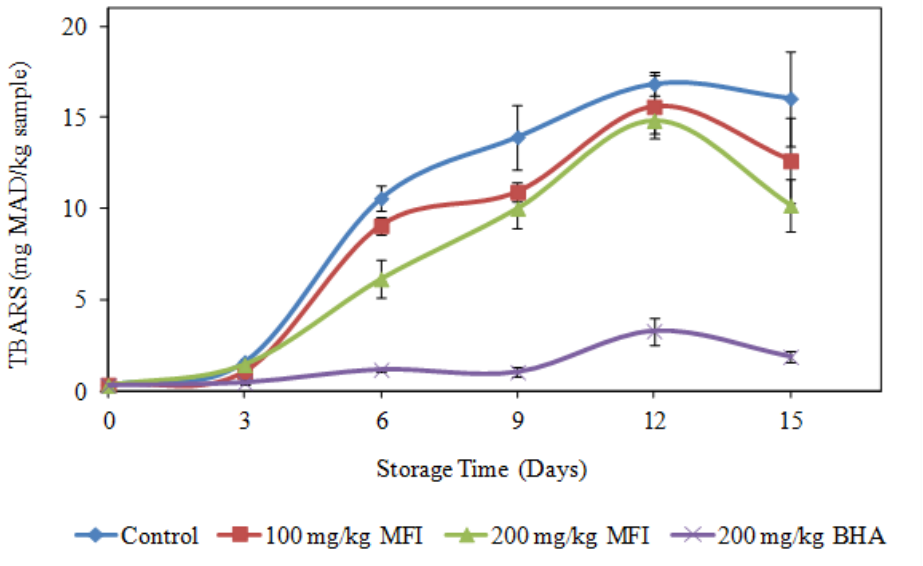


Figure 7. Effect of MFI from splendid squid on the formation of lipid oxidation products in mackerel mince during iced storage of 15 days. Peroxide value (A), and thiobarbituric acid-reactive substances (TBARS) values (B). Bars represent the standard deviation (n=3).

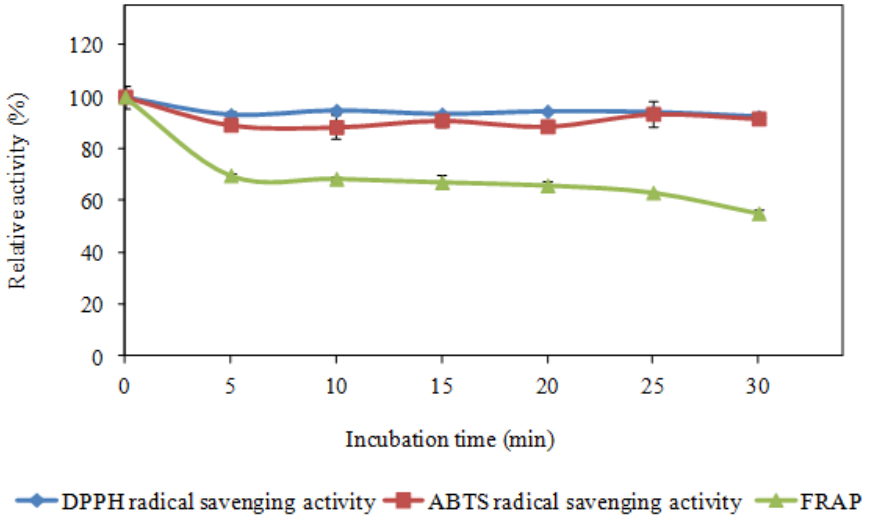


Figure 8. Thermal stability of MFI from splendid squid at 90° C. Bars represent the standard deviation (n=3).

2.4.5 Antioxidant activities of fraction with different MWs

DPPH radical scavenging activity of fractions obtained from ultrafiltration with various MW ranges is shown in Figure 9A. Higher activity was observed as MW decreased. The <3 KDa fraction had higher activity than those with MW of 3-10 KDa and >10 KDa, respectively. The <3 KDa fraction had five-fold higher activity than MFI. It was noted that MFI showed similar DPPH radical scavenging activity to that with MW of >10 KDa fraction (P<0.05). ABTS radical scavenging activity (Figure 9B) had the similar result to that of DPPH radical scavenging activity but ABTS radical scavenging activity of >10 KDa fraction was lower than that of MFI (P<0.05). The <3 KDa fraction showed two-fold higher activity than MFI (P<0.05). FRAP of the fractions decreased as MW of fractions increased (Figure 9C). Nevertheless, FRAP of >10 KDa fraction was higher than that of MFI (P<0.05). FRAP of <3 KDa fraction was seven-fold higher than that of MFI. Metal chelating activity of different fractions varied with MW (P<0.05) (Figure 9D). It was found that chelating activity of >10 KDa fraction was similar to that of MFI (P<0.05). In general, peptides or proteins with lower MW showed higher

antioxidative activity (Bernardini *et al.*, 2011). Liu *et al.* (2010) reported that <3 *KDa* fraction from porcine plasma protein hydrolysate exhibited the highest DPPH radical scavenging activity and reducing power. The low MW fraction (<1 *KDa*) from the protease N hydrolysate of royal jelly proteins had the highest antioxidative activity (Guo et al. 2009). Park *et al.* (2001) also noted that <5 *KDa* hydrolysate from egg yolk protein had the highest antioxidative activity. Wang et al. (2007) reported that the antioxidant activity of wheat gluten hydrolysate UF fraction (<5 KDa) was higher than its original hydrolysate. The <1 KDa fraction of conger eel muscle protein hydrolysates obtained from ultrafiltration exhibited the highest inhibition activity of linoleic acid peroxidation (Ranathunga *et al.*, 2006). Therefore, MW distribution was an important factor governing antioxidative activity of MFI from splendid squid.

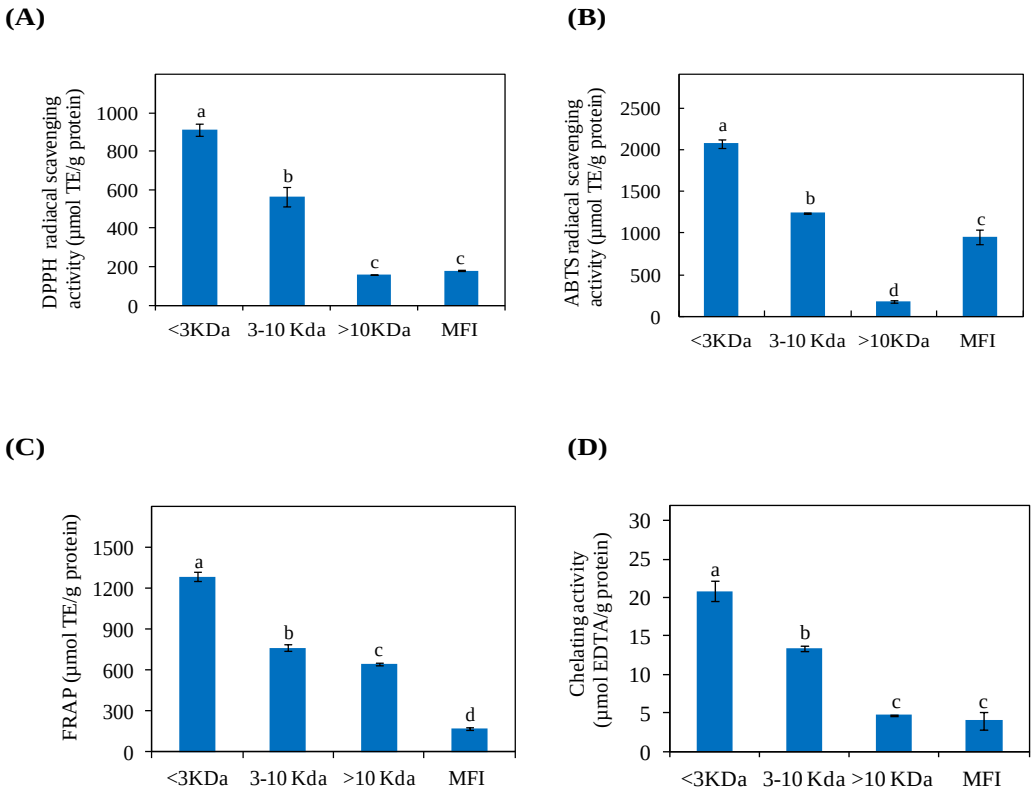


Figure 9. DPPH (A), ABTS (B), FRAP (C) and Chelating activity toward Fe^{2+} (D) of ultrafiltration fractions from MFI of splendid squid with different MWs. Bars represent the standard deviation (n=3). Different letters on the bars for each assay indicate significant differences ($p < 0.05$).

2.5 Conclusion

Squid ink, which is discarded as byproduct, could be used as the good source for natural antioxidant after melanin removal. MFI with high thermal stability possessed radical scavenging and metal chelating activities. After ultrafiltration, fraction with lower MW had the greater antioxidative activity. The small MW fraction could be further used as potential antioxidant.

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CHAPTER 3

APPLICATION OF MELANIN-FREE INK AS A NEW ANTIOXIDATIVE GEL ENHANCER IN SARDINE SURIMI GEL

3.1 Abstract

The squid ink which is discarded as waste during processing can be effectively utilized as gel enhancer in surimi gels, especially those prepared from dark fleshed fish which has poor gel properties. It also acts as an antioxidant inhibiting lipid oxidation. This study aimed to study the effect of melanin-free ink (MFI) from splendid squid (*Loligo formosana*) on properties and oxidative stability of surimi gel from sardine (*Sardinella albella*). MFI (0-0.1 g kg^{-1} surimi) increased breaking force and deformation of sardine surimi gel in a dose-dependent manner ($P<0.05$). The addition of MFI had no effect on whiteness of surimi gels ($P>0.05$). The expressible moisture content of gels decreased as the levels of MFI increased ($P<0.05$). Based on microstructure study, gel added with MFI at a level of 0.08 g kg^{-1} surimi was denser and finer than that of the control (without MFI). Surimi gels with MFI had lower peroxide values (PV), thiobarbituric acid reactive substances (TBARS), nonanal and 2-decenal. MFI could improve the properties of sardine surimi gel. Additionally, it was able to prevent lipid oxidation in surimi gels during refrigerated storage.

3.2 Introduction

Surimi is the washed fish mince and can be used as raw material for several products with elastic texture. During washing with cold water, fat and other water-soluble components are removed, whereas myofibrillar proteins become concentrated (Benajkul *et al.*, 1996). Traditionally, lean fish have been used for surimi production owing to the superior gelling property and whiteness to those from dark fleshed fish. Due to the limited fish resources, especially lean fish, pelagic dark-fleshed fish have increasingly gained attention as a potential alternative raw material for surimi production (Arfat and Benjakul, 2012). Apart from the dark color, surimi from sardine generally has poorer gel properties than those produced from lean fish (Kudre *et al.*, 2013). To increase gel forming ability of surimi from dark fleshed fish, the uses of many

additives which are capable of increasing gel strength have been implemented. Various food grade ingredients have been used to increase the gel strength of surimi such as whey protein concentrate (WPC), several plasma proteins, egg white (EW), etc (Rawdkuen *et al.,* 2007). However, beef plasma proteins have been prohibited for the use in surimi due to the religious constraint as well as possible disease transmission (Rawdkuen and Benjakul, 2008). Egg white is expensive and associated with allergy problems (Balange and Benjakul, 2009; Kudre *et al.,* 2013).

Squid and cuttlefish are important fishery in Thailand and serve as one of major parts in marine exports. Ink is one of the by-products produced during processing of squid or cuttlefish. The ink in squid is primarily used as a defence mechanism to escape from the predators (Wood *et al.,* 2010). The ink of the squid, *Illex argentines,* was reported to contain tyrosinase and possess antitumor activity (Naraoka *et al.,* 2003). Tyrosinase, an enzyme present in squid ink, is known to play a key role in the defence against microbes (Takai *et al.,* 1992). A protein extracted from cuttlefish (*Sepioteuthis lessoniana*) ink exhibited the inhibitory effect toward the growth of *Staphylococcus aureus* (Mochizuki, 1979). The ink from *Sepioteuthis lessoniana* and *Sepia pharaonis* showed potential antibacterial activity against biofilm bacteria (Ramasamy and Muruga, 2005). The addition of squid ink to the feed had the effect on the growth performance, antioxidant functions and immunity of broilers (Liu *et al.,* 2011). Although whole ink from squid or cuttlefish has been widely used in foods, especially pasta, etc, the addition of ink can exhibit the negative effect on color and acceptability of foods. Therefore, the removal of melanin of ink could conquer such a problem. Recently, melanin-free ink (MFI) from splendid squid (*Loligo formosana*) has been reported to have the antioxidant activity (Vate and Benjakul, 2013). Since tyrosinase has been known to induce the cross-linking of protein (Buchert *et al.,* 2010), MFI from squid may be used as the natural additive to enhance gel strength of surimi as well as to increase the oxidative stability of gel during the storage. Thus the objectives of this study were to investigate the effect of MFI at different levels on gelling properties of sardine surimi and to evaluate the antioxidative effect of MFI on gel from sardine surimi during the extended refrigerated storage.

3.3 Materials and methods

3.3.1 Chemicals and surimi

2- thiobarbituric acid, *β*-mercaptoethanol (*β*-ME) and wide range molecular weight protein markers were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cumene hydroperoxide was procured from Fluka (Buchs, Switzerland). Acetic acid, methanol, and chloroform were purchased from RCI Labscan Limited (Bangkok, Thailand). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, *N*,*N*,*N'*,*N'*-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

Frozen surimi grade AA from sardine (*Sardinella albella*) was obtained from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand) and kept at -20 °C until use, but not longer than one month.

3.3.2 Preparation of melanin-free ink

Squids were purchased from a local market in Hat Yai, Thailand stored in ice using a squid/ice ratio 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Thailand. Upon arrival, ink sac was separated from the squid by cutting the ink duct and ink was squeezed out from the ink sac. The squid ink was diluted ten-fold using the cold deionized water (2-4 ° C). Then it was subjected to centrifugation at 18,000×g for 30 min at 4 °C using a refrigerated centrifuge (Allegra 25 R centrifuge, Beckman Coulter, Palo Alto, CA, USA) to remove the melanin. The supernatant obtained was used as melanin-free ink (MFI).

3.3.3 Measurement of tyrosinase activity

Tyrosinase activity was assayed using L-DOPA (3,4-dihydroxy-L-phenylalanine) as a substrate according to the method of Simpson *et al.* (1987) with a slight modification. Reaction mixtures consisted of 600 µl of 15 mM L-DOPA in deionized water, 400 µl of 0.05 M phosphate buffer (pH 6.0) and 100 µl of deionized water. To initiate the reaction, 100 µl of MFI were added and the reaction was run for 3 min. The formation of dopachrome at 475 nm was monitored using a UV-160

spectrophotometer (Shimadzu, Kyoto, Japan). One unit of tyrosinase activity was defined as an increase in the absorbance by 0.001 at 475 nm/min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and deionized water was used instead.

3.3.4 Study on the impact of MFI at different levels on properties of surimi gel

3.3.4.1 Gel preparation

Frozen surimi was partially thawed at 4 °C for 6 h prior to cutting into small pieces. The sample was ground for 2 min using a Moulinex Masterchef 350 mixer (Paris, France). NaCl (2.5%, w/w) was then added and the mixture was chopped for 1 min in a walk-in cold room. The surimi paste was added with MFI (0.02, 0.04, 0.06, 0.08 and 0.1 g kg^{-1} paste) and the mixtures were chopped at a high speed (2200 rpm) for 1 min. Final moisture content was adjusted to 80% using cold distilled water (1-2 °C). All mixtures were chopped for another 4 min to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

3.3.4.2 Textural analysis

Gel samples were subjected to textural analysis using a Model TA-XT2i texture analyser (Stable Micro Systems, Surrey, England). Gels were equilibrated and evaluated at room temperature (28–30 °C) for approximately 30 min. Cylinder-shaped samples of 2.5 cm in length were prepared and subjected to determination. Breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a spherical plunger (diameter 5 mm, depression speed of 60 mm/min).

3.3.4.3 Determination of whiteness

Whiteness of gels was measured using a Hunterlab (ColorFlex, Hunter Associates Laboratory, Reston, VA). Illuminant C was used as the light source of measurement. *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by NFI (1991) as follows:

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

3.3.4.4 Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al.* (2001) with slight modifications. A gel sample with a thickness of 0.5 cm was weighed (X g) and placed between two pieces of Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, England) at the top and three pieces of the same type of filter paper at the bottom. The standard weight (5 kg) was placed on the top of the sample and maintained for 2 min. The sample was then removed and weighed again (Y g). Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

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

3.3.4.5 SDS-polyacrylamide gel electrophoresis

Protein patterns of gels were analysed by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution (85 °C) were added to the sample (3 g). The mixture was then homogenized at a speed of 11,000 rpm using a homogenizer (IKA Labortechnik, Selangor, Malaysia) for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. 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 in the supernatant was determined as per the method of Lowry *et al.* (1951). Solubilized samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% β-ME) 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, the gel was 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, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid.

3.3.4.6 Scanning electron microscopy (SEM)

Microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces ($0.25 \times 0.25 \times 0.25$ cm^3) and fixed with 2.5 % (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50%, 70%, 80%, 90%, and 100%. Samples were critical point dried (Balzers mod. CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on a JSM 5800 scanning electron microscope (JEOL, Ltd., Tokyo, Japan) at an acceleration voltage of 20 kV.

3.3.5 Study on oxidative stability of surimi gel added with MFI during the refrigerated storage

Surimi gels containing different MFI levels were packaged in polyethylene bag. The samples were stored at 4 °C and analysed at day 0, 5, 10, 15 and 20 of storage.

3.3.5.1 Measurement of peroxide value

Peroxide value (PV) was determined according to the method of Richards and Hultin (2002) with slight modifications. The surimi gel samples (1 g) were mixed with 11 ml of chloroform/methanol (2:1, v/v) and homogenized at a speed of 12,000 rpm for 2 min. Homogenate was then filtered using Whatman no. 1 filter

paper. Two millilitres of 0.5 % NaCl were then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 6,000×g for 3 min at 4 °C using a refrigerated centrifuge to separate the sample into two phases. To 3 ml of lower phase, 2 ml of cold chloroform/methanol (2:1, v/v), 25 µl of ammonium thiocyanate and 25 µl of iron (II) chloride were added. The reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at the concentration range of 0.5–2 ppm. PV was expressed as milligrams of cumene hydroperoxide equivalent per kilogram of surimi gel.

3.3.5.2 Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) of surimi gel samples were determined according to the method of Egan *et al.* (1981). Sample (10 g) was added with 50 ml of distilled water and the mixture was transferred into a distillation flask containing 47.5 ml of distilled water. Thereafter, 2.5 ml of 4 M HCl were added to bring the pH to 1.5. A few glass beads were added into the mixture before heating using an electric mantle (Kando electric, Samutprakarn, Thailand). Distillation was performed until the distillate of 50 ml was obtained. An aliquot (5 ml) of distillate was placed in a glass-stoppered tube, and 5 ml of TBA reagent (0.2883 g/100 ml of 90 % glacial acetic acid) were added. The mixture was heated in boiling water for 35 min. Distilled water (5 ml) was used as a blank. After heating, the reaction mixture was cooled in water for 10 min and the absorbance at 532 nm (A_{532}) was measured. TBARS value was calculated using the following equation and expressed as milligrams of malonaldehyde per kilogram of surimi gel.

$$TBARS = 7.8 \times A_{532}$$

3.3.5.3 Determination of volatile compounds

Volatile lipid oxidation compounds in surimi gel without and with the addition of MFI from splendid squid and stored at 4 °C for 0 and 20 days were

determined by solid phase micro extraction–gas chromatography–mass spectrometry (SPME–GC– MS) (Iglesias and Medina, 2008).

3.3.5.3.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 3 g of sample was homogenized at a speed of 13,500 rpm for 2 min with 8 ml of ultra-pure water. The mixture was centrifuged at 2000×g for 10 min at 4 °C. The supernatant (6 ml) was heated at 60 °C with equilibrium time of 10 h in a 20 ml headspace vial. Finally, the SPME fibre (50/30 lm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was exposed to the head space of the vial containing the sample extract and the volatile compounds were allowed to absorb in the SPME fibre for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 °C.

3.3.5.3.2 GC–MS analysis

GC–MS analysis was performed in a HP 5890 series II gas chromatography coupled with HP 5972 mass selective detectors equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m × 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 °C for 3 min, followed by an increase of 3 °C/min to 70 °C, then an increase of 10 °C/min to 200 °C and finally an increase of 15 °C/min to a final temperature of 250 °C and hold for 10 min. Helium was employed as a carrier gas, with a constant flow of 1.0 ml/min. Injector was operated in the splitless mode and its temperature was set at 270 °C. Transfer line temperature was maintained at 265 °C. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250 °C. Initially, a full scan mode data was acquired to determine appropriate masses for the later acquisition in selected ion monitoring (SIM) mode under the following conditions: mass range: 25–500 amu and scan rate: 0.220 s/scan. All the analyses were performed with ionization energy of 70 eV, filament emission current at 150 µA and the electron multiplier voltage at 500 V.

3.3.5.3.3 Analysis of the volatile compounds

Identification of the compounds was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds was performed, based on the retention time and mass spectra in comparison with those of standards from ChemStation Library Search (Wiley 275.L). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds, related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

3.3.6 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

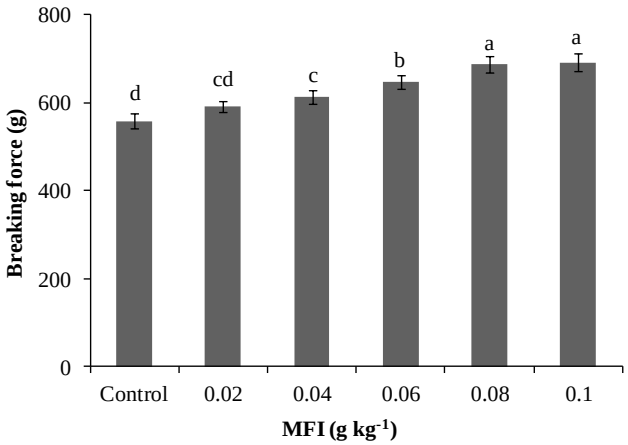
3.4.1 Effect of MFI at different levels on textural properties of surimi gel

3.4.1.1 Breaking force and deformation

Breaking force and deformation of gels from sardine surimi added with MFI at different levels (0-0.1 g kg^{-1}) are depicted in Figure 10. Breaking force and deformation of surimi gel increased with increasing level of MFI up to 0.08 g kg^{-1} (P<0.05). Breaking force and deformation of surimi gels incorporated with MFI at a level of 0.08 g kg^{-1} increased by 23.1% and 12.1%, respectively, compared with those of the control (without addition of MFI). MFI therefore showed gel strengthening effect in sardine surimi. In the present study, MFI contained tyrosinase activity of 1099 ± 80 units/mg. Tyrosinase is able to catalyse the oxidation of tyrosine to DOPA and diquinone (Kim and Uyama, 2005). These diquinones are extremely reactive and can further react with various amino acid side chains such as sulfhydryls, amines, amides,

indoles, and other tyrosines commonly present in proteins (Selinheimo *et al.,* 2008). This resulted in the formation of inter- and intramolecular crosslinks (Mattinen *et al.,* 2008). Onwulata and Tomasula (2010) reported that gelling properties of milk protein was improved through tyrosinase catalysed crosslinking. Gel strengthening effect of

(A)



(B)

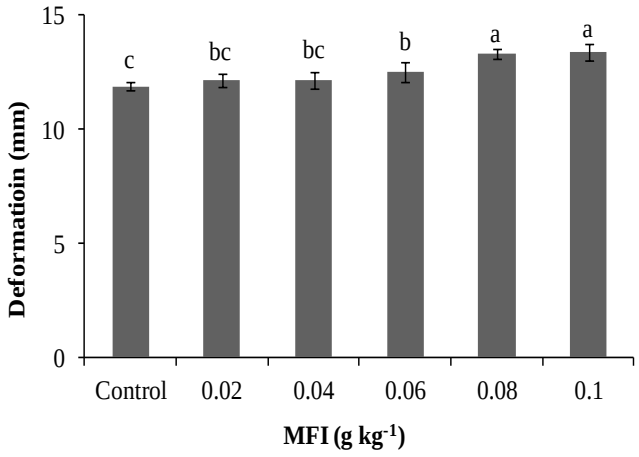


Figure 10. Breaking force (A) and deformation (B) of gels from sardine surimi added with MFI at different levels. Bars represent the standard deviation (n = 3). Different letters on the bars indicate significant differences (P<0.05).

MFI toward sardine surimi depended on the amount of tyrosinase. With increasing MFI levels, the higher tyrosinase activity was obtained. Thus, tyrosinase present in MFI more likely played a role in cross-linking of proteins in surimi, particularly myofibrillar proteins. As a consequence, gel strength of surimi was increased.

3.4.1.2 Whiteness of surimi gel

Whiteness of sardine surimi gels added with different levels of MFI is presented in Table 4. There was no difference in whiteness of all gels, regardless of MFI incorporation (P>0.05). The result indicated that the addition of MFI to surimi gels did not have negative effect on whiteness. Whiteness is one of the quality indices for surimi gel. Since surimi from sardine was quite dark in color due to a large amount of myoglobin, its whiteness was generally lower than that of gel from lean fish surimi. The removal of melanin from the squid ink also improved the color of MFI. Therefore, MFI did not cause the discoloration of resulting surimi gel.

3.4.1.3 Expressible moisture content of surimi gel

The expressible moisture content of gels without and with MFI addition at different levels is given in Table 4. The expressible moisture content of the gels decreased as the levels of MFI increased (P<0.05). The lowest expressible moisture content was observed in gel added with MFI at levels of 0.08 and 0.1 g kg^{-1} (P<0.05). There was no difference in the expressible moisture content between surimi gels added with MFI in the range of 0.02-0.06 g kg^{-1} (P>0.05). This indicated that the surimi gels incorporated with MFI at high levels had higher water holding capacity than those without MFI or added with lower amount of MFI. In general, the fine and strong gel network shows high ability of holding water (Benjakul *et al.,* 2010). The decreased expressible moisture content was in accordance with the increased breaking force and deformation (Figure 10). The results suggested that the higher cross-linking of protein molecules in the surimi, especially during setting aided by the tyrosinase, more likely enhanced water holding capacity of gels added with MFI.

Table 4. Whiteness and expressible moisture content of gels from sardine surimi added with MFI at different levels

MFI (g kg^{-1})	Expressible moisture content (%)	Whiteness
0	4.96±0.12[a]	69.49±1.28[a]
0.02	4.35±0.14[b]	69.29±0.41[a]
0.04	4.31±0.17[b]	69.05±0.74[a]
0.06	4.30±0.13[b]	69.53±0.38[a]
0.08	4.01±0.10[c]	69.70±0.56[a]
0.1	3.97±0.13[c]	69.47±0.75[a]

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

3.4.1.4 Protein patterns of surimi gel

Protein patterns of surimi gels added without and with MFI at different levels (0.02-0.1 g kg^{-1}) are shown in Figure 11. Myosin heavy chain (MHC) was dominant in surimi paste. Actin and tropomyosin could be observed in surimi paste but their band intensity was lower than MHC. For surimi gels, negligible band of MHC was found, regardless of MFI addition. The decrease in MHC band intensity of gel, in comparison with that found in the surimi paste, was more likely due to the polymerization mediated by endogenous transglutaminase. Benjakul and Visessanguan (2003) suggested that the decreases in MHC band intensity in bigeye snapper surimi gels were most likely caused by polymerization of MHC, especially during setting. No differences in MHC band intensity were observed amongst gels with different MFI levels. Similar band intensity of actin and tropomyosin between all gels was noticeable. The result revealed that the addition of MFI more likely led to the formation of larger aggregates in gel matrix. Quinone or diquinone, intermediate from tyrosinase reaction, might induce the cross-linking of proteins in the network along with the formation of non-disulfide covalent bonds mediated by endogenous transglutaminase. As a result, the strength of the gel was increased, particularly when MFI at higher levels was added (Figure 10), whilst no much change in protein pattern was observed (Figure 11). Thus,

tyrosinase in MFI plausibly had the combined effect with endogenous transglutaminase in gel strengthening of sardine surimi.

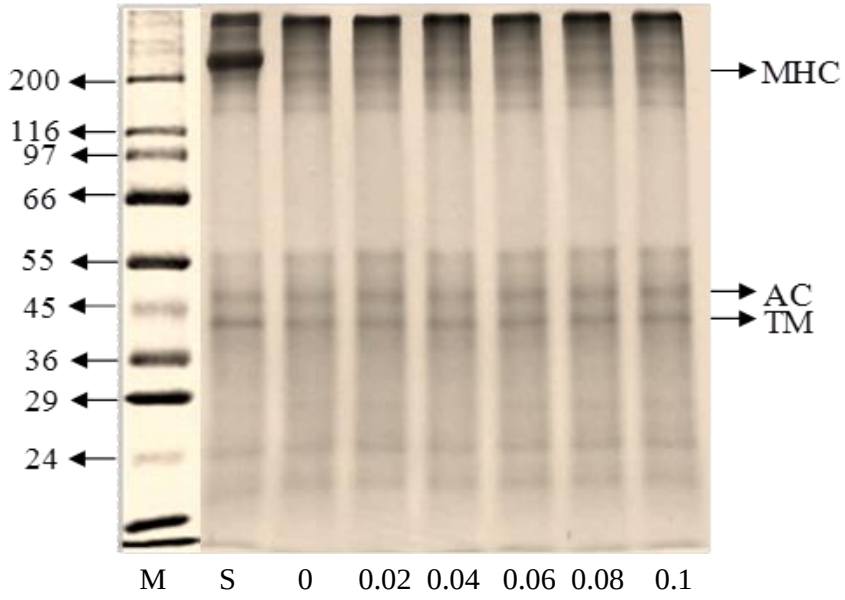


Figure 11. Protein pattern of gels from sardine surimi added without and with MFI at different levels (0-0.1 g kg^{-1}). MHC: myosin heavy chain; AC: actin; TM: tropomyosin; S: surimi. Numbers designate the level of MFI added (g kg^{-1}).

3.4.1.5 Microstructure of surimi gel

Microstructures of surimi gel from sardine without and with MFI addition (0.08 g kg^{-1}) are shown in Figure 12. More compact and denser gel network with finer strand was observed in the surimi gel containing 0.08 g kg^{-1} MFI, compared to the control gel (without MFI). This was coincidental with the higher breaking force and deformation of surimi gel added with 0.08 g kg^{-1}. The higher interconnected three-dimensional protein network of surimi gel was observed with addition of MFI, suggesting that MFI was effective in cross-linking of protein molecules. This resulted in the formation of fine gel network with improved gel strength and water holding capacity. Balange and Benjakul (2009) reported that the mackerel surimi added with oxidized tannic acid possessed a more ordered fibrillar structure with finer strands and high capacity of imbibing water. The result suggested that tyrosinase in MFI more

likely induced the cross-linking of protein along with endogenous transglutaminase during setting at 40 °C.

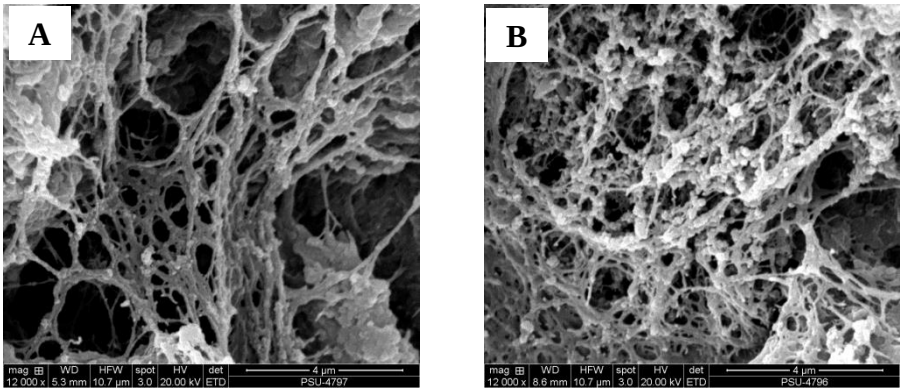


Figure 12. Scanning electron microscopic images of gel from sardine surimi. Gel without MFI (A) and with MFI at a level of 0.08 g kg^{-1} (B). Magnification: ×12,000.

3.4.2 Effect of MFI at different levels on oxidative stability of surimi gel during refrigerated storage

During the storage of 20 days, gel containing MFI at different levels underwent lipid oxidation at various degrees.

3.4.2.1 Peroxide value (PV)

Surimi gels added with MFI at different levels had the lower PV than the control gel throughout the storage (P<0.05) (Figure 13A). The lower PV of surimi gels was observed as the MFI levels increased (P<0.05). The result suggested that MFI acted as antioxidant and inhibited the lipid oxidation in surimi gels in a dose dependent manner. PV of surimi gels without and with MFI addition increased up to 10 day of storage (P<0.05). Thereafter, PV gradually decreased, suggesting that hydroperoxides formed were decomposed to other secondary oxidation compounds. Hydroperoxides break down in several steps, yielding a wide variety of decomposition products, including aldehydes, etc (Maqsood and Benjakul, 2013). The surimi gel added with MFI at 0.1 g kg^{-1} showed the lowest peroxide value. Vate and Benjakul (2013) reported

that MFI had radical scavenging and metal chelating activities and was capable of inhibiting the formation of lipid oxidation in food model systems.

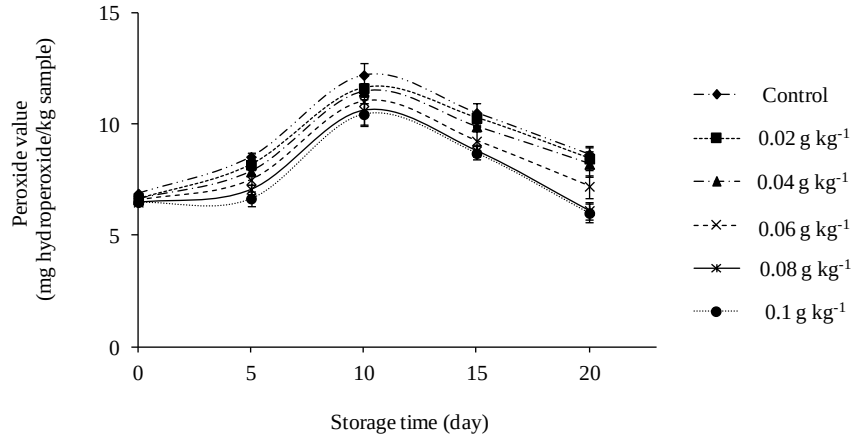
3.4.2.2 Thiobarbituric acid- reactive substances (TBARS)

Changes in TBARS in sardine surimi gels during the storage are depicted in Figure 13B. The TBARS values gradually increased up to 15 days. At day 20, slight decrease in TBARS was noticeable (P<0.05). The decrease in TBARS value at day 20 was more likely due to the loss of low MW volatile secondary products. Surimi gels incorporated with MFI had lower TBARS values than that without MFI. As the MFI level in surimi gel increased, TBARS values decreased (P<0.05). MFI was reported to exhibit DPPH and ABTS radical scavenging activities, ferric reducing antioxidant power and chelating activity (Vate and Benjakul, 2013). The lower TBARS value of surimi gel added with MFI indicated that antioxidative compounds in MFI acted as antioxidant during the storage. MFI could therefore act as the natural antioxidant, apart from serving as gel enhancer.

3.4.2.3 Effect of MFI on the formation of volatile lipid oxidation products in sardine surimi gel

Volatile compounds in sardine surimi gel without and with MFI at a level of 0.1 g kg^{-1}, stored at 4 °C for 0 and 20 days, are shown in Figure 14. At day 0, the sample without MFI had higher abundance of volatile compounds compared to the sample with MFI at a level of 0.1 g kg^{-1} surimi. 1-octen-3-ol was the most dominant compound, followed by 1-penten-3-ol. The result suggested that lipid oxidation took place in the surimi gel during preparation, especially during thermal gelation. The volatile compounds in both samples were higher at 0 day than after 20 days. Amongst the volatile compounds, alcoholic compounds were markedly decreased, plausibly due to volatilization. Alcohols are known as the secondary products produced by the decomposition of hydroperoxides (Girand and Durance, 2000). On the other hand, aldehydes were formed to a high extent after 20 days of storage. Aldehydic volatile compounds such as nonanal, 2-octen-1-al, and 2-decenal were found mainly in samples

(A)



(B)

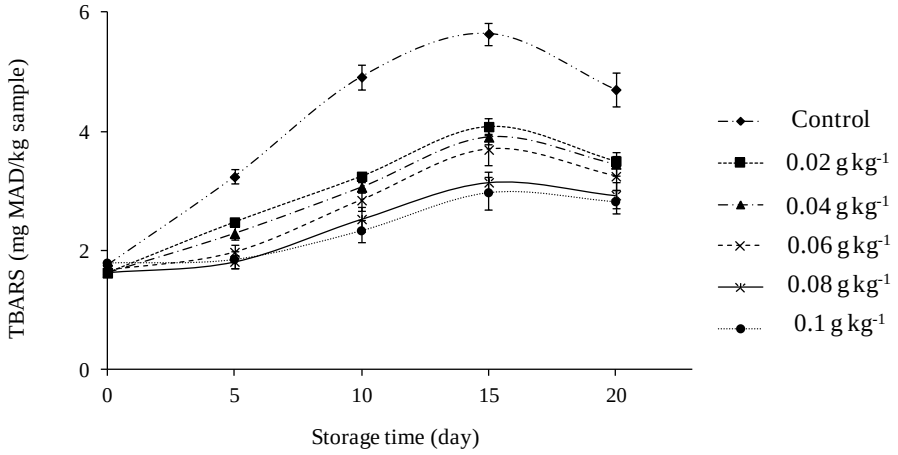


Figure 13. Changes in peroxide value (A) and thiobarbituric acid-reactive substances (TBARS) values (B) of surimi gels without and with MFI at different levels during the storage at 4 °C for 20 days. Bars represent the standard deviation (n=3).

stored for 20 days. Aldehydes are the most prominent volatile compounds during lipid oxidation and successfully used to follow lipid oxidation in many food products including muscle foods (Ross and Smith, 2006). Maqsood and Benjakul (2011) reported that the washed fish mince added with haemoglobin stored in ice for 10 days had more aldehydic volatile compounds and had high peak areas of volatile compounds,

compared to the control. At day 20, the abundance of volatile compounds was lower in the sample added with MFI at 0.1 g kg^{-1}, indicating that MFI could retard lipid oxidation of surimi gel during the refrigerated storage. This result was in accordance with PV and TBARS values where the sample incorporated with MFI at a level of 0.1 g kg^{-1} had the lowest PV and TBARS values.

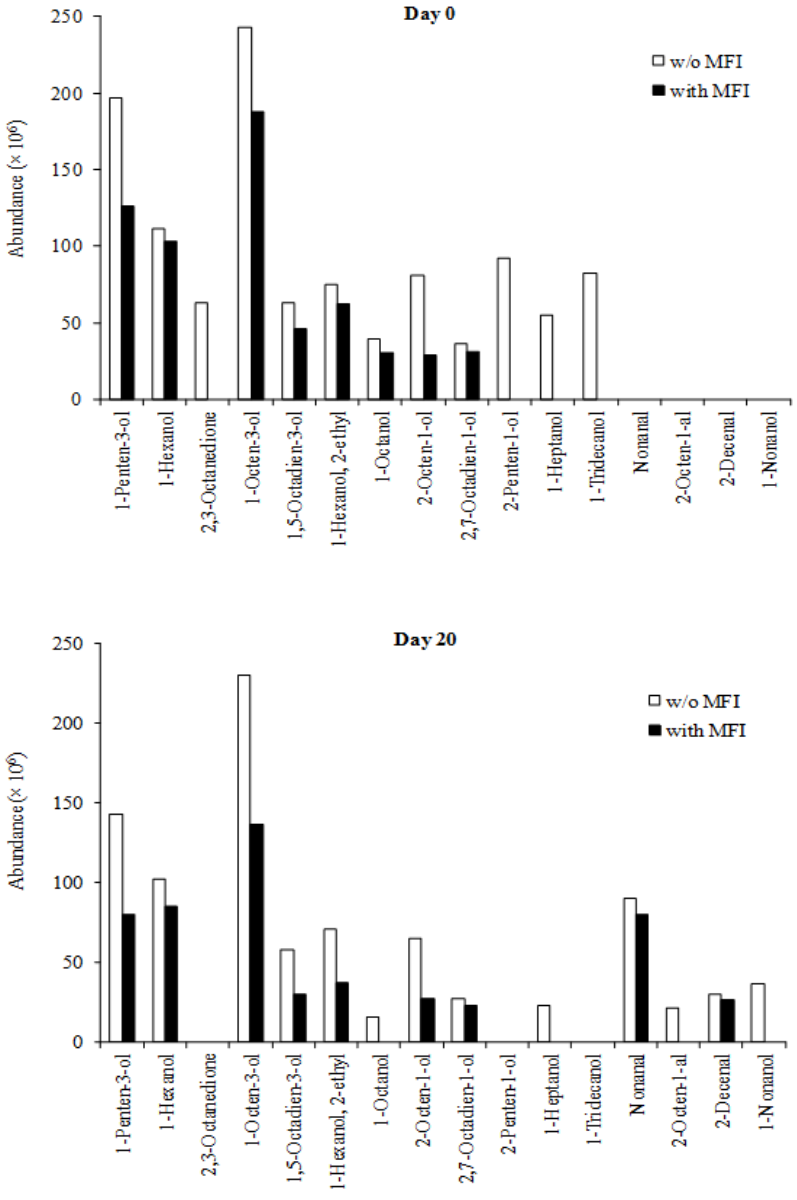


Figure 14. Abundance of lipid oxidation compounds identified by SPME-GC-MS technique in sardine surimi gel without and with MFI at a level of 0.1g kg^{-1}, stored at 4 °C for 0 and 20 days.

3.5 Conclusion

The addition of MFI improved textural properties of gels of sardine surimi without negative effect on whiteness. It also increased water holding capacity of surimi gel. Also MFI inhibited lipid oxidation in surimi gels during the extended refrigerated storage. Hence squid ink, a by-product from squid processing, could be effectively utilized as an alternative multi-functional additive in surimi or other related food products.

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CHAPTER 4

EFFECT OF MIXTURES OF SQUID INK TYROSINASE AND TANNIC ACID ON PROPERTIES OF SARDINE SURIMI GEL

4.1 Abstract

Effect of the mixture of squid ink tyrosinase (SIT) at 300 and 500 U/g protein and tannic acid (TA) at 0.5 and 1% (based on protein) with different reaction times (90 and 180 min) on gel properties of sardine surimi was investigated. Surimi gel incorporated with mixture of SIT (500 U/g protein) and 1% TA with a reaction time of 90 min had the highest breaking force and deformation ($P< 0.05$), in which the increases by 29.3% and 11.9% were observed, in comparison with the control. However, gels added with SIT/TA mixture had the lower whiteness, compared to the control ($P<0.05$). Gel added with SIT/TA mixture showed more compact and finer network with higher connectivity of strands, compared to the control. This was coincidental with decreased expressible moisture content. Based on sensory evaluation, the highest overall likeness score was found in gel added with the mixture of SIT (500 U/g protein) and 1% TA ($P<0.05$). Therefore the mixture of tyrosinase from squid ink and tannic acid could be used as additives to improve the properties of surimi gel.

4.2 Introduction

Surimi is the concentrated myofibrillar proteins, mainly prepared via washing the fish mince in order to remove water soluble sarcoplasmic proteins, lipids and pigments. Gelation is an important property of surimi and determines the quality of surimi. Gelation involves the ordered aggregation of proteins, forming a three dimensional network with water entrapment (Ko *et al.*, 2007). Lean fish has been extensively used for surimi production. Owing to their overexploitation, dark fleshed fish have been used as an alternative raw material for surimi production. Nevertheless, the dark fleshed mince has high contents of lipid and myoglobin, leading to poor gel forming ability (Chaijan *et al.*, 2004). To improve the properties of surimi gel from dark fleshed fish, various food-grade ingredients have been used. Microbial transglutaminase (MTGase) has shown the potential in increasing gel strength of surimi

by introducing non-disulfide covalent bond (Benjakul *et al.,* 2008), whereas protein additives have been widely used to alleviate the softening (modori) induced by endogenous thermostable proteases (Benjakul *et al.,* 2004). However, some additives such as bovine plasma protein, porcine plasma protein and egg white have been prohibited due to safety concern. Additionally, the use of cross-linking enzymes, especially MTGase, is still costly for surimi manufacturing. Therefore, the novel and cheap additives capable of improving gel quality of mince or surimi has been paid increasing attention.

Polyphenols are the natural compounds which are abundant in plants. Tannic acid (TA) belongs to the polyphenol consisting of a central carbohydrate (glucose) and 10 galloyl groups (Lopes *et al.,* 1999). Different kinds of foods such as red wine, coffee, chocolate, tea, sorghum, spinach and fruits (Bananas, grapes and persimmons) contain tannic acid (Lopes *et al.,* 1999; Naczk and Shahidi, 2004). Depending on the type of food to which it is added, tannic acid can be used as a food additive in the range of 10-400 mg/l (Chen and Chung, 2000). TA contains sufficient hydroxyls and other suitable groups (such as carboxyls) to form strong complexes with proteins and other macromolecules. Phenols may be oxidized easily to their corresponding quinones (Hurrell and Finot, 1984). The quinone, a reactive electrophilic intermediate, can readily undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan residues in a protein chain (Hurrell and Finot, 1984). The formation of rigid molecular structures of proteins by ortho-quinones has been demonstrated by Strauss and Gibson (2004).

Tyrosinase is a copper containing enzyme, belonging to the group of polyphenol oxidases (PPOs). Tyrosinase catalyses the oxidation of phenolic ring of tyrosine side chain, inducing the conversion of L-dopa (3,4-dihydroxy-Lphenylalanine) intermediate to diquinone (Kim and Uyama, 2005). These diquinones are extremely reactive and can further react with various amino acid side chains such as sulfhydryls, amines, amides, indoles, and other tyrosines commonly present in proteins, resulting in the formation of inter- and intramolecular crosslinks (Bittner, 2006). Recently, tyrosinase has been reported in squid ink (Vate *et al.,* 2014). The squid ink is a good source of tyrosinase, responsible for synthesis of melanin in squid ink. The tyrosinase

from squid ink can be alternatively used for improving the properties of surimi along with the phenolic compound, which more likely acts as substrate for the enzyme. The quinone formed could serve as the protein cross-linker, thereby improving gel strength of surimi. Hence this study aimed to investigate the effect of tyrosinase from squid ink in combination with tannic acid on the properties of sardine surimi gel.

4.3 Materials and methods

4.3.1 Chemicals and surimi

Tannic acid, 2-thiobarbituric acid, β-mercaptoethanol (β-ME) and wide range molecular weight protein markers were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, *N*,*N*,*N*,*N*-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

Frozen surimi from sardine (*Sardinella albella*) with grade AA was obtained from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand) and kept at -20 °C until use, but not longer than two months.

4.3.2 Preparation of squid ink tyrosianse

4.3.2.1 Preparation of melanin-free ink

Melanin-free ink was prepared according to the method of Vate and Benjakul (2013). Squids were purchased from a local market in Hat Yai, Thailand, stored in ice using a squid/ice ratio 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Thailand. Upon arrival, ink sac was separated from the squid by cutting the ink duct and ink was squeezed out from the ink sac. The squid ink was diluted ten-fold using the cold deionized water (2-4 °C). Thereafter, it was subjected to centrifugation at 18,000×g for 30 min at 4 °C to remove the melanin using a refrigerated centrifuge (Allegra 25 R centrifuge, Beckman Coulter, Palo Alto, CA, USA). The supernatant obtained was used as melanin-free ink (MFI).

4.3.2.2 Fractionation of tyrosainase

Tyrosinase from MFI was fractionated as per the method of Simpson *et al.* (1987) with a slight modification. MFI (50 mL) was mixed with 50 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). The mixture was stirred continuously at 4 °C for 30 min. Solid ammonium sulfate was added into the mixture to obtain 60% saturation. The mixture was allowed to stand at 4 °C for 30 min. The precipitate was collected by centrifugation at 12,500×g at 4 °C for 30 min. The pellet obtained was dissolved in a minimum volume of 0.05 mM sodium phosphate buffer, pH 7.2 and dialysed with 15 volumes of the same buffer with three changes overnight at 4 °C. The fraction containing tyrosinase referred to as 'squid ink tyrosinase, SIT' was kept at -20 °C until used.

4.3.2.3 Measurement of tyrosianse activity

Tyrosinase activity was assayed using L-DOPA (3,4-Dihydroxy-L-phenylalanine) as a substrate according to the method of Simpson *et al.* (1987) with a slight modification. Reaction mixtures consisted of 600 µl of 15 mM L-DOPA in deionized water, 400 µl of 0.05 M phosphate buffer (pH 6.0) and 100 µl of deionized water. To initiate the reaction, 100 µl of SIT were added and the reaction was run for 3 min at room temperature. The formation of dopachrome at 475 nm was monitored using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of tyrosinase activity was defined as the amount of enzyme that induced an increase in the absorbance by 0.001 at 475 nm/min.

4.3.3 *In vitro* oxidation of TA by SIT

Oxidation of TA by SIT was determined at different temperatures. TA solution was prepared in deionized water and pH was adjusted to 7 using 1 M NaOH. TA solution was mixed with SIT to obtain final concentrations of 0.5% and 30 U/mL respectively. The mixture (1 mL) was incubated at various temperatures (25, 30, 35, 40, 50 and 60 °C) for 30 min. For blank, distilled water was used instead of SIT. The increase in the absorbance at 475 nm, representing the formation of quinone, was

recorded after blank subtraction. The temperature yielding the highest quinone formation was selected.

The formation of quinone in the assay system containing SIT and TA was monitored as the function of time. The assay mixture prepared as previously described was incubated at 25 °C. The increase in absorbance at 475 nm was recorded every 10 min up to 180 min.

4.3.4 The impact of SIT/TA mixtures on properties of surimi gel

4.3.4.1 Preparation of SIT/TA mixtures

TA solution (2% w/v) was firstly prepared in deionized water and pH was adjusted to 7 using 1 M NaOH. TA solution was mixed with SIT (2500 U/ mL) to obtain the different TA and SIT working concentrations and incubated at 25 °C for 90 and 180 min. The reaction was terminated by boiling the mixture for 3 min. Thereafter, the obtained mixtures were cooled and used for surimi gel preparation.

4.3.4.2 Gel preparation

Frozen surimi was partially thawed at 4 °C for 6 h prior to cutting into small pieces. The sample was ground for 2 min using a Moulinex Masterchef 350 mixer (Paris, France). NaCl (2.5%, w/w) was then added and the mixture was chopped for 1 min. The surimi paste was added with the prepared SIT/TA solutions to obtain various TA (0.5% and 1% based on protein) and SIT (300 and 500 U/g protein) levels in the surimi paste. The mixtures were then chopped for 1 min. Final moisture content was adjusted to 80% using cold distilled water (1-2 °C). All mixtures were chopped for another 4 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

4.3.4.3 Textural analysis

Gel samples were subjected to textural analysis using a Model TA-XT2i texture analyser (Stable Micro Systems, Surrey, England). Gels were equilibrated and evaluated at room temperature (28–30 °C) for approximately 30 min. Cylinder-shaped samples of 2.5 cm in length were prepared and subjected to determination. Breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a spherical plunger (diameter 5 mm, depression speed of 60 mm/min).

4.3.4.4 Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al.* (2001). Expressible moisture content was expressed as percentage of sample weight.

4.3.4.5 Determination of whiteness

Whiteness of gels was measured using a Hunterlab (ColorFlex, Hunter Associates Laboratory, Reston, VA). Illuminant C was used as the light source of measurement. L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness) were measured and whiteness was calculated as described by NFI (1991) as follows:

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

4.3.4.6 SDS-polyacrylamide gel electrophoresis

Protein patterns of gels were analysed by SDS–PAGE according to the method of Laemmli (1970). Samples solubilized in SDS according to the method described by Benjakul *et al.* (2008) and were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% β-ME) 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, the gel was 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, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid.

4.3.5 Characterization of the selected surimi gels added with SIT/TA mixtures

4.3.5.1 Rheological property

Surimi pastes containing different SIT/TA mixtures were prepared as previously described and were subjected to dynamic rheological measurement following the method of Rawdkuen *et al.* (2008) with a slight modification. A rheometer (HAAKE RheoStress1, ThermoFisher Scientific, Karlsruhe, Germany) with 35 mm, 4^{o} slope cone and plate geometry was used for monitoring the changes in storage or elastic modulus (G'). An oscillation of 1 Hz with 1% deformation was used for testing. This condition yielded a linear response in the viscoelastic region. The temperature sweep was recorded during heating up from 10 to 90 ^{o}C with heating rate of 1 ^{o}C/min. To minimize water evaporation of surimi pastes during measurement, silicon oil was applied to cover the samples.

4.3.5.2 Scanning electron microscopy

Gels were cut into small pieces ($0.25 \times 0.25 \times 0.25$ cm^3) and fixed with 2.5 % (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50%, 70%, 80%, 90%, and 100%. Samples were critical point dried (Balzers mod. CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on a JSM 5800 scanning electron microscope (JEOL, Ltd., Tokyo, Japan) at an acceleration voltage of 20 kV.

4.3.5.3 Sensory evaluation

Gel samples were cut into a bite-size (1 cm thick and 2.5 cm in diameter), equilibrated at room temperature (28-30 ºC) for 30 min and coded with 3-digit random numbers. Gel samples were served on the white paper dishes at room temperature under the fluorescent daylight-type illumination. Eighty non-trained panelists (aged between 20 and 45) were the students and staffs at the Department of Food Technology, who were acquainted with surimi products. The panelists were asked to evaluate for color, taste, texture and overall liking of gel samples using 9-point hedonic scale (Meilgaard *et al.,* 1999). Between samples, the panelists were asked to rinse their mouth with distilled water.

4.3.6 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 *In vitro* oxidation of TA by SIT

Oxidation of TA by SIT as influenced by temperatures was monitored by the increase in A_{475} as depicted in Figure 15A. The highest formation of quinone as indicated by the highest increase in A_{475} was observed at 25 °C. At higher temperature, tyrosinase might undergo denaturation, thereby losing its activity. Yang and Wu (2006) reported that tyrosinase from fungi *Agaricus bisporus* showed the optimal temperature at 27 °C. Nevertheless, phenoloxidase purified from heads of shrimp (*Penaeus setiferous)* had optimum temperature of 45 °C (Simpson *et al.,* 1987). Increase in A_{475} indicates the formation of dopachrome (Simpson *et al.,* 1987). This was most likely caused by the oxidation of TA by SIT. The formation of quinone decreased with

increasing incubation temperatures. Hence the optimum temperature for the oxidation of TA by SIT was 25 °C.

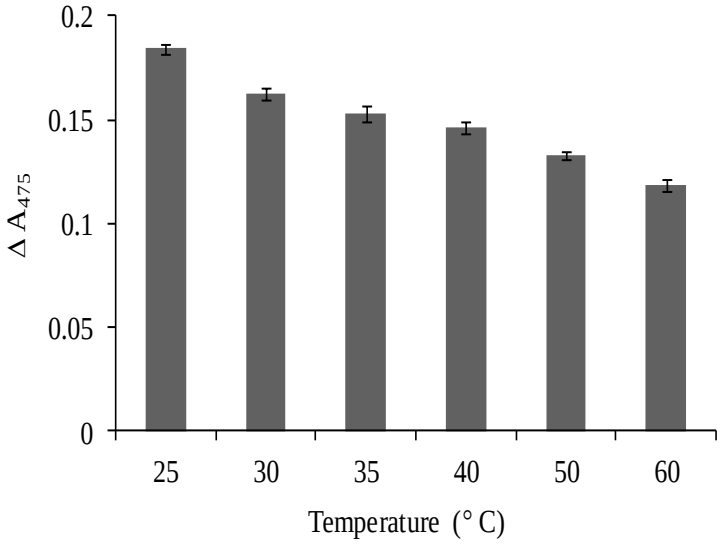
Formation of quinone from TA induced by SIT at 25 °C as a function of time was investigated (Figure 15B). The formation of quinone increased as the incubation time increased and the highest A_{475} was observed after 180 min. This indicated that TA was oxidized by SIT to a higher extent as the incubation time at the optimal temperature increased. Generally phenolic ring of compound is oxidized by tyrosinase, resulting in the formation of L-DOPA (3,4-dihydroxy-Lphenylalanine), which is further converted to the dopachromes (Buchert *et al.*, 2010). Thus the conversion of TA to quinones by SIT required the sufficient time, in which oxidation took place effectively.

4.4.2 Effect of SIT/TA mixtures on textural properties of sardine surimi gel

4.4.2.1 Breaking force and deformation

Breaking force and deformation of sardine surimi gels added without and with SIT/TA mixtures at various concentrations with different reaction times, are depicted in Table 5. Surimi gels added with SIT/TA mixtures had higher breaking force and deformation, compared to the control (without SIT and TA) (P<0.05). In the presence of mixture of 0.5% TA and 300 U SIT/g protein, breaking force of gels increased as the reaction times increased from 90 to 180 min (P<0.05). Tyrosinase is able to react on various monophenolic and diphenolic compounds, such as phenol and catechol or phloretic acid and hydrocaffeic acid. Tyrosine in the side chain of proteins can be oxidized by tyrosinase to quinone, which can further crosslink with lysyl, tyrosyl, and cysteinyl residues in proteins (Buchert *et al.*, 2010). The result suggested that quinone was formed to a higher extent when the incubation time increased. Within the reaction time used, the level of quinone was not excessive for self-aggregation. As a consequence, the quinones formed were available for protein cross-linking, as evidenced by the increased breaking force. The quinones are extremely reactive and can react with various amino acid side chains, resulting in the formation of inter- and intra-molecular protein crosslinks (Mattinen *et al.*, 2008). On the other hand, breaking force and deformation of gels decreased with increasing reaction time when added with

(A)



(B)

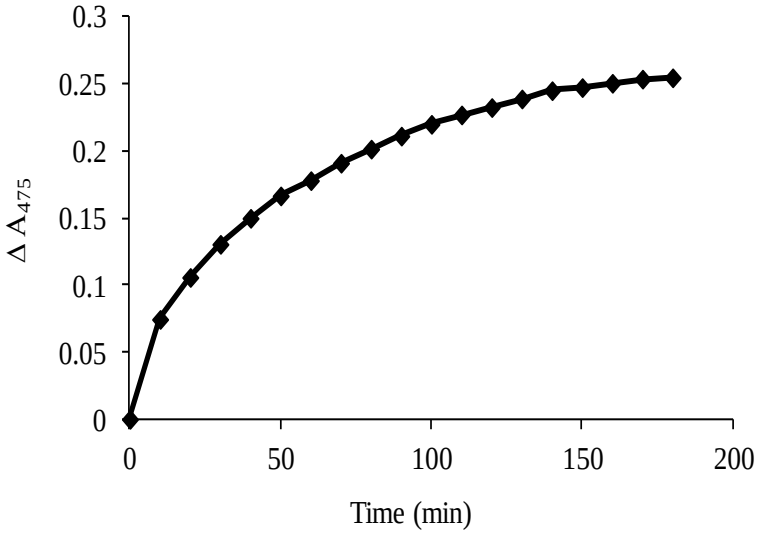


Figure 15. Increase in A_{475} of TA solution (2 % w/v) induced by SIT (2500 U/mL) as the function of temperature (A) and incubation time at 25 °C (B). Bars represent the standard deviation (n=3).

the mixture of 1% TA and 500 U SIT/g protein. With higher level of TA, a larger amount of quinone was generated. This might favour the self-aggregation of oxidized TA, quinone. As a result, the cross-linking sites of oxidized TA were reduced. This led to the poorer gel properties, especially when the reaction time increased (180 min). The concentration of substrate, tyrosinase and the reaction times played an important role in formation of quinone, a protein cross-linking agent. Amongst all samples, surimi gel added with the mixture of 1% TA and 500 U SIT/g protein and reaction time of 90 min had the highest breaking force (P<0.05). Significant increase in the gel strength of bigeye snapper surimi was found when oxidized phenolic compounds and oxidized kiam wood extract were added (Balange and Benjakul, 2009a; 2011). Tyrosinase in combination with caffeic acid increased the cross-linking of whey proteins such as α-lactalbumin and β-lactoglobulin (Thalmann and Lötzbeyer, 2002). Amongst all oxidized phenolic compounds, the oxidized TA exhibited the highest gel strengthening effect, compared with oxidized ferulic acid, catechin and caffeic acid. The use of TA in combination with appropriate washing process was shown to markedly improve the gel property of surimi from dark fleshed fish (Balange and Benjakul, 2009b). Due to the plenty of hydroxyl group in TA, it could induce the hydrogen bonding with hydrogen acceptor in surimi proteins. Both reduced and oxidized forms of TA were able to improve gel property of surimi via the enhanced protein cross-linking.

4.4.2.2 Expressible moisture content

Expressible moisture content of sardine surimi gels without and with different mixtures of SIT and TA is given in Table 5. Expressible moisture content of sardine surimi gels with SIT/TA mixtures was lower, compared to that of control (P<0.05). This indicated that water holding capacity of surimi gels increased when added with the SIT/TA mixture. Lower expressible moisture content of the gels suggests more water retained in the gel network (Niwa, 1992). In general, no differences in expressible moisture content were observed amongst all gel added with different SIT/TA mixtures (P>0.05). The ordered aggregation of finer protein network imbibes more water, as indicated by lowered expressible moisture content. Vate *et al.* (2014) reported that sardine surimi gels added with melanin free ink at levels of 0.08 and 0.1 g/kg surimi had the improved water holding capacity as evidenced by the lowest

expressible moisture content. For gels prepared from mackerel mince washed by conventional process, the lowest expressible moisture content was found with the addition of 0.5% oxidized TA (Balange and Benjakul, 2009b). The results suggested that TA, both reduced and oxidized forms, was able to enhance the formation of ordered gel network, in which water could be more imbibed.

4.4.2.3 Whiteness

Whiteness of sardine surimi gels decreased slightly when added with different SIT/TA mixtures (Table 5). The dark color of TA had significant effect on lowering whiteness of sardine surimi gels. When TA was oxidized by SIT, quinones were formed. This resulted in the darker color of surimi gel. It is also known that the oxidation products of phenolic compounds are colorful, and the shade of these colors is varying from purple to black (Bittner, 2006; Monogioudi *et al.*, 2009). Phenolic compounds were responsible for discoloration in cheese products (O'Connell and Fox, 2001). Balange and Benjakul (2009b) reported that addition of 0.75% oxidized TA resulted in the decrease in whiteness of gels prepared from mackerel surimi. Since gel from sardine was quite dark in color, the slight decrease in whiteness of gel did not result in the obvious decrease in color perception.

4.4.2.4 Protein patterns

Protein patterns of sardine surimi gels without and with different SIT/TA mixtures are shown in Figure 16. Myosin heavy chain (MHC) band was prominent in surimi. No MHC band was observed in gels, regardless of SIT/TA incorporation. It was noted that slight decrease in the intensity of actin band was noticeable in the surimi gels with addition of SIT/TA mixtures. For the control gel (without addition of SIT/TA mixtures), MHC almost completely disappeared. MHC has been known as a preferable substrate for endogenous TGase, which played a major role in protein crosslinking during setting. This result was in agreement with Balange and Benjakul (2009a) who reported that the decrease in MHC band intensity was found in surimi gel from bigeye snapper, regardless of oxidized phenolic addition. Therefore,

Table 5. Breaking force, deformation, expressible moisture content and whiteness of gels from sardine surimi added without and with different SIT/TA mixtures having different reaction times.

SIT (U/g protein)	TA (%) (based on protein)	Incubation time (min)	Breaking force (g)	Deformation (mm)	Expressible moisture content (%)	Whiteness
0	0	0	413.90±3.21^{f}	10.34±0.03^{d}	4.84±0.01^{a}	69.85±0.50^{a}
300	0.5	90	445.25±16.36^{e}	10.73±0.04^{c}	4.55±0.06^{b}	64.56±0.53^{bc}
		180	469.15±11.39^{d}	10.80±0.13^{c}	4.51±0.06^{bc}	65.18±0.47^{bc}
	1	90	506.49±7.66^{bc}	11.24±0.25^{b}	4.51±0.01^{bc}	64.78±1.00^{bc}
		180	489.72±9.34^{c}	10.84±0.18^{c}	4.58±0.01^{b}	65.18±0.52^{bc}
500	0.5	90	512.06±3.12^{b}	11.15±0.13^{b}	4.49±0.11^{bc}	65.43±0.37^{b}
		180	521.91±2.12^{ab}	11.34±0.06^{ab}	4.48±0.06^{c}	64.51±0.42^{c}
	1	90	535.22±19.37^{a}	11.58±0.17^{a}	4.50±0.05^{bc}	64.55±0.52^{c}
		180	466.95±10.79^{d}	10.75±0.18^{c}	4.54±0.01^{b}	64.77±0.37^{bc}

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

the complete disappearance of MHC indicated the superior setting phenomenon of sardine surimi gel. However, actin was still retained, plausibly due to the structural constraint for cross-linking induced by TGase. In the presence of SIT/TA mixture, quinone formed might induce the polymerization of actin to some degrees. Ou *et al.* (2005) reported the polymerization of protein molecules caused by the reaction of different proteins with phenolic substances. Oxidized phenolic compounds, electrophilic in nature, could induce the formation of non-disulfide covalent bonds between proteins (Benjakul and Vissessangan, 2003). Thus, the SIT/TA mixture containing quinones more likely induced the cross-linking of both MHC and actin in sardine surimi. This contributed to the increased gel strength of surimi.

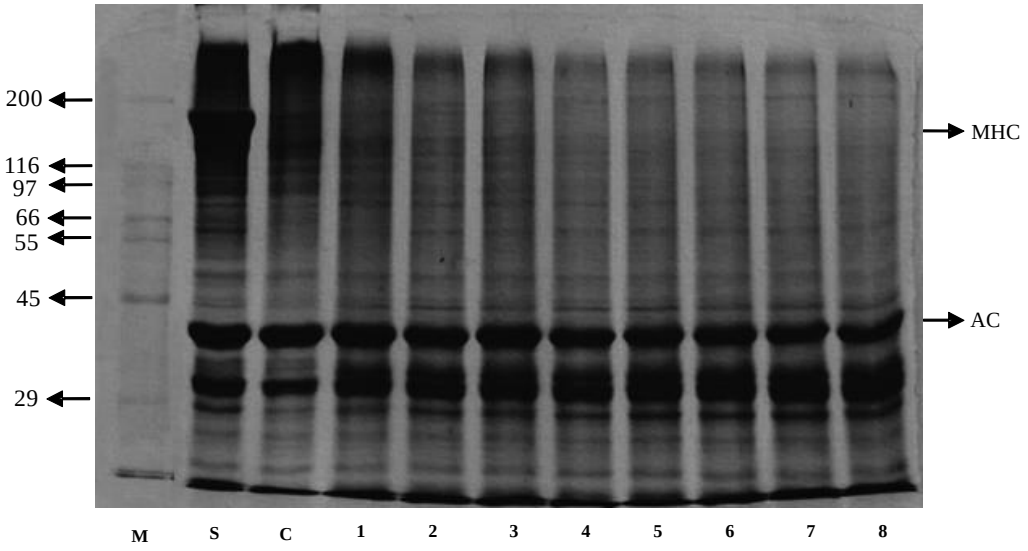


Figure 16. Protein pattern of gels from sardine surimi without and with different SIT/TA mixtures having different reaction times**. M**: Marker; **S**: Surimi; **C**: Control (without SIT/TA mixture); **1**: 0.5%TA, 300U SIT/g, 90min; **2**: 0.5% TA, 300U SIT/g, 180min; **3**: 0.5% TA, 500U SIT/g, 90min; **4**: 0.5% TA, 500U SIT/g, 180min; **5**: 1% TA, 300U SIT/g, 90min; **6**: 1% TA, 300U SIT/g, 180min; **7**: 1% TA, 500U SIT/g, 90min; **8**: 1% TA, 500U SIT/g, 180min; MHC: myosin heavy chain; AC: actin.

4.4.3 Characteristics of the selected surimi gel

4.4.3.1 Rheological property

Changes in storage modulus (G') of surimi paste without and with addition of SIT/TA mixtures during heating from 10 to 90 °C are depicted in Figure 17. The control had the lowest G' throughout the heating. The sample with the mixture of 1% TA and 500U SIT/g protein and the reaction time of 90 min had the highest G', especially during heating at temperature lower than 60 °C. This was in accordance with the highest gel strength. For all the samples, G' increased as the temperature increased up to 35 °C. Subsequently, G' decreased sharply to the lowest value when heated at temperature about 50 °C. This was more likely due to the action of endogenous proteases and dissociation of actomyosin complex. Rawdkuen *et al.* (2007) reported that G' of Pacific whiting surimi reached the minimal value at the temperature of 55 °C. The optimum temperature for proteolytic enzymes in surimi was in the range of 50- 60 °C (Klomklao *et al.*, 2008). G' values of surimi added with SIT/TA mixture increased as the temperature increased from 55 °C to 90 °C, whereas G' value of the control remained constant when heated from 65 to 90 °C. During heating at higher temperature, the unfolded proteins underwent more aggregation. As result, the cross-links with higher molecular weight were formed as evidenced by higher G' values. Higher G' indicated the higher stiffness or firmness of gel (Gordon, 1984). Cross-linking between the dissociated protein molecules mediated by quinones more likely caused the increase in G'. In the presence of 0.5% TA, the incorporation of 500U SIT/g protein with reaction time of 90 min yielded the lower G' value than that added with mixture prepared with reaction time of 180 min. This was in agreement with the breaking force (Table 5). Oxidation of TA by SIT resulted in the formation of quinones which might interact with the reactive side chains of unfolded protein molecules. It was noted that G' values of surimi paste added with SIT/TA mixtures were higher than the control even without heating. This suggested that the quinones formed by the oxidation of TA by SIT in the mixture readily interacted with muscle proteins, in which protein cross-links were formed rapidly. At higher temperatures, TA oxidized by SIT most likely interacted with the unfolded proteins more effectively. This led to the higher polymerization of proteins as indicated by the higher G'.

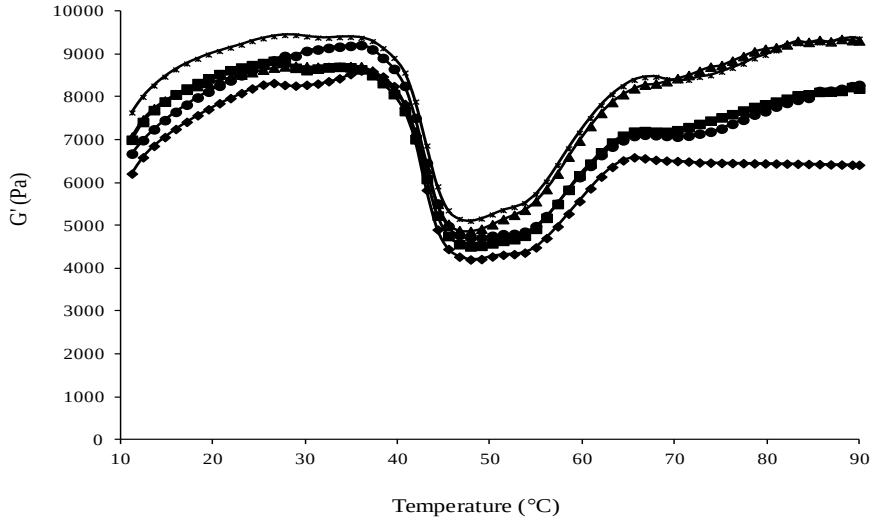


Figure 17. Storage modulus (G') of sardine surimi paste without and with different SIT/TA mixtures having different reaction times. Control (without SIT/TA mixture); 0.5% TA, 500U SIT/g, 90min; 0.5% TA, 500 U SIT/g, 180 min; 1% TA, 300 U SIT/g, 180 min; 1% TA, 500 U SIT/g, 90min.

4.4.3.2 Microstructure of sardine surimi gel

Scanning electron microscopic images of control gel (a), gel with the mixture of 0.5% TA and 500U SIT/g protein with reaction time of 90 min (b), the mixture of 0.5% TA and 500 U SIT/g protein with reaction time of 180 min (c), the mixture of 1% TA and 300 U SIT/g protein with reaction time of 180 min (d) the mixture of 1% TA and 500 U SIT/g protein with reaction time of 90 min (e) are given in Figure 18. Surimi gels containing the SIT/TA mixture had a finer and more interconnected matrix with higher density of strands than the control. This suggested that TA oxidized by SIT plausibly induced the cross-linking of proteins, in which the denser network with a larger number of finer strands was formed. Amongst surimi gels, the gel added with the mixture 1% TA and 500 U SIT/g protein with 90 min incubation time possessed fibrillar structure with more interconnected strands (Figure 18e). This might be attributed to the cross-linking ability of TA oxidized by SIT, which in turn caused the development of aggregated proteins. The gel added with mixture of 1% TA and 300 U SIT/g protein (180 min reaction time) had less compact structure with lower

density of strands than that containing mixture of 1% TA and 500 U SIT/g protein having the shorter incubation time (90 min). The result indicated the paramount role of tyrosinase in protein cross-linking. A large amount of quinones might induce the excessive protein cross-linking. As a result, the coarse network with larger voids could be formed. The finer and ordered gel network with smaller voids was observed in gels with the higher gel strength, whilst the looser network with larger voids was formed in the gels with lower gel strength (Balange and Benjakul, 2009b). Microstructure of gel confirmed that oxidation of TA by SIT proceeded under the optimum condition could provide the appropriate level of quinone for protein cross-linking at the proper level, in which the matrix with interconnected strands could be formed.

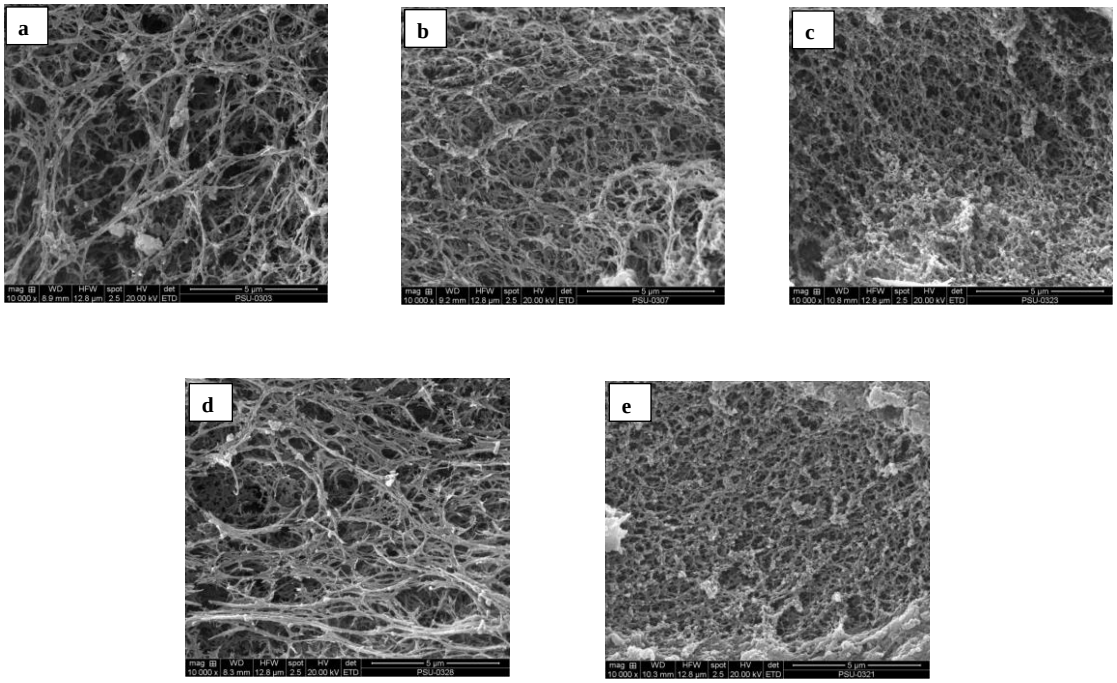


Figure 18. Scanning electron microscopic images of gel from sardine surimi without and with different SIT/TA mixtures having different reaction times. **a**: Control (without SIT/TA mixture); **b**: 0.5% TA, 500U SIT/g, 90min; **c**: 0.5% TA, 500 U SIT/g, 180 min; **d**: 1% TA, 300 U SIT/g, 180 min; **e**: 1% TA, 500 U SIT/g, 90 min. Magnification: ×10,000.

4.4.3.3 Sensory property

Likeness score of sardine surimi gels without and with varying SIT/TA mixtures is shown in Table 6. There was no significant difference in score of appearance likeness amongst all gel samples ($P>0.05$). Surimi gels added with SIT/TA mixtures had lower color likeness score, compared to the control ($P<0.05$). This could be attributed to the darker color of the gels as influenced by oxidized TA induced by SIT. It was noted that gel added with 1% TA and 500U SIT/g protein with 90 min reaction time showed the higher texture and overall likeness score than others ($P<0.05$). This was coincidental with the increased breaking force and deformation and decreased expressible moisture content of the corresponding gel (Table 5). However, the taste likeness score for the samples added with SIT/TA mixtures was lower, compared to that of control. This was more likely due to the bitter taste mediated by the tannic acid. O'Connell and Fox (2001) stated that phenolic compounds play a role in the sensory attributes of many food products. There was no detrimental effect on the acceptability when chestnut and grape seed extracts, containing a high proportion of polyphenols, were added into dry cured sausages. The incorporation of seaweed extract into lesser sardine surimi had no impact on sensory property (Shitole *et al.*, 2014). Thus, the addition of SIT/TA mixture more likely improved sensory property by enhancing gel strength.

Table 6 Sensory properties of gels from sardine surimi added without and with different SIT/TA mixtures having different reaction times.

Samples	Appearance	Color	Odor	Texture	Taste	Overall
a	7.06±0.83^{a}	7.27±0.76^{a}	6.94±0.61^{a}	6.67±0.69^{c}	7.36±0.49^{a}	6.91±0.76^{b}
b	6.85±0.56^{a}	6.64±0.60^{b}	6.97±0.73^{a}	7.12±0.60^{b}	6.97±0.68^{b}	6.91±0.68^{b}
c	6.91±0.68^{a}	6.67±0.54^{b}	6.85±0.62^{a}	7.00±0.61^{b}	6.82±0.63^{b}	7.03±0.58^{ab}
d	7.03±0.58^{a}	6.70±0.47^{b}	7.03±0.77^{a}	6.91±0.52^{bc}	6.73±0.57^{b}	6.94±0.66^{b}
e	7.00±0.66^{a}	6.66±0.48^{b}	6.97±0.68^{a}	7.48±0.71^{a}	6.97±0.73^{b}	7.33±0.54^{a}

Mean ± S.D (n =80). Different superscripts in the same column indicate significant differences (P<0.05).

a: Control (without SIT/TA mixture); **b**: 0.5% TA, 500U SIT/g, 90min; **c**: 0.5% TA, 500 U SIT/g, 180 min; **d**: 1% TA, 300 U SIT/g, 180 min; **e**: 1% TA, 500 U SIT/g, 90 min.

4.5 Conclusion

The mixture of SIT and TA improved the breaking force and deformation of sardine surimi gels. Water holding capacity of gels was increased with the addition of mixtures, but the whiteness was slightly decreased. The use of 500U SIT/g protein and 1% TA mixture with reaction time of 90 min increased the gel strength of sardine surimi gel effectively. The resulting gel with finer and compact structure had the higher texture and overall likeness scores, compared with control. Hence the mixture of tyrosinase from squid ink and tannic acid could be utilized as the additive to improve the gel strength of sardine surimi.

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CHAPTER 5

COMBINED EFFECT OF SQUID INK TYROSINASE AND TANNIC ACID ON HEAT INDUCED AGGREGATION OF NATURAL ACTOMYOSIN FROM SARDINE

5.1 Abstract

The mixture of squid ink tyrosinase (SIT) at 300 and 500 U/g protein and tannic acid (TA) at 0.5 and 1% (based on protein) increased the turbidity and surface hydrophobicity of natural actomyosin (NAM) from sardine (*Sardinella albella)* in a dose dependent manner. Total sulfydryl group content decreased in the NAM solutions with coincidental increase in disulfide bond content, when added with SIT/TA mixture. Ca^{2+}-ATPase activity was also lower in NAM solutions added with SIT/TA mixture. Higher aggregation of protein filaments was noticeable in NAM added with SIT at 500 U/g protein and 1% TA, which also had the highest storage modulus (G') and largest particle size. Negative charge of NAM was decreased when SIT/TA mixture was incorporated. Therefore, the mixture of SIT and TA, particularly at higher level, could be used as the novel protein cross-linker in fish mince and surimi.

5.2 Introduction

Myofibrillar proteins including myosin and actin, found as actomyosin complex, play an important role in gelation of fish mince and surimi (Niwa, 1992). Gelation is the main parameter determining the quality of surimi. Thermal gelation of fish muscle proteins has been reported to occur in a three-step process including (1) dissociation of myofibril structures by protein solubilization in the presence of salt; (2) partial unfolding of protein structure induced by heat treatment; and (3) aggregation of unfolded protein via both covalent and non-covalent bonds to form a three-dimensional network (Stone & Stanley, 1992). The conformational change of actomyosin caused by heat correlates with the exposure of functional groups such as sulfydryl groups and hydrophobic domains (Benjakul *et al.*, 2001). Subsequently, those groups most likely undergo disulfide bond formation and hydrophobic interaction, respectively. Additionally, gelation is dependent upon temperature (Sano *et al.*, 1994), heating rate

(Yongsawatdigul and Park, 1999), pH and type of actomyosin (Lefevre *et al.,* 2007). Generally lean fish are used for surimi production due to their high gelation and whiteness. Because of their overexploitation, dark fleshed fish have been used as an alternative source. The dark fleshed mince has high contents of lipid, myoglobin as well as proteases and generally exhibits poor gel forming ability (Chaijan *et al.,* 2004). Therefore, various food-grade additives such as microbial transglutaminase (MTGase), bovine plasma protein, porcine plasma protein and egg white have been used to improve the properties of surimi from dark fleshed fish (Benjakul *et al.,* 2008; Benjakul *et al.,* 2004; Benjakul *et al.,* 2004). However, the use of cross-linking enzymes such as MTGase in surimi may not be economical because of its high cost and other protein additives have been prohibited due to safety concern. Hence there is a need for novel, cheap and effective additives, which are capable of improving the properties of fish mince and surimi.

Tannin belongs to the polyphenol group abundantly found in plants. Tannic acid (TA) is a specific commercial form of tannin consisting of a central carbohydrate (glucose) and 10 galloyl groups (Lopes *et al.,* 1999). Tannin contains sufficient hydroxyls and other groups such as carboxyls to form strong complexes with the proteins and other macromolecules (Kroll *et al.,* 2003). TA can be oxidized to corresponding quinones by enzymatic and non-enzymatic reaction (Hurrell and Finot, 1984; Balange and Benjakul, 2010). The quinones are extremely reactive and can further react with various amino acid side chains such as sulfydryls, amines, amides, indoles, and other tyrosines commonly present in proteins, resulting in the formation of inter and intramolecular crosslinks (Ito *et al.,* 1984; Burzio and Waite, 2000; Bittner, 2006; Mattinen *et al.*, 2008).

Polyphenol oxidases (PPOs) are copper containing enzymes, which are found in mammals, plants, fungi, and other microorganisms. Tyrosinase, belonging to the group of PPOs, is a bifunctional enzyme as it catalyses ortho-hydroxylation of monophenols (monophenolase or cresolase activity) and subsequent oxidation of diphenols (diphenolase or catecholase activity) to quinones. Tyrosinase can oxidize tyrosine in the side chain of proteins to the quinone, which can further crosslink with lysyl, tyrosyl, and cysteinyl residues of proteins (Selinheimo *et al.,* 2008). Tyrosinase

also reacts on various monophenolic and diphenolic small compounds, such as phenol and catechol or phloretic acid and hydrocaffeic acid (Buchert *et al.*, 2010). Recently, squid ink tyrosinase (SIT) has been extracted from squid melanin-free ink (Vate and Benjakul, 2015). This tyrosinase along with some polyphenols, which act as substrates for the enzyme, can be used as the protein cross-linker in gelly food products. Therefore, the objectives of this study were to elucidate the combined impact of SIT and TA on the heat induced aggregation and to investigate physicochemical changes of natural actomyosin from sardine (*Sardinella albella*) during heating at various temperatures.

5.3 Materials and methods

5.3.1 Chemicals

Adenosine-5′-triphosphate (ATP), 8-anilino-1-naphthalenesulfonic acid (ANS), guanidine thiocyanate, sodium hydrogen sulfite, β-mercaptoethanol (β-ME) and Tris-maleate were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Potassium chloride, sodium chloride, calcium chloride, trichloroacetic acid, potassium dihydrogen phosphate and ammonium molybdate were procured from Merck (Darmstadt, Germany). 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Bovine serum albumin (BSA) was obtained from Fluka (Buchs, Switzerland).

5.3.2 Collection and preparation of fish

Sardines (*Sardinella albella*) with an average weight of 50-60 g were caught from Songkhla coast along the Gulf of Thailand. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. The fish were headed, gutted and washed with water. The flesh was separated manually from skin and bone and kept on ice not longer than 24 h.

5.3.3 Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Balange and Benjakul (2010) with a slight modification. Sardine mince was homogenized in chilled 0.6 M KCl, pH 7.0 at a ratio of 1:10 (w/v) using a homogenizer (IKA, Labortechnik, Selangor, Malaysia). To avoid overheating, the sample was placed in ice and homogenized for 20 s, followed by a 20 s rest interval for a total extraction time of 4 min. The homogenate was centrifuged at 5000g for 30 min at 4 ℃ using a refrigerated centrifuge (Avanti J-E Centrifuge; Beckman Coulter, Fullerton, CA, USA). Three volumes of chilled deionized water (0–2 °C) were added to precipitate NAM. The NAM was collected by centrifuging at 5000g for 20 min at 4 ℃. The NAM pellet was dissolved in chilled 0.6 M KCl, pH 7.0 for 30 min at 4 ℃ and then centrifuged at 5000g for 20 min at 4 ℃. The supernatant was collected and used as NAM.

5.3.4 Preparation of squid ink tyrosinase

5.3.4.1 Preparation of melanin-free ink

Melanin-free ink was prepared according to the method of Vate and Benjakul (2013). Squids were purchased from a local market in Hat Yai, Thailand, stored in ice using a squid/ice ratio of 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Thailand. Upon arrival, ink sac was separated from the squid by cutting the ink duct and ink was squeezed out from the ink sac. The squid ink was diluted ten-fold using the cold deionized water (2-4 °C). Then it was subjected to centrifugation at 18,000×g for 30 min at 4 °C to remove the melanin using a refrigerated centrifuge (Allegra 25 R centrifuge, Beckman Coulter, Palo Alto, CA, USA). The supernatant obtained was used as melanin-free ink (MFI).

5.3.4.2 Fractionation of tyrosinase

Tyrosinase from MFI was fractionated as per the method of Simpson, Marshall and Otwell (1987) with a slight modification. MFI (50 mL) was mixed with 50 mL of 0.05 M sodium phosphate buffer (pH 7.2), containing 1.0 M NaCl and 0.2% Brij 35. The mixture was stirred continuously at 4 ℃ for 30 min. Solid ammonium sulfate was added into the mixture to obtain 60% saturation. The mixture was allowed

to stand at 4 °C for 30 min. The precipitate was collected by centrifugation at 12,500×g at 4 °C for 30 min. The pellet obtained was dissolved in a minimum volume of 0.05 mM sodium phosphate buffer (pH 7.2) and dialysed with 15 volumes of the same buffer with three changes overnight. The fraction containing tyrosinase referred to as 'squid ink tyrosinase, SIT' was kept at -40 °C until used.

5.3.4.3 Measurement of tyrosinase activity

Tyrosinase activity was assayed using L-DOPA (3,4-Dihydroxy-L-phenylalanine) as a substrate according to the method of Simpson *et al.* (1987) with a slight modification. Reaction mixtures consisted of 600 µl of 15 mM L-DOPA in deionized water, 400 µl of 0.05 M phosphate buffer (pH 6.0) and 100 µl of deionized water. To initiate the reaction, 100 µl of SIT was added and the reaction was run for 3 min at room temperature. The formation of dopachrome was monitored by reading at 475 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of activity was defined as the enzyme causing an increase in the absorbance at 475 nm by 0.001/min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and deionized water was used instead.

5.3.5 Study on the effect of SIT/TA mixture at different concentrations on heat-induced aggregation of NAM

5.3.5.1 Preparation of SIT/TA mixtures

TA solution (2% w/v) was firstly prepared in deionized water and pH was adjusted to 7 using 1 M NaOH. TA solution was mixed with SIT (2500 U/ mL) to obtain the different TA (0.5 and 1%) and SIT (300 and 500 U) as the working concentrations in NAM.

5.3.5.2 Effect of heat treatment on aggregation and physiochemical changes of NAM added with SIT/TA mixtures

NAM was diluted to 1 mg/mL with chilled 0.6 M KCl (pH 7.0). NAM solutions added with the prepared SIT/TA mixtures to obtain various TA (0.5% and 1% based on protein) and SIT (300 and 500 U/g protein) levels, were heated at a heating

rate of 0.65 °C/min from 20 to 75 °C using a temperature controlled water bath (Memmert, Schwabach, Germany). NAM without the addition of SIT/TA mixture was used as the control. During the heating, the samples were taken every 5 °C of temperature increment. At the designated temperature, the samples were cooled immediately with iced water. The samples obtained were subjected to analyses.

5.3.5.3 Analysis

5.3.5.3.1 Turbidity

Different NAM solutions (1 mg protein/mL) were placed in the cuvette (light path length of 1 cm). Turbidity indicating the degree of protein aggregation was measured by reading the absorbance at 660 nm (Benjakul *et al.,* 2001) using a UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

5.3.5.3.2 Surface hydrophobicity

Surface hydrophobicity was measured as per the method of Benjakul *et al.* (2001) using 8-anilo-1-naphthalenesulfonic acid (ANS) as a probe. Heated NAM solutions were diluted to 0.125, 0.25, 0.5 and 1 mg/mL using 0.6 M KCl (pH 7.0). To 2.0 mL of diluted NAM solution, 10 µL of 10 mM ANS dissolved in 50 mM potassium phosphate buffer (pH 7.0) were added and the mixtures were mixed thoroughly using a vortex mixture (Vortex Genie-2, Scientific Industries, Inc. New York, USA). Sample blanks of each protein concentration were prepared in the same manner, except the same volume of 50 mM potassium phosphate buffer (pH 7.0) was used instead of ANS solution. Fluorescence intensity was measured using a RF-1501 spectrofluorometer (Shimadzu, Kyoto, Japan) at the excitation and emission wavelength of 374 and 485 nm, respectively. Surface hydrophobicity was calculated from initial slope of plot between fluorescence intensity against protein concentration using a linear regression analysis. The initial slope was referred to as S_0ANS.

5.3.5.3.3 Total sulfydryl group and disulfide bond contents

Total sulfydryl group content was measured using 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) following the method of Ellman (1959) as modified by

Benjakul et al. (2001). To 1.0 mL of sample solutions, 9 mL of 0.2 M Tris–HCl buffer, pH 8.2, containing 8 M urea, 2% SDS and 10 mM ethylenediaminetetraacetic acid (EDTA) were added. To 4 mL of the mixture, 0.4 mL of 0.1% DTNB was added and incubated at 40 °C for 25 min. The absorbance at 412 nm was then measured. A sample blank was conducted in the same manner except that distilled water was used instead of DTNB. Total sulfydryl group content was calculated using the extinction coefficient of 13,600 M^{-1} cm^{-1}.

Disulfide bond content was determined using 2-nitro-5-thiosulfobenzoate (NTSB) (Thannhauser *et al.*, 1987). To 0.5 mL of sample solution, 3.0 mL of freshly prepared NTSB assay solution were added. The mixture was incubated in the dark at room temperature (26–28 °C) for 25 min. A sample blank was prepared in the same manner but the distilled water was used instead of NTSB assay solution. The absorbance at 412 nm was read. The disulfide bond content was calculated using the extinction coefficient of 13,900 M^{-1} cm^{-1}.

5.3.5.3.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of NAM solutions added without and with SIT/TA mixture at 20 °C and after heating to reach the temperature of 75 °C were analysed using SDS–PAGE according to the method of Laemmli (1970). The samples were mixed with sample buffer (4 ml of 10% SDS, 2 ml of glycerol, 1 ml of β-mercaptoethanol, 2.5 ml of 0.5 M Tris– HCl (pH 6.8), and 0.03 g Bromophenol blue) at 3:1 ratio (v/v). The samples (12 μg protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit (Bio-Rad Laboratories Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid. Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems).

5.3.5.4 Study on the effect of SIT/TA mixtures on Ca^{2+}-ATPase activity of NAM during heating

Firstly, NAM solutions (1 mg protein/mL) were heated and reached different temperatures (20, 30, 40, 50 and 60 °C), followed by cooling as previously described. Ca^{2+}-ATPase activity of NAM samples was determined according to the method of Benjakul *et al.* (1997). One mL of solution was mixed with 0.6 mL of 0.5 M Tris-maleate (pH 7.0). The mixture was further added with $CaCl_2$ solution to obtain a final concentration of 10 mM $CaCl_2$ with the total volume of 9.5 mL. To initiate the reaction, 0.5 mL of 20 mM ATP was added. The reaction was conducted for exactly 10 min at 25 °C and was terminated by adding 5 mL of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500g for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Ca^{2+}-ATPase activity was expressed as µmoles of inorganic phosphate released per mg protein per min. A blank solution was prepared by adding chilled trichloroacetic acid prior to ATP addition.

5.3.5.5 Study on rheological property of NAM added with SIT/TA mixtures during heating

NAM solution (20 mg protein/mL) containing different SIT/TA mixtures were subjected to dynamic rheological measurement following the method of Rawdkuen *et al*. (2008) with a slight modification. A rheometer (HAAKE RheoStress1, ThermoFisher Scientific, Karlsruhe, Germany) with 60 mm parallel plate geometry was used for monitoring the changes in storage modulus (G'). An oscillation of 2.1 Hz with 0.05% strain was used for testing. This condition yielded a linear response in the viscoelastic region. The temperature sweep was recorded during heating from 20 to 75 ^{o}C with heating rate of 1 ^{o}C/min.

5.3.5.6 Study on the effect of SIT/TA mixtures on charge, size and microstructure of heated NAM

5.3.5.6.1 Zeta potential and particle size

NAM solutions (1 mg protein/mL) added with the selected SIT/TA mixtures were heated to reach the final temperature of 75 °C. The mixtures were cooled and then stirred gently for 6 h in ice. Thereafter, the mixtures were allowed to stand at 4 °C for 30 min prior to analysis. The zeta (ζ) potential and particle size of NAM solutions were measured using a ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY, USA) at room temperature.

5.3.5.6.2 Transmission electron microscopy

NAM solutions (1 mg protein/mL) added with the selected SIT/TA mixtures, were heated to reach the final temperature of 75 °C, followed by cooling. The prepared samples were diluted to 0.2 mg/mL with 50 mM potassium phosphate buffer containing 0.6 M KCl (pH 7). A drop of sample was fixed for 5 min on a carbon-coated grid, negatively stained with 4% uranyl acetate for 5 min and washed with distilled water until the grid was cleaned. The specimens were visualized using a JEOL JEM-2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) (×60,000) at an accelerating voltage of 160 kV.

5.3.5.7 Protein determination

The protein content of NAM was determined by the Biuret method (Robinson and Hogden, 1940) using bovine serum albumin as the standard.

5.3.6 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

5.4 Results and discussion

5.4.1 Effect of SIT/TA mixture at different concentrations on heat induced aggregation of NAM

5.4.1.1 Turbidity

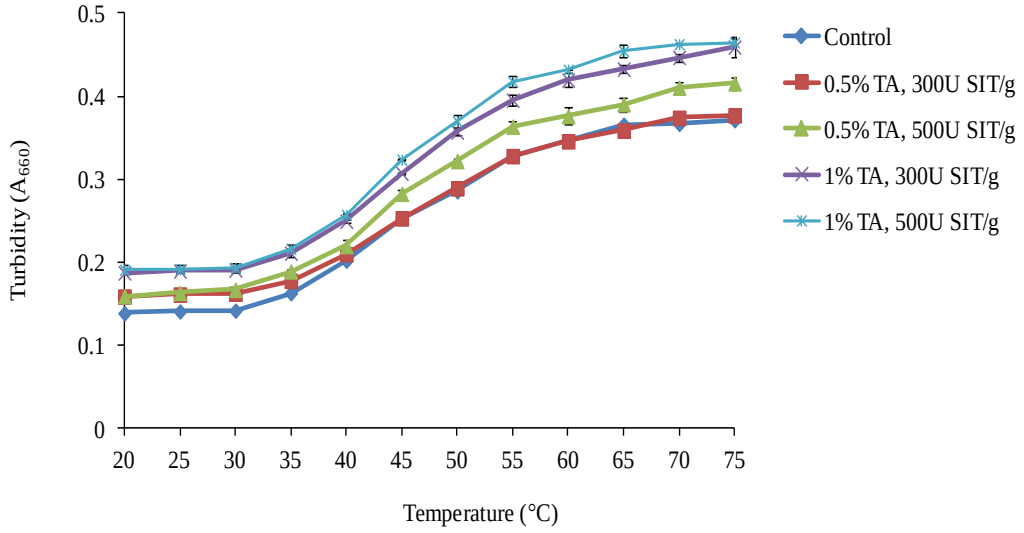
Changes in turbidity of NAM solutions added without and with different SIT/TA mixtures during heating from 20 to 75 °C are given in Figure 19A. Turbidity expressed as absorbance at 660 nm is commonly used to monitor the extent of protein aggregates (Benjakul *et al.*, 1997; Yarnpakdee *et al.*, 2009). Turbidity of NAM solutions increased as the temperature increased. Myosin molecule is bound to the actin filament at its head portion with its tail portion sticking out, known as actomyosin (Sano *et al.*, 1988). Upon heating, NAM molecules tend to interact with each other and form protein aggregates. The NAM solutions added with SIT/TA mixtures had more turbidity, compared to the control (without SIT/TA mixture) (P<0.05). At the same concentrations of TA, higher turbidity was observed in NAM samples having higher level of SIT (500 U/g). Furthermore, higher turbidity was found in NAM added with higher level of TA (P<0.05) when the same SIT level was used. The result indicated the essential role of both SIT and TA in the formation of quinone, a protein cross-linker. Turbidity has been used as a rough estimate of the degree of aggregation and is affected by several factors such as pH and/or ionic strength (Hermansson, 1986). Amongst all treatments, the highest turbidity was found in NAM solution added with mixture of 1% TA and 500 U/g SIT throughout the heating up to 75 °C (P<0.05). It was noted that the turbidity of NAM solutions added with SIT/TA mixture was higher, compared to the control even at 20 °C (P<0.05). The result indicated that quinones formed via the oxidation of TA by SIT readily reacted with the protein molecules, leading to the formation of aggregates. The tyrosinase from melanin free squid ink showed the optimal temperature at 25 °C (Vate and Benjakul, 2015). Marked increase in turbidity was observed when temperatures were above 30 °C. At temperature higher than 30 °C, unfolding of protein molecules occurred, more likely due to the instability of hydrogen bonds. As a consequence, the greater numbers of hydrophobic portions were exposed (Niwa, 1992). Those unfolded proteins subsequently underwent aggregation via

hydrophobic-hydrophobic interaction as indicated by increased turbidity. When both TA and SIT were present, TA served as a substrate for SIT, in which quinones could be formed and released into NAM solution. These quinones could induce cross-linking of proteins via reactive groups exposed during heating, thereby enhancing the protein cross-linking. Quinone, an electrophilic group, in oxidized TA was able to interact with the unfolded proteins, mainly via the amino group, a nucleophilic counterpart (Balange and Benjakul, 2010). Thus, the SIT/TA mixture could induce the aggregation of NAM and the efficacy was augmented as the concentrations of both SIT and TA used increased.

5.4.1.2 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) of NAM samples added without and with SIT/TA mixtures at different concentrations during heating from 20 to 75 °C is illustrated in Figure 19B. S_0ANS of all NAM samples increased as the temperature increased (P<0.05). Increase in S_0ANS indicated that the hydrophobic domains of NAM were exposed during heating. The higher S_0ANS was observed for NAM added with SIT/TA mixtures, particularly when high levels of both SIT and TA were used (P<0.05). It was noted that NAM added with SIT/TA mixtures showed the higher S_0ANS than the control, even in unheated samples. The results indicated that TA oxidized by SIT more likely induced the conformational changes of NAM to some extent, as evidenced by the increase in surface hydrophobicity. ANS, fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as phenylalanine and tryptophan (Benjakul *et al.*, 1997). The increase in ANS binding was related with the presence of exposed hydrophobic sites of unfolded proteins. The aggregated proteins via those exposed hydrophobic domains subsequently led to the decrease in surface hydrophobicity (Benjakul *et al.*, 2001). The exposure of hydrophobic domains has been suggested as a prerequisite for the formation of large myosin aggregates via hydrophobic interaction (Chan *et al.*, 1992; Visessanguan *et al.*, 2000). In the presence of SIT/TA mixture, the attachment of oxidized tannic acid with NAM also provided the hydrophobic benzene ring into the proteins as indicated by the higher surface hydrophobicity (P<0.05). With the low amount of quinones associated with low concentrations of both SIT and TA (mixture of 0.5%TA and SIT at 300 U/g),

(A)



(B)

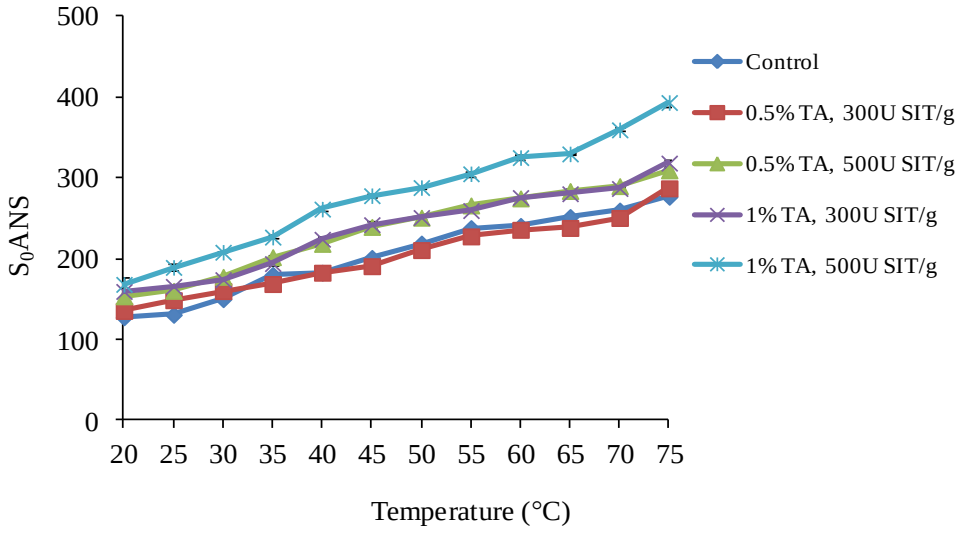


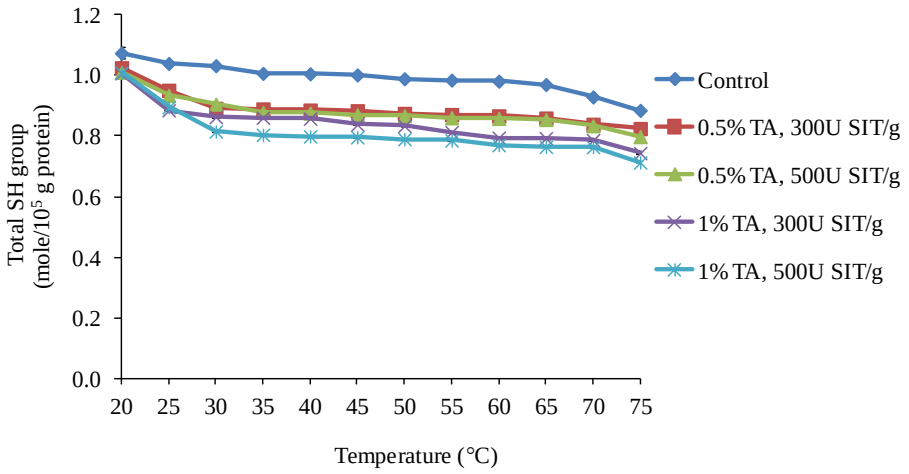
Figure 19. Turbidity (A) and surface hydrophobicity (B) of NAM solutions (1 mg protein/mL) without and with SIT/TA mixtures at different concentrations during heating from 20 to 75 °C. Heating rate was 0.65 °C/ min. Bars represent the standard deviation (n=3).

the reactivity of quinones towards NAM was insufficient. This was evidenced by no difference in S_0ANS between this sample and the control throughout the heating up to 75 °C. Therefore, the SIT/TA mixture more likely provided quinones, which could either induced the conformational change of NAM or attached directly to NAM.

5.4.1.3 Total sulfydryl group and disulfide bond contents

Changes in total sulfydryl group and disulfide bond contents of NAM without or with the addition of SIT/TA mixtures at different concentrations after heating to reach the temperature range of 20 to 75 °C are shown in Figure 20. In general, total sulfydryl groups of all NAM solutions decreased as the temperature increased with concomitant increase in disulfide bond formation (P<0.05). Nevertheless, the control (without SIT/TA mixture) had higher sulfydryl group and lower disulfide bond contents, compared to the samples added with SIT/TA mixtures (P<0.05). A decrease in total sulfydryl group content was reported to be due to the formation of disulfide bonds through oxidation of sulfydryl groups or disulfide interchanges (Hayakawa and Nakai, 1985). Oxidation of two cysteine molecules on neighbouring protein chains leads to the formation of an inter-molecular disulfide bond (Lanier, 2000). At high temperature, oxidation of sulfydryl groups occurred to a higher extent, leading to the formation of disulfide bonds. The NAM solution added with mixture of 1% TA and SIT at 500 U/g protein had the lowest sulfydryl group content but highest disulfide bond content (P<0.05). TA oxidized by SIT might induce the changes in conformation of NAM, in which sulfydryl groups could be exposed and favoured the oxidation process. This was evidenced by the higher disulfide bond contents in NAM added with SIT/TA mixtures. Additionally, quinone could interact directly to sulfydryl groups (Strauss and Gibson, 2004). As a result, these sulfydryl groups were masked by those quinones. This led to lower sulfydryl group content. The lower sulfydryl group and higher disulfide bond in NAM added with SIT/TA mixture at the beginning (20 °C) reconfirmed the reactivity of quinone in the mixture toward sulfydryl groups of NAM. At higher concentrations of SIT and TA, quinones formed likely resulted in the conformational changes in protein, which promoted the formation of disulfide bonds.

(A)



(B)

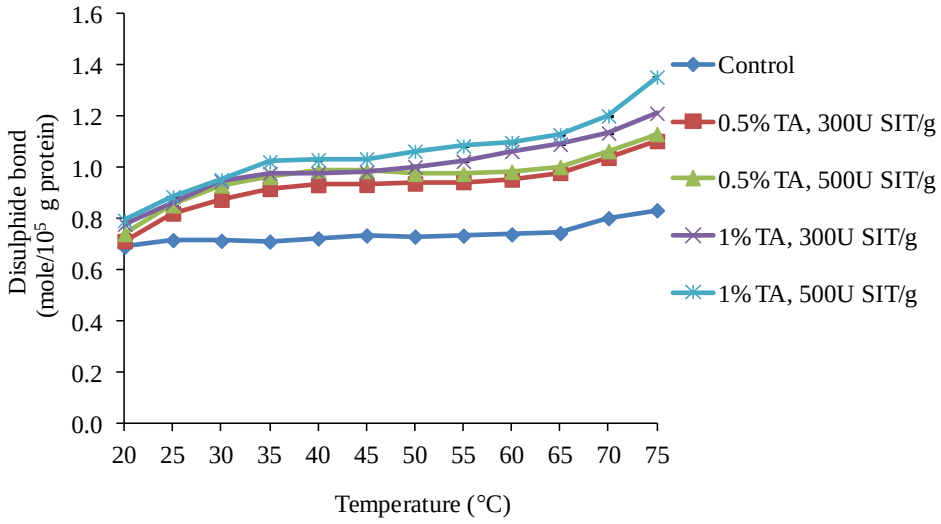
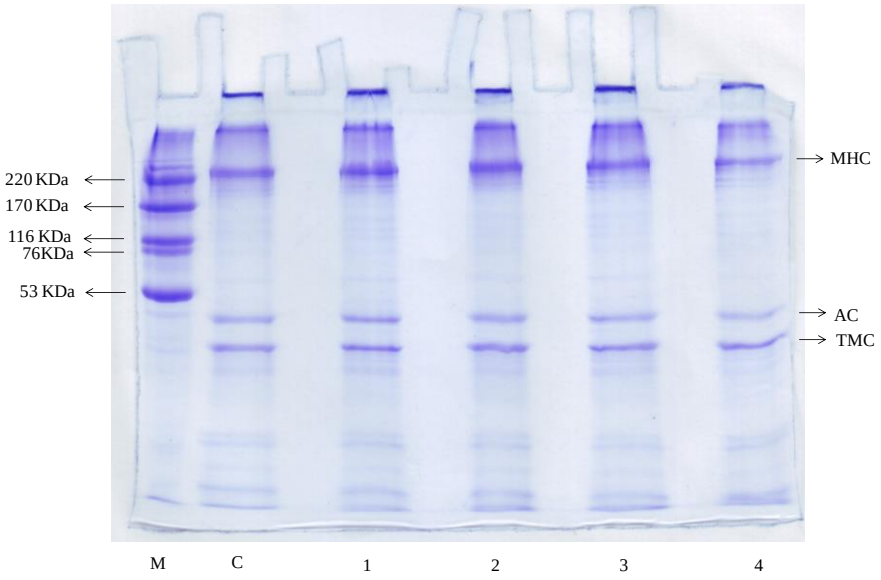


Figure 20. Total sulfydryl group content (A) and disulfide bond content (B) of NAM solutions (1 mg protein/mL) without and with SIT/TA mixtures at different concentrations during heating from 20 to 75 °C. Heating rate was 0.65 °C/min. Bars represent standard deviation (n=3).

5.4.1.4 SDS-polyacrylamide gel electrophoresis

Protein patterns of NAM solutions without and with the addition of SIT/TA mixture heated before (20 °C) and after heating to obtain the temperature of 75 °C are shown Figure 21. At 20 °C (Figure 21A), the sample added with the mixture of 500 U SIT/g protein and TA at 1% (based on protein) had the lowest band intensity of myosin heavy chain (MHC) (lane 4), which was 30.5% lower than that of control. This suggested that the quinones readily formed induced the cross-linking of MHC in NAM solution even at low temperatures. However, no marked change in actin band intensity was observed. Balange and Benjakul (2010) found that MHC was susceptible to cross-linking by quinone. After heating to 75 °C, no MHC band was observed in all samples treated with the mixture of SIT and TA, regardless of levels of SIT and TA (Figure 21B). MHC band intensity of the control (without SIT and TA) heated to 75 °C was lower than that found at 20 °C. This might be governed by remaining endogenous transglutaminase in NAM, which induced the formation of non-disulfide covalent bond during heating, particularly at temperature around 40-50 °C, which was the optimal temperature for endogenous transglutaminase (Benjakul *et al.*, 2004). During heating up to 75 °C, MHC might be partially unfolded and quinones from the SIT/TA mixture could interact with those proteins to a higher extent. As a result, a larger proportion of polymerized MHC was formed as indicated by the pronounced decrease in MHC band. Conversely, at lower temperature (20 °C), native proteins were present and their reactive groups might not be available for cross-linking induced by quinones. In general, TA in reduced form could interact with protein via hydrogen bond. Hydroxyl group of TA more likely acted as hydrogen donor and side chains of amino acids in protein chains could be hydrogen acceptor. Nevertheless, this weak bond could be destroyed by SDS used for electrophoresis. The disappearance of MHC band confirmed the formation of cross-link via non-disulfide covalent bond induced by quinones, the products from tyrosinase and TA. Covalent modification of proteins by phenolic oxidation products was reported by Rawel *et al.* (2002). Thus, the efficacy in protein cross-linking of quinones was maximized during heating for gelation process. The cross-linking efficiency depended on both TA and tyrosinase levels, which were more likely related with the amount of quinones formed.

(A)



(B)

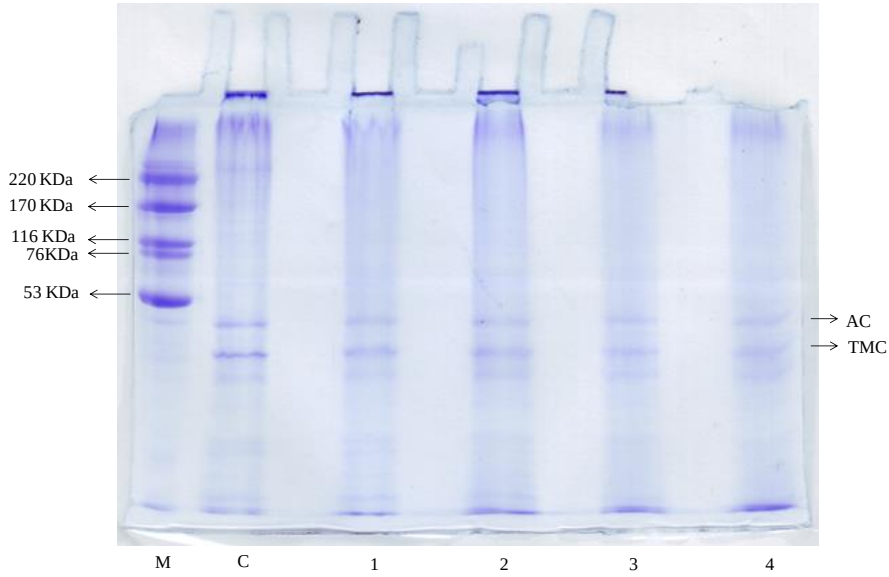


Figure 21. SDS–PAGE patterns of proteins of NAM without and with SIT/TA mixtures at different concentrations at 20 °C (A) and after heating to 75 °C (B). M, high molecular weight protein markers; C, control; MHC, myosin heavy chain; AC, actin; TMC, tropomyosin; 1, 0.5% TA and 300 U SIT/g protein; 2, 0.5% TA and 500 U SIT/g protein; 3, 1% TA and 300 U SIT/g protein; 4, 1% TA and 500 U SIT/g protein.

5.4.2 Effect of SIT/TA mixtures on Ca^{2+}-ATPase activity of NAM during heating

Ca^{2+}-ATPase activity of NAM solutions added with SIT/TA mixtures at various concentrations is given in Table 7. As the temperature increased, the Ca^{2+}-ATPase activity of NAM solutions decreased ($P<0.05$). At the same temperature, NAM solutions added with SIT/TA mixtures had lower Ca^{2+}-ATPase activity, compared to the control ($P<0.05$). No activity was detectable in NAM when heated at temperature above 40 °C, indicating the complete denaturation of myosin molecule when sufficient heat was applied. Ca^{2+}-ATPase is used as a good indicator of the integrity of myosin molecule (Benjakul *et al.,* 1997). Foegeding *et al.* (1996) reported that Ca^{2+}-ATPase activity is a sensitive indicator of myosin denaturation. Lower Ca^{2+}-ATPase activity in NAM solution added with SIT/TA mixtures indicated that quinone formed via the oxidation of TA by SIT could induce the structural changes in myosin molecule, especially at head portion, thus lowering the activity. The SH groups located in the head portion (SH1 and SH2) play an essential role in the Ca^{2+}-ATPase activity (Kielley and Bradley, 1956). Quinones were able to interact with sulfydryl group of myosin, especially at head portion. This was also associated with the decrease in Ca^{2+}-ATPase. The denatured myosin head, induced by SIT/TA mixture could undergo aggregation with ease. This correlated well with the increased turbidity (Figure 19).

Table 7. Ca^{2+}-ATPase activity of NAM solutions (1 mg protein/mL) containing SIT/TA mixtures at different concentrations after heating from 20 to 60 ℃.

Temperature (°C)	Control	0.5% TA, 300 U SIT/g	0.5% TA, 500 U SIT/g	1% TA, 300 U SIT/g	1% TA, 500 U SIT/g
20	0.20±0.002aA	0.18±0.005aB	0.18±0.002aB	0.18±0.008aC	0.17±0.001aC
30	0.18±0.004bA	0.17±0.008bB	0.17±0.006bB	0.16±0.001bC	0.16±0.003bC
40	0.04±0.002cA	0.04±0.004cA	0.03±0.006cB	0.03±0.001cB	0.02±0.002cC
50	ND	ND	ND	ND	ND
60	ND	ND	ND	ND	ND

Different lowercase letters in the same column indicate the significant difference ($P<0.05$).

Different uppercase letters in the same row indicate the significant difference ($P<0.05$).

Values are given as mean±SD (n=3).

ND: Not detectable.

5.4.3 Rheological property of NAM added with SIT/TA mixtures during heating

Changes in storage modulus (G') of NAM samples without and with SIT/TA mixtures during heating with temperature range of 20 to 75 °C, are depicted in Figure 22. Control had the lowest G' during heating up to 75 °C. Changes in G' was more pronounced in NAM samples containing SIT/TA mixtures. The sample with the mixture of 1% TA and SIT at 500 U/g protein had the highest G' upon heating (P<0.05). Generally, G' value is a measure of deformation energy stored in the sample during shear process, representing the elastic behaviour of a sample (Tabilo-Munizaga and Barbosa-Cánovas, 2005). The increase in G' indicated the onset of gelation or the formation of elastic protein network. For all the samples, the G' was constant up to 30 °C and increased continuously until the end of the heating (75 °C). At higher temperature, the protein molecules more likely unfolded, thereby facilitating the interaction between several reactive groups or domains. NAM could undergo heat induced gelation via several bonding, e.g. disulfide bond, hydrophobic interaction, etc. It was noted that the protein interaction in NAM solutions containing SIT/TA mixtures was enhanced as indicated by higher G'. The unfolding of actomyosin helical structure, hydrophobic interaction and disulfide formation took place and became greater at high temperature (Yongsawatdigul and Park, 2003). It was noted that G' values of NAM solutions added with SIT/TA mixtures were higher than the control even without heating. This suggested that the quinones formed via the oxidation of TA by SIT in the SIT/TA mixture readily interacted with NAM, in which protein cross-links were formed rapidly. This result was in accordance with the higher turbidity of NAM added with SIT/TA mixtures (Figure 1). At higher temperatures, quinones formed were able to interact with the unfolded proteins more effectively. This led to the higher polymerization of proteins as indicated by the higher G'. Rheological study confirmed that the addition of SIT/TA mixture could enhance the interaction or entanglement of protein molecules in NAM upon heating. The effectiveness was dependent on the concentration of both SIT and TA, associated with the quinones formed.

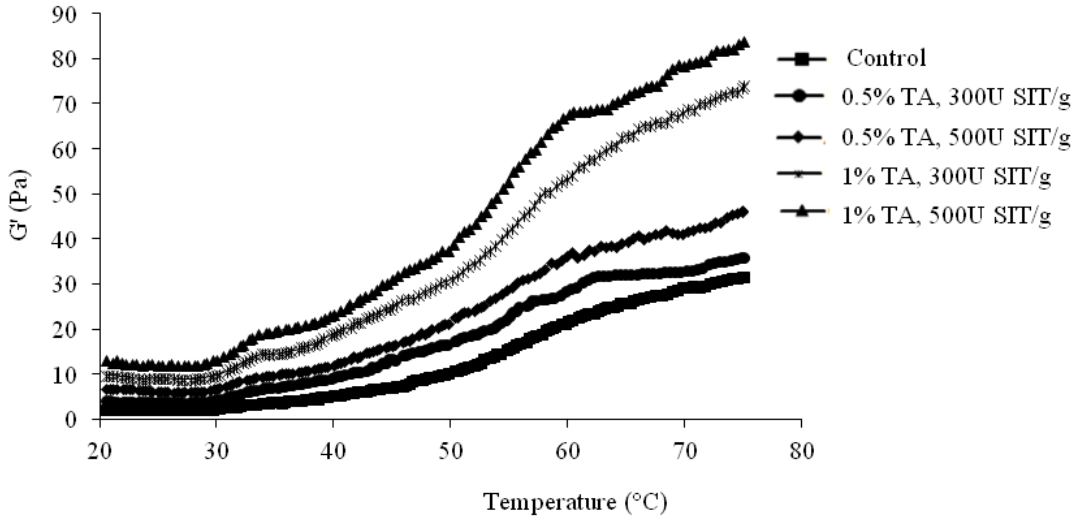


Figure 22. Storage modulus (G') of NAM solutions (20 mg protein/mL) without and with SIT/TA mixtures at different concentrations during heating from 20 to 75 °C.

5.4.4 Effect of SIT/TA mixtures on charge, size and microstructure of heated NAM

5.4.4.1 Zeta potential and particle size

Zeta potential (ζ) values of the control NAM solution and NAM solutions added with the mixture of 0.5% TA and SIT at 500 U/g and with the mixture of 1% TA and SIT at 500 U/g protein after heating to 75 °C, are given in Table 8. The control NAM had the highest negative value (P<0.05). Fish muscle proteins are rich in acidic amino acids, e.g. glutamic acid or aspartic acid (Chalamaiah *et al.,* 2012). At neutral pH (pH 7.0), those amino acids in NAM became deprotonated, providing negative charge. When NAM was added with SIT/TA mixtures, the lower negative values were observed. The NAM added with 0.5% TA and SIT at 500 U/g had more negative charge, compared to that containing 1% TA and SIT at 500 U/g. In the presence of SIT/TA mixture, protein aggregate induced by the oxidized TA plausibly masked the charged amino acids present in the NAM. The burial of charged amino acids inside the protein complex resulted in less negative charge on the surface. Also

quinones might react directly with charged amino acids during protein interaction, thereby reducing the surface charge.

The average particle sizes of NAM solutions containing the mixture of 0.5% TA and SIT at 500 U/g protein and with the mixture of 1% TA and SIT at 500 U/g protein and the control are presented in Table 8. The NAM solution added with 1% TA and SIT at 500 U/g protein had the highest particle size (P<0.05). Control had the average particle size of 513.37 nm and the sizes increased to 607.9 nm and 645.63 nm in the presence of 0.5% TA and SIT at 500 U/g protein and with the mixture of 1% TA and SIT at 500 U/g protein, respectively. The result was in accordance with the turbidity, where the mixture of 1% TA and 500 U/g SIT exhibited the highest turbidity (Figure 1). The interaction between the protein molecules in the presence of SIT/TA mixture, led to the formation of larger particles. Thus, the aggregation of the heated protein molecules not only affected the size of particulates formed, but also had an impact on the surface charge of the aggregates.

Table 8. Zeta potential and particle size of NAM solutions (1 mg protein/mL) containing the mixture of 0.5% TA and 500 U SIT/g and with the mixture of 1% TA and 500 U SIT/g after heating to 75 °C.

Treatment	Zeta potential (mV)	Particle size (nm)
Control	-13.01 ± 0.71c	513.37 ± 1.10c
0.5% TA, 500 U SIT/g	-10.32 ± 0.43b	607.90 ± 3.97b
1% TA, 500 U SIT/g	- 5.32 ± 0.72a	645.63 ± 1.62a

Different lowercase letters in the same column indicate significant differences (P < 0.05).

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

5.4.4.2 Microstructure

The microstructures of control NAM solution and NAM solutions added with the mixture of 0.5% TA and SIT at 500 U/g protein and with the mixture of 1% TA and SIT at 500 U/g after heating to 75 °C are shown in Figure 23. The marked differences in microstructure between the control and NAM with SIT/TA mixture were

noticeable. The highest aggregation with denser cluster was found in NAM solutions added with 1% TA and SIT at 500 U/g protein. This result was in accordance with the highest turbidity (Figure 19) and the largest particle size (Table 8). Gelation is the result of protein denaturation, followed by the aggregation via inter-molecular covalent bonds and noncovalent interactions (Lee and Lanier, 1995). The heat treatment provided sufficient energy for the unfolding of protein molecules, which allowed quinones formed by oxidation of TA by SIT, to interact easily. As a result, the large aggregates were formed amongst proteins in NAM. For the control, the heat denatured protein molecules underwent aggregation to a lower degree. This was indicated by the looser cluster of filamentous proteins. Balange and Benjakul (2010) reported that a highly interconnected, finer and denser network structure was observed in NAM from mackerel added with 0.2% oxidized TA incubated at 40 °C for 30 min. Microstructure revealed that addition of SIT/TA mixture increased the protein interaction or aggregation as evidenced by denser network with higher interconnection.

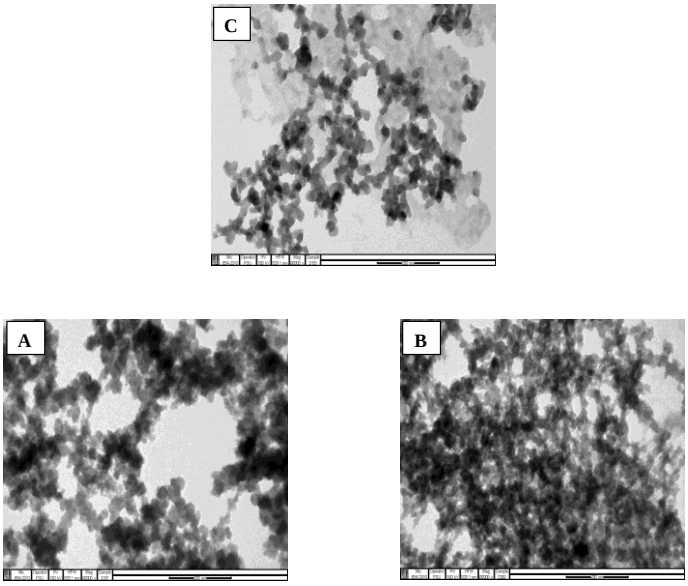


Figure 23. Transmission electron micrograph of NAM solutions containing the mixture of 0.5% TA and 500 U SIT/g protein (A) and with the mixture of 1% TA and 500 U SIT/g protein (B) after heating at 0.65 °C/min to 75 °C. C: control NAM solution (without SIT/TA mixture). Heated NAM was cooled rapidly in iced water prior to TEM analysis. Magnification: ×60,000.

5.5. Conclusion

The mixture of SIT and TA had the profound effect on the heat induced aggregation of NAM. NAM solutions with SIT/TA mixture had increased turbidity and surface hydrophobicity and showed highly interconnected and denser structure with higher G'. Disulfide bond formation was also induced by SIT/TA mixture. Heat-induced aggregation was mainly influenced by heating temperature and the concentration of both SIT and TA. Hence the SIT/TA mixture could be effectively utilized as gel enhancer in fish protein based products.

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CHAPTER 6
ENHANCEMENT OF GEL PROPERTIES OF SARDINE SURIMI USING SQUID INK TYROSINASE IN COMBINATION WITH COCONUT HUSK EXTRACT

6.1 Abstract

Squid ink tyrosinase (SIT) at levels of 300 and 500 KU kg^{-1} protein in combination with ethanolic extract of coconut husk (EEC) at concentrations of 1 and 1.25 g kg^{-1} protein was added into sardine surimi and the resulting gels were examined. The use of SIT (500 KU kg^{-1} protein) and EEC (1.25 g kg^{-1} protein) yielded the gel with the highest breaking force and deformation (P<0.05). Nevertheless, the expressible moisture content and whiteness scores were markedly decreased. Protein cross-linking of surimi gels increased as evidenced by the decreased band intensity of myosin heavy chain (MHC). This coincided with the increased storage modulus (G'). Based on microstructure study, finer and more compact structure of surimi gels added with both SIT and EEC were obtained, compared to the control. Thus, SIT in conjunction with EEC could be used as the novel surimi gel strengthener.

6.2 Introduction

Surimi is an important proteinaceous material for preparing seafood products with elastic texture. In general, lean fish have been used for surimi production but their amount is decreasing due to overexploitation. Hence pelagic dark-fleshed fish has gained more attention as a potential alternative raw material (Chaijan *et al.*, 2004). However, those pelagic dark-fleshed fish have high contents of lipid, myoglobin and sarcoplasmic proteins, thereby limiting their use for surimi production (Chaijan *et al.*, 2010). To improve the gel properties of surimi from dark-fleshed fish, various food-grade additives and cross-linking enzymes such as microbial transglutaminase (MTGase) have been used (Kaewudom *et al.*, 2013; Oujifard *et al.*, 2012). Nevertheless, MTGase is expensive and other additives such as egg white and bovine serum albumin possess negative effects such as allergy and religious constraints, respectively. Hence natural and cheap additives, which can improve the gel properties

of surimi, have been searched. Phenolic compounds, either commercial or from natural sources, both in reduced and oxidized form are shown to improve the gel properties of surimi from dark fleshed fish (Balange and Benjakul, 2009a; 2009b; Buamard and Benjakul, 2015; Vate and Benjakul, 2016).

Coconut husk, generated as a waste during processing, is rich in phenolic compounds such as 4-hydroxybenzoic acid (4-HBA), ferulic acid, tannic acid and lignin phenols including vanillic acid, *p*-coumaric acid and syringic acid (Lobbes *et al.*, 1999; Rodrigues and Pinto, 2007). Phenolic compounds from various sources, particularly in the oxidized form, have been utilized as a protein cross-linking agent, which was able to strengthen surimi gels (Balange and Benjakul, 2009a).

Squids are important fishery in Thailand and many Southeast Asian countries. During processing of squid, viscera along with ink sac are discarded as waste. Recently, tyrosinase has been extracted from squid melanin-free ink (Vate and Benjakul, 2016). Tyrosinase can induce oxidation of various phenolic compounds to quinones. These quinones can react with various amino acid side chains including sulfhydryls, amines, amides, indoles, and other tyrosines commonly present in proteins, resulting in the formation of inter and intramolecular crosslinks (Mattinen *et al.*, 2008). Squid ink tyrosinase in combination with tannic acid has been shown to improve the properties of sardine surimi gel (Vate and Benjakul, 2016). Since coconut husk extract is the potential source of phenolic compounds, it can act as substrate for quinone formation induced by tyrosinase. Therefore, the objective of this study was to investigate the impact of mixture of squid ink tyrosinase and ethanolic extract of coconut husk on the properties of sardine (*Sardinella albella*) surimi gel.

6.3 Materials and methods

6.3.1 Chemicals/Surimi

β-mercaptoethanol (*β*-ME) and wide range molecular weight protein markers were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

Frozen surimi from sardine (*Sardinella albella*) with grade AA was obtained from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand) and kept at -20 °C until use, but not longer than two months.

6.3.2 Preparation of coconut husk extract

Coconut husk extract was prepared according to the method of Buamard and Benjakul (2015). Dried husk powder with the particle size of 80 mesh was extracted with 60% ethanol (v/v) and filtered. The filtrates were evaporated at 40 °C, followed by freeze drying. Dried extracts were powdered using a mortar and pestle. The ethanolic extract of coconut husk obtained was referred to as 'EEC'. The powder was kept in an amber bottle and stored in a desiccator until use.

6.3.3 Preparation of squid ink tyrosinase

6.3.3.1 Preparation of melanin-free ink

Melanin-free ink was prepared according to the method of Vate and Benjakul (2013). The collected squid ink was diluted ten-fold using the cold deionized water (2-4 °C). The prepared ink was centrifuged at 18,000×g for 30 min at 4 °C to remove the melanin using a refrigerated centrifuge (Allegra 25 R centrifuge, Beckman Coulter, Palo Alto, CA, USA). The supernatant obtained was used as melanin-free ink (MFI).

6.3.3.2 Fractionation of tyrosinase

Tyrosinase from MFI was fractionated as per the method of Simpson *et al.* (1987) with a slight modification. MFI (50 mL) was mixed with 50 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). The mixture was stirred continuously at 4 °C for 30 min. Solid ammonium sulfate was added into the mixture to obtain 60% saturation. The mixture was allowed to stand at 4 °C for 30 min. The precipitate was collected by centrifugation at 12,500×g at 4 °C for 30 min. The pellet obtained was dissolved in a minimum volume of 0.05 mM sodium phosphate buffer, pH 7.2 and dialysed with 15 volumes of the same

buffer with three changes overnight. The fraction containing tyrosinase referred to as 'squid ink tyrosinase, SIT' was kept at -20 °C until used.

6.3.3.3 Measurement of tyrosinase activity

Tyrosinase activity was assayed using L-DOPA (3,4-Dihydroxy-L-phenylalanine) as a substrate according to the method of Simpson *et al.* (1987). One unit of tyrosinase activity was defined as the enzyme causing an increase in the absorbance by 0.001 at 475 nm min^{-1}.

6.3.4 *In vitro* oxidation of EEC by SIT

EEC solution was dissolved in deionized water to obtain various concentrations (0.5, 0.75, 1, 1.25 and 1.5 g kg^{-1}) and pH was adjusted to 7 using 1 M NaOH. Thereafter, SIT (0.5 mL) was mixed with EEC solutions (0.5 mL) to obtain the final concentration of 100 KU L^{-1}. Quinone formation was monitored using a spectrophotometer at room temperature. For blank, distilled water was used instead of SIT. The increase in the absorbance at 475 nm, representing the formation of quinone, was recorded after blank subtraction. The mixtures of SIT and EEC yielding the highest quinone formation were selected for the study.

6.3.5 The impact of SIT/EEC mixtures on properties of surimi gel

6.3.5.1 Gel preparation

Frozen surimi was partially thawed at 4 °C for 6 h prior to cutting into small pieces. The sample was ground for 2 min using a Moulinex Masterchef 350 mixer (Paris, France). NaCl (2.5%, w/w) was then added and the mixture was chopped for 1 min. The surimi paste was added with the prepared SIT/EEC solutions containing various levels of SIT (300 and 500 KU kg^{-1} protein) and EEC (1 and 1.25 g kg^{-1} protein). The mixtures were then chopped for 1 min. Final moisture content was adjusted to 80% using cold distilled water (1-2 °C). All mixtures were chopped for another 4 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step

heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

6.3.5.2 Textural analysis

Gel samples were subjected to textural analysis using a Model TA-XT2i texture analyser (Stable Micro Systems, Surrey, England) as described by Benjakul *et al.* (2001). Breaking force and deformation (elasticity/deformability) were recorded.

6.3.5.3 Determination of expressible moisture content and whiteness

Expressible moisture content was measured according to the method of Benjakul *et al.* (2001). Expressible moisture content was expressed as percentage of sample weight. Whiteness of gels was determined following the method of Vate and Benjakul (2016).

6.3.5.4 SDS-polyacrylamide gel electrophoresis

Protein patterns of gels were analysed by SDS–PAGE according to the method of Laemmli (1970). Prior to loading, surimi gel samples were solubilized in SDS according to the method described by Benjakul *et al.* (2008). 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, the gel was stained and destained as per Benjakul *et al*. (2008). Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems).

6.3.6 Characterization of the selected surimi gels added with SIT/EEC mixtures

6.3.6.1 Rheological property

Surimi pastes containing the selected SIT/EEC mixtures were prepared as previously described and were subjected to dynamic rheological measurement following the method of Rawdkuen *et al.* (2008) with a slight modification. A rheometer (HAAKE RheoStress1, ThermoFisher Scientific, Karlsruhe, Germany) with 35 mm, 4^{o} slope cone and plate geometry was used for monitoring the changes in storage or elastic modulus (G'). An oscillation of 1 Hz with 1% deformation was used for testing. This condition yielded a linear response in the viscoelastic region. The temperature sweep was recorded during heating up from 10 to 90 ^{o}C with heating rate of 1 ^{o}C min^{-1}. A sample cover was used to minimize water evaporation of surimi pastes during measurement.

6.3.6.2 Scanning electron microscopy

Gels were cut into small pieces (0.25 × 0.25 × 0.25 cm^{3}) and fixed with 2.5 % (v/v) glutaraldehyde, followed by dehydration as described by Vate and Benjakul (2016). Samples were critical point dried, sputter-coated with gold and examined on a scanning electron microscope (Vate and Benjakul, 2016).

6.3.7 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan’s multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

6.4 Results and discussion

6.4.1 *In vitro* oxidation of EEC by SIT

Oxidation of EEC by SIT as influenced by EEC concentrations was monitored by the increase in A_{475} as depicted in Figure 24. The increases in A_{475} of the mixture were observed as EEC concentrations increased up to 1.25 g kg^{-1} (P<0.05). This was most likely caused by the oxidation of EEC by SIT. Increase in A_{475} indicates the formation of dopachrome (Simpson *et al.*, 1987). With sufficiently high EEC concentration, phenolics might be oxidized by SIT to a high extent. However, the formation of quinone decreased when EEC was present at the concentration greater than 1.25 g kg^{-1}. Oxidized phenols might undergo polymerization and the availability of free phenols as a substrate was lowered. As a result, the quinone formation was decreased. Generally phenolic ring is oxidized by tyrosinase, resulting in the formation of L-DOPA (3,4-dihydroxy-Lphenylalanine), which is further converted to the dopachromes (Buchert *et al.*, 2010). The result indicated that the EEC concentration was another key factor affecting quinone production mediated by SIT.

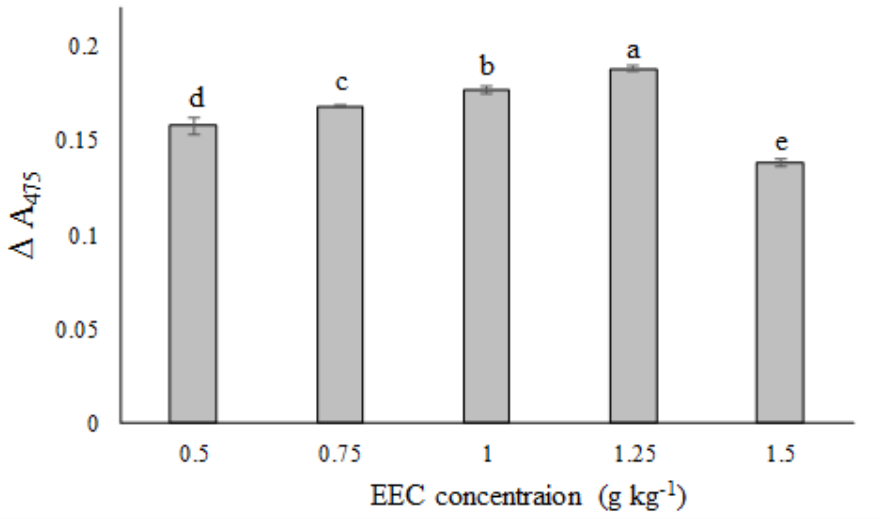

Figure 24. Increase in A_{475} of EEC solution at different concentrations in the presence of SIT (100 KU L^{-1}). Bars represent the standard deviation (n=3). Different letters on the bars indicate significant differences (P<0.05).

6.4.2 Effect of SIT/EEC mixture at different concentrations on the properties of sardine surimi

6.4.2.1 Textural properties

Breaking force and deformation of sardine surimi gels added without and with SIT/EEC mixtures at various concentrations are shown in Table 9. Surimi gels added with SIT/EEC mixtures had higher breaking force and deformation, compared to the control (without SIT and EEC) and those added with either SIT or EEC alone (P<0.05). Surimi gels added with only SIT at 300 and 500 KU kg^{-1} protein (sample No. 1 and 2) had higher breaking force, compared to the control (P<0.05). This was more likely due to the oxidation of tyrosine present in the surimi by SIT, leading to the production of quinones. Tyrosine of proteins can be oxidized by tyrosinase to quinone, which can further crosslink with lysyl, tyrosyl, and cysteinyl residues present in the adjacent proteins (Selinheimo *et al.*, 2008; Buchert *et al.*, 2010). Vate *et al.* (2014) reported that the addition of MFI containing tyrosinase increased the gel strength of sardine surimi gel in a dose dependent manner. When EEC alone was incorporated, the breaking force and deformation of surimi gels also increased, indicating that phenolics in EEC were able to induce the cross-linking of myofibrillar proteins. Phenolics in coconut husk included tannic acid (24 mg g^{-1} dry extract), which has several hydroxyl groups (Buamard and Benjakul, 2015). Those phenolics could cross-link proteins via hydrogen bonding. As a consequence, the network of surimi gel was strengthened as indicated by the increased breaking force. The efficacy was in dose-dependent manner.

The mixture of SIT and EEC was generally more effective in improving the gel strength of surimi gel. SIT was able to oxidize the tannins present in EEC into quinones, which were able to induce protein cross-linking. Tyrosinase is able to react on various monophenolic and diphenolic compounds, such as phenol and catechol or phloretic acid and hydrocaffeic acid (Selinheimo *et al.*, 2008). The result suggested that quinone was formed to a higher extent when EEC was mixed with SIT. The quinones formed were involved in protein cross-linking, as evidenced by the increased breaking force. The quinones are reactive and can react with various amino acid side chains, resulting in the formation of inter- and intramolecular protein crosslinks (Mattinen *et*

al., 2008). With higher levels of SIT and EEC, a larger amount of quinones was generated. The enzyme and substrate concentrations therefore played an important role in formation of quinone. Amongst all samples, surimi gel added with the mixture of 1.25 g EEC kg^{-1} and 500 KU SIT kg^{-1} protein had the highest breaking force and deformation (P<0.05). Significant increase in gel strength of bigeye snapper surimi was found when oxidized phenolic compounds and oxidized kiam wood extract were added (Balange and Benjakul, 2009a; 2011). Tyrosinase in combination with caffeic acid increased the cross-linking of whey proteins, α-lactalbumin and β-lactoglobulin (Thalmann and Lotzbeyer, 2002). Vate and Benjakul (2016) recently reported the increases in gel strength of sardine surimi when the mixture of tannic acid and SIT was added. Result suggested that both reduced and oxidized forms of phenolics in EEC were able to improve gel property of surimi via the enhanced protein cross-linking. However, the oxidized phenolics in EEC were formed as induced by SIT and showed higher efficiency than the reduced form (ECC without SIT). As a result, the higher increases in breaking force and deformation were observed in sample No. 5, 6, 7 and 8, in comparison with sample No. 3 and 4 (Table 9).

6.4.2.2 Expressible moisture content

Expressible moisture contents of sardine surimi gel added without and with SIT/EEC mixtures are shown in Table 9. The expressible moisture content is an indirect measure of water holding capacity of surimi gel. The control gel had the highest expressible moisture content, compared with the others (P<0.05). There was no difference in expressible moisture content of surimi gels added with SIT at 300 and 500 KU kg^{-1} protein (P>0.05), but the values were lower than that of control. This was more likely due to the cross-linking capacity of SIT via oxidising the side chains of amino acids, particularly tyrosine, in which inter- chain connection was enhanced. Surimi gels added with EEC also had lower expressible moisture content, indicating that EEC contributed to the formation of gel network with increased water holding capacity. This result confirmed the study of Buamard and Benjakul (2015) who reported that addition of coconut husk extract at 0.1% increased the water holding capacity of sardine surimi gels. The lowest expressible moisture content was observed in gels added with 1.25 g

EEC kg^{-1} protein in the presence of SIT either at 300 or 500 KU kg^{-1} protein. Both SIT and EEC could induce the formation of quinone at higher level, leading to higher interconnection of proteins in the surimi gel with coincidently increased water holding capacity. Lower expressible moisture content of the gels suggests more water retained in the gel network (Benjakul *et al.*, 2008).

6.4.2.3 Whiteness

Whiteness of sardine surimi gels decreased slightly ($P<0.05$) when added with SIT, EEC and different SIT/EEC mixtures (Table 9). There was no difference between whiteness of control gel and gel added with SIT at 300 KU kg^{-1} protein ($P>0.05$). The surimi gel added with EEC had lower whiteness, compared to the gels added with SIT. This was more likely due to the dark color of EEC, which had significant effect on lowering the whiteness of gels. Sardine surimi added with coconut husk extract had lower whiteness, compared to the control ($P<0.05$) (Buamard and Benjakul, 2015). The gels added with SIT/EEC mixtures had the lowest whiteness and no differences in the whiteness were observed between gels added with different SIT/EEC mixtures ($P>0.05$). When EEC was oxidized by SIT, dopachromes, which are dark brown in color, were formed. Balange and Benjakul (2009b) reported that addition of 0.75% oxidized tannic acid resulted in the decrease in whiteness of gels prepared from mackerel surimi. The slight decrease in whiteness of gels was still acceptable since the sardine surimi gel was generally dark in color.

6.4.2.4 Protein patterns

Protein patterns of sardine surimi gels without and with addition of SIT/EEC mixtures are shown in Figure 25. Myosin heavy chain (MHC) was the most prominent protein in surimi, followed by actin. MHC band intensity in the control was much lower than that of surimi paste. Band intensity of MHC was drastically decreased when gelation took place, regardless of SIT, EEC or SIT/EEC mixtures added. The decrease in MHC in gel suggested the formation of protein cross-link via non-disulfide bond, especially induced by endogenous transglutaminse (TGase).

Table 9. Breaking force, deformation, expressible moisture content and whiteness of sardine surimi gels added without and with different SIT/EEC mixtures.

Sample	Breaking force (g)	Deformation (mm)	Expressible moisture content (%)	Whiteness
C	391.09±39.58^{i}	10.30±0.88^{d}	4.17±0.12^{a}	67.98±0.42^{a}
1	487.09±14.49^{h}	11.02±0.81^{d}	3.77±0.11^{b}	67.40±0.63^{ab}
2	557.99±13.19^{g}	11.98±0.48^{c}	3.71±0.07^{b}	66.83±0.70^{bc}
3	598.74±5.59^{f}	11.89±0.27^{c}	3.62±0.03^{bc}	66.33±0.38^{c}
4	622.12±13.29^{e}	12.27±0.25^{c}	3.57±0.15^{c}	66.56±0.46^{c}
5	657.19±3.58^{d}	12.98±0.42^{b}	3.46±0.05^{cd}	65.26±0.48^{d}
6	683.95±8.17^{c}	13.04±0.25^{b}	3.28±0.024^{d}	65.46±0.58^{d}
7	732.66±7.53^{b}	13.15±0.53^{b}	3.18±0.53^{de}	65.09±0.56^{d}
8	810.50±10.99^{a}	14.57±0.34^{a}	3.08±0.01^{e}	65.43±0.46^{d}

Mean ± S.D (n = 3). Different superscripts in the same column indicate significant differences (P<0.05). C: Control (without SIT/EEC mixture); 1: 300KU SIT kg^{-1}; 2: 500KU SIT kg^{-1}; 3: 1g EEC kg^{-1}; 4: 1.25g EEC kg^{-1}; 5: 1g EEC+300KU SIT kg^{-1}; 6: 1g EEC+500KU SIT kg^{-1}; 7: 1.25g EEC+300KU SIT kg^{-1}; 8: 1.25g EEC+500KU SIT kg^{-1}.

The disappearance of MHC was the combined effect of setting mediated by TGase and the heat-induced aggregation (Wan *et al.*, 1994). It was noted that the lowest band intensity of MHC band was noticeable in the surimi gels with addition of SIT (500 KU kg^{-1}) and EEC (1.25 g kg^{-1}). The band intensity of MHC was 32.2% lower, compared to that of control. It was suggested that quinones formed in the mixture of SIT and EEC induced covalent bonding to some extent. However, actin was still retained in all gel samples. Actin might have structural constraint for cross-linking induced by TGase or tyrosinase or phenolic, both reduced and oxidized forms. Ou *et al.* (2005) reported the polymerization of protein molecules caused by the reaction of different proteins with phenolic substances. Thus, SIT/EEC mixture containing quinones more likely induced the cross-linking of MHC in sardine surimi, thereby increasing gel strength of surimi.

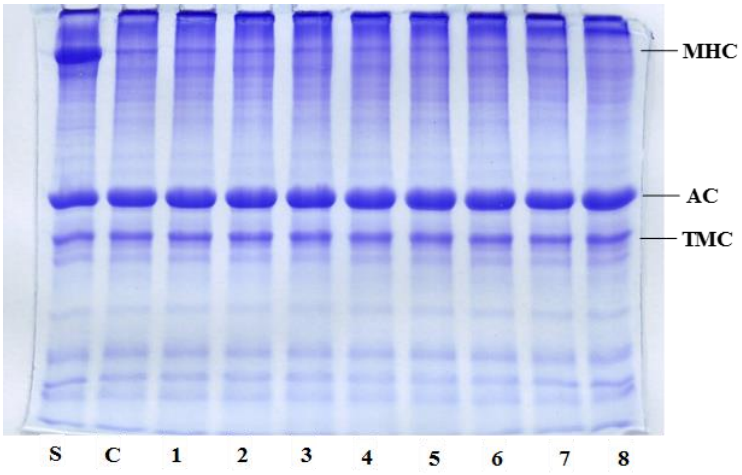

Figure 25. Protein pattern of gels from sardine surimi without and with different SIT/EEC mixtures. S: Surimi; MHC myosin heavy chain, AC actin chain, TMC tropomyosin chain; C: Control (without SIT/EEC mixture); 1: 300KU SIT kg^{-1}; 2: 500KU SIT kg^{-1}; 3: 1g EEC kg^{-1}; 4: 1.25g EEC kg^{-1}; 5: 1g EEC+300KU SIT kg^{-1}; 6: 1g EEC+500KU SIT kg^{-1}; 7: 1.25g EEC+300KU SIT kg^{-1}; 8: 1.25g EEC+500KU SIT kg^{-1}.

6.4.3 Characteristics of the selected surimi gel

6.4.3.1 Rheological property

Changes in storage modulus (G') of surimi paste without and with addition of different SIT/EEC mixtures during heating from 10 to 90 °C are depicted in Figure 26. The G' gradually increased and reached the maximum at 30 °C. The decrease was noticeable and the lowest G' was found at 50 °C. Gelation likely occurred at a temperature around 30 °C. The lowest G' observed at 50 °C was more likely due to the action of endogenous proteases and dissociation of actomyosin complex. Rawdkuen *et al.* (2007) reported that G' of Pacific whiting surimi reached the minimal value at 55 °C. The optimum temperature for proteolytic enzymes in surimi was in the range of 50-60 °C (Klomklao *et al.*, 2008). When heating up to 90 °C, the sharp increase in G' was observed and reached the maximum at 75 °C. During heating at higher temperature, the unfolded proteins underwent more aggregation. As a result, the cross-links with higher molecular weight were formed as evidenced by higher G' values. Higher G' indicated

the higher stiffness or firmness of gel (Gordon, 1984). Amongst all samples, the control had the lowest G' throughout the heating. The sample with the mixture of 1.25 g EEC kg^{-1} and 500 KU SIT kg^{-1} protein had the highest G', especially during heating at temperature higher than 50 °C. This was in accordance with the highest breaking force and deformation (Table 9). Cross-linking between the dissociated protein molecules mediated by quinones plausibly caused the increase in G'. Oxidation of EEC by SIT resulted in the formation of quinones, which induced the interaction of protein chains via the reactive groups of unfolded protein molecules. It was noted that G' values of surimi paste added with SIT/EEC mixtures were higher than the control even without heating. This suggested that the quinones formed in the EEC/SIT mixture readily interacted with muscle proteins, in which protein cross-links were formed rapidly. At higher temperatures, quninones interacted with the unfolded proteins more effectively. This led to the higher polymerization of proteins.

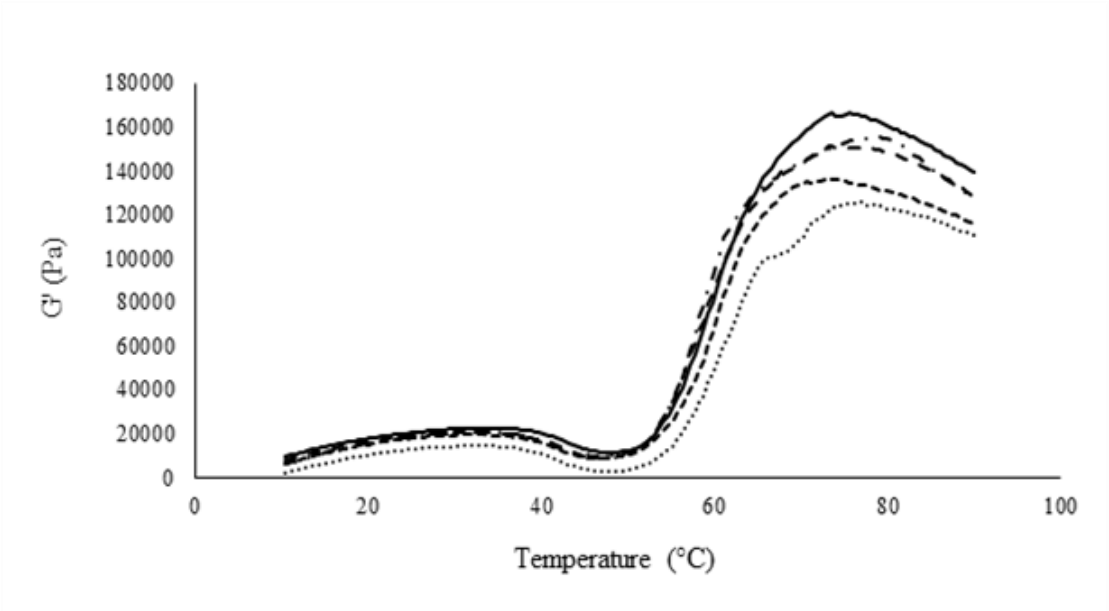

Figure 26. Storage modulus (G') of sardine surimi paste without and with different SIT/EEC mixtures during heating process. ····· Control (without SIT/EEC mixture); ----- 500KU SIT kg^{-1}; - - - 1.25g EEC kg^{-1}; - · - 1g EEC+500KU SIT kg^{-1}; —— 1.25g EEC+500KU SIT kg^{-1}.

Additionally, it was noted that G' of paste containing 1.25 g EEC kg^{-1} protein was higher than that added with SIT alone (500 KU kg^{-1}) throughout heating. This indicated the cross-linking activity of phenolics in EEC toward surimi proteins during thermal induced gelation process. Nonetheless, SIT in combination with EEC was able to enhance the interchain or entanglement of proteins in surimi more effectively than using SIT or EEC alone.

6.4.3.2 Microstructure of sardine surimi gel

Scanning electron microscopic images of selected surimi gels are given in Figure 27. The control gel had less dense structure and more voids than those containing the SIT/EEC mixture. The surimi gel added with 500 KU SIT kg^{-1} protein had similar structure to the control. More compact and finer network with small voids was observed in gel containing 1.25 g EEC kg^{-1} protein. This indicated that phenolic compounds in EEC were effective in protein cross-linking (Buamard and Benjakul, 2015). The highest interconnected gel network was observed in surimi gels added with SIT/EEC mixture (Figure 27D & 27E). This result was in correlation with the highest breaking force (Table 9) and storage modulus (Figure 26). The addition of SIT along with EEC led to the increased oxidation of tannins present in EEC by SIT. As a result, the formation of quinones was augmented, thus causing the increased protein interaction. The finer and ordered gel network with smaller voids was generally observed in gels with the higher gel strength, whilst the looser network with larger voids was formed in the gels with lower gel strength (Balange and Benjakul, 2009b). Hence the finer and more interconnected network was obtained when quinones are formed at a larger extent from the oxidation of EEC by SIT.

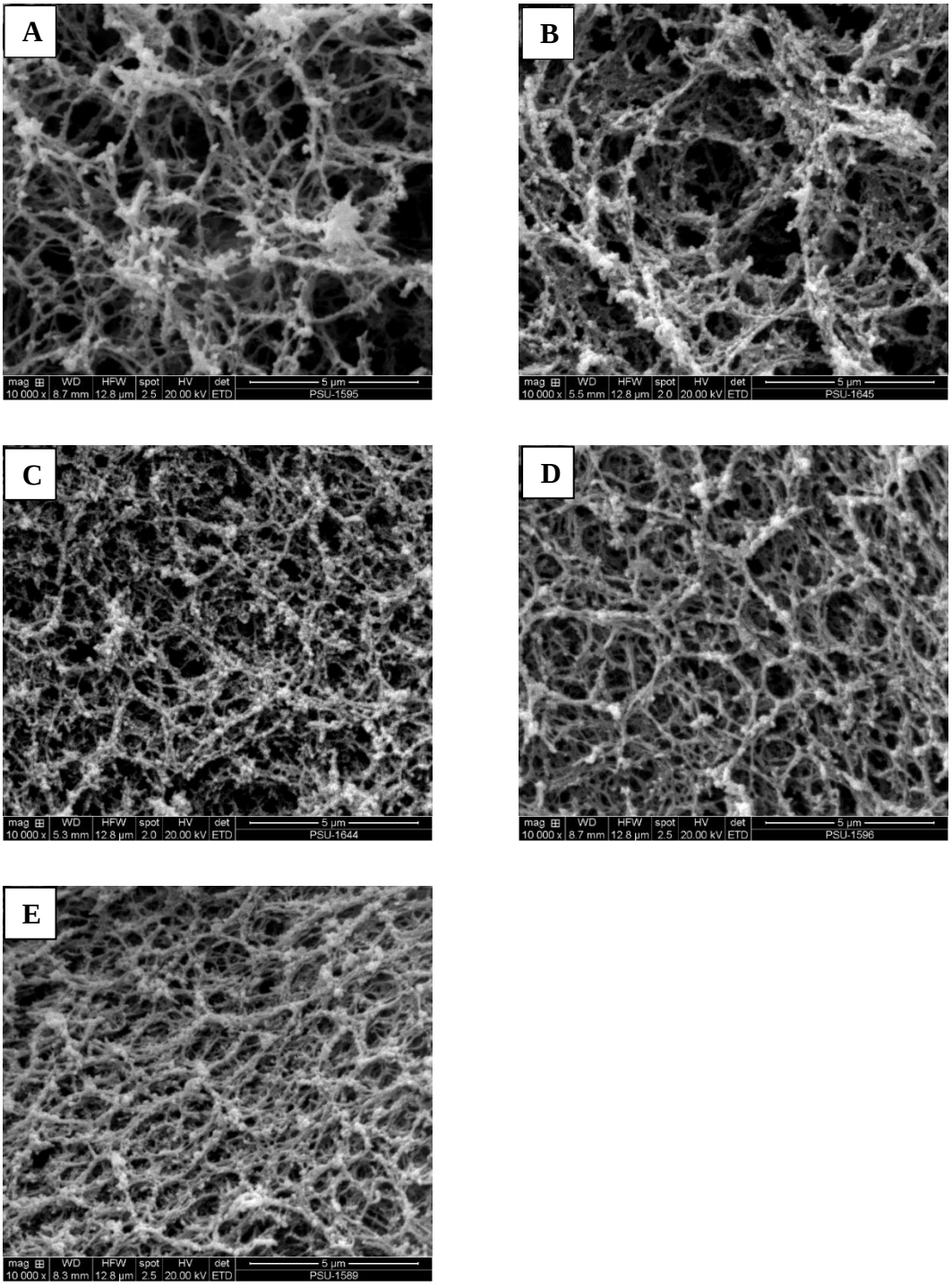

Figure 27. Scanning electron microscopic images of gel from sardine surimi without and with different SIT/EEC mixtures. **A**: Control (without SIT/EEC mixture); **B**: 500KU SIT kg^{-1}; **C**: 1.25g EEC kg^{-1}; **D**: 1g EEC+500KU SIT kg^{-1}; **E**: 1.25g EEC+500KU SIT kg^{-1}.
Magnification: ×10,000.

6.5 Conclusion

SIT in combination with EEC effectively enhanced the gel properties of sardine surimi. The combination of SIT and EEC increased breaking force, water holding capacity and storage modulus of surimi gel via enhanced protein cross-linking. Surimi gel with fine and compact network having more interconnection could be obtained by the addition of SIT and EEC mixture. Therefore, both by-products, squid ink and coconut husk, can be valorized as the processing aids to improve the properties of surimi from dark-fleshed fish.

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CHAPTER 7
EFFECT OF MELANIN FREE INK ON MECHANICAL PROPERTIES AND YELLOW DISCOLORATION OF PROTEIN FILM FROM WASHED SARDINE MINCE

7.1 Abstract

The effect of the melanin free ink (MFI) at different concentrations (0, 0.1, 0.2, 0.3 and 0.4 g L^{-1} of film forming solution (FFS)) on the properties and yellow discoloration of films from washed sardine mince was studied. Tensile strength (TS) of the film increased with increasing MFI concentration (P<0.05). Conversely water vapor permeability (WVP) of films decreased as the concentration of MFI increased (P<0.05). Fourier transform infrared (FTIR) spectra of films showed a slight shift to lower wavenumber of amide-B band of film added with MFI, indicating protein cross-linking. The microstructure showed slightly rough surface of the films when amount of MFI increased. However, cracks in the films were much decreased when MFI at higher levels was incorporated. Films added with MFI had lower thiobarbituric acid reactive substances (TBARS) value, indicating the lowered lipid oxidation. The addition of MFI also decreased the yellow discoloration but increased the transparency of film in a dose-dependent manner.

7.2 Introduction

Synthetic polymers are extensively used for food packaging but they have negative impact on environment associated with non-biodegradable nature. Hence, the focus has been diverted to biodegradable packaging from biopolymers, which are environmental friendly. Biopolymers such as polysaccharide, protein, lipid etc. have been used for packaging materials. Proteins are abundantly available and have good film-forming ability. Myofibrillar proteins from different fish species have been studied for their film forming ability (Shiku *et al.,* 2004; Chinabhark *et al.,* 2007; Tongnuanchan *et al.,* 2011). The main disadvantage in myofibrillar protein films especially from sardine is the yellow discoloration during prolonged storage (Tongnuanchan *et al.,* 2011). Fish muscle contains a large amount of polyunsaturated

fatty acids, especially ω3 fatty acids, which are susceptible to oxidation (Chen-Huei and Yih-Ming, 1998). Lipid oxidation is the main reason for yellow discoloration in myofibrillar protein films (Tongnuanchan *et al.*, 2011). The use of antioxidants in the protein films could reduce the yellow discoloration. The synthetic antioxidant such as trolox was used to prevent yellow discoloration in film based on fish protein isolate (Tongnuanchan *et al.*, 2012). Nevertheless, a number of natural antioxidants, particularly from marine byproducts, can serve as an alternative to alleviate the undesirable discoloration of myofibrillar protein based films.

Thailand is a major exporter of squid and cuttlefish. Squid is eviscerated and only head and mantle are exported, mainly as frozen blocks. During processing, viscera along with ink sac are discarded as waste. Squid ink is known to have many bioactivities such as anti-tumour, antimicrobial, antioxidant activities etc. (Ramasamy and Muruga, 2005; Liu *et al.*, 2011; Vat and Benjakul, 2013). However, melanin from squid ink with dark color is a drawback for further utilization. The removal of melanin could widen the applications of squid ink. The melanin-free ink (MFI) has been known to possess strong antioxidant activity (Vate and Benjakul, 2013). MFI was shown to increase the storage stability of sardine surimi gel by decreasing the lipid oxidation (Vate *et al.*, 2015). Thus, MFI can be used as an alternative natural antioxidant to reduce the yellow discoloration in myofibrillar protein-based films. In addition, tyrosinase present in MFI could improve the mechanical properties of the film (Vate *et al.*, 2015). Therefore, the objective of this investigation was to study the effect of MFI on the properties and yellow discoloration of sardine myofibrillar protein-based film.

7.3 Materials and methods

7.3.1 Chemicals

Glycerol, sodium chloride (NaCl), sodium dodecylsulfate (SDS), thiobarbituric acid and malondialdehyde bis (dimethyl acetal) were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was procured from Fluka (Buchs, Switzerland). All chemicals were of analytical grade.

7.3.2 Preparation of melanin-free ink (MFI)

MFI was prepared according to the method of Vate and Benjakul (2013). Squids were purchased from a local market in Hat Yai, Thailand, stored in ice using a squid/ice ratio of 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Thailand. Upon arrival, ink sac was separated from the squid by cutting the ink duct and ink was squeezed out from the ink sac. The squid ink was diluted ten-fold using the cold deionized water (2-4 °C). Prepared ink was centrifuged at 18,000×g for 30 min at 4 °C to remove the melanin using a refrigerated centrifuge (Allegra 25 R centrifuge, Beckman Coulter, Palo Alto, CA, USA). The supernatant obtained was used as MFI.

7.3.3 Preparation of washed mince

Sardines (*Sardinella albella*) with an average weight of 50-60 g/ fish were caught from Songkhla coast along the Gulf of Thailand. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported in polystyrene box to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. The fish were headed, gutted and washed with water. The flesh was separated manually from skin and bone and kept on ice prior to mincing and washing. The storage time was not longer than 24 h.

Flesh was minced using a mincer having the hole diameter of 5 mm. Mince obtained was washed according to the method of Toyohara *et al.* (1990) with slight modifications. Fish mince was homogenized with 5 volumes of cold 0.05 M NaCl (2-4 °C) at a speed of 13,000 rpm for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The washed mince was filtered through two layers of cheesecloth. The washing process was repeated twice. Washed mince obtained was stored on ice until used for film preparation.

7.3.4 Preparation of film forming solution

The film-forming solution (FFS) from washed mince was prepared according to the method of Chinabhark *et al.* (2007). The washed mince (200 g) was added with 3 volumes of distilled water and homogenized at 13,000 rpm for 1 min. The protein concentration of the mixture was fixed at 2% (w/v). Glycerol, used as a plasticizer, was added at 50% (w/w) of protein. The pH of the mixture was adjusted to 3 using 1 M HCl to solubilize the protein. MFI was added into FFS at different levels (0, 0.1, 0.2, 0.3 and 0.4 g L^{-1}). FFS was degassed using the sonicating bath (Elmasonic S 30 H, Singen, Germany) for 10 min. FFS obtained was filtered through a layer of cheesecloth to remove undissolved debris. The filtrate was used for film casting.

7.3.5 Film casting and drying

To prepare the film, FFS (4 g) was cast onto a rimmed silicone resin plate (50 × 50 mm^2) and air-blown for 12 h at room temperature prior to further drying at 25 °C and 50±5% RH for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting films were manually peeled off and used for analyses.

7.3.6 Determination of film properties

7.3.6.1 Film thickness

The thickness of film was measured using a micrometer (Gotech, Model GT-313-A, Gotech testing machines Inc, Tawai). Average thickness was determined using five random locations around each film samples.

7.3.6.2 Mechanical properties

Prior to testing for the mechanical properties, films were conditioned for 48 h at 25 °C and 50±5 % RH. Tensile strength (TS) and elongation at break (EAB) were examined as per the method of Iwata *et al.* (2000) with a slight modification using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK) equipped with

tensile load cell of 100 N. Ten samples (2×5 cm^2) with initial grip length of 3 cm were used for testing. Cross-head speed was set at 30 mm/min.

7.3.6.3 Water vapor permeability (WVP)

WVP was measured, using a modified ASTM method (1989) as described by Shiku *et al.* (2004). The film was sealed on an aluminium permeation cup containing dried silica gel (0% RH) with silicone vacuum grease and a rubber gasket to hold the film in place. The cups were placed in a desiccator containing the distilled water at 30 °C. The cups were weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$\text{WVP (g m}^{-1}\text{ s}^{-1}\text{ Pa}^{-1}) = w\, l\, A^{-1}\, t^{-1}\, (P_2 - P_1)^{-1}$$

where w is the weight gain of the cup (g); l is the film thickness (m); A is the exposed area of film (m^2); t is the time of gain (s); (P_2–P_1) is the vapor pressure difference across the film (Pa).

7.3.6.4 Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

Films were scanned with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at room temperature as described by Nuthong *et al.* (2009). Films were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 400-4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ºC.

7.3.6.5 Scanning electron microscopy (SEM)

Morphology of surface and cross-section of the film samples was visualized using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, Netherlands). For cross-section, samples were fractured under liquid nitrogen prior to

morphology visualization. Then, the samples were mounted on bronze stub and sputtered with gold (Sputter coater SPI-Module, PA, USA) in order to make the sample conductive. The photographs were taken at an acceleration voltage of 15 kV.

7.3.7 Effect of MFI on yellow discoloration of film during storage

Films without and with MFI at various levels were stored at room temperature (28-30 °C) for 0, 5, 10, 15 and 20 days. Films were randomly taken for analyses.

7.3.7.1 TBARS value

TBARS value of samples was determined as described by Buege and Aust (1978). Film was ground using mortar and pestle in the presence of liquid nitrogen and analysed. Standard curve was prepared using 1,1,3,3- tetramethoxypropane (malonaldehyde; MAD) at the concentrations ranging from 0 to 10 ppm and TBARS was expressed as mg MAD equivalents/kg sample.

7.3.7.2 Color

Color of the film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA), using D_{65} (day light) and a measure cell with opening of 30 mm. The color of the films was expressed as b^* (yellow/ blueness) and the difference of color (ΔE^*) was calculated as follows:

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

where ΔL^*, Δa^* and Δb^* are the differentials between the color parameter of the samples and the color parameter of the white standard (L*= 93.60, a*= -0.99, b*= 0.49) used as the film background.

7.3.7.3 Light transmittance and transparency value

Light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using UV–vis spectrophotometer (Shimadzu UV-1800, Kyoto,

Japan) as described by Shiku *et al.* (2004). The transparency value of film was calculated using the following equation (Han and Floros, 1997):

$$\text{Transparency value} = -\log T_{600}/x$$

where T_{600} is the fractional transmittance at 600 nm and x is the film thickness (mm). The greater transparency value represents the lower transparency of film.

7.3.8 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

7.4 Results and discussion

7.4.1 Effect of MFI on properties of myofibrillar protein-based films from washed mince of sardine

7.4.1.1 Thickness and mechanical properties

Thickness and mechanical properties of films prepared from washed sardine mince added without and with MFI at different concentrations are given in Table 10. There was no difference in the thickness between films without and with MFI, regardless of concentrations (P>0.05). Thus, MFI in the level range tested had no impact on the thickness of resulting films. No changes in TS of film were found when MFI was added up to 0.2 g L^{-1} FFS (P>0.05). TS of the film increased as MFI concentrations were above 0.2 g and similar TS was found for the film added with MFI at 0.3 and 0.4 g L^{-1} FFS (P>0.05). The increase in TS was more likely due to the tyosinase present in MFI, which was able to oxidize the side chain of amino acids, particularly OH group of tyrosine (Selinheimo *et al.*, 2008). The quinone formed could act as protein cross-linker. Vate *et al.* (2015) reported that tyrosinase activity in squid

MFI increased the gel properties of sardine surimi via the enhanced protein cross-linking. The tyroisnase oxidizes the side chains of the amino acids such as tyrosine to dihydroxy phenyl alanine (DOPA) and diquinone (Kim and Uyama, 2005). These diquinones are very reactive and can further react with various amino acid side chains such as sulfhydryls, amines, amides, indole and tyrosine commonly present in proteins (Selinheimo *et al.,* 2008). However, the activity of squid ink tyrosinase was more likely lower at the pH 3 of FFS used in the present study. The optimal pH of tyrosinase was around 6 (Simpson *et al.,* 1987). Hence the activity of tyrosinase in resulting film was decreased, in which the lower content of quinone was formed. Nevertheless, the addition of higher amount of MFI (0.3 and 0.4 g L^{-1} FFS) could provide more tyrosinase retained in FFS or films. As a result, quinone generated at higher content could strengthen the film as evidenced by the increased TS.

EAB of the films decreased as the MFI concentration increased (P<0.05). However, MFI at 0.1 g L^{-1} FFS had no effect on EAB (P>0.05). Amongst all films, that added with 0.4 g L^{-1} FFS showed the lowest EAB (P<0.05). In general, the increased TS of film was concomitant with the decreased EAB. The higher EAB reflected the increased flexibility. The flexibility of the films was reduced by the addition of MFI, plausibly due to enhanced cross-linking of proteins in film network. Lower EAB of film is associated with the presence of stronger bonds stabilising film matrix (Tongnuanchan *et al.,* 2011). This indicated that films added with MFI had higher interaction between protein molecules. Hence the addition of MFI increased the TS but decreased the flexibility as indicated by lower EAB.

7.4.1.2 Water vapor permeability (WVP)

WVP of films prepared from washed sardine mince without or with MFI at various concentrations is shown in Table 10. The control film (without MFI) and film with 0.1 g L^{-1} FFS had the highest WVP (P<0.05). This suggested that these films had the lower barrier property toward water vapor migration. The films added with MFI more than 0.1 g L^{-1} FFS had the decreased WVP (P<0.05), in comparison with the control film. The decreased WVP was in agreement with the increased TS. Increased compactness of the film structure by high protein cross-linking induced by MFI more

likely reduced the permeability of water vapor through the films. Tyrosinase in MFI increased the protein-protein interaction in the films, especially at higher concentration. This led to the decrease in hydrophilic moieties of protein molecules available for interacting with water and thus resulting in decreased water adsorptivity and water vapor permeability in the films added with MFI. Protein films generally possess poor water vapor barrier properties because of their hydrophilic nature (Guilbert, 1986; Kester and Fennema, 1986). Paschoalick *et al.* (2003) reported that the muscle protein of Nile tilapia had high content of polar ionic amino acids (aspartic acid, glutamic acid, arginine and lysine). pH of FFS also had the impact on WVP of film based on fish muscle protein. Tongnuanchan *et al.* (2011) reported that films from washed mince from red tilapia prepared at pH 3 had the lower WVP than those prepared at pH 11. Thus, MFI had the influence on WVP of fish protein film, apart from pH used for FFS preparation.

7.4.1.3 Fourier-transform infrared (FTIR) spectroscopy

FTIR spectra of films from washed mince in the absence and presence of MFI at various levels are illustrated in Figure 28. All the films showed the similar major peaks. Generally, all films had the similar spectra in the range of 1800-400 cm^{-1}, covering amide-I, II and III bands. Films exhibited the amide-I band at around 1646 cm^{-1} (representing C=O stretching/hydrogen bonding coupled with COO) (Veeruraj *et al.*, 2015). For amide-II band, the peak was found at wavenumber 1539 cm^{-1}, arising from bending vibration of N-H groups and stretching vibrations of C-N groups (Muyonga *et al.*, 2004). Amide-III band, representing the vibrations in plane of C- N and N-H groups of bound amide or vibrations of CH_2 groups of glycine (Sionkowska *et al.*, 2004), was found at wavenumber 1234 cm^{-1}. The band situated at the wavenumber of 1038-1039 cm^{-1} was found in all film samples, possibly corresponding to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo and Sobral, 2007). An amide-A band was observed at the wavenumber of around 3275 cm^{-1} for all film samples representing the NH-stretching coupled with hydrogen bonding (Elliott and Ambrose, 1950). Amide-B band representing the asymmetric stretching vibration of CH as well as NH_3^+ (Muyonga *et al.*, 2004) was observed at 2930 cm^{-1}, depending on samples.

Table 10: Properties of films from washed sardine mince added with different concentrations of MFI

Samples (g MFI L^{-1} FFS)	TS (MPa)	EAB (%)	WVP ($\times 10^{-11}$ g m^{-1} s^{-1} Pa^{-1})	Thickness (mm)
0	7.95 ± 0.27^{b}	33.07 ± 3.55^{a}	3.00 ± 0.07^{a}	0.040 ± 0.004^{a}
0.1	8.06 ± 0.45^{b}	30.75 ± 2.48^{ab}	2.96 ± 0.01^{a}	0.044 ± 0.005^{a}
0.2	8.49 ± 0.82^{b}	27.76 ± 1.43^{bc}	2.64 ± 0.02^{b}	0.042 ± 0.003^{a}
0.3	9.51 ± 0.25^{a}	26.64 ± 1.54^{c}	2.58 ± 0.01^{b}	0.045 ± 0.003^{a}
0.4	9.52 ± 0.60^{a}	24.72 ± 1.25^{d}	2.54 ± 0.07^{b}	0.043 ± 0.008^{a}

Values indicate mean ± SD (n = 3). Different superscript lowercase letters in the same column indicate the significant differences (P<0.05).

Amide-B band was shifted from 2931 cm^{-1} in the control film to 2928 cm^{-1} in film with the addition of MFI at 0.4 g L^{-1} FFS. The shift in the amide-B band indicated that there was interaction between the protein molecules mainly via NH_3 group of protein chains. This interaction more likely caused by the oxidation of the amino acid side chains in the protein molecules, particularly tyrosine by tyrosinase in the MFI.

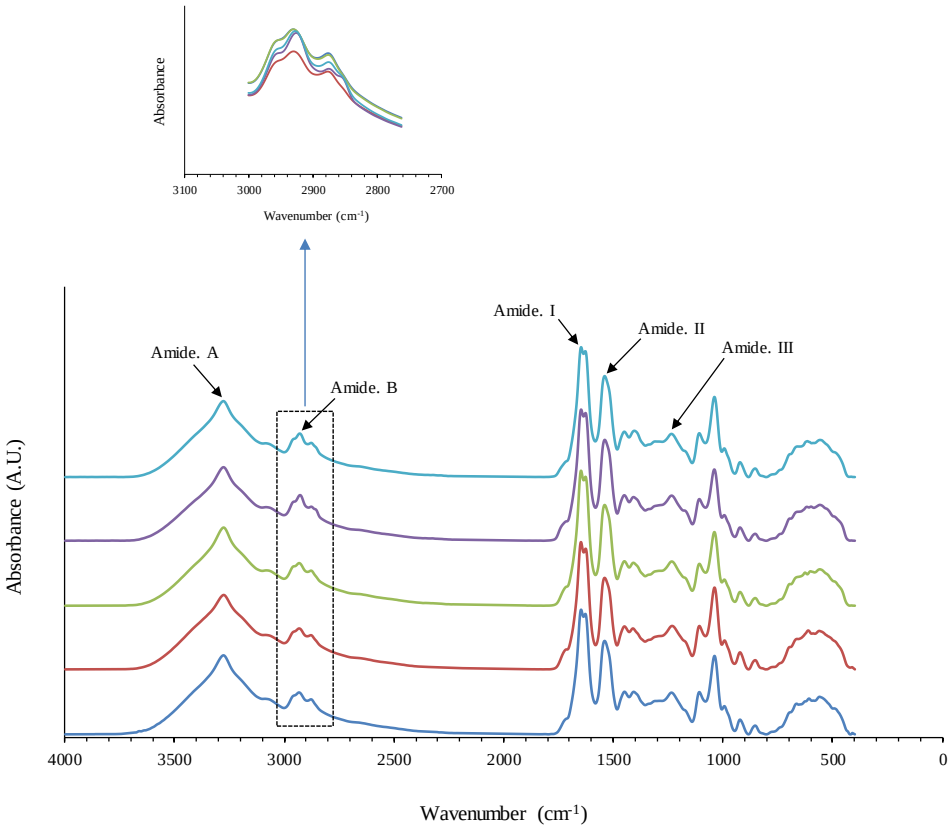

Figure 28. ATR-FTIR of films from washed mince of sardine without and with MFI at different concentrations. 0 g MFI L^{-1} FFS, 0.1 g MFI L^{-1} FFS, 0.2 g MFI L^{-1} FFS, 0.3 g MFI L^{-1} FFS, 0.4 g MFI L^{-1} FFS.

7.4.1.4 Light transmittance

Transmission of UV and visible light at wavelength range of 200-800 nm of films from washed sardine mince added without and with MFI at various concentrations is presented in Table 11. Transmittance was very low at 200 and 280 nm

for all the films. This indicated that the films had the barrier property against UV light. Protein-based films had good UV barrier properties owing to their high content of aromatic amino acids that absorb UV light (Hamaguchi *et al.,* 2007). Light transmittance of the films in visible range increased as the MFI concentrations increased. Light transmission of visible range (350–800 nm) of control film ranged from 39.81% to 78.64%, whereas highest values were found for film added with MFI at 0.4 g L^{-1} FFS (48.75–81.99%). Light transmission of film was most likely governed by the arrangement or alignment of polymer in film network (Limpan *et al.,* 2010). This indicated that the addition of MFI led to the arrangement of proteins in the film matrix via protein cross-linking in the way which enhanced light transmission through films.

Table 11: Light transmittance values of the films from washed sardine mince added with different concentrations of MFI

Samples	**Light transmittance (%)**							
(g MFI L^{-1} FFS)	200	280	350	400	500	600	700	800
0	0.01	0.06	39.81	55.35	71.64	75.48	77.34	78.64
0.1	0.02	0.07	46.44	59.86	71.93	76.62	78.83	80.38
0.2	0.02	0.07	47.08	60.74	72.59	76.81	78.96	80.42
0.3	0.02	0.09	47.28	60.76	74.27	78.54	80.58	81.98
0.4	0.02	0.12	48.75	60.84	74.49	78.74	80.67	81.99

7.4.1.5 Scanning electron microscopy

Scanning electron microscopic images of surface and cross-section of films from washed mince incorporated without and with MFI at different concentrations are depicted in Figure 29. The surface of the control film and film with 0.1 g MFI L^{-1} FFS was smoother, compared to that of the films added with MFI at higher levels. The increased cross-linking in network might lead to the protruded structure, as indicated by the rough surface.

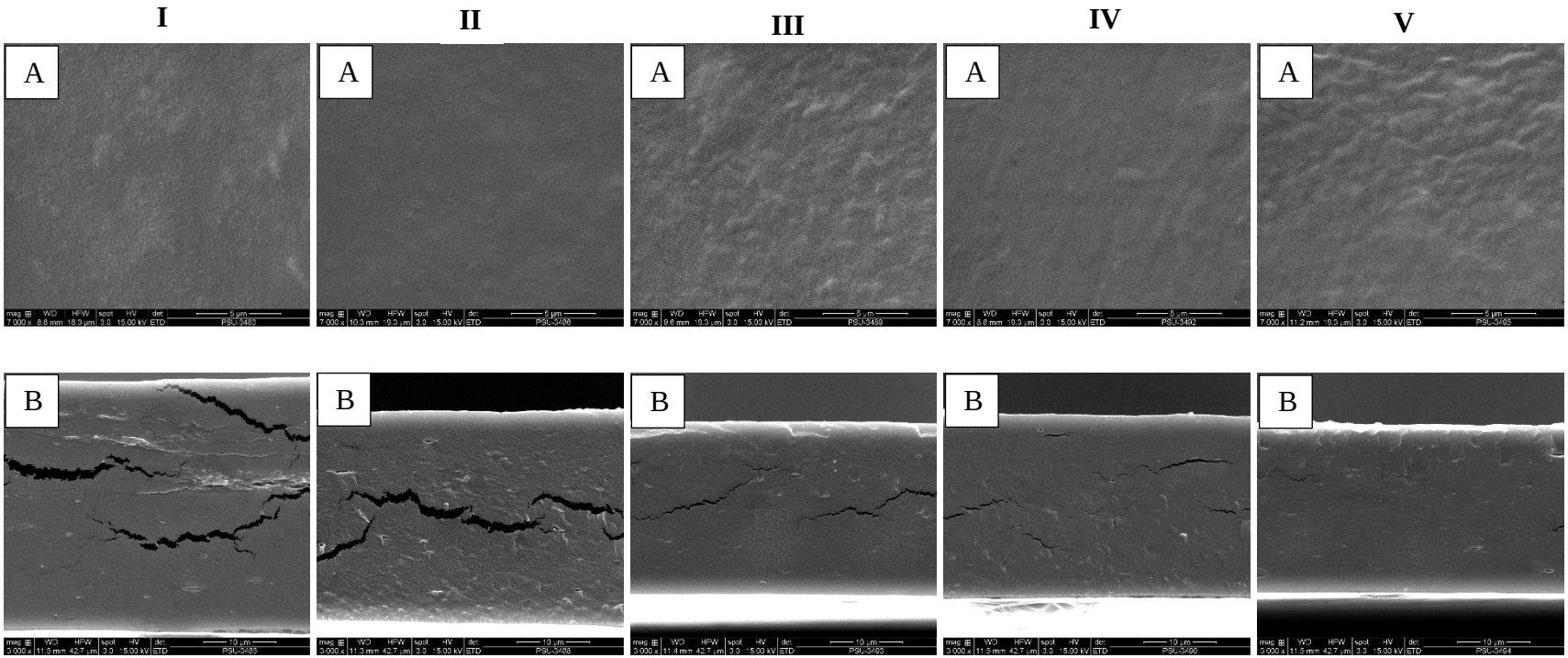

Figure 29. Scanning electron micrograph of surface (A) and cross-section (B) of films from washed mince of sardine without and with MFI at different concentrations. **I**, 0 g MFI L^{-1} FFS; **II**, 0.1 g MFI L^{-1} FFS; **III**, 0.2 g MFI L^{-1} FFS; **IV**, 0.3 g MFI L^{-1} FFS; **V**, 0.4 g MFI L^{-1} FFS.

The freeze-fractured cross-section of the film without MFI and with 0.1 g L^{-1} FFS showed the larger cracks or gap, compared to those of films with higher MFI concentrations. Smaller cracks were observed in the films added with 0.2 and 0.3 g MFI L^{-1} FFS, suggesting the higher protein cross-linking induced by MFI.No cracks were found in film containing MFI at 0.4 g L^{-1} FFS. This reconfirmed the immense protein cross-linking in film network. The result was in accordance with the highest TS and lowest WVP of film added with 0.4 g L^{-1} FFS.

7.4.2 Effect of MFI on oxidation and yellow discoloration of films from washed sardine mince during storage

7.4.2.1 TBARS

TBARS values of films prepared from washed mince added without and with MFI at various concentrations during 20 days of storage are shown in Figure 30. At day 0 of storage, TBARS value was higher for the control film (without MFI), compared with other samples (P<0.05). The TBARS values of films with MFI decreased as the concentration of MFI increased and the lowest value was observed for the film added with MFI at 0.4 g MFI L^{-1} FFS (P<0.05). The films were prone to lipid oxidation during casting and drying as large surface area was exposed to the atmospheric oxygen. TBARS values of the films with MFI were lower, indicating that the MFI inhibited lipid oxidation during casting and drying. The MFI was able to scavenge radicals or pro-oxidants, thereby preventing lipid oxidation in films. Vate and Benjakul (2013) reported that MFI had DPPH and ABTS radical scavenging activities and activities were governed by size of peptides in MFI. Heme iron, pro-oxidant in the fish muscle, might be involved in lipid oxidation in the films. The MFI was able to chelate the metal ions in the FFS (Vate and Benjakul, 2013). As a result, lipid oxidation during casting and drying could be decreased.

During the extended storage, TBARS values of all films increased up to 10^{th} day. Thereafter, TBARS values decreased until the end of storage (day 20). The loss of low molecular weight volatile secondary oxidation products might occur. However, films with MFI had lower TBARS than did the control film throughout the

storage for 20 days (P<0.05). Mackerel mince added with MFI and stored in ice for 15 days had lower TBARS values than the control (without MFI) (Vate and Benjakul, 2013). MFI also increased the storage stability of gels from sardine surimi by lowering the lipid oxidation during the 20 days of storage at 4 ℃ (Vate *et al.*, 2015).

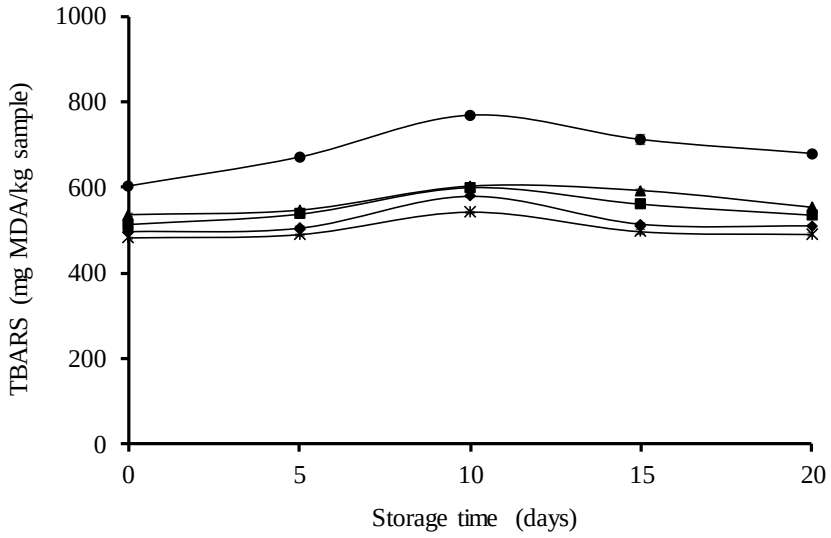

Figure 30. Changes in TBARS of the films from washed mince of sardine without and with MFI at different concentrations during the storage of 20 days. 0 g MFI L^{-1} FFS, 0.1 g MFI L^{-1} FFS, 0.2 g MFI L^{-1} FFS, 0.3 g MFI L^{-1} FFS, 0.4 g MFI L^{-1} FFS. Bars represent the standard deviation (n = 3).

7.4.2.2 Color

Color expressed as b*-value (yellowness) and ΔE*-value (total color difference) of protein films from washed mince added without and with MFI at different concentrations during 20 days of storage are depicted in Figure 31A and 31B. At day 0, the control film (without MFI) had higher b* and ΔE* -values, compared to those added with MFI at concentrations of 0.3 and 0.4 g L^{-1} FFS (P<0.05). It indicated that lipid oxidation was more pronounced in the control film, even during casting and drying. The films added with 0.1 and 0.2 g MFI L^{-1} FFS had similar b* and ΔE* values throughout the storage time of 20 days (P>0.05). The lowest b* and ΔE* values were observed for the films added with MFI at 0.4 g L^{-1} FFS throughout the storage (P<0.05).

Conversely, the control film showed the highest b* and ΔE*-values during the storage of 20 days. It was noted that the control film became more yellowish, compared with others. The result suggested that MFI acted in a dose dependent manner in lowering the yellow discoloration. At higher concentration, MFI was able to act as antioxidant and reduce the lipid oxidation more effectively. This result was in agreement with the TBARS values of the films (Figure 30). Cuq *et al.* (1996) reported that fish myofibrillar protein-based films from Atlantic sardines showed yellow discoloration during storage for 8 weeks at 20 °C. Therefore, MFI could prevent yellow discoloration of film based on washed sardine mince during prolonged storage.

7.4.2.3 Transparency value

Transparency values of protein films from washed mince added without and with MFI at different levels during 20 days of storage are given in Figure 31C. The control film had higher transparency values throughout the storage, compared to those added with MFI ($P<0.05$). At day 0, the transparency values were decreased as the MFI concentration in the films increased ($P<0.05$). The lower transparency value indicated that the film was more transparent. Thus, the films added with MFI were more transparent, compared to the film without MFI. Lipid oxidation taken place during casting and drying might result in the decrease in transparency of films. Lipid oxidation products formed might be related with increased yellowness of control films. The increase in transparency of the films added with MFI, especially at higher levels, could be caused by the decreased lipid oxidation. Nevertheless, there was no change in the transparency values of all films during the storage. This result was in agreement with Tongnuanchan *et al.* (2013) who reported that protein based films from red tilapia protein isolate had no marked change in transparency value during 40 days of storage at room temperature.

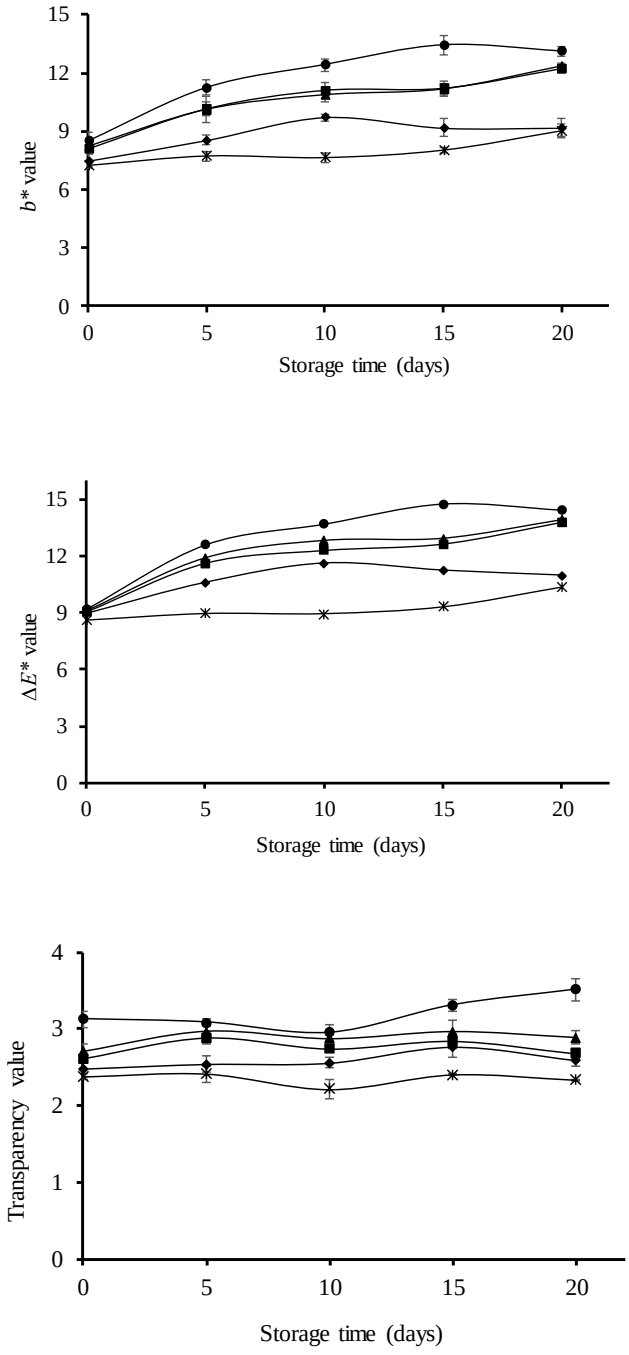

Figure 31. Changes in b^* (A), ΔE^* (B) and transparency value (C) of films from washed mince of sardine without and with MFI at different concentrations during 20 days of storage. —●— 0 g MFI L^{-1} FFS, —▲— 0.1 g MFI L^{-1} FFS, —■— 0.2 g MFI L^{-1} FFS, —◆— 0.3 g MFI L^{-1} FFS, —✱— 0.4 g MFI L^{-1} FFS. Bars represent the standard deviation (n = 3).

7.5 Conclusion

MFI increased the mechanical properties and decreased WVP of protein films from washed sardine mince. Yellow discoloration was lowered by incorporation with MFI, especially at higher levels. This was related with decreased lipid oxidation as indicated by lower TBARS values. Hence MFI acted as antioxidant and decreased the yellow discoloration, whilst tyrosinase in MFI improved the mechanical properties of the film.

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CHAPTER 8
IMPROVEMENT OF PROPERTIES OF SARDINE MYOFIBRILLAR PROTEIN FILMS USING SQUID INK TYROSINASE IN COMBINATION WITH TANNIC ACID

8.1 Abstract

The effect of squid ink tyrosinase (SIT) and tannic acid (TA) on the properties of myofibrillar protein films from washed sardine mince was studied. The film made from film forming solution added with SIT (5 k units L^{-1}) and TA (1 g L^{-1}) had the highest tensile strength (TS) but lowest elongation at break (EAB) (P<0.05). The addition of mixture of SIT and TA reduced the water vapor permeability (WVP) of films (P<0.05). Fourier transform infrared (FTIR) spectra of films containing both SIT and TA showed a shift in the amide-III band to the lower wavenumber. The decrease in myosin heavy chain (MHC) band intensity in the film added with the mixture of SIT and TA was observed, indicating the increased cross-linking. The films added with the mixture of SIT and TA were less transparent, compared to the control film. The microstructure study revealed that the surface of films added with only SIT or the mixture of SIT and TA became slightly rougher. Thus, the use of SIT in conjunction with TA could improve mechanical property and water barrier property of resulting film.

8.2 Introduction

Biodegradable packaging has gained more attention recently since it is environment friendly. Biopolymers can be potential alternatives to non-biodegradable synthetic polymers. Biodegradable/edible packagings can be prepared from various biopolymers such as polysaccharides, proteins, lipids etc. Among the various sources, proteins have been extensively used for the preparation of biodegradable films because of its good film-forming ability. Films have been prepared using myofibrillar proteins from different fish species (Shiku *et al.,* 2004; Chinabhark *et al.,* 2007; Tongnuanchan *et al.,* 2011). However, myofibrillar protein films still have poor mechanical and water vapor barrier properties, compared to their synthetic counterparts.

Various methods including chemical and enzymatic reactions, irradiation treatments or incorporation of hydrophobic material or synthetic polymers have been implemented to improve the properties of protein based films (Benjakul *et al.*, 2016). The addition of tannic acid, caffeic acid and ferulic acid increased the tensile strength of porcine plasma protein-based films (Nuthong *et al.*, 2009). Mechanical properties of films from myofibrillar proteins of bigeye snapper (*Priacanthus tayenus*) were improved by the addition of phenolic compounds such as caffeic acid, catechin, ferullic acid and tannic acid (Prodpran *et al.*, 2012). Weng and Zheng (2015) studied the effect of transglutaminase on properties of tilapia scale gelatin incorporated with soy protein isolate. Prior enzymatic cross-linking of bovine gelatin via horseradish peroxidase, glucose oxidase and glucose improved the microstructure and mechanical properties of target film (Han and Zhao, 2016).

Melanin-free ink from squid has been reported to show antioxidative and gel strengthening properties (Vate and Benjakul, 2013; Vate *et al.*, 2015). Tyrosinase extracted from squid melanin-free ink in combination with tannic acid improved the gel properties of sardine surimi (Vate and Benjakul, 2016). Combination of squid ink tyrosinase and tannic acid also increased the protein cross-linking in heat induced aggregation of natural actomyosin (Vate and Benjakul, 2016a). Protein cross-linking induced by tyrosinase from melanin-free ink in the presence of appropriate phenolic compound might improve the properties of fish myofibrillar protein films. Nevertheless, no information on the use of this approach for improvement of fish myofibrillar protein films exists. Therefore, the aim of this study was to investigate the effect of mixture of squid ink tyrosinase and tannic acid on the properties of myofibrillar protein film from washed sardine mince.

8.3 Materials and methods

8.3.1 Chemicals

Tannic acid, glycerol, sodium chloride (NaCl), Brij 35, L-DOPA (3,4-Dihydroxy-L-phenylalanine), β-mercaptoethanol and wide range molecular weight protein markers were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide

(NaOH) and hydrochloric acid (HCl) were obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, *N*,*N*,*N*,*N*-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

8.3.2 Preparation of squid ink tyrosinase

8.3.2.1 Preparation of melanin-free ink

Melanin-free ink was prepared according to the method of Vate and Benjakul (2013). Squids were purchased from a local market in Hat Yai, Thailand, stored in ice using a squid/ice ratio of 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Thailand. Upon arrival, ink sac was separated from the squid by cutting the ink duct and ink was squeezed out from the ink sac. The squid ink was diluted ten-fold using the cold deionized water (2-4 °C). Then it was centrifuged at 18,000×g for 30 min at 4 °C to remove the melanin using a refrigerated centrifuge (Allegra 25 R centrifuge, Beckman Coulter, Palo Alto, CA, USA). The supernatant obtained was used as melanin-free ink (MFI).

8.3.2.2 Fractionation of tyrosinase

Tyrosinase from MFI was fractionated as per the method of Simpson *et al.* (1987) with a slight modification. MFI (50 mL) was mixed with 50 mL of 0.05 M sodium phosphate buffer (pH 7.2), containing 1.0 M NaCl and 0.2% Brij 35. The mixture was stirred continuously at 4 °C for 30 min. Solid ammonium sulfate was added into the mixture to obtain 60% saturation. The mixture was allowed to stand at 4 °C for 30 min. The precipitate was collected by centrifugation at 12,500×g at 4 °C for 30 min. The pellet obtained was dissolved in a minimum volume of 0.05 mM sodium phosphate buffer (pH 7.2) and dialyzed with 15 volumes of the same buffer with three changes overnight. The fraction containing tyrosinase referred to as ‘squid ink tyrosinase, SIT’ was kept at -40 °C until used.

8.3.2.3 Measurement of tyrosinase activity

Tyrosinase activity was assayed using L-DOPA as a substrate according to the method of Simpson *et al.* (1987) with a slight modification. Reaction mixtures consisted of 600 µl of 15 mM L-DOPA in deionized water, 400 µl of 0.05 M phosphate buffer (pH 6.0) and 100 µl of deionized water. To initiate the reaction, 100 µL of SIT was added and the reaction was run for 3 min at room temperature. The formation of dopachrome was monitored by reading at 475 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of activity was defined as the enzyme causing an increase in the absorbance at 475 nm by 0.001/min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and deionized water was used instead.

8.3.3 Collection and preparation of fish

Sardines (*Sardinella albella*) with an average weight of 50-60 g were caught from Songkhla coast along the Gulf of Thailand. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. The fish were headed, gutted and washed with water. The flesh was separated manually from skin and bone and kept on ice prior to washing. The storage time was not longer than 24 h.

8.3.3.1 Preparation of washed mince

Washed mince was prepared according to the method of Toyohara *et al.* (1990) with slight modifications. Fish mince was homogenized with 5 volumes of cold 0.05 M NaCl (2-4 °C) at a speed of 13,000 rpm for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The washed mince was filtered through two layer of cheese-cloth. The washing process was repeated twice. Washed mince obtained was stored on ice until used for film preparation.

8.3.4 Preparation of film-forming solution

The film-forming solution (FFS) from washed mince was prepared according to the method of Chinabhark *et al.* (2007). The washed mince (200 g) was added with 3 volumes of distilled water and homogenized at 13,000 rpm for 1 min. The protein concentration of the mixture was fixed at 2% (w/v). Glycerol, used as a plasticizer, was added at 50% (w/w) of protein. The pH of the mixture was adjusted to 3 using 1 M HCl to solubilize the protein. FFS was added with SIT (5 k units L^{-1}) or TA (0.5 and 1 g L^{-1}), referred to as SIT, TA-I and TA-II, respectively. SIT/TA mixtures including 5 k units L^{-1} SIT + 0.5 g L^{-1} TA or the mixtures of 5 k units L^{-1} SIT + 1 g L^{-1} TA named as SIT/TA-I and SIT/TA-II, respectively, were also added into FFS. FFS was degassed using the sonicating bath (Elmasonic S 30 H, Singen, Germany) for 10 min. FFS obtained was filtered through a layer of cheese-cloth to remove undissolved debris. The filtrate was used for film casting.

8.3.4.1 Film casting and drying

To prepare the film, FFS (4 g) was cast onto a rimmed silicone resin plate (50 × 50 mm^2) and air-blown for 12 h at room temperature prior to further drying at 25 °C and 50±5% RH for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting films were manually peeled off and used for analyses.

8.3.5 Determination of film properties

8.3.5.1 Film thickness

The thickness of film was measured using a micrometer (Gotech, Model GT-313-A, Gotech testing machines Inc, Taichung city, Taiwan). Five random locations around each film samples were used for average thickness determination.

8.3.5.2 Mechanical properties

Prior to testing, films were conditioned for 48 h at 25 °C and 50±5 % RH. Tensile strength (TS) and elongation at break (EAB) were determined as described

by Iwata et al. (2000) with a slight modification using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK) equipped with tensile load cell of 100 N. Ten samples (2×5 cm^2) with initial grip length of 3 cm were used for testing. Cross-head speed was set at 30 mm/min.

8.3.5.3 Water vapor permeability (WVP)

WVP was measured following a modified ASTM method (American Society for Testing and Materials, 1989) as described by Shiku *et al.* (2004). The film was sealed on an aluminium permeation cup containing dried silica gel (0% RH) with silicone vacuum grease and a rubber gasket to hold the film in place. The cups were placed in a desiccator containing the distilled water at 30 °C. The cups were weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$\text{WVP (g m}^{-1}\text{ s}^{-1}\text{ Pa}^{-1}) = w\, l\, A^{-1}\, t^{-1}\, (P_2 - P_1)^{-1}$$

where w is the weight gain of the cup (g); l is the film thickness (m); A is the exposed area of film (m^2); t is the time of gain (s); (P_2–P_1) is the vapor pressure difference across the film (Pa).

8.3.5.4 Color

Color of films was determined using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA), using D_{65} (day light) and a measure cell with opening of 30 mm. The color of the films was expressed as L^* (lightness), a^* (redness) and b^* (yellowness/greenness). The difference of color (ΔE^*) was calculated as follows:

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

where ΔL^*, Δa^* and Δb^* are the differences between the color parameters of the samples and those of the white standard.

8.3.5.5 Light transmittance and transparency value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using UV–vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) as described by Shiku *et al.* (2004). The transparency value of film was calculated using the following equation (Han and Floros, 1997):

$$\text{Transparency value} = -\log T_{600}/x$$

where T_{600} is the fractional transmittance at 600 nm and x is the film thickness (mm). The greater transparency value represents the lower transparency of film.

8.3.6 Protein pattern

Protein patterns of films was determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). To solubilize the films prior to SDS-PAGE analysis, films were mixed with 5% SDS and heated at 85 °C for 1 h. Proteins (15 μg) determined by the Biuret method (Robinson and Hodgen, 1940) were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-PROTEAN II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant 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 for 12 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h. Molecular weights of proteins were estimated from protein markers.

8.3.7 Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

Films were scanned with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45 º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at room temperature as described by Nuthong *et*

al. (2009). Films were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 400-4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ºC.

8.3.8 Scanning electron microscopy (SEM)

Morphology of surface and cross-section of film samples was visualized using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, Netherlands). For cross-section, samples were fractured under liquid nitrogen prior to morphology visualisation. Then, the samples were mounted on bronze stub and sputtered with gold (Sputter coater SPI-Module, PA, USA) in order to make the sample conductive. The photographs were taken at an acceleration voltage of 15 kV.

8.3.9 Statistical analysis

All experiments were run 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 tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

8.4 Results and discussion

8.4.1 Effect of SIT/TA mixtures on properties of sardine myofibrillar protein films

8.4.1.1 Thickness and mechanical properties

Thickness and mechanical properties of films prepared from washed sardine mince added without and with SIT, TA or the SIT/TA mixtures are given in Table 12. The film added with only SIT (5 k units L^{-1} FFS) had the highest thickness amongst all film samples (P<0.05). The presence of SIT might reduce the interaction between protein molecules in the film, in which the compact network was not formed. However, films added with only TA or SIT/TA-II had the lowest thickness (P<0.05).

TS of film containing only SIT was similar to that of control film (film without SIT, TA or SIT/TA mixture addition) but was lower than that added with only TA (P<0.05). This was more likely due to the lower activity of SIT in FFS at pH 3. The optimum pH of tyrosinase was around 6 (Simpson *et al.,* 1987). The addition of TA increased TS of the film in a dose dependent manner (P<0.05). TA was reported to exhibit multidentate mechanism, in which phenolic compounds could react with several protein sites and led to the augmented cross-links between proteins (Haslam, 1989). Amongst different phenolic compounds (caffeic acid, catechin, ferullic acid and tannic acid), tannic acid yielded the film from myofibrillar proteins of bigeye snapper (*Priacanthus tayenus*) with the highest TS (P<0.05) (Prodpran *et al.,* 2012). TS of the film increased with the addition of SIT/TA mixtures and the highest TS was found for the film added with the SIT/TA-II. Oxidation of TA by SIT take could place and the resulting quinones could induce the cross-linking of proteins in washed sardine mince. Vate and Benjakul (2016) reported that the mixture of SIT and TA improved the gel properties of sardine surimi. The quinones formed via the oxidation of TA by SIT mostly increased the cross-linking between the actomyosin molecules during heat induced aggregation (Vate and Benjakul, 2016a). These diquinones formed during diphenolase activity of tyrosinase are very reactive and can react with various amino acid side chains such as sulfhydryls, amines, amides, indoles and other tyrosines commonly present in proteins (Selinheimo *et al.,* 2008).

EAB of films decreased with the addition of TA or SIT/TA mixtures. In general, the increased TS of film was concomitant with the lowered EAB. The higher EAB reflected the higher extensibility of films. Extensibility of films was reduced by the addition of TA or SIT/TA mixture. This was plausibly due to the increased cross-linking of proteins, particularly in the presence of SIT and TA. EAB of film was decreased when the strong bonds were introduced in film matrix (Tongnuanchan *et al.,* 2011). The result suggested that the films added with SIT/TA mixture had stronger bonds, compared to those added with only SIT and only TA. The addition of SIT/TA-II yielded the film with higher protein cross-linking than SIT/TA-I as evidenced by the lower EAB and higher TS of the former. Quinone or diquinone, intermediate from oxidation of TA by SIT more likely induced the cross-linking of proteins in the network

along with the formation of non-disulfide covalent bonds (Vate and Benjakul, 2016). Hence the addition of mixture of SIT and TA, especially in the presence of higher level of TA, increased TS but decreased the extensibility as indicated by lower EAB values.

8.4.1.2 Water vapor permeability (WVP)

WVP of films prepared from washed sardine mince as affected by the addition of SIT, TA and their mixtures is shown in Table 12. The control film had the highest WVP (P<0.05). This suggested that these films had lower barrier toward water vapor permeability. The film added with only SIT had higher WVP than those incorporated with only TA and the mixtures of SIT and TA (P<0.05). This indicated that there was lower protein interaction in the film added with SIT, compared with those added with TA or the SIT/TA mixture. Both the film samples with only TA (0.5 g and 1 g L^{-1} FFS) had the similar WVP (P>0.05). Thus, TA addition could improve water vapor barrier property more effectively than SIT in this study. The films added with mixture of SIT and TA had the lower WVP values (P<0.05). These results were in agreement with the increased TS of resulting films (Table 12). Increased compactness of the film structure by high protein cross-linking more likely reduced the permeability of moisture vapor through the films. Despite having good mechanical properties, protein films generally possess poor moisture barrier properties because of their hydrophilic nature (Guilbert, 1986; Kester and Fennema 1986). Fish muscle is hydrophilic, associated with polar amino acids. Paschoalick *et al.* (2003) reported that the muscle protein of Nile tilapia had polar ionic amino acids (aspartic acid, glutamic acid, arginine and lysine) at high content. The cross-linked proteins mediated by TA and SIT/TA mixture could reduce the hydrophilic moieties in the matrix of film, resulting in decreased film hydrophilicity and thus decreasing WVP of the film.

Table 12: Thickness, mechanical properties and water vapor permeability of films from washed sardine mince added with SIT, TA or SIT/TA mixtures

Samples	TS (MPa)	EAB (%)	WVP ($\times 10^{-11}$ g m^{-1} s^{-1} Pa^{-1})	Thickness (mm)
Control	7.32 ± 0.20^{e}	38.17 ± 2.75^{a}	2.98 ± 0.07^{a}	0.056 ± 0.004^{b}
SIT	7.34 ± 0.19^{e}	37.75 ± 3.01^{ab}	2.69 ± 0.04^{b}	0.061 ± 0.004^{a}
TA-I	7.66 ± 0.14^{d}	34.27 ± 2.58^{bc}	2.60 ± 0.06^{c}	0.049 ± 0.002^{c}
TA-II	7.83 ± 0.10^{c}	32.46 ± 1.92^{c}	2.56 ± 0.09^{cd}	0.055 ± 0.004^{b}
SIT/TA-I	8.44 ± 0.44^{b}	27.18 ± 1.27^{d}	2.39 ± 0.08^{e}	0.054 ± 0.003^{b}
SIT/TA-II	9.49 ± 0.11^{a}	25.91 ± 1.81^{e}	2.21 ± 0.02^{f}	0.049 ± 0.003^{c}

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

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

8.4.1.3 Color

L*, a* and b*-values of protein films from washed sardine mince added without and with SIT, TA and their mixtures are presented in Table 13. The control film and film containing 0.5 g TA L^{-1} FFS had higher L* (lightness) values, compared to other films (P<0.05). There was a coincidental increase in a*-value (redness) in the sample added with the SIT/TA mixture. The b*-value (yellowness) was highest for the films added with SIT. This might be due to the indigenous pigment of SIT. In general, films added with TA at both levels had the lower b*-value than those containing SIT (P<0.05). Decreased L* values were in accordance with the increases in a*-values and b*-values of myofibrillar protein films incorporated with phenolic compounds (Prodpran *et al.*, 2012).

8.4.1.4 Transparency

Transparency values of protein films from washed sardine mince added without and with SIT, TA and SIT/TA mixtures are given in Table 13. The control film had the lowest transparency value, compared to others (P<0.05). The lower transparency value indicated that the film was more transparent. The film added with SIT (5 k units L^{-1}) had the highest transparency value (P<0.05), indicating the lowest transparency. This result was coincidental with the lower light transmittance of the film (Table 14). There were no differences in transparency values amongst all films added with TA, regardless of SIT addition. The films added with SIT/TA mixtures were less transparent, compared to the control. This was more likely due to the brownish color of quinones formed via the oxidation of TA induced by SIT. In addition, the higher protein cross-linking induced by the quinone might also lead to less transparent films. Films from porcine plasma added with phenolic compounds had higher transparency values (Nuthong *et al.*, 2009). The result suggested that the incorporation of SIT, TA or their mixtures affected the transparency of resulting films to varying degrees.

Table 13: Color and transparency value of the films from washed sardine mince added with SIT, TA or SIT/TA mixtures

Samples	L*	a*	b*	Transparency
Control	88.49 ± 0.05 [a]	-2.02 ± 0.04 [e]	7.27 ± 0.16[c]	1.97 ± 0.23[c]
SIT	86.52 ± 0.27 [c]	-1.72 ± 0.09 [d]	8.72 ± 0.45[a]	2.69 ± 0.51[a]
TA-I	88.45 ± 0.06 [a]	-1.58 ± 0.03 [c]	5.71 ± 0.10[e]	2.32 ± 0.20[b]
TA-II	87.81 ± 0.08 [b]	-1.44 ± 0.05 [b]	6.06 ± 0.12[d]	2.16 ± 0.20[bc]
SIT/TA-I	85.86 ± 0.07 [d]	-1.37 ± 0.03 [a]	8.07 ± 0.15[b]	2.29 ± 0.15[b]
SIT/TA-II	85.55 ± 0.16 [e]	-1.36 ± 0.04 [a]	8.80 ± 0.18[a]	2.39 ± 0.18[b]

Values indicate mean ± SD (n = 3). Different superscript lowercase letters in the same column indicate the significant differences (P<0.05).

SIT: 5 k units L^{-1} SIT; **TA-I**: 0.5 g L^{-1} TA; **TA-II**: 1 g L^{-1} TA; **SIT/TA-I**: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; **SIT/TA-II**: 5 k units L^{-1} SIT+ 1 g L^{-1} TA.

8.4.1.5 Light transmittance

Light transmittance in both UV and visible ranges of protein films from sardine washed mince with different treatments is given in Table 14. Light transmittance decreased as SIT was added. Only slight decrease of transmittance was found in films added with TA at both levels. Nevertheless, the decrease in transmittance of films was also noticeable in the wavelength of 350-600 nm when the SIT/TA mixtures were incorporated. Light transmittance in the UV range (200 and 280 nm) was lower for all the films. Protein-based films had good UV barrier properties owing to their high content of aromatic amino acids that absorb UV light (Hamaguchi *et al.*, 2007). The enhanced cross-linking of the protein molecules in the film matrix might also lower the light transmittance throughout the films. This indicated that the films added with SIT/TA mixture, especially in the presence of 1 g L^{-1} TA, were more compact and were able to prevent the light to pass through the film. As a result, it could lower the oxidation of lipids in food induced by light.

Table 14: Light transmittance values of the films from washed sardine mince added with SIT, TA or SIT/TA mixtures

Samples	Light transmittance (%)							
	200	280	350	400	500	600	700	800
Control	0.01	0.02	47.95	62.03	74.24	77.96	79.74	81.03
SIT	0.01	0.08	38.72	51.42	64.01	68.54	71.06	72.97
TA-I	0.01	0.01	50.58	62.43	73.02	76.77	78.92	80.43
TA-II	0.01	0.01	48.16	60.94	72.31	76.08	78.27	79.70
SIT/TA-I	0.01	0.01	44.10	58.01	70.99	75.44	78.06	79.92
SIT/TA-II	0.01	0.01	41.59	56.85	71.61	76.54	79.46	81.48

SIT: 5 k units L^{-1} SIT; **TA-I**: 0.5 g L^{-1} TA; **TA-II**: 1 g L^{-1} TA; **SIT/TA-I**: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; **SIT/TA-II**: 5 k units L^{-1} SIT+ 1 g L^{-1} TA.

8.4.2 Protein pattern of films

Protein patterns of films prepared from washed sardine mince incorporated with SIT, TA or their mixtures, under reducing conditions are shown in Figure 32. Similar protein patterns were observed among all film samples. Some cross-links between stacking gel and separating gel were found in the control films, suggesting the cross-linking of proteins in film via covalent bonds. Increase in polymers found in the stacking gel was found in the film samples added with SIT, TA and the mixture of SIT and TA. This suggested that MHC was more susceptible to crosslinking induced by the quinones formed via the oxidation of TA by SIT. The formation of cross-link of proteins was more likely stabilized by non-disulfide covalent bond, especially during film drying process. This was in accordance with increased TS of those samples (Table 12). In general, non-disulfide covalent bond, disulfide bond as well as other weak bonds most likely contributed to the film strengthening. The addition of phenolic compounds, especially oxidized form, contributed to the improved mechanical property of fish myosin protein based films (Prodpran *et al.*, 2012).

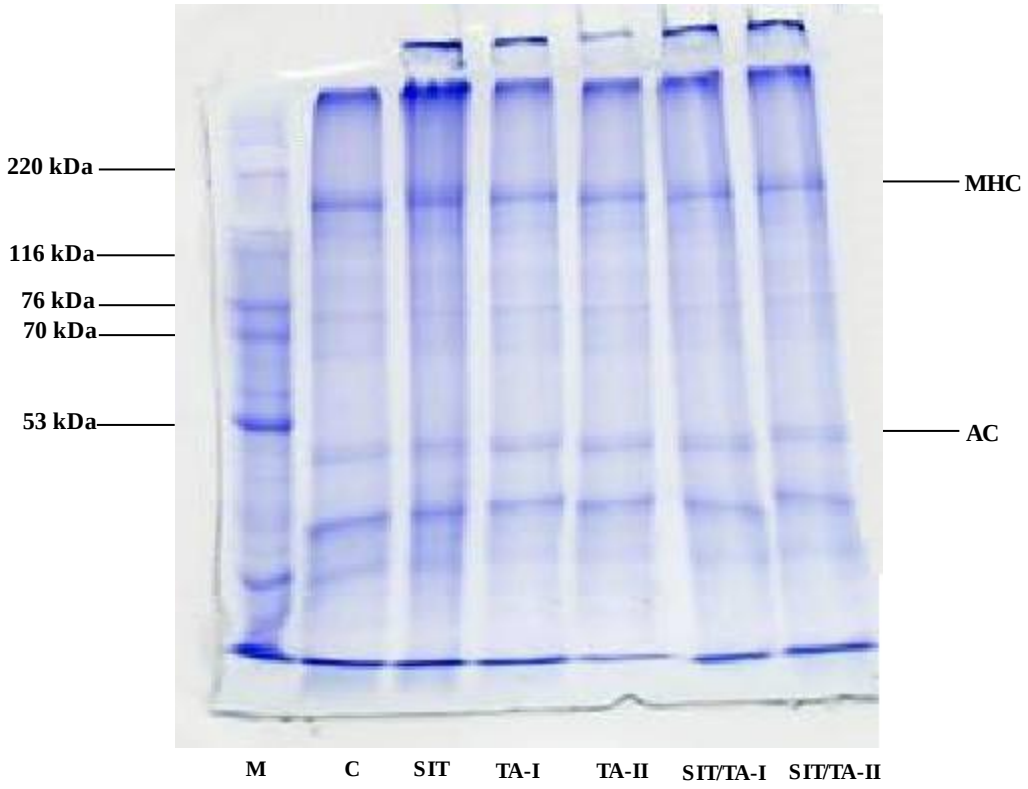

Figure 32. SDS–PAGE patterns of proteins of films from washed sardine mince without and with SIT, TA or SIT/TA mixtures at different concentrations. **M**, high molecular weight protein markers; **C**, control; **MHC**, myosin heavy chain; **AC**, actin; **SIT**, 5 k units L^{-1} SIT; **TA-I**, 0.5 g L^{-1} TA; **TA-II**, 1 g L^{-1} TA; **SIT/TA-I**, 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; **SIT/TA-II**, 5 k units L^{-1} SIT+ 1 g L^{-1} TA.

8.4.3 Fourier-transform infrared (FTIR) spectroscopy

FTIR spectra of films from sardine washed mince with various treatments in comparison with control film are illustrated in Figure 33. All films exhibited the major bands such as amide-I at around 1646 cm^{-1} representing C=O stretching/hydrogen bonding coupled with COO and amide-II at 1539 cm^{-1}, which is arising from bending vibration of N-H groups and stretching vibrations of C-N groups (Aewsiri *et al.*, 2009; Muyonga *et al.*, 2004). Amide-III band representing the vibrations in plane of C- N and N-H groups of bound amide or vibrations of CH_2 groups of glycine, shifted from around 1234 cm^{-1} in the control film to 1230 cm^{-1} in film added with SIT/TA-II. This indicated protein interaction between C- N and N-H groups induced

by the mixture of SIT and TA. The band situated at the wavenumber of around 1038 cm^{-1} was found in all film samples, corresponding to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo and Sobral, 2007; Hoque *et al.*, 2011). Amide-A and amide-B bands were observed at the wavenumber of 3275 cm^{-1} and 2930 cm^{-1}, respectively. The amide-A band represents the NH-stretching coupled with hydrogen bonding and amide-B band represents the asymmetric stretching vibration of CH as well as NH_3^+ (Ahmad and Benjakul, 2011; Muyonga *et al.*, 2004).

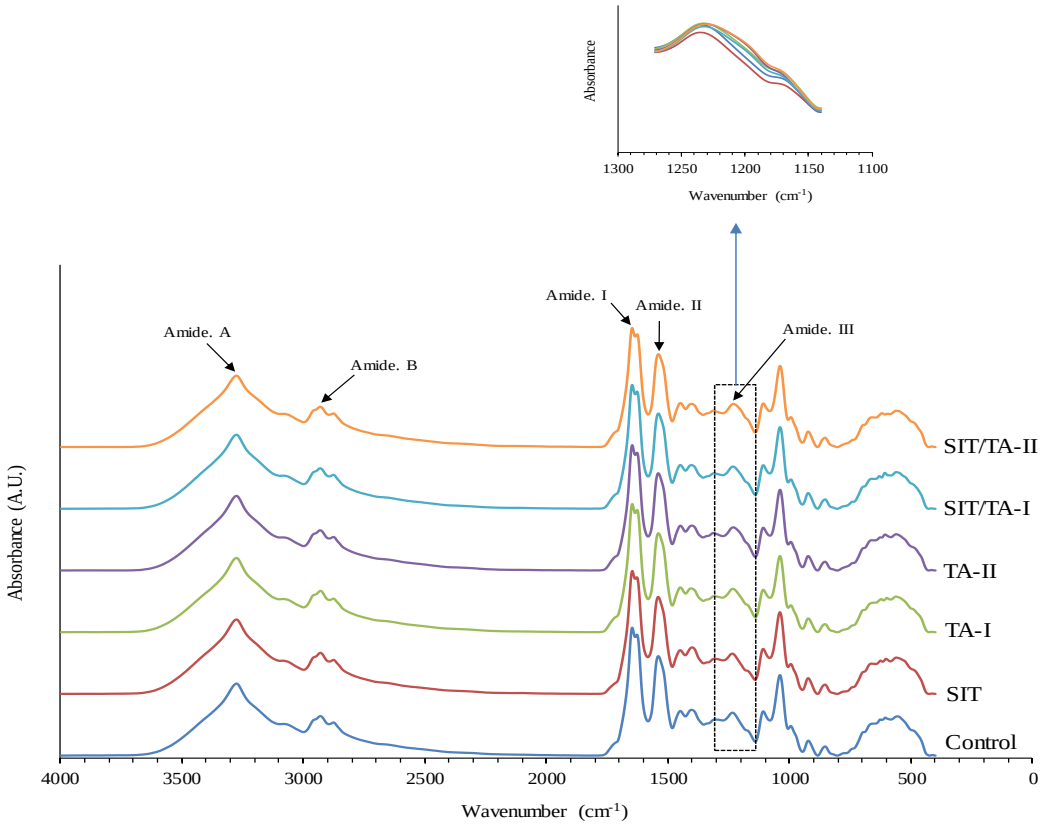

Figure 33. ATR-FTIR of films from washed sardine mince without and with SIT, TA or SIT/TA mixtures at different concentrations. **SIT**: 5 k units L^{-1} SIT; **TA-I**: 0.5 g L^{-1} TA; **TA-II**: 1 g L^{-1} TA; **SIT/TA-I**: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; **SIT/TA-II**: 5 k units L^{-1} SIT+ 1 g L^{-1} TA.

8.4.4 Scanning electron microscopy

Scanning electron microscopic images of surface and freeze-fractured cross-section of films from washed sardine mince without and with SIT, TA or SIT/TA mixture are depicted in Figure 34. The surface of the control film and film added with TA at 0.5 g L^{-1} FFS was smoother, compared to those of other film samples. The surface of the film added with SIT at 5 k units L^{-1} FFS had rough surface. This was more likely due to the poorer cross-linking of the protein molecules owing to the lower activity of SIT at pH 3. The surfaces of other films were smoother, owing to the presence of ordered and homogeneous network. The freeze-fractured cross-section of the control film or those added with only TA or SIT showed the larger cracks, compared to those of films added with the SIT/TA mixture. The compact network of those films added with SIT/TA mixtures was in agreement with higher TS and lowered WVP. The result suggested the higher protein cross-linking induced via quinones produced by oxidation of TA by SIT.

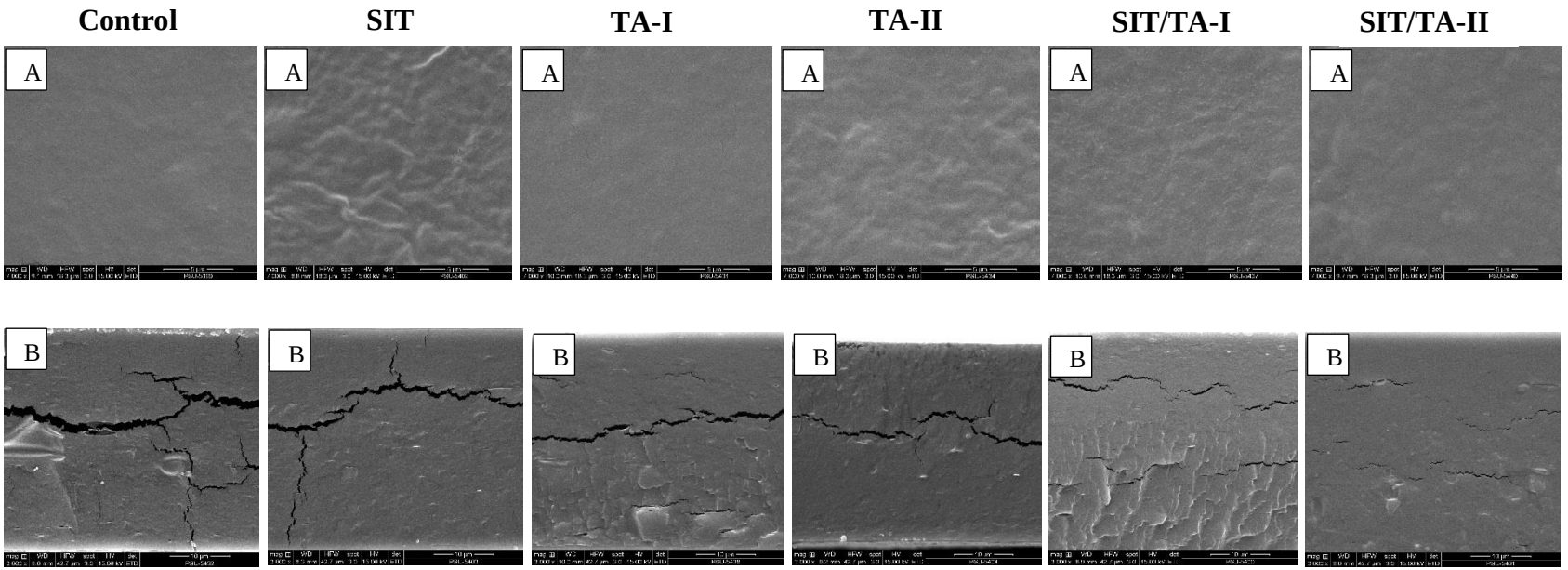

Figure 34. Scanning electron micrograph of films without and with SIT, TA or SIT/ TA mixtures at different concentrations. Surface (A) and freeze-fractured cross-section (B). **SIT**: 5 k units L^{-1} SIT; **TA-I**: 0.5 g L^{-1} TA; **TA-II**: 1 g L^{-1} TA; **SIT/TA-I**: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; **SIT/TA-II**: 5 k units L^{-1} SIT+ 1 g L^{-1} TA.

8.5 Conclusion

The mixture of SIT and TA increased the mechanical properties of protein films from washed sardine mince. The addition of SIT/TA mixture also decreased WVP in the resulting films. However, the films added with mixture of SIT and TA were less transparent than the control. Higher crosslinking associated with film strengthening was induced by the mixture of SIT and TA. Less cracks in cross-section of the films added with SIT/TA mixture was observed. Hence, the SIT in combination with TA could improve the properties of myofibrillar protein films from washed fish mince.

8.6 References

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CHAPTER 9
CONCLUSION AND SUGGESTION

9.1 Conclusions

1. Squid ink, which is discarded as byproduct, could be used as the good source for natural antioxidant after melanin removal. MFI with high thermal stability possessed radical scavenging and metal chelating activities. MFI fraction with lower MW had the greater antioxidative activity.

2. The addition of MFI improved textural properties of gels of sardine surimi without negative effect on whiteness. It also increased water holding capacity of surimi gel. Also, MFI inhibited lipid oxidation in surimi gels during the extended refrigerated storage.

3. The mixture of SIT and TA improved the breaking force and deformation of sardine surimi gels. Water holding capacity of gels was increased with the addition of mixtures, but the whiteness was slightly decreased. The use of 500U SIT/g protein and 1% TA mixture with reaction time of 90 min increased the gel strength of sardine surimi gel effectively. The resulting gel with finer and compact structure had the higher texture and overall likeness scores, compared with control.

4. The mixture of SIT and TA had the profound effect on the heat induced aggregation of NAM. SIT/TA mixture increased turbidity and surface hydrophobicity of NAM solution and showed highly interconnected and denser structure with higher G'. Disulphide bond formation was also induced by SIT/TA mixture. Heat-induced aggregation was mainly influenced by heating temperature and the concentration of both SIT and TA.

5. SIT in combination with EEC effectively enhanced the gel properties of sardine surimi. The combination of SIT and EEC increased breaking force, water holding capacity and storage modulus of surimi gel via enhanced protein cross-linking. Surimi gel with finer and more compact network having more interconnection was formed.

6. MFI increased the mechanical properties and decreased WVP of protein films from washed sardine mince. Yellow discoloration was lowered by incorporation with MFI, especially at higher levels. This was related with decreased lipid oxidation.

7. The mixture of SIT and TA increased the mechanical properties of protein films from washed sardine mince. The addition of SIT/TA mixture also decreased WVP in the resulting films. However, the films added with mixture of SIT and TA were less transparent than the control. Higher crosslinking associated with film strengthening was induced by the mixture of SIT and TA.

9.2 Suggestions

1. Isolation and characterization of antioxidative peptides from MFI should be carried out.

2. Purification and characterization of tyrosianse from squid ink should be done.

3. Effect of SIT in combination with other phenolic compounds on the properties of other muscle food gels or emulsion based foods should be studied.

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