Enhancement of Gel Strength of Surimi Using Oxidized Phenolic Compounds

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ABSTRACT

Effects of different oxidized phenolic compounds (ferulic acid, OFA; tannic acid, OTA; catechin, OCT and caffeic acid, OCF) at different levels (0-0.25% of protein content) on the properties of gels from bigeye snapper (*Priacanthus tayenus*) surimi were investigated. Breaking force and deformation of surimi gel varied with types and amounts of oxidized phenolic compounds. Gels added with 0.20% OFA, 0.05% OTA, 0.15% OCF and 0.05% OCT exhibited the increases in breaking force by 28.98, 39.52, 29.78 and 29.69% and in deformation by 38.06, 38.97, 38.63 and 38.40%, respectively compared with the control (without oxidized phenolic compounds) ($P < 0.05$). Those increases were associated with lower expressible moisture content. No changes in the whiteness of gel were found with addition of OFA ($P > 0.05$), but the decreases in whiteness were noticeable as other oxidized phenolics were incorporated ($P < 0.05$). Different microstructures were obtained among gels added with different oxidized phenolics. Based on physicochemical study of natural actomyosin (NAM), oxidized phenolics could induce conformational changes and the cross-linking through amino groups or the induction of disulphide bond formation.

When the oxidized phenolic compounds were added into mackerel surimi gels, those added with 0.40% OFA, 0.50% OTA, 0.50% OCF or 0.10% OCT had the increases in breaking force by 45, 115, 46.1 and 70.3 and in deformation by 12.2, 27.5, 28.1 and 28.4%, respectively, compared with the control. Similar impact on whiteness and expressible moisture content of resulting surimi gel was noticeable, compared to that found in bigeye snapper surimi gel. Sensory study indicated that

addition of oxidized phenolic compounds with the concentration range used had no negative impact on the color and taste of resulting gels ($P > 0.05$).

To maximize the gel improvement of mackerel mince and surimi, different washing process in conjunction with OTA incorporation were applied. In general, surimi from different washing processes required the varying levels of OTA. The gel of alkaline-saline washing process surimi (ASWPS) added with 0.25% OTA had the increases in breaking force and deformation by 166.2 and 45.9%, respectively, compared with that of conventional washing process surimi (CWPS) without OTA addition. For CWPS gel, the addition of 0.50% OTA resulted in the highest breaking force and deformation ($P < 0.05$). For unwashed mince, the addition of 0.25 and 0.50% OTA had no influence on breaking force and deformation of resulting gel ($P > 0.05$).

The effect of OTA (0 - 0.3%) on aggregation and physicochemical properties of mackerel NAM after setting at room temperature (26-28 ^{o}C) or 40 ^{o}C for 30 min was elucidated. After setting at both temperatures, the increases in turbidity, surface hydrophobicity and disulfide bond contents were observed in NAM as OTA added increased up to 0.2%. When the same level of OTA was incorporated, the higher formation of aggregate of NAM was found when NAM solution was set at 40 ^{o}C, compared with at room temperature. The lower aggregation of NAM was noticeable in the presence of sarcoplasmic protein (SP), which was more preferably cross-linked by OTA mainly via weak bonds. Thus, SP showed the interfering effect on NAM cross-linking induced by OTA. Myosin heavy chain band intensity was decreased and highly ordered and dense protein network of NAM was obtained when 0.2 % OTA was added. Conversely, the protein coagulate was formed in NAM/SP mixture added with 0.2% OTA.

Kiam (*Cotylelobium lanceotatum* Craih) wood was extracted using water or ethanol. Water kiam wood extract (WKWE) and ethanolic kiam wood extract (EKWE) contained tannin at levels of 251.90 and 456.30 mg/g dry extract, respectively. Gels added with 0.30% WKWE, 0.15% EKWE or 0.30% commercial tannin (CT) had the increases in breaking force by 134.81, 136.09 and 121.34% and in deformation by 52.60, 54.96 and 33.53%, respectively, compared with the control

(without addition of extracts or CT). The addition of WKWE, EKWE or CT at the optimal level had no negative impact on the sensory properties of resulting gels. At the same level (0.15%), gel added with EKWE showed the higher breaking force and deformation compared with those added with WKWE or CT ($P < 0.05$). This might be attributed to the presence of other components along with tannin in the extracts. The pH for oxygenation of phenolic compounds in EKWE had the impact on the gel strength of mackerel surimi. Gels added with 0.15% EKWE oxygenated at alkaline pH had the finer matrix with smaller strands and voids, compared with those containing EKWE oxygenated at pH 3 or 7.

Gel strengthening effect of EKWE or CT on surimi prepared from mackerel stored in ice for various times (0-12 days) was studied. Gel-forming ability of surimi, without and with the addition of EKWE or CT, decreased as storage time increased ($P < 0.05$). However, superior breaking force and deformation of surimi gel, added with 0.30% CT or 0.15% EKWE, to those of the control gel were observed when surimi was prepared from mackerel stored in ice up to 6 days. Whiteness of surimi gel generally decreased and expressible moisture increased especially when the storage time of mackerel increased. When surimi was prepared from mackerel stored in ice for more than 6 days, both EKWE and CT showed no gel enhancing effect. Therefore, efficacy of EKWE or CT in improving gel properties depended on the quality of surimi.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Surimi is a Japanese term which can be defined as the minced fish flesh, washed to remove most of lipids, blood, enzymes and sarcoplasmic proteins and stabilized for frozen storage by cryoprotectants. The myofibrillar proteins, which possess gel forming ability, are concentrated in the resulting product (Benjakul *et al.,* 2003). These gel forming properties make surimi become a valuable texture binding agent in formulated muscle foods. Surimi can be used to bind natural muscle fibres together or may be the base material for the forming of artificial meat fibres in analog products. Thailand is one of the largest surimi producers in Southeast-Asia. About 16 surimi factories are located in Thailand, with a total production of 96,500 to 1,13,500 metric tons per year of which, 80% exported to Japan & Korea and the remainder to Singapore and other countries (Hong and Eong, 2005). In general, lean fish have been used for surimi production in Thailand. Those include threadfin bream (*Nemipterus spp*.), bigeye snapper (*Priacanthus spp*.), croaker (*Pennahia* and *Johnius spp.*) and barracuda (*Sphyraena spp.*). However, dark fleshed fish have been used for surimi production even at much smaller portion. This is associated with the poor gel forming ability of the dark fleshed fish compared with lean fish. There is a great interest in using the large quantities of these low value fatty pelagic fish for human food, particularly for surimi production. Problems faced with producing surimi from small pelagic species, such as sardine and mackerel is the high content of dark muscle associated with high content of lipid and myoglobin. Those contribute to the difficulties in making high-quality surimi (Chen, 2002; Ochiai *et al.,* 2001). Due to the limited fish resources, especially lean fish, dark muscle fish have been paid more attention as a potential alternative raw material, especially for surimi production (Wu *et al.,* 2000; Chen *et al.,* 1997; Chaijan *et al.,* 2004). So far, sardine and mackerel

have been used for surimi production (Ochiai *et al.,* 2001). Recently, round scad (*Decapterus maruadsi*) meat of a dark fleshed fish have been used to produce protein based film to increase the potential use of this species (Benjakul *et al.,* 2008b; Artharn *et al.,* 2007; Prodpran *et al.,* 2007). Protein hydrolysate of yellow stripe trevally have been used as functional ingredients, antioxidants and microbial nutrient (Klompong *et al.,* 2007).To exploit the better benefit from these fish, the improvement of surimi gel quality either by development of new processing or by direct enhancement of gel strength has been paid more attention.

Plant phenolic compounds, also denoted polyphenols, are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s), which are derived from the secondary metabolism of plants (Parr and Bolwell, 2000; Robards *et al.,* 1999).The interactions between phenolic compounds and proteins play a very important role in the processing of certain food products. A better understanding of phenolic compound-protein interactions would help to control the functional properties of proteins in food products and the production of protein ingredients. Additionally, the use of phenolic compound in the appropriate form at a proper concentration would be a possible means to improve the surimi gel property, especially from low quality fish like mackerel. Therefore, the novel natural additive for surmi gel improvement can be used as the processing aid in surimi idustry. The outcome of this research will be of great benefit for the surimi and surimi based products industry.

1.2 Review of literature

1.2.1 Chemical composition of fish

The main constituents of fresh fish are water (65-85 %), protein (15-24 %), fat (0.1-22 %), carbohydrate (1-3 %) and inorganic substances (0.8-2 %). The amount of fish meat varies according to the species, age, part of body, pre or post-spawning season and the feeding conditions (Suzuki, 1981). Protein is a major composition of fish muscle with the range of 15-20 % (wet weight). Protein compositions of fish vary, depending upon muscle type, feeding period, and spawning, etc. Hashimoto *et al.* (1979) determined the protein compositions of the

dark and the white muscle from sardine (*Sardinops melanosticta*). The dark muscle contained 23-29% sarcoplasmic protein, 62-66% myofibrillar protein, 6-9% alkali-soluble protein and 2-3% stromal protein. The white muscle comprised 33-37% sarcoplasmic protein, 59-61% myofibrillar protein, 1-5% alkali-soluble protein and 1-2% stromal proein. Generally, protein content is reduced in spawning period (Almas, 1981).

1.2.2 Muscle protein

1.2.2.1 Myofibrillar protein

Myofibrillar proteins are the major proteins in fish muscle. Normally, these proteins account for 65-75 % of total protein in muscle, compared with 52-56 % in mammals (Mackie, 1994). These proteins can be extracted from the muscle tissue with neutral salt solutions of ionic strength above 0.15, usually ranging from 0.30 to 0.70. The myofibrillar proteins are related with the water hoding capacity and other functional properties of proteins such as gelation etc (McCormick, 1994).

Contractile proteins, which are different in size and location in the muscle, are listed in Table 1 (Ashie and Simpson, 1997).

Table1. Contractile protein in food myosystems.

Protein	Relative abundance (%)	Size (kDa)	Location
Myosin	50-60	470	Thick filaments
Actin	15-30	43-48	Thin filaments
Tropomyosin	5	65-70	Thin filaments
Troponins	5		Thin filaments
Troponin-C		17-18	
Troponin-I		20-24	
Troponin-T		37-40	
C- protein	-	140	Thick filaments
α - Actin	-	180-206	Z-disc
Z-nin	-	300-400	Z-disc
Connective / Titin	5	700-1000	Gap filaments
Nebulin	5	~ 600	N_2-line

a. Myosin

Myosin is the protein which forms the thick filament. A molecular weight is about 500,000 daltons. It is the most abundant myofibrillar component, constituting approximately 40-60 % of total protein content. Myosin consists of six polypeptide subunits, two large heavy chains and four light chains arranged into an asymmetrical molecule with two pear-shaped globular heads attached to a long helical rod-like tail (Xiong, 1997) (Figure 1).

When myosin is digested by trypsin or chymotrypsin for a short period, myosin is divided into two components, a rapid sediment component called heavy-meromyosin (HMM) and slow sediment called light-meromyosin (LMM). When HMM is treated with papain, it is divided into a head and a neck part. A head is called S-1 and the neck part is S-2 (Suzuki, 1981). The myosin head contains the actin binding site, ATP site, alkali light chain site and DTNB [(5,5-dithiobis)-2-(nitrobenzoic acid)] light chain site. The light chains bind to the alpha-helical regions of the heavy chain. The tail portion of the heavy chain molecule is responsible for its association into thick filaments (Foegeding *et al.,* 1996).

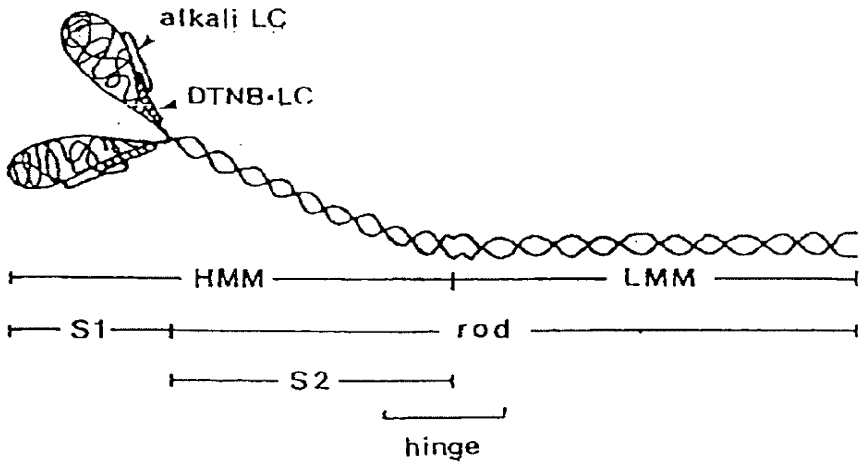


Figure 1. Model of myosin molecule.
Source: Xiong (1997)

b. Actin

Actin constitutes about 22 % of myofibrillar mass with a molecular weight of 42,000 daltons. Normally, actin in muscle tissue is associated with troponin

and tropomyosin complex. It also contains a myosin binding site, which allows myosin to form temporary complex with it during muscle contraction or the permanent myosin-actin complex during rigor mortis in postmortem (Xiong, 1997). Monomer form of actin is called G-actin and after polymerization, actin filaments are formed and referred to as F-actin. Two F-actin in helix form is called super helix (Foegeding *et al.,* 1996) (Figure 2).

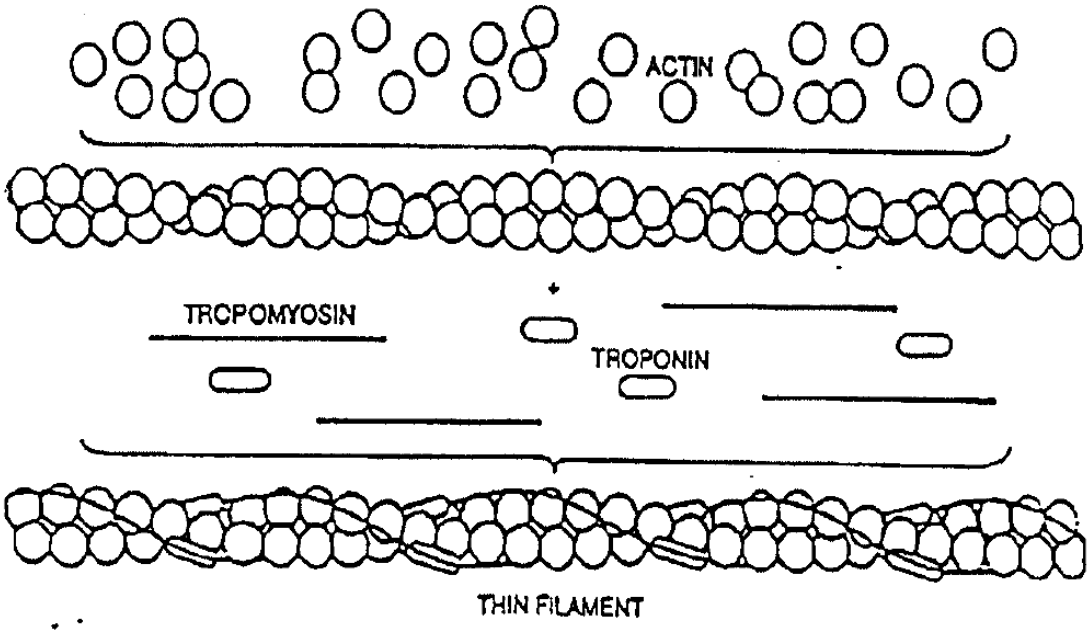


Figure 2. Structure of actin, troponin and tropomyosin.

Source: Foegeding *et al.* (1996)

c. Troponin

Troponin and tropomyosin regulate muscle contraction. Troponin accounting for 8-10 % of myofibrillar proteins consists of three subunits such as troponin C, which is a calcium binding protein and confers calcium regulation to the contraction process via the thin filament; troponin I, which strongly inhibits ATPase activity of actomyosin; and troponin T, which provides a strong association site for binding of tropomyosin (Foegeding *et al.,* 1996).

d. Tropomyosin

Tropomyosin represents approximately 8-10 % of myofibrillar protein. It has two subunit chains (Suzuki, 1981). In skeletal muscle, two polypeptides, alpha- and beta-tropomyosin can combine to form a tropomyosin dimer.

Tropomyosin aggregates end-to-end and binds to actin filaments along each groove of the actin double helix such that each molecule interacts with seven G-actin monomers (Foegeding *et al.,* 1996).

1.2.2.2 Sarcoplasmic protein

Sarcoplasmic protein contains many kinds of water soluble proteins called myogen. It represents 20-35 % of the total protein content in muscle (Mackie, 1994). It can be obtained simply by pressing fish meat, or by extracting with low ionic strength salt solution (Mackie, 1994). The content of sarcoplasmic protein in fish meat varies with fish species, but is generally higher in pelagic fish such as sardine and mackerel and lower in demersal fish (Suzuki, 1981). Haard *et al.* (1994) suggested that the sarcoplasmic proteins from fish included myoglobin, enzymes and other albumins. The presence of sarcoplasmic proteins of dark muscle has often been cited as one of the reasons for the poorer gelation characteristics of dark muscle fish compared with light muscle (Haard *et al.,* 1994). Sarcoplasmic proteins bind to the myofibrillar proteis and thus interfere with the formation of gels. Hultin and Kelleher (2000) and Haard *et al.* (1994) reported that small quantities of sarcoplasmic proteins had an adverse effect on the strength and deformability of myofibril protein gels. These proteins may be interfered with myosin crosslinking during gel matrix formation because they did not form gels and had poorer water holding capacity. The presence of sarcoplasmic proteins may change the rheological properties of the fish gels (Sikorski, 1994). Benjakul *et al.* (2004a) reported that the sarcoplasmic fraction from bigeye snapper muscle possessed cross-linking activity towards myosin heavy chain (MHC).

1.2.2.3 Stroma protien

Stroma is the protein, which forms connective tissue, representing approximately 3% of total protein content of fish muscle. It cannot be extracted by water, acid, or alkali solution and neutral salt solution of 0.01-0.1 M concentration. The component of stroma is collagen, elastin or both (Suzuki, 1981). Elastin is very resistant to moist heat and cooking. Normally, it is a reflection of the different structural arrangements of muscle cells in fish, compared to mammals (Mackie, 1994). Collagen is almost totally insoluble in water or saline and does not participate in gel formation. Collagen can convert to gelatin when heated, depending

on the structure of the collagen present. This soluble gelatin can interfere with the gelation of myofibrillar proteins (Park, 2000).

1.2.3 Lipids

Dark muscle fish are often referred to as fatty fish. This is a reflection of their high lipid content. The presence of high lipid content has important implications in the storage, processing, stability and nutritional value of fish muscle (Hultin and Kelleher, 2000). Dark muscle relies on the oxidative metabolism of lipid as its principal source of energy. This is the reason for the high content of oil in the muscle (Hultin and Kelleher, 2000)**.** Dark muscle contains the greater quantities of mitochondria, myoglobin, fats, glycogen and cytochromes and has a more abundant vascular supply. Chaijan *et al.* (2007) found a high content of myoglobin in sardine (*Sardinella gibbosa*) dark muscle. Chaijan *et al.* (2006) also investigated about the changes of lipids in sardine (*Sardinella gibbosa*) muscle during iced storage. Inhibitory effect of oxidized lipid on the thermal gelation of Alaska pollack (*Theragra chalcogramma*) surimi were reported by Murakawa *et al.* (2003).

Lipid content in fish varies with species. Also, lipid compositions can be different. Sarma *et al.* (1998) reported that the crude lipid content in sardine muscle (*Sardinella longiceps*) was 3.99%. Pacheco-Aguilar *et al.* (2000) found that the fat content in monterey sardine muscle (*Sardinops sagax caerulea*) from the winter period was 8.4%, while the fat content of 1.1% was found in the spring period. Fatty acid composition is not the only different between the neutral oils and the polar membrane lipids. Because the polar phospholipids of the membrane exist primarily as a bilayer, they have a very large surface area exposed to the aqueous phase of the cell. In fish muscle, which contains 10% neutral lipid and 1% phospholipids, the phospholipids fraction would have 10 times more exposure to prooxidants in the aqueous phase than the triacylglycerols at the surface of the oil droplets (Hultin and Kelleher, 2000). In addition to their greater surface area, membrane lipids are found in association with components that can accelerate their oxidation. Mitochondrial inner membrane possesses most of the molecular oxygen of the cell and reactive oxygen species may escape from its electron transport chain. Other membrane systems also have electron transport systems that, although they may not be as active as the

mitochondrial inner membrane, can still produce reactive oxygen species. In addition, membrane components, such as cytochromes or nonheme iron proteins, can convert species like superoxyl radicals (or the protonated HOO°) into more reactive species such as the hydroxyl radical. The juxtaposition of these membrane components and the highly unsaturated fatty acids encourage oxidation of the fatty acids (Hultin and Kelleher, 2000).

1.2.4 Surmi and factors affecting surmi quality

Surimi is a Japanese term for mechanically deboned fish flesh washed with water and mixed with cryoprotectants. Myofibrillar proteins will lose their functional properties rapidly when they are frozen. Therefore, the raw surimi is generally mixed with cryoprotectants such as sucrose, sorbitol, and polyphosphate (Park and Morrissey, 2000). Surimi is used as an intermediate product for a variety of fabricated seafoods, such as the crab legs and flakes. Washing is the important step for surimi production. Washing not only removes fat and undesirable matters, such as blood, pigments and odorous substances (Suzuki, 1981), but also increases the concentration of myofibrillar protein. Myosin has been known as the dominant protein involved in gelation. The use of fresh fish for the surimi production is also essential since tissue autolysis lowered the gelstrength (Benjakul *et al.,* 2002; Benjakul *et al.,* 2003). Surimi can be produced from different fish species. Quality of surimi is governed by many factors.

Factors influencing surmi quality

a. Effects of species

Depending on the species used, the functional and compositional properties of the surimi vary. Muscle proteins from two species of bigeye snapper showed the different gelling property. *Priacanthus tayenus* protein exhibited the larger aggregation stabilized by hydrophobic interaction and disulfide bond than *P. macracanthus* (Benjakul *et al.,* 2002). Surimi from tropical species had the different gel properties when set at medium and high temperature prior to heating (Benjakul *et al.,* 2004b). Generally, shrimp meat has the poor gel forming ability, which is possibly associated with the low setting phenomenon or the inappropriate

protein structure for cross-linking or gelation (Tammatinna *et al.,* 2007). The gel-forming ability of dark muscle fish meat has been known to be lower than that of ordinary muscle (Chen, 2002; Ochiai *et al.,* 2001).

b. Effects of freshness and handling

Prolonged holding times and elevated temperatures can cause severe proteolysis of myofibrillar proteins, which is directly associated with inferior gel quality (Suzuki, 1981). Degradation of myosin heavy chain (MHC) generally occurred during iced storage of Pacific whiting (Benjakul *et al.,* 1997). During handling, leakage of digestive enzymes into the muscle also results in subsequent hydrolysis of muscle proteins. Therefore pretreatment of fish, including beheading and evisceration prior to handling, can be another means to retard the deterioration caused by proteolysis (Benjakul *et al.,* 2002). Surimi gel quality can be influenced by many factors affecting protein structure.

Freshness of fish is primarily time/temperature-dependent. Generally, surimi produced from fish stored in ice for a longer time showed the decrease in gel-forming ability. Bigeye snapper and lizardfish kept in ice with extending storage time had the continuous decrease in breaking force and deformation (Benjakul *et al.,* 2002; Benjakul *et al.,* 2003). On at-sea vessels, processing of Alaska pollock occurs within 12 h, whereas at shore-side operations processing occurs within 24-48 h. Because of endogenous enzymes activated by rising temperatures, Pacific whiting is processed within a shorter period; immediately on at-sea vessels and within 20 h after harvest at shore-side plants (Park and Morrissey, 2000). The length of time that fish can be held in ice or refrigeration before processing varies depending on the species. Time and temperature of the fish between capture and processing can be considered two of the most important factors in final surimi quality. Holding temperatures at 4-6°C can make a significant difference in surimi quality compared with fish held close to 0°C (Park and Morrissey, 2000). Degradation of MHC was also affected by storage temperatures. Fish kept at 5°C showed higher degradation than those stored at 0°C, suggesting that ice water was more efficient than refrigeration in controlling proteolysis (Park and Morrissey, 2000). However, Benjakul *et al.* (1997) found the degradation of MHC of Pacific whiting during iced storage.

With prolonged storage time, severe degradation occurred, although the storage temperature had been maintained at 0°C (Lin and Park, 1996). According to An *et al.* (1994), in the temperature range of 0-5°C, the activity of cathepsin L was insignificant, whereas cathepsin B exhibited half of its maximal activity and cathepsin H retained about a fifth of its maximal activity. Therefore, cathepsins B and H might contribute to the degradation occurring at low-temperature storage. Consequently, to minimize proteolysis, fish should be processed promptly on landing or kept at 0°C if holding is necessary.

1.2.5 Surimi gelation

Gelation is an aggregation of proteins forming a three-dimensional network in which water is entrapped (Hermansson, 1979; Pomeranz, 1991). 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. In addition, the head portions associate to form "super junctions" which provide extra cross-linking to the gel network. Generally, myosin alone forms excellent gels. Actin has a synergistic or antagonistic effect on myosin gelation, depending upon the myosin/actin ratio in the gelling system (Grabowska and Sikorski, 1976; Matsumoto, 1979). Gel-forming ability of frozen surimi is the most important functional requirement for imposing good quality of surimi-based products (Saeki *et al.,* 1995). Differences in cross-linking of MHC contribute to the differences in gel-forming ability among the muscles of various fish (Benjakul *et al.,* 2001a).

During heating of surimi pastes, the proteins unfold, exposing reactive surfaces of neighboring protein molecules, which then interact to form intermolecular bonds (Lanier, 2000). When the temperature rises, hydrogen bonds become less stable and hydrophobic hydration becomes favored. Thus the hydrophobic amino acid residues become more exposed and subsequent hydrophobic interaction occurs. During the slow-setting upon incubation near 40°C, hydrophobic groups are introduced onto their molecular surface and hydrophobic interactions proceed and play an important role in the setting phenomenon (Niwa *et al.,* 1981). Benjakul

et al. (2001a) reported the increase in surface hydrophobicity of actomyosin from bigeye snapper during thermal gelation, suggesting that hydrophobic interaction involved in gelation. Chan *et al.* (1992) reported that thermal aggregation of fish myosin was coincidental with an increase in the surface hydrophobicity of the unfold domains of myosin molecules and was affected by the temperature at which these domains unraveled. Extent of aggregation for fish myosin seems to depend on the amount of hydrophobic surface exposed on the headed molecules (Chan *et al.,* 1992; Wicker *et al.,* 1986). Hydrogen bonds are weaker dipole bonds not responsible for the gelation of myofibrillar proteins but are important in the stabilization of bound water within the hydrogel **(**Niwa *et al.,* 1981; Niwa, 1992**).** The aggregation of the head portions of the myosin molecules, in which the sulfhydryl groups are located, is mainly through disulfide bond formation. This is followed by cross-linking of the rod portion myosin molecules which accompanies the conversion of the α-helix to a random coil. Such a contribution of the sulfhydryl groups to gelation was suggested from the finding that the thermally induced increase in rigidity and turbidity of myosin subfragment S1 and heavy meromyosin (HMM) solution was suppressed upon the addition of dithiothreitol, and that SH content was remarkably decreased upon heating the S1 fragments (Samejima *et al.,* 1981). It has been demonstrated that the formation of S-S bonds is more intensive for carp (Itoh *et al.,* 1979) and Atlantic croaker actomyosin (Liu *et al.,* 1983) at the higher temperatures of cooking (80°C or above) than at the lower temperatures. Benjakul *et al.* (2001a) also reported that disulfide bonds were found in bigeye snapper actomyosin during thermal gelation process. Covalent bonds such as disulfide bonds are dominant when heating at high temperatures (>40°C) (Lanier, 2000). However, a variety of crosslinks, including ε-(γ-glutamyl) lysine [ε-(γ-Glu) Lys] crosslinks and crosslinks through aldol condensation, are believed to be closely related to texture (Sakamoto *et al.,* 1995).

1.2.6 Setting of surimi gel

Setting or suwari is a well known occurrence in the surimi pastes during the incubation at temperatures lower than 40°C. Generally, gel strengthening of surimi can be achieved by subjecting surimi sol to setting below 40°C prior to

cooking (An *et al.,* 1996). Gelation of fish paste during setting has been reported to have a close relationship to the formation of cross-linking between myosin heavy chains induced by endogenous transglutaminase (TGase) (Kumazawa *et al.,* 1995; Seki *et al.,* 1990). TGase has been reported to catalyze an acyl transfer reaction between γ-carboxamide groups of glutamyl residues in proteins as the acyl donors and a variety of primary amines and water as the acyl acceptor (Kumazawa *et al.,* 1995). The increase in gel strength of surimi from Alaska pollack was associated with the increased cross-linking of MHC and ε-(γ -glutamyl) lysine content formed *(*Kumazawa *et al.,* 1995). Conversely, inhibition of endogenous TGase resulted in complete suppression of myosin cross-linking of walleye pollack surimi gel and the cross-linking was also inhibited above 45°C due to the inactivation of TGase (Takeda and Seki, 1996). The ε-(γ -glutamyl) lysine formation in Alaska pollack gel was suppressed by addition of EDTA and ammonium chloride (Kumazawa *et al.,* 1995*)*. Wan *et al.* (1995) found that the poorer gel-forming ability of salmon was due to the lower TGase activity as well as the lower contents of myosin and calcium ion, compared with walleye pollack surimi. Benjakul and Visessanguan (2003) found that setting of surimi paste without and with subsequent heating resulted in the increase in both breaking force and deformation of suwari and kamaboko gel from bigeye snapper surimi, respectively. Rawdkuen *et al.* (2005) reported the synergistic effects of chicken plasma protein and setting phenomenon on gel properties and cross-linking of bigeye snapper muscle proteins. Benjakul *et al.* (2004b) reported the effect of medium temperature setting on the improvement of gelling characteristics of surimi from some tropical fish. Moreover, the reactivity of TGase to various fish actomyosin was markedly different (Araki and Seki, 1993) and depended on the conformation of actomyosin. Fish TGase has been found to be Ca^{2+}-dependent, however, the requirement of Ca^{2+} ion varies among fish species (Nozawa *et al.,* 1997). The addition of calcium compounds to surimi enhanced TGase -mediated setting, resulting in stronger gels (Lee and Park, 1998). Setting phenomenon caused by endogenous TGase may be varied between species and contributes to the different gelation characteristics.

1.2.7 Softening of surimi gel

Gel softening or gel weakening termed "modori" is a problem found in surimi, especially for some 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.,* 2000). 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 has a detrimental effect on surimi quality, and substantially lowers the gel strength (Morrissey *et al.,* 1993). Proteinases associated with gel weakening can be categorized into two major groups: cathepsin (Seymour *et al.,* 1994; Toyohara *et al.,* 1993) and heat-stable alkaline proteinase (Wasson *et al.,* 1992).

High level of cysteine proteinase activity mediated by cathepsin B, H, and L have been found in Pacific whiting and arrowtooth flounder (An *et al.,* 1994; Wasson *et al.,* 1992), chum salmon during spawning migration (Yamashita and Konagaya, 1990), and mackerel (Lee *et al.,* 1993). Softening of arrowtooth flounder gel is due to a cysteine proteinase that has 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). Modori is attributed to proteolysis by heat stable alkaline proteinases (Lanier *et al.,* 1981; Kinoshita *et al.,* 1990). Benjakul *et al.* (2004b) reported that suwari gel from some tropical fish, prepared by setting at 25°C showed lower degradation although the setting time increased up to 8 h compared with setting at 40°C. Kamath *et al.* (1992) found that proteolysis in croaker paste increased with increasing temperature of setting, especially in the temperature range of 40-50°C. Proteolytic degradation of surimi gels is increased at temperatures above 50°C with the rapid and severe degradation of myofibrillar proteins, particularly myosin (Jiang *et al.,* 2000). Klomklao *et al.* (2007) also reported the presence of prtoeinases 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). Rawdkuen *et al.* (2007) reported that cysteine proteinase inhibitor containing fraction from chicken plasma could inhibit autolysis and improve gelation of Pacific whiting surimi.

1.2.8 Gel strengthening agents in surimi

Gelation of fish proteins is the most important step in forming desired texture in many seafood products, particularly those from surimi. To strengthen the gel, various chemical additives as well as enzymes have been successfully used in surimi.

1.2.8.1 Microbial Transglutaminase

TGase from microorganisms or microbial TGase (MTGase) have been successfully produced by fermentation process. TGase from *Streptoverticillium mobarense* is Ca^{2+} in-dependent in a hydroxamate assay and also differs from mammalian TGase in molecular weight, thermal stability, isoelectric point, and substrate specificity (Ando *et al.,* 1989; Tsai *et al.,* 1996*).* MTGase can be obtained from *Streptoverticillium ladakanum* and *Streptoverticillium mobaraense* (Seki *et al.,* 1998; Shann *et al.,* 1998).

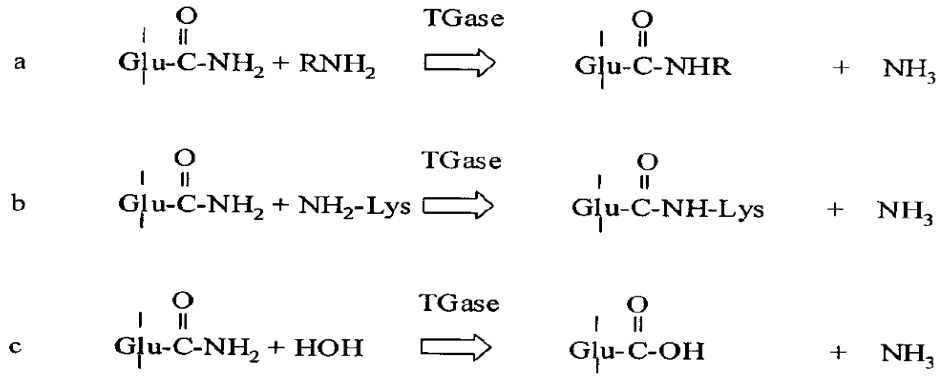


Figure 3. Reaction catalyzed by TGase.

Source: Ashie and Lanier (2000)

MTGase offers a means of upgrading the gelling quality of surimi (Ando *et al.,* 1989). Lanier and Kang (2000) reported that, in Pacific whiting gels, the addition of MTGase has more pronounced effects, especially when combined

with beef plasma (1 %), which inhibits heat stable protease. TGase from *Streptoverticillium ladakanum* and *Streptoverticillium mobaraense* catalyzed the crosslinking of fish myosin heavy chain and substantially increased the gel strength of mackerel surimi (Tsai *et al.,* 1996), threadfin bream surimi, pollock surimi (Jiang *et al.,* 2000), silver carp surimi (Ramirez *et al.,* 2000) and Alaska pollock surimi (Seguro *et al.,* 1995). Addition of MTGase to surimi significantly increased the gel strength, particularly when the surimi has lower natural setting ability (Lee *et al.,* 1997; Seguro *et al.,* 1995; Kumazawa *et al.,* 1993). An increase in non-disulfide polymerization and formation of ε (γ-glutamyl) lysine isopeptides was found with an increase in setting time and MTGase concentration (Tsukamasa and Shimizu, 1990; Benjakul and Visessanguan, 2003). The amount of MTGase added highly depended on fish species and also other factors such as freshness, protein quality, and harvesting season (Asagami *et al.,* 1995*).*

1.2.8.2 Oxidizing agents

L-ascorbic acid is widely used as an improver in kamaboko manufacture. The improvement mechanism has been explained in the way that after being added to surimi, L-ascorbic acid is rapidly oxidized to dehydroascorbic acid (DHA) by oxidase or other factors, and DHA has been proposed to oxidize sulfhydryl groups on muscle proteins to disulfides and to form intermolecular bonds (Yoshinaka *et al.,* 1972). Nishimura *et al.* (1996) found that L-ascorbic acid significantly enhanced the decrease in the amount of MHC from fish gel, by promoting the polymerization of MHC via disulfide bridging. The addition of L-ascorbic acid to surimi enhanced the oxidation of protein sulfhydryl groups to form disulfide-bridged polymers by producing the superoxide radical (Figure 4).

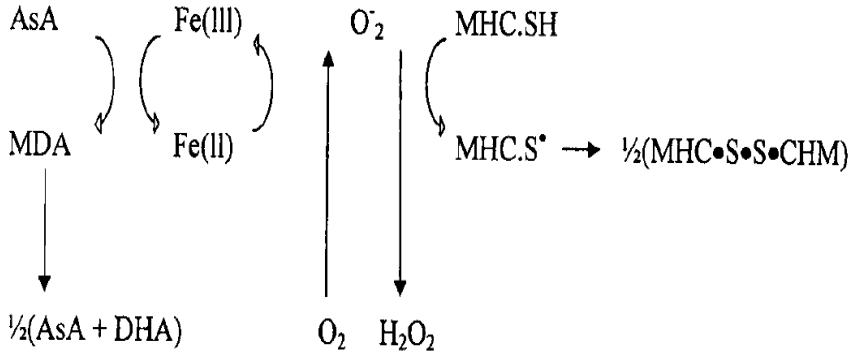


Figure 4. Scheme for the superoxide radical-dependent polymerization of MHC.
Source: Nishimura *et al.* (1996)

Some oxidizing agents were found to increase gel strength of surimi. Phatcharat *et al.* (2006) found that fish mince washed with H_2O_2 showed the increase in breaking force, most likely due to the formation of disulfide bonds.

1.2.8.3 Calcium compounds

Calcium compounds are commonly added as a gel enhancer. Yamamoto *et al.* (1991) used a mixture of sodium bicarbonate, calcium citrate and calcium lactate as gel quality-improving agents. Low gel strength of Pacific whiting surimi compared with Alaska pollock surimi was thought to be due to a lower concentration of calcium ions in the flesh (Park, 2000). Gordon and Roberts (1977) reported that the calcium content of Pacific whiting was 8.7 mg/ 100g meat, whereas pollock contained 63 mg calcium ions per 100 g meat (Sidwell, 1981). Lee and Park (1998) concluded that the textural properties of surimi can be improved maximally with a 25°C pre-incubation and the addition of 0.1 % calcium lactate or 0.05 % calcium acetate for Alaska pollock and 0.2 % calcium lactate for Pacific whiting. Benjakul *et al.* (2004d) also found that addition of $CaCl_2$ increased the breaking force and deformation of gel from bigeye snapper, threadfin bream, barracuda and bigeye croaker surimi. The gel strengthening effect was depending upon the concentration used.

1.2.9 Phenolic compounds

Plant phenolic compounds, also denoted polyphenols, are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s), and which are derived from the secondary metabolism of plants (Parr and Bolwell, 2000; Robards *et al.,* 1999). In plants, phenolic compounds play a role in numerous processes, such as plant growth and reactions to stress and pathogen attack (Parr and Bolwell, 2000). Plant phenolic compounds are present in products ranging from food to sunblockers and paper. As an example, the yellowing of paper over the years is caused by photochemical reactions of phenolic compounds (Zhu and Gray, 1995). Phenolic compounds can be found in many foods and drinks from plant origin, e.g. fruits, vegetables, coffee (Clifford, 1999), tea (Lakenbrink *et al.,* 2000), beer, wine and chocolate (Arts *et al.,* 1999). Red wine for example has a total content of phenolic compounds of 1-4 g/L (Shahidi and Naczk, 1995). These high amounts have led to the hypothesis that phenolic compounds would be one of the responsible factors for the beneficial effect of wine consumption on cardiovascular diseases (Wallerath *et al.,* 2005). Dark chocolate contains approximately 1.6 g/kg of oligomeric phenolic compounds, called proanthocyanidins (USDA database, 2004), while a member of the proanthocyanidin sub-class, the procyanidins, is present in particularly high concentrations in apples and cider (2-3 g/L) (Shahidi and Naczk, 1995). The content of phenolic compounds in foods may change during storage as induced by light and temperature (Friedman, 1997). Apart from being naturally present in the raw materials used for foods, phenolic compounds are also added to some foods for their coloring properties and for their antioxidant effects (O'Connell and Fox, 2001; Richelle *et al.,* 2001).

The presence of phenolic compounds may be easily observable due to the chromophoric groups that some phenolic compounds bear, e.g. the red-purple anthocyanins (Bakowska *et al.,* 2003), or by the brown and green reaction products of phenolic compounds with themselves or with proteins (Montavon *et al.,* 2003; Yabuta *et al.,* 2001). The presence of phenolic compounds can also affect the taste of food. Low concentrations of phenolic compounds may be responsible for desirable sweet, smoky or caramel flavours in foods e.g. dairy products (O'Connell and Fox, 2001). High concentrations of phenolic compounds in tea and wine provide the astringent

sensation. The latter results from the precipitation of saliva proteins on the tongue by interactions with specific phenolic compounds (Baxter *et al.,* 1997; Charlton *et al.,* 2002). If milk is added to tea, the proteins present in milk will bind most of the present phenolic compounds, leaving the saliva proteins unaffected. On the other hand, the interactions between phenolic compounds and proteins may lead to a decrease of protein digestibility, by blocking the substrate and/or inhibiting certain proteases (Kroll *et al.,* 2003).

1.2.9.1 Classification of phenolic compounds

Phenolic compounds represent a wide range of molecules with a molecular mass from about 100 to 3,000-4,000 Da (Haslam, 1996). They are produced by two principal pathways: the shikimate and the polyketide pathway (O'Connell and Fox, 2001). Different classifications of phenolic compounds have been proposed (O'Connell and Fox, 2001). For example, plant "tannins" are proposed to be broadly divisible into two majors groups: the proanthocyanidins and the polyesters based on gallic and/or hexahydroxydiphenic acid (Haslam, 1989). However, this classification does not include simple phenols. In Figure 5, a more complete classification according to the number of carbon atoms is given. It is adapted from the one given by O'Connell and Fox (2001) and divides the phenolic compounds into five groups: 1) the C_6 group, comprising simple phenols and benzoquinones; 2) the C_6C_n group, which includes phenolic acid derivatives and hydroxycinnamic acid derivatives (Figures 6 and 7) the C_6-C_n-C_6 group, which includes flavanoids (C_6-C_3-C_6); 4) the $(C_6$-$C_3)_{n,}$ group consisting of lignans and lignins; and 5) the tannin group, which are divided into hydrolysable tannins and condensed tannins (O'Connell and Fox, 2001). The hydrolysable tannins are formed by gallic acid, 3-digallic acid or hexahydroxydiphenic acid, esterified to a polyol such as glucose or quinic acid (O'Connell and Fox, 2001). Proanthocyanidins are oligomers and polymers of flavanols (Figure 6), which are members of the flavanoid sub-class (O'Connell and Fox, 2001). It has to be noted that, therefore, some authors classify proanthocyanidins in the same class as their monomeric units, i.e. the C_6-C_n-C_6 class. Among these monomeric units, (+)-catechin, (-)-epicatechin, (+)-gallocatechin and

(-)-epigallocatechin are the most common (Haslam, 1989). Their 2S enantiomers, e.g. (-)-catechin and (+)-epicatechin, may also be present (Haslam, 1989).

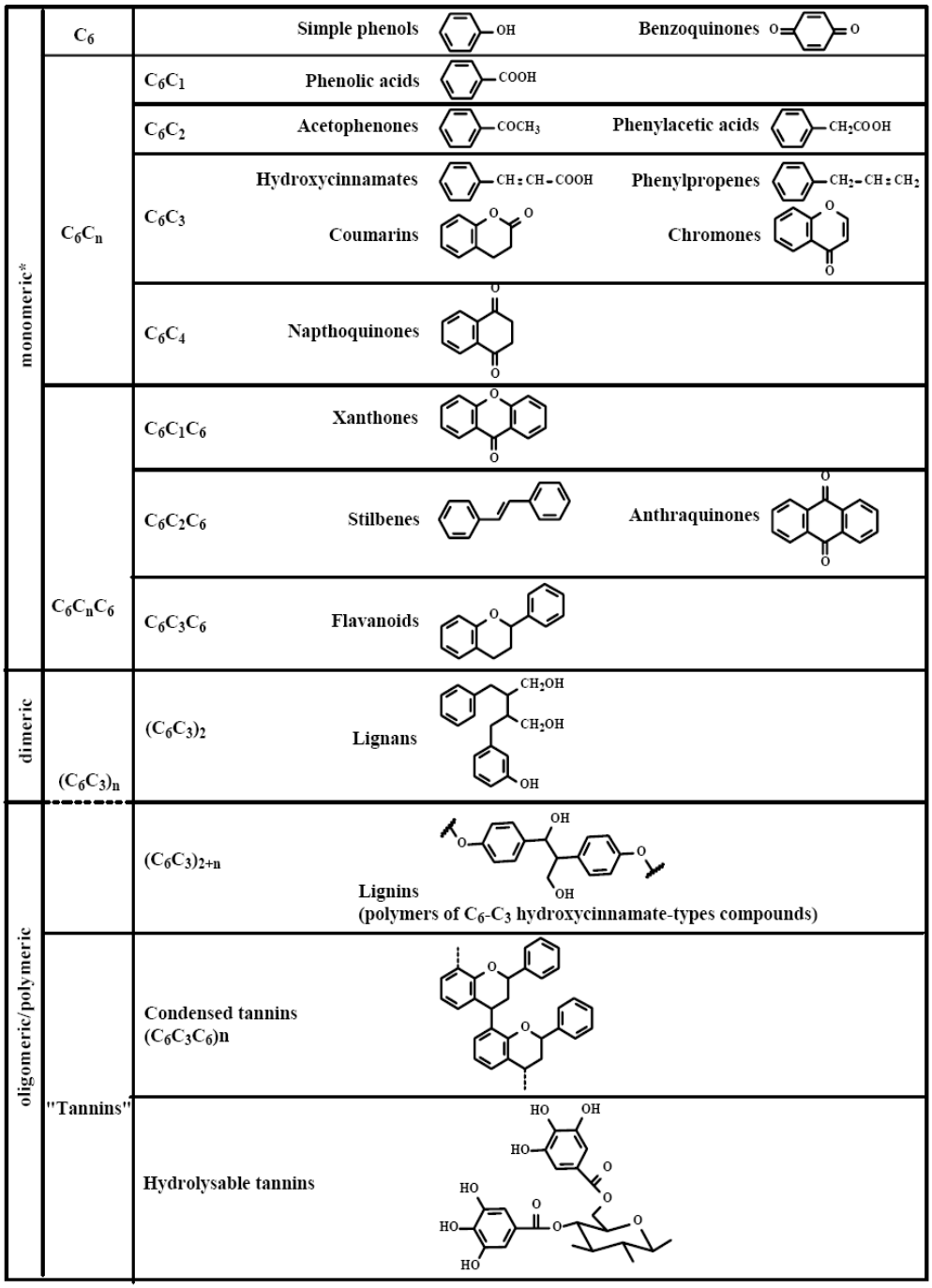


Figure 5. Classification of phenolic compounds. *Oligomers are occasionally formed in these groups.

Source: O'Connell and Fox (2001)

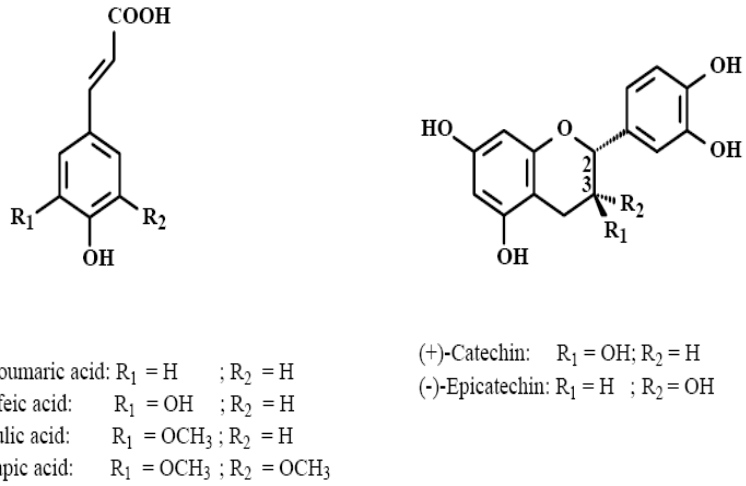


Figure 6. Structure of some hydroxycinnamic acids and flavanols.
Source: O'Connell and Fox (2001)

5'-Caffeoylquinic acid (CQA; 1',3',4',5'-tetrahydroxycyclohexanecarboxylic acid 3-(3,4)-dihydroxypropenyldihydroxyphenyl-1-propenoate, (Figure 7) is composed of a molecule of caffeic acid esterified with quinic acid (Clifford, 2000). Its molecular mass is 354 Da. Caffeic acid and its esters are usually the predominant hydroxycinnamic acids in fruits (Shahidi and Naczk, 1995). Caffeoylquinic acid is present in high concentrations in certain foods and is considered to be the main phenolic compound responsible for browning (Shahidi and Naczk, 1995). Because of its high concentration in some foods (up to 700 mg per cup of coffee) (Clifford, 1999) and its ubiquitous character, CQA is often chosen as a model compound for studying hydroxycinnamic acid derivatives and the simple phenolic compounds. CQA is often referred to as "chlorogenic acid" (Clifford, 2000), although the chlorogenic acids are actually a group of compounds, that contains also 3'-caffeoylquinic acid ("neochlorogenic acid") and 4'-caffeoylquinic acid ("cryptochlorogenic acid"). Additionally, depending on the position of the hydroxyl group in the quinic acid moiety, several R and S configurations can be encountered (Haribal *et al.,* 1998). Whereas CQA can be oxidized at alkaline pH, it is stable at acidic pH at moderate temperatures (Friedman and Jurgens, 2000).

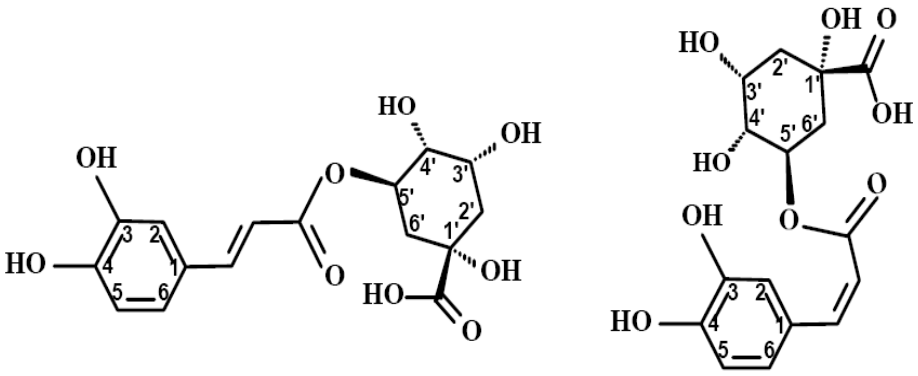
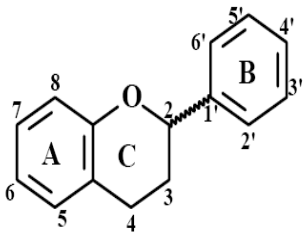


Figure 7. Structure of 5' caffeoylquinic acid (CQA).
Source: O'Connell and Fox (2001)

1.2.9.2 Condensed tannins

The most common sub-class of condensed tannins in food is the proanthocyanidins (Haslam, 1989). The term "proanthocyanidins" is derived from the fact that they are cleaved into anthocyanidins upon heat treatment at acidic pH (Swain and Hillis, 1959). Proanthocyanidins can be classified based on their hydroxylation pattern. They are always hydroxylated at the 3, 5 and 7 position, while hydroxylation on the B ring varies. The most common members of the proanthocyanidins are the procyanidins, formed from catechin units (Figure 8), and the prodelphinidins, formed from gallocatechin units (USDA database, 2004; Haslam, 1989; Shahidi and Naczk, 1995). Having the structure of a catechin molecule that is dehydroxylated at the 3'-position, afzelechin is the sub-unit of propelargonidins (Figure 8), which are a rare member of the proanthocyanidins (Haslam, 1989). In addition to these proanthocyanidins, other members of condensed tannins exist and are usually not called proanthocyanidins (Hemingway, 1989). These condensed tannins have different hydroxylation patterns and are only rarely encountered. In contrast to the proanthocyanidins, these condensed tannins are dehydroxylated on their 5-position (proguibourtinidins, profisetinidins, prorobinetinidins) or on their 3-position (proapigeninidins, proluteolinidins) (Haslam, 1989) (Figure 8). Two additional, particularly rare, members exist: proteracacidins and promelacacidins, which are

dehydroxylated on their 5-position and hydroxylated on their 8-position (Hemingway, 1989) (Figure 8). Proanthocyanidins are difficult to analyse directly and to quantify in foods, because of their multimeric and polydisperse nature (Santos-Buelga and Scalbert, 2000).



Members	Hydroxylation pattern	
Procyanidin	3, 3', 4', 5, 7	Proanthocyanidins
Prodelphinidin	3, 3', 4', 5, 5', 7	
Propelargonidin	3, 4', 5, 7	
Proguibourtinidin	3, 4', 7	
Profisetinidin	3, 3', 4', 7	
Prorobinetinidin	3, 3', 4', 5', 7	
Proteracacidin	3, 4', 7, 8	
Promelacacidin	3, 3', 4', 7, 8	
Proapigeninidin	4', 5, 7	
Proluteolinidin	3', 4', 5, 7	

Figure 8. Structure of the monomeric units of condensed tannins.
Source: Hemingway (1989)

Proanthocyanidins are classified not only according to their constitutive units, but also according to the nature and number of interflavan bond(s), and according to their degree of polymerization. The consecutive units of proanthocyanidins are linked through interflavan bond(s), usually between C4 and C6 or between C4 and C8 (Shahidi and Naczk, 1995). Double linkages, which occasionally occur, are between C4-C6 or C4-C8 and C2-O-C5 or C2-O-C7. This linkage may be an axial (Figure 9) or an equatorial bound. The degree of polymerization is symbolized by a letter, e.g. B = dimer, C = trimer, etc. The letter A is used to name the proanthocyanidins possessing double linkages. The letter is followed by an arbitrarily chosen number to indicate the stereochemistry of the proanthocyanidins.

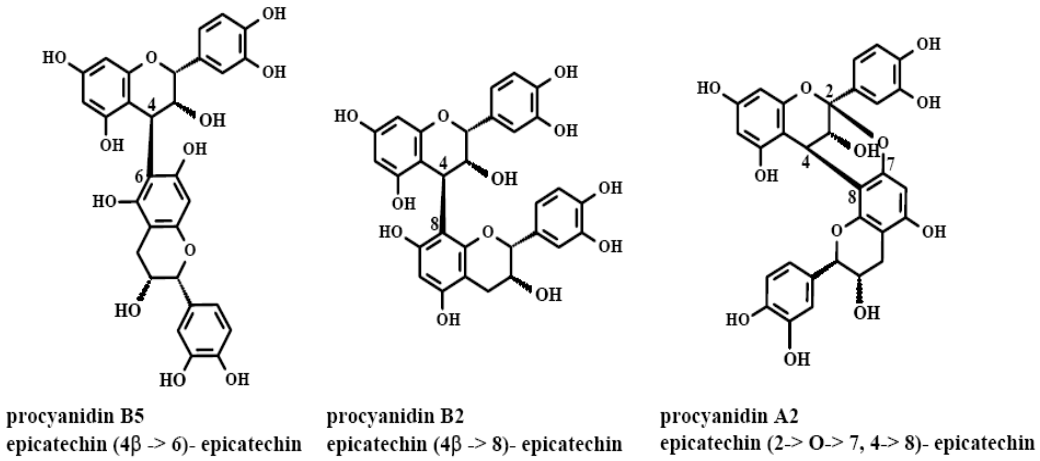


Figure 9. Structure of some dimeric proanthocyanidins.

Source: Prigent (2005)

1.2.9.3 Oxidation of phenolic compounds

Formation of quinone

Ortho-diphenolic compounds can be oxidized into *ortho*-quinones (Figure 10). These quinones can be formed enzymatically or non-enzymatically. Non-enzymatic oxidation occurs readily at alkaline pH (Yabuta *et al.,* 2001).

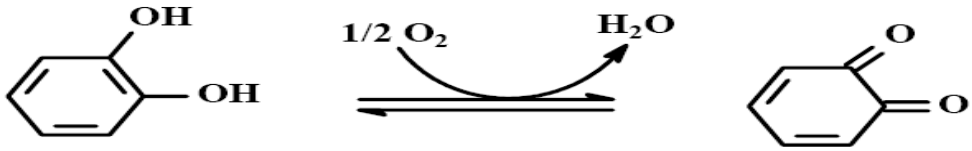


Figure 10. Oxidation of an *ortho*-diphenol into quinone.

Source: Prigent (2005)

1. Enzymatic oxidation

Quinones can be formed via the action of two types of enzymes: polyphenol oxidases and peroxidases. Quinones are produced by peroxidases via the formation of radicals. Peroxidases require the presence of hydrogen peroxide (Matheis and Whitaker, 1984), which thus makes their role in foods limited compared to the action of polyphenol oxidases. Polyphenol oxidases (EC 1.14.18.1) are divided

into catechol oxidases and laccases. Both enzymes can oxidize phenolic substrates using molecular oxygen (Mayer and Harel, 1979; Osuga *et al.,* 1994). Catechol oxidases can catalyze the oxidation of *ortho*-diphenols to *ortho*-quinones using their catecholase activity (Mayer and Harel, 1979) (Figure 10). Furthermore, when they also possess the so-called cresolase activity, catechol oxidases may convert monophenols to *Ortho* -diphenols (Mayer and Harel, 1979; Rodriguez-Lopez *et al.,* 2001) (Figure 11). Laccases are able to oxidize a broader range of substrates than catechol oxidases, including *ρ*-diphenols (Mayer and Harel, 1979; Mayer and Staples, 2002) and non phenolic compounds e.g. phosphorothiolates (Amitai *et al.,* 1998). They are also able to catalyze other reactions than oxidation, such as demethylation and (de-)polymerisation of phenolic compounds (Mayer, 1987).

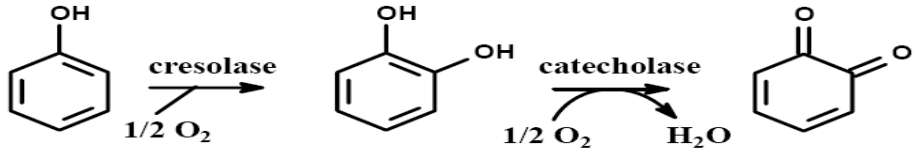


Figure 11. Cresolase and catecholase mechanism of polyphenol oxidase.
Source: Matheis and Whitaker (1984)

Catechol oxidase is usually called tyrosinase in mammals and mushrooms. Tyrosinases from mammals are relatively specific for tyrosine and DOPA (dihydroxyphenylalanine), whereas catechol oxidases from fungi and higher plants show activity on a wider range of mono- and *o*-diphenols (Mayer and Harel, 1979). The pH optimum of most catechol oxidases is between pH 5.0 and 7.0. The activity of mushroom tyrosinase was reported to be optimal at pH 7.0 and to be negligible at pH 4.0 (McCord and Kilara, 1983). The enzyme can also be inactivated by the direct interaction with an inhibitor, e.g. EDTA, which binds to copper in the active site (Mayer and Harel, 1979). Another way of neutralizing the phenolic oxidation is the addition of reducing compounds, like vitamin C or certain thiol compounds, which revert the oxidized phenolic to the original compound (Negishi and Ozawa, 2000; Richard-Forget *et al.,* 1992a).

2. Non-enzymatic oxidation

The formation of quinones may also occur in the absence of an enzyme. Increasing the pH induces the deprotonation of the phenolic hydroxyl group, eventually leading to the formation of quinones. This method is used in food industry e.g. to produce black olives by treating them with diluted NaOH in order to oxidize the caffeic acid and hydroxytyrosol present in olives (Garcia *et al.,* 1996). An additional possibility to form quinones is the use of an oxidizing reagent such as periodate (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.

1.2.9.4 Reactions of quinones with phenolic compounds

Quinones have the ability to oxidize other phenolic compounds via the oxido-reduction mechanism, depending on the redox potential of the phenolics (Cheynier *et al.,* 1988). This coupled oxidation can thus induce the oxidation of compounds, which are otherwise not substrates for catechol oxidases.

The oxido-reduction mechanism is the main phenomenon at low pH, whereas at higher pH values quinones tend to covalently react with other phenolic molecules leading to formation of dimers (Cheynier *et al.,* 1988; Richard-Forget *et al.,* 1992b). Via this mechanism, higher molecular weight condensation products can be formed (Cheynier *et al.,* 1988). The formation of dimers induced by oxidation of phenolic compounds was observed with gallic acid (Kawabata *et al.,* 2002; Tulyathan *et al.,* 1989) and caffeic acid (Cilliers and Singleton, 1991; Fulcrand *et al.,* 1994; Rompel *et al.,* 1999; Tazaki *et al.,* 2001; Yabuta *et al.,* 2001), CQA (Antolovich *et al.,* 2004) and catechin (Guyot *et al.,* 1996; Oszmianski and Lee, 1990). Various dimeric structures were formed upon oxidation of caffeic acid, as presented in Figure 12 (Cilliers and Singleton, 1991; Fulcrand *et al.,* 1994; Rompel *et al.,* 1999; Tazaki *et al.,* 2001; Yabuta *et al.,* 2001).

CQA was also proven to dimerize (Antolovich *et al.,* 2004), leading to the formation of various dimers as observed by reverse-phase chromatography (Bernillon *et al.,* 2004). The structure of one of these CQA dimers

has been elucidated and corresponds to the same structure as presented in Figure 9 (Prigent , 2005).

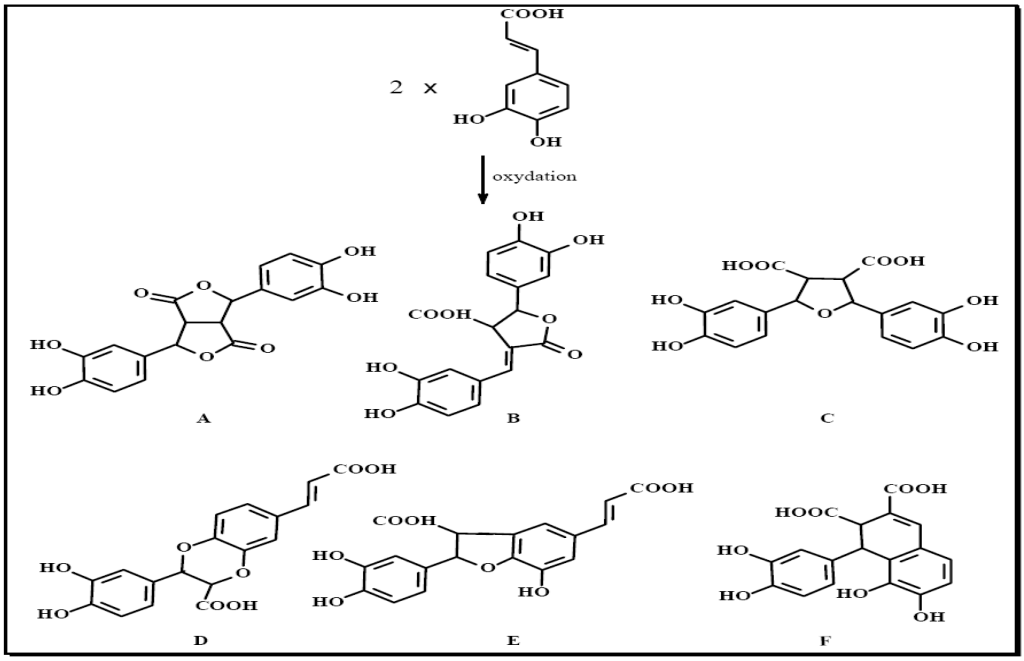


Figure 12. Dimers formed from caffeic acid oxidation.

Source: Prigent (2005)

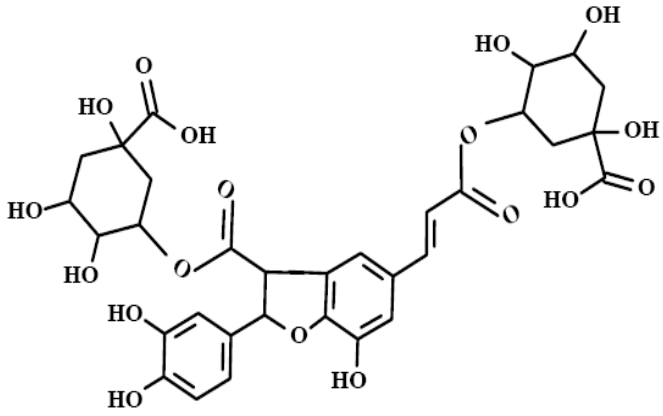


Figure 13. Dimers formed from CQA oxidation**.**

Source: Prigent (2005)

1.2.9.5 Protein-phenolic interactions

Phenolic compounds can interact with proteins in two different ways: via non-covalent (reversible) interactions and via covalent interactions, which in most cases are irreversible. Two types of complexation mechanisms can be distinguished: a monodentate and a multidentate mechanism (Haslam, 1989). "Monodentate" means that a phenolic compound interact with only one protein site. At a high phenolic compound to protein ratio, phenolics can form a layer around a protein molecule, thereby more or less covering its surface, via a monodentate mechanism (Figure 14). The layer at the surface of the protein makes it less hydrophilic, which may lead to aggregation. The other mechanism, the multidentate mechanism, applies only to phenolic compounds with sufficient size to be able to interact with more than one site, thus being able to form cross-links between proteins (Figure 14). Both complexation mechanisms may lead to aggregation and precipitation (Charlton *et al.,* 2002; Haslam, 1989). The multidentate mechanism requires a much lower phenolic compound / protein molar ratio and thus a lower phenolic compound concentration than the monodentate mechanism. Proanthocyanidins, therefore, would decrease protein solubility at much lower ratios than monomeric phenolic compounds, such as CQA. Phenolic compounds-protein interaction can be summarized in Figure 15.

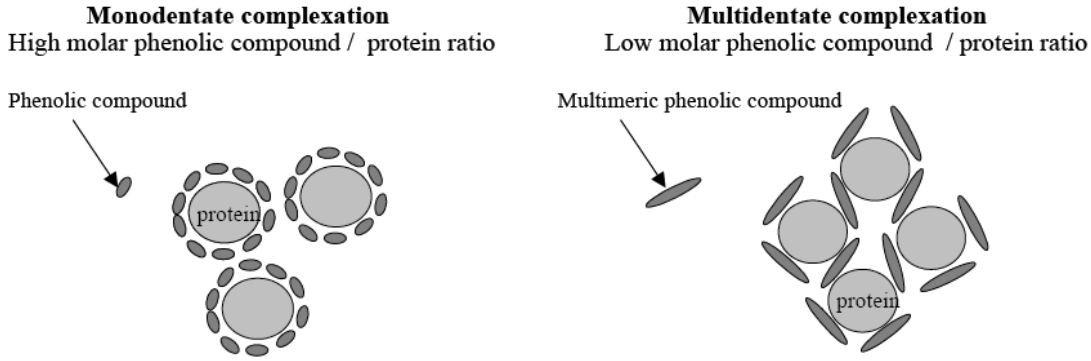


Figure 14. Monodentate and multidentate mechanism.

Source: Haslam (1989)

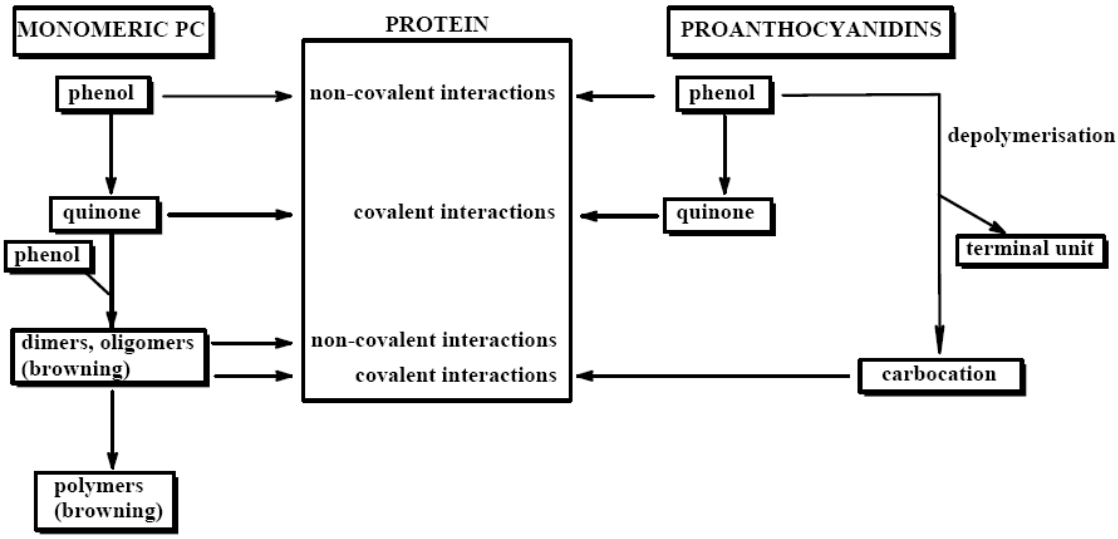


Figure 15. Overview of the phenolic compound (PC) – protein interactions.
Source: Prigent (2005)

The non-covalent and covalent interactions between phenolic compounds and proteins do not only depend on the phenolic compound / protein ratio but also on factors such as steric hindrance and the polarity of both the protein and the phenolic compound involved (Prigent, 2005). Therefore, the nature and the sequence of amino acids residues in the protein chain are of particular importance (Prigent, 2005). The interactions between phenolic compounds and proteins have also consequences for the production of plant protein ingredients, as these interactions may hinder protein extraction. Removing phenolic compounds for example is one of the main issues for the production of protein products from sunflower (Gonzalez-Perez *et al.,* 2002) and phenolic compounds may be responsible for the low solubility of some potato protein preparations (van Koningsveld *et al.,* 2002). Because protein insolubility also hinders other protein functional properties, protein solubility is an important factor in protein functionality.

a. Non-covalent interactions

Non-covalent interactions can be divided into five types: electrostatic interactions, van der Waals interactions, hydrogen bonds, hydrophobic interactions and π bonds. In the case of phenolic compound-protein interactions, hydrogen bonding and hydrophobic interactions are recognized as the main driving forces. Hydrogen bonds may involve the interactions between hydroxyl groups of

phenolic compounds and the nitrogen or oxygen of lysine, arginine, histidine, asparagine, glutamine, serine, threonine, aspartic acid, glutamic acid, tyrosine, cysteine and tryptophan (Prigent, 2005). Hydrophobic interactions may occur between phenolic compounds and amino acids such as alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine residues (Prigent, 2005).

Two theories exist to explain the relative importance of hydrogen bonding and hydrophobic interactions between phenolic compounds and proteins. In the first hypothesis, two stages occur: the associations are driven by hydrophobic interactions, after which hydrogen bonding enhances the interactions (Haslam, 1989). In the second hypothesis, the nature of the phenolic compound is considered: hydrophobic interactions are the main forces responsible for the interactions with nonpolar phenolic compounds such as pentagalloylglucose, whereas hydrogen bonding is the main force driving the interactions with more polar phenolic compounds such as procyanidins (Hagerman *et al.,* 1998). Heating increases the interaction between BSA and the nonpolar pentagalloylglucose, suggesting dominant hydrophobic interactions, but has no effect on the interactions with the more polar procyanidin dimer (Hagerman *et al.,* 1998). The latter suggests a balance between hydrophobic and hydrophilic interactions. Therefore, both hydrogen bonding and hydrophobic interactions are involved, while the nature of the phenolic compound, the protein and the environment determine which kind of interactions is the most important.

Considering the types of phenolic compounds, not only the polarity of the phenolic compound influences the binding, but also the size and the flexibility of the phenolic compound. The larger the phenolic compound, or more exactly the more binding sites the phenolic compound possesses, the stronger the association is expected (Hagerman *et al.,* 1998), i.e. proanthocyanidin trimers bind more tightly to BSA than proanthocyanidin dimers (Artz *et al.,* 1987). Less protein is precipitated with procyanidins > 3.5 kDa than with smaller procyanidins (De Freitas and Mateus, 2001). The lower solubility of large phenolic compounds causes the difficulty to interact with proteins (de Freitas and Mateus, 2001). In addition, the size of the phenolic compound can decrease its conformational flexibility, which is

observed to be an important parameter in protein-phenolic compound interactions (Frazier *et al.,* 2003). With respect to the type of protein, it has been shown that globular proteins, which are small and compact, have a lower affinity for phenolic compound than proteins with a more open conformation, e.g. proline-rich proteins (PRP), such as collagen (Prigent, 2005). When a proline residue interacts with a phenolic compound, a specific interaction takes place. This binding is controlled by weak forces occurring at short distances between the aromatic groups of phenolic compounds and proline (Bianco *et al.,* 1997). Such interactions may also occur between aromatic groups of two phenolics (Baxter *et al.,* 1997).

b. Covalent interactions

Covalent interactions between phenolic compounds and proteins can occur via oxidation of phenolic compounds to radicals or quinones. In addition to this mechanism, the reaction of a protein with a carbocation derived from proanthocyanidin degradation is a theoretical possibility for occurrence of protein-phenolic compound interactions.

1. Covalent interactions of quinones with proteins and other nucleophilic molecules

Quinones have been indirectly proven to react with amino acids incorporated in a peptide chain. The terminal amino group of proteins (Pierpoint, 1969a; Pierpoint, 1969b), as well as cysteine (Felton *et al.,* 1989), lysine (Pierpoint, 1982; Rawel *et al.,* 2000), tryptophan (Rawel *et al.,* 2002a) and histidine (Hurrell *et al.,* 1982) side-chains are able to react with quinones. Increasing the pH to alkaline conditions induces the deprotonation of the phenolic hydroxyl group, leading to quinone formation. This method is used in food industry e.g. to produce ripe olives by treating them with dilute NaOH in order to oxidize caffeic acid and hydroxytyrosol (Garcia *et al.,* 1996). The covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been studied extensively (Kroll *et al.,* 2003; Rawel *et al.,* 2000; Rawel *et al.,* 2002a). The reactions involved primary amino groups, thiol groups and tryptophan residues. Covalent protein modification by phenols oxidized at alkaline pH induced protein cross-linking and a decrease of the isoelectric pH of the proteins. Direct evidence for reaction products with the side-chains of amino acids has been demonstrated by the covalent reaction between CQA

and cysteine (Pierpoint, 1966; Richard *et al.,* 1991), caffeic acid and cysteine (Cilliers and Singleton, 1990), oxidized catechols and histidine (Kerwin *et al.,* 1999; Kramer *et al.,* 2001; Xu *et al.,* 1996) and oxidized catechols and methionine (Vithayathil and Satyanarayana Murthy, 1972).

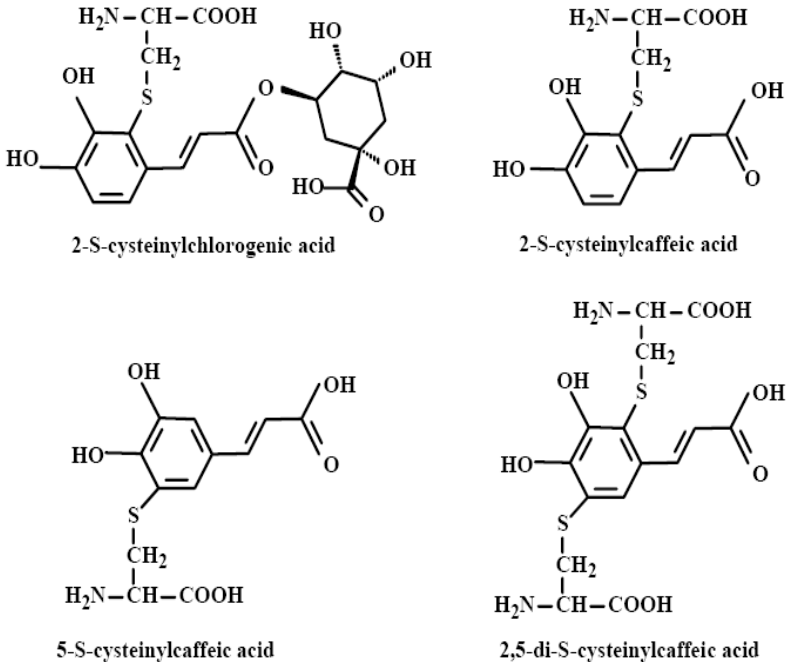


Figure 16. Reaction products of cysteine and oxidized CQA and oxidized Caffeic acid.

Source: Cilliers and Singleton (1990)

The reaction between CQA and cysteine leads to the formation of 2-S-cysteinylchlorogenic acid (Richard *et al.,* 1991) (Figure 16). The 2-position of the aromatic ring of oxidized CQA is the most electrophilic position and, therefore, it is the most susceptible site to react with nucleophiles, such as cysteine (Cilliers and Singleton, 1990). The reaction between caffeic acid and cysteine also occurs at this position. In addition, the 5-position of oxidized caffeic acid was found to be able to react with cysteine yielding to 5-S-cysteinylcaffeic acid, resulting in the binding of two cysteine molecules with the subsequent formation of 2, 5-di-S-cysteinylcaffeic acid (Cilliers and Singleton, 1990). A reaction product formed by the binding of cysteine to the 5-position of CQA can also be expected. No reaction products with dimers of CQA or caffeic acid with cysteine have been detected. The

mechanism would differ with the amino acid involved, because when they react with primary amino groups, phenolic compounds are supposed to dimerize prior to reacting with the amino acid (Namiki *et al.,* 2001). This mechanism, in which a cyclization step occurs after binding of the amino acid, is shown in Figure 17.

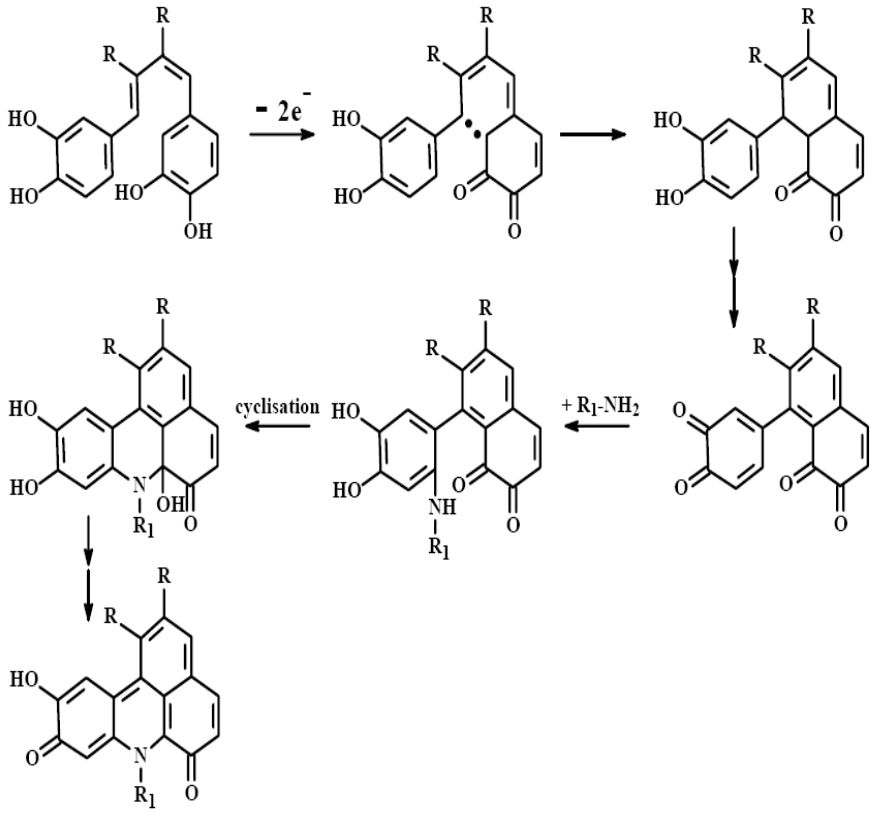


Figure 17. Proposed mechanism of reaction of dimerised caffeic acid ester (R=COO-CH_2-CH_3) with an amino compound (R_1-NH_2).

Source: Namiki *et al.* (2001)

2. Covalent interactions of proanthocyanidins and proteins at acidic pH

As mentioned previously, proanthocyanidins can be degraded at low pH usually only upon heating into anthocyanidins (Beart *et al.,* 1985; Haslam, 1989; Porter *et al.,* 1986; Swain and Hillis, 1959) (Figure 18). The affinity of proteins for procyanidins increases when the pH is brought close to the isoelectric pH of the protein. This change in affinity as a function of pH is in agreement with other studies that have shown that the optimum pH for complex formation generally is 0.3-3 units

below the pI of the protein (Naczk *et al.,* 1996). In order to reach saturation of α-lactalbumin by procyanidins, the protein concentration used at pH 3.0 was four times lower than at higher pHs. Because more phenolics are required to precipitate protein in diluted protein solutions than in concentrated protein solutions (Haslam, 1996), the affinity observed at pH 3.0 is likely being under-estimated compared to the affinity measured at higher pHs.

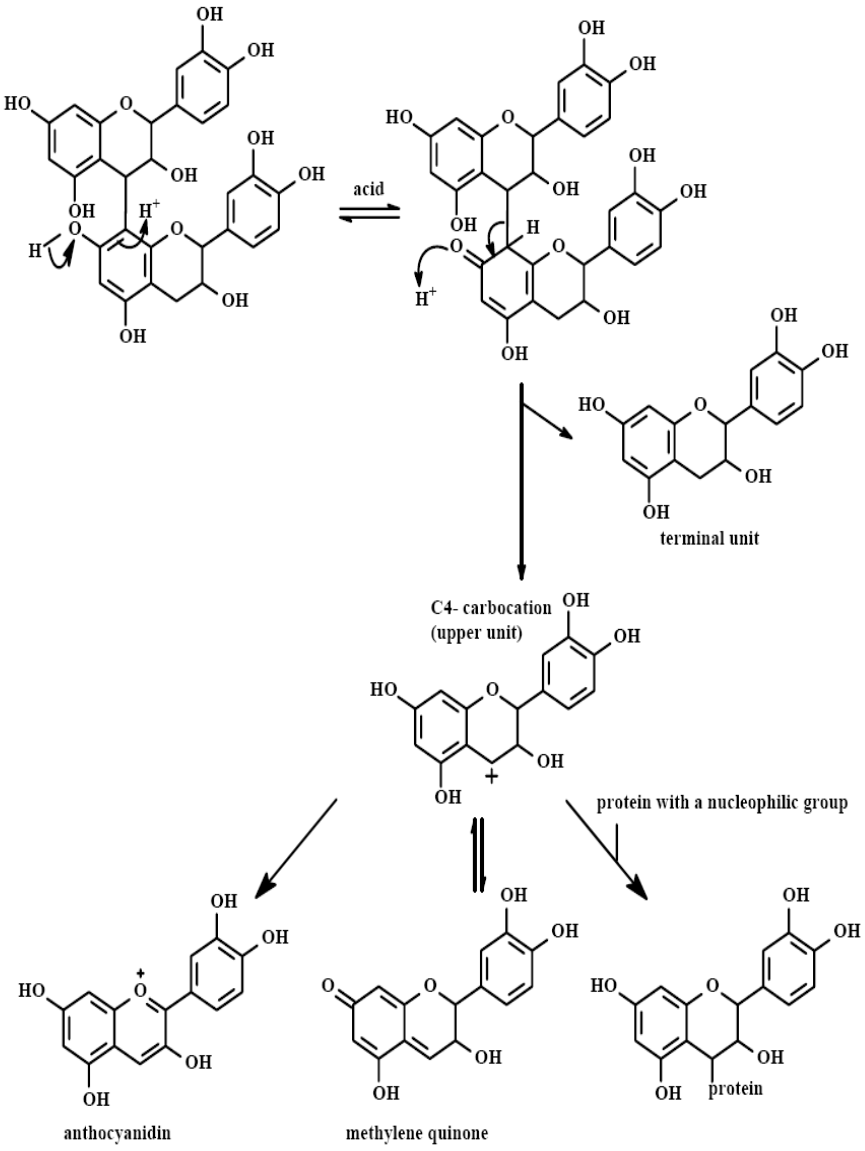


Figure 18. Acidic degradation of proanthocyanidins and reaction with nucleophilic groups.

Source: Haslam (1989)

1.2.10 Kiam tree (*Cotylelobium lanceotatum* craih)

Kiam (*Cotylelobium lanceotatum* craih) trees are very common in the southern part of Thailand. Pieces of wood from the kiam tree have been traditionally submerged in sugar palm sap to prevent or retard microbial fermentation (Chanthachum and Beuchat, 1997). The kiam wood is either burned for energy production or simply disposed. The preparation of kiam extract containing phenolic compounds could increase the value of kiam woods and the novel natural additives can be applied in food industry, especially surimi industry.

1.3 Objectives of study

1. To study the effects of commercial grade phenolic compounds on the gel properties of bigeye snapper surimi as well as mackerel mince and surimi prepared by different washing processes.
2. To elucidate the effect of phenolic compounds on physicochemical changes of mackerel muscle proteins.
3. To extract phenolic compounds from kiam wood and to use as gel strengthener in mackerel surimi.
4. To investigate the effect of oxygenation and pHs of phenolic compounds and kiam extract on protein crosslinking and gel enhancing ability of mackerel surimi.
5. To study the effect of phenolic compounds on gel enhancement of surimi prepared from mackerel stored in ice for different times.

CHAPTER 2

ENHANCEMENT OF GEL STRENGTH OF BIGEYE SNAPPER (*PRIACANTHUS TAYENUS*) SURIMI USING OXIDIZED PHENOLIC COMPOUNDS

2.1 Abstract

Effects of different oxidized phenolic compounds (ferulic acid, OFA; tannic acid, OTA; catechin, OCT and caffeic acid, OCF) at different levels (0-0.25% of protein content) on the properties of gels from bigeye snapper (*Priacanthus tayenus*) surimi were investigated. Breaking force and deformation of surimi gel varied with types and amounts of oxidized phenolic compounds. Gels added with 0.20% OFA, 0.05% OTA, 0.15% OCF and 0.05% OCT exhibited the marked increases in both breaking force and deformation, compared with the control ($P < 0.05$). Those increases were associated with lower expressible moisture content. No increases in both breaking force and deformation were observed when ferulic acid without oxygenation at alkaline pH was added, regardless of amount added ($P > 0.05$). No changes in the whiteness of gel were found with addition of OFA ($P > 0.05$), but the decreases in whiteness were noticeable as other oxidized phenolics were incorporated ($P < 0.05$). Different microstructures were obtained among gels with different oxidized phenolics. The physicochemical properties of natural actomyosin suggest that oxidized phenolics could induce conformational changes and the cross-linking through amino groups or the induction of disulfide bond formation. Therefore, the addition of oxidized phenolic compounds at the optimum level could increase the gel strength of surimi gel.

2.2 Introduction

Surimi is minced fish flesh, washed to remove most of lipids, blood, enzymes and sarcoplasmic proteins and stabilized for frozen storage by cryoprotectants. Surimi possesses the functionalities including gelling, 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). To increase the gel strength of surimi, various food grade ingredients have been used. However, the addition of these ingredients poses adverse effects on the surimi gel, particularly on off flavor or off color development (Rawdkuen and Benjakul, 2008). Addition of bovine plasma protein has been prohibited due to the mad cow disease, while addition of egg white is associated with allergy problems.

Polyphenols, which are widely distributed as minor components but are functionally important constituents of plant tissues, occur mainly in rigid tissues, such as the hulls of cereal grains, cell walls of fruits (e.g. grapes, apples), coffee beans, tea leaves, and tubers (e.g. potatoes) (Shahidi and Naczk, 2004; Naczk and Shahidi, 2004). The most common polyphenols are hydroxylated cinnamic acids such as caffeic acid (3, 4-dihydroxycinnamic acid), chlorogenic acid (its quinic acid ester), caftaric acid (its tartaric acid ester), and flavonols such as quercetin and rutin (its rutinoside) (Spanos and Wrolstad, 1992). These compounds have an ortho-diphenol (or a 1-hydroxy-2-methoxy) structure (Strauss and Gibson, 2004). The formation of rigid molecular structures by reactions of ortho-quinones with proteins is demonstrated by Strauss and Gibson (2004). Diphenol moiety of a phenolic acid or other polyphenol is readily oxidized to an ortho-quinone, either enzymatically as in plant tissues, or by molecular oxygen. The quinone forms a dimer in a side reaction, or reacts with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds with the phenolic ring, with regeneration of hydroquinone. The latter can be reoxidized and bind a second polypeptide, resulting in a cross-link. Alternatively, two quinones, each carrying one chain, can dimerise, also producing a cross-link (Strauss and Gibson, 2004). 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). Rawel *et al.* (2002a) reported that the phenolic compounds react with proteins, resulting in the formation of cross-links. Nevertheless, information regarding the effect of phenolic compounds on the gel property of surimi is very scarce. Thus, the study aimed to investigate the effect of phenolic compounds, including ferulic acid, tannic acid, catechin and caffeic acid on the properties of surimi gel from bigeye snapper, a species commonly used as a raw material for surimi production in Thailand.

2.3 Materials and Methods

Chemicals /surimi

Ferulic acid (FA), tannic acid (TA) and β-mercaptoethanol (βME) were obtained from Sigma (St. Louis, MO, USA). Caffeic acid (CF) and catechin (CT) were purchased from Fluka (Buchs, Switzerland). Sodium dedocylsulphate (SDS), *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

Frozen surimi grade B (breaking force of 300–400 g; deformation of <8 mm), produced from bigeye snapper (*Priacanthus tayenus*) were purchased from Man A Frozen Foods Co., Ltd. (Songkhla, Thailand) and kept at −20 °C not more than two months before use.

Effect of phenolic compounds on the properties of surimi gel

Preparation of oxidized phenolic solutions

Four phenolic compounds namely ferulic acid, tannic acid, caffeic acid and catechin were dissolved in distilled water as per the method of Strauss and Gibson (2004) with slight modifications. Phenolic solution (100 ml; 1%, w/v) was adjusted to pH 8 using 6 M NaOH or 6 M HCl. The prepared solution was placed in a temperature-controlled water bath (40^{o}C) and subjected to oxygenation for 1 h by bubbling the solution with oxygen to convert the phenolic compounds to quinones.

After being oxygenated for 1 h, the solution was then adjusted to pH 7 by using 6 M HCl and was referred to as 'oxidized phenolic compound'. For ferulic acid, another portion (pH 7) was also prepared without oxygenation.

Surimi gel preparation

To prepare the gel, frozen surimi was tempered for 30 min in running water (26-28°C) until the core temperature reached 0-2°C. The surimi was then cut into small pieces with an approximate thickness of 1 cm. The surimi was placed in a mixer (National Model MK-K77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% salt was added. Different oxidized phenolic compounds at various concentrations (0, 0.05, 0.10, 0.15, 0.20 and 0.25% protein content) were added. The mixture was chopped for 4 min at 4 °C to obtain a homogeneous sol. The sol was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Sols were incubated at 40 °C for 30 min, followed by heating at 90 °C for 20 min (Benjakul *et al.,* 2004b). All gels were cooled in iced water and stored for overnight at 4 °C prior to analyses.

To study the effect of oxygenation of ferulic acid on the properties of surimi gel, ferulic acid with and without oxygenation at different levels (0, 0.05, 0.10, 0.15, 0.20 and 0.25% protein content) was added into surimi sol. Surimi gels in five replicates were then prepared as previously described.

Texture analysis

Texture analysis of surimi gels was performed using a texture analyser Model TA-XT2 (Stable Micro Systems, Surrey, England). Gels were equilibrated and tested at room temperature. Five cylinder-shaped samples of 2.5 cm in length were prepared. The breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a spherical plunger (5 mm diameter; 60 mm/min deformation rate).

Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al.* (2001b) with a slight modification. Gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between 3 pieces of Whatman paper No. 4 at the bottom and 2 pieces on the top of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The sample was then removed from the papers and weighed again (*Y*). Expressible moisture content was calculated using the following equation:

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

Determination of whiteness

Color of surimi gels was determined using a JP7100F colorimeter (Juki Corporation, Tokyo, Japan). *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by Lanier *et al.* (1991) as follows:

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

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of surimi gels were analyzed by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85 °C were added to the sample (3 g). The mixture was then homogenized 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 3,500x*g* for 20 min to remove undissolved debris. The samples (20 μg protein) were loaded into 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.

Protein determination

Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Solubility determination

Solubility of protein in surimi gel was determined as described by Benjakul *et al.* (2001b). Finely chopped gel sample (1 g) was solubilized with various solvents including S1 (0.6 M KCl), S2 (20 mM Tris–HCl, pH 8.0), S3 (20 mM Tris–HCl, pH 8.0 containing 1% SDS), S4 (20 mM Tris–HCl, pH 8.0 containing 1% SDS and 8 M urea) and S5 (20 mM Tris–HCl, pH 8.0 containing 1% SDS, 2% β-mercaptoethanol and 8 M urea). The mixture was homogenized for 1 min, boiled for 2 min and stirred for 4 h at room temperature (28–30 °C) using a magnetic stirrer (IKA-Werke, Staufen, Germany). The mixture was centrifuged at 10,000×*g* for 30 min. Two ml of 50% (w/v) cold trichloroacetic acid (TCA) were added to 10 ml of supernatant. The mixture was kept at 4 °C for 18 h prior to centrifugation at 10,000×*g* for 20 min. The precipitate was washed with 10% (w/v) TCA, followed by solubilizing in 0.5 M NaOH. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940). Solubility of protein in surimi samples was expressed as the percentage of total protein in surimi gels solubilized directly in 0.5 M NaOH.

Scanning electron microscopy (SEM)

Microstructure of surimi gels was determined using SEM (JEOL JSM-5800 LV, Tokyo, Japan). The control gel (without oxidized phenolic compound) and those containing oxidized phenolic compounds with a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub, and sputter-coated with gold (Sputter coater SPI-Module, PA, USA). The specimens were observed with a scanning electron microscope at an acceleration voltage of 10 kV.

Effect of phenolic compounds on physico-chemical properties of natural actomyosin from bigeye snapper surimi

Preparation of natural actomyosin (NAM)

Natural actomyosin (NAM) was prepared according to the method of Benjakul *et al.* (1997) with a slight modification. Surimi (10g) was homogenized in 100 ml of chilled 0.6 M KCI, pH 7.0 for 4 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia). The container with sample was placed in ice. Each 20 sec of homogenization was followed by a 20 sec rest interval to avoid overheating during extraction. The homogenate was centrifuged at 5,000xg for 30 min at 4°C. Three volumes of chilled water (0-2°C) were added to precipitate NAM and NAM was then collected by centrifuging at 5,000xg for 20 min at 4°C. The pellets were then dissolved by stirring in an equal volume of chilled 0.6 M KCI, pH 7.0 for 30 min at 4°C.

Incorporation of oxidized phenolic compounds into NAM

NAM was diluted to a concentration of 4 mg/ml. Each oxidized phenolic compound was added into NAM to obtain the final concentration, which was equivalent to that rendering the highest breaking force in surimi gel. The control (without oxidized phenolic compound) was also prepared. The mixture was subjected to heating with two conditions involving i) 40°C for 30 min or ii) 40^{o}C for 30 min followed by 90^{o}C for 20 min. Heated samples were cooled rapidly in iced water and subjected to analyses.

Determination of surface hydrophobicity

Surface hydrophobicity was determined as described by Benjakul *et al.* (1997) using 1-anililonaphthalene-8-sulphonic acid (ANS) as a probe. Different NAM mixtures were diluted to protein contents of 0.1, 0.2, 0.3 and 0.5 % (w/v) using 10 mM phosphate buffer, pH 6.0 containing 0.6 M NaCl. The diluted protein solution (2 ml) was added with 20 µl of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0. The fluorescence intensity of ANS-conjugates was measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. Blanks

were prepared using oxidized phenolic compounds at the concentrations equivalent to those found in differently diluted NAM. Net fluorescence intensity of NAM at each concentration was obtained after blank correction. The initial slope of the plot of fluorescence intensity versus NAM concentration was referred to as SoANS.

Determination of total sulfhydryl group and disulfide bond contents

Total sulfhydryl group content was determined using 5, 5'-dithio-bis (2nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul *et al.* (1997). To 1.0 ml NAM solution (4.0 mg/ml), 9 ml of 0.2 M Tris-HCI buffer, pH 6.8, 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. Absorbance at 412 nm was then measured. A blank was conducted by replacing the sample with 0.6 M KCI. Sulfhydryl group content was calculated using the extinction coefficient of 13,600 $M^{-1}cm^{-1}$.

Disulfide bond content was determined by using 2-nitro-5-thiosulphobenzoate (NTSB) assay according to the method of Thannhauser *et al.* (1987). To 0.5 ml of NAM (4.0 mg/ml), 3.0 ml of freshly prepared NTSB assay solution was added. The mixture was incubated in the dark at room temperature (26-28°C) for 25 min. Absorbance at 412 nm was measured. Disulfide bond content was calculated using the extinction coefficient of 13,900 $M^{-1}cm^{-1}$.

Determination of free amino group contents

Free amino group content was determined according to the method of Benjakul and Morrissey (1997). Diluted samples (125µl) were mixed thoroughly with 2.0 ml of 0.2 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01% TNBS solution. The mixture was then placed in a water bath at 50°C for 30 min in dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixture was cooled to room temperature for 15 min. The absorbance was measured at 420 nm and free amino group content were expressed in terms of L-leucine.

Statistical analysis

One-way ANOVA was used and mean comparison was performed by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was carried out using the SPSS statistic programme (Version 10.0) for Windows (SPSS Inc. Chicago, IL).

2.4 Results and Discussion

Effect of oxidized phenolic compounds on the properties of surimi gel

Gels from bigeye snapper surimi added with different oxidized phenolic compounds at various levels had varying breaking force and deformation (Figure 19). Gels added with oxidized ferulic acid (OFA) or oxidized caffeic acid (OCF) had the increases in both breaking force and deformation when the levels added increased up to 0.20 and 0.15%, respectively *($P < 0.05$)*. With the addition of 0.20% OFA, breaking force and deformation of gel increased by 28.98 and 38.06%, respectively, compared with that of the control. The addition of OCF at a level of 0.15% resulted in the increases in breaking force and deformation of gel by 29.78 and 38.63%, respectively, compared with that of the control. For gels added with oxidized tannic acid (OTA) or oxidized catechin (OCT), the highest breaking force and deformation were obtained when both phenolic compounds were added at 0.05% ($P<0.05$). The continuous decreases in both breaking force and deformation were noticeable as the levels added increased up to 0.25% *($P < 0.05$)*. OTA at 0.05% resulted in the increase in breaking force and deformation of surimi gel by 39.52 and 38.97%, respectively. The addition of OCT at the same level increased breaking force and deformation of surimi gels by 29.69 and 38.40%, respectively. These results indicate that oxidized phenolic compounds at the optimum concentration showed the enhancing effect on gel formation. Phenolic compounds can interact with proteins in two different ways: via non-covalent (reversible) interactions and via covalent interactions, which in most cases are irreversible (Prigent *et al.,* 2003). Two types of complexation mechanisms can be distinguished: a monodentate and a multidentate

mechanism (Haslam, 1989). Both complexation mechanisms lead to aggregation and precipitation of proteins (Haslam, 1989). Lower amounts of TA and CT (0.05%) were required to increase the breaking force and deformation of surimi gel, compared with FA and CF. The multidentate mechanism generally requires a much lower phenolic compound / protein molar ratio and thus a lower phenolic compound concentration (Haslam, 1989). On the contrary, "Monodentate" means that a phenolic compound interacts with only one protein site at a higher phenolic compound concentration (Haslam, 1989). From the result, both OFA and OCF having 1 and 2 hydroxyl groups, respectively, at higher levels (0.20 and 0.15%) were required to increase breaking force and deformation of surimi gel. The larger the phenolic compound or the more binding sites the phenolic compound possesses, the stronger the association is expected (Hagerman *et al.,* 1998). In the present study, at the lower level (0.05%), higher breaking force and deformation was observed when OTA was added *(*$P < 0.05$*)*. OTA has a greater number of hydroxyl groups attached to the aromatic benzene ring as compared to others. Nevertheless, the lower solubility of large phenolic compounds at high concentration causes the difficulty to interact with proteins (De Freitas and Mateus, 2001). In addition, the size of the phenolic compound can decrease its conformational flexibility, which is observed to be an important parameter in protein-phenolic compound interactions (Frazier *et al.,* 2003). The decreased breaking force and deformation with increasing concentrations of phenolic compounds in the present study might be associated with self-aggregation of phenolic compounds, leading to the loss in capability of protein cross-linking.

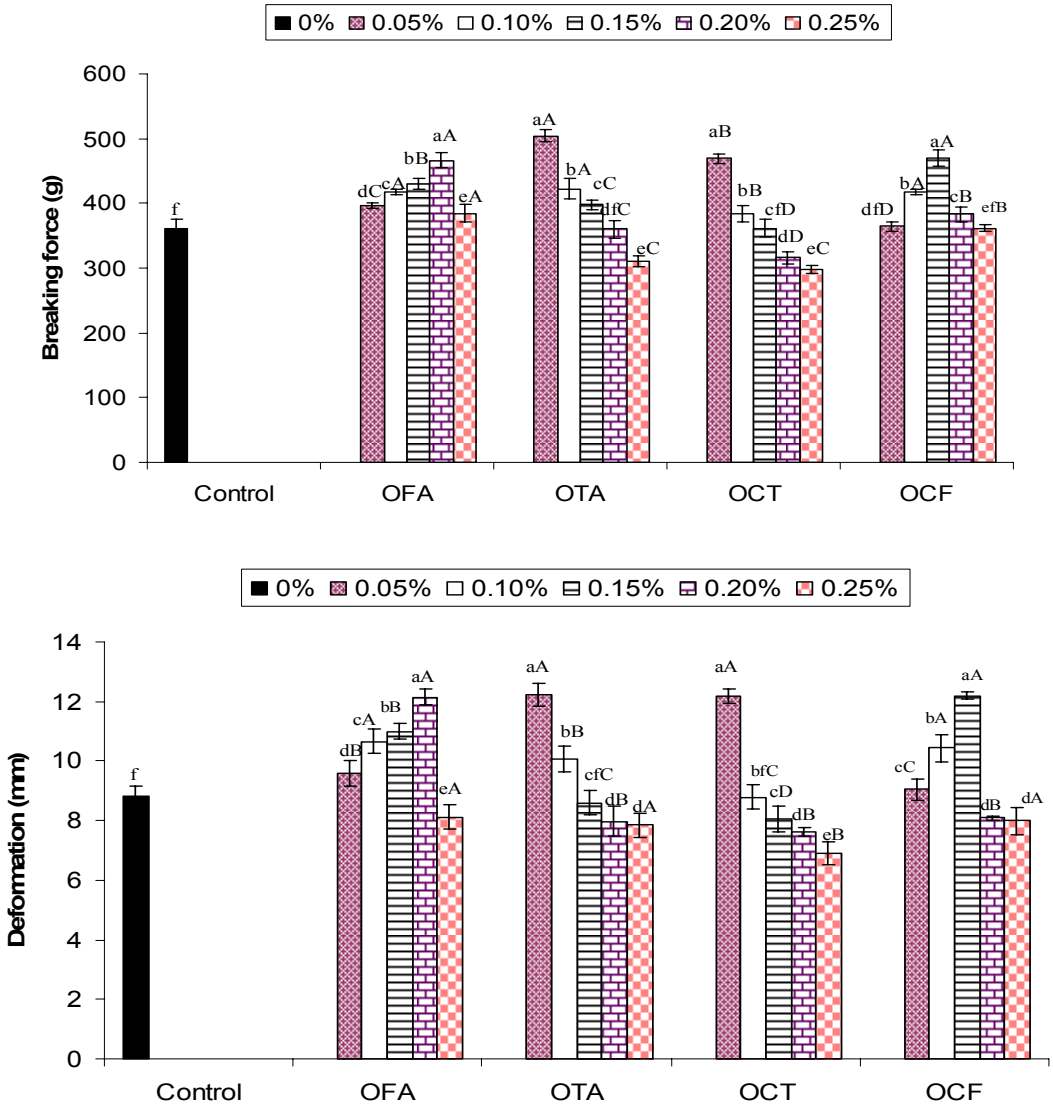


Figure 19. Breaking force and deformation of gels from bigeye snapper surimi added with oxidized phenolic compounds at different levels. OFA, OTA, OCT and OCF represent oxidized ferulic acid, tannic acid, catechin and caffeic acid, respectively. Bars represent the standard deviation from three determinations. Different letters on the bars within the same oxidized phenolic compounds together with the control indicate the significant differences *($P < 0.05$)*. The different capital letters on the bars within the same levels of oxidized phenolic compounds indicate the significant differences *($P < 0.05$)*.

Effect of oxygenation of ferulic acid on the properties of surimi gel

FA, without and with oxygenation under alkaline pH prior to neutralisation at different levels was added in surimi gels. It was noted that no changes in breaking force and deformation were observed with the addition of FA without prior oxygenation (Figure 20). On the other hand, the continuous increase in breaking force was found when the oxidized FA (OFA) was added up to 0.20%. The highest deformation was also obtained as 0.20% OFA was added *($P < 0.05$)*. Oxygenation under alkaline conditions induces the deprotonation of phenolic hydroxyl group, leading to quinone formation. Quinones have been indirectly proven to react with amino acids in a peptide chain. This method is used in food industry e.g. to produce ripe olives by treating them with dilute NaOH in order to oxidise caffeic acid and hydroxytyrosol (Garcia *et al.,* 1996). The covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been studied extensively (Rawel *et al.,* 2002a). Covalent protein modification by phenols oxidized at alkaline pH induced protein cross-linking and a decrease of the isoelectric pH of proteins (Prigent, 2005).

Direct evidence for reaction products with the side-chains of amino acids was demonstrated by the covalent reaction between CQA and cysteine (Richard *et al.,* 1991), caffeic acid and cysteine (Cilliers and Singleton, 1990) and oxidized catechols and histidine (Kerwin *et al.,* 1999). In the present study, OFA at a level of 0.20% increased breaking force and deformation effectively after the oxidation took place. This reveals that the formation of quinone was necessary for the cross-liking of proteins. As a consequence, the protein gel network could be strengthened. The result was in agreement with Strauss and Gibson (2004) who found the increase in bloom strength of gelatin gels incorporated with oxygenated phenolics.

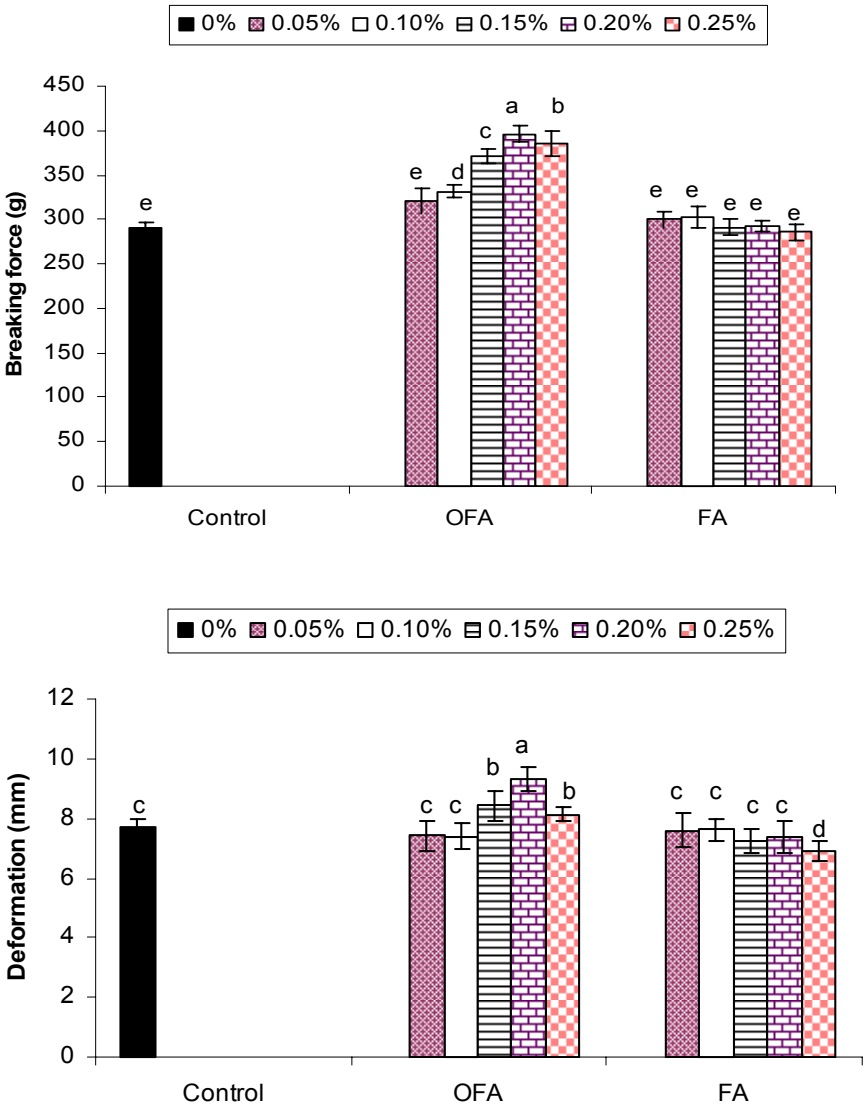


Figure 20. Breaking force and deformation of gels from bigeye snapper surimi added with ferulic acid with and without oxygenation at different levels. Bars represent the standard deviation from three determinations. Different letters on the bars indicate the significant differences *(P* < 0.05*)*.

Effect of oxidized phenolic compounds on expressible moisture of surimi gels

Expressible moisture content of surimi gels added with different oxidized phenolic compounds at various levels is shown in Table 2. Gel added with OFA or OCF had the decreased expressible moisture content as the levels added increased up to 0.20 and 0.15%, respectively. The lowered expressible moisture content was in accordance with the increased breaking force and deformation of resulting surimi gels (Figure 19). The lowest expressible moisture content was observed in gels added with 0.05% OTA or 0.05% OCT, compared with gels added with higher levels of oxidized phenols. During setting at 40^{o}C, proteins underwent some denaturation and aligned themselves gradually to form the network, which can imbibe water (Benjakul and Visessanguan, 2003). When the optimal level of oxidized phenolic compound was added, the cross-linking of proteins could be enhanced, resulting in the formation of stronger network with greater water holding capacity.

Effect of oxidized phenolic compounds on whiteness of surimi gels

Whiteness of all surimi gels added with OTA, OCT and OCF decreased as the concentrations increased *($P < 0.05$)* (Table 2). Phenolic compounds were responsible for discoloration in cheese products (O'Connell and Fox, 2001). On the contrary, no changes in whiteness were observed in surimi gels added with different levels of OFA, compared with the control ($P > 0.05$). At alkaline-neutral pH values, FA is inherently unstable and is converted to carbinol base, which is colorless (Asen *et al.,* 1979). As a result, no changes in whiteness of surimi gels were noticeable when different levels of OFA were added. Phenolic compounds like tannic acid, catechin and caffeic acids are stable at neutral pH values and thus are suitable colorants (Asen *et al.,* 1979).

Table 2. Expressible moisture content and whiteness of gels from bigeye snapper surimi added with various oxidized phenolic compounds at different levels.

Oxidized phenolic compounds	Amount added (%)	Expressible moisture content (%)	Whiteness
Control	-	3.93 + 0.57a*	73.15 + 0.28a*
OFA	0.05	2.97+0.75bA**	72.85+0.25aA**
	0.10	2.92+0.72 bA	73.18+0.31aA
	0.15	2.62+0.48cC	72.50+0.24aA
	0.20	2.56+0.51dC	72.53+0.33aA
	0.25	2.62+0.48cD	73.08+0.26aA
OTA	0.05	2.55+0.07dB	65.66+1.68bC
	0.10	2.61+0.43cB	65.30+2.13bB
	0.15	3.02+0.11bB	62.82+0.43dB
	0.20	3.05+0.39bB	63.76+1.70cB
	0.25	3.12+0.15bB	61.89+0.26eD
OCT	0.05	2.62+0.15dB	67.45+0.52bB
	0.10	2.94+0.34cA	65.83+0.57cB
	0.15	3.48+1.12cA	63.88+0.50dC
	0.20	3.71+1.17bA	63.64+0.43dB
	0.25	3.90+1.09bA	62.64+0.32eC
OCF	0.05	2.81+0.47bA	67.29+0.36bB
	0.10	2.65+0.72bB	65.70+0.74cB
	0.15	2.06+0.12cD	63.88+0.50dC
	0.20	2.40+0.45cD	63.62+0.44dB
	0.25	2.84+1.43bC	63.32+0.79dB

* Different letters in the same colomn within the same oxidized phenolic compound together with the control indicate the significant differences ($P < 0.05$). ** Different capital letters in the same colomn within the same level of oxidized phenolic compounds used indicate the significant differences ($P < 0.05$).
Values are mean + standard deviation (n=3).

Effect of oxidized phenolic compounds on protein patterns of surimi gels

No differences in MHC band intensity were noticeable when oxidized phenolic compounds at the optimum level were used, compared with that of the control (without oxidized phenolic addition) (data not shown). Generally, no changes in actin band intensity were observed. Decrease in MHC band intensity was found in surimi gel, regardless of oxidized phenolic addition, when compared with that observed in sol. This suggests the formation of cross-linking stabilized by non-disulfide covalent bond, especially during setting. MHC was most susceptible to cross-linking during setting (Benjakul and Visessanguan, 2003). Benjakul and Visessanguan (2003) reported the decrease in MHC of surimi gel from bigeye snapper, particularly when the setting was implemented. Since no differences in MHC band intensity among the control gel and those added with oxidized phenolic compounds, non-disulfide covalent bonds induced by oxidized phenolic compounds might be formed at low level. Weak bonds most likely contributed to the gel strengthening caused by the addition of oxidized phenolic compounds. Nevertheless, covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been reported extensively (Rawel *et al.,* 2002a). Thus, bonding involved in protein cross-linking on gel strengthening could be varied with types of phenolics and proteins, etc.

Effect of oxidized phenolic compounds on solubility of surimi gels

Solubility of surimi gels added with different oxidized phenolic compounds at the optimum level in different solubilising solutions is shown in Figure 21. Solubility was found to be lower than 10% in all gels with and without oxidized phenolic compounds when solubilized with 0.6M KCl (S_1) and 20 mM Tris-HCl (pH 8.0) (S_2). The decrease in solubility suggests the formation of protein aggregates during setting and heating. When the gels were solubilized in 20 mM Tris-HCl (pH 8.0) containing 1% SDS (S_3), solubility was increased up to 50% in the control, while gels added with OFA, OTA, OCT and OCF had the increases in solubility by 36, 34, 40 and 38%, respectively. SDS is capable of destroying hydrogen and some

hydrophobic interactions (Hamada, 1992). Further increases in solubility were observed in S_4, containing urea and SDS, indicating the presence of hydrophobic and hydrogen bonds in surimi gels. Hydrogen bonds might involve in the interactions between hydroxyl groups of phenolic compounds and the nitrogen or oxygen of lysine, arginine, histidine, asparagine, glutamine, serine, threonine, aspartic acid, glutamic acid, tyrosine, cysteine and tryptophan as hydrogen acceptor (Prigent, 2005). Protonation of quinone during oxygenation could take place to some extent after neutralisation. As a consequence, hydroxyl groups could be regenerated partially. Hydrophobic interactions may occur between phenolic compounds and hydrophobic amino acids such as alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine residues (Prigent, 2005). Surimi gels added with oxidized phenolic compounds had the lower solubility than the control when S_4 was used. This suggests that covalent bonds were formed in gels added with phenolic compounds. When the samples were solubilized in S_5, containing urea, SDS and βME, it was noticeable that a higher increase in solubility was observed, indicating the presence of disulfide bonds in the gel. When quinones attached to protein molecules, the conformational changes of protein possibly occurred in the way that favored the oxidation of sulfhydryl groups, leading to the increases in disulfide bond formation. Gels added with oxidized phenolic compounds had a slightly lower solubility in S_4, compared with the control gel. Thus, some non-disulfide covalent bonds induced by quinone, oxidized phenolic, could play a partial role in gel strengthening. It was reported that quinone probably attached to amino groups of protein molecules in which the subsequent intermolecular cross-linking could be formed (Strauss and Gibson, 2004; Rubino *et al.,* 1996; Rawel *et al.,* 2002a).

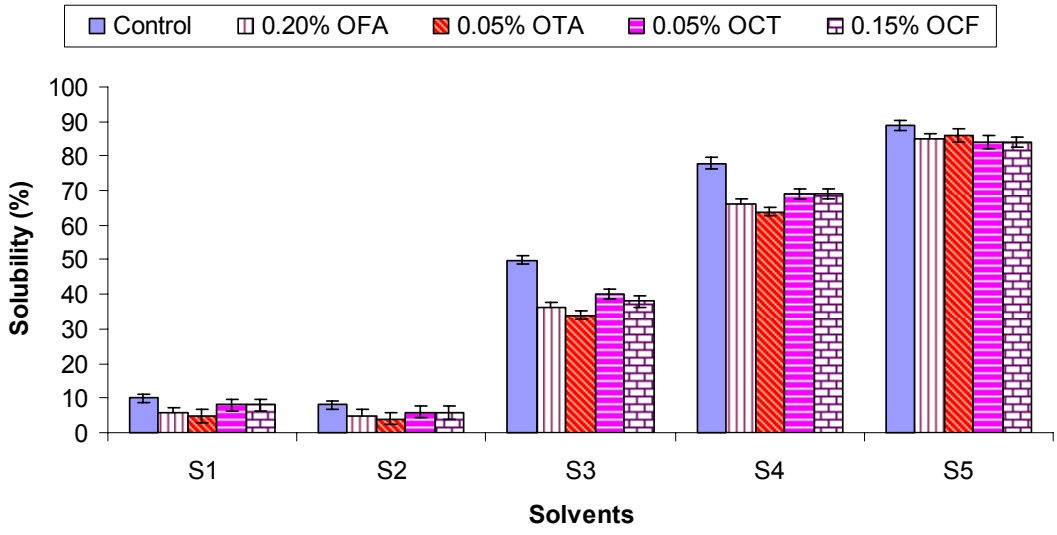
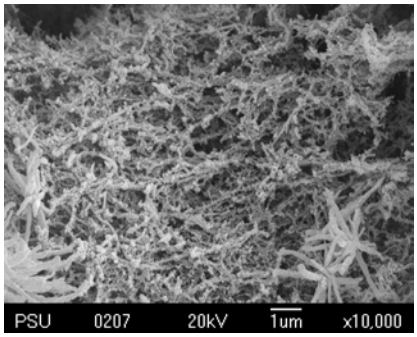


Figure 21. Solubility of gels added with oxidized phenolic compounds at the selected levels. Samples were solubilized in different solvents and soluble protein was determined by the biuret assay. S1 (0.6 M KCl), S2 (20 mM Tris–HCl pH 8.0), S3 (20 mM Tris–HCl, pH 8.0, containing 1% SDS), S4 (20 mM Tris–HCl, pH 8.0, containing 1% SDS and 8 M urea) and S5 (20 mM Tris–HCl, pH 8.0, containing 1% SDS, 2% β-mercaptoethanol and 8 M urea). Bars represent the standard deviation from triplicate determinations.

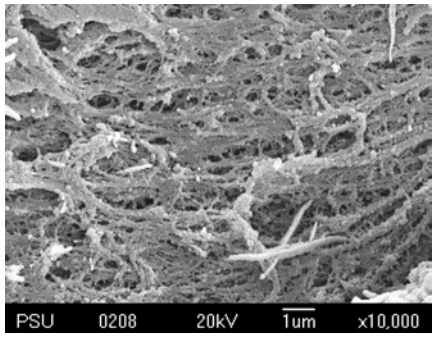
Effect of phenolic compounds on microstructure of surimi gels

Microstructures of gels from bigeye snapper surimi with 0.20% OFA (A), 0.05% OTA (B), 0.05% OCT (C), 0.15% OCF (D) and control (without oxidized phenolic compounds) (E) are shown in Figure 22. In general, a network with fibrous structure was observed for bigeye snapper surimi gel. However, the surimi gel without oxidized phenolic compound had a larger void, compared with those containing different oxidized phenolic compounds. These observations suggest that the addition of oxidized phenolic compounds resulted in the formation of an ordered structure with finer strands. However, surimi gel added with 0.05% OTA exhibited more compact structure with larger bundles in the matrix as compared to others. For gel added with 0.05% OCT, bead type aggregates were found in the gel matrix. Both OTA and OCT

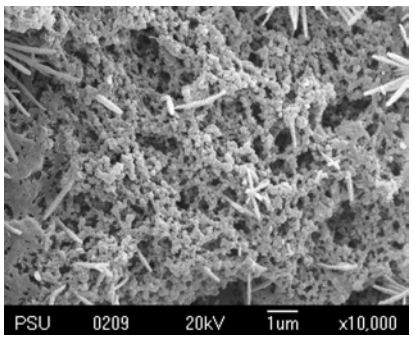
might have a greater number of binding sites and in turn caused a higher aggregation. As a result, different structures of gels were obtained, compared with those added with OFA or OCF.



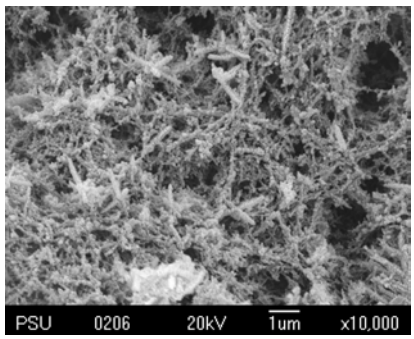
A) 0.20% OFA



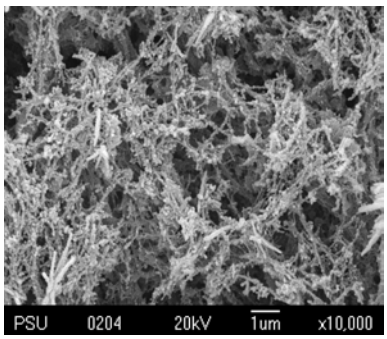
B) 0.05% OTA



C) 0.05% OCT



D) 0.15% OCF



E) Control

Figure 22. Electron microscopic images of gels from bigeye snapper surimi added without and with different oxidized phenolic compounds at the selected levels. OFA, oxidized ferulic acid; OTA, oxidized tannic acid; OCT, oxidized catechin; OCF, oxidized caffeic acid. (Magnification: 10,000X).

Effect of oxidized phenolic compounds on physicochemical properties of natural actomyosin

Surface hydrophobicity

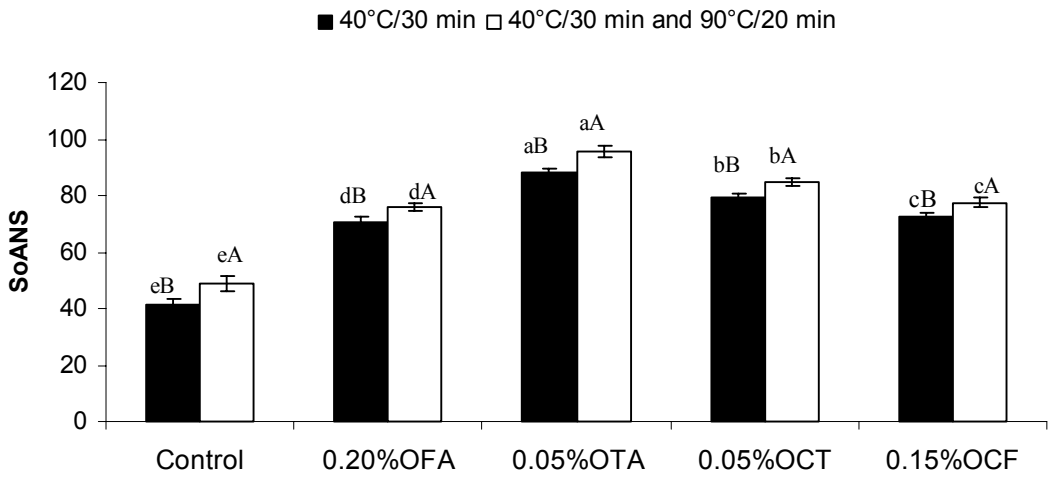
Surface hydrophobicity of NAM extracted from surimi of bigeye snapper added with different oxidized phenolic compounds at the optimum level and subjected to heating under different conditions: 1) 40^{o}C for 30 min and ii) 40^{o}C for 30 min / 90^{o}C for 20 min, is shown in Figure 23A. Generally, NAM added with oxidized phenolic compounds had an increase in surface hydrophobicity, compared with the control NAM (without oxidized phenolic compounds) ($P < 0.05$). The results indicate that oxidized phenolic compounds likely induced the conformational changes of NAM to some extent, as evidenced by the increase in surface hydrophobicity. ANS, a fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as phenylalanine and tryptophan, and can be used to indicate the conformational changes occurring in the proteins (Benjakul *et al.,* 1997). The increase in surface hydrophobicity was possibly caused by the exposure of hydrophobic groups of the protein molecules. Among all oxidized phenolic compounds, OTA even at a lower level caused the greatest increase in surface hydrophobicity. Multifunctional groups of this compound possessed a higher potential to bind or attach to protein molecules, in which the alteration of protein conformation could be more enhanced. Higher surface hydrophobicity of NAM without and with oxidized phenolic compounds was observed when heated at both 40^{o}C for 30 min and 90^{o}C for 20 min, compared with that found in NAM heated at 40^{o}C *($P < 0.05$)*. Unfolding of protein molecules was due to the instability of hydrogen bonds at higher temperature, exposing greater numbers of hydrophobic portions (Niwa, 1992). When NAM was heated at 90^{o}C, more reactive groups including amino groups became more available. As a consequence, quinone or phenolic could bind more effectively and might induce the conformational change to a greater extent, as indicated by the higher surface hydrophobicity *($P < 0.05$)*.

Total sulfhydryl group and disulfide bond contents

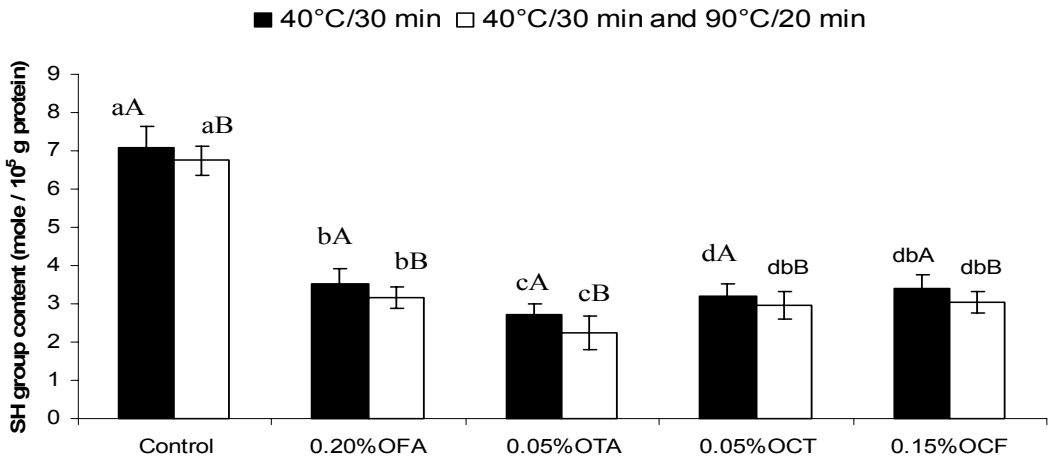
Total sulfhydryl group content of NAM extracted from bigeye snapper surimi and added with different oxidized phenolic compounds at the optimum level and subjected to heating under two different conditions is shown in Figure 23B. NAM added with different oxidized phenolic compounds had lower sulfhydryl group content than that of the control *(P* < 0.05*)*. Phenolic compound might induce the conformation, in which oxidation of sulfhydryl group could occur more easily as evidenced by the lowered sulfhydryl group content. Additionally, quinone could interact directly to sulfhydryl group (Strauss and Gibson, 2004). As a result, these sulfhydryl groups could be masked by those quinones. A decrease in total sulfhydryl group content was reported to be due to the formation of disulfide bonds through oxidation of sulfhydryl groups or disulfide interchanges (Hayakawa and Nakai, 1985). Sulfhydryl group content of NAM without and with phenolic compounds was lower *(P* < 0.05*)* when NAM mixtures were heated at 40^{o}C for 30 min and 90^{o}C for 20 min in comparison with those heated at 40^{o}C for 30 min *(P* < 0.05*)*. Elevated temperature during heating most likely resulted in further oxidation of sulfhydryl groups with the accompanied disulfide bond formation. The decrease in sulfhydryl group content was in accordance with the increase in disulfide bond formation in all treatments and heating conditions used (Figure 23C). NAM had lower disulfide bond content than those added with all oxidized phenolic compounds *(P* < 0.05*)* (Figure 23C). Therefore, the addition of oxidized phenolic compounds was associated with the increased formation of disulfide bonds. An intermolecular disulfide bond is formed by the oxidation of two cysteine molecules on neighbouring protein chains (Lanier, 2000). The formation of disulfide bonds might contribute to the improved gel property of surimi added with the oxidized phenolic compounds at the optimum level (Figure 19). Among all oxidized phenolic compounds used, OTA at a level of 0.05% caused the greater decrease in sulfhydryl group content with the concomitant increase in disulfide bond. This was coincidental with the highest increase in both breaking force and deformation of gel added with 0.05% OTA (Figure 19).

Free amino group content

Free amino group contents of NAM from surimi of bigeye snapper added without and with different oxidized phenolic compounds at optimum level and subjected to heating under different conditions are shown in Figure 23D. NAM of surimi added with all oxidized phenolic compounds had lower free amino group content than that of control *(P* < 0.05*)*. This indicates that the amino groups might undergo cross-linking via quinone. The rate of loss in free amino group was generally higher in NAM added with 0.05% OTA *(P* < 0.05*)*. This was associated with the more number of hydroxyl groups present in the tannic acid compared with others. The hydroxyl groups were presumably converted to quinone, which functioned as cross-linkers. Additionally, remaining hydroxyl groups might interact with amino groups. The interactions between hydroxyl groups of phenolic compounds and the nitrogen or oxygen of some amino acids were reported by Prigent (2005). Hydrophobic interactions may occur between phenolic compounds and amino acids (Prigent, 2005). Free amino group content of NAM without and with oxidized phenolic compounds decreased to a higher degree when heated at 40^{o}C for 30 min and 90^{o}C for 20 min, compared with that of NAM heated at 40^{o}C for 30 min *(P* < 0.05*)*. Unfolding and exposing of free amino groups at elevated temperature allowed the oxidized phenolic compounds to react with amino groups more effectively, thereby lowering free amino group contents. The lower free amino group content in NAM without oxidized phenolic compound indicates that cross-linking of protein via remaining TGase in NAM could occur, particularly during heating at 40^{o}C. TGase from bigeye snapper exhibited the optimal temperature at 40^{o}C (Benjakul and Visessanguan, 2003).

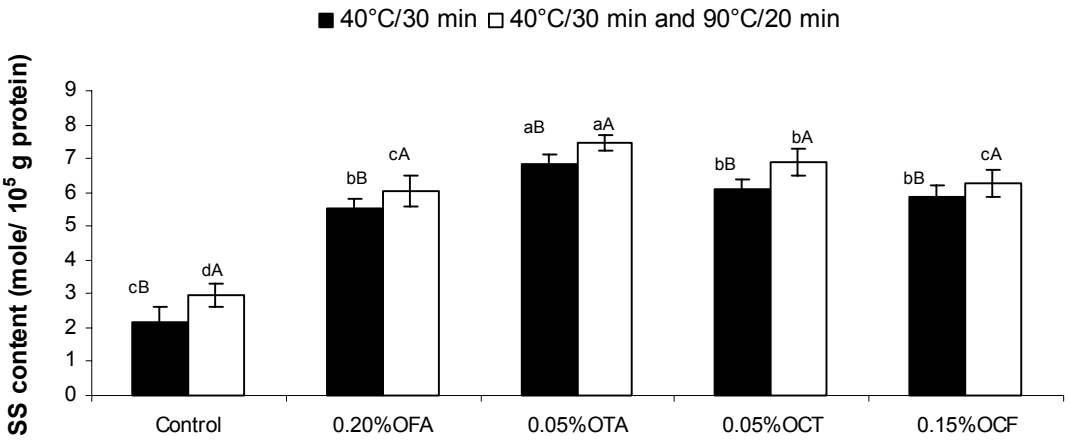


A)

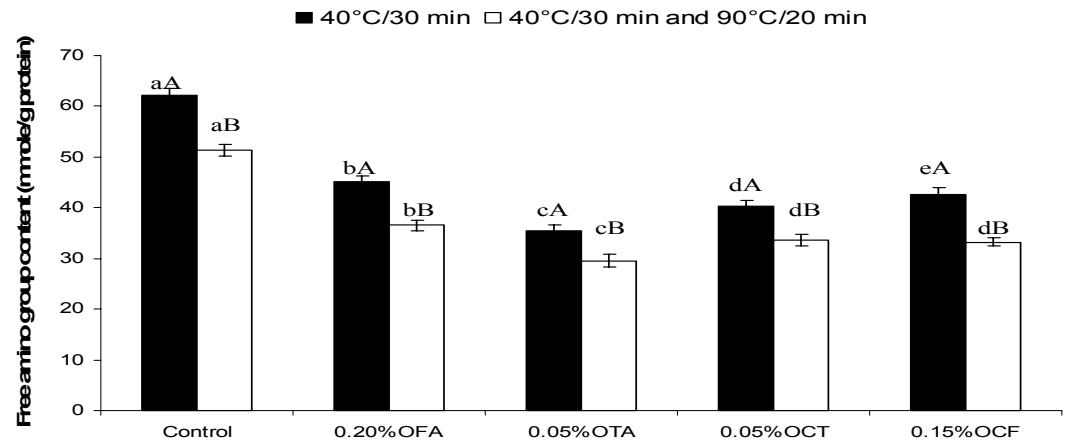


B)

Figure 23. Effect of oxidized phenolic compounds on surface hydrophobicity, (A); sulfhydryl group content, (B); disulfide bond content (C) and free amino group content (D) of natural actomyosin from bigeye snapper surimi heated at two conditions: 1) 40^0C for 30 min and 2) 40^0C for 30 min and 90^0C for 20 min. OFA, oxidized ferulic acid; OTA, oxidized tannic acid; OCT, oxidized catechin; OCF, oxidized caffeic acid. Different letters within the same heating conditions indicates significant difference (P <0.05). Different capital letters within the same treatment of oxidized phenolic compound indicates significant difference (P < 0.05).



C)



D)

Figure 23 (Cont). Effect of oxidized phenolic compounds on surface hydrophobicity, (A); sulfhydryl group content, (B); disulfide bond content (C) and free amino group content (D) of natural actomyosin from bigeye snapper surimi heated at two conditions: 1) 40^0C for 30 min and 2) 40^0C for 30 min and 90^0C for 20 min. OFA, oxidized ferulic acid; OTA, oxidized tannic acid; OCT, oxidized catechin; OCF, oxidized caffeic acid. Different letters within the same heating conditions indicates significant difference (P <0.05). Different capital letters within the same treatment of oxidized phenolic compound indicates significant difference (P < 0.05).

2.5 Conclusions

Type and concentration of oxidized phenolic compounds had varying influence on surimi gels. OTA exhibited superior gel strengthening effect to others. However, addition of OTA, OCT and OCF caused the decreased whiteness, especially with increasing amount. Addition of OFA had no adverse effect on whiteness of resulting gel.

CHAPTER 3

EFFECT OF OXIDIZED PHENOLIC COMPOUNDS ON THE GEL PROPERTY OF MACKEREL (*RASTRELLIGER KANAGURTA*) SURIMI

3.1 Abstract

Effects of different oxidized phenolic compounds (ferulic acid, OFA; tannic acid, OTA; catechin, OCT and caffeic acid, OCF) at different levels (0-0.60% of protein content) on the properties of gels from mackerel (*Rastrelliger kanagurta*) surimi were investigated. Gels added with 0.40% OFA, 0.50% OTA, 0.50% OCF or 0.10% OCT had the increases in breaking force by 45%, 115%, 46.1% and 70.3% and in deformation by 12.2, 27.5, 28.1 and 28.4%, respectively, compared with the control (without addition of oxidized phenolics). Lowered expressible moisture contents without any change in the whiteness of resulting gels were found. Slightly lower myosin heavy chain (MHC) band intensity of gels added with oxidized phenolics at the optimal level was noticeable compared with that of the control. Sensory evaluation study indicated that addition of oxidized phenolic compounds had no negative impact on the color and taste of resulting gels ($P > 0.05$). Gels added with all oxidized phenolics at optimum level had the finer matrix with smaller strands.

3.2 Introduction

Phenolic compounds are a diverse group of chemicals possessing one or more aromatic rings to which at least one hydroxyl group is attached. Phenolic compounds are produced as secondary metabolites by most plants and probably function as natural antimicrobial agents and inhibitors of pre-harvest seed germination

(O'Connell and Fox, 2001). These compounds generally have an ortho-diphenol (or a 1-hydroxy-2-methoxy) structure (Strauss and Gibson, 2004).

Surimi is a Japanese term which can be defined as washed fish mince. With washing process, myofibrillar proteins, which mainly contribute to gel formation, are concentrated in the resulting surimi (Benjakul *et al.,* 2003). Thailand is one of the largest surimi producers in Southeast-Asia. About 16 surimi factories are located in Thailand, with a total production of 96,500 to 1, 13,500 metric tons per year of which, 80% exported to Japan and Korea and the remainder to Singapore and other countries (Hong and Eong, 2005). In general, lean fish have been used for surimi production in Thailand. Due to the limited fish resources, especially lean fish, dark flesh fish have been paid more attention as a potential alternative raw material for surimi production, due to its high potential for capture and low price (Chaijan *et al.,* 2004). However, problems faced with producing surimi from dark flesh fish, such as mackerel, is the high content of dark muscle associated with high content of lipid and myoglobin. This results in the difficulties in making high quality surimi as evidenced by poor gel forming ability of those species (Chaijan *et al.,* 2004). To increase the gel strength of surimi, various food grade ingredients have been used but the addition of these ingredients poses adverse effects on the surimi gel, particularly off flavour or off color (Rawdkuen and Benjakul, 2008). Bovine plasma protein has been prohibited due to the mad cow disease, while egg white is associated with allergy problems. Therefore, alternative food-grade ingredients are still needed to increase the gel strength of surimi**,** particularly those produced from dark flesh fish.

Naturally derived plant phenolic compounds, especially in the oxidized form, have been shown to be the potential protein cross-linker (Rawel *et al.,* 2002a). 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.* (2002b). 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). Addition of phenolic compounds at very low amount might have no negative effect on color of resulting gel from dark flesh fish surimi, which is generally dark in color. Nevertheless, the

information regarding the effect of phenolic compounds on the gel property of dark flesh fish surimi is very scarce. Thus, the study aimed to investigate the effect of oxidized phenolic compounds, including ferulic acid, tannic acid, catechin and caffeic acid on the properties of mackerel surimi gel.

3.3 Materials and Methods

Chemicals

Ferulic acid (FA), tannic acid (TA) and β-mercaptoethanol (βME) were obtained from Sigma (St. Louis, MO, USA). Caffeic acid (CF) and catechin (CT) were purchased from Fluka (Buchs, Switzerland). Sodium dedocyl sulphate (SDS), *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

Fish sample preparation

Mackerel (*Rastrelliger kanagurta*) with an average weight of 85-90g were caught from Songkhla coast along the Gulf of Thailand during March-April, 2008, stored in ice and off-loaded approximately 36 h after capture. Upon the arrival to the dock in Songkhla, fish were iced 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 immediately washed and drained before using for surimi preparation.

Surimi preparation

Surimi was prepared according to the method of Benjakul and Visessanguan (2003) with slight modifications. Mackerel skin and bones were removed manually and the flesh was minced to uniformity using a mincer with a hole diameter of 5 mm. The mince was then washed with cold water (5^{o}C) at a mince/water ratio of 1:3 (w/w). The mixture was stirred gently for 4 min and washed

mince was filtered with a layer of nylon screen. The washing process was repeated twice. For the third washing, cold 0.5% NaCl solution was used. Finally, the washed mince was subjected to centrifugation using a Model CE 21K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700*xg* for 10 min. To the washed mince, 4% sucrose and 4% sorbitol were added and mixed well. The mixture (500g) was packed in polyethylene bag and frozen using an air-blast freezer (-20^{o}C). The resulting surimi with pH of 6.8 contained 78.41% moisture, 14.0% protein, 0.30% lipid as determined by the method of AOAC (1999). The gel testing was performed within one week during frozen storage.

Effect of oxidized phenolic compounds on the properties of surimi gel

Preparation of oxidized phenolic solutions

Four phenolic compounds including ferulic acid, tannic acid, caffeic acid and catechin were dissolved according to the method of Strauss and Gibson (2004) with slight modifications. Phenolic solution (100 ml; 1% w/v) was adjusted to pH 8 using 6 N NaOH or 6 N HCl. The prepared solution was placed in a temperature-controlled water bath (40^{o}C) and subjected to oxygenation for 1 h by bubbling the solution with oxygen to convert the phenolic compounds to quinone. After being oxygenated for 1 h, the solution was then adjusted to pH 7 by using 6 N HCl and was referred to as ‘oxidized phenolic compound’.

Surimi gel preparation

To prepare the gel, frozen surimi was tempered for 30 min in running water (26-28°C) until the core temperature reached 0-2°C. The surimi was then cut into small pieces with an approximate thickness of 1 cm. The surimi was placed in a mixer (National Model MK-K77, Tokyo, Japan). The moisture content was adjusted to 80% and 2.5% salt was added. Different oxidized phenolic compounds at various concentrations (0%, 0.10%, 0.20%, 0.30%, 0.40%, 0.50% and 0.60% of protein content of surimi) were added. The mixture was chopped for 4 min at 4°C to obtain a homogeneous sol. The sol was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were

sealed tightly. Sols were incubated at 40°C for 30 min, followed by heating at 90°C for 20 min (Benjakul and Visessanguan, 2003). The control gels were prepared by adding distilled water (pH 7) of same volume as that of oxidized phenolic solutions. All gels were cooled in iced water and stored overnight at 4°C prior to analyses.

Texture analysis

Texture analysis of surimi gels was performed using a texture analyser Model TA-XT2 (Stable Micro Systems, Surrey, England). Gels were equilibrated and tested at room temperature. Five cylinder-shaped samples of 2.5 cm in length were prepared. The breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a spherical plunger (5 mm diameter; 60 mm/min plunger speed).

Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al.* (2001b) with slight modifications. Gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between 3 sheets of Whatman paper No. 4 at the bottom and 2 sheets on the top of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The sample was then removed from the papers and weighed again (*Y*). Expressible moisture content was calculated using the following equation:

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

Determination of whiteness

Color of surimi gels was determined using a JP7100F colorimeter (Juki Corporation, Tokyo, Japan). *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by Lanier *et al.* (1991) as follows:

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

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of surimi 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 heated to 85°C were added to the sample (3 g). The mixture was then homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11000 rpm for 2 min. The homogenate was incubated at 85°C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500x*g* for 20 min to remove undissolved debris. The samples (20 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.

Protein determination

Protein concentration was measured by the method of Lowry *et al.,* (1951) using bovine serum albumin as standard.

Sensory evaluation

Mackerel gels containing oxidized phenolic compounds at optimal levels were evaluated for color, taste, texture, odor and overall liking by 30 non-trained panelists, in comparison with the control gel (without oxidized phenolic compound). A nine-point hedonic scale, in which a score of 1= not like very much, 5= neither like nor dislike and 9= like extremely, was used for evaluation (Meilgaard *et al.,* 1990).

Scanning electron microscopy (SEM)

Microstructure of surimi gels was observed using SEM. Gels containing oxidized phenolic compounds and the control gel (without oxidized phenolic compound) with a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed

for1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50%, 70%, 80%, 90% and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

Statistical analysis

Analysis of variance (ANOVA) was performed and the mean comparisons were carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 10.0 for Windows: SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

Effect of oxidized phenolic compounds on the properties of surimi gel

Breaking force and deformation of gels from mackerel surimi added with different oxidized phenolic compounds at various levels are shown in Figure 24. Breaking force and deformation of gels increased as the oxidized phenolic compounds were added up to a particular level ($P < 0.05$). Gels added with 0.40% oxidized ferulic acid (OFA) or 0.50% oxidized caffeic acid (OCF) had the increases in breaking force by 45% or 46.1% and in deformation by 12.2% or 28.1%, respectively, compared with that of the control ($P < 0.05$). For gels added with 0.50% oxidized tannic acid (OTA) or 0.10% oxidized catechin (OCT), breaking force was increased by 115.0% or 70.4% and deformation was increased by 27.5% or 28.4%, respectively. Nevertheless, the continuous decreases in both breaking force and deformation were noticeable when all oxidized phenolic compounds at the greater levels were added ($P < 0.05$). The results revealed that oxidized phenolic compounds at the optimum concentration were effective in increasing gel strength of mackerel surimi. Prigent *et al.* (2003) found that phenolic compounds can interact with proteins via non-covalent interactions and via covalent interactions. Two types of complexation mechanisms

can be distinguished, monodentate and multidentate mechanisms (Haslam, 1989). Multidentate mechanism generally requires a much lower phenolic compound/protein molar ratio and thus a lower concentration of phenolic compound is needed. For "Monodentate" mechanism, a phenolic compound interacts with only one protein site and a higher concentration of phenolic compound is required. OTA, OFA and OCF at higher levels (0.50%, 0.40% and 0.50%, respectively) were required to increase breaking force and deformation of surimi gels, while OCT at a lower level (0.10%) effectively increased both breaking force and deformation of surimi gels. The larger the phenolic compound or the more binding sites the phenolic compound possesses, the stronger the association with proteins is expected (Hagerman *et al.,* 1998). Proanthocyanidin trimers bind more tightly to BSA than proanthocyanidin dimers (Artz *et al.,* 1987). In the present study, the highest breaking force was observed when 0.5% OTA was added ($P < 0.05$). OTA has a greater number of hydroxyl groups attached to the aromatic benzene ring as compared to others. As a consequence, a larger amount of quinone could be formed via oxidation process. Quinones were reported to be able to cross-link the protein molecules (Strauss and Gibson, 2004). The decreased breaking force and deformation of surimi gel with increasing concentrations of phenolic compounds might be associated with self-aggregation of phenolic compounds, leading to the loss in capability of protein cross-linking. De Freitas and Mateus (2001) found that the high concentration of phenolic compounds showed the lower efficiency in interacting with proteins.

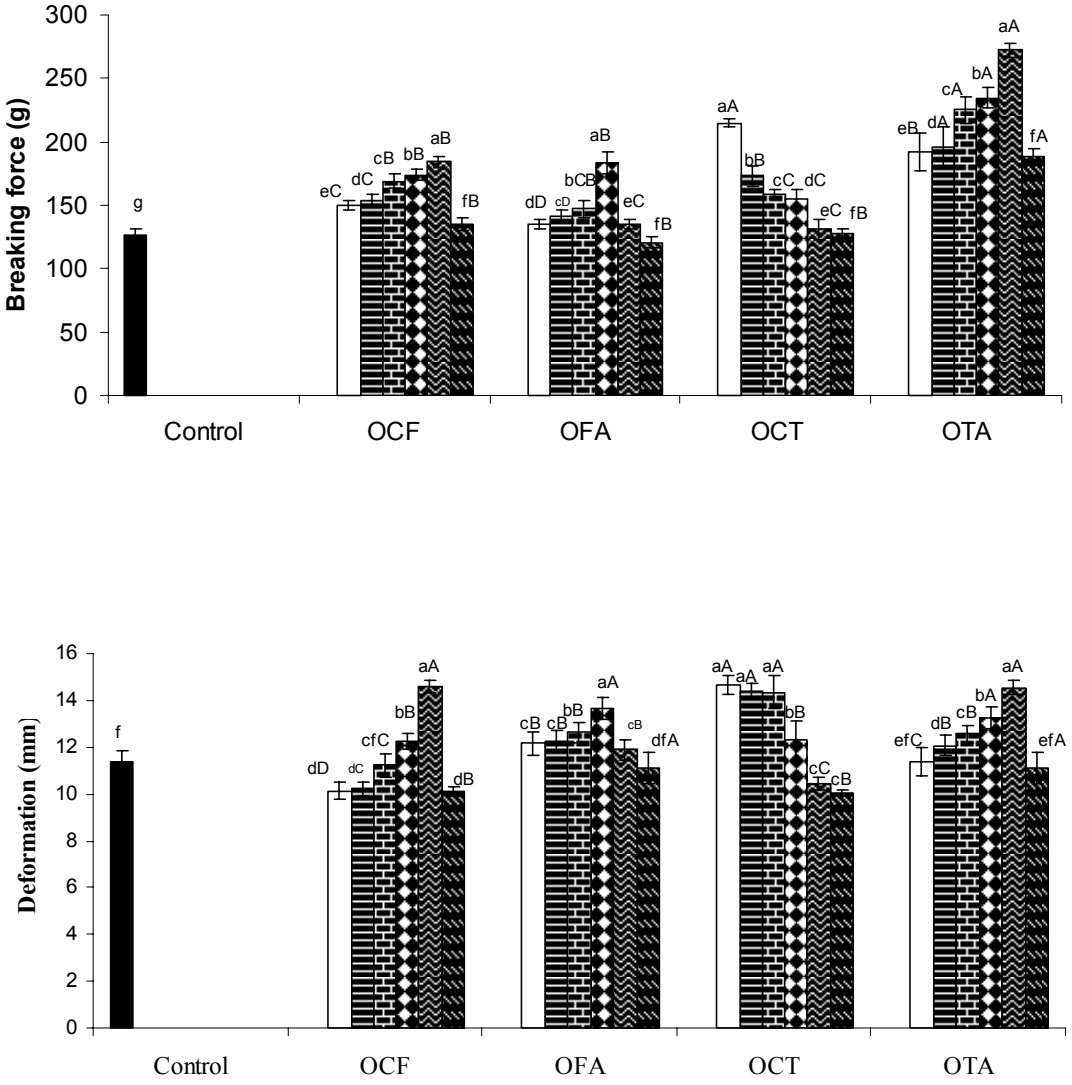


Figure 24. Breaking force and deformation of gels from mackerel surimi added with oxidized phenolic compounds at different levels. OCF, OFA, OCT and OTA represent oxidized caffeic acid, ferulic acid, catechin and tannic acid, respectively. ■, 0%; □, 0.10%; ▤, 0.20%; ▦, 0.30%; ▩, 0.40%; ▨, 0.50%; ▧, 0.60%. Bars represent the standard deviation (n=3). Different letters on the bars within the same oxidized phenolic compounds together with the control indicate the significant differences ($P < 0.05$). The different capital letters on the bars within the same levels of oxidized phenolic compounds indicate the significant differences ($P < 0.05$).

Effect of oxidized phenolic compounds on expressible moisture content of surimi gels

The expressible moisture content of surimi gels added with different oxidized phenolic compounds at various levels is shown in Table 3. When OFA, OCT, OCF or OTA at the optimal levels were added, the expressible moisture content of mackerel surimi gels significantly decreased as compared to that of control ($P < 0.05$). The lowest expressible moisture content was observed in gels added with 0.50% OTA. This indicated that water-holding capacity of surimi gels could be improved with the addition of phenolic compounds at optimal levels. During setting at 40^{o}C, proteins underwent some denaturation and aligned themselves gradually to form the network, which can imbibe water (Benjakul and Visessanguan, 2003). When the optimal level of oxidized phenolic compound was added, the cross-linking of proteins could be enhanced, resulting in the formation of stronger network with a greater water holding capacity. In general, water holding capacity of gel added with oxidized phenolic compounds varied with the type of phenolics.

Effect of oxidized phenolic compounds on whiteness of surimi gels

No changes in whiteness were observed in surimi gels added with all four oxidized phenolic compounds at optimal levels, compared with the control ($P > 0.05$). When oxidized phenolic compounds at a level of 0.6% was added, all gels except that added with OFA had the decrease in whiteness ($P < 0.05$) (Table 3). Phenolic compounds were responsible for discoloration in cheese products (O'Connell and Fox, 2001). However, mackerel fish has high content of dark muscle associated with high content of myoglobin. This results in natural dark color of mackerel surimi (Chen, 2002). Thus, oxidized phenolic compounds at optimum level could be used in mackerel surimi to improve the gel strength without any effect on the whiteness of surimi gel.

Table 3. Expressible moisture content and whiteness of gels from mackerel surimi added with various oxidized phenolic compounds at different levels.

Oxidized phenolic compounds	Amount added (%)	Expressible moisture content (%)	Whiteness
Control	-	17.25 ± 1.69a*	65.19 ± 1.12a*
OCF	0.1	12.43±0.22cA**	65.12±0.47aA**
	0.2	12.03±0.68cA	65.10±0.92aA
	0.3	9.79±0.28dA	65.08±0.57aA
	0.4	8.81±1.05eB	65.05±0.59aA
	0.5	7.12±0.76fC	65.00±0.23aA
	0.6	14.23±0.21bA	64.76±0.66bB
OFA	0.1	11.76±0.31cB	65.20±0.48aA
	0.2	8.16±1.06dB	65.18±0.37aA
	0.3	7.99±0.63edC	65.15±0.27aA
	0.4	7.76±0.92eB	65.15±0.35aA
	0.5	8.33±0.32dB	65.10±0.72aA
	0.6	12.32±0.31bB	65.00±0.12aA
OCT	0.1	7.10±0.74eD	65.16±0.54aA
	0.2	7.63±0.47eC	65.16±0.63aA
	0.3	8.44±0.75dB	65.12±0.70aA
	0.4	9.16±1.06cA	65.04±0.45aA
	0.5	9.80±0.50cA	65.01±0.67aA
	0.6	10.79±0.99bC	64.86±0.26bB
OTA	0.1	8.46±0.20cC	65.14±0.54aA
	0.2	4.59±0.91dD	65.13±0.22aA
	0.3	3.67±0.51eD	65.04±0.47aA
	0.4	3.54±0.22eD	65.03±0.32aA
	0.5	3.26±1.00eD	65.00±0.97aA
	0.6	10.38±0.21bC	64.73±0.21bB

* Different letters in the same column within the same oxidized phenolic compound together with the control indicate the significant differences ($P < 0.05$). ** Different capital letters in the same coloumn within the same level of oxidized phenolic compounds used indicate the significant differences ($P < 0.05$).
Values are mean ± standard deviation (n=3).

Effect of oxidized phenolic compounds on protein patterns of surimi gels

Protein patterns of surimi gels without and with the addition of different oxidized phenolic compounds at the optimum level yielding the highest breaking force and deformation are depicted in Figure 25. Surimi paste contained MHC and actin as the major proteins. Decrease in MHC band intensity was found in the control gel (without addition of oxidized phenolic compounds), when compared with that observed in sol. The result suggested that the formation of cross-linking stabilized by non-disulphide covalent bond took place, especially during setting. MHC was most susceptible to cross-linking during setting (Benjakul and Visessanguan, 2003). Benjakul and Visessanguan (2003) reported the decrease in MHC of surimi gel from bigeye snapper, particularly when the setting was implemented. No MHC band was remained in gels added with 0.50% OCF and 0.40% OFA. For gels added with 0.10% OCT or 0.5% OTA, MHC band disappeared almost completely. The result suggested that MHC was cross-linked by oxidized phenolic compounds to high extent via non-disulphide covalent bond. Coincidental occurrence of polymerised proteins in the stacking gel was noticeable. No marked changes in actin band intensity were observed between the control gel and those added with 0.10% OCT or 0.50% OTA. However, lower band intensity of actin was found in gels added with 0.5% OCF or 0.4% OFA. Due to the slight differences in protein patterns among gels added with different oxidized phenolic compounds, the protein molecules might be cross-linked differently in term of degree and site/domains of interaction induced by oxidized phenolic compounds. Additionally, dark flesh fish were reported to possess high autolytic activity (Shimizu *et al.,* 1992), which is associated with the poor gel properties. Kroll *et al.* (2003) reported that the interactions between phenolic compounds and proteins may lead to a decrease of protein digestibility, by blocking the substrate and/or inhibiting certain proteases. Covalent modification of proteins by phenolic oxidation products generated at alkaline pH was reported extensively (Rawel *et al.,* 2002a). It was postulated that oxidized phenolic compounds might partially lower the proteolysis caused by endogenous proteinases. Cross-linked proteins were more likely less susceptible to proteolysis. This might be associated with gel strengthening in addition to enhanced protein cross-linking.

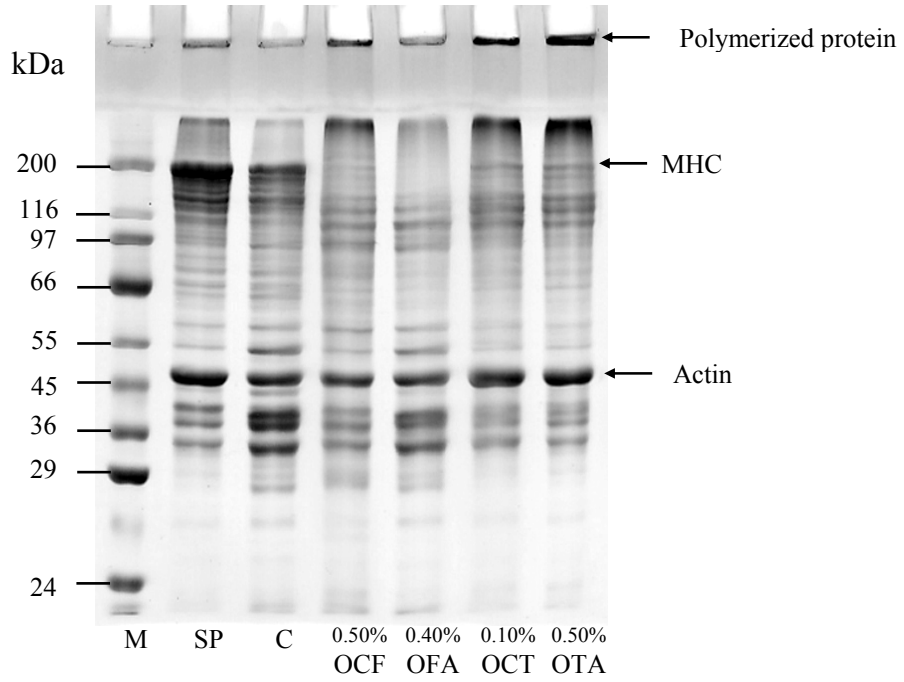


Figure 25. SDS-PAGE patterns of protein in gels from mackerel surimi added with various oxidized phenolic compounds at the selected levels. MHC, myosin heavy chain; M, marker; SP, surimi paste; C, control (without oxidized phenolic addition); OCF, oxidized caffeic acid; OFA, oxidized ferulic acid; OCT, oxidized catechin; OTA, oxidized tannic acid

Effect of oxidized phenolic compounds on sensory properties of surimi gel

Likeness scores of the control gels (without oxidized phenolic compounds) and those containing oxidized phenolic compounds at selected levels are depicted in Figure 26. Addition of oxidized phenolic compounds had no negative impact on the color and taste of resulting gels. The addition of oxidized phenolic compounds at selected levels yielded the gel with the higher texture score, compared with that of control ($P < 0.05$). This was coincidental with the increased breaking force and deformation in the surimi gels added with oxidized phenolic compounds (Figure 24). Among surimi gels added with different phenolic compounds, that containing 0.5% OTA had the highest texture score ($P < 0.05$). However, no differences in texture score were found between the gels added with 0.40% OFA, 0.10% OCT and 0.50% OCF ($P > 0.05$). Phenolic compounds play a major role in

the sensory attributes of many food products (O'Connell and Fox, 2001). The role of phenolic compounds in astringency has been established and is thought to be associated with the precipitation of salivary glycoproteins and mucopolysaccharides onto the tongue, resulting in the development of a feeling of constriction, roughness and dryness on the palate (Haslam and Lilley, 1988). The addition of tea-extracts containing a high proportion of polyphenols to sherbet mixes, yoghurt and acidified milk drinks was reported (Ioki and Suzuki, 1992). Addition of 0.10% OCT or 0.50% OCF had no effect on taste score ($P > 0.05$). Furthermore, gels added with 0.40% OFA or 0.50% OTA showed the highest taste score ($P < 0.05$). For the color score, no differences were found between the control gel and that containing 0.50% OTA ($P > 0.05$). Moreover, the addition of 0.40% OFA, 0.10% OCT or 0.50% OCF yielded the gel with increased color score ($P < 0.05$). The results suggested that the addition of oxidized phenolic compounds at very low levels had no adverse effect on color of resulting gel. Among all gel samples added with oxidized phenolic compounds, those containing 0.5% OTA had the highest overall likeness score, followed by those added with 0.40% OFA or 0.50% OCF. Gel added with 0.1% OCT exhibited similar overall likeness score to the control gel ($P > 0.05$). No differences in odor score were found between the control gel and those added with the oxidized phenolic compounds ($P > 0.05$) (data not shown). Therefore, the addition of oxidized phenolic compounds at low concentration in this study did not negatively affect the sensory properties of mackerel surimi gel.

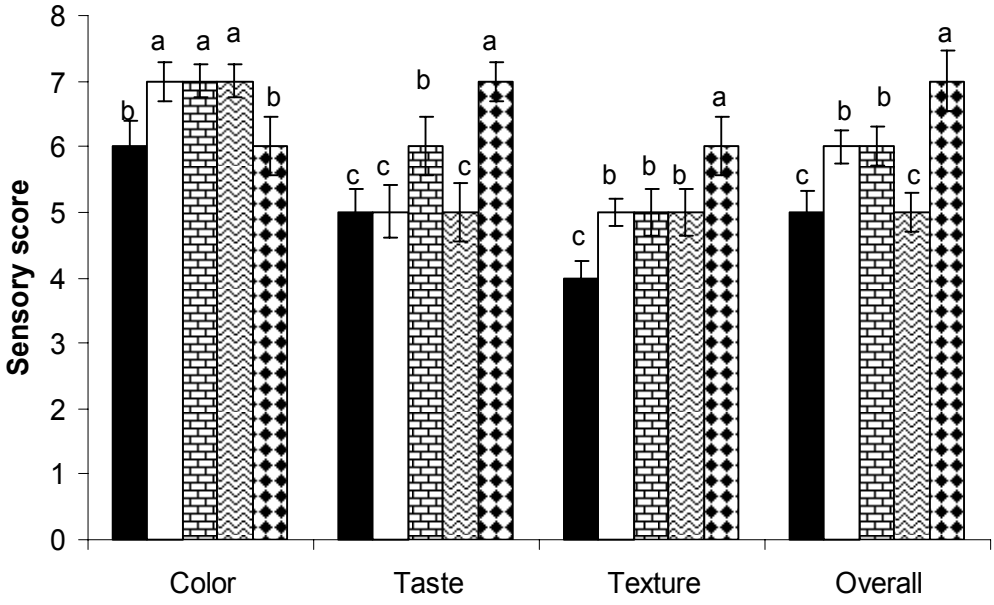
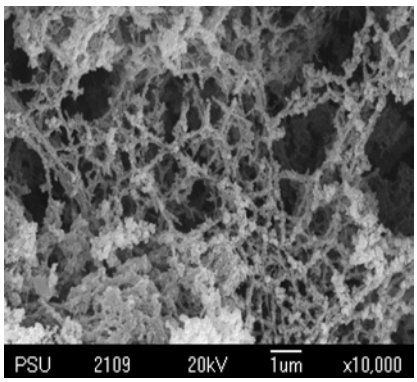


Figure 26. Likeness score of surimi gels from mackerel added without and with different oxidized phenolic compounds at selected levels. C, control (without oxidized phenolic addition); OCF, oxidized caffeic acid; OFA, oxidized ferulic acid; OCT, oxidized catechin; OTA, oxidized tannic acid**.** ■, Control;□, 0.50% OCF, 0.40% OFA;, 0.10% OCT;, 0.50% OTA. Bars represent standard deviation (n=30). Different letters on the bars within the same sensory attribute indicate significant differences (P <0.05).

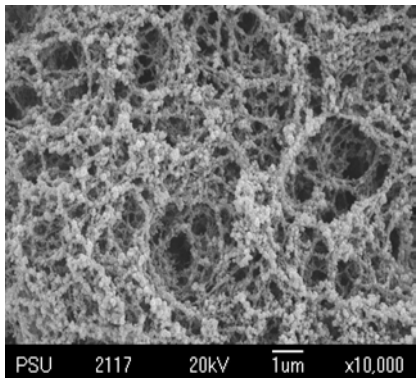
Effect of oxidized phenolic compounds on microstructure of surimi gels

Microstructures of control (without oxidized phenolic compounds) gels from mackerel surimi (A), with 0.50% OCF (B), 0.40% OFA (C), 0.10% OCT (D) and 0.50% OTA (E) are illustrated in Figure 27. Surimi gels containing all oxidized phenolic compounds had finer and continuous matrix than the control. This suggested that oxidized phenolic compounds might induce the cross-linking of protein, in which the protein filaments could be formed. Those filaments further underwent polymerization effectively, leading to gel network with fibrillar structure. Among four oxidized phenolic compounds, the gel added with 0.50% OTA possessed more

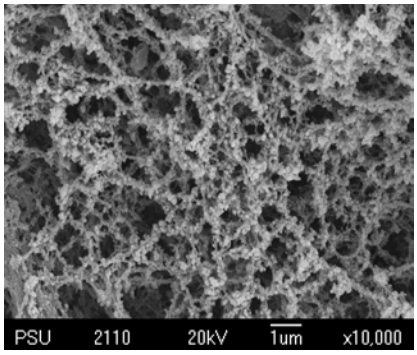
ordered fibrillar structure with finer strands and high capacity of imbibing the water. This might be attributed to the greater number of binding sites in OTA, which in turn caused a higher aggregation. The finer and more ordered structure of OTA added gel correlated with the highest breaking force and deformation (Figure 24) as well as the lowest expressible moisture content (Table 3).



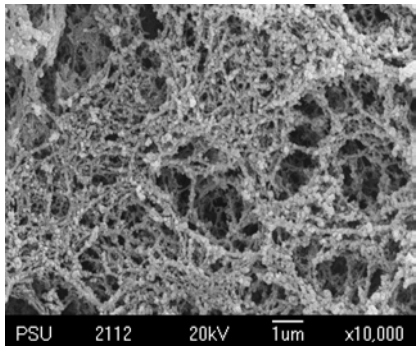
A) Control



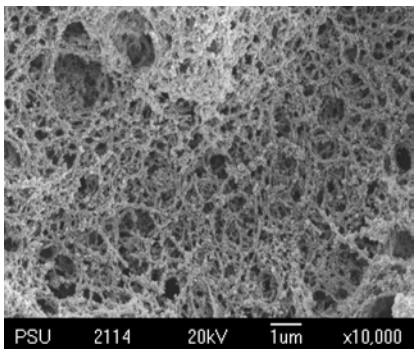
B) 0.50% OCF



C) 0.40% OFA



D) 0.10% OCT



E) 0.50% OTA

Figure 27. Electron microscopic images of gels from mackerel surimi added without and with different oxidized phenolic compounds at the selected levels. OCF, oxidized caffeic acid; OFA, oxidized ferulic acid; OCT, oxidized catechin; OTA, oxidized tannic acid (Magnification: 10,000X).

3.5 Conclusions

Type and concentration of oxidized phenolic compounds had varying influence on mackerel surimi gels. OTA, at a level of 0.5%, exhibited gel strengthening effect without affecting the whiteness of mackerel surimi gels. Therefore, the use of oxidized phenolic compounds can be a suitable option to improve the gel strength of surimi manufactured from dark flesh fish.

CHAPTER 4

EFFECT OF OXIDIZED TANNIC ACID ON THE GEL PROPERTIES OF MACKEREL (*RASTRELLIGER KANAGURTA*) MINCE AND SURIMI PREPARED BY DIFFERENT WASHING PROCESSES

4.1 Abstract

Effect of oxidized tannic acid (OTA) at different levels (0, 0.25, 0.50 and 0.75% of protein content) on the gel properties of mackerel (*Rastrelliger kanagurta*) mince and surimi prepared by different washing processes was investigated. Breaking force and deformation of gels varied with washing processes and concentrations of OTA. The gel of alkaline-saline washing process surimi (ASWPS) added with 0.25% OTA had the increases in breaking force and deformation by 166.2 and 45.9%, respectively, compared with that of conventional washing process surimi (CWPS) without OTA addition. Those increases were associated with the lowered expressible moisture content. Electrophoretic studies revealed that the greater polymerization was found in ASWPS added with 0.25% OTA. Slight retention of myosin heavy chain (MHC) with lowered trichloroacetic acid (TCA) soluble peptide contents were observed in ASWPS gel added with 0.25% OTA, suggesting the decreased degradation induced by indigenous proteases. The microstructure of ASWPS gels became more ordered, compact and denser with the addition of 0.25% OTA. The use of OTA in conjunction with alkaline-saline washing process could improve the properties of gel from mackerel surimi without adverse effect on sensory properties.

4.2 Introduction

Surimi technology has been widely developed to improve the gelling properties of fish mince. Basically surimi is produced by repeated washing the mechanically separated fish mince with chilled water (5-10°C) until most of the water-soluble protein is removed. The washing procedure is of great importance for surimi quality, not only for removing fat and undesirable materials, such as blood, pigments and odorous substances but also for increasing the concentration of myofibrillar proteins, the major proteins contributing to gel formation (Chaijan *et al.,* 2004). In general, lean fish have been used for surimi production worldwide. Due to the limited fish resources, dark muscle fish have been paid more attention as a potential alternative raw material for surimi production. However, problem faced with producing surimi from those dark fleshed species, such as mackerel (*Rastrelliger kanagurta*), is the high content of dark muscle associated with high content of lipid and myoglobin, resulting in the difficulties in making high quality surimi (Chen, 2002). Due to the lowered pH of dark fleshed fish during postmortem handling or storage, the gel forming ability decreases gradually. To alleviate this problem, alkaline leaching has been developed to raise the pH of muscle and to increase the efficacy in removing sarcoplasmic proteins, lipid, pigments, etc. (Shimizu, 1965). Shimizu (1965) also reported that surimi produced by alkaline leaching exhibited higher breaking force and deformation, when compared with surimi produced by conventional method.

To enhance the gel strength of surimi or fish mince, various food-grade ingredients and cross-linking enzymes such as microbial transglutaminase have been used (Benjakul *et al.,* 2004c; Benjakul and Visessanguan, 2003; Benjakul, *et al.,* 2004b). However, the addition of some ingredients poses the adverse effects on the surimi gel, particularly on its flavour or color. Addition of the bovine plasma protein has been prohibited due to the mad cow disease, while egg white is associated with allergy problems. Hence, the need of natural additives with an ability of protein cross-linking has been paid increasing attention for the surimi industry.

Polyphenols are the natural compounds which are abundant in the plants. They can be classified into two forms, hydrolysable polyphenols and condensed polyphenol (Shahidi and Naczk, 2004). Tannins, also referred to as tannic acid (TA), belong to the first group and have a structure consisting of a central carbohydrate (glucose) and 10 galloyl groups (Lopes *et al.,* 1999). Red wine, coffee, chocolate, tea, sorghum, spinach and fruits (Bananas, grapes and persimmons) are the different kinds of foods containing tannins (Lopes *et al.,* 1999; Naczk and Shahidi, 2004). Tannins can be used as a food additive with the range of 10 to 400 mg/l, depending on the type of food to which it is added (Chen and Chung, 2000). Tannins contain sufficient hydroxyls and other suitable groups (such as carboxyls) to form strong complexes with the proteins and other macromolecules. The interactions between phenolic compounds and proteins play a very important role in the processing of certain food products. Phenols may be oxidized easily, in an alkaline solution, 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 by reactions of ortho-quinones with proteins has been demonstrated by Strauss and Gibson (2004). Interactions of different phenolic acids and flavonoids with soy proteins were reported by Rawel *et al.* (2002b). Significant increase in the gel strength of bigeye snapper surimi was found when oxidized phenolic compounds were added (Balange and Benjakul, 2009a). Among the all used oxidized phenolic compounds, the oxidized tannic acid (OTA) exhibited the highest gel strengthening effect, compared with oxidized ferulic acid, catechin and caffeic acid. The use of tannic acid in combination with appropriate washing process would be an effective means to fully improve the gel property of surimi from dark fleshed fish. However, no information regarding the effect of tannic acid on the properties of surimi gel of mackerel prepared by different washing processes has been reported. Therefore, this study aimed to investigate the effect of oxidized tannic acid (OTA) as a protein cross-linker on the textural, physical and sensory properties of mackerel (*Rastrelliger kanagurta*) unwashed mince and surimi prepared by conventional washing process (CWPS) and alkaline-saline washing process (ASWPS).

4.3 Materials and Methods

Chemicals

Tannic acid (TA), L-tyrosine and β-mercaptoethanol (βME) were obtained from Sigma (St. Louis, MO, USA). Sodium dedocyl sulphate (SDS), *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium chloride, tricholoroacetic acid and ethanol were obtained from Merck (Darmstadt, Germany).

Fish sample

Mackerel (*Rastrelliger kanagurta*) with an average weight of 85-90 g were caught from Songkla coast along the Gulf of Thailand, stored in ice and off-loaded approximately 36 h after capture. Upon the arrival to the dock in Songkhla province, fish were iced with a fish/ice ratio of 1:2 (w/w) and transported to the laboratory within 1 h. The fish were immediately washed and drained. The flesh was removed manually and used for the preparation of mince and surimi.

Preparation of mince and surimi by different washing processes

Preparation of unwashed mince

Mince was prepared according to the method of Benjakul and Visessanguan (2003) with a slight modification. The flesh was minced to uniformity using a mincer with a hole diameter of 5 mm. This was referred to as “Unwashed mince”. Cryoprotectants (4% sucrose and 4% sorbitol) were added to the unwashed mince and mixed well. Sample (0.5 kg) was packed in polyethylene bag, sealed and frozen using an air-blast freezer at -18 ^{o}C.

Preparation of surimi by conventional and alkaline-saline washing processes

Surimi was prepared by conventional washing process according to the method of Chaijan *et al.* (2004) with a slight modification. Mince was suspended in cold water (5^{o}C) at a mince/water ratio of 1:3 (w/w). The mixture was stirred gently for 4 min and washed mince was filtered with a layer of nylon screen (Material: Nylon 1010, nylon 66, polyamide and polyester fiber (Butterfly, Lao Hah Seng Lee Co., Ltd., Bangkok, Thailand). The washing process was repeated twice. For the third washing, cold 0.5% NaCl solution was used. Finally, the washed mince was subjected to centrifugation using a Model CE 21K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700 x g at 4^{o}C for 10 min. Washed mince was added with cryoprotectants, packaged and frozen as previously described. This was referred to as "Conventionally washed process surimi" (CWPS).

For alkaline-saline washing process, surimi was prepared according to the method of Shimizu (1965) with a slight modification. For first washing, the mince was suspended in cold (5^{o}C) alkaline-salt solution (0.15% NaCl in 0.2% $NaHCO_3$) at a mince/solution ratio of 1:4 (w/w). The mixture was stirred gently for 15 min and washed mince was filtered with a layer of nylon screen. For second washing, only cold water with a mince/solution ratio of 1:3 (w/w) was used. For the third washing, cold 0.5% NaCl solution was used. Finally, the washed mince was subjected to centrifugation using a Model CE 21K basket centrifuge with a speed of 700 x g at 4^{o}C for 10 min. To the washed mince, cryoprotectants were added. The mixed sample was packaged and frozen as mentioned before. This was referred to as "Alkaline-saline washing process surimi" (ASWPS).

The stabilized mince and surimi were stored at -18 ^{o}C not longer than 2 months. All samples were subjected to analysis of protein patterns using SDS-PAGE.

Effect of oxidized tannic acid (OTA) on gel properties of mince and surimi prepared by different washing processes

Preparation of OTA solution

Tannic acid was dissolved in distilled water as per the method of Strauss and Gibson (2004) with slight modification. The solution (100 ml; 1% w/v) was adjusted to pH 8 using 6 N NaOH or 6 N HCl. The prepared solution was placed in a temperature-controlled water bath (40^{o}C) and subjected to oxygenation for 1 h by bubbling the solution with oxygen with the purity of 99.5-100% (TTS Gas Agency, Hat Yai, Songkhla, Thailand) to convert tannic acid to quinone. The solution was adjusted to pH 7 by using 6 N HCl and referred to as 'oxidized tannic acid' (OTA).

Surimi gel preparation

To prepare the gels, frozen mince and surimi prepared by different washing processes, were tempered for 30 min in running water (26-28°C) until the core temperature reached 0-2°C. The mince and surimi was then cut into small pieces with an approximate thickness of 1 cm and placed in a mixer (National Model MK-K77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% sodium chloride was added. OTA at various concentrations (0, 0.25, 0.50 and 0.75% of protein content) was added into the sols. The mixture was chopped for 4 min at 4°C to obtain a homogeneous sol. The sol was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Sols were incubated at 40°C for 30 min, followed by heating at 90°C for 20 min (Benjakul *et al.,* 2003). All gels were cooled in iced water and stored overnight at 4°C prior to analyses.

Measurement of gel properties

Texture analysis

Texture analysis of gels was performed using a texture analyser Model TA-XT2 (Stable Micro Systems, Surrey, England) according to the method of Benjakul *et al.* (2003). Gels were equilibrated and tested at room temperature. Five

cylinder-shaped samples of 2.5 cm in length were prepared. The breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a spherical plunger (5 mm diameter). The probe was pressed into the cut surface of a gel specimen perpendicularly at a constant depression speed (60 $mm.min^{-1}$) until the puncture occurred. The force in gram (g) required to puncture into the gel (breaking force) and the distance (in mm) at which the ball probe punctured into the gel (deformation) were recorded.

Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al.* (2001b) with a slight modification. Gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between 3 pieces of Whatman paper No. 4 at the bottom and 2 pieces on the top of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The samples were then removed from the papers and weighed again (*Y*). Expressible moisture content was calculated using the following equation:

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

Determination of whiteness

Color of gels was determined in triplicate using a JP7100F colorimeter (Juki Corporation, Tokyo, Japan). CIE *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by Lanier *et al.* (1991) as follows:

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

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of gels were analysed under reducing condition, in comparision with unwashed mince and surimi prepared by different washing processes by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85°C were added to the sample (3 g). The mixture was then homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11000 rpm for 2 min. The

homogenate was incubated at 85°C for 1 h to dissolve total proteins. The samples were centrifuged at 3500 x *g* for 20 min to remove undissolved debris. Protein concentration of the supernatant was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard. The sample was then 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 1:1 ratio (v/v). The samples (20 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.

Solubility determination

Solubility of protein in surimi gel was determined as described by Benjakul *et al.* (2001b). Finely chopped gel sample (1 g) was solubilized with various solvents including 0.6 M KCl (S1); 20 mM Tris–HCl, pH 8.0 (S2); 20 mM Tris–HCl, pH 8.0 containing 1% SDS (S3); 20 mM Tris–HCl, pH 8.0 containing 1% SDS and 8 M urea (S4) and 20 mM Tris–HCl, pH 8.0 containing 1% SDS, 2% β-mercaptoethanol and 8 M urea (S5). The mixture was homogenized for 1 min, boiled for 2 min and stirred for 4 h at room temperature (28–30 °C) using a magnetic stirrer (IKA-Werke, Staufen, Germany). The mixture was centrifuged at 10000 × *g* for 30 min at 25 °C using a centrifuge (Sorvall Model RC-B plus, Newtown, CT, USA). Two ml of 50% (w/v) cold trichloroacetic acid (TCA) were added to 10 ml of supernatant. The mixture was kept at 4 °C for 18 h prior to centrifugation at 10000 × *g* for 20 min. The precipitate was washed with 10% (w/v) TCA, followed by solubilising in 0.5 M NaOH. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940). Solubility of protein in surimi samples was expressed as the percentage of total protein in surimi. To completely solubilise the total proteins, gels were solubilized directly in 0.5 M NaOH.

Determination of TCA-soluble peptides

To 2 g of finely chopped gel samples, 18 ml of 5% TCA were added and the mixture was homogenized at a speed of 11000 rpm for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was incubated at 4°C for 1 h and centrifuged at 8000 x g for 5 min (25°C) using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). TCA-soluble peptide content in the supernatant was measured according to the Lowry method (Lowry *et al.,* 1951) and expressed as mmol tyrosine/g sample.

Scanning electron microscopy (SEM)

Microstructure of gels from unwashed mince, CWPS and ASWPS without and with OTA at the optimum level was determined using SEM. Samples with a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

Sensory evaluation

ASWPS gels without and with 0.25% OTA were evaluated for color, appearance, odor, taste, texture and overall liking by 30 non-trained panelists. A nine-point hedonic scale, in which a score of 1= not like very much, 5= neither like nor dislike and 9= like extremely, was used for sensory evaluation (Meilgaard *et al.,* 1990).

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range tests (Steel and Torrie, 1980). To

compare the differences in sensorial data, T-test was used. Analysis was performed using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA).

4.4 Results and Discussion

Protein patterns of unwashed mince and surimi prepared by different washing processes

Differences in protein patterns were noticeable between unwashed mince and surimi prepared from different washing processes (Figure 28). The lowest myosin heavy chain (MHC) and actin band intensity was found in unwashed mince. Nevertheless, the higher band intensity of proteins with molecular weight of 39, 37 and 32 kDa was observed in unwashed mince. Higher MHC and actin band intensity was noticeable in ASWPS, compared with CWPS. The increase in MHC band intensity in ASWPS must be attributed to the effective removal of interfering and low molecular weight components, especially sarcoplasmic proteins. Protein with the molecular weight of 55 kDa almost disappeared in ASWPS and the lower amount of this protein was retained in CWPS, in comparison with that found in unwashed mince. For proteins with the molecular weight ranging from 55 to 120 kDa, slightly lower band intensity was observed in ASWPS. Sarcoplasmic proteins are soluble in water and salt solutions of low ionic strength of 0.05 (Govindan, 1985). Solubility of sarcoplasmic proteins of dark fleshed species is increased in “alkaline saline leaching” solution (Shimizu, 1965). Mince from dark flesh fish has lower pH which can cause rapid denaturation of protein. However, after washing with alkaline saline solution, there was slight increase in pH which resulted in decreased rate of denaturation. Furthermore, the addition of salt reulted in the removal of heme pigments. The greater removal of sarcoplasmic proteins from the mince by alkaline-saline washing resulted in the higher concentration of myofibrillar proteins including MHC and actin, compared with typical washing process as evidenced by the larger band intensity of those proteins. After washing, the proteins with MW lower than 35 kDa almost disappeared.

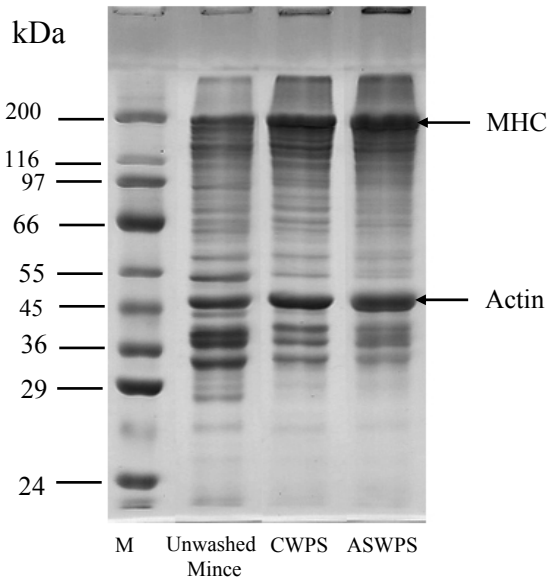


Figure 28. SDS-PAGE patterns of proteins of unwashed mackerel mince and surimi from different washing processes. MHC, myosin heavy chain; M, protein markers; CWPS, conventional washing process surimi; ASWPS, alkaline-saline washing process surimi.

Effect of OTA on breaking force and deformation of gel from unwashed mince and surimi prepared by different washing processes

Different breaking force and deformation were observed between gels from unwashed mince, CWPS and ASWPS ($P < 0.05$) (Figure 29). ASWPS showed the highest breaking force and deformation, followed by CWPS and unwashed mince, respectively. The higher MHC content of ASWPS (Figure 28) might be associated with higher gel forming ability because it has been established that myofibrillar proteins, mainly MHC, contribute to gel formation (Benjakul and Visessanguan, 2003). Therefore, washing process, particularly alkaline-saline washing, played a role in the improvement of gel strength of mackerel surimi. Shimizu *et al.* (1979) reported that Pacific mackerel mince washed by alkaline saline leaching method had the increase in gel strength by 10-fold, compared with unwashed mince. Only a two to

three fold increase in gel strength was found in mince washed by conventional method.

Addition of OTA at various levels resulted in varying breaking force and deformation of resulting gels (Figure 29). For unwashed mince, the addition of 0.25 and 0.5% OTA had no impact on breaking force of resulting gel ($P > 0.05$). OTA might preferably interact with sarcoplasmic proteins instead of myofibrillar proteins. However, the decrease in breaking force was found in unwashed mince gel added with 0.75% OTA ($P < 0.05$). The larger aggregate of sarcoplasmic protein formed, when 0.75% OTA was added, might impede the interaction between myofibrillar proteins. This led to the slight decrease in breaking force of resulting gel. Addition of OTA up to 0.75% had no effect on deformation of the gel from unwashed mince ($P > 0.05$). For CWPS, breaking force increased when OTA was added up to 0.5% ($P < 0.05$). Thereafter, the decrease was found in gel added with 0.75% OTA ($P < 0.05$). The similar result was observed for deformation. CWPS gel added with 0.5% OTA had the increases in breaking force and deformation by 96.7 and 24.8%, respectively, compared with that without OTA addition.

Gels from ASWPS added with 0.25% OTA showed the increases in breaking force and deformation by 166.2 and 45.9%, respectively, compared with those of gel from CWPS without OTA. No differences in breaking force and deformation were noticeable between gels added with 0.25 and 0.5% OTA ($P > 0.05$). The result suggests that OTA addition had the synergistic effect on gel strengthening with alkaline-saline washing process. ASWPS gel added with 0.75% OTA had the lower breaking force than those added with 0.25% or 0.5% OTA ($P < 0.05$) , while gel with 0.75% OTA showed similar deformation to those with lower OTA levels ($P > 0.05$). The decreased breaking force and deformation with increasing concentrations of OTA in the present study might be associated with self-aggregation of phenolic compounds, leading to the loss in capability of protein cross-linking. The lower solubility of large phenolic compounds at high concentration causes the difficulty to interact with proteins (De Freitas and Mateus, 2001). In addition, the size of the phenolic compound can decrease its conformational flexibility, which is observed to be an important parameter in protein-phenolic compound interactions (Frazier *et al.,* 2003).

Alkaline-saline washing process could remove most interfering components including myoglobin, lipid and other impurities more effectively than conventional washing process. This possibly facilitated the better interaction of OTA with MHC. Tannic acid (TA) has a number of hydroxyl groups attached to the aromatic benzene ring which provide more binding sites for proteins (Lopes *et al.,* 1999). Balange and Benjakul (2009a) reported an increased breaking force and deformation of bigeye snapper surimi with the addition of oxidized tannic acid. Therefore, 0.5 and 0.25% were the optimal levels of OTA for CWPS and ASWPS, respectively.

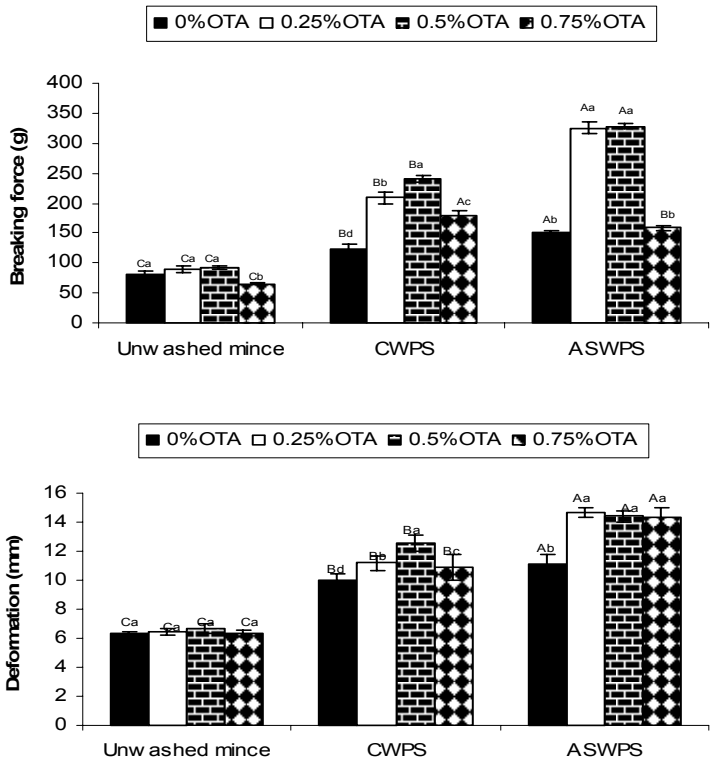


Figure 29. Breaking force and deformation of gels from unwashed mackerel mince and surimi from different washing processes. CWPS and ASWPS represent conventional washing process surimi and alkaline-saline washing process surimi, respectively. Bars represent the standard deviation from three determinations. Different letters on the bars within the same washing treatment indicate the significant differences ($P < 0.05$). The different capital letters on the bars within the same levels of OTA added indicate the significant differences ($P < 0.05$).

Effect of OTA on expressible moisture content of gels from unwashed mince and surimi prepared by different washing processes

For CWPS gel, the lowest expressible moisture content was found with the addition of 0.5% OTA ($P < 0.05$) (Table 4). The lowest expressible moisture content was found in ASWPS gel added with 0.25 or 0.50% OTA ($P < 0.05$). The decreases in expressible moisture contents were in accordance with the increased breaking force and deformation of resulting surimi gels (Figure 29). The higher expressible moisture contents were observed in the gels prepared from unwashed mince, irrespective of the levels of OTA incorporated (Table 4). The result suggested that the formation of stronger network induced by OTA might imbibe more water.

Effect of OTA on whiteness of gels from unwashed mince and surimi prepared by different washing processes

Gels prepared from unwashed mince had the lowest whiteness, compared with those from CWPS and ASWPS (Table 4). Gel from ASWPS had the higher whiteness than that from CWPS ($P < 0.05$). This indicates that alkaline-saline washing process was more effective in removing pigments, especially myoglobin and hemoglobin from the mince than typical washing process. The decreases in whiteness of gels from unwashed mince and ASWPS were observed as OTA levels increased ($P < 0.05$) (Table 4). These results are in agreement with O'Connell and Fox (2001) who reported that phenolic compounds were responsible for darkening in cheese products. For CWPS gels, OTA in the range of 0-0.5% had no impact on the whiteness ($P > 0.05$). However, the addition of 0.75% OTA resulted in the decrease in whiteness ($P < 0.05$). From the result, the addition of OTA caused the decrease in whiteness of resulting gels. Since the plant phenolic compounds naturally have dark color, the addition of those compounds may cause the darkening of the final products.

Table 4. Expressible moisture content and whiteness of gels of unwashed mackerel mince and surimi prepared by different washing processes and added with OTA at different levels.

Samples	OTA amount added (%)	Expressible * moisture content (%)	Whiteness*
Unwashed mince	0	17.43+0.78bA	61.21+0.67aC
	0.25	17.04+0.21bA	60.42+0.21bC
	0.50	17.12+0.62bA	59.15+0.07cC
	0.75	18.02+0.13aA	57.72+0.36dC
CWPS	0	14.67 + 1.09aB	65.87 + 0.58aB
	0.25	4.84+0.76cB	65.69+0.19aA
	0.50	3.35+0.82 dB	65.54+0.44aA
	0.75	12.30+0.73bB	63.22+0.16bA
ASWPS	0	11.14+0.79aC	67.95+0.46aA
	0.25	3.69+0.43cC	63.66+0.46bB
	0.50	3.14+0.27cB	61.36+0.51cB
	0.75	7.62+0.32bC	59.82+0.44dB

*Mean + SD (n=3).

Different letters in the same column within the same sample indicate the significant differences ($P < 0.05$). Different capital letters in the same coloumn within the same level of OTA added indicate the significant differences ($P < 0.05$).

Effect of OTA on protein patterns of gels from unwashed mince and surimi prepared by different washing processes

Protein patterns of gels from unwashed mince, CWPS and ASWPS without and with addition of OTA at the optimum level yielding the highest breaking force and deformation are depicted in Figure 30. For unwashed mince gel, that containing 0.5% OTA, which had non-significant increase in both breaking force and deformation, was also determined for protein pattern. MHC completely disappeared in the control gel samples (without OTA addition). Actin was found to be the dominant protein in the gel, suggesting that actin was more resistant to proteolysis or could not

be polymerised during gelation. The result was in agreement with Benjakul *et al.* (1997) who reported that actin in Pacific whiting muscle was more resistant to proteolysis than MHC. Slight retention of MHC and the increase in actin band intensity were observed in the gels of CWPS and ASWPS added with OTA at the optimum level. The result suggests that OTA might be able to inhibit the degradation of MHC to some extent, as evidenced by the more retained MHC and the lower band intensity of peptides with MW lower than 34 kDa. Benjakul *et al.* (2003) reported that degradation of muscle proteins, especially MHC, in both washed and unwashed lizardfish mince, occurred at temperatures ranging from 60 to 65^{o}C. Thus it was postulated that OTA may protect myofibrillar proteins of surimi by binding with indigenous proteases, leading to the loss in their activity. Polymerized proteins induced by quinone might be more resistant to degradation. Kroll *et al.* (2003) reported that the interactions between phenolic compounds and proteins may result in inhibiting certain proteases.

For gels of CWPS and ASWPS added with optimal OTA level, a large amount of polymerised proteins as appeared on stacking gel was noticeable (Figure 30). It indicated that non-disulphide covalent bonds were formed to a higher extent when OTA was incorporated. Protein cross-links might be more resistant to proteolysis caused by indigenous proteases. Additionally, the cross-links mainly contributed to the increases in gel strength of surimi added with OTA at optimal level. Ou *et al.* (2005) and Cao *et al.* (2007) also reported the polymerisation of protein molecules as a possible subsequent reaction of different proteins with phenolic substances.

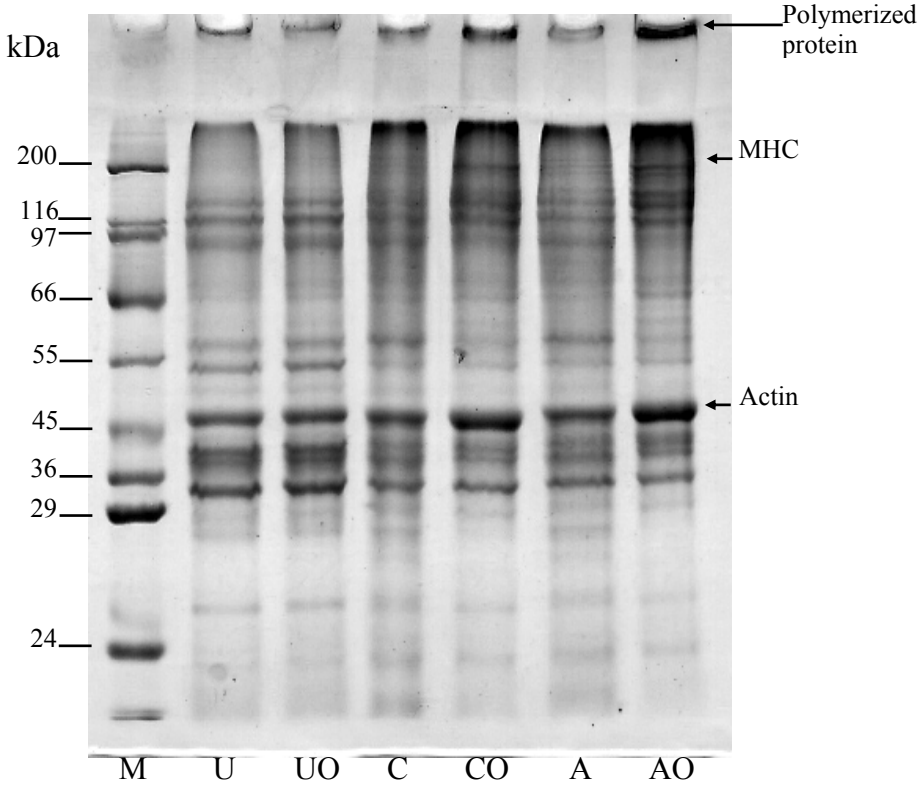


Figure 30. SDS-PAGE patterns of proteins in gels from unwashed mackerel mince and surimi from different washing processes. MHC, myosin heavy chain; M, protein markers; U, unwashed mince gel; UO, unwashed mince gel with 0.50% OTA; C, CWPS gel; CO, CWPS gel with 0.50% OTA ; A, ASWPS gel; AO, ASWPS gel with 0.25% OTA.

Effect of OTA on degradation of gels from unwashed mince and surimi prepared by different washing processes

Protein degradation of gels from unwashed mince, CWPS and ASWPS added without and with OTA at the optimum concentration was monitored as TCA-soluble peptide content (Figure 31). The highest TCA-soluble peptide content was observed in gels of unwashed mince ($P < 0.05$). This result was in accordance with the lowest gel strength in the unwashed mince gel (Figure 29). The degradation occurred during heat-induced gelation is considered to result from the action of indigenous proteinases (An *et al.,* 1994; Visessanguan *et al.,* 2001). ASWPS gel had the lower TCA soluble peptide content than CWPS ($P < 0.05$). In the presence of

OTA, the decreases in TCA soluble peptide content were found in both ASWPS and CWPS gels ($P < 0.05$). It was, therefore, concluded that TCA-soluble peptide formation was slightly inhibited by addition of OTA. This was in agreement with the more retained MHC and actin in gels added with OTA (Figure 30).

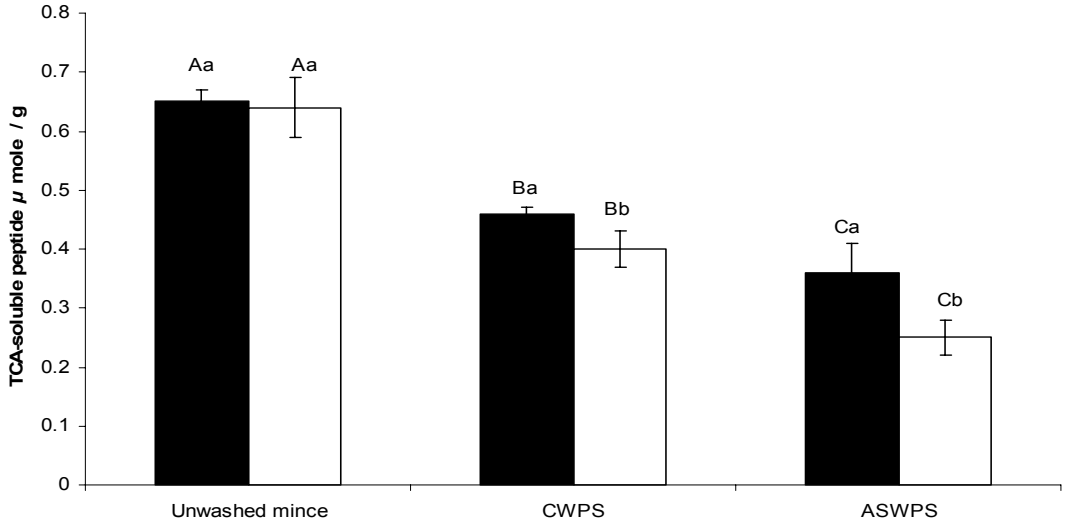


Figure 31. TCA-soluble peptide content of gels of unwashed mackerel mince and surimi from different washing processes added without and with OTA at optimum level. CWPS, conventional washing process surimi; ASWPS, alkaline-saline washing process surimi. Bars represent the standard deviation from three determinations. Different letters on the bars within the same washing treatment indicate the significant differences ($P < 0.05$). The different capital letters on the bars within the same levels of OTA added indicate the significant differences ($P < 0.05$) .■ without OTA; □ with OTA (0.50% for CWPS and unwashed mince; 0.25% for ASWPS)

Effect of OTA on solubility of gels from surimi prepared by different washing processes

Solubility of gels from, ASWPS and CSWP added with OTA at the optimum level in different solubilising solutions is shown in Figure 32. Solubility was found to be lower than 20% in all gels with and without OTA when solubilized with 0.6M KCl (S_1) and 20 mM Tris-HCl (pH 8.0) (S_2). Native myofibrillar proteins are

normally soluble in high ionic strength buffer (Suzuki, 1981). The decrease in solubility suggests the formation of protein aggregates during gelation process. During heating, proteins underwent denaturation and aggregation to form a three dimensional structure (Stone and Stanley, 1992). When the gels were solubilized in 20 mM Tris-HCl (pH 8.0) containing 1% SDS (S_3), solubility was increased up to 34.9 and 31.1% in the control gels of CWPS and ASWPS, respectively, while gels of CWPS and ASWPS added with OTA at the optimum level had the increases in solubility by 29.5% and 27.6%, respectively. SDS is capable of destroying hydrogen and some hydrophobic interactions (Hamada, 1992). Further increases in solubility were observed in S_4, containing urea and SDS, indicating the presence of hydrophobic and hydrogen bonds in surimi gels. This was in accordance with Balange and Benjakul (2009a) who reported an increase in the solubility in S_4 of bigeye snapper surimi gels added with optimum concentration of pheolic compounds, indicating the formation of hydrogen and hydrophobic interactions. CWPS and ASWPS gels added with optimum concentration of OTA also showed the lower solubility in S_4, indicating the formation of covalent bonds in the gels. When the samples were solubilized in S_5, containing urea, SDS and βME, it was noticeable that a higher increase in solubility was observed, indicating the presence of disulphide bonds in the gel. The S-S interchanges and disulphide interchange (SH-SS) between the protein molecules involve in the development of gel structure (Itoh *et al.,* 1979). Quinones might have attached to protein molecules which caused unfolding of protein molecule that facilited the oxidation of sulfhydryl groups, leading to the increase in disulfide bond formation. Gels added with OTA had a slightly lower solubility in S_4, compared with the control gels. This suggests that some non-disulfide covalent bonds were formed in gels added with phenolic compounds. Intermolecular cross-linking of protein molecules could be induced by quinone (Strauss and Gibson, 2004).

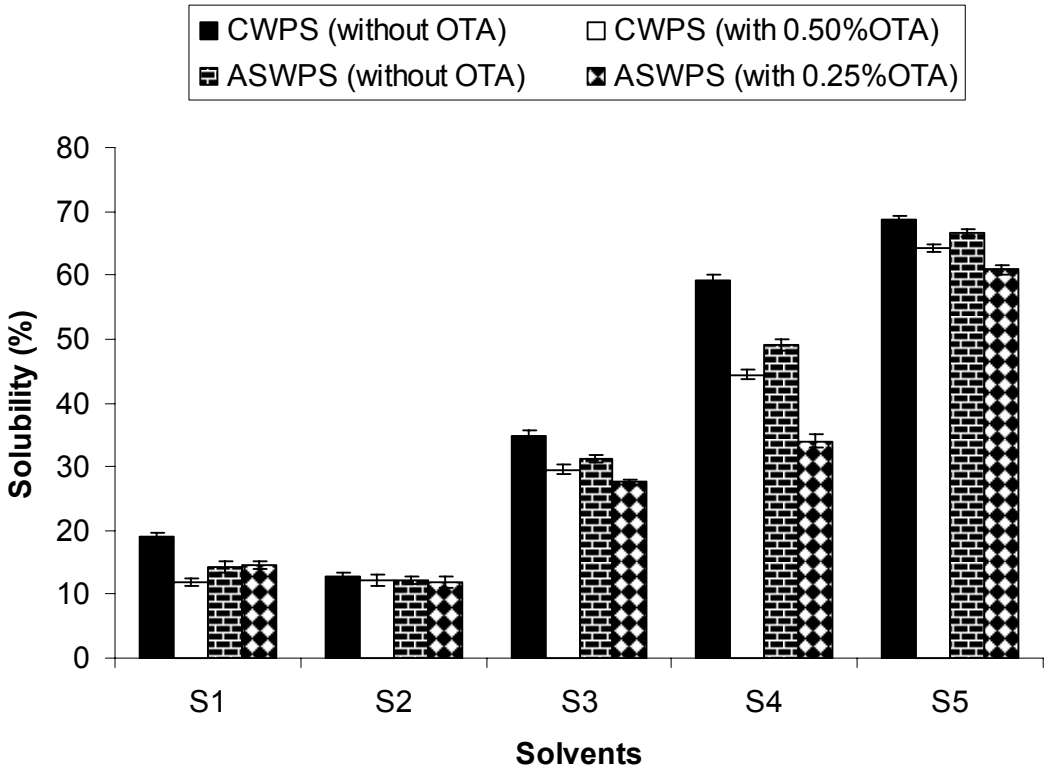
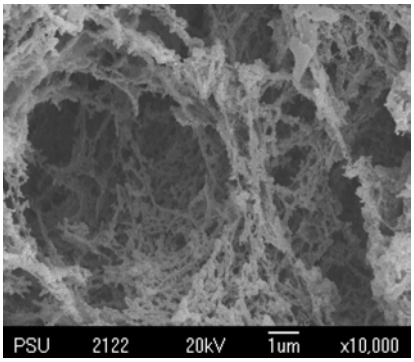


Figure 32. Solubility of mackerel surimi gels from different washing processes added without and with OTA at optimum level. Samples were solubilised in different solvents and soluble protein was determined by the biuret assay. 0.6 M KCl (S1); 20 mM Tris–HCl pH 8.0 (S2); 20 mM Tris–HCl, pH 8.0, containing 1% SDS (S3); 20 mM Tris–HCl, pH 8.0, containing 1% SDS and 8 M urea (S4) and 20 mM Tris–HCl, pH 8.0, containing 1% SDS, 8 M urea and 2% β-mercaptoethanol (S5). CWPS, conventional washing process surimi; ASWPS, alkaline saline washing process surimi. Bars represent the standard deviation from triplicate determinations.

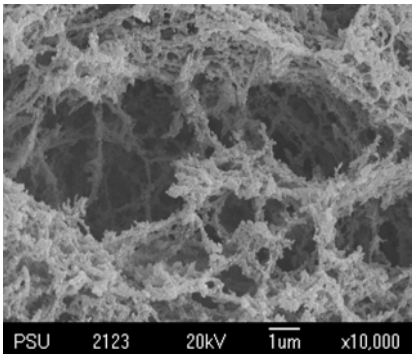
Effect of OTA on microstructure of gels from unwashed mince and surimi prepared by different washing processes

Microstructures of gels from unwashed mince and surimi prepared by different washing processes are shown in Figure 33. The control gel of ASWPS exhibited better gel network with less void as compared to those of CWPS and unwashed mince. This was mainly due to the removal of most interfering components for gelation by alkaline-saline washing process, which resulted in the increased concentration of myofibrillar proteins. Those myofibrillar proteins could undergo the aggregation more effectively in the presence of OTA, which induced the protein cross-linking, to yield the more compact and dense gel network.

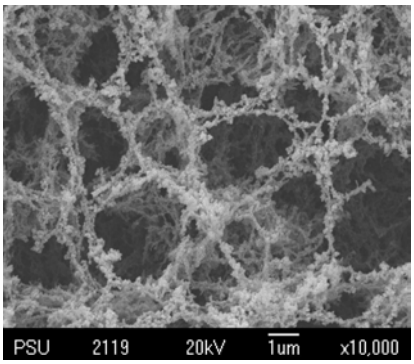
When OTA was incorporated into the gel, the finer structure with the smaller voids was found in all gels, compared with gels without OTA. Very loose network with larger voids was observed in the unwashed mince gels, irrespective of OTA addition. This was coincidental with the lowest gel strength (Figure 29) and highest expressible moisture content (Table 4) obtained in the gel of unwashed mince. Gels of CWPS added with 0.50% OTA exhibited the finer and ordered gel network with smaller voids, when compared with the control gel. The compact and dense gel network with finer strand was observed in the gels of ASWPS when 0.25% OTA was added.



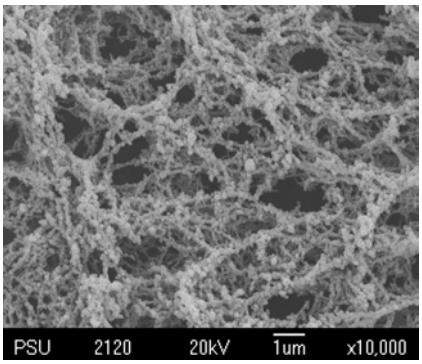
A) Unwashed mince (without OTA)



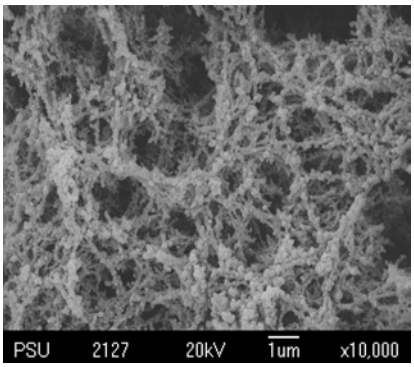
B) Unwashed mince (with 0.50% OTA)



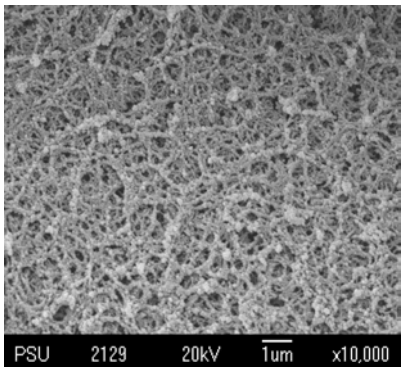
C) CWPS (without OTA)



D) CWPS (with 0.50% OTA)



E) ASWPS (without OTA)



F) ASWPS (with 0.25% OTA)

Figure 33. Electron microscopic images of gels of unwashed mackerel mince and surimi from different washing processes added without and with OTA at optimum level. CWPS, conventional washing process surimi; ASWPS, alkaline-saline washing process surimi. (Magnification: 10,000X).

Effect of OTA on sensory properties of ASWPS gels

Likeness scores of the ASWPS gels without and with 0.25% OTA addition are shown in Table 5. No differences in likeness scores for color, appearance, odor, taste and overall were noticeable between gels without and with 0.25% OTA ($P > 0.05$). Although the addition of 0.25% OTA lowered the whiteness of ASWPS gel (Table 4), it showed no effect on color likeness ($P > 0.05$). For texture likeness, gel containing 0.25% OTA had the higher score than that without OTA ($P < 0.05$). This was coincidental with the increased breaking force and deformation in the ASWPS gels added with 0.25% OTA (Figure 29). These results were in agreement with Balange and Benjakul (2009b), who reported no adverse effect on the sensory properties of conventionally washed mackerel surimi gels added with oxidized phenolic compounds at optimum concentration. Thus, OTA, at optimum level can be used in mackerel surimi to improve the gel strength without causing the negative effect on sensory property.

Table 5. Likeness score of alkaline-saline washing process surimi (ASWPS) gels from mackerel added without and with 0.25% OTA.

Attributes	Likeness score*	
	ASWPS (without OTA)	ASWPS (with 0.25% OTA)
Color	6.96+ 0.12a**	6.96+ 0.13a
Appearance	6.62+ 0.15a	6.50+ 0.14a
Odor	6.48+ 0.12a	6.41+ 0.36a
Taste	7.09+ 1.02a	7.18+ 0.89a
Texture	6.19+ 0.12b	7.28+ 0.95a
Overall	6.67+ 0.15a	6.87+ 0.21a

*Mean + SD (n=30).

**Different letters in the same row indicate significant differences ($P < 0.05$)

4.5 Conclusions

The addition of OTA in the mackerel surimi at optimum level enhanced the interaction between myofibrillar proteins, which was associated with the formation of an ordered gel microstructure with finer strands. Thus, oxidized tannic acid showed the synergistic effect with alkaline washing process in improving the gel properties of mackerel surimi without any adverse effect on sensory properties.

CHAPTER 5

CROSS-LINKING ACTIVITY OF OXIDIZED TANNIC ACID TOWARDS MACKEREL MUSCLE PROTEINS AS AFFECTED BY PROTEIN TYPES AND SETTING TEMPERATURES

5.1 Abstract

Cross-linking activity of oxidized tannic acid (OTA) at different levels (0-0.3% of protein content) towards natural actomyosin (NAM), sarcoplasmic protein (SP) and NAM/SP (65:35) mixture from mackerel (*Rastrelliger kanagurta*) muscle incubated at different temperatures for 30 min was investigated. NAM solution had the increases in turbidity, surface hydrophobicity and disulfide bond contents as OTA added increased up to 0.2%. The higher formation of aggregate of NAM was found when NAM solution containing 0.2% OTA was incubated at 40 ^{o}C, compared with at room temperature (26-28 ^{o}C). The lower aggregation of NAM was noticeable in the presence of SP, which was more preferably cross-linked by OTA via weak bonds. Thus, SP showed the interfering effect on NAM cross-linking induced by OTA. MHC band intensity was decreased and highly ordered dense protein network of NAM were obtained when 0.2 % OTA was incorporated. Conversely, coagulation was formed in NAM/SP mixture added with 0.2% OTA. Thus, the cross-linking efficiency of OTA varied with the type of muscle protein and setting temperature.

5.2 Introduction

Gel-forming ability is one of the most important attributes of fish mince and surimi. It can be affected by both intrinsic and extrinsic factors including species, freshness, endogenous enzymes, additives as well as cooking procedure

(Benjakul *et al.,* 2001a, 2003, 2004b, d). Three major groups of proteins are found in the fish muscles: water-soluble sarcoplasmic proteins (about 30 wt %) consisting of albumins, myoglobin and enzymes; salt-soluble myofibrillar protein (60–70 wt %) containing principally myosin, actin and less amount of tropomyosin and troponin; and insoluble stromal proteins representing 3-10% of total proteins (Suzuki, 1981). Myosin is an important myofibrillar protein, mainly responsible for fish gel formation (Niwa, 1992). On the other hand, sarcoplasmic proteins, which possess a relatively simple globular structure, have poor gelling ability and contribute very little to food texture (Xiong, 1997). Fish mince generally have poorer gel forming ability, compared with washed mince due to the presence of sarcoplasmic proteins, lipid, pigments, etc (Balange and Benjakul, 2009b). To improve the properties of gel from mince and washed mince, many approaches have been implemented. "Setting" is a well known phenomenon in the surimi paste during the incubation at temperatures lower than 40^{o}C. This phenomenon involves network formation of myosin due to the cross-linking induced by endogenous transglutaminase (TGase) (Seki *et al.,* 1990). Microbial transglutaminase (MTGase) has shown potential to increase the gel strength of surimi by inducing non-disulfide covalent bond formation (Benjakul *et al.,* 2008a), whereas protein additives have been widely used to alleviate the softening (modori) induced by endogenous thermostable proteinases (Benjakul *et al.,* 2004d). However, some additives such as bovine plasma protein, etc. have been prohibited due to the safety concern. Additionally, the use of cross-linking enzymes, especially MTGase, is still costly in 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 (Shahidi and Naczk, 2004). The interactions between phenolic compounds and proteins play an essential role in the processing of certain food products. Tannin can be used as a food additive with the range of 10 to 400 mg/l, depending on the type of food to which it is added (Chen and Chung, 2000). Tannin contains sufficient hydroxyls and other groups such as carboxyls to form strong complexes with the proteins and other macromolecules (Kroll *et al.,* 2003). Recently, Balange and Benjakul (2009a, b, c) found the increase in gel strength of bigeye snapper and mackerel surimi with the addition of oxidized tannic acid (OTA). Nevertheless, OTA

had no gel strengthening effect on unwashed mince (Balange and Benjakul, 2009c). OTA may react more effectively with sarcoplasmic proteins in unwashed mince, thereby being less available in cross-linking myosin heavy chain. However, the reactivity of OTA towards fish myofibrillar proteins and sarcoplasmic proteins has not yet been elucidated. Therefore, the objectives of this research were to compare the cross-linking ability of OTA towards natural actomyosin (NAM) and sarcoplasmic protein (SP) from mackerel muscle and to investigate the impact of OTA on physicochemical changes of both proteins at different setting temperatures.

5.3 Materials and Methods

Chemicals / Fish

Potassium chloride and sodium chloride were purchased from Merck (Darmstadt, Germany). Tannic acid, β-mercaptoethanol (βME), 8-anilino-1-naphthalenesulphonic acid (ANS), guanidine thiocyanate, sodium hydrogen sulphite and tris-maleate were procured from Sigma Chemical Co. (St. Louis, MO, USA). 5, 5' –dithio-bis (2-nitrobenzoic acid) (DTNB) was obtained from Wako Pure Chemical Industries (Tokyo, Japan). Sodium dedocylsulfate (SDS), N,N,N′,N′-tetramethyl ethylenediamine (TEMED) and all chemicals for electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Mackerel (*Rastrelliger kanagurta*) with an average weight of 85-90 g were caught from Songkhla coast along the Gulf of Thailand during January-February, 2009, stored in ice and off-loaded approximately 36 h after capture. Upon the arrival to the dock in Songkhla, fish were iced 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 immediately washed and drained. Fish were filleted and the ordinary muscle was collected, minced and used for extraction of natural actomyosin (NAM) and sarcoplasmic protein (SP).

Preparation of natural actomyosin (NAM) and sarcoplasmic protein (SP) from mackerel muscle

Preparation of NAM

NAM was prepared according to the method of Benjakul *et al.,* (1997) with a slight modification. Fish mince (10g) was homogenized in 100 ml of chilled 0.6 M KCl, pH 7.0 for 4 min at a speed of 11000 rpm using a homogenizer (IKA Labortechnik, Selangor, Malaysia). The container with sample was placed in ice. Each 20 sec of homogenization was followed by a 20 sec rest interval to avoid overheating during extraction. The homogenate was centrifuged at 5,000 *x g* for 30 min at 4 °C. Three volumes of chilled water (0-2 °C) were added to precipitate NAM and NAM was then collected by centrifuging at 5,000 *x g* for 20 min at 4 °C using a refrigerated centrifuge (Beckman Coulter Inc., Avanti –J-E Centrifuge, Fullerton, CA, USA). The pellets were then dissolved by gradually stirring in an equal volume of chilled 0.6 M KCl, pH 7.0 for 30 min at 4 °C.

Preparation of SP

SP was prepared according to the method of Benjakul *et al.* (2004a) with a slight modification. Fish mince was homogenized with 2 volumes of extraction buffer (20 mM Tris HCl, pH 7.5) using a homogenizer at a speed of 11,000 rpm for 2 min. The homogenate was centrifuged at 16,000 x *g* for 20 min at 4°C using a Beckman refrigerated centrifuge. The supernatant obtained was further centrifuged at 18,000 x *g* for 60 min at 4 °C. The final supernatant was referred to as "SP; sarcoplasmic protein".

Preparation of oxidized tannic acid (OTA)

Tannic acid was dissolved in distilled water as per the method of Strauss and Gibson (2004) with a slight modification. The solution (100 ml; 1% w/v) was adjusted to pH 8 using 6 M NaOH or 6 M HCl. The prepared solution was placed in a temperature-controlled water bath (40 ^{o}C) and subjected to oxygenation for 1 h by bubbling the solution with oxygen with the purity of 99.5-100% (TTS Gas Agency,

Hat Yai, Songkhla, Thailand) to convert tannic acid to quinone. The solution was then adjusted to pH 7 by using 6 M HCl and referred to as 'oxidized tannic acid' (OTA).

Study on the cross-linking ability of OTA towards mackerel muscle proteins at different temperatures

NAM, SP, and NAM/SP (65:35) mixture were diluted to obtain the protein concentration of 5 mg/ml. NAM (65%) was also prepared by mixing NAM with 20 mM Tris HCl (pH 7.5) at ratio of 65:35 (v/v). To these protein solutions, OTA at various concentrations (0, 0.1, 0.2 and 0.3% of the protein) was added. The resulting mixtures were incubated in a temperature-controlled water bath (Memmert, D-91126, Schwabach, Germany) at 40 ^{o}C or at room temperature (26-28 ^{o}C) for 30 min, followed by cooling in iced water for 30 min. The prepared samples were then subjected to analyses.

Turbidity measurement

Different protein solutions (5 mg protein/ml) were placed in the cuvette and the turbidity was measured by monitoring the absorbance at 660 nm (Benjakul *et al.,* 2001b).

Protein solubility

Different protein solutions with different treatments were centrifuged at 3500 x *g* for 20 min at 4 °C to remove undissolved debris. Protein concentration of the supernatant was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard. Solubility of protein in the samples was expressed as the percentage of protein in the supernatant, relative to that found in the initial solution (5 mg protein /ml).

Surface hydrophobicity

Surface hydrophobicity of all samples with different treatments was determined as described by Benjakul *et al.,* (2001b) using 1-anililonaphthalene-8-sulphonic acid (ANS) as a probe. Different solutions were diluted to obtain the

protein contents of 0.1, 0.2, 0.3 and 0.5 % (w/v) using 10 mM phosphate buffer, pH 6.0 containing 0.6 M NaCl. The diluted protein solution (2 ml) was added with 20 µl of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0. The fluorescence intensity of ANS-conjugates was measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. Blanks were prepared for all samples containing different protein contents by adding 0.1 M phosphate buffer, pH 7.0, instead of ANS solution. Net fluorescence intensity of protein solutions at each concentration was obtained after blank substraction. For each sample, the initial slope of the plot of fluorescence intensity versus protein concentration was referred to as SoANS.

Determination of total sulfhydryl groups and disulfide bond contents

Total sulfhydryl group content was determined using 5, 5'-dithio-bis (2nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul *et al.,* (2001b). To 1.0 ml of sample solutions, 9 ml of 0.2 M Tris-HCl buffer, pH 6.8, 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. Absorbance at 412 nm was then measured. A sample blank of each sample was conducted in the same manner except that distilled water was used instead of DTNB. Sulfhydryl group content was calculated using the extinction coefficient of 13,600 $M^{-1}cm^{-1}$.

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

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of different protein solutions added with OTA at various concentrations and incubated at different temperatures were analyzed under reducing condition by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 9 ml of 5% (w/v) SDS solution heated to 85°C were added to the sample (1 ml). The mixture was incubated at 85°C for 1 h to dissolve total proteins. The samples were centrifuged at 3500 x *g* for 20 min to remove undissolved debris. Protein concentration of the supernatant was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard. The sample was then 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 1:1 ratio (v/v). The samples (20 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.

Transmission electron microscopy (TEM)

Selected protein solutions 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 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) (×80,000) at an accelerating voltage of 160 kV.

Statistical analysis

The experiments were run in triplicate. Analysis of variance (ANOVA) was performed and the mean comparisons were carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using

the Statistical Package for Social Sciences (SPSS 10.0 for windows: SPSS Inc., Chicago, IL, USA).

5.4 Results and Discussion

Turbidity

Changes in turbidity of different protein solutions in the absence and in the presence of OTA at different concentrations after incubation at room temperature (26-28°C) or at 40 ^{o}C for 30 min are shown in Figure 34A and 34B, respectively. After incubation at both temperatures, NAM solution became more turbid, compared with the control (without OTA), as OTA at the level of 0.1 and 0.2% was added ($P < 0.05$). Nevertheless the lower turbidity was found when OTA at a level of 0.3% was incorporated ($P < 0.05$). Increased turbidity of NAM solution indicated the formation of protein aggregate induced by OTA added. Absorbance reading is commonly used to monitor the extent of protein aggregates (Benjakul *et al.,* 1997; Yarnpakdee *et al.,* 2009). At all levels of OTA incorporated, NAM solutions incubated at 40 ^{o}C became more turbid, compared with those incubated at room temperature ($P < 0.05$). In the absence of OTA, the higher turbidity was also found in NAM solution incubated at higher temperature ($P < 0.05$). The results indicated that OTA likely functioned as a protein cross linker, as evidenced by the increase in turbidity, especially when OTA at higher level was used. Multifunctional groups of OTA possessed a higher potential to bind or attach to protein molecules, in which a large protein aggregate could be formed (Balange and Benjakul, 2009a).

When NAM solution was incubated at 40 ^{o}C, hydrophobic domains as well as reactive groups were more exposed and underwent aggregation readily. Unfolding of protein molecules was due to the instability of hydrogen bonds at higher temperature, exposing the greater numbers of hydrophobic portions (Niwa, 1992). As a consequence, the aggregate could be formed via hydrophobic interaction. In the presence of OTA, quinone, an electrophilic group, in OTA was able to interact with the unfolded proteins, mainly via amino group, a nucleophilic counterpart. However, the turbidity of NAM solution at both incubation temperatures decreased with the

addition of 0.3% OTA ($P < 0.05$). This might be associated with self-aggregation of OTA, resulting in the loss in capability of protein cross-linking. These results were in agreement with Balange and Benjakul (2009b) who reported that the lower breaking force of mackerel surimi gel was obtained when the excessive amount of OTA was added.

At both incubation temperatures, SP solution had the higher turbidity when OTA ranging from 0.1 – 0.3 % was added, compared with the control ($P < 0.05$). Nevertheless, at the same incubation temperature, no differences in turbidity were found between SP solution incorporated with OTA at all levels tested ($P > 0.05$). Generally, the absorbance of SP solution incubated at both temperatures was much lower than that of NAM solutions, irrespective of OTA addition. The results suggested that sarcoplasmic protein underwent aggregation to a lower extent than did NAM. Additionally, SP aggregate formed might have the smaller size than NAM aggregate. However, OTA was able to induce cross-linking of SP to some degree as shown by the increased turbidity. Turbidity of SP solution was higher when incubated at 40 ^{o}C. Unfolding of SP incubated at 40 ^{o}C might favor the cross-linking of SP induced by OTA. OTA in the range of 0.1-0.3% had no differences in cross-linking activity as indicated by the similar turbidity of NAM solution. The result suggested that the level of OTA above 0.1% might be excessive for a limited number of reactive groups, mainly amino groups of SP.

For NAM/SP mixture (65:35) incubated at both temperatures, no changes in turbidity were observed when OTA at all levels was added ($P > 0.05$). SP, which had the smaller size, might be more prone to cross-linking induced by OTA. As a result, the cross-linking of NAM in the mixture induced by OTA became lowered. When NAM solution at the same amount (65%) found in NAM/SP mixture was used, the similar result was obtained, compared with NAM solution at higher protein concentration (100%) for both temperatures. This further confirmed the interfering effect of SP on NAM aggregation induced by OTA. The results were in agreement with Balange and Benjakul (2009c) who reported no changes in the gel strength of mackerel mince with the addition of OTA at levels ranging from 0 to 0.75%. Therefore, gel strengthening effect of OTA can be found in surimi rather than mince, which contains a larger amount of sarcoplasmic proteins.

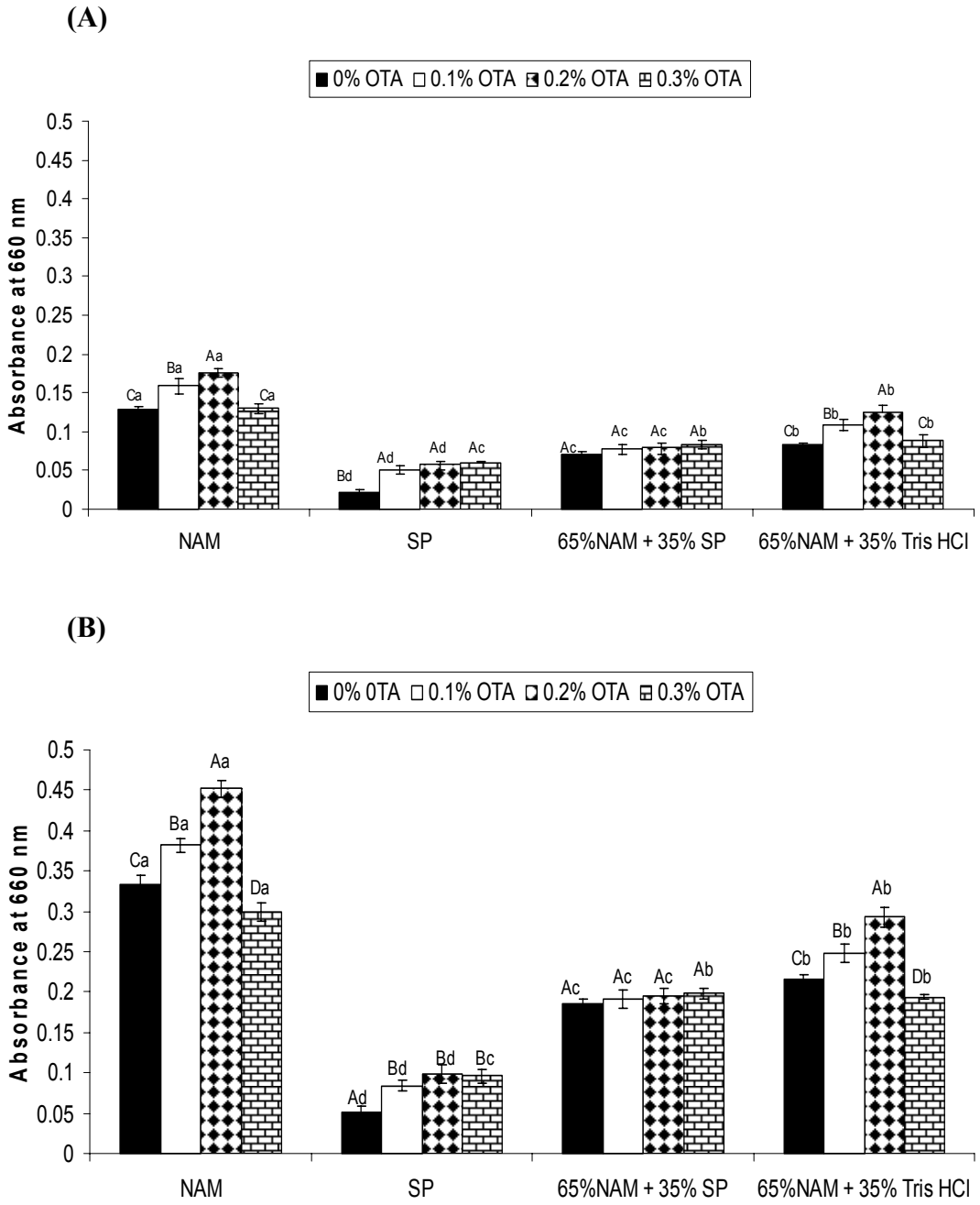


Figure 34. Turbidity of different protein solutions without and with OTA at different concentrations after incubation at room temperature (26–28 ^{o}C) (A) or at 40 ^{o}C (B) for 30 min. NAM, SP and OTA represent natural actomyosin, sarcoplasmic protein and oxidized tannic acid, respectively. Different capital letters on the bars within the same protein solution indicate the significant differences ($P < 0.05$). The different letters on the bars within the same levels of OTA indicate the significant differences ($P < 0.05$).

Protein solubility

Solubility of different protein solutions in the absence and presence of OTA at various levels after incubation at room temperature or at 40 ^{o}C for 30 min is shown in Figure 35A and 35B, respectively. The solubility of NAM solution at both incubation temperatures decreased as the concentration of OTA increased up to 0.2%, compared with that of the control ($P < 0.05$). The decrease in solubility, suggesting the formation of protein aggregates, was in accordance with the increased turbidity (Figure 34). A slight increase in solubility was found when 0.3% OTA was incorporated, compared with that of solution added with 0.1 or 0.2% OTA, regardless of incubation temperatures. During heating, proteins underwent denaturation and aggregation to form a three dimensional structure (Stone and Stanley, 1992). At the same level of OTA used, the lower solubility was observed in solution incubated at 40 ^{o}C, where proteins underwent conformational changes to a higher extent. Hydrophobic interactions might occur between phenolic compounds and hydrophobic amino acids such as alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine residues (Prigent, 2005). Furthermore, the quinones in OTA might induce the formation of strong non-disulfide covalent bonds between NAM molecules. This result was in accordance with the higher turbidity of NAM solution when incubated at 40 ^{o}C, compared with at room temperature (Figure 34).

SP solution incubated at both incubation temperatures with the addition of OTA ranging from 0.1-0.3%, had the lower solubility compared with that of the control ($P < 0.05$). Nevertheless, no differences in solubility were found between samples when OTA at all concentrations range was added ($P > 0.05$). When incubated at 40 ^{o}C, SP solutions had the lower solubility, compared with those incubated at room temperature, regardless of OTA addition. For NAM/SP mixture, no changes in solubility were noticeable when OTA was incorporated, irrespective of OTA levels used ($P > 0.05$). The result was in agreement with that of turbidity, in which OTA addition had no impact on turbidity of NAM/SP mixture. This confirmed that SP most likely exhibited the interfering effect on aggregation of NAM induced by OTA. OTA at all concentrations used in this study showed similar cross-linking

activity towards NAM. The similar result was noticeable between NAM solutions for both concentrations (65 and 100% NAM), in which 0.2% OTA exhibited the highest cross-linking activity towards NAM.

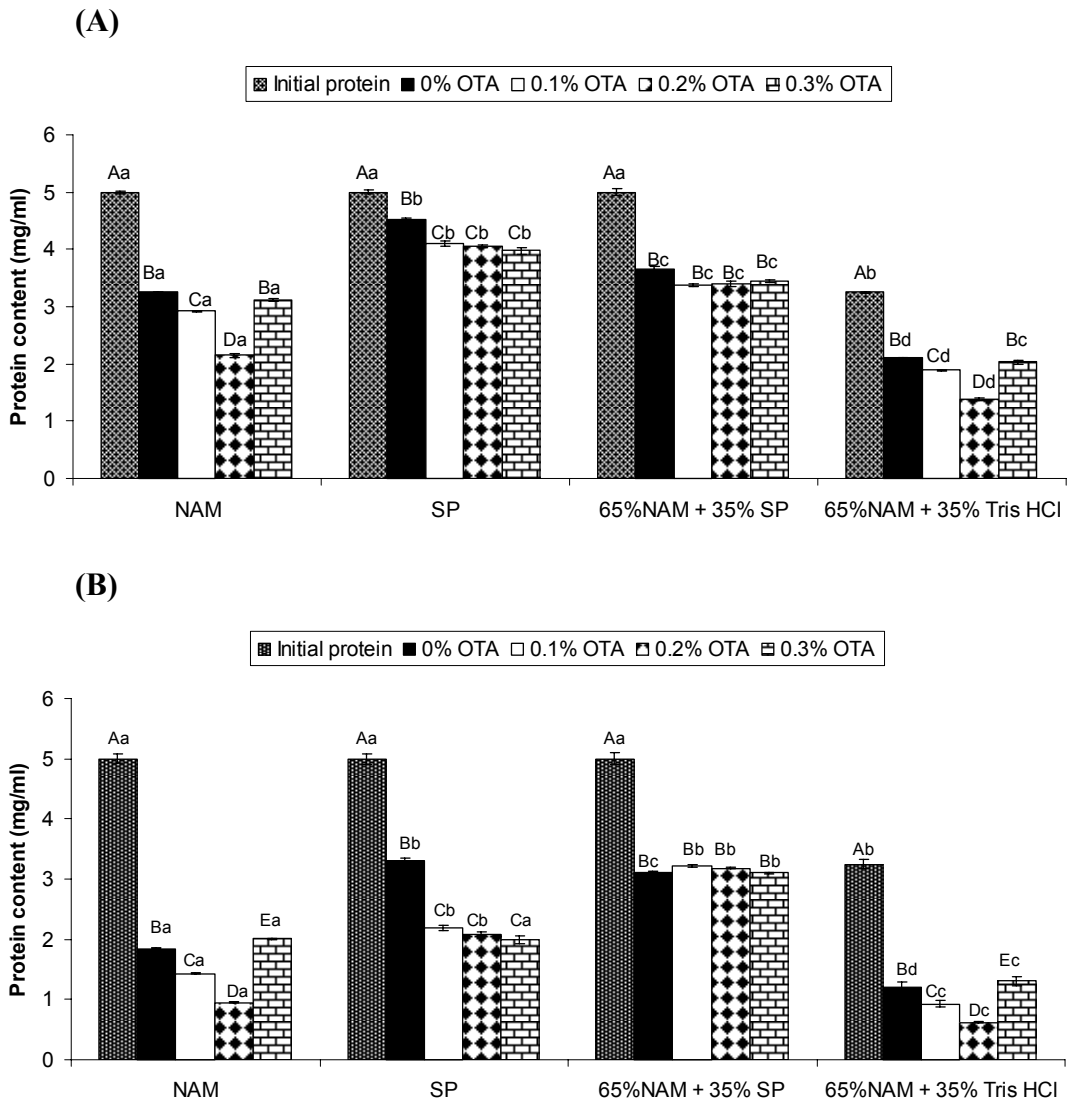


Figure 35. Solubility of different protein solutions without and with OTA at different concentrations after incubation at room temperature (26–28 ^{o}C) (A) or at 40 ^{o}C (B) for 30 min. NAM, SP and OTA represent natural actomyosin, sarcoplasmic protein and oxidized tannic acid, respectively. Different capital letters on the bars within the same protein solution indicate the significant differences ($P < 0.05$). The different letters on the bars within the same levels of OTA indicate the significant differences ($P < 0.05$).

Surface hydrophobicity

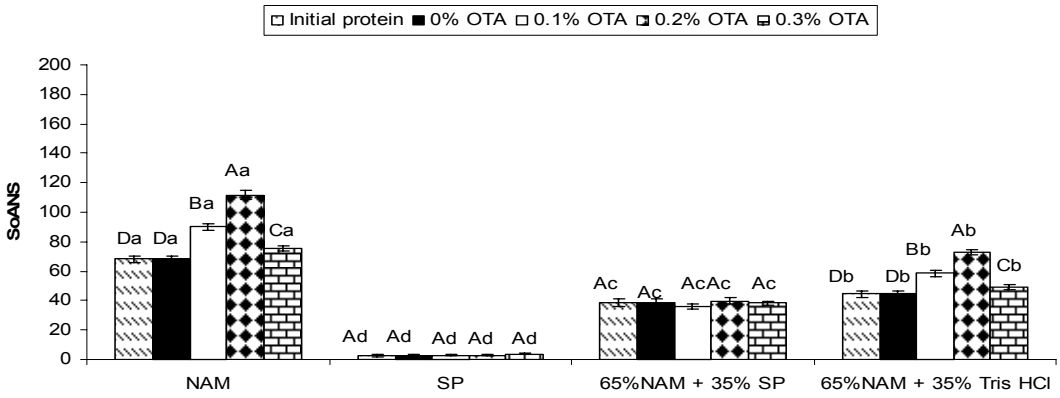
Surface hydrophobicity of different protein solutions added without and with OTA at various levels after incubation at room temperature or at 40 ^{o}C for 30 min is illustrated in Figure 36A and 36B, respectively. At both incubation temperatures, NAM solution had the higher increase in surface hydrophobicity as the concentration of OTA increased up to 0.2% ($P < 0.05$). The results indicated that OTA likely induced the conformational changes of NAM to some extent, as evidenced by the increase in surface hydrophobicity. ANS, a fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as phenylalanine and tryptophan, and can be used to indicate the conformational changes occurring in the proteins (Benjakul *et al.,* 1997). The increase in surface hydrophobicity was possibly associated with the exposure of hydrophobic groups of the protein molecules. When OTA attached to protein molecules, it might cause the alteration of protein conformation to some degree. Nevertheless the slightly lower surface hydrophobicity was found when NAM solution was incorporated with 0.3% OTA and incubated at both temperatures. This was postulated to be due to self-aggregation of OTA at high concentrations, which led to the less efficacy in protein cross-linking. When NAM solution was heated at 40 ^{o}C, more amounts of reactive groups including hydrophobic amino acid became more exposed. In the presence of OTA, the attachment of OTA with NAM could enhance the conformational change as indicated by the higher surface hydrophobicity ($P < 0.05$). Balange and Benjakul (2009a) also found the increase in surface hydrophobicity of bigeye snapper NAM with OTA addition.

SP solution without and with OTA at all levels used had no changes in surface hydrophobicity when incubated at both temperatures (Figure 36). SP is a water soluble protein and most of the hydrophobic amino acids might be localized inside the globular molecules, which might require temperature higher than 40 ^{o}C to open up or unfold the molecules.

For NAM/SP mixture, the addition of OTA had no effect on surface hydrophobicity, regardless of OTA levels ($P > 0.05$). However, higher surface hydrophobicity was noticeable in the solution incubated at higher temperature. OTA

might preferably bind with SP, leading to less availability in binding with NAM. As a result, NAM was less attached or bound with OTA and the alteration in NAM conformation became lessened or negligible. For NAM solution containing lower protein content (65%), the similar result was observed, in comparison with that found in NAM solution at higher protein concentration (100%). This indicated the effect of SP on physicochemical change of NAM, which might be associated with aggregation of NAM.

(A)



(B)

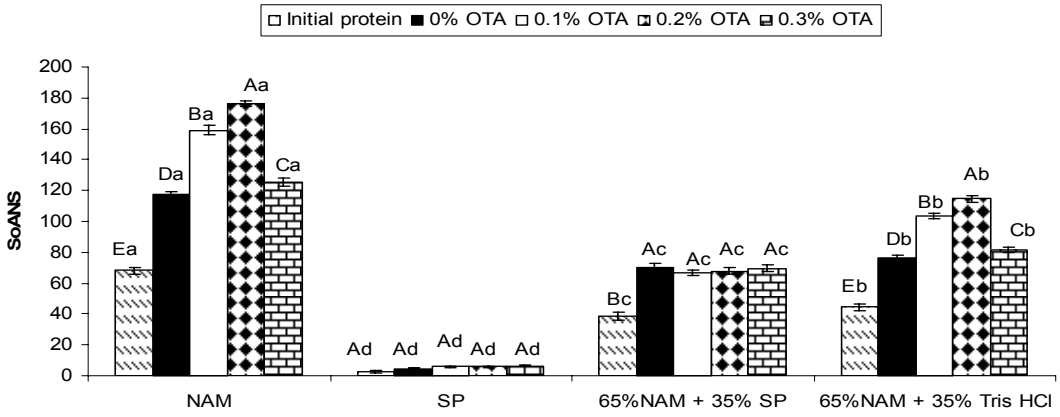


Figure 36.Surface hydrophobicity of different protein solutions without and with OTA at different concentrations after incubation at room temperature (26–28 ^{o}C) (A) or at 40 ^{o}C (B) for 30 min. NAM, SP and OTA represent natural actomyosin, sarcoplasmic protein and oxidized tannic acid, respectively. Different capital letters on the bars within the same protein solution indicate the significant differences ($P < 0.05$). The different letters on the bars within the same levels of OTA indicate the significant differences ($P < 0.05$).

Total sulfhydryl group and disulfide bond contents

Total sulfhydryl group and disulfide bond contents of different protein solutions without and with addition of OTA at different levels after incubation at room temperature or at 40 ^{o}C for 30 min are shown in Table 6. Total sulfhydryl group content of NAM solution decreased with concomitant increase in disulfide bond at both incubation temperatures as the concentration of OTA increased up to 0.2% ($P <$ 0.05). However, no differences in total sulfhydryl group content and disulfide bond content were found in NAM solution added with 0.3% OTA, compared with that of the control at both incubation temperatures ($P >$ 0.05). At higher temperature, the higher disulfide bond content with the lower sulfhydryl groups retained was noticeable in NAM solutions. A decrease in total sulfhydryl group content was reported to be due to the formation of disulfide bonds through oxidation of sulfhydryl groups or disulfide interchanges (Hayakawa and Nakai, 1985). An intermolecular disulfide bond is formed by the oxidation of two cysteine molecules on neighboring protein chains (Lanier, 2000). Elevated temperature most likely resulted in the enhanced oxidation of sulfhydryl groups with the accompanied disulfide bond formation. OTA might induce the conformation of NAM, in which sulfhydryl groups might be exposed and favored oxidation process. This was evidenced by the higher disulfide bond contents in NAM added with OTA. Additionally, quinone could interact directly to sulfhydryl group (Strauss and Gibson, 2004). As a result, these sulfhydryl groups could be masked by those quinones. At high concentration of OTA (0.3%), self-aggregation of OTA might lower its ability in interaction with sulfhydryl groups directly or in induction of sulfhydryl group oxidation.

Addition of OTA in SP at all levels had no impact on both total sulfhydryl group and disulfide bond contents ($P >$ 0.05). Therefore, it can be inferred that OTA was not able to induce the conformational changes of SP molecule in the way which favored the oxidation of sulfhydryl groups. Furthermore, sulfhydryl groups of SP might be present inside molecules and could not readily interact with OTA. It was noted that after incubation at 40 ^{o}C, SP had the lower sulfhydryl group content with higher disulfide bond content than those obtained after incubation at room temperature.

No changes in both total sulfhydryl group and disulfide bond contents were observed in NAM/SP mixture when OTA at all levels was added, except for the mixture incorporated with 0.3% OTA and incubated at 40 ^{o}C, in which the lowest disulfide bond content was obtained. The result of sulfhydryl group and disulfide bond contents correlated well with those of turbidity and solubility (Figure 34 and 35).

NAM solution with a lower protein content (65%) showed the similar result of sulfhydryl group and disulfide bond contents to that found for NAM with higher protein concentration (100%). Higher disulfide bonds were formed when 0.2% OTA was added, especially when incubated at 40 ^{o}C. Therefore, the addition of OTA was associated with the increased formation of disulfide bonds in NAM. The formation of disulfide bonds might contribute to the formation of NAM aggregate, particularly when NAM was incubated at 40 ^{o}C, which is commonly used for high-temperature setting for surimi or mince gel preparation (Benjakul *et al.,* 2004b).

Table 6. Total sulfhydryl groups and disulfide bond contents of different protein solutions without and with OTA at different concentrations after incubation at room temperature (26-28 ^{o}C) or at 40 ^{o}C for 30 min.

Protein	% OTA	Incubation at room temperature (26-28 ^{o}C)		Incubation at 40 ^{o}C	
		Total SH group (mole/10^5 g protein)	SS content (mole/10^5 g protein)	Total SH group (mole/10^5 g protein)	SS content (mole/10^5 g protein)
NAM	0	5.40± 0.18aA	3.53± 0.09aA	4.20± 0.31aA	3.92± 0.35cA
	0.1	5.20± 0.16aA	3.65± 0.25aA	3.82± 0.15bA	4.51± 0.12bA
	0.2	4.81± 0.32bA	4.20± 0.05bA	3.21± 0.21cA	4.92± 0.16aA
	0.3	5.24± 0.18aA	3.49± 0.20aA	4.10± 0.28aA	3.82± 0.30cA
SP	0	1.14± 0.09aD	0.48± 0.03aD	0.95± 0.08aD	0.68± 0.05aD
	0.1	1.16± 0.05aD	0.51± 0.05aD	0.78± 0.05aD	0.79± 0.07aD
	0.2	1.10± 0.11aD	0.47± 0.08aD	0.75± 0.07aD	0.85± 0.06aD
	0.3	1.22± 0.15aD	0.52± 0.04aD	0.71± 0.04aD	0.92± 0.03aD
65% NAM + 35% SP	0	4.28± 0.18aB	1.61± 0.08aC	3.95± 0.38aB	2.12± 0.23aC
	0.1	4.24± 0.21aB	1.55± 0.19aC	3.84± 0.23aB	2.15± 0.35aC
	0.2	4.35± 0.11aB	1.59± 0.21aC	3.79± 0.20aB	2.21± 0.49aC
	0.3	4.42± 0.19aB	1.42± 0.15aC	3.92± 0.26aB	1.88± 0.20bC
65% NAM + 35% Tris HCl	0	3.51± 0.21aC	2.29± 0.09aB	2.73± 0.08aC	2.54± 0.09aB
	0.1	3.35± 0.31aC	2.50± 0.20aB	2.48± 0.09bC	2.93± 0.20bB
	0.2	2.67± 0.05bC	2.73± 0.08bB	1.98± 0.05cC	3.19± 0.08aB
	0.3	3.40± 0.19aC	2.27± 0.18aB	2.66± 0.11aC	2.48± 0.18aB

*Mean ± SD (n=3).

Different letters within the same column within the same protein solution indicate the significant differences ($P < 0.05$). The different capital letters within the same row for the same parameter determined indicate the significant differences ($P < 0.05$).

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of different protein solutions without and with addition of OTA at various concentrations after incubation at room temperature or at 40 ^{o}C for 30 min are shown in Figure 37. No differences in MHC and actin band intensity were noticeable when NAM solutions were incubated at room temperature, regardless of OTA addition (Figure 37A). However, the lower MHC and actin band intensity was found in NAM solution added with OTA up to 0.2% when incubated at 40 ^{o}C for 30 min. The result suggested that the formation of cross-linking stabilized by non-disulfide covalent bond took place, especially during setting. MHC was most susceptible to cross-linking during setting (Benjakul and Visessanguan, 2003). Benjakul and Visessanguan (2003) reported the decrease in MHC band intensity of surimi gel from bigeye snapper, particularly when the setting at 40 ^{o}C was implemented. NAM without OTA incubated at 40 ^{o}C showed the slightly lower MHC band intensity compared with those found in NAM incubated at room temperature. This might be governed by remaining endogenous TGase in NAM. At high temperature (40 ^{o}C), MHC might be partially unfolded and OTA could interact with those proteins to a higher extent via non-disulfide covalent bond. This was evidenced by the lowest MHC band intensity found in NAM added with 0.2% OTA and incubated at 40 ^{o}C. Conversely, room temperature (26-28^{o}C) might not be enough for protein unfolding and the reactive groups of NAM might not be available for cross-linking induced by OTA. Ou *et al.* (2005) reported the polymerization of soy protein molecules by ferulic acid. Mechanical properties of gelatin films were also improved when ferulic acid and tannic acid were used as cross-linkers (Cao *et al.,* 2007). Covalent modification of proteins by phenolic oxidation products generated at alkaline pH was reported extensively (Rawel *et al.,* 2002a). Thus, the efficacy in protein cross-linking of OTA was maximized when setting at temperature high enough for conformational alteration of proteins was applied. As a result, the polymerization of NAM could be enhanced.

SP solution without and with OTA after incubation at both temperatures had no marked differences in protein patterns. However, the band intensity of all proteins decreased slightly at both incubation temperatures when 0.3%

OTA was added. This indicated that non-disulfide covalent bond induced by OTA was negligible. SDS and β-mercaptoethanol used for electrophoresis could destroy all weak bonds and disulfide bond. It was suggested that weak bonds were involved in SP cross-linking especially hydrophobic interaction or hydrogen bond. The results indicated that OTA, especially at high concentration, was able to cross-link SP to some degree. Nevertheless, the aggregate might have the small size which did not show positive response for turbidity or solubility studies. For NAM/SP mixture, MHC band intensity decreased slightly when incubated at 40 ^{o}C for 30 min as compared with that of mixture incubated at room temperature. This might be a result of the formation of non-disulfide cross-link induced by endogenous TGase prevalent in SP. The sarcoplasmic fraction from bigeye snapper muscle possessed cross-linking activity towards MHC (Benjakul *et al.,* 2004a). Benjakul and Visessanguan (2003) reported the role of TGase in setting of bigeye snapper surimi. However, no differences in band intensity of MHC and other proteins were observed when OTA at all levels was incorporated. The results indicated the interfering effect of SP on NAM cross-linking. OTA might prefer to bind with SP, thus it was not available to cross-link MHC. The result was in accordance with no changes in turbidity, solubility, total sulfhydryl groups and disulfide bond contents of NAM/SP mixture (Figure 34, 35 and Table 6, respectively).

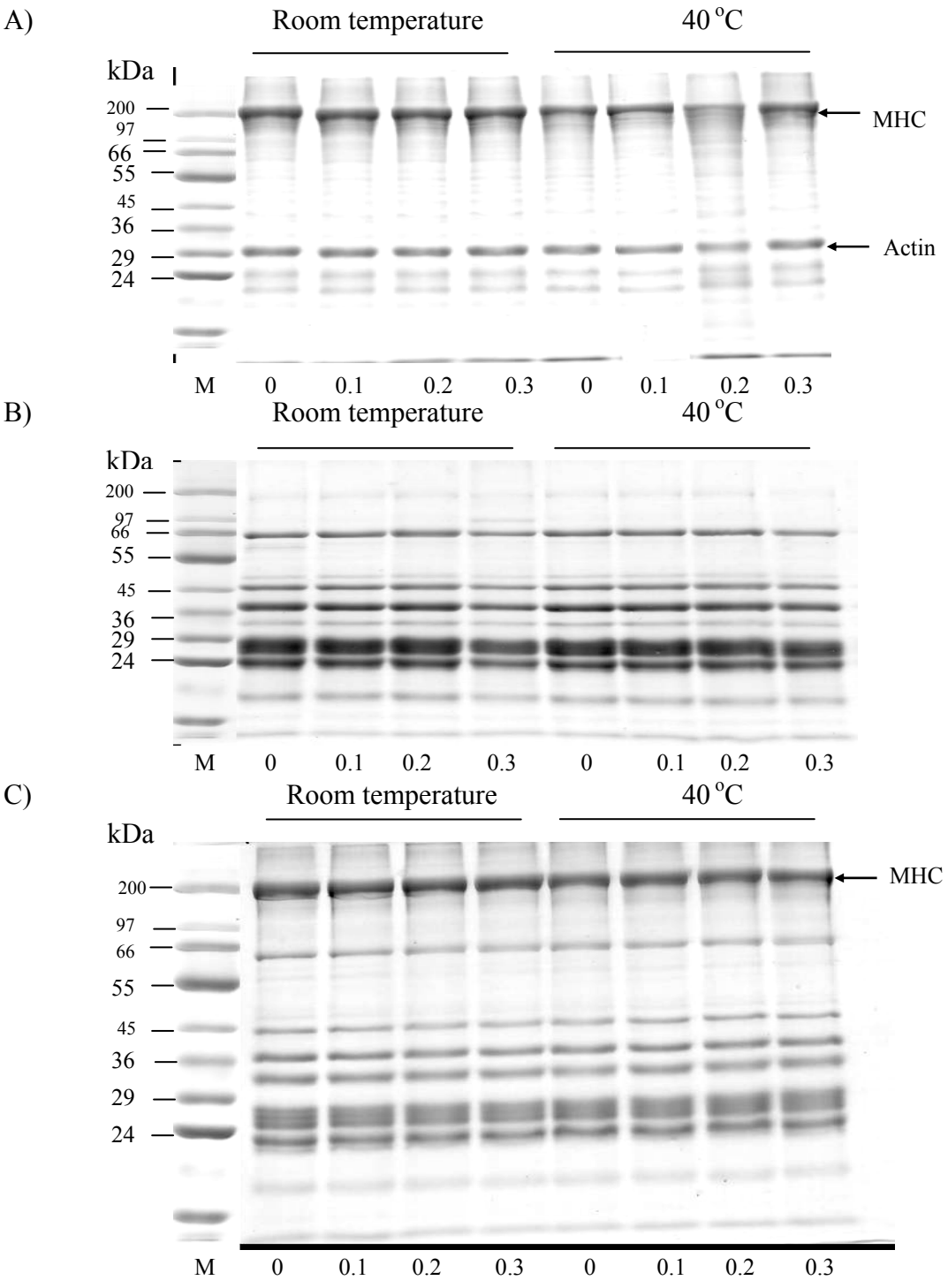
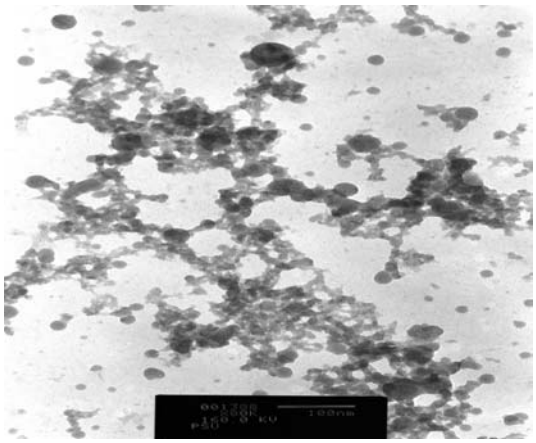


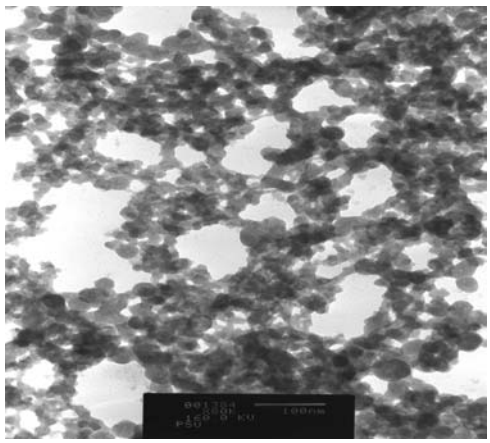
Figure 37. SDS-PAGE patterns of proteins of NAM (A), SP (B) and NAM/SP (65:35) mixture (C), without and with OTA at different concentrations after incubation at room temperature (26–28 °C) or at 40 °C for 30 min. MHC, myosin heavy chain; M, high MW protein markers; Numbers denote the concentration of OTA added (%).

Microstructure

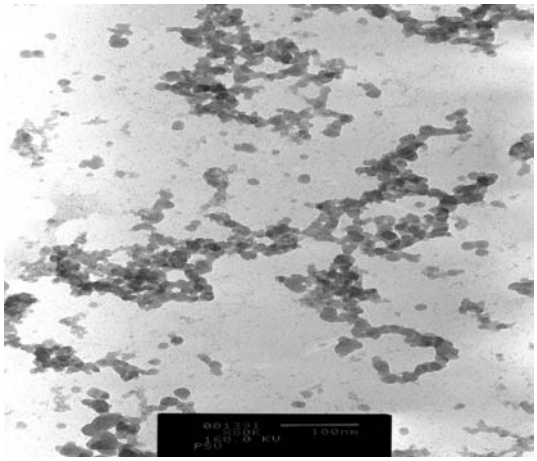
Microstructures of NAM or NAM/SP (65:35) mixture incubated at 40 ^{o}C for 30 min without and with 0.2% OTA are illustrated in Figure 38. NAM aggregate had the larger strand with the denser structure, compared with the aggregate of NAM/SP mixture. SP with a lower molecular weight might interfere the interaction between NAM molecules, leading to the formation of looser cluster. A highly interconnected, finer and denser network structure was observed in NAM with 0.2% OTA incubated at 40 ^{o}C for 30 min. Gelation is the result of protein denaturation, followed by the aggregation via inter-molecular covalent bonds and non-covalent interactions (Lee and Lanier, 1995). The incubation at 40 ^{o}C might provide the sufficient energy for unfolding protein molecules, which allowed OTA to interact easily. This resulted in highly ordered three dimensional protein networks. This was coincidental with the highest turbidity development and disulfide bond formation of NAM added with 0.2% OTA. (Figure 34B and Table 6). Looser protein network was formed in NAM/SP mixture incubated at 40 ^{o}C for 30 min without OTA (Figure 38B). In the presence of OTA, the coagulum with denser structure was formed. Very less interconnected network was observed. Therefore, SP at a level of 35% exhibited the dilution effect on NAM, which had more fibrous structure (Figure 38C). Furthermore, OTA most likely preferred to bind SP than NAM, mainly via weak bonds. This resulted in the poor protein network formation.



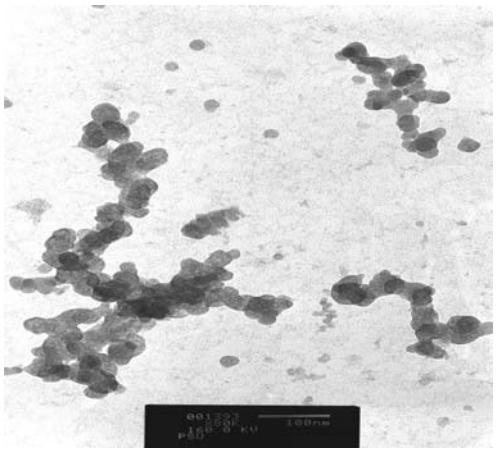
A) NAM without OTA



B) NAM with 0.2% OTA



C) NAM/SP mixture without OTA



D) NAM/SP mixture with 0.2% OTA

Figure 38. Transmission electron micrograph of NAM or NAM/SP mixture without and with 0.2% OTA after incubation at 40 ^{o}C for 30 min. NAM, SP and OTA represent natural actomyosin, sarcoplasmic protein and oxidized tannic acid respectively. Magnification: 80,000×.

5.5 Conclusions

OTA could be used as a cross-linker of NAM when the incubation temperature was high enough to induce the conformational change. Nevertheless, SP exhibited the interfering effect on cross-linking activity of OTA towards NAM, a major contributor for gel formation. OTA might preferably interact with SP instead of NAM, in which a small aggregate or cluster was formed and impede the aggregation of NAM.

CHAPTER 6

USE OF KIAM WOOD EXTRACT AS GEL ENHANCER FOR MACKEREL (*RASTRILLEGER KANAGURTA*) SURIMI

6.1 Abstract

Kiam (*Cotylelobium lanceotatum* Craih) wood was extracted using water or ethanol. Water kiam wood extract (WKWE) and ethanolic kiam wood extract (EKWE) contained tannin at levels of 251.90 and 456.30 mg/g of dry extract, respectively. Effects of WKWE and EKWE at different levels (0-0.60% of protein content) on the properties of gels from mackerel (*Rastrelliger kanagurta*) surimi were investigated in comparison with commercial tannin (CT). Gels added with 0.30% WKWE, 0.15% EKWE or 0.30% CT had the increases in breaking force by 134.81, 136.09 and 121.34% and in deformation by 52.60, 54.96 and 33.53%, respectively, compared with the control (without addition of extracts or CT). Those increases were associated with the lowered expressible moisture content and the disappearance of myosin heavy chain (MHC) band intensity of resulting gels. Thus, the extract of kiam wood can be used as surimi gel enhancer without affecting its sensory properties.

6.2 Introduction

Surimi gel is a three-dimensional myofibrillar protein network. The textural properties developed during gelation are normally expressed in terms of gel strength, which is the basic parameter for determining the quality and price of surimi (Benjakul *et al.,* 2004b). In general, the lean fish have been used for the surimi production but overexploitation of the lean fish has resulted in the insufficiencies of

those species as raw material. The use of under-utilized small pelagic fish species, such as sardine and mackerel could be a better alternative for the lean fish but their use for surimi production is limited mainly due to the large quantity of lipids and myoglobin in the muscle tissue (Chaijan *et al.,* 2004). Furthermore, pelagic fish has been found to possess the high proteolytic activity, causing the gel softening of those species. To alleviate the problem, protein additives have been widely used to enhance the gel strength of the surimi via inhibition of proteolysis caused by an endogenous proteinase (Benjakul *et al.,* 2004c). To strengthen the gel, the cross-linking enzyme such as microbial transglutaminase has been used (Tammatinna *et al.,* 2007). Recently, the interactions between phenolic compounds and proteins have been paid more attention in the processing of certain food products. There have been a few studies describing the cross-linking ability of phenolic compounds with proteins (Strauss and Gibson, 2004; Rawel *et al.,* 2002b). Balange and Benjakul (2009a) reported a significant increase in the gel strength of bigeye snapper surimi when commercial phenolic compounds in oxidized forms were added. Among all oxidized phenolic compounds used, oxidized tannic acid (OTA) exhibited the highest gel strengthening effect, compared with oxidized ferulic acid, oxidized catechin and oxidized caffeic acid (Balange and Benjakul, 2009a).

Tannins are polyphenolic compounds occurring in the barks and fruits of many kinds of plants. Extraction of tannins from the bark of different trees has been carried out (Fradinho *et al.,* 2002; Yazaki and Collins, 1994). Kiam (*Cotylelobium lanceotatum* craih) trees are very common in the southern part of Thailand. Pieces of wood from the kiam tree have been traditionally submerged in sugar palm sap to prevent or retard microbial fermentation (Chanthachum and Beuchat, 1997). The kiam wood is either burned for energy production or simply disposed. The preparation of kiam extract containing phenolic compounds could increase the value of those wood and the novel natural additives can be applied in food industry, especially surimi industry.

However, there is a little information on the utilization of kiam wood extract as the cross-linking agents in food proteins, particularly myofibrillar proteins. Therefore the objectives of this research were to extract and quantify tannin in the

wood of kiam (*Cotylelobium lanceotatum* craih) and to use the extracts as gel strengthener in surimi from mackerel (*Rastrelliger kanagurta*).

6.3 Materials and Methods

Chemicals

Tannic acid (TA), sodium hydroxide, hydrochloric acid, sodium carbonate, $CuSO_4.5H_2O$, bovine serum albumin, Folin-Ciocalteu reagent and β-mercaptoethanol were obtained from Sigma (St. Louis, MO, USA). Sodium dedocyl sulfate (SDS), *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium chloride, trichloroacetic acid and ethanol were obtained from Merck (Darmstadt, Germany). HPLC-grade tannic acid was obtained from Sigma (St. Louis, MO, USA).

Preparation of kiam wood extracts

Collection and preparation of kiam wood

The kiam wood was obtained from the forest of the Phattalung province in the Southern Thailand. The tree was about 15–20 years old and harvested in the month of June-2008. The tree was cut by using a sawing machine, the leaves and branches were separated manually by cutting and the trunk was kept for sun drying for three months. The trunk was chopped into smaller flakes of wood and then dried in an oven at 70 ^{o}C for 8 h and cut into pieces with an average size of 1.5 x 1.5 cm^2 and referred to as “Intact form”. Those pieces were ground using a portable grinding machine (Spong-90, Leeds, England) with a sieve size of 6 mm. The resulting ground bark was referred to as “Coarse form”. This coarse form was then subjected to a blender (National Model MK-K77, Tokyo, Japan) and finally sieved using a stainless steel sieve of 80 mesh size and referred to as “fine form”.

Extraction of crude phenolics from kiam wood

Extraction using ethanol

Three different forms of kiam wood were extracted according to the method of Santoso *et al.* (2004) with slight modifications. The kiam wood samples (10 g) were mixed with 150 ml of absolute ethanol. The mixtures were stirred at room temperature (28-30^{o}C) using a magnetic stirrer (IKA-Werke, Staufen, Germany) for 3 h. The mixture was then centrifuged at 5000 x *g* for 10 min at room temperature using a Sorvall Model RC-B plus centrifuge (Newtown, CT, USA). The supernatant was filtered using Whatman filter paper No.1 (Whatman Schleicher & Schuell, Maidstone, England). The filtrate was then evaporated at 40 ^{o}C using Eyela rotary evaporator (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan). The volume was made to 10 ml with ethanol in a volumetric flask. The resulting extracts were referred to as "ethanolic intact wood extract", "ethanolic coarse wood extract" and "ethanolic fine wood extract".

Extraction using water

Three different forms of kiam wood were extracted following the method of Chanthachum and Beuchat (1997) with slight modifications. Kiam (10 g) was mixed with 80 ml of distilled water. The mixtures were stirred continuously at 70 ^{o}C for 2 h. The mixtures were allowed to stand until the temperature decreased to room temperature. The mixtures were then centrifuged at 5000 x *g* for 10 min at room temperature using a Sorvall Model RC-B plus centrifuge (Newtown, CT, USA). The supernatant was filtered using Whatman filter paper No 1. The filtrate was then evaporated on a hot plate (EGO, Model-18715, 1500w, Germany). The volume was made to 10 ml with distilled water in a volumetric flask. The resulting extracts were referred to as "water intact wood extract", "water coarse wood extract" and "water fine wood extract" respectively.

Determination of total phenolic compounds in different kiam wood extracts

Quantification of total phenolic compounds in different kiam wood extracts was carried out according to the method of Slinkard and Singletone (1977). The extract (0.5 ml) was mixed with 0.5 ml of distilled water. To the mixture, 0.5 ml of Folin-ciocalteu reagent (1:1 with water) and 2.5 ml of 2% sodium carbonate solution were added sequentially. The reaction mixture was mixed thoroughly and placed in dark for 40 min and the absorbance was recorded at 725 nm. The total phenolic content was calculated from the standard curve of tannins (0.1 mg/ml) and expressed as mg tannin / g dry kiam after blank substraction. Blank for each extract was prepared in the same manner, except that distilled water was used instead of Folin-ciocalteu reagent.

Reverse- phase High Performance Liquid Chromatography (HPLC) of different wood extracts

Qualitative analysis of kiam wood extracts was performed using an HPLC equipped with VWD detector following the method of Tian *et al.* (2009) with slight modifications. The HPLC system consisted of an Agilent 1100 series HPLC (Alginet, Wilmington, DE, USA), quaternary pump with seal wash option, degasser, solvent, cabinet and preparative autosampler with thermostat equipped with a diode array detector. The separation was performed on a column (Hypersil ODS C18 4.0*250 mm, 5 µm, Cole – Parmer, Hanwell, London). HPLC conditions were as follows: mobile phase: 0.4% Formic acid: Acetonitrile (85:15), flow rate: 0.8 ml/min; temperature; 25 ^{o}C. The detection was carried out at 280 nm. The concentration of extracts was 25 mg/ml and each injection volume was 20 µl. Standard tannin was used for peak identification.

Preparation of surimi by alkaline saline washing process

Mackerel (*Rastrelliger kanagurta*) with an average weight of 85-90 g were caught during September – October, 2008, from Songkhla coast along the

Gulf of Thailand, stored in ice and off-loaded approximately 36 h after capture. Fish were iced 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. Upon arrival, fish were immediately washed and used for surimi preparation.

Surimi was prepared according to the method of Shimizu (1965) with a slight modification. Mackerel flesh was removed manually and minced to uniformity using a mincer with a hole diameter of 5 mm. The mince was then suspended in four volumes of cold (5 ^{o}C) washing solution (0.15% NaCl in 0.2% $NaHCO_3$). The mixture was stirred gently for 15 min and washed mince was filtered with a layer of nylon screen (Butterfly, Lao Hah Seng Lee Co., Ltd., Bangkok, Thailand). The washing process was repeated twice with cold water only. For the third washing, cold 0.5% NaCl solution was used as washing medium. Finally, the washed mince was subjected to centrifugation using a Model CE 21K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700 x g at 4 ^{o}C for 10 min. To the washed mince, 4% sucrose and 4% sorbitol were added, mixed well and frozen using an air-blast freezer. Resulting surimi was kept at -20 ^{o}C for no longer than 1 month.

Effect of oxidized kiam wood extracts on the property of mackerel surimi gel

Preparation of oxidized kiam wood extracts

WKWE and EKWE containing the highest tannin content were oxidized according to the method of Strauss and Gibson (2004) with slight modifications. The extract solutions (100 ml; 1% w/v) were adjusted to pH 8 using 6 N NaOH or 6 N HCl. The prepared solutions were placed in a temperature-controlled water bath (Memmert, Schwabach, Germany) at 40 ^{o}C and subjected to oxygenation for 1 h by bubbling the solution with oxygen to convert phenolic compound to quinone. After being oxygenated for 1 h, the solutions were then adjusted to pH 7 by using 6 N HCl. CT was also oxidized in the same manner. Prepared extracts and prepared tannin solution were used as the additives in surimi gels.

Surimi gel preparation

To prepare surimi gels, frozen surimi was tempered for 30 min in running water (26-28 °C) until the core temperature reached 0-2 °C. The surimi was then cut into small pieces with an approximate thickness of 1 cm and placed in a mixer (National Model MK-K77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% salt was added. WKWE, EKWE or CT (pH 7.0) at various concentrations (0, 0.15, 0.30, 0.45 and 0.60% of protein content) was added into the sols. The mixtures were chopped for 4 min at 4 °C to obtain homogeneous sols. The sols were then stuffed into polyvinylidine casings with a diameter of 2.5 cm and both ends of casings were sealed tightly. Sols were incubated at 40 °C for 30 min, followed by heating at 90 °C for 20 min (Benjakul *et al.,* 2003). All gels were cooled in iced water and stored for overnight at 4 °C prior to analyses.

Measurement of gel properties

Textural analysis

Textural analysis of gels was performed using a texture analyser Model TA-XT2 (Stable Micro Systems, Surrey, England). Gels were equilibrated and tested at room temperature. Five cylinder-shaped samples of 2.5 cm in length were prepared. The breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a spherical plunger (5 mm diameter). The probe was pressed into the cut surface of a gel specimen perpendicularly at a constant depression speed (60 mm min^{-1}) until the puncture occurred. The force in gram (g) required to puncture into the gel (breaking force) and the distance (in mm) at which the ball probe punctured into the gel (deformation) were recorded.

Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al.* (2001b) with a slight modification. Gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between 3 pieces of Whatman paper No. 4

(Whatman Schleicher & Schuell, Maidstone, England) at the bottom and 2 pieces on the top of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The sample was then removed from the papers and weighed again (*Y*). Expressible moisture content was calculated using the following equation:

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

Determination of whiteness

Color of gels was determined using a JP7100F colorimeter (Juki Corporation, Tokyo, Japan). *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by Lanier *et al.* (1991) as follows:

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

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

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 heated to 85 °C were added to the gel samples (3 g). The mixture was then homogenised using a homogeniser (IKA Labortechnik, Selangor, Malaysia) at a speed of 11000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500 x *g* for 20 min to remove undissolved debris. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940), using bovine serum albumin as a standard. The samples (20 µg protein) were loaded into 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 for 15 min, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid for 3 h.

Sensory evaluation

Mackerel surimi gels without and with WKWE, EKWE or CT at the optimal level were evaluated for colour, appearance, odour, taste, texture and overall liking by 30 non-trained panelists. A nine-point hedonic scale, in which a score of 1= not like very much, 5= neither like nor dislike and 9= like extremely, was used for evaluation (Meilgaard *et al.,* 1990).

Scanning electron microscopy (SEM)

Microstructure of gels was determined using SEM. Mackerel surimi gels, without and with WKWE, EKWE or CT at the optimal level were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub, and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

Statistical analysis

The experiments were run in triplicate. All chemical analyses were performed in triplicate. For physical analyses, e.g. expressible moisture, whiteness and textural properties, at least 5 determinations were conducted for each 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). T-test was used for pair comparison. Analysis was performed using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA).

6.4 Results and Discussion

Composition of different kiam wood extracts

Total phenolic contents of different kiam wood extracts are shown in Table 7. Ethanolic extracts contained the higher total phenolic content than did the water extracts for all forms of kiam wood used for extraction ($P < 0.05$). Generally, the extraction of phenolic compounds depends on the source of raw material, type of solvent and its polarity (Tian *et al.,* 2009). The higher total phenolic content in the ethanolic extract of kiam wood might be due to the similarity in polarity between tannin in the kiam wood and ethanol. Total phenolic content of fine wood extracts was higher than that of coarse wood extract and intact wood extract, respectively ($P < 0.05$). Wood with finer particles had a larger surface area, in which the solvent could contact the sample more effectively. As a consequence, the higher extraction efficacy was obtained for finely ground wood, especially when ethanol was used as an extracting medium. Ethanolic and water fine wood extracts had the total phenolic content of 498.44 and 198.99 mg tannin/g dry wood, respectively.

Table 7. Total phenolic contents of different kiam wood extracts.

Extraction media	Total phenolic content (g tannin kg^{-1} dry wood)		
	Intact form	Coarse form	Fine form
Water	29.33 ± 0.49cB	99.22 ± 0.41bB	198.99 ± 0.21aB
Ethanol	78.83 ± 0.22c*A**	249.14 ± 0.57bA	498.44± 0.98aA

* Different letters in the same row indicates the significant differences ($P < 0.05$).

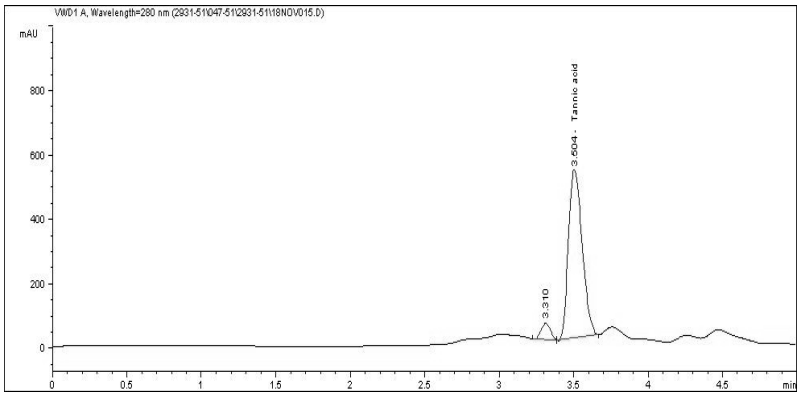
** Different capital letters in the same column indicates the significant differences ($P < 0.05$).

Values are mean ± standard deviation (n=3).

Water and ethanolic extracts from finely ground wood were analysed by HPLC-DAD and tannin was found as the major component (Figure 39). Wood of different trees mainly contains tannins (Fradinho *et al.,* 2002; Yazaki and Collins,

1994). Water extract contained the lower tannin (251.90 mg tannin/g of dry wood extract) than did ethanolic extract (456.30 mg tannin/g of dry wood extract). This was in agreement with the lower total phenolic content in the former. The result indicated that kiam wood extract was an important source of tannin and the reduction of wood prior to extraction increased the efficacy for tannin extraction. Thus, both ethanolic kaim wood extract (EKWE) and water kaim wood extract (WKWE) were used for improving the gel properties of surimi from mackerel, in comparison with commercial tannin (CT)

(A)



(B)

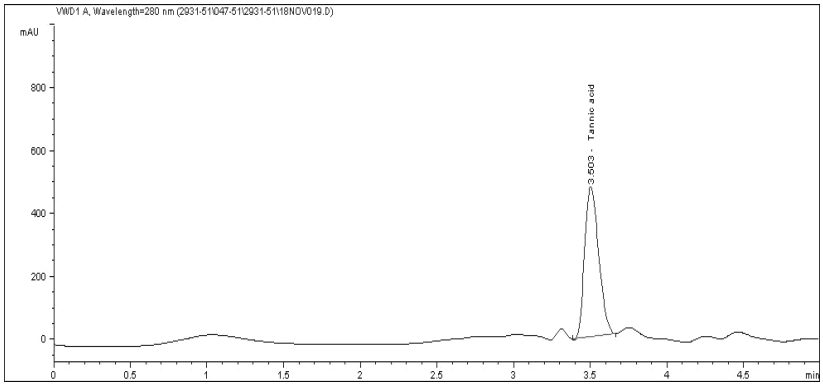


Figure 39. HPLC-DAD chromatograms of water kiam wood extract (A) and ethanolic kiam wood extract (B).

Effect of kiam wood extracts on mechanical properties of mackerel surimi gel

Breaking force and deformation of mackerel surimi gels added with EKWE, WKWE or CT at different levels are depicted in Figure 40. Breaking force and deformation of gels increased as EKWE, WKWE or CT were added up to a particular level ($P < 0.05$). Gels added with 0.30% WKWE or CT had the increases in breaking force by 134.81% or 121.34% and in deformation by 52.60% or 33.53%, respectively, compared with that of the control ($P < 0.05$). For gels added with 0.15% EKWE, breaking force and deformation increased by 136.09 and 54.96%, respectively. Nevertheless, the continuous decreases in both breaking force and deformation were noticeable when both extracts and CT at the greater levels were added ($P < 0.05$). The lower solubility of large phenolic compounds at high concentration causes the difficulty to interact with proteins (De Freitas and Mateus, 2001). In addition, the size of the phenolic compound can decrease its conformational flexibility, which is observed to be an important parameter in protein-phenolic compound interactions (Frazier, *et al.,* 2003). The decreased breaking force and deformation with increasing concentrations of the extracts or CT in the present study might be associated with self-aggregation of phenolic compounds, leading to the loss in capability of protein cross-linking. At a level of 0.15%, gel added with EKWE showed the higher breaking force and deformation as compared with gel added with WKWE or CT ($P < 0.05$). Water is highly polar solvent as compared with ethanol and is able to extract highly polar tannins, whereas, ethanol extracts the tannins with weaker polarity (Tian *et al.,* 2009). The major chemical constituents in the wood from different trees were reported to be tannin with small amount of lignin (Fradinho *et al.,* 2002; Yazaki and Collins, 1994). The weak polar tannins were found to have higher molecular weight and larger size than the strong polar tannins (Tian *et al.,* 2009). Therefore, tannin extracted by ethanol with the larger size might interact more effectively with the myofibrillar proteins in surimi, mainly due to the multiple binding site of the tannin. Tannic acid has a number of hydroxyl groups attached to the aromatic benzene ring which provide more binding cites for proteins (Lopes *et al.,* 1999). Balange and Benjakul (2009a) reported the increases in breaking force and deformation of bigeye snapper surimi with the addition of oxidized tannic acid. It was

noted that surimi gel added with WKWE and EKWE at their optimal concentrations had the higher breaking force and deformation as compared with those of gel added with CT ($P < 0.05$). This might be attributed to the presence of lignin along with tannin in the extracts. This lignin, also being a polyphenolic compound with two hydroxyl groups and two benzene rings, might function as a synergist to tannin in improving the gel strength of mackerel surimi. Therefore, the optimal levels of WKWE, EKWE and CT were 0.3, 0.15 and 0.15%, respectively.

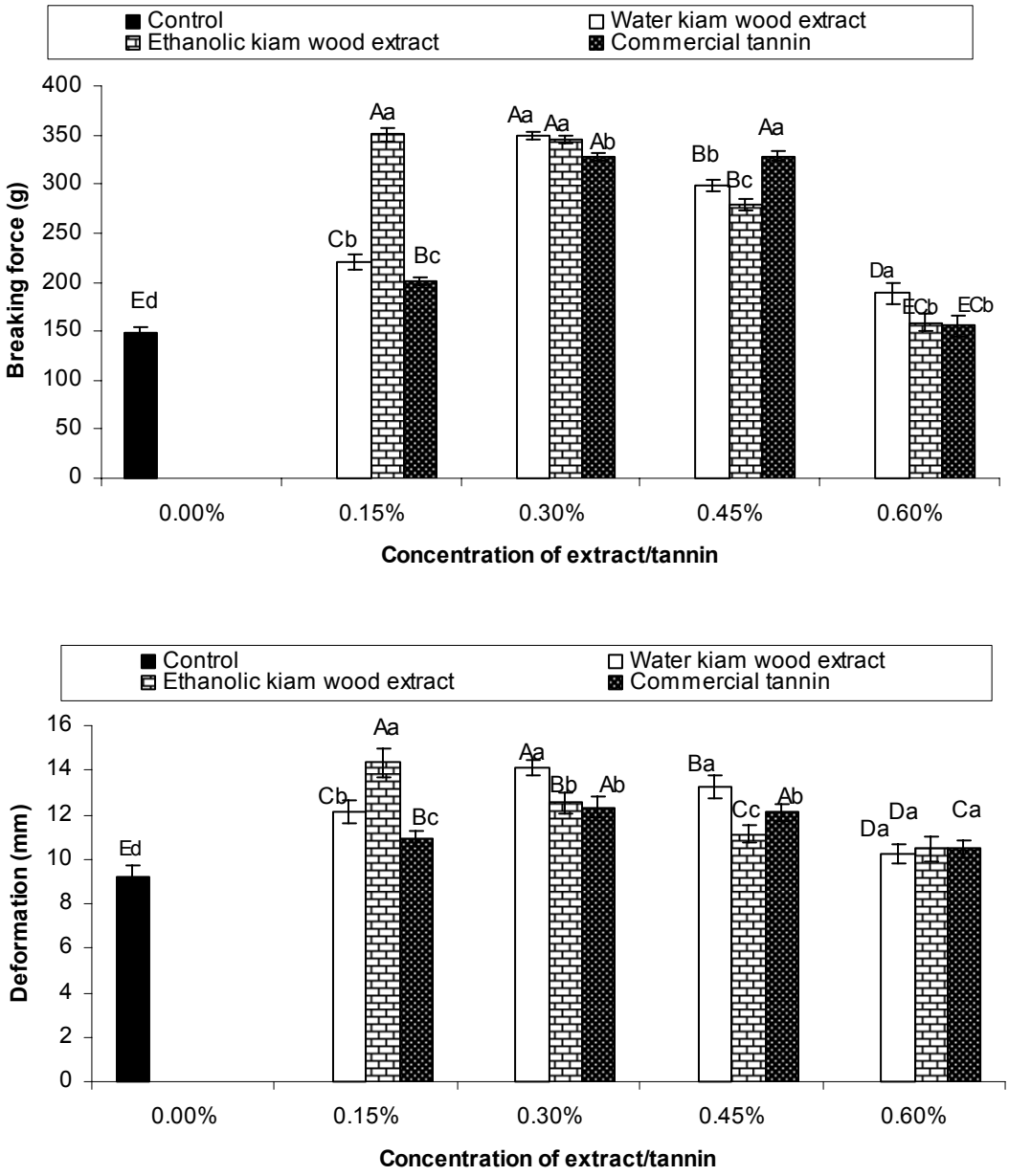


Figure 40. Breaking force and deformation of gels from mackerel surimi added with extracts or commercial tannin (CT). Bars represent the standard deviation (n=3). Different capital letters on the bars within the same additives together with the control indicate the significant differences ($P < 0.05$). The different letters on the bars within the same levels of additives indicate the significant differences ($P < 0.05$).

Effect of kiam wood extracts on expressible moisture content of mackerel surimi gel

The lowest expressible moisture content of mackerel surimi gel was found when WKWE, EKWE or CT at the optimal level were added (P<0.05) (Table 8). The increases in expressible moisture content were found in surimi gels added with WKWE, EKWE or CT above the optimal level (P<0.05). The decreases in expressible moisture contents of surimi gel added with both extract or CT were in accordance with the increased breaking force and deformation of resulting surimi gels (Figure 40). At the optimal level, the cross-linking of proteins in the mackerel surimi gels could be enhanced. This resulted in the formation of stronger network with greater water holding capacity. Among the extracts and CT, EKWE at a level of 0.15% yielded the gel with the lowest expressible moisture content. This reconfirmed that EKWE addition resulted in gel strengthening. As a result, gel network with capability of imbibing water could be obtained.

Effect of kiam wood extracts on whiteness of mackerel surimi gel

The decreases in whiteness of mackerel surimi gels were observed as the levels of the extracts or CT were increased (P<0.05) (Table 8). These results were in agreement with O'Connell and Fox (2001) who reported that phenolic compounds were responsible for discoloration in the cheese products. However the decrease in whiteness was more pronounced in the mackerel surimi gels with the addition of WKWE (P<0.05). Evaporation of water extract at high temperature for a long time enhanced the darkening of water extract. Pansera *et al.* (2004) used the process of hydrosolubilisation at 100 ^{o}C for the extraction of tannin and found that the extraction process at high temperature motivates a hydro cracking of sugar and other organic compounds with darkening of the final product. From the result, surimi gel added with EKWE at a level of 0.15% had a slight decrease in whiteness.

Table 8. Expressible moisture content and whiteness of gels from mackerel surimi added with water and ethanolic kiam wood extracts or commercial tannin at different levels.

Oxidized phenolic compounds	Amount added (%)	Expressible moisture content (%)	Whiteness
Control	0	9.48±0.59a*	68.15±0.36a*
WKWE	0.15	6.69±0.23bB**	62.36±0.46bC**
	0.30	4.14±0.45cC	60.76±0.51cC
	0.45	5.62±0.22dB	57.12±0.44dC
	0.60	10.53±0.73aA	55.07±0.44eC
EKWE	0.15	3.16±0.33bC	66.36±0.46bA
	0.30	4.84±0.37cB	64.76±0.51cA
	0.45	6.12±0.42dA	63.12±0.44dA
	0.60	9.38±0.59aB	63.07±0.44dA
CT	0.15	7.56±0.43bA	64.16±0.46bB
	0.30	5.14±0.57cA	62.26±0.51cB
	0.45	5.02±0.32cC	61.65±0.51dB
	0.60	10.57±0.59dA	60.12±0.44eB

* Different letters in the same column within the same additive together with the control indicates the significant differences (P<0.05). ** Different capital letters in the same column within the same level of additive used indicates the significant differences (P<0.05).
Values are mean ± standard deviation (n=3).

Effect of kiam wood extracts on the protein pattern of mackerel surimi gel

Protein patterns of gels added with WKWE, EKWE or CT at different concentrations are shown in Figure 41. Surimi paste contained MHC and actin as the major proteins. Decrease in MHC band intensity was found in the control gel (without addition of extracts or CT), when compared with that observed in surimi paste. The result suggested that the formation of cross-linking stabilized by non-disulfide covalent bond took place, especially during setting. MHC was most susceptible to

cross-linking during setting (Benjakul and Visessanguan, 2003). Benjakul and Visessanguan (2003) reported the decrease in MHC of surimi gel from bigeye snapper, particularly when the setting was implemented.

For surimi gels added with WKWE, EKWE or CT at the different concentrations, MHC band intensity decreased significantly as compared with the control gel (Figure 41A, 41B and 41C, respectively). No MHC band was found in all gels added with the extracts or CT ranging from 0.15 to 0.45%. The results suggested that MHC was cross-linked by oxidized phenolic compounds effectively via non-disulfide covalent bond. The disappearance of MHC was in accordance with the increases in breaking force and deformation of mackerel surimi gels added with the extracts or CT at the optimum level (Figure 40). Ou *et al.* (2005) and Cao *et al.* (2007) also reported the polymerization of protein molecules as a possible subsequent reaction of different proteins with phenolic substances. Phenols may be oxidized with ease, in an alkaline solution, to their corresponding quinones (Hurrell and Finot, 1984). The quinone, a reactive electrophilic intermediate, can readily undergo attack by nucleophiles such lysine, methionine, cysteine and tryptophan residues in a protein chain (Hurrell and Finot, 1984). For gels added with 0.6% CT or 0.6% EKWE, MHC band was more retained. This suggested that the lower ability of those EKWE or CT in cross-linking proteins, especially MHC. This was in agreement with the lower gel strengthening effect of the extract or CT at the level above the optimal one. This was mainly due to the self-aggregation of quinones at the higher levels. Due to the lower content of tannin in WKWE, a level of 0.6% water extract might not be sufficient to induce the self-aggregation. As a result, the polymerization of MHC induced by WKWE at such a level could take place effectively.

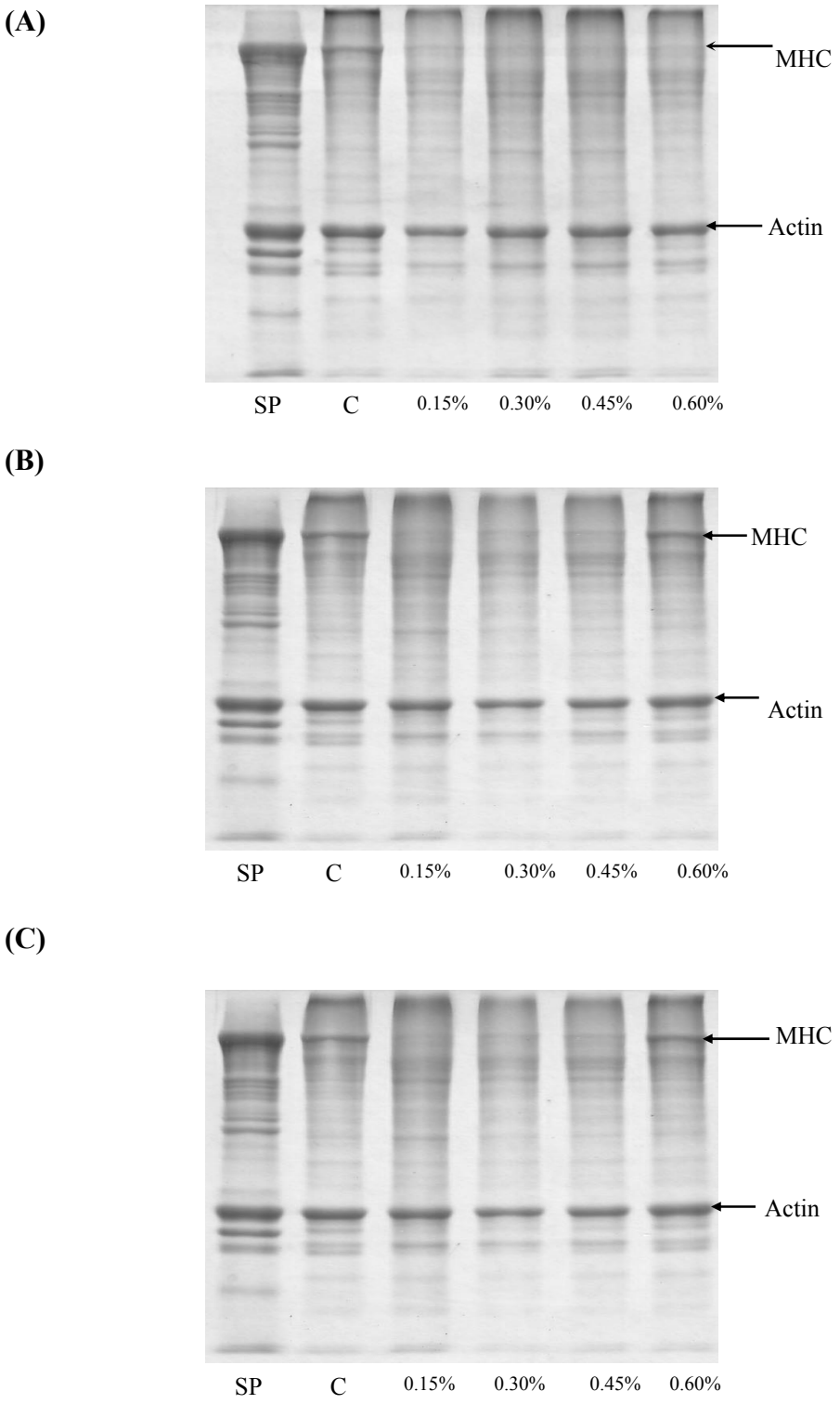


Figure 41. SDS-PAGE patterns of protein in gels from mackerel surimi added with water kiam wood extract (A), ethanolic kiam wood extract (B) or commercial tannin (C) at different concentrations. SP, surimi paste; MHC, myosin heavy chain; C, control gel (without extracts/CT solution).

Effect of kiam wood extracts on the sensory properties of mackerel surimi gels

Likeness scores of the control gels (without extracts or CT) and those added with the extracts or CT at the optimal level are depicted in Figure 42. Addition of CT and EKWE had no negative impact on the color, appearance, odor and taste of resulting gels. However, the addition of WKWE resulted in significantly lower likeness score for the appearance and color, compared with other treatments including the control (P<0.05). Dark color of WKWE most likely contributed to the darker color of resulting gels. Pansera *et al.* (2004) used the process of hydrosolubilization at 100 ^{o}C for the extraction of tannin and found that the extraction process at high temperature resulted in the darkening of the final product. Nevertheless, addition of WKWE, EKWE or CT yielded the gel with the higher texture score, compared with that of control (P<0.05). This was coincidental with the increased breaking force and deformation in the surimi gels added with those extracts or CT (Figure 40). Among all surimi gels tested, those containing 0.15% EKWE or 0.3% WKWE had the highest texture score (P<0.05). According to O'Connell and Fox (2001), addition of phenolic compounds affected the sensory properties of many food products. Recently, Balange and Benjakul (2009b, c) successfully used oxidized phenolic compounds in mackerel surimi gel to increase its gel strength without affecting sensory properties of gels. Surimi gel containing 0.15% EKWE exhibited the highest overall likeness score. Quinone in the extracts or CT underwent cross-linking with myofibrillar protein or self-aggregation. As a result, no quinones were available for binding with salivary proteins. Thus they could not have the impact on taste or flavor of surimi gel. Therefore, the addition of extracts or CT at low concentration did not negatively affect the overall sensory properties of resulting mackerel surimi gel.

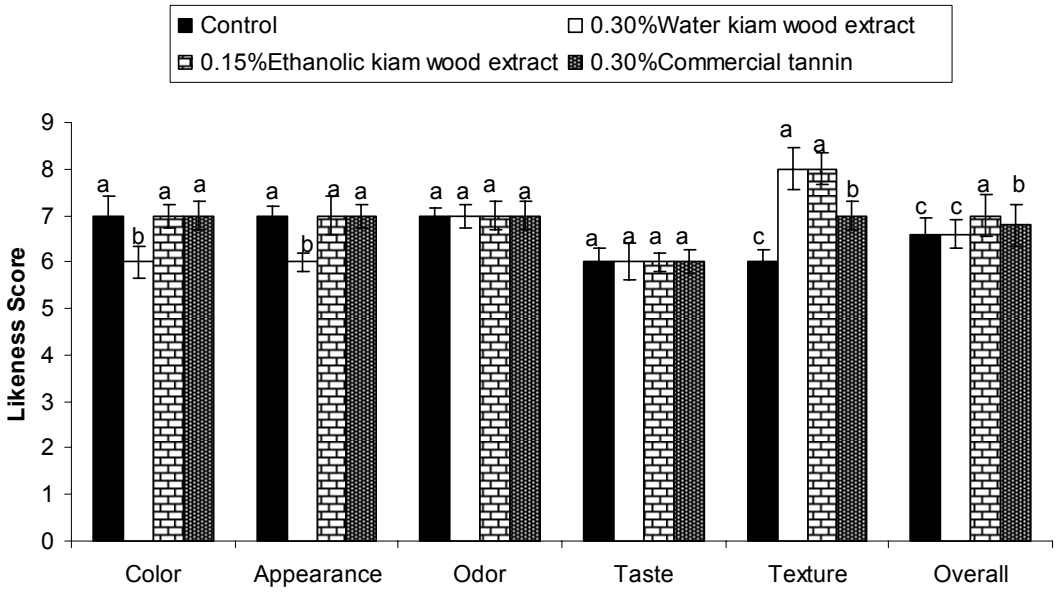
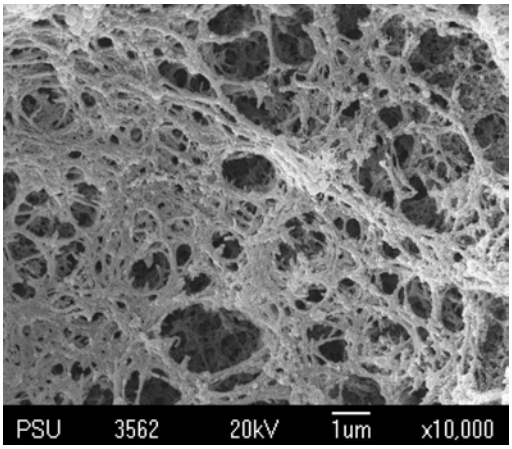


Figure 42. Likeness score of surimi gels from mackerel added without and with water and ethanolic kiam wood extracts or commercial tannin at selected levels. Bars represent standard deviation (n=30). Different letters on the bars within the same sensory attribute indicate significant differences (P<0.05).

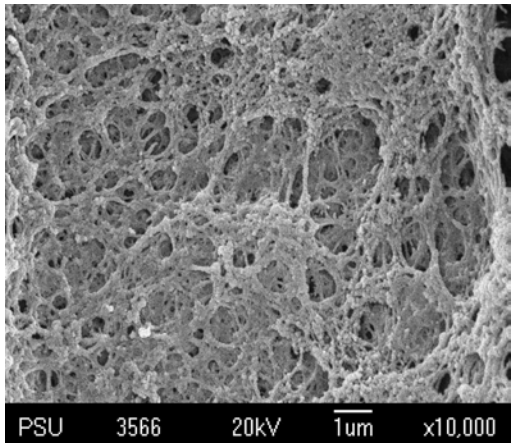
Effect of kiam wood extracts on microstructure of mackerel surimi gel

Microstructures of control gel (A), gel added with 0.30% WKWE (B), gel added with 0.15% EKWE (C) and gel added with 0.30% CT (D) are illustrated in Figure 43. Surimi gels containing both extracts or CT had finer and more continuous matrix than the control gel. This suggested that oxidized phenolic compounds in the extract might induce the cross-linking of proteins, in which the filamental network could be formed orderly. In the presence of oxidized phenolic compounds, mostly quinone, the inter-junctions between fibrillar protein molecules could be enhanced. This led to the formation of finer gel matrix. Among all gel, the gel added with 0.15% EKWE possessed more ordered fibrillar structure with larger strands and had high capacity of imbibing the water (Table 8). The presence of tannin with a greater amount in the EKWE was more likely associated with a higher aggregation between protein molecules. This was in agreement with the markedly improved gel strength of

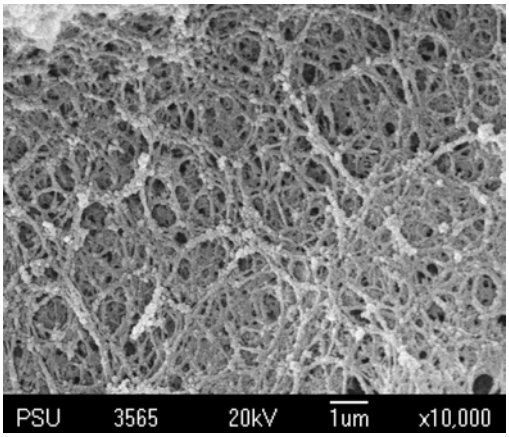
mackerel surimi added with EKWE. Additionally, this might lead to the increase in overall liking score of this gel (Figure 42).



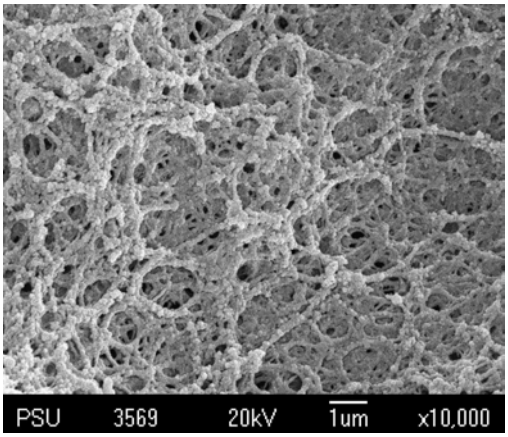
A) Control



B) 0.30% Water kiam wood extract



C) 0.15% Ethanolic kiam wood extract



D) 0.30% Commercial tannin

Figure 43. Electron microscopic images of gels from mackerel surimi (Magnification: 10,000X).

6.5 Conclusions

Ethanolic kiam wood extract had a potential in strengthening the gel of mackerel surimi when the optimum level (0.15%) was introduced. Addition of ethanolic extract had no detrimental effect on sensory properties of surimi gel but slightly increased overall likeness of the gel. Furthermore, ethanolic kiam wood extract was more effective than costly commercial tannin. Thus, the extract from kiam wood can be used as a natural gel enhancer for surimi industry.

CHAPTER 7

SURIMI GEL STRENGTHENING EFFECT OF KIAM WOOD EXTRACT AS INFLUENCED BY pH DURING OXYGENATION PROCESS

7.1 Abstract

Impact of ethanolic kiam wood extract (EKWE) oxygenated at various pHs on the properties of gels from mackerel (*Rastrelliger kanagurta*) surimi were studied in comparison with commercial tannin (CT). Surimi gels, added with 0.30% CT or 0.15% EKWE oxygenated at pH 3 or 7, had the slight increases in breaking force and deformation ($P < 0.05$). When CT and EKWE oxygenated at alkaline pHs (8 and 9) were incorporated, surimi gel had the increases in breaking force by 123.4 - 135.7% and 136.9-157.5%, respectively, while the deformation was increased by 20.9-38.1% and 33.7-48.2%, respectively, compared with those of the control ($P < 0.05$). Those increases were associated with the lowered expressible moisture content and the disappearance of myosin heavy chain (MHC) band intensity. Gels added with 0.15% EKWE oxygenated at alkaline pH had the finer matrix with smaller strands and voids. Thus, the pH for oxygenation of phenolic compounds in EKWE had the impact on the gel strength of mackerel surimi.

7.2 Introduction

Surimi, a minced and washed fish flesh, has now become a very popular term. It has been used as a base material for the production of various imitation products such as crab stick, shrimp analogue, lobster analogue, etc. Lean fish with white meat containing low fat have been generally used to make surimi. Nowadays, over-exploitation of lean fish has resulted in their shortage. Dark flesh fish

species currently make up 40-50% of the total fish catch in the world and could be a better alternative for lean fish for surimi production (Chaijan *et al.,* 2004). Due to its high fat and myoglobin content, dark flesh fish commonly have the low gel forming ability and less whiteness. To overcome these problems, different washing methods have been developed (Shimizu, 1965; Chen *et al.,* 1997; Karayannakidis *et al.,* 2007). A variety of additives have been used to improve the gel strength of surimi including beef plasma protein (Benjakul *et al.,* 2004c), phosphate compounds (Julavittayanukul *et al.,* 2006) and whey protein concentrate (Rawdkuen and Benjakul, 2008).

Phenolic compounds have become increasingly important because of their dynamic properties as a cross-linker, antioxidant, antimicrobial agent, etc (Nuthong *et al.,* 2009; Nirmal and Benjakul, 2009). Protein-phenolic interactions had the impact in various food systems (Strauss and Gibson, 2004; Rawel *et al.,* 2002b). Recently, Balange and Benjakul, (2009a) reported the cross-linking activity of phenolic compounds toward surimi proteins, which were associated with the increase in gel strength of bigeye snapper (*Priacanthus tayenus*) surimi. Among different commercial phenolic compounds used, tannic acid in its oxidized form exhibited the highest gel strengthening effect, compared with others. Oxidation of phenolic compounds results in the formation of quinone which is highly reactive and involve in the formation of strong covalent bonds with the proteins (Kroll *et al.,* 2003). This oxidation can be achieved enzymatically or non-enzymatically. Enzymatic oxidation requires technical expertise and is costly. On the contrary, non-enzymatic oxidation is simple and not expensive (Prigent, 2005). The covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been studied extensively (Rawel *et al.,* 2002a; Kroll *et al.,* 2003). Phenolic compounds could be oxidized non-enzymatically to quinone at pH 8 (Srauss and Gibson, 2004; Balange and Benjakul, 2009a). Nevertheless, protein modification with plant phenolic compounds at neutral or mildly acidic pH conditions, which are much more relevant to foods than alkaline pH, have not been intensively investigated.

Kiam (*Cotylelobium lanceotatum* craih) trees are very common in the southern part of Thailand. Pieces of wood from kiam tree have been traditionally submerged in sugar palm sap in Thailand to prevent or retard microbial fermentation (Chanthachum and Beuchat, 1997). Kiam wood extract contained tannin as the major

constituent and could be used as the natural protein cross-linker (Balange and Benjakul, 2009d). To maximize the use of this wood extract as the processing aid, the preparation condition including pH adjustment prior to oxidation should be optimized. The objective of this research was to study the effect of different pHs on the oxidation of kiam wood phenolic compounds and to determine the properties of mackerel (*Rastrelliger kanagurta*) surimi gel added with kiam wood extracts oxidized at different pHs in comparison with commercial tannin.

7.3 Materials and Methods

Chemicals

Tannic acid (TA), sodium hydroxide (NaOH), hydrochloric acid, sodium carbonate, $CuSO_4.5H_2O$, bovine serum albumin, Folin-Ciocalteu reagent and β-mercaptoethanol (βME) were obtained from Sigma (St. Louis, MO, USA). Sodium dedocyl sulfate (SDS), *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA**).** Sodium chloride and ethanol were obtained from Merck (Darmstadt, Germany).

Preparation of kiam wood extracts

Collection of kiam wood

Kiam wood was obtained from a forest of the Phattalung province in the Southern Thailand. The tree was about 15 – 20 years old and harvested in June, 2008. The tree was cut by using a sawing machine; the leaves and branches were separated manually by cutting and the trunk was sun-dried for three months. The pieces of wood with an average thickness of 1.5 cm were dried in an oven at 70 ^{o}C for 8 h. Prepared wood was then subjected to a portable grinding machine (Spong-90, England) with a sieve size of 6 mm. This coarse form was blended using a blender

(National Model MK-K77, Tokyo, Japan) and finally sieved using a stainless steel sieve of 80 mesh size.

Extraction of phenolic compounds from kiam wood using ethanol

Ethanol extract from powdered kiam wood was prepared as per the method of Santoso *et al.* (2004) with slight modifications. Wood powder (10 g) was mixed with 150 ml of absolute ethanol. The mixture was homogenized for 2 min at 15000 rpm using a homogenizer (IKA Labortechnik, Selangor, Malaysia) and stirred at room temperature using a magnetic stirrer (IKA-Werke, Staufen, Germany) at room temperature (28-30 ^{o}C) for 3 h. The homogenate was then centrifuged at 5000 x g for 10 min at 25 ^{o}C using a Sorvall Model RC-B plus centrifuge (Newtown, CT, USA). The supernatant was filtered using Whatman filter paper No.1 (Whatman Schleicher & Schuell, Maidstone, England). The filtrate was then evaporated at 40 ^{o}C using a rotary evaporator model N-1000 (Eyela, Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) until ethanol was almost removed. The extract was then dried in a forced-air oven at 70 ^{o}C for 4 h. Dried extract was powdered using a mortar and pestle. Extract powder referred to as ethanolic kiam wood extract, EKWE, was kept in a desiccator at 28-30 ^{o}C until used.

Preparation of surimi

Mackerel (*Rastrelliger kanagurta*) with an average weight of 85-90 g were caught from Songkhla coast along the Gulf of Thailand during September – October, 2008. Fish were stored in ice and off-loaded approximately 36 h after capture. Upon the arrival to the dock in Songkhla, fish were iced 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 immediately washed and used for surimi preparation.

Surimi was prepared according to the method of Shimizu (1965) with a slight modification. After the flesh was removed manually and minced to uniformity using a mincer with a hole diameter of 5 mm, the mince was then suspended in four volumes of cold (5 ^{o}C) diluted alkaline salt solution (0.15% NaCl in 0.2% $NaHCO_3$ of

which the ionic strength was 0.05 and the final pH was 7.0-7.1). The mixture was stirred gently for 20 min and the washed mince was filtered with a layer of nylon screen (Butterfly, Lao Hah Seng Lee Co., Ltd., Bangkok, Thailand). The washing process was repeated twice. Finally, the washed mince was subjected to centrifugation using a Model CE 21K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700 x g at 4 ^{o}C for 10 min. To the washed mince, 4% sucrose and 4% sorbitol were added and mixed well. The portion of mixture (500 g) was packaged in polyethylene bag and frozen using an air-blast freezer at -20 ^{o}C (Patkol, Patanakolkarn Co.Ltd., Bangkok, Thailand). Frozen surimi was stored at -20 ^{o}C for no longer than 2 weeks.

The effect of pHs on the oxidation of CT or EKWE solutions and its subsequent effect on the gel properties of mackerel surimi

Oxidation of commercial tannin (CT) and ethanolic kiam wood extract (EKWE) at different pHs

CT and EKWE were subjected to oxidation at different pHs as per the method of Strauss and Gibson (2004) with slight modifications. The solutions (100 ml; 1% w/v) were adjusted to pHs 3, 7, 8 and 9, using 6 M NaOH or 6 M HCl. The prepared solutions were placed in a temperature-controlled water bath (40 ^{o}C) and subjected to oxygenation for 1 h by bubbling the solution with oxygen with the purity of 99.5-100% (TTS Gas Agency, Hat Yai, Songkhla, Thailand) to convert phenolic compounds to quinone, an oxidized form. After being oxygenated for 1 h, the solutions without and with neutralization were then determined for the content of remaining phenolic compound with the reduced form.

Determination of total phenolic content in CT or EKWE oxidized at different pHs

The conversion of phenolic compounds in CT and EKWE to quinone, an oxidized form of CT and EKWE at different pHs was monitored indirectly by

determining total phenolic content in CT or EKWE before and after oxidation according to the method of Slinkard and Singletone (1977) with slight modifications. CT or EKWE solutions (0.5 ml; 50 µg/ml) bubbled at different pHs were mixed with 0.5 ml of distilled water. To the mixture, 0.5 ml of Folin-ciocalteu reagent (1:1 with water) and 2.5 ml of 2% sodium carbonate solution were added sequentially. The reaction mixture was mixed thoroughly and placed in dark for 40 min and the absorbance was read at 725 nm. The total phenolic content was calculated from the standard curve of tannins (100 µg/ml) and expressed as µg tannin / ml after blank substraction. Blank for each extract was prepared in the same manner, except that distilled water was used instead of Folin-ciocalteu reagent. The differences in the total phenolic content before and after oxidation indicated the formation of quinones.

Surimi gel preparation

Frozen surimi was tempered for 30 min in running water (26-28 ^{o}C) until the core temperature reached 0-2 ^{o}C. The surimi was then cut into small pieces with an approximate thickness of 1 cm and placed in a mixer (National Model MK-K77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% salt was added. Neutralized CT and EKWE solutions oxygenated at different pHs (3, 7, 8 and 9) were added into the sols at levels of 0.30 and 0.15% (based on protein content), respectively (Balange and Benjakul, 2009d). The mixtures were chopped for 4 min at 4 ^{o}C to obtain homogeneous sols. The sols were then stuffed into polyvinylidine casings with a diameter of 2.5 cm and both ends of the casings were sealed tightly. Sols were incubated at 40 ^{o}C for 30 min, followed by heating at 90 ^{o}C for 20 min (Benjakul *et al.,* 2003b). All gels were cooled in iced water and stored overnight at 4 ^{o}C prior to analyses.

Measurement of surimi gel properties

Texture analysis

Texture analysis of gels was performed using a texture analyzer Model TA-XT2 (Stable Micro Systems, Surrey, England). Gels were equilibrated and tested at room temperature. Five cylinder-shaped samples of 2.5 cm in length were prepared. The breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyzer equipped with a spherical plunger (5 mm diameter). The probe was pressed into the cut surface of a gel specimen perpendicularly at a constant plunger speed (60 mm min^{-1}) until the puncture occurred. The force in gram (g) required to puncture into the gel (breaking force) and the distance (mm) at which the spherical probe punctured into the gel (deformation) were recorded.

Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al.* (2001b) with a slight modification. Gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between 3 pieces of Whatman paper No. 4 at the bottom and 2 pieces on the top of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The sample was then removed from the papers and weighed again (*Y*). Expressible moisture content was calculated using the following equation:

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

Determination of whiteness

Color of gels was determined using a JP7100F colorimeter (Juki Corporation, Tokyo, Japan). *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by Lanier *et al.* (1991) as follows:

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

Sodium dedocyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of samples were analyzed under a reducing condition by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85 ^{o}C were added to the sample (3 g). The mixture was then homogenized at a speed of 11000 rpm for 2 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia) for 2 min. The homogenate was incubated at 85 ^{o}C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500 x g for 20 min to remove undissolved debris. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard. The sample was then 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 1:1 ratio (v/v). The samples (20 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.

Determination of solubility

Solubility of protein in surimi gel added with EKWE oxygenated at different pHs was determined as described by Benjakul *et al.* (2001b). Finely chopped gel sample (1 g) was solubilized with various solvents including S1 (0.6 M KCl), S2 (20 mM Tris–HCl, pH 8.0), S3 (20 mM Tris–HCl, pH 8.0 containing 1% SDS), S4 (20 mM Tris–HCl, pH 8.0 containing 1% SDS and 8 M urea) and S5 (20 mM Tris–HCl, pH 8.0 containing 1% SDS, 2% β-mercaptoethanol and 8 M urea). The mixture was homogenized for 1 min, boiled for 2 min and stirred for 4 h at room temperature (28–30 ^{o}C) using a magnetic stirrer (IKA-Werke, Staufen, Germany). The mixture was centrifuged at 10,000 x g for 30 min. Two ml of 50% (w/v) cold trichloroacetic acid (TCA) were added to 10 ml of supernatant. The mixture was kept at 4 ^{o}C for 18 h prior to centrifugation at 10,000 x g for 20 min. The precipitate was washed with 10% (w/v) TCA, followed by solubilizing in 0.5 M NaOH. Protein concentration was

determined by the Biuret method (Robinson and Hodgen, 1940). Solubility of protein in surimi samples was expressed as the percentage of total protein in surimi gels solubilized directly in 0.5 M NaOH.

Scanning electron microscopy (SEM)

Microstructure of gels was determined using scanning electron microscope (SEM). Surimi gels added, without and with EKWE (0.15%) solutions oxygenated at different pHs were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub, and sputter-coated with gold (Sputter coater SPI-Module, West Chester PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

Sensory evaluation

Mackerel surimi gels added with EKWE oxygenated at different pHs at a level of 0.15% were evaluated for color, appearance, odor, taste, texture and overall liking by 30 non-trained panelists. A nine-point hedonic scale, in which a score of 1= not like very much, 5= neither like nor dislike and 9= like extremely, was used for evaluation (Meilgaard *et al.,* 1990). The control gel (without addition of EKWE) was also assessed for likeness.

Statistical analysis

All experiments were run in triplicate. For each run, chemical analyses were preformed in triplicate. For physical analyses, e.g. expressible moisture, whiteness and textural properties, 5 determinations were conducted. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range tests (Steel and Torrie, 1980). T-test was used for pair comparison. Analysis was performed using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA).

7.4 Results and Discussion

Conversion of phenolic compounds in CT and EKWE to oxidized forms after oxygenation at different pHs

Oxidized form, mainly quinones, in CT and EKWE solutions oxygenated at different pHs were determined indirectly by using Folin-ciocalteu reagent (Table 9). The amount of quinone formed in both CT and EKWE increased with increasing pHs of solutions ($P < 0.05$). Non-significant increases in quinone content were found in CT and EKWE solutions when oxygenated at pH 3 and 7 ($P > 0.05$). The amount of quinone in CT and EKWE solutions increased by 50.42% and 59.24%, respectively when oxygenated at pH 8. The highest amount of quinone was formed in CT and EKWE solutions when oxygenated at pH 9, in which the increases by 68.96% and 75.08% were obtained ($P < 0.05$). Under the alkaline conditions, the hydroxyl groups are readily oxidized to hydroxyl radical (Huang and Chu, 1995). Those radicals would react with the neutral molecules and cause the benzene ring to undergo hydrolization. Increasing pH induces the deprotonation of the phenolic hydroxyl group, eventually leading to the formation of quinones (Prigent, 2005). Therefore, the conversion of phenolic compounds into quinones was more pronounced at alkaline conditions, compared with the acidic or neutral conditions. Furthermore, the content of quinone in CT and EKWE solutions oxygenated at different pHs, slightly decreased after neutralization ($P > 0.05$). The quinones formed during oxidation are highly unstable and are prone to dimerization (Huang and Chu, 1995). Additionally, the reduction of quinone formed could take place to some extent during neutralization. This resulted in the lower content of quinone detected in the solutions.

Table 9. Total phenolic content (μg/ml) of commercial tannin (CT) and ethanolic kiam wood extract (EKWE) oxygenated at different pHs

Source of phenolic compounds	Treatments	pHs			
		3	7	8	9
CT	Without treatment	50a	50a	50a	50a
	After oxygenation	47.5± 0.51aA (5%)	46.87± 0.48aA (6.26%)	24.79± 0.61bB (50.42%)	15.52± 0.46bC (68.96%)
	After neutralization	47.15± 0.46aA (5.7%)	46.87± 0.48aA (6.26%)	25.81± 0.25bB (48.38%)	16.93± 0.16bC (66.14%)
EKWE	Without treatment	50a	50a	50a	50a
	After oxygenation	49.48± 0.56aA (1.04%)	47.73± 0.16aA (4.54%)	20.38± 0.53bB (59.24%)	12.46± 0.26bC (75.08%)
	After neutralization	48.65± 0.31aA (2.70%)	47.73± 0.16aA (4.54%)	21.13± 0.26bB (57.74%)	13.14± 0.12bC (73.72%)

* Different letters in the same column within the same source of phenolic compound indicate the significant differences ($P < 0.05$). ** Different capital letters in the same row indicate the significant differences ($P < 0.05$). Values in parenthesis indicate the % conversion of phenolic compounds to quinone.

Values are mean ± standard deviation (n=3).

CT, commercial tannin; EKWE, ethanolic kiam wood extract

Effect of CT or EKWE oxygenated at different pHs on properties of mackerel surimi gel

Breaking force and deformation of mackerel surimi gels added with 0.30% CT or 0.15% EKWE oxygenated at different pHs are shown in Figure 44. Surimi gels added with CT or EKWE oxygenated at pH 3 or 7 had the increases in breaking force by 49.3% or 52.5% and in deformation by 20.9% or 38.2 %, respectively, compared with those of the control. For surimi gels added with 0.30% CT or 0.15% EKWE oxygenated at pH 8, the increases in breaking force by 123.4 or 135.7 % and in deformation by 35.9 or 56.2% were obtained, respectively, compared with that of the control ($P < 0.05$). The highest breaking force was observed in the surimi gels added with 0.30% CT or 0.15% EKWE oxygenated at pH 9, in which the increases by 136.9 and 157.5% were found, respectively. Similar deformation was noticeable in comparison with that found in gel added with either CT or EKWE oxygenated at pH 8 ($P > 0.05$). The results revealed that pH for the oxidation of phenolic compounds had a pronounced effect on gel properties of mackerel surimi. Breaking force and deformation of gels increased when both CT and EKWE oxygenated at alkaline pHs, were incorporated ($P < 0.05$). This might be due to the fact that the greater formation of quinones took place at alkaline pHs (Table 9). Quinones formed were electrophilic and could react with amino acids in a peptide chain via covalent bond. The covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been studied extensively (Rawel *et al.,* 2002a). On the contrary, the deprotonation of phenolic hydroxyl groups occurred at a low extent at acidic or neutral pHs. As a consequence, hydroxyl groups were mostly present in the reduced forms. Those hydroxyl groups could interact with peptide chain, particularly carbonyl group of peptide backbone via hydrogen bond. Moreover, phenolic compound could cross-link proteins via hydrophobic interaction (Prigent, 2005). All surimi gels added with CT and EKWE oxygenated at all pHs had higher breaking force and deformation as compared with the control gels ($P < 0.05$). It was noted that breaking force of surimi gel could be much improved by addition of CT or EKWE oxygenated at pHs 8 or 9, most likely due to the formation of covalent bonds. The major chemical constituents in wood or bark from different trees were reported to

be tannin with small amount of lignin (Fradinho *et al.,* 2002; Yazaki and Collins, 1994). At pHs 8 and 9, EKWE exhibited the higher gel strengthening effect than did CT, even though the lower amount of the former was incorporated. Apart from tannin, ethanol might extract other phenolic compounds such as lignin from kiam wood. The presence of lignin and other phenolic compounds along with tannin might have the synergistic effect on the improvement of gel strength of surimi. Recently, Balange and Benjakul (2009d) reported that tannin was the major constituent in both water and ethanolic kiam wood extract.

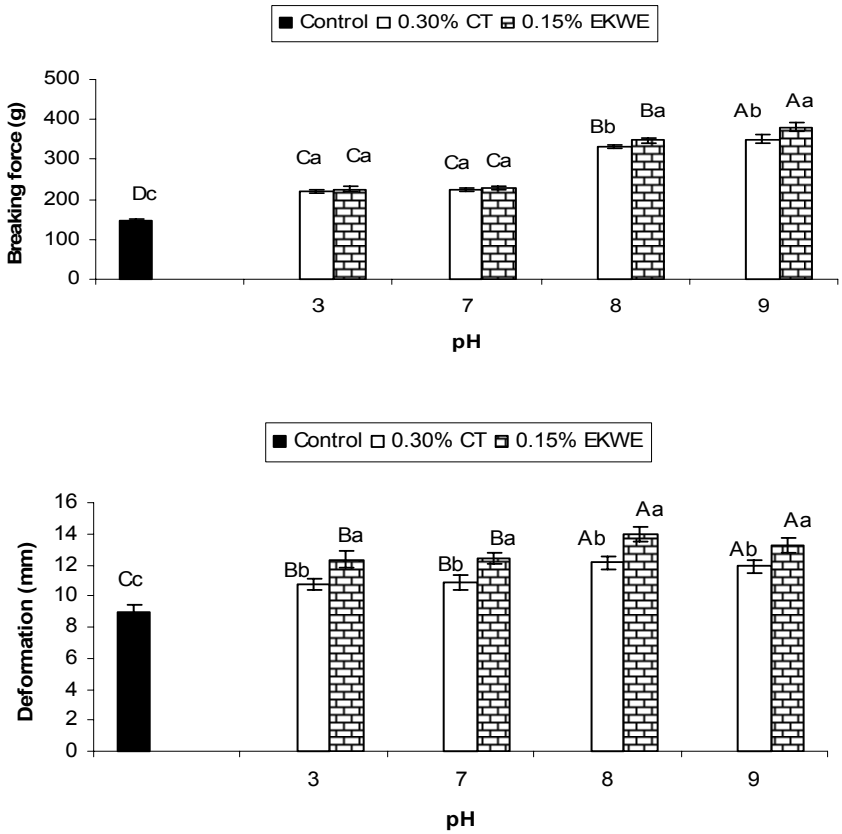


Figure 44. Breaking force and deformation of gels from mackerel surimi added with 0.30% commercial tannin (CT) or 0.15% ethanolic kiam wood extract (EKWE) oxygenated at different pHs. Bars represent the standard deviation (n=3). Different capital letters on the bars within the same source of phenolic compounds together with the control indicate the significant differences ($P < 0.05$). The different letters on the bars within the same pH indicate the significant differences ($P < 0.05$). Both CT and EKWE were neutralized after oxygenation prior to the addition into surimi sol.

Effect of CT and EKWE oxygenated at different pHs on the expressible moisture content of mackerel surimi gel

When CT or EKWE oxygenated at different pHs was added into surimi gel, the expressible moisture content gradually decreased when pHs of both CT and EKWE subjected to oxygenation increased (Table 10). The decreases in expressible moisture contents were in accordance with the increased breaking force and deformation of resulting surimi gels (Figure 44). During setting at 40 ^{o}C, proteins underwent some denaturation and aligned themselves gradually to form the network, which can imbibe water (Benjakul and Visessanguan, 2003). In the presence of CT or EKWE, tannin and other components could interact with unfolded muscle proteins in which the network capable of holding water could be formed. It was suggested that quinones formed at alkaline pHs effectively cross-linked proteins or amino acids, resulting in the formation of stronger network with greater water holding capacity. For gel incorporated with CT or EKWE oxygenated at pHs 3 or 7, the lower expressible moisture content was obtained compared with the control gel. Weak bonds stabilizing gel network including hydrogen bond or hydrophobic interaction might play a role in building up the network which could imbibe the water. However, the efficacy in water holding of gel added with CT or EKWE oxygenated at both pHs was lower than that of gel incorporated with CT or EKWE oxygenated at pH 8 and 9 ($P < 0.05$). Thus, pH of CT or EKWE during oxygenation had the impact on water holding capacity of gel. At the same pH used, EKWE yielded the gel with the lower expressible moisture content than did CT ($P < 0.05$). This result was in agreement with the higher breaking force of gel added with EKWE, particularly those oxygenated at the alkaline pH range.

Effect of CT and EKWE oxygenated at different pHs on whiteness of mackerel surimi gel

The decreases in whiteness of mackerel surimi gels were observed as the levels of pH for oxygenation of CT and EKWE increased up to pH 9 ($P < 0.05$) (Table 10). These results were in agreement with O'Connell and Fox (2001) who

reported that phenolic compounds were responsible for discoloration in the cheese products. The decrease in whiteness was more pronounced in the mackerel surimi gels with the addition of CT or EKWE oxygenated at pHs 8 and 9, compared with pH 3 and 7 ($P < 0.05$). The color of solution becomes darker after being oxygenated in the alkaline pH range, probably due to the dimerization or polymerization of oxidized phenolic compounds (Huang and Chu, 1992). Furthermore, carbonyl of quinone might undergo Maillard reaction with amino group of surimi to a greater extent, compared with the reduced phenolic compounds in both CT or EKWE. Thus, it caused the increased darkening in surimi gel added with CT or EKWE oxygenated at alkaline pH, as indicated by the lowered whiteness. When CT and EKWE with the same pH for oxygenation were added, the resulting surimi gel added with the former had the lower whiteness than that added with the latter. This indicated that other components in EKWE might be less susceptible to darkening than tannin. As a consequence, the darkening was lower in surimi gel added with EKWE.

Table 10. Expressible moisture content and whiteness of gels from mackerel surimi added with commercial tannin (CT) and ethanolic kiam wood extract (EKWE) oxygenated at different pHs.

Sources of phenolic compounds	Amount added (%)	pH of solution	Expressible moisture content (%)	Whiteness
Control	0	-	11.18±0.48a*	69.25±0.36a*
CT	0.30	3	8.46±0.34bA**	67.26±0.34bB**
		7	8.56±0.37bA	67.06±0.42bB
		8	5.37±0.21cA	62.26±0.42cB
		9	4.45±0.39dA	58.22±0.34dB
EKWE	0.15	3	8.10±0.23bA	68.16±0.46bA
		7	8.02±0.45bA	68.76±0.51bA
		8	4.32±0.33cB	66.25±0.55cA
		9	3.12±0.57dB	64.17±0.34dA

* Different letters in the same column within the same source of phenolic compounds together with the control indicate the significant differences ($P < 0.05$).
** Different capital letters in the same column within the same pH used indicate the significant differences ($P < 0.05$).
Values are mean ± standard deviation (n=3).
CT, commercial tannin; EKWE, ethanolic kiam wood extract

Effect of EKWE oxygenated at different pHs on the protein patterns of mackerel surimi gels

Due to the higher efficacy of EKWE in strengthening the surimi gel with the less adverse effect on whiteness, only gels incorporated with 0.15% EKWE oxygenated at different pHs were determined for protein patterns (Figure 45). Surimi sol contained MHC and actin as the major proteins. Decrease in MHC band intensity was found in the control gel (without addition of EKWE), compared with that observed in sol. The result suggested that the formation of cross-linking stabilized by non-disulfide covalent bond mediated by indigenous transglutaminase took place, especially during setting. MHC was most susceptible to cross-linking during setting (Benjakul and Visessanguan, 2003). Benjakul and Visessanguan (2003) reported the decrease in MHC of surimi gel from bigeye snapper, particularly when the setting was implemented. Additionally, autolysis might take place during setting at 40^{o}C. Dark flesh surimi contained higher amount of proteinase, which was associated with modori (Shimizu *et al*., 1992).

For surimi gels added with 0.15% EKWE oxygenated at alkaline pH, MHC band intensity decreased to a higher extent, compared with that found in the control gel. The results suggested that MHC underwent cross-linking induced by oxidized phenolic compounds in EKWE via non-disulfide covalent bonds effectively. For gels added with EKWE oxygenated at pH 3 and pH 7, MHC band was decreased slightly, compared with the control gels. However, MHC band disappeared almost completely when the gels were added with EKWE oxygenated at pHs 8 and 9. The coincidental formation of polymerized proteins was noticeable in the stacking gel. The results reconfirmed that enhanced cross-linking of proteins in surimi was related with the formation of quinones, which acted as the potential protein cross-linkers. The similar protein pattern was observed in case of surimi gels added with CT oxygenated at alkaline pHs (data not shown). Phenols could be oxidized with ease, in an alkaline solution, 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, forming strong covalent bonds (Hurrell and Finot, 1984).

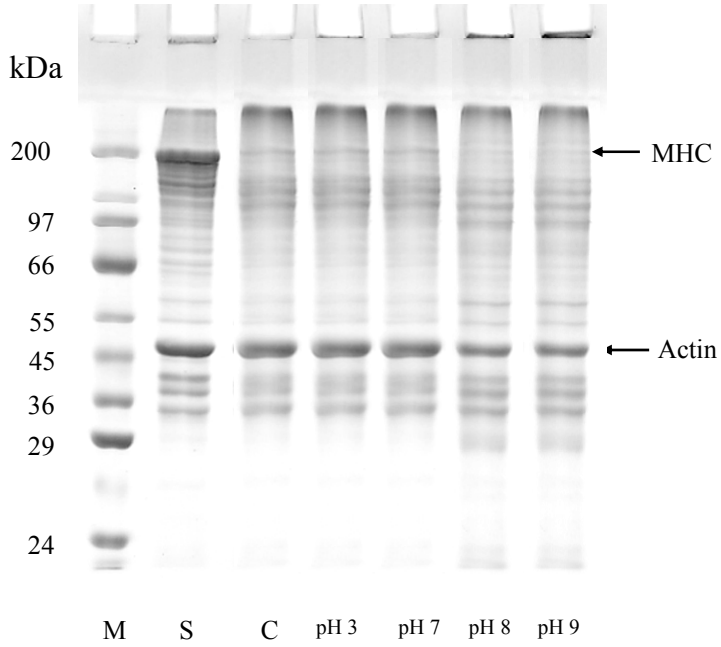


Figure 45. SDS-PAGE patterns of protein in gels from mackerel surimi added with 0.15% ethanolic kiam wood extract (EKWE) oxygenated at different pHs. M, marker; S, surimi sol; MHC, myosin heavy chain; C, the control gel.

Effect of EKWE oxygenated at different pHs on the solubility of mackerel surimi gels

Solubility of surimi gels added with 0.15% EKWE oxygenated at different pHs in different solubilizing solutions is shown in Figure 46. Solubility was found to be 28 and 25% in the control gels without EKWE when solubilized with 0.6M KCl (S_1) and 20 mM Tris-HCl (pH 8.0) (S_2), respectively. Solubility was lower than 20% in the gels added with 0.15% EKWE oxygenated at pH 3 and pH 7 when solubilized with S_1 and S_2. Nevertheless, the lowest solubility was observed in the gels added with 0.15% EKWE oxygenated at pHs 8 and pH 9, when solubilized with S_1 and S_2. Native myofibrillar proteins are normally soluble in high ionic strength buffer (Suzuki, 1981). The decrease in solubility of surimi gel suggested the formation of protein aggregates during setting and heating. When the gels were

solubilized in 20 mM Tris-HCl (pH 8.0) containing 1% SDS (S_3), the solubility was increased up to 65% in the control gel, while gels added with 0.15% EKWE oxygenated at pH 3, 7, 8 and 9 had the increases in solubility by 50, 48, 38 and 28%, respectively. SDS is capable of destroying hydrogen and some hydrophobic interactions (Hamada, 1992). Further increases in solubility were observed in S_4, containing urea and SDS, indicating the presence of hydrophobic and hydrogen bonds in surimi gels. During neutralization of oxidized phenolic solutions before adding into surimi sol, partial regeneration of hydroxyl groups might occur (Table 9). Presence of hydroxyl groups around the benzene rings of tannin facilitated the formation of hydrogen bonds with nitrogen or oxygen of amino acids of the unfolded protein (Prigent, 2005). Furthermore, benzene ring of phenolic compounds facilitated interaction with hydrophobic amino acids, leading to the formation of hydrophobic interaction (Prigent, 2005). Surimi gels added with EKWE oxygenated at pHs 8 and 9 had the lower solubility than those incorporated with EKWE oxygenated at pHs 3 and 7 when S_4 was used. This suggested that covalent bonds were formed in gels added with EKWE oxygenated at pH 8 and 9 to a high extent. When gel samples were solubilized in S_5 containing urea, SDS and βME, it was noticeable that a higher increase in solubility was observed in surimi gels added with EKWE oxygenated at pH 8 and 9 in comparison with that found in S_4. The result indicated the presence of disulfide bonds in the respective gels. When quinones attached to protein molecules, the conformational changes of protein possibly occurred in the way that favored the oxidation of sulfhydryl groups, leading to the increases in disulfide bond formation. Gels added with EKWE oxygenated at alkaline pHs, particularly at pH 9, had a lower solubility in S_5, compared with other gels ($P < 0.05$). Thus, the non-disulfide covalent bonds induced by quinones, an oxidized phenolic compound, could play a partial role in gel strengthening. It was reported that quinone probably attached to amino groups of protein molecules in which the subsequent inter-molecular cross-linking could be formed (Strauss and Gibson, 2004; Rawel *et al.,* 2002a). The solubility result reconfirmed the gel enhancing effect of oxidized phenolic compounds in EKWE via both disulfide and non-disulfide covalent bonds.

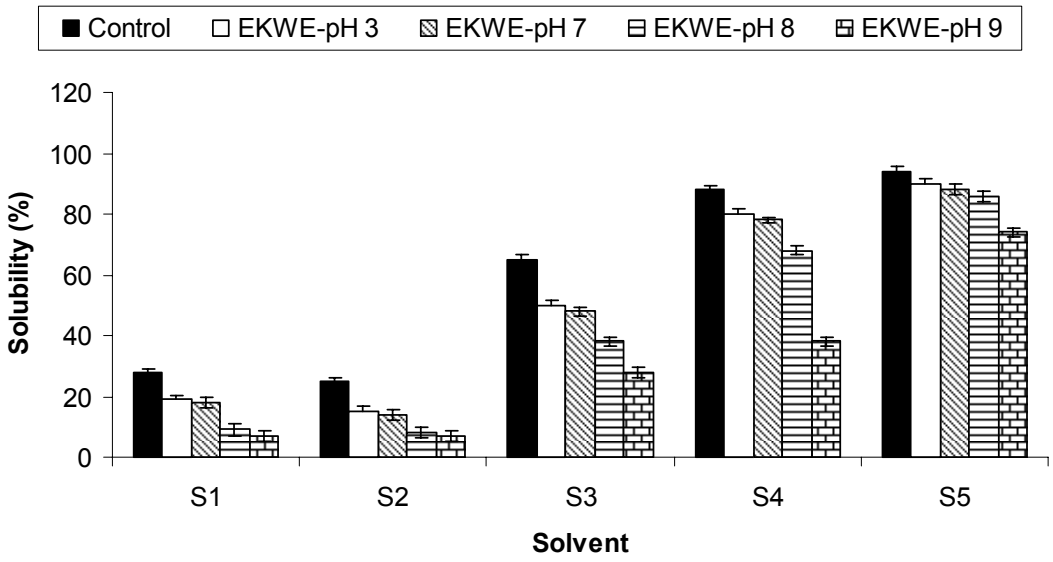
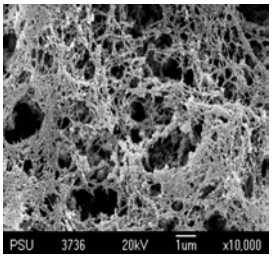


Figure 46. Solubility of mackerel surimi gels added with 0.15% ethanolic kiam wood extract (EKWE) oxygenated at different pHs. Samples were solubilized in different solvents and soluble protein was determined by the biuret assay. 0.6 M KCl (S1); 20 mM Tris–HCl, pH 8.0 (S2); 20 mM Tris–HCl, pH 8.0, containing 1% SDS (S3); 20 mM Tris–HCl, pH 8.0, containing 1% SDS and 8 M urea (S4) and 20 mM Tris–HCl, pH 8.0, containing 1% SDS, 8 M urea and 2% β-mercaptoethanol (S5). Bars represent the standard deviation (n=3)**.**

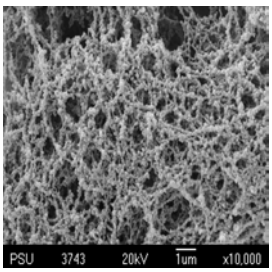
Effect of EKWE oxygenated at different pH on the microstructures of gels from mackerel surimi

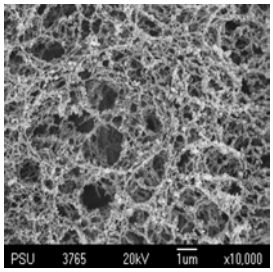
Microstructures of gels from mackerel surimi without (A) and with 0.15% EKWE oxygenated at different pHs (B) are illustrated in Figure 47. Surimi gels containing 0.15% EKWE oxygenated at pHs 3 and 7 had a finer gel network with less number of voids, compared with the control gel. This might be attributed to the formation of hydrophobic interaction and hydrogen bonds between phenolic compounds and muscle proteins in gel network. Surimi gels containing 0.15% EKWE oxygenated at pHs 8 and 9 had a finer gel and continuous matrix than other treatments. Nevertheless, the gel added with 0.15% EKWE oxygenated at pH 9 had very compact and denser network. This suggested that phenolic compounds oxidized

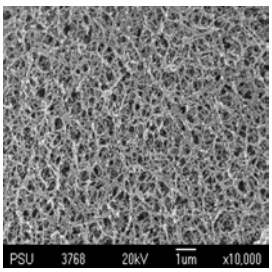
at alkaline pH range effectively induced the cross-linking of proteins, in which inter-connection between protein molecules could be formed to a higher extent. Those cross-links further underwent polymerization, leading to the formation of gel network with fibrillar structure. In addition to tannin, other polyphenolic compounds in EKWE might function synergistically in developing the gel matrix. The finer and more ordered structure of gels added with EKWE oxygenated at alkaline pHs correlated with the higher breaking force and deformation (Figure 44) as well as the lower expressible moisture content of the resulting gels (Table 10).

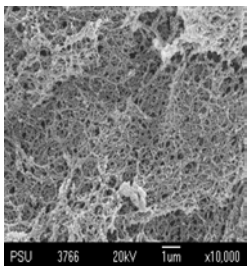


A) Control









pH-3 pH-7 pH-8 pH-9

B) 0.15% EKWE

Figure 47. Electron microscopic images of gels from mackerel surimi added without and with 0.15% ethanolic kiam wood extract (EKWE) oxygenated at different pHs (Magnification: 10,000X). EKWE were neutralized after oxygenation prior to the addition into surimi sol.

Effect of EKWE oxygenated at different pHs on the sensory properties of mackerel surimi gels

Likeness scores of the control gels (without EKWE) and those containing 0.15% EKWE oxygenated at different pHs are depicted in Figure 48. Addition of 0.15% EKWE oxygenated at pHs 3, 7 and 8 had no negative impact on the color, appearance, odor, taste, texture and overall likeness of resulting gels ($P > 0.05$). However, the addition of 0.15% EKWE oxygenated at pH 9 resulted in the lower score for the appearance and color of resulting surimi gels, compared with other treatments including the control ($P < 0.05$). The result was in accordance with the lowest whiteness of gel added with EKWE oxygenated at pH 9 (Table 10). Nevertheless, the addition of 0.15% EKWE oxygenated at pHs 8 and 9 yielded the gel with the higher texture score, compared with other gels ($P < 0.05$). Higher texture score was also found in gels added with EKWE oxygenated at pHs 3 and 7, compared with that of the control gel ($P < 0.05$). This was coincidental with the increased breaking force and deformation in the surimi gels added with EKWE oxygenated at pHs 8 and pH 9 (Figure 44). Natural extracts containing polyphenols have been used in many food products and their effects on the sensory properties vary with the types of food (Ioki and Suzuki, 1992; O'Connell and Fox, 2001; Balange and Benjakul, 2009d). Among all gel samples, those added with 0.15% EKWE oxygenated at pH 8 had the highest overall likeness score. Therefore, the addition of 0.15% EKWE oxygenated at pH 8 in this study had the positive impact on the overall sensory properties of mackerel surimi gels.

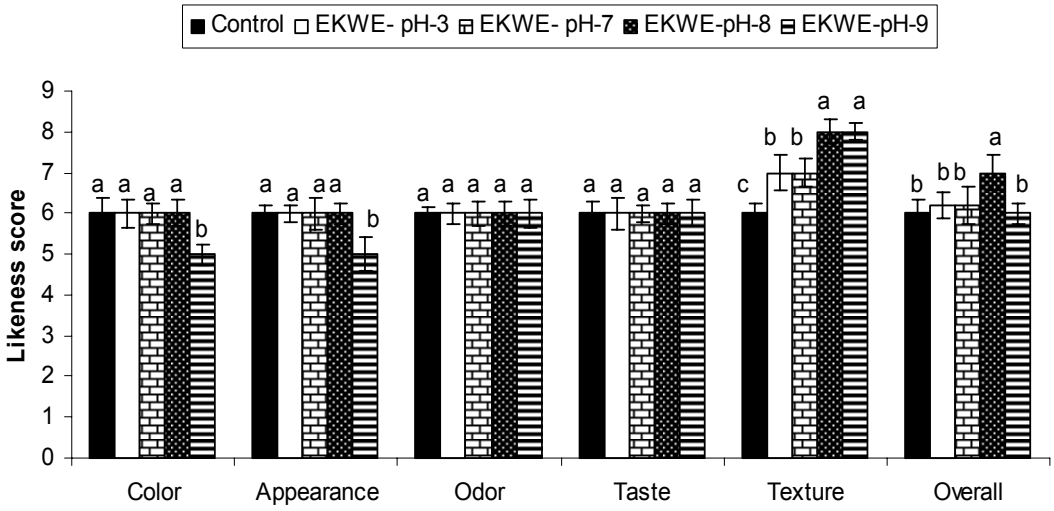


Figure 48. Likeness score of surimi gels from mackerel added with 0.15% ethanolic kiam wood extract (EKWE) oxygenated at different pHs. Bars represent standard deviation (n=30). Different letters on the bars within the same sensory attribute indicate significant differences ($P < 0.05$). EKWE were neutralized after oxygenation prior to the addition into surimi sol.

7.5 Conclusions

The pH of CT and EKWE solution subjected to oxygenation was the key factor determining the oxidation of phenolic compounds, in which quinones were formed. Alkaline condition favored the formation of quinones which enhanced the cross-linking of proteins via both disulfide and non-disulfide covalent bonds. Therefore, incorporation of 0.15% EKWE oxygenated at pH 8 into the surimi yielded the gels with the improved mechanical and sensory properties.

CHAPTER 8

GEL STRENGTHENING EFFECT OF WOOD EXTRACT ON SURIMI PRODUCED FROM MACKEREL STORED IN ICE

8.1 Abstract

The effect of ethanolic kiam wood extract (EKWE) and commercial tannin (CT) on the gel properties of surimi produced from mackerel (*Rastrelliger kanagurta*) stored in ice for different times (0-12 days) was studied. During 12 days of iced storage, pH, total volatile base (TVB), trimethylamine (TMA) and trichloroacetic acid (TCA)-soluble peptide contents as well as thiobarbituric reactive substances (TBARS) of mackerel mince increased while myosin heavy chain (MHC) band intensity decreased continuously ($P < 0.05$). The result suggested that deterioration, protein degradation and lipid oxidation proceeded with increasing storage time. For corresponding surimi, TVB and TMA were almost removed and TBARS and TCA soluble peptide content were decreased. Conversely, MHC became more concentrated. Decreases in gel forming ability of surimi were observed when fish used as raw material were stored in ice for a longer time, regardless of EKWE or CT addition. Whiteness of surimi gel decreased and expressible moisture increased especially when the storage time increased. However, superior breaking force and deformation of surimi gel, added with 0.15% EKWE or 0.30% CT, to those of the control gel were observed during the first 6 days of the storage. Thereafter, EKWE and CT had no gel enhancing effect on surimi. Therefore, freshness was a crucial factor determining gel enhancing ability of EKWE or CT towards mackerel surimi.

8.2 Introduction

Freshness of fish is generally considered as the most important factor determining the gel-forming ability of surimi. Time and temperature of post-mortem storage of fish can affect the final surimi quality (Park and Morrissey, 2000). The rate of deterioration is associated with many factors such as fish species, size, lipid content, state at the moment of capture, microbial load, and storage temperature. Fresh fish are extremely perishable and should be handled with a great care. Low-temperature storage, especially iced storage, is one of the primary methods to maintain fish freshness (Benjakul *et al*., 2002). However, microbiological, chemical and physical changes of fish muscle still occur during iced storage (Benjakul *et al.*, 2003; Riebroy *et al.*, 2007). Lower gel quality is generally associated with extended storage times in ice and the rate of loss of gel forming ability appears to vary among species (Benjakul *et al.*, 2002; Benjakul *et al.*, 2003). The gel strength of kamaboko made from lizardfish kept in ice for 3 days was 50% of that made from fresh fish (Kurokawa, 1979). Northern squawfish surimi could be made from fish stored for up to 9 days (Lin and Morrissey, 1995). Generally, the prolonged holding times and elevated temperatures can cause severe proteolysis of myofibrillar proteins, which directly results in an inferior gel quality (Suzuki, 1981). During handling, leakage of digestive enzymes into the muscle contributes to the subsequent hydrolysis of muscle proteins. Degradation of myosin heavy chain (MHC) also occurred in Pacific whiting muscle during iced storage (Benjakul *et al.*, 1997).

Mackerel (*Rastrelliger kanagurta*) is a pelagic fish species containing a high amount of dark muscle, fat and myoglobin. This makes it less appealing for human acceptance and is considered as underutilized species (Chaijan *et al.*, 2004). When dark fleshed fish are used for surimi production, the dark color and low gel forming ability of surimi still limit the manufacturing and marketing. To improve surimi gel properties, several approaches such as the addition of protein additives (Benjakul *et al.*, 2004c; Julavittayanukul *et al.*, 2006), an appropriate setting (Benjakul *et al.*, 2004b), and the use of microbial transglutaminase (Benjakul *et al.*,

2008a) have been implemented. Also, the washing process was shown to enhance the gel strength of surimi (Park and Morrissey, 2000).

Phenolic compounds are natural additives and can be derived from different parts of the plants (Shahidi and Naczk, 2004). Recently, phenolic-protein interaction has been used extensively for improving the food properties (Kroll *et al.,* 2003). Balange and Benjakul (2009a, b, c) used the phenolic compounds in oxidized form to improve the gel strength of mackerel surimi. Since the oxidized phenolic compounds can induce the cross-linking of proteins, they might be able to cross-link the degraded proteins found in surimi with poor quality. Therefore, the gel-forming ability can be recovered in surimi produced from low quality fish associated with the degradation during extended iced storage. Recently, kiam wood extracts containing tannin was reported to improve the gel strength of mackerel surimi (Balange and Benjakul, 2009d). The objective of this study was to investigate the changes in chemical composition of mackerel during iced storage and to study the impact of oxidized kiam wood extract and oxidized tannic acid on gel properties of surimi prepared from mackerel kept in ice for different times.

8.3 Materials and Methods

Chemicals

Tannic acid (TA), sodium hydroxide (NaOH), hydrochloric acid, sodium carbonate, $CuSO_4.5H_2O$, bovine serum albumin, Folin-Ciocalteu reagent and β-mercaptoethanol (βME) were obtained from Sigma (St. Louis, MO, USA). Sodium dedocyl sulfate (SDS), *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA**).** Sodium chloride and ethanol were obtained from Merck (Darmstadt, Germany).

Preparation of kiam wood extracts

Collection of kiam wood

Kiam wood was obtained from a forest of the Phattalung province in the Southern Thailand. The tree was about 15 – 20 years old and harvested in June, 2008. The tree was cut by using a sawing machine; the leaves and branches were separated manually by cutting and the trunk was sun-dried for three months. The pieces of wood with an average thickness of 1.5 cm were dried in an oven at 70 ^{o}C for 8 h. Prepared wood was then subjected to a portable grinding machine (Spong-90, Leeds, England) with a sieve size of 6 mm. This coarse form was further blended using a blender (National Model MK-K77, Tokyo, Japan) and finally sieved using a stainless steel sieve of 80 mesh size. The kiam wood powder obtained was further subjected to extraction.

Extraction of phenolic compounds from kiam wood using ethanol

Ethanol extract from kiam wood powder was prepared as per the method of Santoso *et al.* (2004) with slight modifications. Wood powder (10 g) was mixed with 150 ml of absolute ethanol. The mixture was homogenized for 2 min at 15000 rpm using a homogenizer (IKA Labortechnik, Selangor, Malaysia) and stirred at room temperature using a magnetic stirrer (IKA-Werke, Staufen, Germany) at room temperature (28-30 ^{o}C) for 3 h. The homogenate was then centrifuged at 5000 x g for 10 min at 25 ^{o}C using a Beckman centrifuge (Beckman Coulter Inc., Avanti –J-E Centrifuge, Fullerton, CA, USA). The supernatant was filtered using Whatman filter paper No.1 (Whatman Schleicher & Schuell, Maidstone, England). The filtrate was then evaporated at 40 ^{o}C using a rotary evaporator model N-1000 (Eyela, Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) until ethanol was almost removed. The extract was then dried in a forced-air oven at 70 ^{o}C for 4 h. Dried extract was powdered using a mortar and pestle. Extract powder referred to as ‘ethanolic kiam wood extract, EKWE’ was kept in a desiccator at 28-30 ^{o}C until used.

Preparation and iced storage of fish samples

Mackerel (*Rastrelliger kanagurta*) with an average weight of 85-90 g were caught from Songkhla coast along the Gulf of Thailand during February-March, 2009. Fish were stored in ice and off-loaded approximately 36 h after capture. Upon the arrival to the dock in Songkhla, fish were iced 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 immediately washed and kept in a styrene foam box containing crushed ice. The fish were placed and distributed uniformly between the layers of ice using a fish/ice ratio of 1:2 (w/w). The box was kept at room temperature (27–30 ^{o}C) for up to 12 days. To maintain the ice content, the molten ice was removed and replaced with an equal amount of ice every 2 days. At the time designated (0, 3, 6, 9 and 12 days), fish were washed and filleted manually. The flesh was minced to uniformity using a mincer with a hole diameter of 5 mm. The mince was subjected to chemical analyses and surimi production.

Preparation of surimi

Surimi was prepared according to the method of Shimizu (1965) with a slight modification. Mince was suspended in four volumes of cold (5 ^{o}C) diluted alkaline salt solution (0.15% NaCl in 0.2% $NaHCO_3$ of which the ionic strength was 0.05 and the final pH was 7.0-7.1). The mixture was stirred gently for 20 min and the washed mince was filtered with a layer of nylon screen (Butterfly brand, Lao Hah Seng Lee Co., Ltd., Bangkok, Thailand). The washing process was repeated twice. Finally, the washed mince was subjected to centrifugation at a speed of 700 x g at 4 ^{o}C for 10 min using a Model CE 21K basket centrifuge (Grandiumpiant, Belluno, Italy). To the washed mince, 4% sucrose and 4% sorbitol were added and mixed well. A portion of mixture (500 g) was packaged in polyethylene bag and frozen using an air-blast freezer at -20 ^{o}C (Patkol, Patanakolkarn Co.Ltd., Bangkok, Thailand). Frozen surimi was stored at -20 ^{o}C for no longer than 2 weeks. Surimi obtained was subjected to chemical analyses and gel preparation.

Chemical analyses

Determination of pH

The pH was determined according to the method of Benjakul *et al.* (1997). Sample was added with 10 volumes of deionized water (w/v) and homogenized at 11,000 rpm for 60 s using an IKA homogenizer (Model T25, Selangor, Malaysia). The pH of homogenate was measured using a pH meter (Sartorious, PB 10, Goettingen, Germany)**.**

Determination of total volatile base (TVB) and trimethylamine (TMA) contents

TVB and TMA contents were determined using the Conway microdiffusion assay as described by Ng (1987). A sample (2 g) was added to 8 ml of 4% trichloroacetic acid (TCA) (w/v) and homogenized with an IKA homogenizer at a speed of 11,000 rpm for 2 min. The homogenate was centrifuged at 3000x g for 15 min using a Beckman centrifuge (Beckman Coulter Inc., Avanti –J-E Centrifuge, Fullerton, CA, USA) at room temperature. The supernatant referred to as 'sample extract' (1 ml) was placed in the outer ring of the Conway apparatus. The inner ring solution (1% boric acid containing the Conway indicator) was then pipetted into the inner ring. To initiate the reaction, K_2CO_3 (1 ml) was mixed with the sample extract. The Conway unit was closed and incubated at 37 ^{o}C for 60 min. The inner ring solution was then titrated with 0.02 N HCl until the green color turned to pink. Determination of TMA content was done in the same manner except that 1 ml of 10% formaldehyde was added to the sample extract to fix ammonia present in the sample prior to the assay.

Determination of thiobarbituric acid reactive substances (TBARS)

TBARS value was determined according to the method of Buege and Aust (1978). A sample (5 g) was homogenized with 25 ml of TBARS solution (0.375% TBA, 15% TCA, and 0.25 N HCl) at a speed of 11000 rpm for 1 min using an IKA homogenizer. The homogenate was heated for 10 min in boiling water (95–100 ^{o}C) to develop a pink color. Then the mixture was cooled with running water and

centrifuged at 5500x g for 25 min at 25 ^{o}C using a Beckman centrifuge.The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The TBARS value was calculated from the standard curve of malonaldehyde and expressed as mg malonaldehyde/kg sample.

Determination of TCA-soluble peptides

TCA-soluble peptide content was determined according to the method of Green and Babbitt (1990). A sample (3 g) was homogenized with 27 ml of 15% TCA at a speed of 11000 rpm for 1 min using an IKA homogenizer. The homogenate was kept in ice for 1 h and centrifuged at 12,000x g for 5 min at 4 ^{o}C using a Beckman centrifuge. The soluble peptides in the supernatant were measured by the method of Lowry *et al.* (1951) and expressed as μmol tyrosine/g sample.

Effect of oxidized ethanolic kiam wood extract (EKWE) and oxidized commercial tannin (CT) on gel properties of surimi prepared from ice-stored mackerel

Preparation of oxidized kiam wood extract and oxidized tannin

EKWE and CT were oxidized according to the method of Strauss and Gibson (2004) with slight modifications. The extract solutions (100 ml; 1% w/v) were adjusted to pH 8 using 6 M NaOH or 6 M HCl. The prepared solutions were placed in a temperature-controlled water bath (Memmert, Schwabach, Germany) at 40 ^{o}C and subjected to oxygenation for 1 h by bubbling the solution with oxygen with the purity of 99.5-100% (TTS Gas Agency, Hat Yai, Songkhla, Thailand) to convert phenolic compound to quinone. After being oxygenated for 1 h, the solutions were then adjusted to pH 7 by using 6 M HCl. Both oxidized CT and EKWE were used as the additives in surimi gels.

Surimi gel preparation

Frozen surimi was tempered for 30 min in running water (26-28 ^{o}C) until the core temperature reached 0-2 ^{o}C. The surimi was then cut into small pieces with an approximate thickness of 1 cm and placed in a mixer (National Model MK-K77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% salt was added. Oxidized CT and EKWE solutions were added into the sols at levels of 0.30 and 0.15% (based on protein content), respectively (Balange and Benjakul, 2009d). The mixtures were chopped for 4 min at 4 ^{o}C to obtain the homogeneous sols. The sols were then stuffed into polyvinylidine casings with a diameter of 2.5 cm and both ends of the casings were sealed tightly. Sols were incubated at 40 ^{o}C for 30 min, followed by heating at 90 ^{o}C for 20 min (Benjakul and Visessanguan, 2003). All gels were cooled in iced water and stored overnight at 4 ^{o}C prior to analyses.

Measurement of surimi gel properties

Texture analysis

Texture analysis of gels was performed using a texture analyzer Model TA-XT2 (Stable Micro Systems, Surrey, England). Gels were equilibrated and tested at room temperature. Five cylinder-shaped samples of 2.5 cm in length were prepared. The breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyzer equipped with a spherical plunger (5 mm diameter). The probe was pressed into the cut surface of a gel specimen perpendicularly at a constant plunger speed (60 mm min^{-1}) until the puncture occurred. The force in gram (g) required to puncture into the gel (breaking force) and the distance (mm) at which the spherical probe punctured into the gel (deformation) were recorded.

Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al*. (2001b) with a slight modification. Gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between 3 pieces of Whatman paper No. 4

at the bottom and 2 pieces on the top of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The samples were then removed from the papers and weighed again (*Y*). Expressible moisture content was calculated using the following equation:

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

Determination of whiteness

Color of gels was determined using a JP7100F colorimeter (Juki Corporation, Tokyo, Japan). *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured and whiteness was calculated as described by Lanier *et al.* (1991) as follows:

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

Sodium dedocyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of samples were analyzed under a reducing condition by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85 ^{o}C were added to the sample (3 g). The mixture was then homogenized at a speed of 11000 rpm for 2 min using an IKA homogenizer. The homogenate was incubated at 85 ^{o}C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500 x g for 20 min to remove undissolved debris. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard. The sample was then 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 1:1 ratio (v/v). The samples (20 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.

Statistical analysis

All experiments were run in triplicate. For each run, chemical analyses were performed in triplicate. For physical analyses, e.g. expressible moisture, whiteness and textural properties, 5 determinations were conducted. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range tests (Steel and Torrie, 1980). T-test was used for pair comparison. Analysis was performed using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA).

8.4 Results and Discussion

Chemical changes in mince and corresponding surimi from mackerel stored in ice for different times

Changes in pH

Changes in pH of mackerel mince and the resulting surimi during iced storage of 12 days are shown in Figure 49. Mackerel mince had the pH of 6.2 at day 0 of storage. Generally, the dark muscle fish has the low pH values due to the post-mortem glycolytic reaction, in which lactic acid is accumulated (Hultin and Kelleher, 2000). For corresponding surimi, a higher pH (6.9) was obtained. During washing, the acidic compounds, particularly lactic acid might be leached out. This led to the increased pH in surimi. Additionally, the neutralization of acids by sodium bicarbonate in the washing medium might contribute to the raised pH. During iced storage, the continuous increase in the pH of fish mince was observed up to 12 days *(*$P < 0.05$). Nevertheless, no differences in pH of surimi were found within the first 6 days of the storage *(*$P > 0.05$*)*. Thereafter a slight increase in pH was found *(*$P < 0.05$). The increase in pH was postulated to be due to an increase in volatile bases produced by either endogenous or microbial enzymes (Benjakul *et al.*, 2002; Benjakul *et al.*, 2003b; Riebroy *et al.*, 2007). Decomposition of nitrogenous compounds causes an increase in pH in fish flesh (Sikorski *et al.*, 1990). The changes in pH also depend

on the liberation of inorganic phosphate and ammonia due to the enzymatic degradation of ATP (Sikorski *et al.*, 1990).

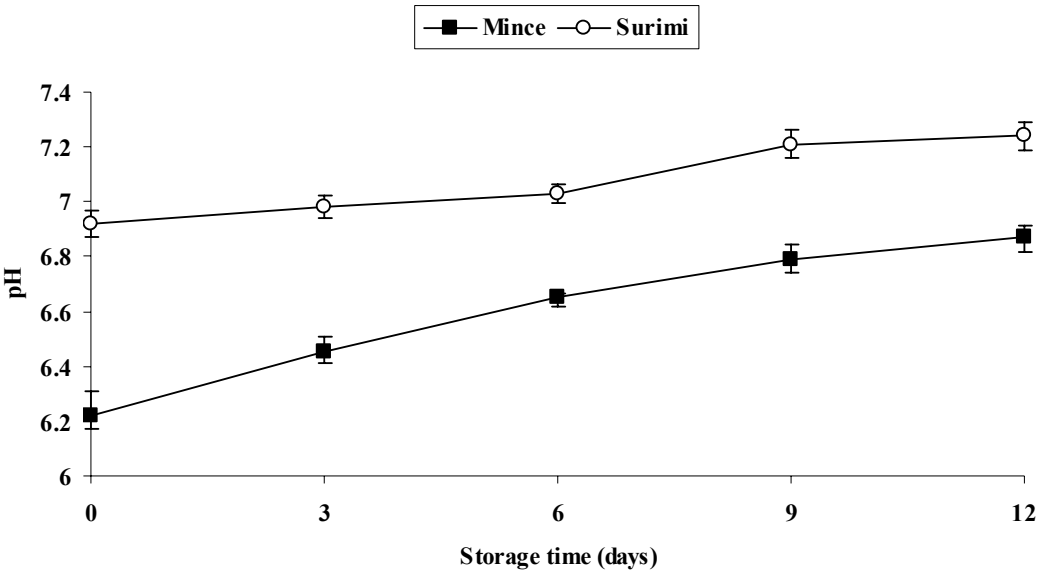


Figure 49. Changes in pH of mince and resulting surimi from mackerel stored in ice for different times. Bars represent standard deviation from three determinations (n=3). The different letters within the same sample indicate the significant differences ($P < 0.05$).

Changes in TVB and TMA contents

TVB and TMA contents of mackerel mince and the corresponding surimi were monitored during iced storage of 12 days as depicted in Figure 50a and b, respectively. At day 0 of storage, the initial TVB content of mince was 4.17 mg N/100 g (Figure 50a), whereas the negligible content of TMA was obtained. Surimi produced from fresh mackerel had TVB and TMA of 0.883 and 0.431 mg N/ 100g sample, respectively. When storage time increased, both TVB and TMA contents in mince increased ($P < 0.05$). For surimi, both TVB and TMA contents remained constant throughout the storage of 12 days ($P > 0.05$) and the contents were much lower than those found in mince ($P < 0.05$). These results indicated that TVB and TMA contents could be reduced markedly by the alkaline saline washing process. The increases in TVB and TMA contents in mince indicated that the decomposition of

nitrogenous compounds became more pronounced with increasing storage time. The increases in both TVB and TMA contents in mince were in accordance with the increase in pH, especially when the storage time increased (Figure 49). The formation of TVB is generally associated with the growth of microorganisms and can be used as an indicator of spoilage (Benjakul *et al.,* 2002). Generally, TVB comprise mainly TMA and ammonia, which are produced by both microbial and endogenous enzymes. A number of specific spoilage bacteria such as *Shewanella putrefaciens, Photobacterium phosphoreum, Vibrionaceae,* etc. typically use TMAO as an electron acceptor in anaerobic respiration, resulting in off-odor and off-flavor due to the formation of TMA (Gram and Huss, 1996). Since fish samples were kept in ice, the formation of TVB and TMA was probably mediated by psychrotropic bacteria (Sasajima, 1974). TVB content of 30 mg N/100 g is generally regarded as the fish acceptability limit (Sikorski *et al.*, 1990). Up to 12 days of iced storage, TVB content of mackerel mince was lower than the limiting level (26.12 mg N/100 g). Washing process used for surimi production could remove volatile base compounds including TMA effectively. This could reduce offensive odor, particularly fishy odor, in resulting surimi.

Changes in TBARS

Changes in TBARS value of mackerel mince and the corresponding surimi during iced storage are shown in Figure 50c. Mince contained TBARS of 5.51 mg MDA/kg sample at day 0, indicating that lipid oxidation took place during handling after capture. However, corresponding surimi had the negligible TBARS. TBARS of mince and surimi increased continuously during storage up to 9 days ($P < 0.05$), indicating that lipid oxidation occurred intensively during the extended iced storage. Fish muscle typically has a high content of polyunsaturated fatty acids and is consequently prone to oxidative reaction (Stamman *et al.*, 1990). TBARS has been widely used to indicate lipid oxidation in meat and meat products (Jo and Anh, 2000). The lipid oxidation can be initiated and accelerated by various mechanisms including the production of singlet oxygen, enzymatic and non-enzymatic generation of free radicals and active oxygen (Kubow, 1992). In general, surimi possessed the lower TBARS than did mince at all storage time used ($P < 0.05$). This was probably due to

the removal of some lipids, pro-oxidants, particularly heme protein, as well as oxidation products from mince during washing. At day 12 of storage, the sharp decrease in TBARS was noticeable ($P < 0.05$). This might be caused by the loss of volatile lipid oxidation products at the extended time of storage.

Changes in TCA-soluble peptide content

TCA-soluble peptide content in mackerel mince and the resulting surimi during iced storage is illustrated in Figure 50d. At day 0, TCA-soluble peptide content in mince and surimi were 0.42 and 0.08 μmol tyrosine/g sample, respectively. TCA-soluble peptides detected in mince at day 0 indicated the indigenous oligopeptides and free amino acids as well as degradation products accumulated during post-harvest handling (Benjakul *et al.*, 1997). During the extended storage, TCA-soluble peptide content in both mince and surimi increased continuously up to 12 days of storage ($P < 0.05$). This suggested that proteolysis caused by either indigenous or microbial proteases, took place during the storage of mackerel. Cathepsins play a role in autolysis of fish myofibrillar protein during the post-mortem storage (An *et al.*, 1994; Riebroy *et al.*, 2007). It was noted that TCA-soluble peptide content in surimi produced from ice-stored fish was lower than that of mince at the same storage time. Washing might remove the small peptides, resulting in the lowered TCA-soluble peptides in surimi. Protease from *Pseudomonas marinoglutinosa* was reported to hydrolyze actomyosin at 0–2 ^{o}C and the optimal pH was above 7.0 (Venugopal *et al.*, 1983). The result suggested that washing process could remove peptides with low molecular weight leading to the concentration of the larger MW proteins or peptides, which generally involve in gelation.

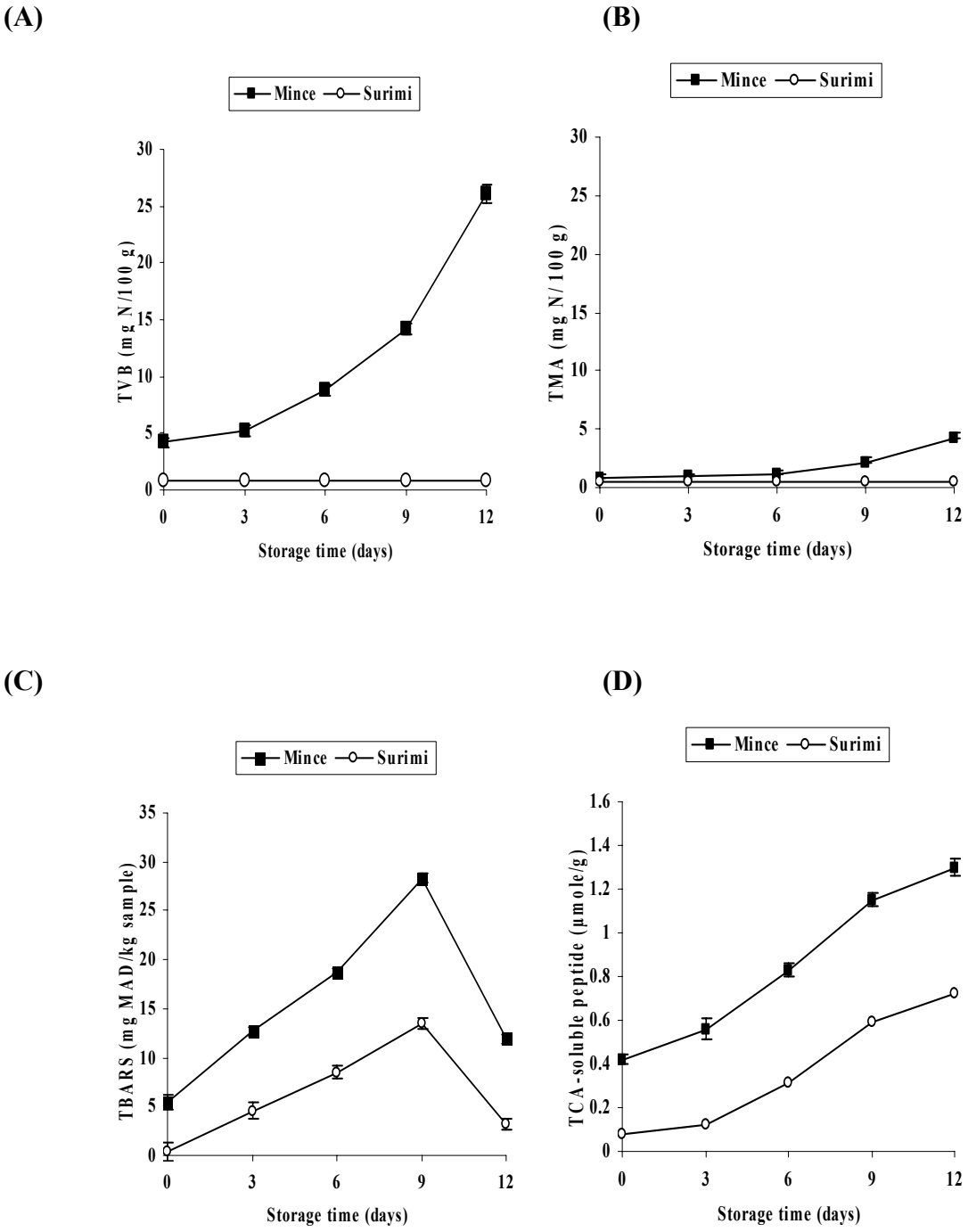


Figure 50. Changes in TVB (A), TMA (B), TBARS (C) and TCA-soluble peptide contents (D) in mince and resulting surimi from mackerel stored in ice for different times. Bars represent standard deviation (n=3). The different letters within the same sample indicate the significant differences ($P < 0.05$).

Changes in protein patterns

Protein patterns of mince and surimi prepared from mackerel stored in ice for different times are shown in Figure 51. The lower band intensity of myosin heavy chain (MHC) and actin was found in mince, in comparison with corresponding surimi. This might be attributed to the effective removal of interfering low molecular weight components, especially sarcoplasmic proteins. Sarcoplasmic proteins are soluble in water and salt solutions of low ionic strength of 0.05 (Govindan, 1985). Solubility of sarcoplasmic proteins of dark fleshed species is increased in "alkaline saline leaching" solution (Shimizu, 1965). The greater removal of sarcoplasmic proteins from the mince by alkaline-saline washing resulted in the higher concentration of myofibrillar proteins including MHC and actin in the surimi.

MHC band intensity of both mince and surimi prepared from mackerel stored in ice decreased continuously with the increasing storage period up to 12 days. Nevertheless, no marked changes were noticeable in the actin band intensity of both mince and surimi prepared from ice-stored mackrel, irrespective of the storage period. SDS–PAGE patterns revealed that MHC was much more susceptible to hydrolysis than was actin. This result was in agreement with Benjakul *et al.* (1997) who reported that MHC was more prone to proteolytic degradation than other muscle proteins, e.g. actin, troponin, and tropomyosin.

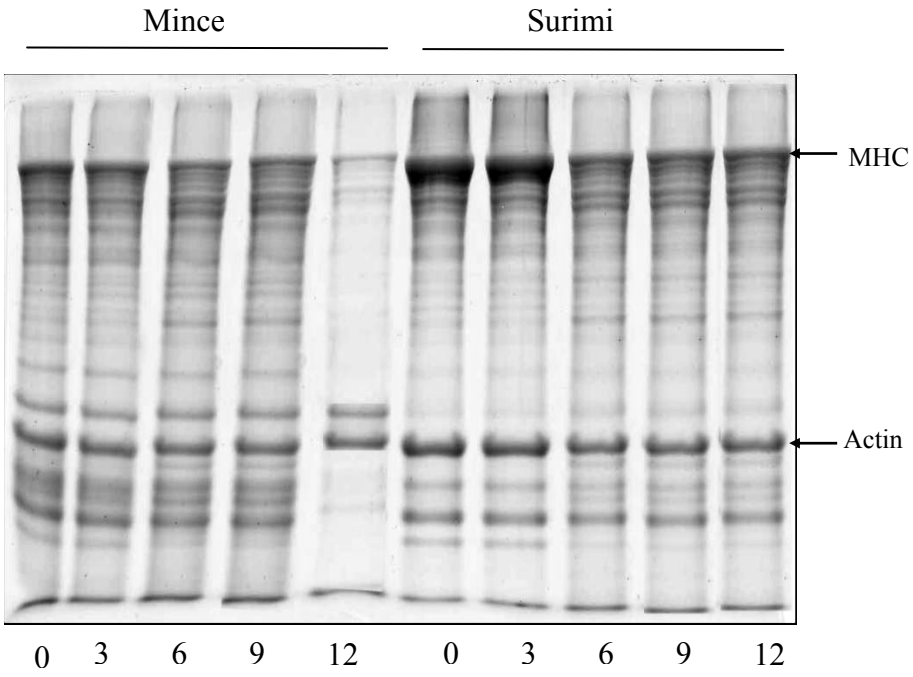


Figure 51. SDS–PAGE patterns of mince and resulting surimi from mackerel stored in ice for different times. Numbers designate storage time (days). MHC, myosin heavy chain.

Effect of oxidized ethanolic kiam wood extract (EKWE) and commercial tannin (CT) on gel properties of surimi prepared from ice-stored mackerel

Breaking force and deformation of mackerel surimi gel

Breaking force and deformation of surimi gels prepared from mackerel stored in ice for different times without and with 0.15% EKWE or 0.30% CT are shown in Figure 52. At day 0 of storage, surimi gels added with EKWE or CT had the increases in breaking force by 112.05 or 130.15% and in deformation by 33.81 or 50%, respectively, compared with those of the control ($P < 0.05$). This might be attributed to the cross-linking activity of oxidized tannin, which could induce the formation of both covalent and non-covalent bonds of gel matrix (Prigent *et al.*, 2003). EKWE has been reported to contain a high amount of tannin (Balange and Benjakul, 2009d). The result was in accordance with Balange and Benjakul (2009a) who reported the increases in breaking force and deformation of bigeye snapper surimi with the addition of oxidized tannin. The decreases in breaking force and

deformation of surimi were observed with increasing storage time of mackerel ($P < 0.05$). The decreases were concomitant with the increase in TCA-soluble peptide content (Figure 50d) and the decrease in MHC band intensity of surimi (Figure 51). Myosin integrity is of paramount importance for gelation (An *et al.*, 1996). The degradation of MHC resulted in an inferior gel network formation, causing a lower elasticity with poor water-holding capacity in the gel matrix. Yean (1993) also found a decrease in gel strength of surimi produced from threadfin bream stored in ice for more than 2 days. Benjakul *et al.* (2003a) reported a decrease in gel strength of surimi from lizardfish and bigeye snapper stored in ice with the extended time.

During 0-6 days of storage, gel added with EKWE showed the higher breaking force and deformation, compared with gel added with 0.30% CT ($P < 0.05$). This might be attributed to the presence of lignin along with tannin in the extracts. Lignin, also being a polyphenolic compound with two hydroxyl groups and two benzene rings, might function as a synergist to tannin in improving the gel strength of mackerel surimi. The major chemical constituents in the bark and wood from different trees were reported to be tannin with small amount of lignin (Fradinho *et al.*, 2002; Yazaki and Collins, 1994). However, no differences in breaking force and deformation of surimi gels without and with EKWE or CT were noticeable when the storage time was greater than 6 days ($P > 0.05$). With increasing storage time, the degradation was more pronounced. Partially hydrolyzed proteins with lower molecular weight were retained after washing, while very low molecular weight peptides were leached out. Those smaller peptides might be preferably cross-linked with the multiple binding sites of quinones in oxidized EKWE or CT. As a consequence, quinones became less available for cross-linking of high molecular weight proteins or peptides. Additionally, the short chain peptides could not form the junction zones, in which the interconnection could be formed, even in the presence of oxidized EKWE or CT.

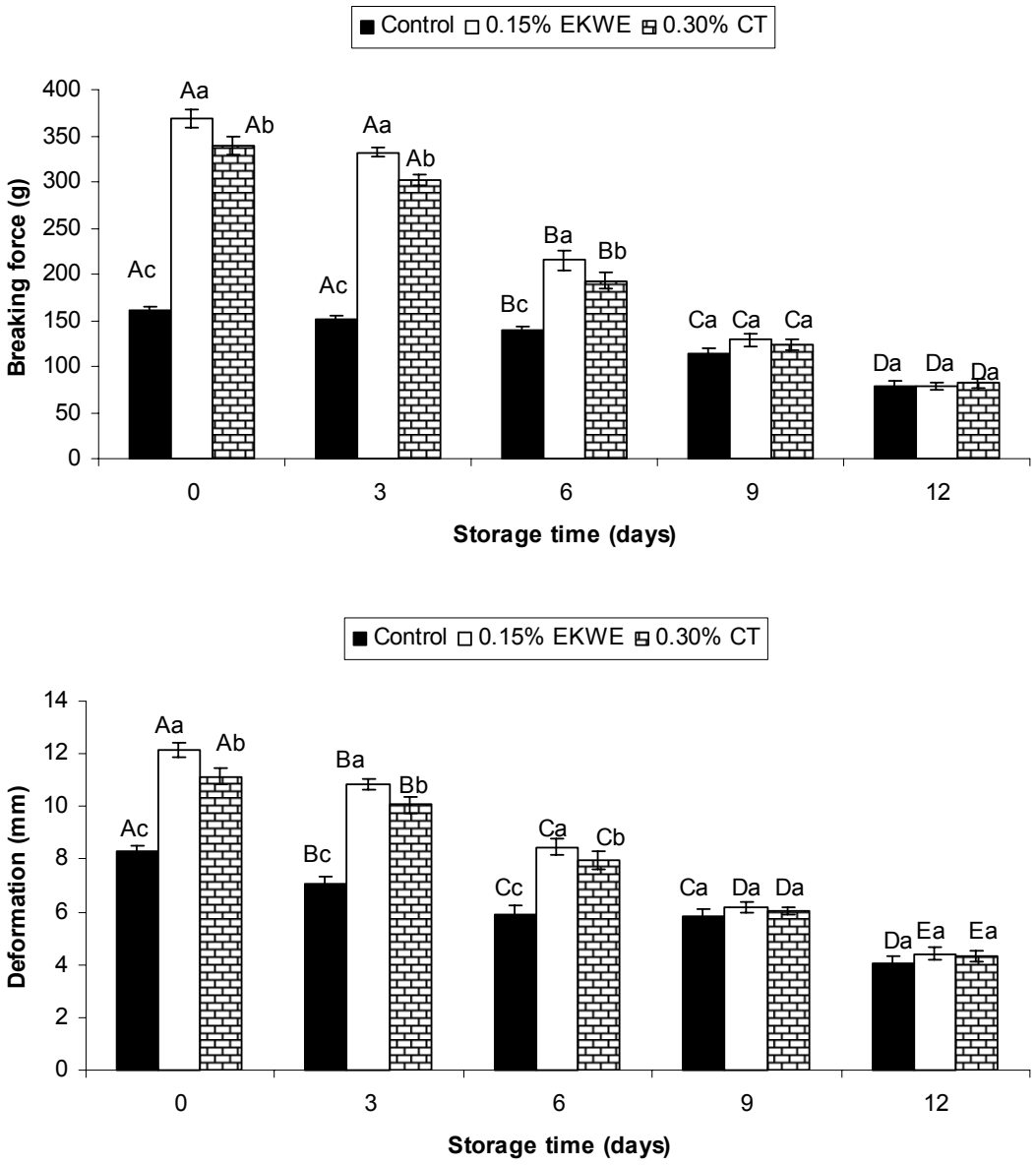


Figure 52. Breaking force and deformation of gels from surimi from mackerel stored in ice for different times without and with 0.30% commercial tannin (CT) or 0.15% ethanolic kiam wood extract (EKWE). Bars represent the standard deviation (n=3). The different letters on the bars within the same storage period indicate the significant differences ($P < 0.05$). Different capital letters on the bars within the same treatment indicate the significant differences ($P < 0.05$). Both CT and EKWE were neutralized after oxygenation prior to addition into surimi sol.

Expressible moisture content of mackerel surimi gel

When 015% EKWE or 0.30% CT was added into gel of surimi prepared from fresh mackerel (day 0), the expressible moisture content was decreased, compared with that of the control gel ($P < 0.05$) (Table 1). The decreases in expressible moisture contents were in accordance with the increased breaking force and deformation of resulting surimi gels (Figure 4). During setting at 40^{o}C, proteins underwent some denaturation and aligned themselves gradually to form the network, which could imbibe water (Benjakul and Visessanguan, 2003). In the presence of oxidized EKWE or CT, quinones of tannin and other components could interact with unfolded muscle proteins, in which the network capable of holding water could be formed. The continuous increases in the expressible moisture content of surimi gels without and with 0.15% EKWE or 0.30% CT were noticeable throughout the storage of 12 days ($P < 0.05$). During the first 6 days of storage, the expressible moisture content of surimi gels with 0.15% EKWE was lowest, followed by gel added with 0.30% CT and the control gel, respectively. The lowered expressible moisture content was in a good agreement with the increased breaking force and deformation during the first 6 days of storage (Figure 4). This might be owing to the formation of an ordered network stabilized by strong bondings, which could possibly imbibe the water effectively than the control gel. Nevertheless, no differences in the expressible moisture content were observed in all gels when surimi was prepared from mackrel stored in ice for 9-12 days ($P > 0.05$). This reconfirmed the role of protein integrity in gel formation, regardless of cross-linkers addition. Surimi with pronounced degradation or denaturation of myofibrillar proteins could not yield the good gel, even when oxidized EKWE or TA was incorporated.

Whiteness of mackerel surimi gel

Whiteness of surimi gels prepared from ice-stored mackerel without or with addition of EKWE or CT decreased continuously as the storage time increased up to day 12 ($P < 0.05$) (Table 11). At day 0, the lower whiteness was observed in surimi gels with the addition of 0.30% CT, compared with the control gel and gel added with 0.15% EKWE ($P < 0.05$). This might be associated with the higher level used (0.30%) of CT, compared with EKWE. These results are in agreement with

O'Connell and Fox (2001) who reported that phenolic compounds were responsible for discoloration in the cheese products. During iced storage, the oxidation of pigments in fish muscle, particularly myoglobin and hemoglobin, occurred. These oxidized products possibly bound tightly with muscle proteins and could not be removed by washing. As a consequence, surimi gel produced from fish kept for a longer time had the lower whiteness. During extended storage, blood and liquid from internal organs in whole samples could penetrate through the muscle, especially when autolysis proceeded and caused a looser muscle structure. Therefore, storage time directly affected the whiteness of surimi gels from mackerel and EKWE could be used as gel enhancer without the adverse effect on whiteness of resulting gel.

Table 11. Expressible moisture content and whiteness of gels of surimi from mackerel stored in ice for different times without and with 0.15% ethanolic kiam wood extract (EKWE) and 0.30% commercial tannin (CT).

Sources of phenolic compounds	Amount added (%)	Storage period (days)	Expressible moisture content (%)	Whiteness
Control	0	0	8.18±0.49eA	67.46±0.51aA
		3	12.32±0.61dA	65.35±0.81bA
		6	17.41±0.52cA	63.71±0.66cA
		9	21.23±0.41bA	61.83±0.32dA
		12	28.14±1.08aA	59.81±0.89eA
EKWE	0.15	0	3.50±0.53eC	66.92±0.57aA
		3	7.12±0.62dB	65.81±0.42bA
		6	11.89±0.71cC	63.20±0.81cA
		9	19.92±0.81bA	61.21±0.91dA
		12	27.89±0.95aA	59.12±0.39eA
CT	0.30	0	4.85±0.39eB	65.34±0.33aB
		3	7.22±0.47dB	63.48±0.45bB
		6	12.35±0.62cB	61.81±0.35cB
		9	20.45±0.71bA	59.63±0.81dB
		12	27.97±0.92aA	57.85±0.52eB

Different letters in the same column within the same source of phenolic compounds indicate the significant differences ($P < 0.05$).
Different capital letters in the same column within the same storage period indicate the significant differences ($P < 0.05$).
Values are mean ± standard deviation (n=3).
CT, commercial tannin; EKWE, ethanolic kiam wood extract.

Protein patterns of mackerel surimi gels

Protein patterns of gels of surimi prepared from mackerel stored in ice for 0, 6 and 12 days without and with the addition of 0.15% EKWE or 0.30% CT are illustrated in Figure 5. Decrease in MHC band intensity was found in the control gel (without addition of EKWE or CT), compared with that observed in surimi sol at day 0 (Figure 3.). The result suggested that the formation of cross-linking stabilized by

non-disulfide covalent bond mediated by indigenous transglutaminase took place, especially during setting. MHC was most susceptible to cross-linking during setting (Benjakul and Visessanguan, 2003). Benjakul and Visessanguan (2003) reported the decrease in MHC of surimi gel from bigeye snapper, particularly when the setting was implemented. For gels of surimi prepared from mackerel stored in ice at day 0 and added with 0.15% EKWE or 0.30% CT, MHC band intensity decreased markedly with the concomitant formation of cross-links with high molecular weight found in the stacking gel. The results suggested that MHC underwent cross-linking induced by oxidized phenolic compounds in EKWE or CT via non-disulfide covalent bonds. The results reconfirmed that the enhanced cross-linking of proteins in surimi was most likely due to the cross-linking activity of quinones in oxidized EKWE or CT. The quinone, a reactive electrophilic intermediate, can readily undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan residues in a protein chain, forming strong covalent bonds (Hurrell and Finot, 1984). Nevertheless, no MHC band was observed in surimi gels prepared from mackerel stored in ice for 6 or 12 days, regardless of the addition of EKWE or CT. The disappearance of MHC in gel of surimi prepared from mackerel stored in ice for 6 or 12 days might be a result of heat activated autolysis or cross-linking of MHC. As a consequence, no MHC was retained in all gels from surimi containing initial low MHC content. Actin was the dominant protein remaining in gel samples. At the same storage time, corresponding surimi gels had the similar actin band intensity, irrespective of EKWE or CT addition. Actin was less susceptible to proteolysis and to cross-linking induced by indigenous transglutaminase (An *et al.,* 1994). Similar protein patterns between different gels correlated well with similar gel properties.

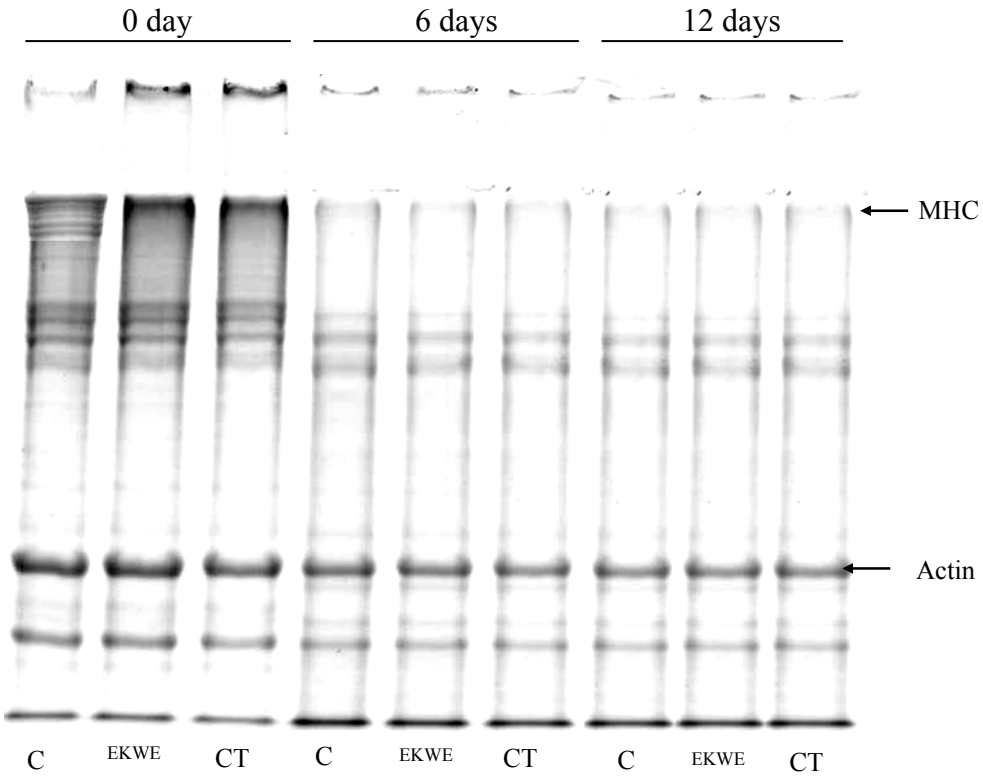


Figure 53. SDS-PAGE patterns of protein in gels from surimi from mackerel stored in ice for 0, 6 and 12 days without and with 0.30% commercial tannin (CT) or 0.15% ethanolic kiam wood extract (EKWE). MHC, myosin heavy chain; C, the control gel.

8.5 Conclusions

Mackerel mince rapidly underwent deterioration and physico-chemical changes especially proteolytic degradation and lipid oxidation during iced storage, leading to the loss in gel-forming ability. The addition of 0.15% EKWE or 0.3% CT effectively improved surimi gel quality, when mackerel kept in ice up to 6 days were used for surimi preparation. Both additives showed no gel strengthening effect towards surimi produced from poor quality fish.

CHAPTER 9

SUMMARY AND FUTURE WORKS

9.1 Summary

1. Use of commercial grade phenolic compounds in their oxidized form at the optimum concentration resulted in the increase in gel strength of both bigeye snapper and mackrel, representing lean fish and dark fleshed fish, respectively. The addition of phenolic compound lowered the whiteness of bigeye snapper surimi gels, but had no negative impact on whiteness as well as color and taste likeness of mackerel surimi gel. OTA generally exhibited the superior gel strengthening effect to others. Therefore, the use of phenolic compounds in their oxidized form at optimum concentration could be a promising alternative in improving the gel strength of surimi.

2. Alkaline-saline washing process for mackerel surimi could remove most interfering components and concentrate myofibrillar proteins, especially MHC more effectively than did conventional washing process. This more likely facilitated the better interaction of OTA with MHC. For unwashed mince, the addition of OTA had no impact on gel strength of resulting gel. OTA might preferably interact with sarcoplasmic proteins instead of myofibrillar proteins, leading to the less availability for cross-linking of myofibrillar proteins, a major contributor to gelation.

3. Kiam wood extract could be used as an important source of tannin and the reduction of wood increased the extraction efficiency with etahnol. Ethanolic kiam wood extract (EKWE) showed the higher efficacy in inceasing breaking force and deformation than did commercial tannin. Addition of EKWE at a level of 0.15% had no detrimental effect on sensory properties of surimi gel but slightly increased overall likeness of the gel. Thus, EKWE could be used as a natural gel enhancer for surimi industry.

4. The pH of solution of phenolic compounds during oxygenation process was the key factor determining the oxidation of phenolic compounds, in which quinones were formed. Alkaline condition favored the formation of quinones,

which enhanced the cross-linking of proteins via both disulfide and non-disulfide covalent bonds. The incorporation of 0.15% EKWE oxygenated at pH 8 into the surimi yielded the gels with the improved mechanical and sensory properties.

5. Mackerel muscle rapidly underwent physico-chemical changes by both denaturation and proteolytic degradation, especially MHC, during iced storage, leading to loss in gel-forming ability. The addition of 0.15% EKWE was able to improve surimi gel quality when the mackerel used as the raw material was kept in ice for no longer than 6 days. Therefore the integrity of myofibrillar proteins was another factor governing gel enhancing ability of oxidized phenolic compound.

9.2 Future works

1. Antioxidative and antimicrobial properties of oxidized phenolic compounds in the resulting gel should be further evaluated.

2. Other components in ethanolic kiam wood extract should be identified and their role as synergists of tannin should be elucidated.

3. The concentration range of oxidized phenolic compounds and its subsequent effect on the sensory properties of surimi prepared from poor quality fish should be further studied.

4. Use of other additives in combination of oxidized phenolic compounds as synergists in improving the gel properties of surimi should be intensively investigated.

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List of Publications and Proceedings

Publications

1. Balange, A. and Benjakul, S. 2009. Enhancement of gel strength of bigeye snapper (*Priacanthus tayenus*) surimi using oxidised phenolic compounds. Food Chem. 113: 61-70.
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4. Balange, A.K. and Benjakul, S. 2009. Use of kiam wood extract as gel enhancer for mackerel (*Rastrilleger kanagurta*) surimi. Int. J. Food Sci. Technol. (Accepted).
5. Balange, A.K. and Benjakul, S. 2009. Surimi gel strengthening effect of kiam wood extract as influenced by pH during oxygenation process. J. Food Biochem. (In review).
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1. Balange, A.K. and Benjakul, S. 2008. Effect of oxidized tannic acid on the gel properties of Mackerel (*Rastrelliger kanagurta*) mince and surimi prepared by different washing process, In 6th IMT-GT UNINET Conference, Penang, Malaysia, 2008.
2. Balange, A.K. and Benjakul, S. 2008. Enhancement of gel strength of bigeye snapper (*Priacanthus tayenus*) surimi using oxidised phenolic compounds**.** International Hydrocolloids Conference 2008, Singapore, June 15-19, 2008.
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