Protease Inhibitors and Hydrolysate from Duck Egg Albumen: Characteristics and Applications in Surimi Gels

Tran Hong Quan

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

Prince of Songkla University

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Major Program	Food Science and Technology

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

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

……Signature

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ABSTRACT

Duck egg albumen (DEA) was monitored for its physicochemical properties, trypsin inhibitory activity, and gelling properties as affected by store conditions (4 °C and room temperature) and salting time (30 days). Haugh unit, moisture content, and trypsin inhibitory activity decreased, while the pH value increased as the storage time increased (P<0.05). The rate of changes was lower at 4 °C. During salting, moisture content and trypsin inhibitory activity were continuously decreased, whereas the salt content and surface hydrophobicity increased as the salting time increased (P<0.05). However, there was no difference in protein patterns during the storage and salting. Protein with MW of 44 kDa acted as trypsin inhibitor in DEA. Based on texture profile analysis, the highest hardness, gumminess, and chewiness were found at day 3 for room temperature and at day 6 for 4 °C. Gel became weaker and had lowered whiteness with increasing storage time, particularly at room temperature. Additionally, as salting time increased, the lowered gel strength with higher whiteness was recorded. Thus, storage conditions and salting directly affected the quality of DEA.

Trypsin inhibitor (TI) from DEA was isolated and purified using ammonium sulfate precipitation at 20%–40% saturation and affinity chromatography using trypsin-CNBr-activated Sepharose 4B column with high purity. Molecular weight of purified TI was 43 kDa based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. TI was stable within the temperature range of 40–60°C and pH of 7–9. The concentration of salt higher than 5% led to the decreases in inhibitory activity of TI. The inhibition mode of TI was classified as uncompetitive type with low Ki value (508 nM).

When the effects of duck and hen egg albumens at different levels (1–4%, w/w) on proteolysis and gel properties of sardine (*Sardinella albella*) surimi were comparatively investigated, the addition of both albumens could inhibit autolysis of surimi proteins in a dose dependent manner as indicated by the lower TCA-peptide content, more retained myosin heavy chain and actin, and increased gel strength of surimi gel (P<0.05). Duck albumen showed higher efficiency in enhancing the gelling properties of sardine surimi than hen albumen. Thus, DEA was considered to replace hen egg albumen as protein additive in surimi gel.

To prepare DEA powder, desugarization for DEA was optimized as follows: glucose oxidase/catalase concentration of 31.24/781 units/mL albumen and incubation time of 6.55 h. Drying methods also affected properties and TI activity. Higher TI activity was found in freeze-dried albumen powder than spray-dried counterpart (P<0.05). However, no marked differences in protein patterns were observed in all the powders, regardless of desugarization and drying methods. The whiteness and foaming property of powder were improved, while the solubility decreased after desugarization (P<0.05). Furthermore, prior desugarization could markedly improve the whiteness and gelling properties of powder. Prior desugarization could lower browning and increased foaming and gelling properties of DEA, particularly when spray dried with inlet temperature of 160 °C.

To widen the exploitation of salted DEA, salted DEA powder (SDEAP) was used as salt replacer at various levels (0.5–2.5%). Autolysis and gelling properties of sardine surimi were investigated. SDEAP had high salt (33.67%) and protein contents (64.52%) with TI activity of 5,975 units/g solid. Sardine surimi gels added with SDEAP had the lowered proteolysis, in which MHC was more retained. The addition of SDEAP could improve the gel strength and sensorial characteristics of surimi gel (P<0.05). SDEAP could be used as a promising ingredient to replace salt used in surimi gel preparation.

Protein hydrolysate from DEA was also prepared and characterized. Influences of different ultrasound treatments combined with heat pre-treatment on enzymatic hydrolysis, emulsifying properties and antioxidant activities of duck albumen hydrolysate (DAH) were studied. Heat treatment of DEA at 95 °C for 30 min

with subsequent ultrasound pretreatment at 60% amplitude for 10 min could effectively increase the DH of DEA. The highest antioxidant activities as well as emulsifying properties of DAH were found after 90 min of hydrolysis. Thus, heat pre-treatment, followed by ultrasonication of DEA under appropriate condition could increase DH, antioxidant activities and emulsifying properties of DAH.

As DAH conjugated with epigallocatechin gallate (EGCG) at various levels (2–5%, w/w) were prepared, the conjugation between DAH and EGCG induced the change of secondary structure and modified functional groups of DAH monitored by FTIR, total sulfhydryl group and carbonyl contents. DAH conjugated with 4% EGCG showed the highest emulsifying properties and enhanced antioxidant activities than DAH. Physical and oxidative stability of fish oil emulsion was improved when DAH-EGCG conjugate was incorporated. Thus, the conjugate between DAH and 4% EGCG could serve as an antioxidant emulsifier for enhancement of physical and oxidative stability of fish oil emulsion.

The impacts of DAH-EGCG conjugate at various levels (0.5-2.0%) on gel properties, sensory characteristics, and storage stability of fish tofu during 21 days of storage at 4 °C were also evaluated. The incorporation of DAH-EGCG conjugate increased gelling properties and sensory likeness scores of fish tofu when the levels of DAH-EGCG conjugate were increased (P<0.05). Moreover, 1.5-2.0% DAH-EGCG conjugate could effectively retard lipid oxidation and microbial growth of fish tofu throughout the storage, compared to the control (P<0.05). DAH-EGCG conjugate was proven as protein cross-linker, antioxidant, and antimicrobial agent in order to prolong shelf-life and maintain quality of fish meat based emulsion products.

Therefore, DEA could be used as a potential protein additive, particularly for surimi, in which gel strength could be improved, regardless of salting. It could be to prepare hydrolysate with improved functional properties and bioactivity, especially after modification with the selected polyphenol, EGCG.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Egg and egg products are consumed by people in many countries, regardless of the culture and religion. Eggs contain almost all essential nutrients including lipids, minerals, proteins, and all essential vitamins (excluding vitamin C) (Abeyrathne *et al.*, 2013). In food industry, eggs have been used not only as nutrients but also for their sensorial and functional properties (Phillips and Williams, 2011). Based on world ranking, Thailand is one of the ten major countries for egg production (Fouad *et al.*, 2018; Huang and Lin, 2011). According to Aendo *et al.* (2018) in 2015, 13,548,366 ducks were used to produce eggs in Thailand, of which 51.86% were from free grazing duck farms located mainly in the western and central areas of Thailand. Globally, hen eggs are most popular, while duck and quail eggs are also utilized for consumption. In general, the compositions of the egg white proteins in the duck and hen eggs are different. Duck albumen contains about 60% methionine, by 30% threonine, by 25% tryptophan and by 45% sulfur amino acids higher than those of hen egg. Thus, duck egg may be a better source of those amino acids (Pikul, 1998). Duck eggs, both fresh and processed, are popular in China and South-East Asia, representing 10–30% of total egg consumption in those regions (Pingle, 2009). Among commercial avian products, salted eggs are popular in Asian countries, especially in South-East Asia and China (Ganesan *et al.*, 2014).

An egg contains two important parts for human consumption, egg yolk and albumen. Albumen contains numerous biological substances including ovotransferrin, ovomucoid, lysozyme, cystatin, and ovoinhibitor (Saxena and Tayyab, 1997). Ovomucoid, ovoinhibitor, and cystatin are known to be protease inhibitors. Ovotransferrin, avidin, and ovoflavoprotein are functionally classified as mineral and vitamin binding agents (Rossi *et al.*, 2013). Duck albumen has several functional properties including water holding capacity, emulsifying, foaming and gelling properties (Chen *et al.*, 2009). Moreover,

albumen proteins and their derivative-proteins have been known to possess antimicrobial (Anton *et al.*, 2006), antioxidant (Ren *et al.*, 2014a) and anti-cancer properties (Lee *et al.*, 2017a). However, eggs have a limited shelf-life and are prone to quality loss. During stockpiling or storage, several changes occur. Those alterations include the thinning of the albumen, an increase in albumen pH, loss of water and carbon dioxide, increase in yolk water content, debilitating and extending of the vitelline membrane, as well as changes in protein conformation (Qiu *et al.*, 2012) and these changes are likely to affect functional properties of albumen. Additionally, processing e.g. salting, drying, and high-pressure processing, also influence the resulting egg albumen proteins (Kaewmanee *et al.*, 2011b; Katekhong and Charoenrein, 2018; Yang *et al.*, 2016).

Additionally, hydrolysate from egg albumen can be another desired product due to its bioactivities. Albumen protein hydrolysates exhibited strong antioxidant activity, including DPPH radical scavenging activity, hydroxyl radical scavenging activity, and superoxide anion scavenging activity (Graszkiewicz *et al.*, 2007; Shuguo *et al.*, 2013). Recently, protein hydrolysates from egg albumen have been reported to reduce blood pressure, improved vascular relaxation and modify aortic angiotensin II receptors expression in spontaneously hypertensive rats (Jahandideh *et al.*, 2016; Ochiai *et al.*, 2017). Moreover, desalted duck albumen peptides and their products could promote the absorption calcium in rats. The functional peptides may facilitate calcium transport by acting as calcium carriers and interacting with the cell membrane to open a special Ca^{2+} channel (Hou *et al.*, 2015; Zhao *et al.*, 2014a). In addition, protein hydrolysates from hen egg albumen (Cho *et al.*, 2014; Singh and Ramaswamy, 2014; Stefanović *et al.*, 2014a), skipjack roe (Intarasirisawat *et al.*, 2014), porcine plasma protein (Chen *et al.*, 2019), shrimp (Ketnawa *et al.*, 2016), rice bran protein (Cheetangdee and Benjakul, 2017) incorporated to phenolic compounds were reported to increase stability of fish emulsion sausage, fish tofu, and fish oil emulsion, respectively.

However, a little information regarding trypsin inhibitor, functional properties, particularly gelation of duck albumen, and protein hydrolysates from duck albumen have been reported. To maximize the application of protease inhibitors and protein hydrolysate

from duck albumen in food products, basic information should be gained. Changes in physicochemical and gelling properties of duck albumen, especially protease inhibitors as affected by storage conditions and salting process should be elucidated in order to exploit duck albumen in surimi with desired qualities. Also, protein hydrolysates from duck albumen and their derivatives with bioactivity and functionality should be produced. The information obtained will be the platform for maximized utilization of duck albumen, either fresh, salted forms, or its hydrolysate.

1.2 Review of literature

1.2.1 Egg compositions and characteristics

Eggs generally consist of three important parts: the yolk, the albumen or white, and the eggshell with the eggshell membrane. The yolk is encompassed by the albumen and both are further wrapped by the eggshell membrane within the outer covering of a hard eggshell (Hincke *et al.*, 2012) (Fig. 1). Because of varieties of laying duck species worldwide, duck egg weight ranges from approximately 60 to 90 g. The egg yolk, albumen, and eggshell account for 28–35%, 45–58%, and 11–13% (based on the whole-egg weight), respectively (Huang and Lin, 2011). Duck eggs have a relatively higher percentage of egg yolk, compared to other avian eggs.

1.2.1.1 Duck albumen composition

Duck albumen has water (88.3%) as its main component and it is rich in protein (8.8%). The remainder includes ash (0.53%), and trace amounts of lipids (0.13%) (Huang and Lin, 2011). In descending order, the main albumen proteins are ovalbumin (40% of dry matter), ovomucoid (10%), ovomucin (3%), ovotransferrin (2.0%), and lysozyme (1.2%). The major proteins in duck albumen are ovalbumin, ovomucoid, ovomucin, conalbumin, and lysozyme. Comparing protein compositions between hen and duck albumen, there are slight differences. Their compositions and biologically functional properties are presented in Table 1 (Belitz *et al.*, 2009; Huang and Lin, 2011). However, Hu *et al.* (2016) did not detect extra-cellular fatty acid-binding protein precursor (ex-

FABP), ovomucoid, prostaglandin D2 synthase (PG D2 synthase) or clusterin in duck albumen proteins. Nevertheless, two main proteins, vitellogenin-2 and the "deleted in malignant brain tumors 1" protein (DMBT1), were found in duck albumen based on a 2-DE gel map. Congjiao *et al.* (2017) studied the divergent proteomic patterns of egg white proteins from chickens, turkeys, ducks, quails, pigeons, and geese using tandem mass tag quantification technology, and totals of 148, 162, 138, 183, 179, and 150 proteins, respectively, were identified. Generally, duck and goose albumen had the highest similarity of protein composition (about 78%). Furthermore, $dBPS_1$ and $dBPS_2$ were the two basic proteins isolated from duck egg albumen, which share a basic lineage with the cygnin and meleagrin of swan, turkey and chicken eggs (Naknukool *et al.*, 2008). The diversity of proteins determines the gelling, emulsifying, and foaming properties of egg albumen, which are the critical factors governing the quality of food using albumen as an ingredient (Congjiao *et al.*, 2017).

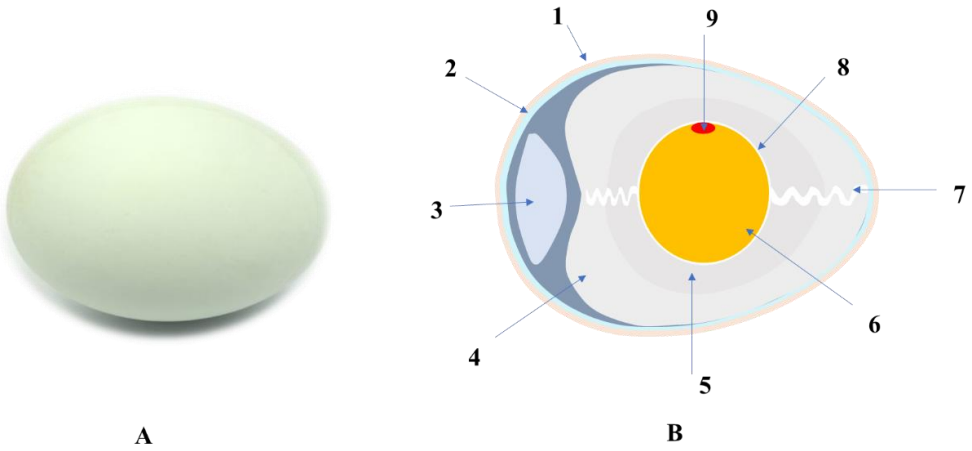

Figure 1. Duck egg (a) and cross section of duck egg (b); 1: eggshell; 2: shell membrane; 3: air cell; 4: thin albumen; 5: thick albumen; 6: egg yolk; 7: chalaza; 8: vitelline membrane; 9: germinal disc.

Source: Hincke *et al.* (2012)

Several protease inhibitors are present in hen egg albumen and can inhibit serine protases such as ovomucoid and ovoinhibitor, while cystatin is an inhibitor of thiol proteases (Réhault, 2007; Stevens, 1991). However, those protease inhibitors could not be

found in duck egg albumen by 2-dimensional polyacrylamide gel electrophoresis (2-DE) (Hu *et al.*, 2016). Takenawa *et al.* (2015) reported that ovalbumin had inhibitory activity toward trypsin. Ovalbumin is known as a member of serpin family and shares sequence homology with α_1-protease inhibitor, antithrombin III and angiotensinogen (Saxena and Tayyab, 1997). In food industry, protease inhibitors have been used as the food additives to improve textural property of several food products e.g. surimi, meat ball, and sausage, etc. (Klomklao *et al.*, 2016; Singh and Benjakul, 2017).

1.2.1.2 Functional properties of egg albumen

Due to the superior foaming and gelling capacities of albumen in food systems, its use is preferred in food products to whole egg or egg yolk.

1.2.1.2.1 Gelling property

Gelation has an essential role in a number of products, e.g. imitation crab, reformulated meat products, fish tofu, and fish ball, etc. (Alleoni, 2006). Some foods (e.g. meringues and angle cakes) require egg albumen as a foaming agent. Egg albumen is a regularly used ingredient for improving gel strength or the water-holding capacity of many food products. The rheological and textural properties of many products depend on the gelling properties or heat coagulation of egg proteins (Ren *et al.*, 2010). However, the gelling characteristics and coagulation temperature of duck and hen eggs are quite different. Pikul (1998) noted that the gelling temperature of duck albumen was 67.5 °C, while that of hen albumen was found to be 75.0 °C. Further, Pikul (1998) found that gel from duck eggs was firmer than that of hen eggs when the same temperature and heating time were applied. The highest hardness of gel from duck albumen was obtained at 80 °C, while that of hen eggs reached its maximum at 85 °C. Moreover, gels from duck albumen showed higher cohesiveness and higher water binding than those from hen eggs (Pikul, 1998). Thus, duck albumen is superior to the hen counterpart in improving gelation in food products. In addition, because egg albumen also contains protease inhibitors, these can help prevent protein degradation during gel formation in some muscle foods, such as surimi, etc.

Table 1. Composition and biological functions of the major duck and hen egg albumen proteins

	Source of albumen	Ovalbumin	Ovomucoid	Ovotransferrin	Ovomucin	Lysozyme	Ovomacro-globulin	Flavo-protein	Avidin	References
Percent of the total protein *	Duck	40.0	10.0	2.0	3.0	1.2	1.0	0.3	0.03	Mine (1995)
	Hen	54	11	12	3.5	3.4	0.5	0.8	0.05	Huang and Lin (2011)
Molecular weight (kDa)	Duck	43.49	28.0	70.0	ND	15.0	ND	33.0	48.1	Hu *et al.* (2016); Miguel *et al.* (2005)
	Hen	44.5	~28.0	77.7	$5.5\text{-}8.3\text{x}10^3$	14.0	769.0	32.0	60.1	Hytönen *et al.* (2003); Miguel *et al.* (2005)
Functions/ functionalities		Inhibit trypsin	Inhibit trypsin	Bind metallic ions	Increase viscosity	Lyse some bacteria	Inhibit serine and cysteine proteases	Bind ribofla-vin	Bind biotin	Belitz *et al.* (2009)

* Average values.

ND: not determined.

Mechanism of egg albumen gel formation

The gelation of egg albumen is considered to be a two-step process. Firstly, some proteins are denatured, while the second step involves the aggregation of the denatured proteins. The extent of denaturation is associated with the unfolding of the proteins, the nature of the interactions or bonding, and the kinetics of the aggregation process. These factors determine the type and characteristics of the resulting gels (Campbell *et al.*, 2003). For gelation, complete denaturation of proteins is not required because of the formation of insoluble precipitates mediated by extensive hydrophobic interactions or hydrogen-bonding between the unfolded protein chains (Campbell *et al.*, 2003). The partially unfolded egg proteins are able to form complexes, resulting in the formation of gel (i.e. the ordered formation of a 3-dimensional network) or a coagulum (a random interaction between egg proteins) depending on the gelling conditions (Ren *et al.*, 2010) (Fig. 2). With varying environmental conditions, such as protein concentration, heating conditions, ionic strength and pH, a transparent/turbid solution and transparent/turbid gel may be formed. Gel hardness is generally maximal under conditions that induce the formation of a slightly turbid or transparent gel (Phillips and Williams, 2011).

The oxidation of sulfhydryl (SH) groups into disulfide (S–S) bonds and the S–S/SH interchange reaction are the key factors determining the intermolecular S–S bonds in the avian protein gel network. Ovalbumin (OVA) contains both S–S bonds and SH groups in its molecule. Native OVA has one S–S bond in each species. Nevertheless, avian species contain different SH groups. OVA hen albumen contains four SH groups (Cys11, Cys30, Cys367, and Cys382), while duck albumen contains two (Cys11 and Cys331). Only one SH radical of the hen OVA molecule is readily available for heat induced aggregation (Sun and Hayakawa, 2002). The differences in number of free SH groups in albumen more likely affect the heat-induced gelation and gelling properties of hen and duck albumen.

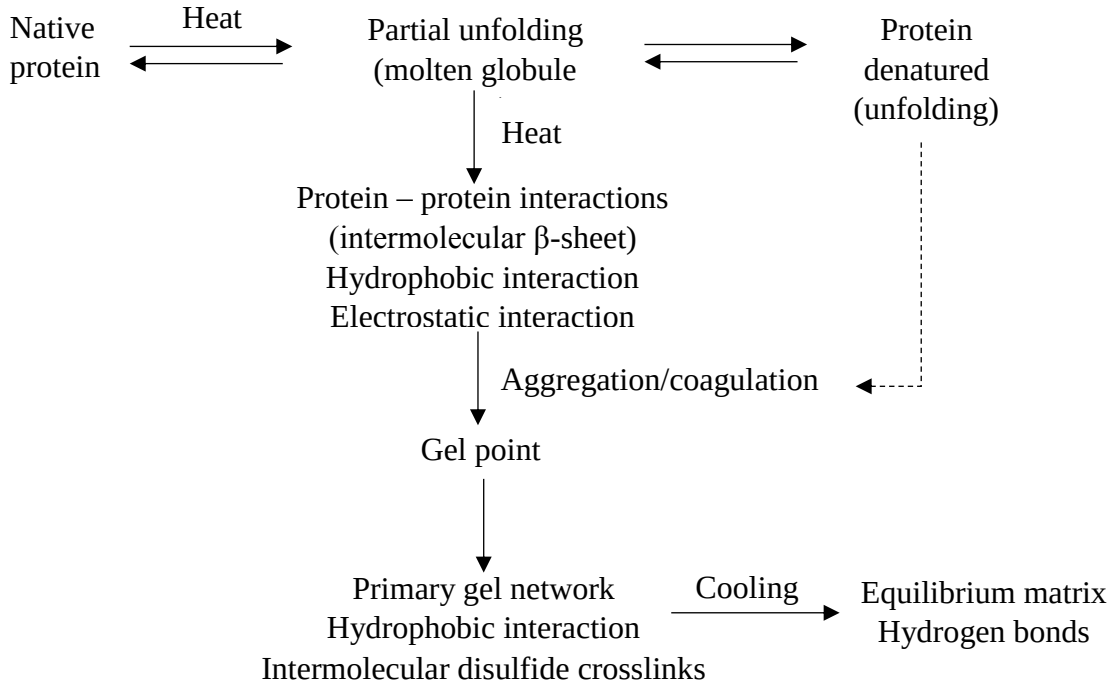

Figure 2. Schematic representation of heat-induced gelation of egg albumen proteins

Source: Campbell *et al.* (2003)

Factors affecting the gelling properties of egg albumen

Albumen forms a 3-dimensional gel network as a result of the interaction between the protein chains. The physicochemical conditions of the medium such as its ionic strength, type of salts, pH, protein concentration, and its interaction with other components have an impact on the quality of the gel (Croguennec *et al.*, 2002; Raikos *et al.*, 2007).

pH

The pH is an essential factor affecting the net charge of proteins. At high pH values, the reactivity of the SH group is augmented (Phillips and Williams, 2011). The pHs of egg albumen increases after eggs are stockpiled and stored. Those changes are coincidental with increases in the viscosity index, penetration force, and gel elasticity (Croguennec *et al.*, 2002). Gel strength increased with increasing pH, and the highest gel

strength for duck albumen was observed at pH 9 by Ren *et al.* (2010), while at a low pH (pH 6), the resulting gels were more brittle but firmer with poor water binding properties and low elasticity. The alkaline pH of egg albumen is able to induce the unfolding of protein molecules as a result of enhanced repulsion between negatively charge domains. As a consequence, the interactions between unfolded proteins favor the formation of inter-junction zones in the gel network (Phillips and Williams, 2011). Houska *et al.* (2004) investigated the effects of dry matter content and pH on the gel strength of native albumen and the gel strength was found to increase with increasing pH and dry matter content. Raikos *et al.* (2007) also reported that a higher gel strength was found among egg samples at pH 5 and 8 than at pH 2. At high ionic strength and near the isoelectric point, coagula or soft/turbid gels of egg albumen were formed. The presence of these coagula within the gel network led to the formation of opaque gels. Nevertheless, the maximum gel hardness was obtained at the critical point, where the interaction between the linear protein chains was balanced by electrostatic repulsive forces (Phillips and Williams, 2011).

Salts and type of salts

Salts can alter the initial ionic nature of a gel. Excessive coagulation of albumen proteins can be induced by NaCl, thus causing the gel to become weaker (Raikos *et al.*, 2007). Gels were found to weaken when 0.9 M NaCl solution was added. However, the inclusion of NaCl up to 0.1 M resulted in slight increases in elasticity and gel strength (Woodward, 1990). Iwashita *et al.* (2015) investigated the effects of inorganic salts including NaCl, Na_2SO_4, and NaSCN on the thermal aggregation of albumen. Na_2SO_4 and NaSCN are kosmotropic and chaotropic, respectively. The surface tension of the solution was increased more by the kosmotrope than the chaotrope. This might be due to the fact that chaotrope destabilizes protein tertiary structure, leading to a decrease in the denaturation temperature, while kosmotropes stabilizes protein structure. At salt concentrations greater than 0.5 M, the salting out of protein molecules dominated. The anions of salts at low concentrations bind to the positively-charged domains of lysozyme, causing an increase in solubility, regardless of the type of ions (Iwashita *et al.*, 2017). Croguennec *et al.* (2002) also reported that the water release of hen albumen gel was higher

with the addition of NaCl at higher levels. The maximum amount of water was liberated in the presence of 120 mM NaCl. The excessive heat-induced aggregation of albumen proteins was related to the higher NaCl concentration, most likely via ionic interaction or hydrophobic interaction. For coagulum type gels from cooked salted duck albumen, NaCl plays a key role in the properties and appearance of the gel. A gel with opaque color and a coarse texture is formed when NaCl at high amounts is present (Kaewmanee *et al.*, 2011a).

The type of salts also affects heat-induced and alkaline induced gelation of egg albumen. Croguennec *et al.* (2002) revealed that Ca^{2+} and Mg^{2+} modified the viscoelastic properties of egg albumen gel, in which the gel was less homogeneous with particles clustered in random aggregates, and had no string of beads structures. The addition of cations increased heat stability of albumen proteins, while $CuSO_4$ markedly softened coagulum gel of albumen. $FeCl_3$ and $A1C1_3$ did not affect gel firmness. These changes were the result of the shielding of the negative charge of albumen proteins (Beveridge and Ko, 1984; Croguennec *et al.*, 2002). Under the extreme pH for pidan production, egg albumen gel could not be formed, properly due to extensive electrostatic repulsion, lowering protein–protein interactions, and preventing gel formation. The addition of selected ions to protein solution diminishes the repulsive forces, and protein–protein association occurs, thus forming a self-supporting gel (Ganesan *et al.*, 2014). Zhao *et al.* (2014c) reported that 0.2% $CuSO_4$ exhibited the optimal effect on the strength of alkaline-induced duck albumen gel. Gel strength was increased by 31.92%. $MgCl_2$, $ZnSO_4$, PbO, and $CaCl_2$ also exhibited gel strengthening effect, while the effect of $Fe_2(SO_4)_3$ was negligible. This was in agreement with the results of Ganasen and Benjakul (2011) who reported that the presence of Pb^{4+}, Zn^{2+} and Ca^{2+} at a level of 0.2% could enhance the solidification of pidan albumen. Those cations might lower the dissociation of protein network, although slightly higher alkaline pH was obtained during aging.

Protein concentration

The gel hardness of albumen generally increases logarithmically with increasing protein concentration. The gel fracturability was found to increase when the

protein concentration was increased, due to favorably enhanced aggregation of albumen (Woodward, 1990). Iwashita *et al.* (2015) found that a high concentration of albumen was easily gelled by heat treatment. They noted that with a restricted attractive hydrophobic interaction but strong electrostatic repulsion, strings of beads described as ordered soluble linear aggregates of denatured ovalbumin molecule, are more likely to be formed. At high protein concentrations, a 3-dimensional gel network is developed via interconnections of these soluble linear aggregates. However, a viscous transparent sol instead of a gel network is formed at low protein concentrations (Ren *et al.*, 2010). In general, conditions that lower the electrostatic repulsive forces between the protein molecules favor gel formation. At a sufficiently high protein concentration, high ionic strength and pH values close to zero net charge (the isoelectric point) are the key factors determining the formation of a 3-dimensional gel network (Campbell *et al.*, 2003). Moreover, protein concentration has also been found to impact the rheological characteristics and gelling temperature of albumen gel. Both gelling temperature and storage modulus (G′) were noted to be concentration-dependent and G′ increased as albumen concentrations increased (Eleya and Gunasekaran, 2002). This increase is generally attributed to a consolidation of attractive forces such as van der Waals and hydrogen bonding between the protein particles within the gel network. Additionally, as the protein concentration increased, the gelation temperature decreased, thus accelerating gel formation (Eleya and Gunasekaran, 2002).

1.2.1.2.2 Foaming property

Egg albumen has been used as a functional protein ingredient in a number of bakery products, such as cookies, bread, meringues cakes as well as ice cream, due to its excellent foaming properties (Phillips and Williams, 2011). The interaction between the thermally induced coagulates and the various constituents are responsible for the unique foaming capacity of albumen, which results in stable foams (Mine, 1995). These properties are governed by the ability of proteins in the albumen to form a cohesive viscoelastic film via their intermolecular interactions, and their rapid adsorption at the air–liquid interface during bubbling or whipping. The foaming capacity and stability of albumen mainly depend on storage time, temperature, egg species, dry heating and pH (Lomakina and

Mikova, 2006). During storage, losses of foam volume and foam stability were related to a lower thick albumen height. This directly caused a decrease in the viscosity of egg albumen (Ren *et al.*, 2010). When the pH increases with extended storage, native ovalbumin is converted to S-ovalbumin, which is more heat-stable (less hydrophobic) than native ovalbumin. This may decrease foam stability as a result of the interference of S-ovalbumin with the cohesive film formation at the air–water interface. Overall, a positive correlation between the volume of drained liquid and S-ovalbumin content has been documented (Lomakina and Mikova, 2006). Nevertheless, the acidity of albumen strongly influences the durability and volume of albumen foam. The lowest drainage and the largest volume were recorded by Bovšková and Mikova (2011) for both pasteurized and non-pasteurized egg albumen in an acidic pH (pH 4.5), close to pI. At such a pH, some proteins precipitate and form thin film surrounding the air bubbles, resulting in the decrease in surface energy and surface tension. In addition, differences of foaming properties between hen and duck albumen have also been reported. Khaki Campbell Duck egg albumen showed a lower foaming ability than hen egg counterpart, however the foam stability of the duck albumen was higher than that of hen egg albumen because of higher hydrophobic amino acid found in duck albumen than hen egg counterpart (Pikul, 1998).

1.2.2 Changes in the quality and physicochemical properties of duck albumen during storage

1.2.2.1 Changes in quality of duck egg

The quality of eggs is influenced by storage conditions and time. Such a quality loss is associated with changes in physicochemical structure and chemical compositions of egg albumen during storage. Good quality egg generally has thick and transparent albumen. The height of albumen is one of the principal characteristics used to evaluate interior egg quality (Lokaewmanee, 2017). Haugh unit (HU) is one of the interior egg quality measurements based on the height of its albumen. An egg is weighed, then broken onto a flat surface, and a micrometer is used to determine the height of the thick albumen surrounding the yolk. The higher the number, the better the quality of the egg

(fresher, higher quality eggs have thicker whites) is gained (Menezes *et al.*, 2012). According to Department of Agriculture (2000), the eggs are graded as AA or A when the HU values are > 72 or 61-72, respectively, whereas, the eggs are classified as grade B or C when the HU value are in a range of 31-59 or < 30, respectively.

Eggs undergo deterioration as storage time increases. As a consequence, they may not be suitable for human consumption. However, loss of quality in eggs can be retarded significantly by lowering the storage temperature. In general, quality deterioration occurs at a faster rate at high temperatures than at refrigerated temperatures. The main factors affecting egg quality are storage time, temperature, humidity, airflow, and handling processes (Akter *et al.*, 2014). Storage time and temperature were found to have a significant impact on the yolk index, yolk color, albumen index and Haugh unit (HU). Pandian *et al.* (2012) studied the influence of various storage periods (1–5 weeks) on duck egg quality at 75% relative humidity and 18 °C. Storage time had significant effects on the yolk index, yolk color, HU and albumen index. The yolk index value decreased significantly when the storage period was lengthened. Huang and Lin (2011) documented that during the storage period of 97–98 days at 44 °C, egg white index and egg yolk index decreased by 56.5% and 66.7%, respectively in hen eggs compared with the storage temperature at 2 °C. Duck egg white and egg yolk indices were decreased by only 22.8% and 15.6%, respectively (Table 2). In addition, the shape index, albumen index, yolk index, shell color, specific gravity, HU and yolk color of Khaki Campbell duck eggs were remarkably affected by storage time (Lokaewmanee, 2017). Khaki Campbell duck eggs still had acceptable quality for human consumption after storage for 11 days at 30 °C and 78% humidity (Lokaewmanee, 2017).

Table 2. Comparison of egg quality during storage between hen and duck eggs

Storage		Hen			Duck		
Temperature (℃)	Period (day)	pH	Egg white index	Egg yolk index	pH	Egg white index	Egg yolk index
2	97	9.0	0.062	0.42	8.9	0.079	0.45
44	46	9.5	0.051	0.23	9.5	0.072	0.40
44	98	9.7	0.027	0.14	9.6	0.061	0.38

Source: Huang and Lin (2011)

During storage, albumen thinning, an important change in albumen, occurs. The degradation of the ovomucin complex results in the breakdown of a thick white to a thin white, especially during improper transportation and prolonged storage (Phillips and Williams, 2011; Woodward, 1990). During storage, the ovomucin–lysozyme complex undergoes destruction. This phenomenon is related to a reduction of thick albumen height and a decrease in HU (Akter *et al.*, 2014). Nevertheless, duck eggs have better stability during storage at room temperature than hen eggs. Storage time was found not to greatly affect duck egg yolk and albumen quality indices, including the HU, while such changes occurred in chicken eggs (Jalaludeen *et al.*, 2009). Duck egg quality was acceptable up to 28 days of storage at 4 °C, while they could be stored for up to only 14 days at room temperature (Akter *et al.*, 2014). Jin *et al.* (2011) revealed that the HU of hen eggs decreased from 87.62 to 60.92 at 29 °C and from 91.30 to 72.63 at 21 °C after 10 days of storage. Nonetheless, this deterioration did not take place at 5 °C and the HU, egg weight, and albumen height were slightly decreased when the cold storage time was extended. However, the average HU value was still in the range of grade A after 10 weeks of storage (Jones and Musgrove, 2005). Hen eggs lost their interior quality much faster than duck eggs during extended storage. This might be due to higher duck eggs' ability to resist bacterial spoilage (Brown *et al.*, 1965). Moreover, the thickness of the domestic duck eggshell, which is considered as a physical and bacterial barrier, is thicker than that of hen

eggs. Therefore, duck eggs are more resistant to an aggressive external environment than hen eggs (Solomon, 2010; Wellman-Labadie *et al.*, 2008).

1.2.2.2 Changes in the chemical composition of duck albumen

Multiple changes in chemical composition of duck albumen take place during storage, especially alteration of the pH, moisture content, and protein patterns. Lokaewmanee (2017) demonstrated that albumen pH of Khaki Campbell duck eggs remained unchanged after 11 days of storage at 30 °C and 78% relative humidity. A decrease in CO_2 of stored quail and hen eggs has however been found (Akter *et al.*, 2014; Jin *et al.*, 2011; Khan *et al.*, 2013). Changes in protein patterns were also found to be accelerated by a high storage temperature and pH. S-ovalbumin, an extremely and irreversibly heat-stable form, is regularly converted from ovalbumin (Deleu *et al.*, 2015; Huang *et al.*, 2012). Qiu *et al.* (2012) reported that the decrease in protease inhibitors became more pronounced when eggs were stored at a higher temperature. Additionally, lipocalin family proteins were decreased, while the formation of ovalbumin complexes was increased with increasing storage temperature. Those phenomena are related to a thermally promoted change in hen eggs. During storage at high temperature, clusterin decreases. This change can be considered as an effective biomarker for egg quality assessment. However, hen albumen protein patterns were not greatly changed after 15 days of storage at low temperature (Qiu *et al.*, 2012). The denaturation of some proteins such as trypsin inhibitors occurred at varying rates during storage for duck and hen eggs, which might cause by differences in stability and the molecular properties of proteins between the two types of eggs (Qiu *et al.*, 2012). Schäfer *et al.* (1999) observed no significant changes in the content of conalbumin and lysozyme in hen albumen during storage. However, S-ovalbumin which is associated with a change in the isoelectric point of ovalbumin, was formed when the pH became slightly more acidic due to the loss in CO_2 (Schäfer *et al.*, 1999). Storage of hen eggs for up to 4 weeks at 15 °C was found to decrease the activity of serine protease inhibitors by 50%, and the activity of lysozyme was decreased by approximately 10%. The activity of cystatin was detected only at a trace level (Kopeć *et al.*, 2005). Eggs stored at

refrigerated temperature could therefore maintain bioactive components, especially trypsin inhibitors during storage.

1.2.3 Egg albumen powder

1.2.3.1 Desugarization before dehydration

Dried egg albumen powder or egg white powder is prone to undesirable browning known as a Maillard reaction, particularly during drying at high temperature. The reaction involves reducing sugars, mainly glucose, which constitute about 4 g/L in the original liquid form, and amino groups in the albumen. The reaction between a cephalic amino group and the aldehydes of glucose (glucose-cephalin reaction) is responsible for the discoloration and off-flavor development in egg albumen. The reaction also causes a loss of protein solubility (Sisak *et al.*, 2006; Wu, 2014). In practice, desugarization is a general requirement before dehydration in order to remove the glucose from the egg albumen. This aims to prevent the browning of the product obtained. Additionally, desugarization improves resistance to microbial attack in the powder obtained, thus increasing the storage stability of the egg albumen powder (Sisak *et al.*, 2006). Desugarization can be conducted either by yeast or bacterial fermentation, in which different organic acids are produced via the conversion of glucose. The reducing sugar content of liquid whole hen egg was completely depleted within 12 h following fermentation by baker's yeast (Sharma *et al.*, 2012). However, these processes pose some bacteriological problems. The use of commercial glucose oxidase containing catalase is a common practice, which converts glucose into gluconic acid (Fig. 3) (Woods and Swinton, 1995). The enzymatic desugarization process is reproducible and straightforward. Also it yields final products with consistently high organoleptic properties (Lechevalier *et al.*, 2013).

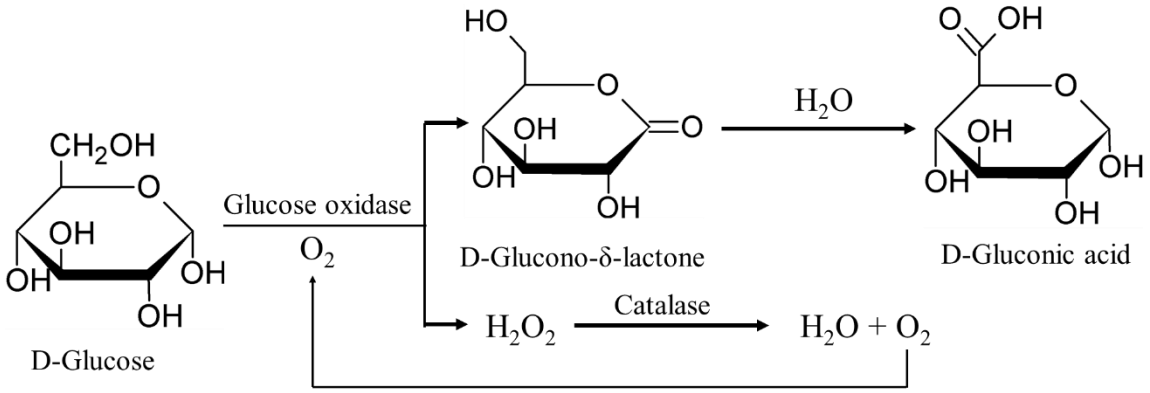

Figure 3. Enzymatic removal of glucose from egg albumen. Firstly, glucose is converted to gluconic acid by glucose oxidase. Hydrogen peroxide formed is further transformed to oxygen and water by catalase.

Source: Woods and Swinton (1995)

In commercial preparations, specifically designed for egg white desugarization, glucose oxidase containing catalase (EC1.11.1.6) is commonly used (Lechevalier *et al.*, 2013). Sisak *et al.* (2006) immobilized a commercial glucose oxidase containing catalase on Amberlite UP 900 anion exchange resin using adsorption and subsequent cross-linking with glutaraldehyde. At least 98% of the initial glucose content was eliminated for 3 h from hen egg albumen by the immobilized enzyme. The glucose concentration in the solution was about 0.4% without desugarization egg albumen and 0.012% in the desugarization egg albumen, after being incubated with glucose oxidase (174 units per milliliter) for 5 h (Reed and Underkofler, 1966).

1.2.3.2 Effect of drying methods on the functional properties of egg albumen powder

Dehydration can retard chemical reactions and microbial growth. Therefore, it is a successful means for preserving egg albumen. Numerous dried egg albumen products are available in the market. Dried egg white products are produced by hot air drying, spray drying, microwave vacuum drying, infrared drying, and freeze-drying (Ma *et al.*, 2013;

Phillips and Williams, 2011). However, the quality, and physical and functional properties of egg albumen powder are directly influenced by the conditions and drying methods used.

1.2.3.2.1 Freeze drying

Freeze drying is considered as being one of the best drying technologies for maintaining the quality of products. This technology also has other advantages, such as good rehydration capacity and the aqueous solubility of the powders obtained. Moreover, it is the most appropriate method for the drying of heat-sensitive materials. Nevertheless, freeze drying involves high capital costs as well as processing costs and the drying process takes a long time (Chen *et al.*, 2012; Zhou *et al.*, 2014). To overcome this limitation, a combined method involving microwave vacuum-drying and freeze drying of duck albumen was developed (Zhou *et al.*, 2014). Drying time of fresh duck albumen and desalted duck albumen was significantly decreased by this combination of methods, compared to a freeze-drying process alone. Fresh duck albumen protein dried in this way had a better color, a higher emulsifying index, and a lower apparent density than that produced by freeze drying alone (Zhou *et al.*, 2014). Katekhong and Charoenrein (2018) dried hen egg albumen with two drying methods, freeze drying and hot air drying. Drying egg albumen using hot air induced changes in color and protein conformation, mainly due to protein aggregation in the resulting powder. Moreover, effects of freeze drying and spray drying processes on functional properties of phosphorylated of egg albumen protein was studied by Liu *et al.* (2015c). Freeze drying was found to be the best method in terms of production of albumen protein powders with superior functional properties. Egg albumen protein hydrolysate was also dried by spray drying and freeze drying (Chen *et al.*, 2012). The foaming properties of albumen protein hydrolysate were improved by freeze drying but reduced by spray-drying. Freeze-drying did not influence the emulsifying properties of albumen protein hydrolysate, while spray-drying had negative effects. Nevertheless, no significant differences in DPPH radical scavenging activity, reducing power, and lipid peroxidation inhibition were found before drying and after freeze drying and spray drying (Chen *et al.*, 2012).

1.2.3.2.2 Spray drying

Spray drying is the most popular drying technology used in the pharmaceutical, chemical, and food industries for the preparation of protein powder (Asghar and Abbas, 2015). During the spray drying process, a foodstuff material in liquid form is exposed to hot air with a temperature in the range of 100–300 °C. The hot air evaporates the liquid in the foodstuff material. The product obtained from spray drying is in dried form, either agglomerates or granules. The shape of the product obtained depends on the physical and chemical properties of the foodstuff material, and the design and operation of the dryer. Evaporation through droplets is facilitated by vapor and heat transfer during the spray-drying process (Asgar and Abbas, 2012).

Egg powder was produced by spray drying at temperature approximately 160 °C, which led to many changes in egg components. This resulted in different functional properties of eggs after reconstitution. Ayadi *et al.* (2008) investigated the optimum operating conditions for drying whole egg and egg albumen using a pilot-scale spray dryer. Spray drying of egg white at moderate conditions (air inlet temperature ranged from 110 to 125 °C) resulted in a product that enhanced water holding capacity of resulting gels. In addition, a gel prepared from the dried samples was firmer than that of the fresh samples. Ma *et al.* (2013) found that the optimized conditions for hen egg albumen spray drying were as follows: flow 22 mL/min, inlet-air temperature 178.2 °C and feeding temperature 39.8 °C.

1.2.4 Salted duck egg

Salted duck egg is a popular traditional egg product in China and other Asian countries. Fresh duck eggs are pickled in a saline solution or in salted clay. During the salting process, the salt diffuses and penetrates into the egg yolk and albumen through the eggshell and the eggshell membrane. Some proteins in the salted duck eggs are degraded to amino acids and small peptides compared to fresh eggs. As a result, a higher amount of inorganic salts can permeate into the eggs (Lian *et al.*, 2014). In general, fresh duck eggs are treated with a high salt concentration (25%, w/v), which is the typical requirement for

salted duck egg production, in order to develop the unique ‘‘fresh, fine, tender, loose, gritty and oily texture’’ yolk characteristic (Kaewmanee *et al.*, 2009a; Xu *et al.*, 2017). Commonly, salted eggs are made from duck eggs because they offer more desirable characteristics with richer flavor than hen eggs (Ji *et al.*, 2013). Salting for 15 days and at least 30 days for ripening are essential to attain the desired features, particularly for aroma and flavor. The pickling period is dependent on the salt content in the pickling solution or salted clay and is also affected by the environmental temperature (Huang and Lin, 2011; Kaewmanee *et al.*, 2009b).

1.2.4.1 Methods for salted egg production

There are two major methods to produce salted eggs. The immersion method is performed by pickling fresh eggs in a salt solution containing wine, tea, etc. or using only saturated salt water. The coating method is conducted by coating the eggs with a macerated paste containing salt, wood ash, red clay, wine (Huang and Lin, 2011). Kaewmanee *et al.* (2009b) studied the changes in chemical composition, textural properties, and microstructure of cooked duck egg salted by 2 methods (immersing and coating) during 4 weeks of salting. At week 3 of salting, egg yolk from coating method had the higher oil exudation than that from immersing method. As the salting times increased, the lower springiness, hardness, chewiness, gumminess, and resilience with higher cohesiveness and adhesiveness were generally found in cooked salted egg albumen (Kaewmanee *et al.*, 2009b). The achievement of salting is generally indicated by the textural development of egg yolk (Kaewmanee *et al.*, 2009b). Wang (2016) produced salted yolk by immersing the separated yolk in solution containing sodium chloride and maltodextrin. This methodology also prevents the curing of albumin as encountered in the normal whole-egg brining protocol, and the spare egg albumen can be used as raw material for other food products. This new brining process accelerated the curing time from 4 weeks to 4 days. Moreover, in order to accelerate salting of duck eggs, Dang *et al.* (2014) applied an ultrasound technique (60 kHz and 140 W) in duck egg salting process. An increased duration of ultrasonication led to an increased effective diffusivity of NaCl through the eggshell during salting, which was much greater (45–46 fold), compared to that of the traditional process.

Pretreatment is also required to accelerate the salting as well as to improve the quality of salted egg. Lian *et al.* (2014) studied the use of sodium dodecyl sulfate (SDS) pretreatment and 2-stage curing for improved quality of salted duck eggs. After pretreatment in 0.5% (w/v) SDS solution at room conditions for 15 min, the eggs were firstly brined in 25% (w/v) or 30% (w/v) saline solutions (defined as Stage I). When the NaCl content of egg albumen reached 3.5% to 4.0% (w/v; about 15 to 18 days), the eggs were moved to a 4.0% NaCl solution (w/v; defined as Stage II). This study indicated that SDS pretreatment was effective to reduce microbial load on the shells of fresh duck eggs and the 2-stage curing can improve physicochemical qualities of the salted duck eggs and shortened curing time to about 7 to 17 days as compared to the traditional 1-step curing method (Lian *et al.*, 2014).

1.2.4.2 Changes in chemical compositions of salted egg

During the salting process, the yolk normally becomes hardened and solidified because of water migration from the yolk to the albumen. Thereafter, water passes through the eggshell and leaves the egg. Meanwhile, the egg albumen becomes watery and loses viscosity when the salt migrates into the egg (Lian *et al.*, 2014). Kaewmanee *et al.* (2009a) reported that salting induced the weight proportion of the egg albumen to increase, while the yolk proportion decreased. With increasing salting time, the moisture contents of both egg yolk and albumen gradually decrease, associated with increases in the ash and salt contents. It was reported that the amount of precipitated protein increased with salting time (Huang *et al.*, 1999). Venkatachalam (2018) noted that particle size, total SH groups, and surface hydrophobicity gradually increased, while moisture and water activity were decreased during 30 days of storage. For the nutritive compositions of salted duck albumen, proteins, fat, carbohydrate, and minerals became higher, as compared to those of fresh duck albumen (Ganesan *et al.*, 2014). Salting process thus alters the nutritive value of salted duck egg albumen.

1.2.4.3 Utilization of salted egg albumen

In general, salted duck egg albumen powder contains a high NaCl content (30%) and possesses hygroscopic properties. This makes it less suitable for application in food products. Thus, the desalination of salted duck albumen could be beneficial for the duck-egg industry and a new source of protein can be gained with the environmental effects being minimized (Zhao *et al.*, 2014b). Salted duck albumen is a potential ingredient for producing food and it has already been used in the production of high protein Frankfurter sausages and noodles (Zhou *et al.*, 2014). Tan *et al.* (2016) studied the utilization of salted duck albumen as an alternative source of salt for manufacturing yellow alkaline noodles. Yellow alkaline noodles with salted duck albumen (15.21 g of salted duck egg albumen/100 g of wheat flour) added had a significantly higher protein content and were more yellow and lighter, compared to a control product. Salted duck albumen-yellow alkaline noodles showed high acceptability because of improved color and textural properties (Tan *et al.*, 2016). Therefore, the potential use of salted duck egg albumen as an alternative source to table salt in some food products such as surimi or meat ball should be more investigated.

1.2.5 The use of egg albumen in surimi

Surimi is myofibrillar protein concentrate obtained by washing fish mince. It has a high commercial value with a wide range of applications, particularly for making a variety of gelling products, e.g. fish ball, imitation crab or and lobster (Jitesh *et al.*, 2011; Zaghbib *et al.*, 2016). The main steps in surimi preparation include heading, gutting and deboning; mincing; washing and dewatering; refining; screw press; stabilizing surimi with cryoprotectants followed by freezing and frozen storage (Park, 2013). Surimi possesses the functionalities including gelling, water and fat binding and emulsifying properties and can be used as a functional protein ingredient in several products. Generally, lean fish are commonly used for surimi preparation due to the superior gelation and white color of resulting gels. In addition, dark fleshed fish had darker color and exhibited poor gelling property. Apart from its higher lipid content and susceptibility to oxidation, higher

concentrations of trimethylamine oxide (TMAO), sarcoplasmic proteins and protease activity are associated with poor quality of gels (Buamard and Benjakul, 2015).

1.2.5.1 Gel weakening (modori)

Modori or gel degradation phenomenon weakens the gel formed with heating (Lee and Min, 2004). Degradation of muscle proteins occurred due to the endogenous heat-activated proteases (An *et al.*, 1996; Benjakul *et al.*, 2003). Degradation of surimi proteins caused by endogenous proteases mainly contributes to gel weakening of surimi and surimi-based foods. Modori phenomenon occurs above the setting temperature (40-50 °C). The proteolytic activity in fish muscle greatly affects the gel-forming ability of surimi. The remaining activity of heat-activated proteases in surimi leads to severe textural degradation upon heating. The proteolysis of fish as a raw material can be minimized by handling and processing at low temperature. Proteases in muscle tissues, particularly those in the sarcoplasmic fraction, can be removed, to a certain extent, during washing. Some proteases are tightly bound to myofibrils and are not removed by washing, posing more severe degradation in surimi (Park, 2013).

Proteolytic activity in muscle is high at temperature above 50 °C and causes the rapid and severe degradation of myofibrillar proteins, particularly myosin (Wasson *et al.*, 1993). The International Union of Biochemistry (IUB) Nomenclature Committee divided proteolytic enzymes into four classes according to the different catalytic residue at the active site; these are serine, cysteine, aspartic, and metallo-proteases. Serine and cysteine proteases most likely appear to be involved in surimi gel degradation during heating (Park, 2013).

1.2.5.2 Inhibition of modori

To alleviate modori or weakening of surimi, numerous food-grade protease inhibitors have been widely used in surimi. Those are beef plasma protein, dried egg white and whey protein concentrate (Benjakul *et al.*, 2004). Beef plasma protein (BPP) is used as a gelling agent and/or protease inhibitor. It will form an elastic irreversible gel when

cooked to above 65 °C and thus is suitable for a variety of restructured meats that require a strong binder to connect between meat chunks and particles. BPP exhibits a remarkable capability to inhibit gel weakening (Nopianti *et al.*, 2011). Lou *et al.* (2000) reported that the incorporation of BPP in paddlefish surimi not only substantially reduced the loss of gel strength but also inhibited the reduction of G′ during extended incubation at 40 °C. BPP also suppressed the degradation of MHC enhances gelation mainly by inhibiting endogenous proteases responsible for the degradation of myofibrillar proteins, particularly myosin. Additionally, BPP could improve texture characteristics of red tilapia surimi gel. However, higher addition levels of BPP significantly affected gel whiteness (Duangmal and Taluengphol, 2010). Whey protein concentrate (WPC) has commonly been used a protein supplement, filler/water binder, emulsifying and gelling agent (Nopianti *et al.*, 2011). WPC (0–3%) showed inhibitory activity against autolysis in surimi produced from tropical fish at both 60 and 65 °C in a concentration-dependent manner. MHC of surimi was more retained in the presence of WPC. Breaking force and deformation of kamaboko gels of all surimi increased as the levels of WPC increased (Rawdkuen and Benjakul, 2008).

Egg albumen possesses several protease inhibitors such as ovostatin, ovomucoid, ovoinhibitor, and cystatin C. The amount of egg white added depends upon the fish species and the quality of the fish used. Egg white added at 10% produced a gel with high yield stress; gels containing up to 20% egg white were softer. However, there was a decrease in gel strength and the gel became brittle at percentage greater than 20%. Egg white contributes to the structure of surimi gels by filling interstitial spaces in fish protein network (Nopianti *et al.*, 2011). Jafarpour *et al.* (2012) compared the effect of egg albumen powder, soy protein isolate and potato starch on functional properties of common carp (*Cyprinus carpio*) surimi gel. All the additives enhanced the functional properties of surimi gel prepared from common carp. Egg albumen (3%) significantly improved texture properties of surimi gel. Additionally, the highest overall liking score was obtained for the surimi gel containing 3% egg albumen, compared to soy protein isolate and potato starch at the same level. Moreover, Benjakul *et al.* (2004) reported that hen egg albumen showed

the inhibitory activity toward heat-activated sarcoplasmic proteases and autolysis of lizardfish mince and washed mince at 60 °C in a concentration-dependent manner. The addition of 2% to 3% spray-dried egg albumen improved textural properties of Pacific whiting and Alaska pollock fish protein gel (Hunt *et al.*, 2009). Jitesh *et al.* (2011) studied the effect of hen egg albumen on surimi prepared from lizardfish (*Saurida tumbil*) during frozen storage. The addition of 3% egg albumen exhibited gel enhancing effect by increasing gel strength, compared to that without egg albumen. Singh and Benjakul (2017) studied serine protease inhibitors from squid ovary (SOSPI) on autolysis of Indian mackerel surimi. Myosin heavy chain was more retained with coincidentally lower trichloroacetic acid soluble peptide content as the level of SOSPI increased. Gel strength and breaking force of surimi were improved with addition of SOSPI. In addition, trypsin inhibitor from the roe of yellowfin tuna also showed inhibitory activity against proteolysis in kamaboko (40/90 °C) and modori (60/90 °C) gels of bigeye snapper in a concentration-dependent manner (Klomklao *et al.*, 2016).

1.2.6 Protein hydrolysates from egg albumen

Enzymatic hydrolysis is a well-known method for increasing the value of food proteins by modifying their physical and nutritional properties. Cleavage of peptide bonds can change these properties in three ways: reducing the molecular weight, increasing the number of ionizable groups and exposing hydrophobic groups (Garcés-Rimón *et al.*, 2016). Functional properties of protein can be improved by enzymatic hydrolysis under suitable conditions. Hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionizable groups of protein hydrolysates (Shuguo *et al.*, 2013). Egg is an inexpensive and nutritious source of proteins, which can be used to generate novel bioactive peptides. The peptides from ovotransferrin and ovalbumin hydrolysates showed anti-inflammatory, strong iron- and copper-binding capacities and antioxidant, anticancer and angiotensin converting enzyme (ACE) inhibitor properties (Abeyrathne *et al.*, 2014; Jahandideh *et al.*, 2016; Lee *et al.*, 2017b). Production of bioactive peptides from egg proteins was carried out using proteases from plant and microbial sources, such as papain, Alcalase and thermolysin (Liu *et al.*, 2017b; Stefanović *et al.*, 2014b).

Proteases can be classified into three broad classes by their effect on ovalbumin (Fig. 4). Those causing extensive cleavage, those causing limited cleavage and those that do not cleave at all are classified as follows:

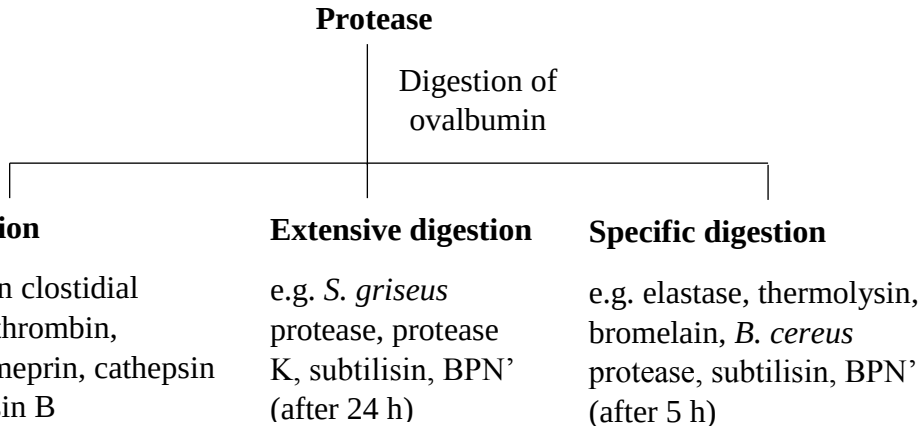

Figure 4. Classification of proteases on the basis of their action on ovalbumin

Source: Saxena and Tayyab (1997)

Generally, Alcalase has been used for hydrolysis of egg albumen protein. Alcalase (EC 3.4.21.14) was initially obtained from *Bacillus subtilis*. It is a serine-endopeptidase with a maximal activity at pH 8.0-9.0 and 50-60 ℃. Typically, the optimal conditions are pH 8.0 and 50 ℃ (Cho *et al.*, 2014; Tavano, 2017). Alcalase is a relatively nonspecific serine type protease with broad specificity, characterized by the presence of an essential serine residue at its active site. A main component of the commercial Alcalase is the serine protease subtilisin having capacity for cleavage of various peptide bonds with some preference for terminal hydrophobic amino acids. It also preferentially cleaves peptide bonds on the C-terminal side of glutamate and aspartate residues (Stefanović *et al.*, 2014b). The hydrolysate from duck egg white protein prepared by two-step enzymatic procedures (the first step with Alcalase or hydrolase specific for egg protein (SEEP) and the second step using trypsin or Alcalase) at degree of hydrolysis (DH) value of 21% (namely HSA_{21}) exhibited high antioxidant capacity in different oxidation systems (Ren *et al.*, 2014a). Bao *et al.* (2017a) reported that Alcalase had better efficiency of hydrolysis of egg yolk protein than Neutrase and Flavourzyme. Moreover, Alcalase treatment improved markedly surface

hydrophobicity and functional properties of egg yolk, such as the solubility, foam properties, emulsifying properties, and water and oil absorption capacity. Ovalbumin from hen egg albumen hydrolyzed by mixture of Alcalase and trypsin showed the highest iron-chelating and antioxidant activities among all the enzyme treatments (Abeyrathne *et al.*, 2014). Noh and Suh (2015) also reported that Alcalase was the most effective protease for hydrolysis of egg white liquid protein to produce antioxidant peptides. Alcalase gave the highest degree of hydrolysis (DH) with the highest radical-scavenging activity. The highest antioxidant properties of obtained hydrolysates from egg white protein were also achieved with Alcalase as reported by Jakovetić *et al.* (2015).

1.2.6.1 Effect of pretreatments on egg albumen hydrolysis

1.2.6.1.1 Thermal Treatment

Technological processes, such as heating, can affect the digestibility of proteins. Since fresh egg albumen may contain protease inhibitors, which greatly reduce the efficiency of the enzymatic reactions, pretreatment strategy for fresh egg albumen was implemented in enzymatic egg albumen hydrolysis (Ren *et al.*, 2014b). Ovalbumin, the major protein in chicken egg white, in its native form, is resistant to digestion by trypsin, whereas heat denatured ovalbumin shows an increased susceptibility to this protease (Van der Plancken *et al.*, 2003). Ren *et al.* (2014b) reported that higher treatment temperature resulted in lower inhibition capability of the fresh duck albumen. After pretreatment of the duck albumen at 95 °C for 30 min, the residual activity of enzyme used was 72.47 %. Besides the heat inactivation effect, thermal treatment may also decrease the α-helix and β-angle structures but increase the β-sheet structures, resulting in looser molecule structures of the proteins and providing more enzyme-binding sites for hydrolysis. Heat pretreatment of proteins before enzymatic hydrolysis can also lead to rearrangements of inter- and intra-molecular linkages, especially disulfide and hydrophobic bonds with concomitant changes in protein conformation and digestibility (Adjonu *et al.*, 2013; Ren *et al.*, 2014b). Hydrophobic groups that had been inwardly-oriented are turned outwards, exposing them to unfavorable interaction with water and encourage increased protein–

protein interactions. The extent of protein unfolding and exposure of hydrophobic patches would increase with increase in heating (Chao *et al.*, 2013). Van der Plancken *et al.* (2003) studied the effect of heating in the temperature range of 50-92 °C on the susceptibility of ovalbumin and albumen solutions to enzymatic hydrolysis by a mixture of trypsin and α-chymotrypsin. Heat treatment resulted in an increase in degree of hydrolysis after 10 min of enzymatic reaction of both ovalbumin and albumen. Thus, these heat-induced changes in protein conformation and polypeptide interactions could dictate degree of exposure of susceptible peptide bonds and influence the type of peptides generated during enzymatic hydrolysis.

1.2.6.1.2 Ultrasonication

Ultrasound is a sound wave with a frequency greater than the threshold for human auditory detection. Ultrasound has recently gained significant interest as it possesses a wide range of applications including extraction, preservation and modification of proteins or other biomolecules (Huang *et al.*, 2014). Ultrasound can mainly be classified into two fields: high frequency low-energy ultrasound in the MHz range, and low frequency high-energy power ultrasound in the kHz range. The former is usually used as an analytical technique for providing information about the physicochemical properties of foods, such as composition, structure and physical state (Jia *et al.*, 2010). However, the latter, which is used in the food industry, is relatively new and has been used in the laboratory or processing plant to modify the physicochemical properties of foods in various areas such as nano-emulsion preparation, ultrasound-assisted extraction, enhancing oxidation, and improvement of functional properties of food proteins (Huang *et al.*, 2014; Singh *et al.*, 2018). More recently the interest of food technologists has turned to the use of power ultrasound in altering enzyme activities. Prolonged exposure to high-intensity ultrasound has been shown to inhibit the catalytic activity of a number of food enzymes (Kadkhodaee and Povey, 2008). However, in some cases, solutions containing enzymes have been found to have increased activity following short exposures to ultrasound (Lee *et al.*, 2008). Yachmenev *et al.* (2009) suggested that the use of ultrasound bio-processing had led to the opening up of substrate surface to the action of enzymes. Ultrasound can

accelerate the enzymatic hydrolysis of corn stover and sugar cane bagasse celluloses. Furthermore, ultrasound treatment affected the yield of soluble protein significantly and also improved the enzymatic hydrolysis of rice protein (Li *et al.*, 2018), whey protein (Abadía-García *et al.*, 2016), soy protein (Chen *et al.*, 2011) and increased the ACE inhibitory activity of the peptides released from wheat germ protein (Huang *et al.*, 2014; Jia *et al.*, 2010). For hen egg white protein hydrolysis, the impact of ultrasound waves on egg white protein susceptibility to hydrolysis compared to both thermal pretreatment and conventional enzymatic hydrolysis was quantitatively investigated. The ultrasound pretreatments at frequency of 40 kHz improved the enzymatic hydrolysis of hen egg white protein under different conditions when compare to other methods (Jovanović *et al.*, 2016). Stefanović *et al.* (2014b) reported that the optimal ultrasound pretreatment at calorimetric power of 21.3 W and frequency of 40 kHz for 15 min at 25 °C resulted in the increased initial rate and equilibrium degree of Alcalase hydrolysis of hen egg white by about 139.8 and 13.86% compared with thermal pretreatment, respectively. Thus, the use of ultrasonic pretreatment improved the degree of hydrolysis and enhanced the release of bioactive peptides from proteinaceous substances.

1.2.6.2 Antioxidant activity and emulsifying property of albumen hydrolysate

1.2.6.2.1 Antioxidant activity

Egg albumen hydrolysate is considered as a rich source of antioxidant peptides. Sixteen antioxidant peptides, which were derived from ovalbumin, ovotransferrin and cystatin were isolated. Three peptides (Ala-Glu-Glu-Arg-Tyr-Pro, Asp-Glu-Asp-Thr-Gln-Ala-Met-Pro, and Asp-His-Thr-Lys-Glu) showed the highest oxygen radical absorbance capacity (ORAC) values (Liu *et al.*, 2015b; Nimalaratne *et al.*, 2015). Adult male spontaneously hypertensive rats fed with egg white hydrolysates for 17 weeks showed increased radical-scavenging capacity of the plasma and lowered malondialdehyde (MDA) concentration in the aorta, and exerted a beneficial effect on the lipid profile, lowering triglycerides and total cholesterol without changing HDL levels (Nimalaratne and Wu,

2015). Recently, the hydrolysate from duck egg white protein (DEWP) prepared by hydrolase, specific enzyme for egg protein (SEEP)-Alcalase at degree of hydrolysis (DH) of 21% (namely HSA21) exhibited high antioxidant capacity in different oxidation systems. A consecutive chromatographic method was then developed for separation and purification of HSA21, including ion-exchange chromatography, macroporous adsorption resin (MAR) and gel filtration chromatography. The final peptides "$P_{21\text{-}3\text{-}75\text{-}B}$" significantly enhanced antioxidant activity. Furthermore, the antioxidant activity of $P_{21\text{-}3\text{-}75\text{-}B}$ kept stable after *in vitro* digestive simulation (Ren *et al.*, 2014a). Kobayashi *et al.* (2017) recently used egg white hydrolysate to prevent oxidative deterioration of lipids in mayonnaise. Hydrolysate had the strongest inhibitory effect on lipid oxidation among the three components (control, 0.01% EDTA, 0.09 – 0.90% egg white hydrolysate). Thus, egg white hydrolysate is considered as natural antioxidant in mayonnaise. Peptides from hen egg lysozyme were also found to efficiently inhibit lipid peroxidation in the Zebrafish model (Carrillo *et al.*, 2016).

1.2.6.2.2 Emulsifying property

Food emulsions generally consist of an aqueous phase and an oil phase. One is dispersed phase, whereas another one is continuous phase (McClements, 2010). Due to the large interfacial area between the two immiscible phases, emulsions have poor thermodynamic stability. Flocculation, coalescence, and phase separation are frequently observed during processing or storage (Mao *et al.*, 2018). In order to stabilize food emulsions, emulsifiers are usually incorporated in the formulations to assure stable emulsions. An emulsifier is an amphiphilic ingredient, which localizes itself at the oil/water interface to reduce the interfacial tension, and to facilitate droplet disruption during emulsification as well as reduce flocculation and coalescence (McClements, 2010). Protein hydrolysates have been considered as effective emulsifiers. Enzymatic hydrolysis is a powerful technique for modification of protein functionalities including emulsifying property, which reflect the ability of the protein in formation of newly created emulsions and stabilization of emulsion by reducing interfacial tension (Shuguo *et al.*, 2013). The emulsifying properties of enzymatic hydrolysates have been evaluated for fish proteins,

casein, rapeseed protein isolates, gluten, soybean proteins, peanut protein, amaranth proteins, potato proteins, chickpea proteins, and hen egg albumen proteins (Amarowicz, 2010). Wang *et al.* (2018) revealed that hen egg albumen protein hydrolysate produced by Thermoase® had higher emulsifying properties than those of native egg albumen. Alcalase hydrolyzed egg proteins exhibited an improvement in emulsifying capability as compared to that of native proteins (Bao *et al.*, 2017b). The increases in surface hydrophobicity of egg albumen protein hydrolysate were linearly related to the enhancement of emulsifying activity (Bao *et al.*, 2017b; Wang *et al.*, 2018). Cho *et al.* (2014) also found that Neutrase hydrolysate showed a significantly higher emulsifying properties than egg albumen powder (without hydrolysis). Thus, hen egg albumen hydrolysate could be used as an effective antioxidant emulsifier in protection of mayonnaise against deterioration, and in inhibition of lipid oxidation (Kobayashi *et al.*, 2017).

1.2.7 Protein–polyphenol conjugates: Antioxidant property and emulsifying property

Protein is one of the major components with distinguished functionalities including emulsifying, gelling and foaming properties (Phillips and Williams, 2011). Apart from their functional properties associated with quality attributes of foods, the improved antioxidant activity of proteins via appropriate modification has gained increasing interest. Thus, the fabrication of antioxidant molecules into protein structure significantly improves the functionalities and bioactive properties of proteins, thus widening applications for pharmaceutical and biomedical industries (Liu *et al.*, 2015a). Phenolic compounds, considered as natural antioxidants, possess at least one aromatic ring attached with one or more hydroxyl groups, which can prevent or delay oxidative damages by reactive oxygen species (Czubinski and Dwiecki, 2017; Liu *et al.*, 2015a). The interaction between proteins and polyphenols plays an important role in quality improvement of certain food products. For example, protein–EGCG conjugates have been reported to exhibit better antioxidant activity than unmodified proteins (Gu *et al.*, 2017a; Yin *et al.*, 2014; You *et al.*, 2014). Furthermore, the conjugation of proteins and nonpolar polyphenols could increase surface hydrophobicity of the modified proteins by providing a large number of hydroxyl groups,

thereby enhancing the emulsifying properties of the native proteins (Ozdal *et al.*, 2013). Thus, the conjugates formed by proteins and nonpolar polyphenols have been considered as potential antioxidant emulsifiers, which could localize and function at the oil-water interface and prevent the oxidation in emulsion-based foods (Fan *et al.*, 2018; Feng *et al.*, 2018).

1.2.7.1 Mechanism for the formation of protein-polyphenol conjugates

Basically, proteins and polyphenols are able to interact together via either noncovalent (hydrophobic, ionic, and hydrogen bondings) or covalent bonds (You *et al.*, 2014). However, the conjugates formed by covalent bonds are more preferably used in food applications owing to their stronger and more permanent interactions with high stability (Liu *et al.*, 2017a). Generally, protein-polyphenol conjugates could be covalently formed by non-enzymatic (alkaline reaction or free radical grafting) or enzymatic (polyphenoloxidase, laccase, tyrosinase, etc.) methods (Gu *et al.*, 2017a; Liu *et al.*, 2017a).

1.2.7.1.1 Non-covalent interaction between proteins and polyphenols

Non-covalent interactions between proteins and polyphenols are normally reversible interactions and weaker than covalent counterparts (Czubinski and Dwiecki, 2017; Jakobek, 2015). In general, hydrogen bonding (Fig. 5a) and hydrophobic-hydrophobic interactions (Fig. 5b) are mainly involved in the formation of protein-polyphenol conjugates (Le Bourvellec and Renard, 2012; Prigent *et al.*, 2009). Phenolic groups have been known as an excellent hydrogen donor, which can form hydrogen bonds with C=O groups of proteins (Buitimea-Cantúa *et al.*, 2018). In addition, hydrogen bonds are also formed via the interactions between OH groups of polyphenols and oxygen or nitrogen, especially hydroxyl (–OH) and amino ($–NH_2$) groups of proteins (Yildirim-Elikoglu and Erdem, 2018).

Hydrophobic interaction is also involved in the conjugation of proteins and polyphenols, wherein hydrophobic amino acids (leucine, isoleucine, glycine, methionine, alanine, phenylalanine, valine, tyrosine, cysteine, and tryptophan) residues of proteins

interact with nonpolar aromatic rings of polyphenols (Fig. 5b) (Kanakis *et al.*, 2011; Ozdal *et al.*, 2013). Other interactions such as ionic bonds (Fig. 5c) could play a minor role in protein-polyphenol interaction, in which positively charged groups of proteins such as the ε-amino groups of lysine reacts with negatively charged hydroxyl groups of polyphenols (Fig. 5c) (Le Bourvellec and Renard, 2012). Non-covalent protein-polyphenol interactions generally result from a combination of different interactions (Liu *et al.*, 2017a). Although the bonds formed are potentially reversible and have low energy, the non-covalent protein-polyphenol interactions may play an important role in food industries for improvement of functional and quality of food products.

1.2.7.1.2 Covalent interaction between proteins and polyphenols

Principles of the common methods being widely used for covalent fabricating proteins and polyphenols are demonstrated in Fig. 6 and 7. Alkaline reaction is one of the common non-enzymatic methods employed for the conjugation of polyphenols and proteins (Chen *et al.*, 2019; Liu *et al.*, 2017a). Polyphenols are easily prone to oxidation under alkaline condition (pH 9.0) in the presence of oxygen to form semi-quinone radicals, which subsequently rearrange to quinones. These reactive intermediate products readily react with nucleophilic residues (methionine, lysine, tryptophan, and cysteine) in protein side chains. Consequently, the covalent cross-link (C–N or C–S) between protein and polyphenol can be formed (Fig. 6a) (Liu *et al.*, 2017a; Rawel *et al.*, 2002; Rawel *et al.*, 2001).

For non-enzymatic method, free-radical grafting, ascorbic acid and hydrogen peroxide are used as a redox pair of initiator system. This method has been proven to be simple and rapid for fabrication of proteins and polyphenols conjugates (Gu *et al.*, 2017a). An illustration of the reaction is shown in Fig. 7. Firstly, amino acids located in the side chain of proteins are oxidized by hydroxyl radicals initially generated by the reaction of redox pair. The radicals localized on protein molecules then react with polyphenols via covalent bond (Liu *et al.*, 2015a; Spizzirri *et al.*, 2009), resulting in the formation of protein-polyphenol conjugates. Gu *et al.* (2017a) documented that the resulting conjugates induced by free radicals exhibited higher antioxidant activity than that formed by the

alkaline method. Additionally, the reaction could be conducted at room temperature without generating toxic products or decomposition of antioxidants (Feng *et al.*, 2018; Liu *et al.*, 2015a). Thus, this method has been considered as an effective synthetic strategy to produce bioactive protein-polyphenol conjugates.

For enzyme-catalyzed method, the first step is the oxidative reaction of mono-phenols into *o*-diphenols induced by enzyme monophenolase (or cresolase). Subsequently, *o*-diphenols are transformed to *o*-quinones using *o*-diphenolase (or catecholase), in the presence of oxygen (Fig. 6b) (Cirkovic Velickovic and Stanic-Vucinic, 2018; Liu *et al.*, 2017a; Rawel *et al.*, 2001). On the other hand, quinones can also be formed by laccases, which oxidize both *o*- and *p*-diphenols (Prigent *et al.*, 2007). The reactive quinones can readily interact with nucleophile amino acid residues in protein chains to form the cross-linked proteins or polymers (Chung *et al.*, 2003; Rawel *et al.*, 2001). The enzymatic approach is often considered as an environmental friendly method with high specificity. Enzymatically synthesized conjugates exhibited strong radical scavenging activity. However, the preparation and procedure used are complicated and costly (Gu *et al.*, 2017a).

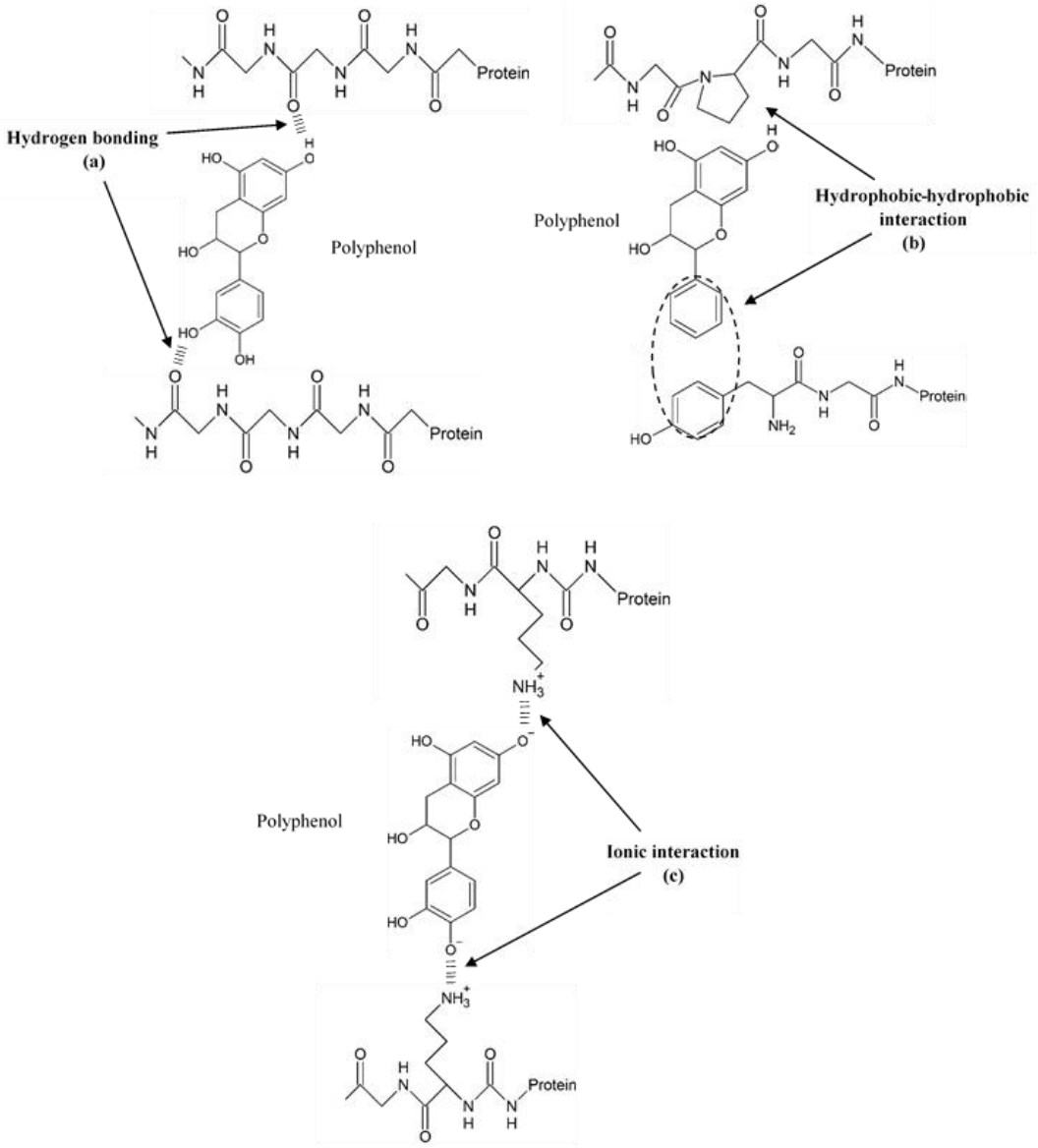

Figure 5. Non-covalent conjugation of proteins and polyphenols and protein cross-linking via hydrogen bonding (a), hydrophobic-hydrophobic interaction (b), and ionic interaction (c)

Source: Le Bourvellec and Renard (2012)

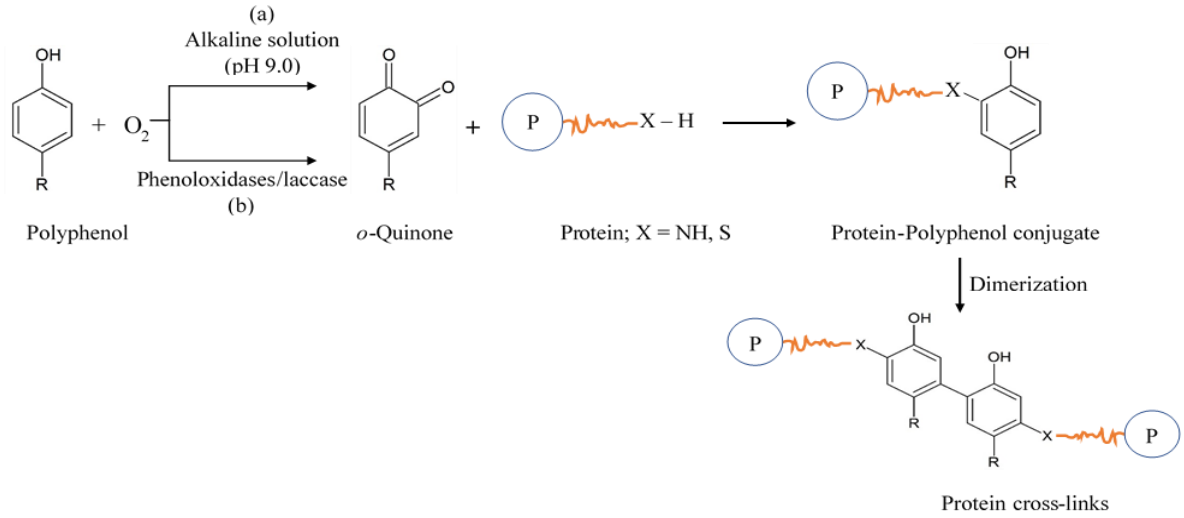

Figure 6. Covalent conjugation of protein and polyphenol and protein cross-linking via alkaline (a) and enzymatic (b) reactions

Source: Prigent *et al.* (2007)

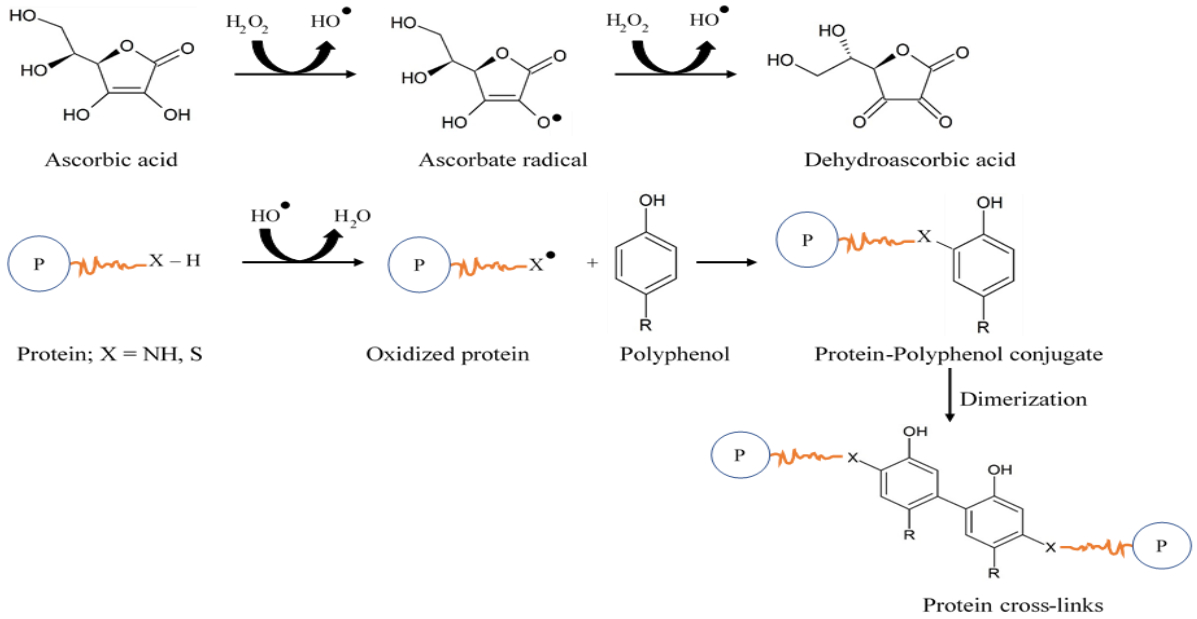

Figure 7. Covalent conjugation of protein and polyphenol and protein cross-linking using free-radical grafting method

Source: Spizzirri *et al.* (2009)

1.2.7.2 Antioxidant activity of protein/peptide-polyphenol conjugates

Antioxidant activity is one of the most important properties of protein-polyphenol conjugate. Numerous studies indicate that protein-polyphenol conjugates exhibited stronger antioxidant activity than original proteins (Fan *et al.*, 2018; Feng *et al.*, 2018; Gu *et al.*, 2017a; Yin *et al.*, 2014; You *et al.*, 2014). Jiang *et al.* (2018) found that radical scavenging capacity was significantly increased in a dose-dependent manner when whey protein and casein were conjugated with chlorogenic acid. Similarly, antioxidant activities of EGCG-α-lactalbumin, α-casein, β-lactoglobulin, and β-casein conjugates were higher than those of native proteins (Almajano *et al.*, 2007). Arts *et al.* (2002) reported the effect of the interaction between flavonoids and proteins on the total antioxidant capacity. It was noticed that addition of catechin to β-casein increased the antioxidant capacity of the β-casein solution. You *et al.* (2014) also found that 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity, ferric reducing activity power (FRAP), and oxygen radical absorbance capacity (ORAC) of ovotransferrin-catechin conjugates were increased by 4−5 times as compared to those of native ovotransferrin. Moreover, ORAC of conjugate formed by radical grafting was higher than that of conjugate generated by alkaline reaction. Feng *et al.* (2018) revealed that conjugates of ovalbumin with epigallocatechin gallate (EGCG), epigallocatechin, or catechin synthesized by free-radical grafting method showed the stronger ORAC, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ABTS radical scavenging activities than ovalbumin. Among all the conjugates, ovalbumin-EGCG conjugate possessed the highest antioxidant activities. The conjugate of catechin and egg albumen protein exhibited augmented scavenging activity against DPPH and ABTS radicals, and had high FRAP (Gu *et al.*, 2017a). Furthermore, the grafting of cuttlefish skin gelatin and tannic acid under alkaline condition also increased antioxidant activities of the resulting conjugate (Aewsiri *et al.*, 2013). Polyphenols exhibited synergistic effect on antioxidant activities with protein hydrolysate when conjugates were formed (Jiang *et al.*, 2018). Chen *et al.* (2019) investigated the antioxidant activities of porcine plasma protein hydrolysates modified with oxidized chlorogenic acid or oxidized tannic acid. DPPH and ABTS radicals scavenging

activity, chelating activity and FRAP of porcine plasma protein hydrolysate-tannic acid and chlorogenic acid conjugates were significantly increased with increasing concentrations of oxidized polyphenols (Chen *et al.*, 2019). The antioxidant activities of protein-polyphenol conjugates were increased because of the introduction of hydroxyl groups from polyphenols into proteins (Czubinski and Dwiecki, 2017; Yildirim-Elikoglu and Erdem, 2018; You *et al.*, 2014). Thus, protein-polyphenol conjugates can be used to enhance the oxidative stability of several lipid-based foods.

1.2.7.3 Emulsifying properties of protein/peptide-polyphenol conjugates

A number of synthesized conjugates of proteins and polyphenols having the excellent emulsifying property have been produced. Liu *et al.* (2015a) synthesized lactoferrin–polyphenols (EGCG, chlorogenic acid and gallic acid) conjugates using free-radical grafting method, which had better emulsifying properties than unmodified lactoferrin. However, lactoferrin-chlorogenic acid conjugate showed the higher emulsifying properties than lactoferrin-EGCG and lactoferrin-gallic acid conjugates (Liu *et al.*, 2015a). Aewsiri *et al.* (2013) also found that conjugation of oxidized tannic acid under alkaline condition could significantly improve the emulsifying properties of cuttlefish skin gelatin. Moreover, conjugate prepared by covalent binding between β-lactoglobulin and catechin via free radical method could stabilize the oil-in-water emulsion more effectively than protein alone (Yi *et al.*, 2015). Abd El-Maksoud *et al.* (2018) covalently coupled caffeic acid to β-lactoglobulin using alkaline method. The resulting conjugates showed better emulsifying properties than native β-lactoglobulin. Recently, Chen *et al.* (2019) also documented that emulsifying activity index (EAI) and emulsifying stability index (ESI) of porcine plasma protein hydrolysate were significantly increased when oxidized chlorogenic acid and oxidized tannic acid were incorporated into hydrolysate. Moreover, porcine plasma protein hydrolysate-oxidized chlorogenic acid conjugate could rapidly absorb and make a thicker interfacial film around oil droplets, thus enhancing the emulsifying properties (Chen *et al.*, 2018b). The covalent attachment of polyphenol moieties could alter protein conformation and increase their surface

hydrophobicity, thus lowering the interfacial tension (Czubinski and Dwiecki, 2017; Gu *et al.*, 2017b). However, protein-polyphenol conjugation might affect emulsion formation negatively with a positive effect on emulsion stability. For instance, in a recent study by Chen *et al.* (2018a), tannic acid was reported to carry out strong interactions with ovalbumin. The conjugates of ovalbumin-tannic acid showed a masking effect on the hydrophobic groups of the ovalbumin and thus reduced the interfacial activity of the ovalbumin. The addition of tannic acid was documented to decrease the emulsifying ability; however, stability of the emulsions was enhanced by retarding creaming of the emulsions at isoelectric point of ovalbumin. The possible explanation for this phenomenon can be due to the enhanced electrostatic repulsion between the emulsion droplets by the presence of tannic acid, which could prevent the aggregation of the droplets near the isoelectric point of ovalbumin (Chen *et al.*, 2018a). Therefore, the effect of protein-polyphenol conjugation on the emulsifying properties depends on the type of protein and polyphenol involved, their structure as well as the pH, at which conjugation occurs.

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1.4 Objectives

1. To investigate the changes of quality, protease inhibitor and gelling property of duck egg albumen as affected by storage conditions.
2. To elucidate the changes of chemical compositions, trypsin inhibitory activity and gelling properties of duck egg albumen separated from salted whole egg during salting of 30 days.
3. To isolate and purify of trypsin inhibitors from duck albumen.
4. To study the impact of duck albumens on proteolysis and gel properties of sardine surimi compared to hen albumen.
5. To determine the changes in physiochemical and gelling properties of duck albumen as affected by desugarization and drying conditions.
6. To evaluate the effect of substitution of salted duck albumen powder on sardine surimi gelling properties.
7. To elucidate the influences of thermal pretreatment in combination with ultrasonication of duck egg albumen on enzymatic hydrolysis, and emulsifying properties, and antioxidant activities of resulting hydrolysates.
8. To study the characteristics and functionalities of duck albumen hydrolysate-EGCG conjugate and its effects on the stability of fish oil emulsion and fish tofu.

CHAPTER 2
QUALITY, PROTEASE INHIBITOR AND GELLING PROPERTY OF DUCK EGG ALBUMEN AS AFFECTED BY STORAGE CONDITIONS

2.1 Abstract

Physicochemical properties, trypsin inhibitory activity, and gelling properties of albumen from duck egg stored at 4 °C and room temperature (28-30 °C) were studied. As the storage time increased, Haugh unit and moisture content decreased, while the pH value increased (P<0.05). The rate of changes was lower at 4 °C. Trypsin inhibitory activity in albumen from egg stored at 4 °C was higher than that kept at room temperature throughout the storage time (P<0.05). Nevertheless, no differences in protein patterns were observed during the storage. Based on texture profile analysis, the highest hardness, gumminess, and chewiness were found at day 3 for room temperature and at day 6 for 4 °C. Higher values were attained for eggs kept at 4 °C. Conversely, albumen gels made from eggs stored at room temperature exhibited higher cohesiveness and springiness than those kept at 4 °C. The gels had the lowered whiteness when eggs were stored for a longer time, particularly at room temperature. Thus, storage condition directly affected the quality of albumen from duck egg.

2.2 Introduction

Over decades, eggs have been used for human consumption as a part of nutritional diet, rich in proteins, lipids, fat soluble vitamins, etc. (Lomakina and Mikova, 2006). Apart from its nutrition value, egg possesses the functional properties of several foods including solubility, water holding capacity, emulsifying, fat binding, foaming and gelling properties (Zayas, 1997). Amongst the commercially produced eggs, duck egg is one of the avian eggs, which has been consumed fresh and produced as the preserved form, especially salted egg, in some countries, e.g. China and South East Asia (Ganesan *et al.*, 2014).

Egg consists of two major edible parts, egg white (albumen) and yolk. Albumen possesses several biological substances such as lysozyme, cystatin, trypsin, ovomucoid and ovoinhibitor (Kopeć *et al.*, 2005). Both cysteine and trypsin protease inhibitors are present in albumen (Machleidt *et al.*, 1989). Cystatin shows a very strong inhibitory capacity towards ficin, papain and cathepsins B, H and L, whereas ovomucoid has the inhibitory activity towards trypsin and chymotrypsin (Kopeć *et al.*, 2005). Therefore, egg albumen has been employed in surimi to alleviate protein degradation caused by the endogenous proteinase, thus improving the properties of surimi gel (Benjakul *et al.*, 2001). In general, hen egg is deteriorated during storage at room temperature and quality loss can be retarded when kept at refrigerated storage (Akter *et al.*, 2014; Jin *et al.*, 2011; Samli *et al.*, 2005). Albumen pH and lipid oxidation of hen egg increased and Haugh unit decreased at higher rate at room temperature. Those changes were lower at refrigerated temperature up to 28 days of storage (Akter *et al.*, 2014).

However, no information regarding quality, composition especially trypsin inhibitor and functional property, particularly gelation of duck albumen, has been reported. Therefore, the purpose of this study was to investigate the changes of some selected quality index and properties of albumen from duck egg as affected by storage conditions.

2.3 Materials and methods

2.3.1 Chemicals

All chemicals used in this study were of analytical grade. Nα-Benzoyl-DL-arginine-*ρ*-nitroanilide (BAPNA), trypsin from bovine pancreas (Type I, ~10,000 BAEE units/mg protein), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO), and high and low molecular protein markers were purchased from GE healthcare UK Limited (Buckinghamshire, UK). Sodium dodecyl sulfate (SDS), mercaptoethanol (β-ME), glutaraldehyde, ethanol and Coomassie blue R-250 were obtained from Merck (Darmstadt, Germany).

2.3.2 Preparation of duck egg albumen

Fresh duck eggs within 24 h after laying were collected randomly at a farm house in Kantang, Trang province, Thailand. Eggs were stored at room temperature (28-30 °C) and 4 °C. Eggs were randomly taken every 3 days and broken. Albumen was separated from egg yolk manually. Albumen was subjected to analyses.

2.3.3 Quality index of duck egg during storage

Haugh unit (HU) was used as quality index of duck egg during storage. HU was determined using Haugh unit tester (Technical Services and Supplies, Technical Services and Supplies Ltd (TSS), York city, England). It was calculated from the recorded egg weights and albumen heights using the following formula (Samli *et al.*, 2005):

$$HU = 100 \log_{10} (H - 1.7 W^{0.37} + 7.56)$$

where HU = Haugh unit, H = height of the albumen (mm), and W = egg weight (g).

2.3.4 Changes in pH and chemical composition of albumen during storage

2.3.4.1 Determination of moisture content, protein content and pH

Moisture and protein contents of albumen samples were determined using oven method (AOAC, 2000) and the Biuret method (Robinson and Hogden, 1940), respectively. The pH of albumen was measured directly using pH meter (pH 700, EUTECH Instruments, Singapore).

2.3.4.2 Determination of trypsin inhibitory activity

Trypsin inhibitory activity was measured according to the method of Benjakul *et al.* (2001). Albumen solution with an appropriate dilution (200 µL) was incubated with 200 µL of porcine pancreas trypsin (0.05 mg/mL) at 37 °C for 15 min. Then, 1000 µL of reaction buffer (50 mM Tris-HCl containing 20 mM $CaCl_2$, pH 8.2) was added. Thereafter, 200 µL of BAPNA (2 mg/mL) was added and incubated at 37 °C for 15 min. To stop the reaction, 200 µL of 30% acetic acid (v/v) were added. The release of *ρ*-nitroalaniline was measured by a spectrophotometer at 410 nm (UV-16001, SHIMADZU, Kyoto, Japan). One unit of trypsin activity was defined as the enzyme

causing an increase of 0.01 absorbance unit/min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduced trypsin activity by one unit.

2.3.4.3 SDS-PAGE

Protein compositions of albumen samples were visualized by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (1970). To prepare protein sample, 2 mL of albumen were mixed with 12 mL of SDS 5% (w/v). The mixture was homogenized and heated at 85 °C for 40 min. Protein concentration was determined by the Biuret method (Robinson and Hogden, 1940). Sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol, with and without 10% β-ME) was added in protein samples and boiled for 3 min. The prepared sample (15 mg protein) was loaded onto gel electrophoresis comprising 12% running gel and 4% stacking gel and subjected to electrophoresis at constant current of 15 mA/gel using electrophoresis unit (Mini-protein II; Bio-Rad Laboratories, Richmond, CA, USA). The gels were stained with Coomassie Brilliant Blue R-125 (0.125%) in 25% methanol and 10% acetic acid. Destaining was performed using 40% methanol and 10% acetic acid. Molecular weight (MW) of protein bands was estimated from the plot of MW standards and Rf.

2.3.5 Changes in gel properties of albumen during the storage

2.3.5.1 Preparation of albumen gel

Albumen gel was prepared following the method of Mmadi *et al.* (2014) with slight modification. Albumen was added with distilled water to obtain the final solid content of 10%. The mixture was stirred gently. Then, the solution was poured into a casing (diameter of 25 mm with the length of 30 cm). Both ends were sealed tightly, and heated at 90 °C for 30 min. Thereafter, the gel was cooled immediately at 4 °C and kept overnight. Finally, gel samples were cut into cylinders (diameter 25 mm, height 30 mm) prior to analyses.

2.3.5.2 Texture profile analysis

Texture profile analysis of gel was performed using a texture analyzer (Model TA-XT2i, Stable Micro System, Surrey, England). The samples were compressed twice to 40% of their original height with a compression cylindrical aluminum probe (50 mm diameter). Force-distance deformation curve was recorded at a cross head speed of 3 mm/s and the recording speed was 3 mm/s. Hardness (N), springiness (cm), cohesiveness, chewiness (N.cm) and gumminess (N) were evaluated. These parameters were recorded using the MicroStable software version 6 (Surrey, England).

2.3.5.3 Determination of color

The color of gel samples was determined by a colorimeter (ColorFlex, Hunter Lab, Reston, VA, USA) and reported in CIE system. L^*, a^*, b^* and ΔE^*, representing lightness, redness/greenness, yellowness/blueness and total difference of color, respectively. The whiteness of gel was calculated (Kaewmanee *et al.*, 2011) using the following equation:

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

The ΔE^* was also calculated by the following formulation:

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

where ΔL^*, Δa^*, Δb^* are the difference between color parameters of the samples and the color parameters of albumen gel at day 0 (L^* = 88.35, a^* = - 2.52, b^* = -2.44).

2.3.5.4 Determination of microstructure

Microstructure of albumen gels stored for the selected time at both storage temperatures was visualized by a scanning electron microscopy as described by Kaewmanee *et al.* (2011). Central part of albumen gels was cut into small pieces (1x1x1 mm^3). Samples were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. Then, fixed samples were rinsed with distilled water for 1 h. Subsequently, samples were dehydrated using ethanol with various concentrations (25, 50, 70, 80, 90,

and 100%) for 15 min at each concentration. The dehydrated samples were subjected to critical point drying (CPD). The samples were coated with 100% gold (sputter coater SPI-Module, West Chester, PA, USA). The gel microstructure was visualized by a scanning electron microscope (JEOL JSM-5800LV, Tokyo, Japan).

2.3.6 Statistical analysis

All the experiments were conducted in triplicate using three lots of samples. Data were presented as mean value with standard deviation. One way variance of analysis (ANOVA) and the Duncan's multiple range tests were carried out to determine the significant difference between samples at $P<0.05$ level using the statistical program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, U.S.A.).

2.4 Results and discussion

2.4.1 Effect of storage temperature and time on quality index of duck egg during storage

Haugh unit (HU) of duck eggs stored at 4 °C and room temperature up to 15 days is shown in Fig. 8. HU is the standard quality index of egg and is considered to be a visually appearance measurement describing the height of egg albumen when it is broken onto a flat surface (Jones and Musgrove, 2005; Samli *et al.*, 2005). HU of fresh duck egg in this study was approximate 81 and graded as AA quality based on USDA (Department of Agriculture, 2000). HU of egg decreased after 3 days of storage ($P<0.05$), regardless of storage temperature. Thereafter, slight change in HU was observed in eggs stored at 4 °C after 15 days ($P<0.05$), while continuous decrease was found in those kept at room temperature ($P<0.05$). At the same storage time, particularly more than 9 days, much higher HU was found in eggs kept at lower temperature ($P<0.05$). After 15 day, HU of 74 was obtained for eggs stored at 4 °C, which was considered as AA. For eggs stored at room temperature, HU was 49, thus being graded as B. The decrease in HU could be explained that the ovomucin – lysozyme complex was destroyed. This was associated with the reduction of thick albumen height during storage (Akter *et al.*, 2014). These results were in accordance with Akter *et al.* (2014); Jin *et al.* (2011); Siyar *et al.* (2007) who reported that HU of hen eggs was appreciably affected by storage time and temperature.

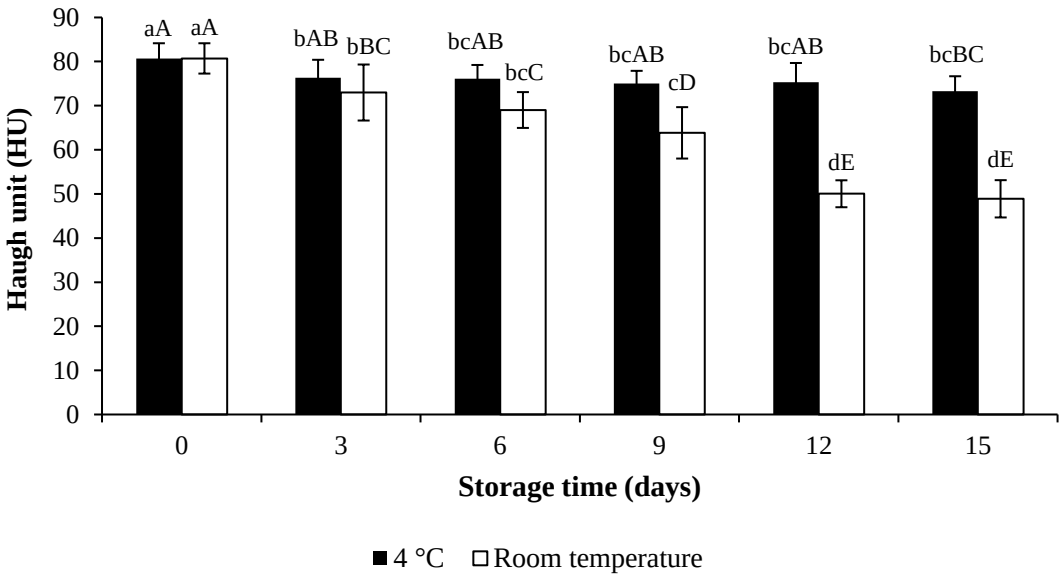

Figure 8. Changes in Haugh unit (HU) of duck egg during 15 days of storage at different temperatures. Mean±SD (*n*=7). Different lowercase letters on the bars within the same storage temperature indicate significant differences. Different uppercase letters on the bars indicate significant differences ($P<0.05$)

2.4.2 Effect of storage temperature and time on pH and chemical compositions of albumen during storage

2.4.2.1 Change in moisture content

Moisture content of albumen from duck egg kept at 4 °C and room temperature are presented in Table 3. Moisture content of albumen of egg stored at room temperature decreased from 87.9% at day 0 to 86.2% after 15 days of storage. Nevertheless, no change in moisture content was observed throughout the storage of 15 days at 4 °C ($P>0.05$). During the extended storage, particularly at room temperature, water was lost from albumen during storage through the shell pores (Bell, 1996; Ren *et al.*, 2010).

2.4.2.2 Change in pH

pH value of albumen of duck eggs increased dramatically ($P < 0.05$) after 3 days from 8.66 to 9.01 and 9.21 when stored at 4 °C and room temperature, respectively (Table 3). The pH increased up to day 6 ($P < 0.05$), and pH remained constant up to

day 12 ($P > 0.05$) within the first 6 days of storage. Carbon dioxide (CO_2) from albumen might be lost continuously via the shell pores. CO_2 in egg is formed when the balance of the carbonate-bicarbonate buffer system was shifted towards production of CO_2 in egg (Akter *et al.*, 2014). The loss in CO_2 of stored hen and quail egg was reported by Akter *et al.* (2014); Jin *et al.* (2011); Silversides and Scott (2001); Itoh *et al.* (1981). Thus, storage temperature and time had the influence on pH of albumen from duck egg.

Table 3. Moisture content, total trypsin inhibitory activity and pH value of albumen from duck egg during 15 days of storage time at different temperatures

Temperature	Storage time (days)	Moisture content (%)	Total trypsin inhibitory activity (kunits/mg solid)	pH
4 °C	0	$87.93\pm0.18^{\dagger aA}$	10.47 ± 0.60^{aA}	8.66 ± 0.07^{cF}
	3	87.90 ± 0.21^{aA}	9.88 ± 0.17^{abB}	9.01 ± 0.03^{bE}
	6	87.89 ± 0.03^{aA}	9.52 ± 0.10^{bBC}	9.16 ± 0.03^{aD}
	9	87.82 ± 0.08^{aAB}	9.20 ± 0.07^{bcC}	9.12 ± 0.02^{aD}
	12	87.69 ± 0.12^{aAB}	8.62 ± 0.07^{cD}	9.13 ± 0.01^{aD}
	15	87.30 ± 0.10^{bC}	7.66 ± 0.01^{dE}	9.13 ± 0.02^{aD}
Room temperature	0	$87.93\pm0.18^{*aA}$	10.47 ± 0.60^{aA}	8.66 ± 0.07^{dF}
	3	87.74 ± 0.04^{bAB}	9.34 ± 0.13^{bBC}	9.21 ± 0.01^{cC}
	6	87.68 ± 0.15^{bAB}	9.30 ± 0.07^{bBC}	9.40 ± 0.01^{aA}
	9	87.60 ± 0.10^{bB}	8.21 ± 0.15^{cD}	9.36 ± 0.02^{aA}
	12	86.86 ± 0.07^{cD}	8.48 ± 0.11^{cD}	9.36 ± 0.02^{aA}
	15	86.27 ± 0.04^{dD}	6.79 ± 0.03^{dF}	9.36 ± 0.01^{aA}

†Mean±SD (n=3). Different lowercase superscripts in the same column within the same storage temperature indicate significant differences ($P<0.05$). Different uppercase superscripts in the same column indicate significant differences ($P<0.05$).

2.4.2.3 Change in trypsin inhibitor

With different storage temperatures and times, trypsin inhibitory activity of albumen was found to be varied (Table 3). Total trypsin inhibitory activity in freshly laid eggs was highest (10.47 kunits/mg solid). The decrease in trypsin inhibitory

activity was found as storage time increased. After 15 days, activities of 7.66 and 6.79 kunits/mg solid were obtained in albumen from egg stored at 4 °C and room temperature, respectively. In general, higher activity was noticeable in albumen of egg stored at 4 °C, compared to room temperature (P<0.05) at the same storage time. The denaturation of ovalbumin and other proteins with protease inhibitory activity might cause the reduction of trypsin inhibitory activity in albumen. Ovalbumin was transferred to ovalbumin isoform (S-ovalbumin) after storage, especially at high temperature (Qiu *et al.*, 2012). Takenawa *et al.* (2015) reported that ovalbumin had inhibitory effect on trypsin activity. Therefore, the change and denaturation of ovalbumin resulted in the loss of its biological activity, especially protease inhibitory activity (Rehault-Godbert *et al.*, 2010). Decrease in antiprotease activity correlated with the deterioration of some pivotal proteins such as clusterin, lysozyme, ovotranferrin during storage at high temperature (Qiu *et al.*, 2012). Rehault-Godbert *et al.* (2010) reported that long storage at 37 °C was associated with reduced antitryptic and antichymotryptic activity of hen egg albumen, which was related with the degradation of ovalbumin and ovotransferrin. Moreover, Kopeć *et al.* (2005) also stated that after 2 weeks of storage at 15 °C, the activity of trypsin inhibitors in hen egg albumen decreased. Thus, trypsin inhibitor was still retained to a higher extent when duck egg was kept at low temperature.

2.4.2.4 Change in protein pattern

Protein is the dominant constituent of albumen with an average amount of 9.7% to 10.6% (w/w) (Phillips and Williams, 2011). Ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), lysozyme (3.5%), and ovomucin (3.5%) are considered as the main proteins found in albumen (Abeyrathne *et al.*, 2013). SDS-PAGE patterns of albumen from duck egg stored at 4 °C and room temperature under non-reducing and reducing condition are illustrated in Fig. 9. Ovalbumin with MW of 45 kDa was found as the most dominant protein. Under non-reducing condition, protein with MW of 81-84 kDa and 70-72 kDa were also observed. Protein with MW of 81-84 kDa was more likely ovotransferrin. Under reducing condition, the thick band of ovotransferrin appeared as the thin band. Ovotransferrin has 15 disulfide bonds and folded into 2 lobes and 4 domains. Each lobe is composed of 2 distinct α- and β-domains (Abeyrathne *et*

al., 2013). Reducing agent (β-ME) most likely broke down disulfide bonds of this protein and those domains were dissociated from each other. Additionally, four bands were inexplicitly observed under reducing condition. Those had MW of 181.48, 151.48, 126.44 kDa. Those proteins might be subunit of egg albumen protein stabilized by disulfide bonds. Moreover, lysozyme was found at MW of 15.0-15.7 kDa in both non-reducing and reducing conditions.

Overall, no remarkable change in protein patterns of albumen from duck egg stored at low temperature for 15 days. Although there was no drastic degradation of most proteins in egg albumen, some proteins such as trypsin inhibitors might undergo denaturation in which the bioactivity could be reduced. Ovalbumin was converted to S-ovalbumin in hen egg during storage (Qiu *et al.*, 2012). When eggs were stored under refrigerated condition, this transformation was negligible (Alleoni, 2006). These results were supported by Rehault-Godbert *et al.* (2010) and Qiu *et al.* (2012) who found the differences in SDS-PAGE pattern of hen egg albumen stored at 20 and 4 °C. However, the intensity of the bands of ovalbumin and ovostranfferin kept at 37 °C seemed to increase after 14 days storage. Itoh *et al.* (1981) reported that SDS-PAGE pattern of thin quail egg white stored at 30 °C showed less clear bands of ovalbumin and ovostranferrin. Higher temperature generally caused the higher changes in proteins of stored egg. Nevertheless, storage temperature in the present study had no profound effect on protein pattern of duck egg kept for up to 15 days. This might be due to the differences in molecular property and stability of proteins between duck egg and hen egg.

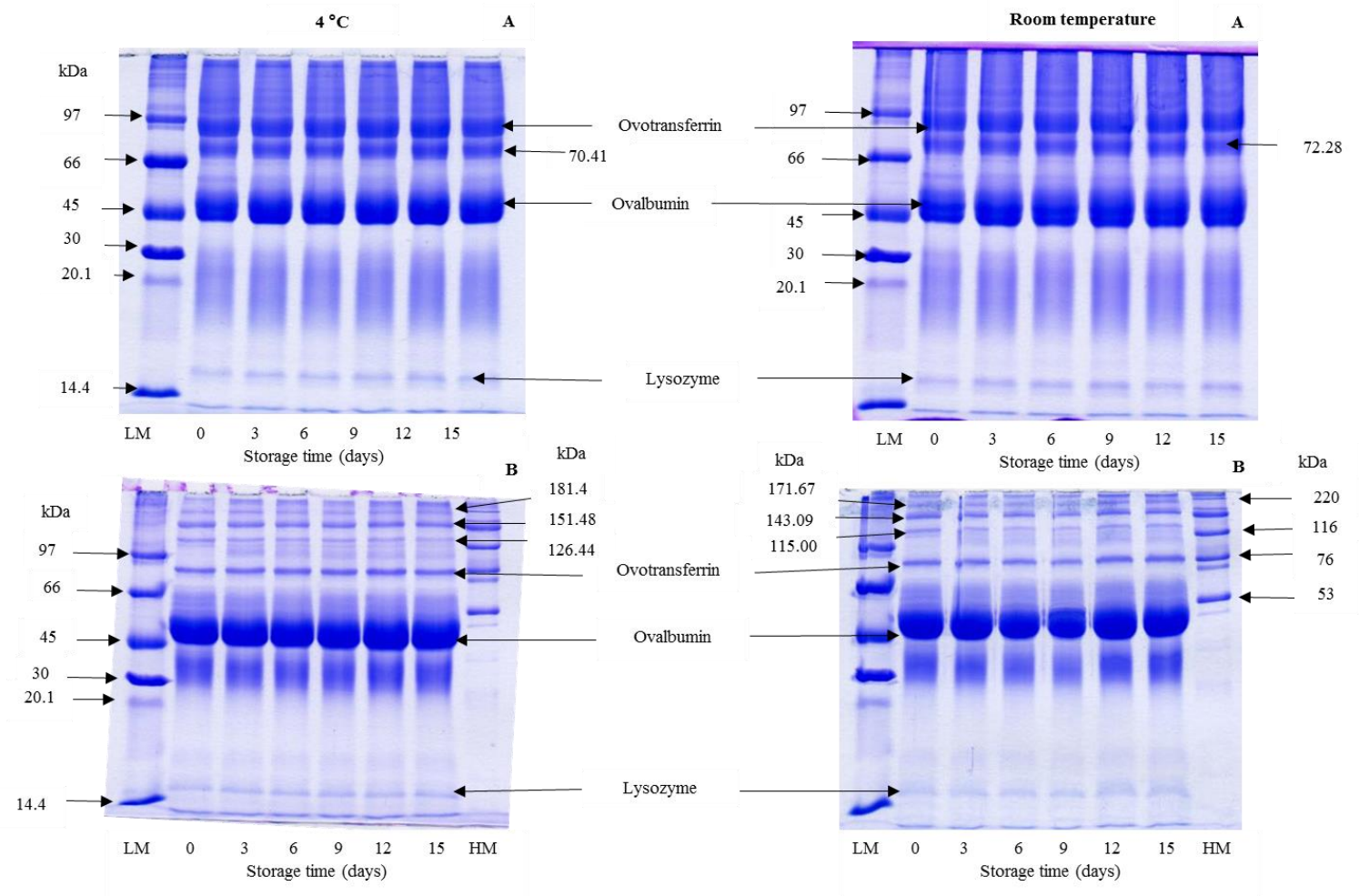

Figure 9. SDS-PAGE patterns of albumen from duck egg during 15 days of storage at different temperatures under non-Reducing (A) and reducing (B) conditions

2.4.3 Effect of storage temperature and storage time on gelling properties of albumen during storage

2.4.3.1 Texture profile analysis

Hardness, springiness, cohesiveness, chewiness and gumminess of albumen from duck egg stored at different temperatures and times are shown in Table 4. Hardness is correlated to the strength of the gel structure under the first compression cycle (Lau *et al.*, 2000). The hardness of albumen gel increased markedly ($P<0.05$) after 3 days of storage for both storage temperatures. However, higher hardness was found for egg kept at room temperature (29.26 N) than that of 4 °C (27.77 N). It was noted that hardness of albumen gel kept at 4 °C increased continuously up to day 6 and reached the maximal value (30.18 N). After 6 and 3 days of storage at 4 °C and room temperature, respectively, hardness of both gels decreased continuously up to the end of storage (15 days). However, these values were still higher than that of freshly laid eggs. pH generally affects turbidity and hardness of heat-induced ovalbumin gels. This was governed by alternation of the net charge of protein and the reactivity of sulfhydryl groups. When pH increases naturally in albumen during the storage of eggs, the gel elasticity and gel rigidity are increased (Phillips and Williams, 2011). Raikos *et al.* (2007) found that the highest hardness values for whole hen egg were obtained at pH 8 and the lowest value was found at pH 2. Additionally, Phillips and Williams (2011) reported that a maximum hardness of the egg white ovalbumin gel was found at pH of 9.0. With increasing storage time, hardness decreased. This might be caused by higher S-ovalbumin content, which could not undergo aggregation effectively on cooking. This lead to the lower gel strengths, compared to that of N-ovalbumin (Hammershøj *et al.*, 2002; Phillips and Williams, 2011).

The gradual increase in cohesiveness of albumen gel was observed with increasing storage time at both storage temperatures up to day 12 (Table 4). At the same time storage, higher values were found in sample kept at room temperature. At day 15, cohesiveness decreased slightly ($P<0.05$), regardless of storage temperature. Cohesiveness is a parameter to measure of the difficult level in breaking down the internal structure of gel (Lau *et al.*, 2000). Springiness of albumen gels is shown in the Table 4. Springiness of albumen gel increased slightly within the first three days

(P<0.05) and remained constant until day 9 and 12 for egg kept at room temperature and 4 °C, respectively. In general, no marked difference in springiness was obtained between albumen from egg kept both temperatures during the first 9 days of storage. Springiness is considered as "elasticity" or "rubberiness" of the gel in the mouth, and is a parameter to show how much the gel structure is broken down by the initial compression (Lau *et al.*, 2000).

Gumminess and chewiness of gels from egg stored at 4 °C and room temperature showed the increase within the first 6 days and 3 days, respectively. Thereafter, the values remained unchanged and decreased at day 12 or 15, respectively. In general, the values were lower in gels from egg stored at room temperature, compared to those kept at 4 °C. The changes in gumminess and chewiness were in agreement with those of hardness. Gumminess and chewiness suggest the resistance to compression force (Yilmaz *et al.*, 2012).

Table 4. Texture profile analysis of albumen gel from duck egg during 15 days at different temperatures

Temperature	Storage time (days)	Hardness (N)	Cohesiveness	Springiness (cm)	Gumminess (N)	Chewiness (N.cm)
4 °C	0	19.08±0.54$^{\dagger dI}$	0.72±0.01^{eI}	0.92±0.01^{dEF}	13.67±0.29^{dF}	12.56±0.44^{dE}
	3	27.77±0.71^{cD}	0.77±0.00^{dH}	0.94±0.01^{aAB}	21.12±0.56^{cD}	19.95±0.59^{cB}
	6	30.18±0.39^{aA}	0.78±0.00^{cG}	0.94±0.01^{aABC}	23.78±0.48^{aA}	21.83±0.61^{aA}
	9	28.71±0.73^{bBC}	0.81±0.01^{aE}	0.94±0.01^{abBCD}	22.79±0.78^{bB}	21.61±0.57^{abA}
	12	28.27±0.37^{bCD}	0.81±0.00^{aE}	0.93±0.01^{bcCDE}	22.89±0.26^{bB}	21.48±0.48^{abA}
	15	27.98±0.70^{bcCD}	0.80±0.01^{bF}	0.93±0.01^{bcDE}	22.71±0.48^{bB}	21.22±0.49^{bA}
Room temperature	0	19.08±0.54^{fI}	0.72±0.01^{fI}	0.92±0.01^{bEF}	13.67±0.29^{dF}	12.56±0.46^{eE}
	3	29.26±0.81^{aB}	0.80±0.01^{eF}	0.94±0.01^{aBCD}	23.30±0.63^{aAB}	21.58±0.97^{aA}
	6	26.68±0.71^{bE}	0.82±0.00^{dD}	0.95±0.01^{aA}	21.78±0.59^{bC}	20.28±1.00^{bB}
	9	24.99±1.07^{cF}	0.86±0.01^{bB}	0.95±0.01^{aA}	21.57±0.76^{bCD}	19.72±0.73^{bcB}
	12	23.23±0.86^{dG}	0.87±0.00^{aA}	0.91±0.02^{bcF}	19.12±0.82^{cE}	18.85±1.03^{cC}
	15	21.80±0.88^{eH}	0.85±0.01^{cC}	0.89±0.00^{cG}	19.82±0.85^{cE}	16.81±1.13^{dD}

†Mean±SD (n=3). Different lowercase superscripts in the same column within the same storage temperature indicate significant differences (P<0.05). Different uppercase superscripts in the same column indicate the significant differences (P<0.05).

2.4.3.2 Color

Color values of albumen gel of duck eggs stored at 4 °C and room temperature are shown in Table 5. The highest lightness (L^*) and whiteness of albumen gels were found at day 0 and decreased continuously as the storage time of duck egg increased ($P<0.05$). The gels color became darker with increasing storage time. Nevertheless, the extent of decrease was higher when egg was kept at higher temperature. There was the decrease in b^*-values of albumen gel throughout the storage. For a^*-value, the value of both gels decreased up to day 15 ($P<0.05$). The sharply increased ΔE^* was found in gel after the eggs were stored for 3–6 days. For gel from egg stored at room temperature, the higher value was observed, compared to that of gel from egg kept at 4 °C ($P<0.05$). These data confirmed that color values of egg albumen gel were significantly affected by both storage time and temperature. Those changes might be related with the dissociation of ovomucin components, especially the soluble β component. Degradation of O-glycosidic bonds following the solubilization of β-ovomucin releases the carbohydrates (hexoses, hexosamine, sialic acid) especially at alkaline pH (Guyot *et al.*, 2013). With increasing storage time, the pH became more increased, which could favour those reactions. As a result, the browning reaction (Maillard reaction) between reducing sugar and amino acid could occur to a higher extent. Refrigerated condition could retard the degradation of O-glycosidic bonds. Coincidentally, Maillard reaction could be retarded. Additionally, the color of gel turned to green as indicated by the lower a*-value. This was plausibly due to iron (II) sulphide formed. Iron from the yolk reacted with hydrogen sulfide released from the albumen by the heat. This reaction occurred more rapidly in older eggs as the albumen became more alkaline in pH (Tinkler and Soar, 1920). On the other hand, the higher storage temperature could augment those changes as evidenced by the darker color of gel of stored duck egg.

2.4.3.3 Microstructure

Scanning electron micrographs of albumen gel from duck eggs stored at 4 °C and room temperature for 3 and 15 days were compared as illustrated in Fig. 10. Fresh duck egg gels were less compact with opened structure. Its network contained random aggregate with less connectivity. After 3 days of storage at 4 °C and room temperature, the denser networks with higher connection were observed. No large voids were

observed. There was no difference in gel network of albumen from duck egg stored at temperatures between 4 °C and room temperature. The denser and compact structure of gel coincided with the increased hardness. With increasing storage time, pH became more alkaline. As a result, unfolding of protein was enhanced, in which the subsequent aggregation induced by heat could be enhanced. The unfolded proteins might align themselves tightly and closely as indicated by compact gel network. Gel with better viscoelastic properties and high water-holding capacity was due to the high cross-linking (Croguennec *et al.*, 2002). After 15 days of storage, the gel network became coarse, especially gel from egg kept at room temperature. The larger voids were obviously observed. The less compactness with the increase voids was related with the decrease in hardness of gel (Table 4). The result revealed that the storage temperature and time had the impact on gel structure of albumen from duck egg.

Table 5. Color and whiteness of albumen gel from duck egg during 15 days of storage at different temperatures

Temperature	Storage time (days)	*L**	*a**	*b**	ΔE*	Whiteness
4 °C	0	88.35±0.01$^{\dagger aA}$	-2.52±0.03^{aA}	-2.44±0.07^{aA}	0.00±0.00^{eH}	87.83±0.13^{aA}
	3	83.38±0.17^{bB}	-3.57±0.04^{bB}	-3.44±0.27^{aA}	5.22±0.16^{dG}	82.64±0.17^{bB}
	6	81.29±0.19^{cC}	-3.57±0.04^{bB}	-3.49±0.12^{aA}	7.21±0.19^{bF}	80.63±0.19^{cC}
	9	81.20±0.25^{cC}	-3.64±0.04^{cBC}	-5.20±0.13^{bB}	7.72±0.22^{bF}	80.18±0.24^{cCD}
	12	80.02±0.26^{dCD}	-3.63±0.06^{cBC}	-5.93±0.11^{cD}	9.08±0.29^{aD}	78.86±0.29^{dE}
	15	79.58±0.24^{dD}	-3.64±0.05^{cD}	-6.25±0.11^{dE}	9.34±0.17^{aC}	78.42±0.17^{dE}
Room temperature	0	88.35±0.01^{aA}	-2.52±0.03^{aA}	-2.44±0.07^{aA}	0.00±0.00^{dH}	87.83±0.13^{aA}
	3	80.35±0.31^{bCD}	-3.98±0.05^{bD}	-3.50±0.25^{aA}	8.21±0.34^{cE}	79.63±0.34^{bD}
	6	80.63±0.22^{bCD}	-4.24±0.09^{cE}	-3.50±0.15^{aA}	8.02±0.20^{cE}	79.82±0.20^{bD}
	9	64.04±0.39^{cE}	-4.47±0.11^{dF}	-5.59±0.18^{bC}	24.59±0.39^{bB}	63.33±0.39^{cF}
	12	62.89±0.67^{cdE}	-4.50±0.09^{dF}	-6.11±0.84^{cDE}	25.81±0.68^{aA}	62.11±0.67^{dG}
	15	63.40±0.53^{dE}	-4.53±0.09^{dF}	-6.74±0.12^{dF}	25.39±0.53^{aA}	62.51±0.53^{dG}

†Mean±SD (n=3). Different lowercase superscripts in the same column within the same storage temperature indicate significant differences (P<0.05). Different uppercase superscripts in the same column indicate the significant differences (P<0.05).

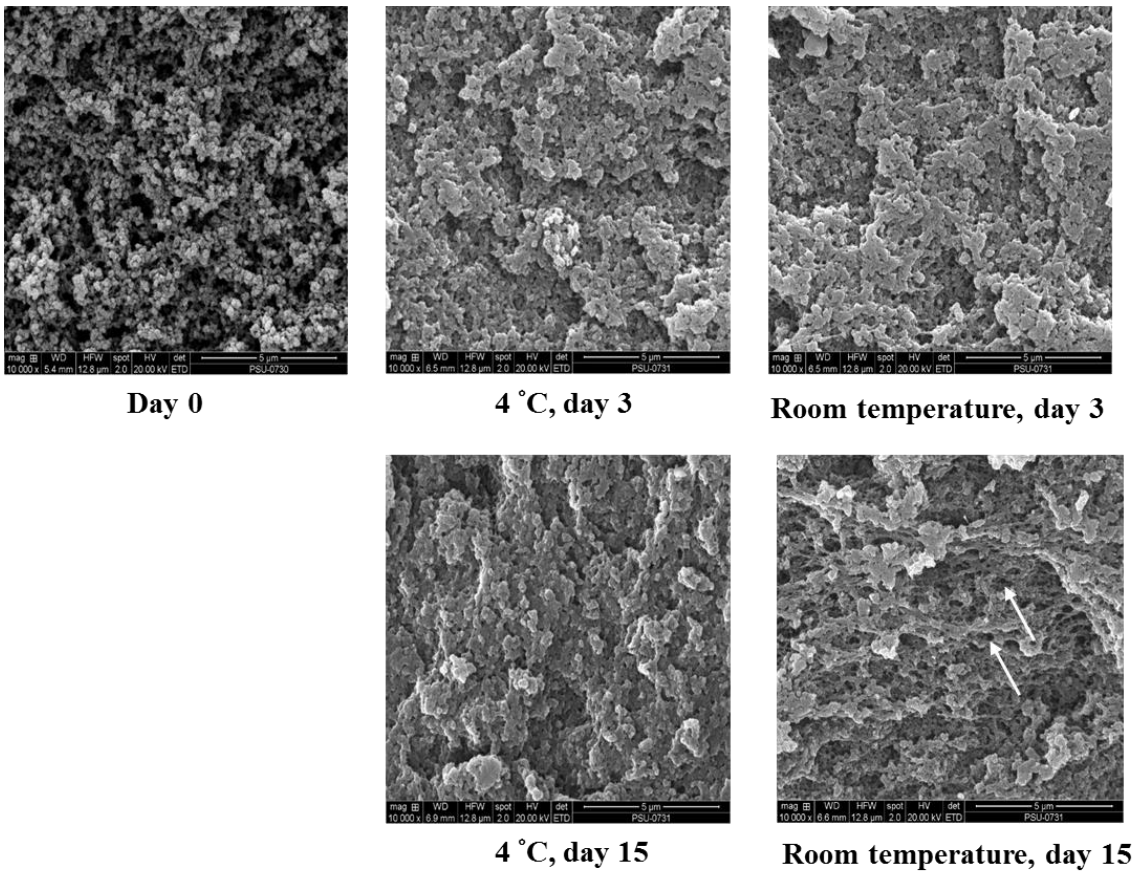

Figure 10. Scanning electron microscopic photograph of albumen gel from duck egg stored at 4 °C and room temperature for selected times. Magnification: 10000×. Scale bar = 5 μm. Arrows sign indicate voids.

2.5 Conclusion

Duck egg albumen quality and functional property was affected by storage conditions. Eggs stored at refrigerated temperature could maintain the bioactive component, especially trypsin inhibitors during storage. Properties of albumen gel could be improved when eggs were kept for 3 days. On the other hand, gel became weaker with the lowered whiteness with increasing storage time, particularly at room temperature. Thus, storage conditions could be optimized to exploit the albumen from duck egg into other food products such as surimi or meat ball.

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CHAPTER 3
COMPOSITIONS, PROTEASE INHIBITOR AND GELLING PROPERTY OF DUCK EGG ALBUMEN AS AFFECTED BY SALTING

3.1 Abstract

Chemical compositions, trypsin inhibitory activity, and gelling properties of albumen from duck egg during salting of 30 days were studied. As the salting time increased, moisture content decreased, the salt content and surface hydrophobicity increased (P<0.05). Trypsin inhibitory activity and specific activity were continuously decreased throughout the salting time of 30 days (P<0.05). This coincided with the decrease in band intensity of inhibitor with molecular weight of 44 kDa as examined by inhibitory activity staining. Nevertheless, no differences in protein patterns were observed in albumen during the salting of 30 days. Based on texture profile analysis, hardness, springiness, gumminess, and chewiness of albumen gel decreased with increasing salting time. Conversely, salted albumen gels exhibited higher cohesiveness, compared to those of fresh albumen. Scanning electron microscopic study revealed that gel of salted albumen showed the larger voids and less compactness. In general, salting lowered trypsin inhibitory activity and gelling property of albumen from duck egg to some extent. Nevertheless, the salted albumen with the remaining inhibitor could be an alternative additive for surimi or other meat products to prevent proteolysis.

3.2 Introduction

Salted egg is a popular traditional product in some Asian countries. To manufacture salted eggs, fresh eggs are soaked in brine solution or coated with salt ash or charcoal (Chi and Tseng, 1998; Yang *et al.*, 2016). Commonly, customers prefer salted duck egg rather than hen counterpart, since its characteristics are better with richer flavour. In general, salted egg yolk is of higher demand than salted albumen, which is commonly discarded. Albumen of salted duck egg contains 4-7% sodium chloride, which is not suitable to apply in food products (Kaewmanee *et al.*, 2011a;

Mmadi *et al.*, 2014). To conquer such a problem, desalination process and electrodialysis were used to remove salt in albumen Furthermore, fresh duck egg yolk was separated from albumen and directly salted. As a consequence, no salt is contaminated in egg albumen and it can be used as raw material for food products or other applications (Wang, 2016).

Albumen is well-known with diversity of bioactive compositions and nutrients. Lysozyme has been functionally prescribed as N-acetylmuramoyl hydrolase (Ren *et al.*, 2010). Ovomucoid, ovoinhibitor, and cystatin are considered as protease inhibitors; ovotransferrin, ovoflavoprotein, and avidin are functionally clarified as mineral and vitamin binding agents (Rossi *et al.*, 2013). In general, physicochemical properties of albumen in duck egg underwent changes during salting. Moisture content decreased gradually, whereas salt, ash, protein and lipid contents increased. Moreover, albumen obtained from cooked salted whole duck egg had lower hardness as the salting time increased (Kaewmanee *et al.*, 2011a; Kaewmanee *et al.*, 2011b).

Albumen from salted duck egg, considered as waste, could be exploited as food additives, particularly in muscle food gel, in which salt is required for solubilization of proteins. Furthermore, the protease inhibitors remaining in albumen could also serve as additive to prevent proteolysis associated with gel softening (Benjakul and Visessanguan, 2000). Additionally, salted albumen with gel forming ability could co-gel with major myofibrillar proteins during gelation of processed muscle foods. This could help strengthen the resulting gel. Thus, salted albumen could be employed as the additives in surimi or muscle food industry. However, a little information regarding trypsin inhibitor, protein pattern, and gelling properties of liquid duck egg albumen as affected by salting time exists. Therefore, the objective of this study was to investigate the changes of chemical compositions, trypsin inhibitory activity and gelling properties of duck egg albumen separated from salted whole egg during salting of 30 days.

3.3 Materials and methods

3.3.1 Chemicals

All chemicals used in this study were of analytical grade. Na-Benzoyl-DL-arginine-ρ-nitroanilide (BAPNA), trypsin from bovine pancreas (Type I, ~10,000

BAEE units/mg protein), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (USA), and high and low molecular protein markers were purchased from GE healthcare UK Limited (UK). Sodium dodecyl sulfate (SDS), β-mercaptoethanol (β-ME), 8-anilino-1-naphthalenesulfonic acid (ANS), glutaraldehyde, ethanol and Coomassie blue R-250 were obtained from Merck (Germany).

3.3.2 Preparation of salted duck egg

Fresh duck eggs within 24 h after laying were collected from a farm in Kantang, Trang province, Thailand. After cleaning with tap water, the eggs were immersed in the brine solution (25%, w/v) using 1 egg/100 mL brine (Kaewmanee *et al.*, 2011b). Brining was performed at room temperature (28-30 °C). Ten eggs were randomly taken every 5 days. The samples were broken and albumen was separated from egg yolk manually. Albumen was subsequently subjected to analyses.

3.3.3 Changes in chemical compositions of albumen

3.3.3.1 Determination of moisture, salt, and protein contents

Moisture and protein contents of albumen samples were determined using oven method (AOAC, 2000) and the Biuret method (Robinson and Hogden, 1940), respectively. Salt content in albumen samples was measured by the AOAC method described as Kaewmanee *et al.* (2011b).

3.3.3.2 Determination of surface hydrophobicity

Surface hydrophobicity of albumen samples was determined according to the method of Kaewmanee *et al.* (2011a) using 8-anilo-1-naphthalenesulfonic acid (ANS) as a probe. Surface hydrophobicity was calculated from the initial slope of the plot of fluorescence intensity against protein concentration determined by the Biuret method using a linear regression analysis. The initial slope was referred to as surface hydrophobicity (S_0ANS).

3.3.3.3 Determination of trypsin inhibitory activity

Trypsin inhibitory activity was measured as per the method of Benjakul *et al.* (2001). Albumen samples with an appropriate dilution (200 μL) were incubated with

200 μL of porcine pancreas trypsin (0.05 mg/mL) at 37 °C for 15 min. Then, 1000 μL of reaction buffer (50 mM Tris-HCl containing 20 mM $CaCl_2$, pH 8.2) were added. Thereafter, 200 μL of BAPNA (2 mg/mL) were added and the mixture was incubated at 37 °C for 15 min. To terminate the reaction, 200 μL of 30% acetic acid (v/v) was added. The release of ρ-nitroaniline was monitored by measuring the absorbance at 410 nm using a spectrophotometer (UV-16001, SHIMADZU, Japan). One unit of trypsin activity was defined as the enzyme causing an increase of 0.01 absorbance unit/min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduced trypsin activity by one unit. At the sampling times, trypsin inhibitor was expressed as kunits/mg solids. Specific activity was also calculated and reported as kunits/mg protein.

3.3.3.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of albumen samples were determined by SDS-PAGE according to the method of Laemmli (1970). To prepare protein sample, 2 mL of albumen were mixed with 12 mL of 5% SDS. The prepared sample (15 mg protein) was loaded onto the gel (12% running gel and 4% stacking gel) and subjected to electrophoresis at constant current of 15 mA/gel using electrophoresis unit (Mini-protein III; Bio-Rad Laboratories, USA). The gels were stained with Coomassie Brilliant Blue R-125 (0.125%) in 25% methanol and 10% acetic acid. Destaining was performed using 40% methanol and 10% acetic acid. Molecular weight (MW) of protein bands was estimated from the plot of MW standards and Rf.

3.3.3.5 Inhibitory activity staining

Inhibitory activity staining was conducted according to the method of Benjakul and Visessanguan (2000). Albumen samples were mixed with the sample buffer without β-ME. Samples without heat treatment (15 mg) were applied onto two identical gels. The proteins were separated via electrophoresis as previously described. To reduce the heat generated, the electrophoresis unit was embedded in crushed ice. One gel was fixed and stained for total proteins with Coomassie Blue R-250 and used as the control gel. Another gel was washed in 2.5% Triton X-100 for 15 min to remove

SDS and then washed in distilled water. The gel was immersed in 50 mL of 0.2 mg/mL trypsin in 50 mM Tris buffer, pH 8.2, containing 20 mM $CaCl_2$. The gel was incubated for 30 min at 0-4 °C and 60 min at room temperature to allow the trypsin to diffuse into the gels. Gel was washed with distilled water and incubated for 90 min at 37 °C in 10 mg/mL casein solution prepared in 50 mM Tris buffer, pH 8.2. The gels were rinsed with distilled water, fixed and stained with Coomassie Blue R-250 to develop inhibitory zones, detected as dark bands on a clear background. The apparent molecular weight of trypsin inhibitor was estimated from the control gels by comparing migration rates with those of protein standards.

3.3.4 Determination of gel properties

3.3.4.1 Preparation of gel

Gels of albumen samples were prepared following the method of Mmadi *et al.* (2014) with a slight modification. Albumen solutions (10% solid) were prepared using distilled water as diluent. The mixture was stirred gently. Then, the solution was poured into a casing (diameter of 25 mm). Both ends were sealed tightly and heated at 90 °C for 30 min. Thereafter, the gels were cooled immediately at 4 °C and kept overnight. Finally, gel samples were cut into cylinders (diameter 25 mm, height 30 mm) prior to analyses.

3.3.4.2 Texture profile analysis

Texture profile analysis of gels was performed using a texture analyzer (Model TA-XT2i, Stable Micro System, England). The samples (diameter 25 mm, height 30 mm) were compressed twice to 40% of their original height with a compression cylindrical aluminum probe (50 mm diameter). Force-distance deformation curve was recorded at a cross head speed of 3 mm/s and the recording speed was 3 mm/s. Hardness, springiness, cohesiveness, chewiness, and gumminess were evaluated using the MicroStable software version 6 (England).

3.3.4.3 Determination of color

The color of gel samples was determined by a colorimeter (ColorFlex, Hunter Lab Reston, USA) and reported in CIE system. L^*, a^*, b^* and ΔE^*, representing

lightness, redness/greenness, yellowness/blueness and total difference of color, respectively, were recorded. The whiteness of gels was calculated (Kaewmanee *et al.*, 2011b) using the following equation:

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

The ΔE^* was also calculated by the following formulation:

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

where ΔL^*, Δa^*, Δb^* are the difference between color parameters of the samples and the color parameters of albumen gel at day 0 (L^* = 88.05, a^* = - 2.44, b^* = -2.40).

3.3.5.4 Determination of microstructure

Microstructure of albumen gels with the salting time of 0, 15, and 30 days was examined by a scanning electron microscopy as described by Kaewmanee *et al.* (2011b). Albumen gels were cut into small pieces (1x1x1 mm^3). Samples were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. Then, fixed samples were rinsed with distilled water for 1 h. Subsequently, samples were dehydrated sequentially using ethanol with various concentrations (25, 50, 70, 80, 90, and 100%) for 15 min at each concentration. The dehydrated samples were subjected to critical point drying (CPD). The samples were coated with 100% gold (sputter coater SPI-Module, USA). The gel microstructure was visualized by a scanning electron microscope (JEOL JSM-5800LV, Japan).

3.3.5 Statistical analysis

All the experiments were conducted in triplicate using three lots of samples. Data were presented as mean value with standard deviation. One way variance of analysis (ANOVA) was performed. The Duncan's multiple range test was carried out to determine the significant difference between samples at $P<0.05$ level using the statistical program (SPSS 11.0 for Windows, SPSS Inc., USA).

3.4 Results and discussion

3.4.1 Effect of salting time on chemical compositions of duck egg albumen

3.4.1.1 Moisture and salt contents

Moisture and salt contents of albumen from duck egg during salting of 30 days are shown in Table 6. Moisture content decreased continuously during salting (P<0.05). After 30 days, the moisture content was decreased to 81.20%. The decrease in moisture content was coincidental with the increase in salt content of albumen. Salt content generally increased as salting time increased (P<0.05) (Table 6). The salt content of freshly laid duck egg albumen increased dramatically from 0.31% up to 8.06% after 30 days of salting. This indicated the migration of salt into albumen during salting. The loss of water from albumen through shell membrane and pore to the outside took place, mainly caused by the osmosis process. This was mediated by a difference in osmotic pressure between albumen and brine. Chi and Tseng (1998) reported that rate of water migration from albumen was also governed by pore sizes and structure of the shell. Although high salt content in albumen might induce water migration from egg yolk, the amount of water loss from albumen to outside was higher (Kaewmanee *et al.,* 2011b). As a consequence, the decrease in moisture content in albumen was noticeable.

3.4.1.2 Surface hydrophobicity

Surface hydrophobicity of duck albumen during 30 days of salting was monitored. The gradual increase in surface hydrophobicity was observed within the first 20 days of salting, in which S_0ANS increased from 719.08 to 821.45 at day 20 (P<0.05) (Table 6). Surface hydrophobicity of a protein is useful in understanding and predicting the effects of manipulation of the sequence of structural or functional protein domains. The number and the relative size of hydrophobic sites on a protein's surface usually dictates its solubility and propensity to aggregate under physiological conditions of pH, temperature, and ionic strength (Cardamone and Puri, 1992). Ji *et al.* (2013) also found that the surface hydrophobicity of duck albumen was increased after 4 days of salting. The increase in S_0ANS indicated protein conformational change induced by salting. Hydrophobic domains originally buried in the protein core were exposed, thus more

readily available for binding with ANS (Huang *et al.*, 1999; Ji *et al.*, 2013). Nevertheless, the significant decrease in S_0ANS was noticeable at day 30 (538.84) (P<0.05). At high salt content with lower moisture content, the interaction of hydrophobic domains might be enhanced. Consequently, those hydrophobic residues were embedded inside the aggregates (Kaewmanee *et al.*, 2011a) as shown by the lowered S_0ANS.

3.4.1.3 Trypsin inhibitory activity

The changes in trypsin inhibitory activity and specific trypsin inhibitory activity in albumen from duck egg as a function of salting time are depicted in Fig. 11. Trypsin inhibitory activity in freshly laid duck egg decreased continuously within the first 15 days of salting, in which 63% of activity was retained (Fig. 11A). The lowest inhibitory activity was found at 30 days of salting (5.68 kunits/mg solid). The similar result was also observed for specific trypsin inhibitory activity, which was decreased with increasing salting time (P<0.05) (Fig. 11B). After 30 days of salting, the specific inhibitory activity was reduced to 7.77 kunits/mg protein from 9.41 kunits/mg protein detected in freshly laid egg. The decrease in trypsin inhibitory activity was coincidental with the increase in salt content in salted albumen. During salting, salt at high concentration in duck egg albumen more likely altered the conformation of proteins including trypsin inhibitors (Huang *et al.*, 1999). Thus, the denaturation of trypsin inhibitors might be associated with the loss of their bioactivity. It can be postulated that the reduction in antiprotease activity correlates with the deterioration of some pivotal proteins such as ovomucoid and ovoinhibitor. Those proteins might undergo denaturation, thus having the lower antiprotease activity under the high salt environment (Qiu *et al.*, 2012). However, the remaining trypsin inhibitor in salted albumen could play a role in controlling proteolysis in surimi or other meat mediated by endogenous protease.

Table 6. Changes in moisture, salt contents, surface hydrophobicity, color and whiteness of albumen gels from duck egg during salting of 30 days

Salting time (days)	Moisture content (%)	Salt content (%)	Surface hydrophobicity (S_0ANS)	*L**	*a**	*b**	ΔE*	Whiteness
0	87.93±0.18$^{\dagger a}$	0.31±0.07^{f}	719.08±23.63^{d}	88.05±0.01^{c}	-2.44±0.02^{e}	-2.40±0.16^{e}	00.00±0.00^{e}	87.86±1.14^{c}
5	86.68±0.45^{b}	2.07±0.08^{e}	750.34±19.74^{c}	94.62±0.14^{a}	-1.63±0.04^{d}	-0.36±0.04^{d}	6.63±0.09^{a}	94.36±0.10^{a}
10	85.55±0.10^{c}	3.42±0.01^{d}	777.65±12.14^{b}	93.87±0.14^{ab}	-1.54±0.02^{c}	0.16±0.01^{c}	6.07±0.13^{b}	93.65±0.16^{ab}
15	84.61±0.04^{d}	5.10±0.08^{c}	809.51±20.89^{a}	93.71±0.20^{ab}	-1.08±0.04^{b}	0.25±0.02^{c}	6.15±0.06^{b}	93.61±0.20^{ab}
20	83.64±0.11^{e}	6.19±0.00^{b}	821.45±16.89^{a}	93.44±0.21^{ab}	-1.04±0.06^{b}	0.55±0.01^{b}	6.07±0.09^{b}	93.36±0.11^{ab}
25	82.36±0.22^{f}	7.78±0.01^{a}	619.07±0.00^{e}	92.62±0.18^{b}	-1.00±0.11^{a}	0.81±0.02^{a}	5.63±0.01^{c}	92.63±0.13^{b}
30	81.20±0.26^{g}	8.06±0.09^{a}	538.84±12.14^{f}	92.56±0.02^{b}	-1.01±0.43^{a}	0.80±0.01^{a}	5.47±0.01^{d}	92.45±0.02^{b}

†Mean±SD (n=3). Different superscripts in the same column indicate significant differences during salting time (P<0.05).

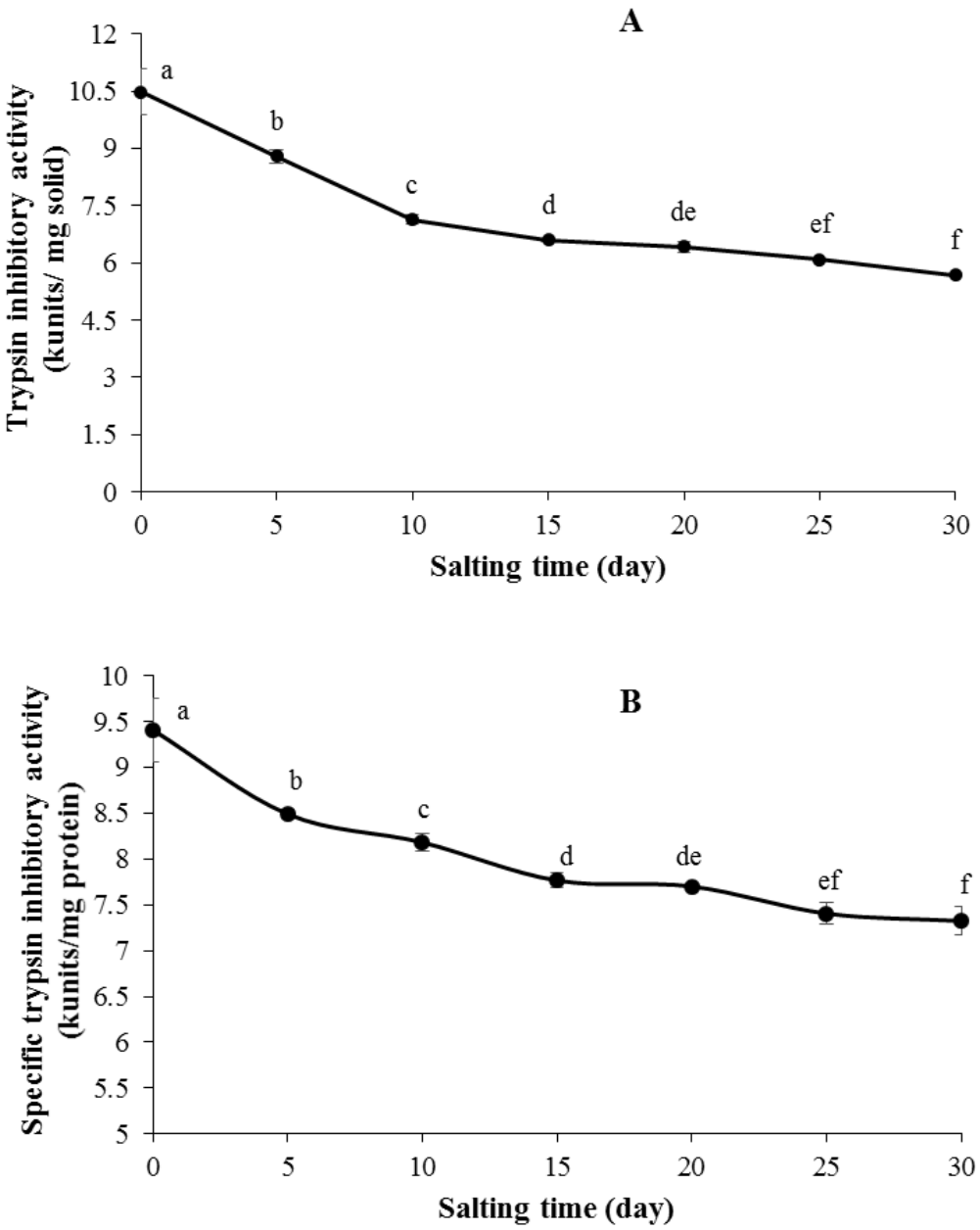

Figure 11. Trypsin inhibitory (A) and specific inhibitory (B) activities of albumen during salting of 30 days. Mean±SD (n=3). Different lowercase letters on the bars indicate significant differences (P<0.05).

3.4.1.4 Protein patterns

SDS-PAGE patterns of albumen from duck egg under reducing and non-reducing condition during salting of 30 days are illustrated in Fig. 12. The most dominant protein was ovalbumin with MW of 44-46 kDa. Under non-reducing condition, proteins with MW of 81, 70, and 16 kDa were observed. Proteins with MW of 81 and 16 kDa were more likely ovotransferrin and lysozyme, respectively. Ovalbumin (54%) and ovotransferrin (12%) are considered as the main proteins found in egg white (Abeyrathne *et al.*, 2013). Hu *et al.* (2016) found that a large number of the identified proteins from duck albumen were belonging to the ovalbumin family using 2-DE gel and MALDI-TOF MS/MS analysis. Those included ovalbumin (OVA), ovalbumin-related protein Y (OVAY), and ovalbumin related protein X (OVAX). Under reducing condition, proteins with MW of 76 and 15 kDa were considered as ovotransferrin and lysozyme, respectively. Nevertheless, some bands were also observed. Those having MW of 179, 153, 125 kDa might be the subunits of egg albumen proteins stabilized by disulfide bonds. In general, no change in protein patterns of albumen was noticeable as the salting time increased up to day 30. In accordance with the results of Ji *et al.* (2013) and Kaewmanee *et al.* (2009), similar protein patterns were found in salted duck egg albumen during 2 weeks of salting. It was reported that the amount of precipitated protein increased with salting time (Huang *et al.*, 1999). Those aggregates might be stabilized by weak bonds such as ionic interaction, etc., which could be destroyed by SDS used for electrophoresis. As a consequence, no differences in protein patterns were observed.

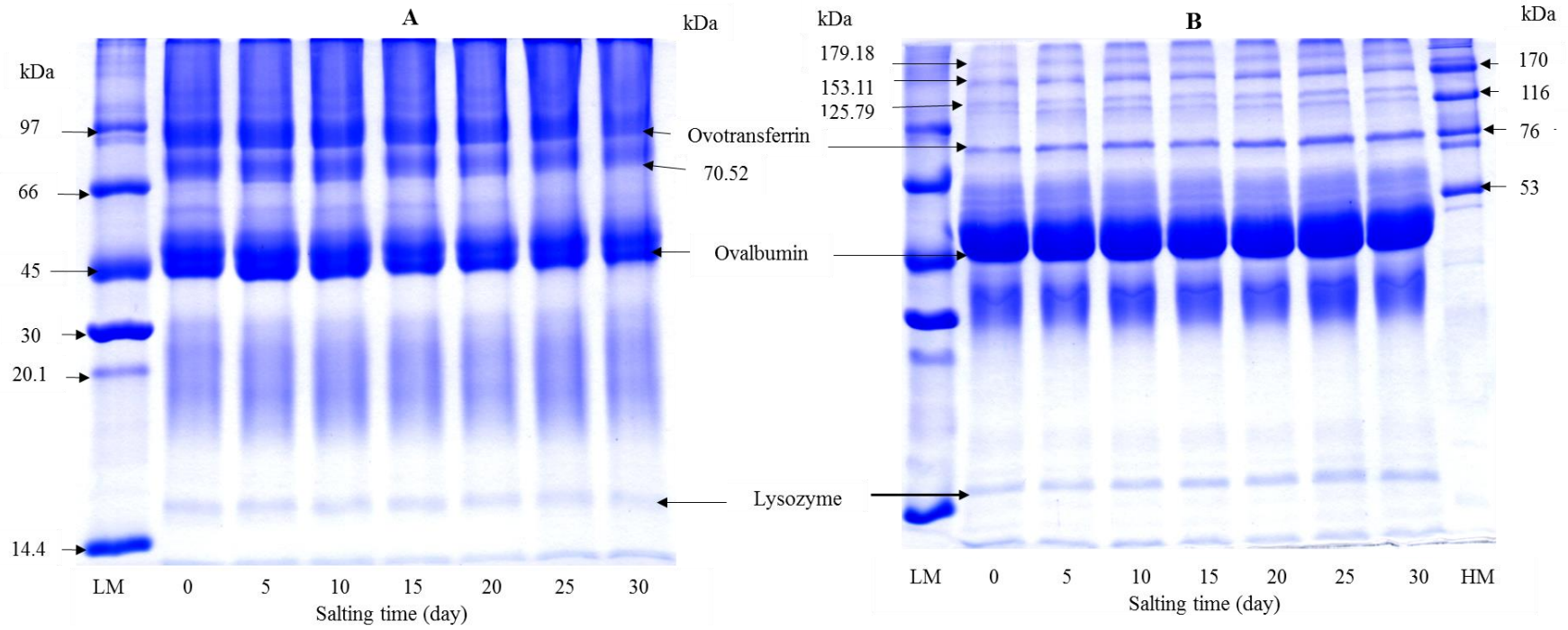

Figure 12. SDS-PAGE patterns of albumen from duck egg during salting of 30 days under non-reducing (A) and reducing (B) conditions. LM: low molecular weight standard; HW: high molecular weight standard.

3.4.1.5 Inhibitory activity staining

Based on inhibitory activity staining, only one band with MW of 44 kDa was retained (Fig. 13). This protein was plausibly ovalbumin or other proteins showing the similar MW to ovalbumin, which had inhibitory activity toward trypsin. Ovalbumin is known as a member of serpin family and shares sequence homology with α_1-protease inhibitor, antithrombin III and angiotensinogen (Saxena and Tayyab, 1997). Takenawa *et al.* (2015) also showed that ovalbumin had ability to inhibit casein degradation induced by trypsin. It was noted that band of lysozyme disappeared after being subjected to inhibitory activity staining. The result indicated that lysozyme was hydrolyzed by trypsin completely. Lysozyme has been reported to exhibit an antibacterial activity against a number of food spoilage bacteria and pathogen (Cegielska-Radziejewska *et al.*, 2008). Hu *et al.* (2016) also reported that ovoinhibitor, ovomucoid, clusterin, ex-FABP, and PG D2 synthase were not detected from duck albumen by 2-dimensional polyacrylamide gel electrophoresis (2-DE). Ovomucoid and ovoinhibitor are found in hen albumen, which are well known as protease inhibitors and are considered to be the main food allergen in egg albumen (Abeyrathne *et al.*, 2015). It was noted that ovomucoid and ovoinhibitor were not detected by inhibitory activity staining. Those inhibitors might lose their inhibitor activity in the presence of SDS used for electrophoresis. As a result, the bands were not retained after trypsin digestion. After 20 days of salting, the band of protein with MW of 44 kDa became less intense than that of freshly laid egg and 10-day salting. It was suggested that protein with inhibitory activity lost in some activity with extended salting time. This might be due to the conformational change of inhibitory proteins induced by high salt concentration. This result was in agreement with the decrease in trypsin inhibitory activity of duck albumen during salting time (Fig. 11). Thus, salting time had the marked impact on the reduction of trypsin inhibitory activity of duck egg albumen.

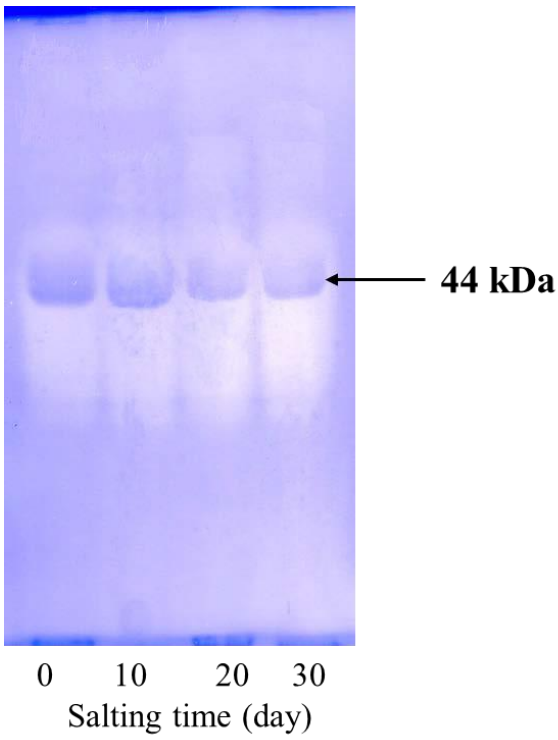

Figure 13. Trypsin inhibitory activity staining of salted duck egg albumen during salting under non-reducing condition. The numbers denote the salting time (day).

3.4.2 Effect of salting time on gelling properties of albumen

3.4.2.1 Texture profile analysis

Texture profile of albumen gel from duck egg during salting of 30 days is presented in Table 7. Hardness of albumen gel decreased with increasing salting time (P<0.05). The highest hardness was found in the gel from freshly laid egg albumen (19.08 N), whereas the lowest value (3.77 N) was obtained in the sample after 30 days of salting (P<0.05). After 5 days of salting, hardness of gel was reduced by 50%, in which the value of 9.26 N was attained. Hardness is related to the strength of gel structure under compression and is the peak force during the first compression cycle (Chandra and Shamasundar, 2015). The increased salt content of albumen plausibly caused the aggregation of albumen proteins, which was associated with a coarser structure and a weakened gel network (Huang *et al.*, 1999). Salts tend to enhance aggregation of egg white proteins, resulting in weaker gels (Woodward, 1990).

Cohesiveness of salted albumen gel increased gradually up to 15 days of salting (P<0.05) (Table 7) and reached the highest value of 0.75. The decrease in cohesiveness was observed for the rest of salting time (P<0.05). Cohesiveness is a parameter to measure the difficulty level in breaking down the internal structure of gel (Lau *et al.*, 2000). Springiness of albumen gel decreased as salting time increased (P<0.05). Springiness is related to the height by which gel recovers during the end of the first bite and the start of the second bite. If springiness is high, it requires more mastication energy in the mouth (Chandra and Shamasundar, 2015). The decreased springiness was concomitant with the decreases in hardness. Gumminess and chewiness of gels from duck egg during salting are shown in Table 7. In general, these values decreased continuously as a function of salting time (P<0.05). The changes in gumminess and chewiness were in correlation with those of hardness. Gumminess and chewiness were calculated based on hardness, which suggests resistance to compression force (Yilmaz *et al.*, 2012). Those changes were mainly mediated by the increasing salt environment. Therefore, gels of albumen had the change in textural characteristics as induced by salting. The change was more pronounced with increasing salting time.

3.4.2.2 Color

Changes in color of albumen gel from duck eggs during salting of 30 days are shown in Table 6. The lowest lightness (L*- value) of gels was found in freshly laid duck egg albumen. L*- value was increased after salting (P<0.05). There was no difference in L*- value between gels from albumen obtained from duck egg salted for 5-20 days (P>0.05). In the presence of salt, the aggregation might be formed to a greater extent when the heat was applied. As a result, the cluster or coagulate was generated. This led to the higher light scattering of egg albumen gel formed (Kaewmanee *et al.*, 2011b). At day 30 of salting, the lower lightness was probably caused by the formation of large coagulate with less surface area, resulting in the decreased light scattering (Kaewmanee *et al.*, 2011b). The whiteness of all albumen gels obtained from salted eggs was higher than that of fresh egg. The increases in both a*- value and b*- value of albumen gel were observed throughout the salting period of 30 days (P<0.05). The values of a* and b* indicate redness/greenness and yellowness/blueness, respectively.

Kaewmanee *et al.* (2011b) reported that the increase in redness and yellowness of salted albumen gel with increasing salting time was due to pigments located at the outer layer of yolk, which might be contaminated into egg white to some extent. Salting process might soften yolk membrane, thereby facilitating the migration of pigment from yolk to albumen. For ΔE^*, the increase in value was obtained for albumen gel from salted egg. The increase in ΔE^* was coincidental with the increase in lightness and whiteness throughout the salting period studied. Thus, salting also affected the color of albumen gel to some degree, apart from textural property (Table 6).

3.4.2.3 Microstructure

Scanning electron micrographs of albumen gel from duck eggs after salting for 15 and 30 days, in comparison with gel from fresh egg are illustrated in Fig. 14. Albumen gel from freshly laid duck egg had the denser network with the smaller voids, compared with those of salted duck egg. For salted albumen gel, the larger voids were observed. After 15 days of salting, the gel network became coarser with less uniformity. With increasing salting time up to 30 days, no marked difference was noted in comparison with that of 15-day salted egg. However, due to the lower protein concentration and higher salt content in salted albumen, especially after 30 days of salting, the interconnection of proteins became less. This was concomitant with the lower strength of protein network (Table 7). Egg white protein at high concentration was easily gelled by heat treatment (Iwashita *et al.*, 2015). The lower hardness of albumen from egg salted for 30 days might be reflected by the coarser network with less protein content. The lower protein content in the salted egg, especially for longer salting time, was mostly associated with the dilution effect by salt and the lower moisture content. In the presence of sodium chloride, the coagulation was induced. During heating, those coagulums underwent interconnection, leading to the formation of coagulum type gel (Kaewmanee *et al.*, 2011b). The result revealed that the salting time had the significant impact on gel structure of albumen from salted duck egg.

Table 7. Changes in texture profile of albumen gels from duck egg during salting of 30 days

Salting time (day)	Hardness (N)	Cohesiveness	Springiness (cm)	Gumminess (N)	Chewiness (N.cm)
0	19.08±0.54[†a]	0.72±0.01[d]	0.92±0.01[a]	13.67±0.29[a]	12.64±0.45[a]
5	9.26±0.21[b]	0.74±0.00[b]	0.89±0.01[b]	6.64±0.13[b]	6.10±0.11[b]
10	6.02±0.20[c]	0.75±0.00[a]	0.87±0.01[c]	4.48±0.10[c]	3.93±0.09[c]
15	4.74±0.13[d]	0.75±0.00[a]	0.86±0.01[d]	3.54±0.09[d]	3.06±0.09[d]
20	4.01±0.05[e]	0.73±0.01[c]	0.85±0.00[d]	2.88±0.06[e]	2.49±0.04[e]
25	3.81±0.09[e]	0.73±0.00[c]	0.85±0.01[d]	2.79±0.08[e]	2.36±0.06[e]
30	3.77±0.10[f]	0.72±0.00[d]	0.82±0.01[e]	2.48±0.07[f]	2.23±0.05[f]

[†]Mean±SD (*n=3*). Different superscripts in the same column indicate significant differences ($p<0.05$)

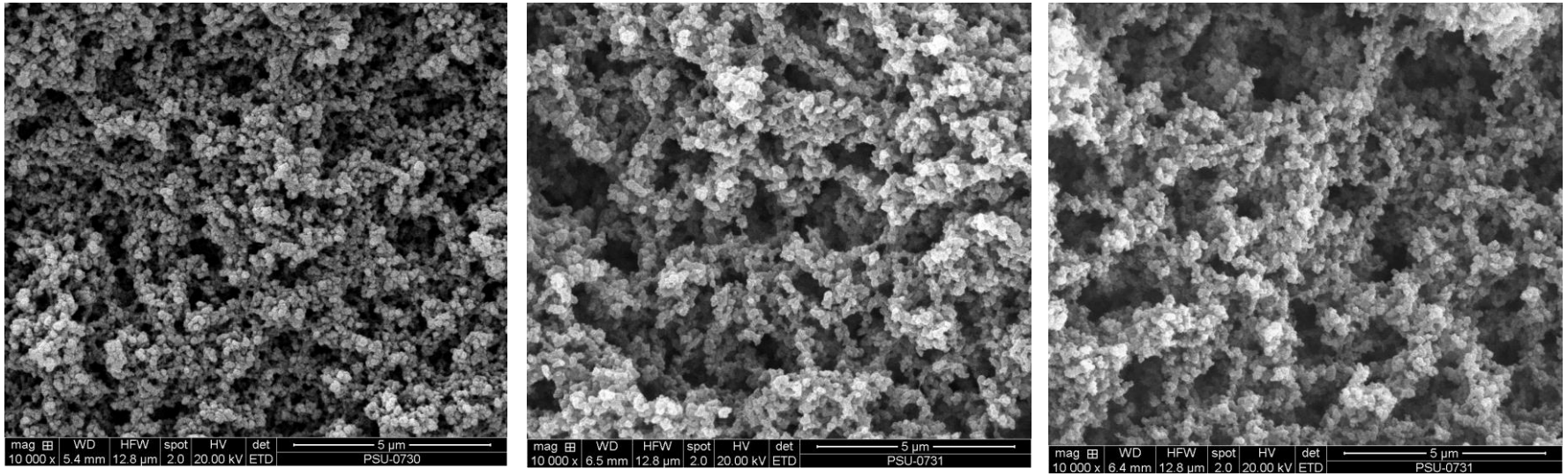

Figure 14. Scanning electron microscopic photograph of albumen gel from fresh duck egg and egg salted for 15 and 30 days. Magnification: 10000X. Scale bar = 5 µm.

3.5 Conclusion

Gelling property and chemical composition of albumen from duck egg, especially protease inhibitor, were affected by salting time. Salting could reduce trypsin inhibitory activity in duck albumen to some degree. Protein with MW of 44 kDa acted as trypsin inhibitor in duck egg albumen. Gel became weaker with the increased whiteness as salting time increased. Due to the remaining gelling property and trypsin inhibitors of albumen from salted duck egg, the salted albumen could be applied in surimi or other meat products to improve their properties via strengthening the gel and inhibiting the proteolysis.

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CHAPTER 4

TRYPSIN INHIBITOR FROM DUCK ALBUMEN: PURIFICATION AND CHARACTERIZATION

4.1 Abstract

Egg albumen is a potential source of trypsin inhibitor (TI), which has been widely used to improve textural property of surimi or surimi-based food products. TI from duck albumen was isolated and purified using ammonium sulfate precipitation at 20%–40% saturation and affinity chromatography using trypsin-CNBr-activated Sepharose 4B column. TI was purified with purity and yield of 111.8-fold and 0.6%, respectively. The purity of inhibitor was confirmed using Native-PAGE as indicated by the presence of single band. Molecular weight of purified TI was 43 kDa based on SDS-GAGE and gel filtration. The purified TI remained unchanged at temperatures below 60 °C and the pH in the range of 7–9. The inhibitory activity of TI was decreased with the addition of salt higher than 5%. Inhibition kinetic study revealed that purified TI from duck albumen was uncompetitive inhibitor and the inhibition constant (Ki) was 508 nM. TI from duck egg albumen could serve as a food grade inhibitor for controlling undesirable proteolysis.

4.2 Introduction

Over decades, bioprocesses especially separation and purification of protease inhibitors have drawn attention. Purification is a critical step prior to molecular characterization of protease inhibitor (Elizeu *et al.*, 2012). Protease inhibitors have physiological functions and possess a key function in the regulation of different biological pathways such as transcription, apoptosis, breakdown of intracellular protein, and invasion of cell cycle, in which proteases are associated (Choi *et al.*, 2002). In food processing, protease inhibitors have been used as the food additives to improve textural property of several food products, e.g., surimi-based products, meat ball, and sausage (Klomklao *et al.*, 2016b). Trypsin inhibitor (TI) is one of the most common serine protease inhibitors, which belong to Kunitz and Bowman-Birk families and could be found in various sources from plants as well as pancreas of animals (Choi *et al.*,

2002; Elizeu *et al.*, 2012). Egg albumen is the cheapest source of several bioactive compounds including protease inhibitors (Datta *et al.*, 2009).

Duck egg albumen or egg white has been considered as a potential source of protein (8.8%) of total weight (Huang and Lin, 2011). Albumen proteins have multiple functional properties including gelling, emulsifying, vitamin, and iron-binding properties, thus widening its use in food industry (Naknukool *et al.*, 2008). Moreover, egg albumen proteins possess several biological activities such as anti-microbial and protease inhibitory activity. Protease inhibitors present in egg albumen were documented in the early part of the twentieth century (Saxena and Tayyab, 1997). Ovomucoid, ovoinhibitor, and ovostatin (ovomacroglobulin) have serine protease inhibitory activity, while cystatin can inhibit cysteine proteases (Saxena and Tayyab, 1997; Ustadi *et al.*, 2005). Duck albumen showed high serine protease inhibitory activity, which could retard proteolysis caused by indigenous proteases in fish muscle during heat-induced gelation (Quan and Benjakul, 2018c). Quan and Benjakul (2018a) documented that the addition of duck albumen in sardine surimi gel resulted in higher breaking force associated with the increased hardness, chewiness, and gumminess than the incorporation of hen albumen.

Protein purification process aims to obtain the specific target protein out of a chosen crude source with adequate yield and purity (Roy *et al.*, 2003). Conventionally, affinity chromatography (AC) has been widely used for purification of protease inhibitors, in which proteases are specifically bound with the ligand or the binding agent (Elizeu *et al.*, 2012). Numerous studies were conducted on isolation and purification of various protein fractions in hen albumen such as ovalbumin, ovomucoid, ovoinhibitor as well as cystatin (Roy *et al.*, 2003; Tankrathok *et al.*, 2009; Wang and Wu, 2014). Moreover, TI from other sources including fish roe (Klomklao *et al.*, 2016a; Ustadi *et al.*, 2005), chicken plasma (Rawdkuen *et al.*, 2005), mung bean (Klomklao *et al.*, 2011), and the seed of *Rhynchosia sublobata* (Schumach.) Meikle (Mohanraj *et al.*, 2019) were also isolated, purified as well as characterized. Recently, protease inhibitors present in duck albumen has been proven to exhibit higher inhibitory activity than hen albumen (Quan and Benjakul, 2018a). However, no information on molecular characteristics of protease inhibitors, particularly TI in duck egg albumen exists. The understanding in

molecular characteristics of inhibitor including molecular weight (MW), stability, and inhibition kinetic studies could help in the usage of inhibitor as a food grade protein additive to prevent the proteolysis occurring in some foods mediated by indigenous proteases, such as surimi, etc. Thus, this investigation aimed to isolate, purify, and characterize TI from duck albumen.

4.3 Materials and methods

4.3.1 Chemicals

All chemicals used were of analytical grade. Coomassie blue R-250, acetic acid, β-mercaptoethanol (β-ME), ethanol, ammonium sulfate (AS), and sodium dodecyl sulfate (SDS) were procured from Merck (Darmstadt, Germany). Cyanogen bromide (CNBr)-activated Sepharose 4B was obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and molecular protein marker was procured from GE healthcare UK Limited (Buckinghams, UK). Bovine pancreas trypsin (~10,000 BAEE units/mg protein, Type I) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4.3.2 Fractionation of TI from duck albumen using AS

Fresh duck eggs were randomly collected after laying within 24 h at a farmhouse in Kantang, Trang province, Thailand. After being broken, egg albumen was manually separated from the yolk. Thereafter, albumen was subsequently subjected to AS precipitation at 20%–40% of saturation. The preliminary result showed that the use of AS at 20%–40% saturation rendered the fraction with the highest specific inhibitory activity toward trypsin as compared with other ranges of AS (0%–20%, 40%–60%, and 60%–80%). After adding AS, the mixture was gradually stirred at 4 °C for 30 min and subsequently centrifuged at 8,000 ×*g* at 4 °C for 30 min using a refrigerated centrifuge (Hitachi centrifuge CR22N, Hitachi Koki Co., Ltd., Tokyo, Japan). Obtained pellet was dissolved in 0.05 M Tris-HCl buffer, pH 8.0 with the minimum volume and dialyzed against 20 volumes of the same buffer at 4 °C overnight with five changes of buffer. “AS fraction, ASF” was named for the dialysate.

4.3.3 Purification of TI from duck albumen

4.3.3.1 Affinity column chromatography

The trypsin-CNBr-activated Sepharose 4B column was prepared as per the guideline of the supplier. A 50-mL of ASF (150 mg protein/mL) was subjected to the trypsin-CNBr-activated Sepharose 4B column (1×10 cm) after equilibrating with 0.05 M Tris-HCl, pH 8.0, containing 0.5 M NaCl. The chromatography system was coupled with a fraction collector (ÄKTAprim plus, GE Healthcare Bio-Science AB, Uppsala, Sweden). Then, the column was washed with the same buffer at a flow rate of 0.5 mL/min until A_{280} was lower than 0.005. HCl (0.005 M) was used for the elution at a flow rate of 1 mL/min. Two mL fractions were taken and rapidly added with 0.5 mL of 0.1 M Tris–HCl containing 0.01 M $CaCl_2$, pH 8.5. Thereafter, the inhibitory activity toward trypsin was tested for all fractions. Substrate used for trypsin activity assay was BAPNA (Benjakul *et al.*, 2001). The fraction showed the highest specific trypsin inhibitory activity (TIA) was pooled and dialyzed against 10 volumes of the same dialysis buffer. During dialysis, five changes of buffer were conducted. The fractions with TIA were pooled and used for further studies. All purification steps were done at 4 °C.

4.3.3.2 Assay for TIA

The procedure tailored by Benjakul *et al.* (2001) was adopted for TIA assay. Firstly, samples were appropriately diluted using 0.05 mM Tris-HCl (pH 8.0). Diluted solution (0.2 mL) was mixed with 0.2 mL of porcine pancreas trypsin (0.05 mg/mL) for 15 min at 37 °C. After adding 1 mL of reaction buffer (50 mM Tris-HCl, pH 8.2, containing 20 mM $CaCl_2$), the mixture was added with 0.2 mL of BAPNA (2 mg/mL) and kept for 15 min at 37 °C. Thereafter, 0.2 mL of acetic acid (30%, v/v) was added to stop the reaction. The released *ρ*-nitroaniline was measured at the A_{410} using a spectrophotometer (UV-16001, Shimadzu, Kyoto, Japan). One unit of trypsin was defined as the enzyme causing an increase in A_{410} by 0.01 unit/min under the assay condition. One unit of TIA was defined as the amount of inhibitor, which decreased trypsin activity by one unit. TIA of samples was expressed as units/mL. Specific TIA

was determined and presented as units/mg protein. All fractions were also determined for protein content using the Lowry method (Lowry *et al.*, 1951).

4.3.3.3 Determination of protein content

Protein concentration of all samples was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard (0–1 mg/mL). Protein sample (0.2 mL) was mixed with 2 mL of Lowry reagents (50 mL of 0.5% $CuSO_4$ in 1% sodium citrate mixed with 1 mL of 2% sodium carbonate in 0.1 N NaOH) for 10 min at room temperature. Then, 0.2 mL of 0.5 N Folin Phenol reagent was added and vortexed immediately. After 30 min, the absorbance at 750 nm was read and protein concentration was calculated from standard curve.

4.3.4 Characterization of purified TI from duck albumen

4.3.4.1 SDS-PAGE and Native-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified TI was conducted following the protocol of Laemmli (1970), in comparison with crude extract and ASF. The sample buffer containing 10% of β-ME was mixed with the samples at a ratio of 1:1 (v/v). Crude albumen, ASF, and affinity column fraction were loaded onto the gel comprising 4% stacking and 15% separating gels and further subjected to electrophoresis (Mini-protein III; Bio-Rad Laboratories, Richmond, CA, USA) at constant current of 15 mA/gel. Protein bands were stained with Coomassie Brilliant Blue R-250 (0.125%) in 10% acetic acid and 25% methanol followed by destaining using 10% acetic acid and 40% methanol.

Native polyacrylamide gel electrophoresis (native-PAGE) was conducted in the same method. However, SDS and a reducing agent were dismissed as well as the sample was not heated. Protein bands were detected by staining with 0.02% formaldehyde and 0.1% silver nitrate (Khantaphant and Benjakul, 2010).

4.3.4.2 Gel filtration

Trypsin-CNBr-activated Sepharose 4B fraction was subjected to size exclusion chromatography using a Sephadex G-50 GF column (GE Healthcare, Bio-Science AB, Uppsala, Sweden) after being equilibrated using Tris-HCl (0.05 mM, pH

8.0). Two milliliters of the fraction were applied onto the column at room temperature (28–30 °C). The elution was then carried out with the same buffer and the fractions (3 mL) were simultaneously collected using a fraction collector at the flow rate of 0.5 mL/min. All fractions were read for A_{280}. Void volume was determined using blue dextran (2,000 kDa). MW markers included cytochrome C (12.4 kDa), albumin (66.5 kDa), and albumin from chicken albumen (44.3 kDa). All fractions were analysed for TIA. Activity peaks were calculated for MW. The plot between the logarithm of the MW of protein standards and the available partition coefficient (Kav) was prepared.

4.3.4.3 Thermal and pH stability of purified TI

The stability of purified TI was assessed by determining the residual activity after incubation under different temperatures and pHs. For thermal stability, purified TI was kept at various temperatures (40–90 °C) for 30 min. Thereafter, the incubated samples were immediately kept in iced water and the remaining TIA was determined as formerly described.

For pH stability, purified TI was mixed with different buffers: 50 mM glycine-NaOH buffer for pHs 10.0–12.0, 50 mM Tris-HCl buffer for pHs 9.0–7.0, and 50 mM sodium acetate-acetic acid for pHs 6.0–4.0 at an equal volume. Then, the mixture was stirred and kept at room temperature (25–28 °C) for 30 min prior to assay for residual TIA as mentioned above.

4.3.4.4 Effect of salt at various levels on TI

Purified TI was mixed with sodium chloride at various concentrations (2.0, 3.5, 5.0, 6.5, and 8.0%) at room temperature (25–28 °C) for 30 min. Then, the mixture was tested for TIA as described above.

4.3.4.5 Kinetic studies

Inhibition kinetic of TI was studied using various BAPNA concentrations (1, 2, 3, 4, and 5 mM) as the substrate. The reaction mixture contained trypsin at a concentration of 1 μM. The mixture was kept for 15 min at 37 °C, before the substrate was added to start the reaction. Residual trypsin activity was spectrophotometrically measured at A_{410}. The velocity of reaction was presented as 1/V (ΔA_{410}/min). The mode

of inhibition was determined using Lineweaver-Burk plots (Lineweaver and Burk, 1934), in which the reciprocal velocity (1/V) versus different inhibitor concentrations [I] (100, 200, 300, 400, and 500 nM) for each substrate concentration [S] was plotted. A single regression line for each [S] was obtained, and the Ki was determined from a secondary plot between 1/Vmax and concentrations of inhibitor (Karbassi *et al.*, 2004).

4.3.5 Statistical analysis

All the analyses were done in triplicate. Completely randomized design was used for stability studies of purified TI. One-way analysis of variance and the Duncan's multiple range test were done to analyze the significant difference among the samples at a level of $p < 0.05$ using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 Purification of TI from duck egg albumen

The purification steps of TI from duck albumen are summarized in Table 8. Overall, TI was purified to homogeneity using AS precipitation, followed by affinity column chromatography. It was found that AS saturation of 20%–40% was efficient for precipitating TI. The specific inhibitory activity of crude TI in duck albumen was 99 units/mg protein. After AS precipitation step, the purity of TI was increased by 1.6 fold with a yield of 7.5%. AS precipitation was commonly used as the first step to isolate other proteins from the crude extract (Khantaphant and Benjakul, 2010). The selected ASF with high TIA were pooled and subjected to trypsin-CNBr activated Sepharose 4B column, in which TI was bound to ligand in the matrix. The pooled fractions of affinity column chromatography showed the drastic increase in specific inhibitory activity (11,067 units/mg protein) with the marked increase in purity (111.8 fold) (Table 15 and Fig. 15). Among the chromatographic techniques, affinity column chromatography has been typically used as the final steps in the purification process of protease inhibitors. This technique offers some advantages, including reduction of purification steps because of high binding specificity between the target protease inhibitor and chromatographic matrix (Elizeu *et al.*, 2012). During purification, other contaminating proteins were removed, as evidenced by lower protein content. This coincided with increased purity of TI during the purification processes.

4.4.2 Purity and MW of TI from duck egg albumen

The purity of TI was examined by SDS-PAGE and native-PAGE. Affinity column chromatography fraction showed a single band when measure using SDS-PAGE under reducing condition (Fig. 16A). The MW of purified TI was approximately estimated to be 43 kDa. As examined by the native-PAGE (Fig. 16B), this fraction was also observed as a single protein band, confirming that TI was purified to homogeneity. MW of the purified TI was also estimated by GF chromatography on a Sephadex G-50 column, in which MW of approximately 43–45 kDa was estimated (Fig. 17A, B). It was concomitant with the MW estimated by SDS–PAGE. The result suggested that TI was present as monomer. According to Quan and Benjakul (2018b), protein band of duck albumen with MW of 44 kDa exhibited TIA when examined using the activity staining. Ovalbumin with MW of 45 kDa shares sequence homology with α1-protease inhibitor and belongs to serpin family, which could inhibit casein digestion mediated by trypsin (Saxena and Tayyab, 1997; Takenawa *et al.*, 2015). However, TI isolated from duck pancreas by affinity column chromatography had the MW of about 7.7 kDa (Wilimowska-Pelc *et al.*, 1999). Additionally, the MW of TI from skipjack tuna roe was 39 kDa as analyzed by SDS-PAGE under reducing condition (Choi *et al.*, 2002). The results indicated that affinity column chromatography effectively purified TI from duck egg albumen with high purity and yield.

Table 8. Purification of trypsin inhibitors from duck egg albumen

Purification steps	Total volume (mL)	Inhibitory activity (units/mL)	Total inhibitory activity (units)	Protein concentration (mg/mL)	Total protein content (mg)	Specific inhibitory activity (units/mg protein)	Purity (fold)	Yield (%)
Crude egg albumen	200	13323	2664.6×10^3	134.8	27.0×10^3	99	1.0	100
Ammonium sulfate fraction	15	13358	200.4×10^3	86.8	1.3×10^3	154	1.6	7.5
Affinity column fraction	3	5527	16.6×10^3	0.5	1.5	11067	111.8	0.6

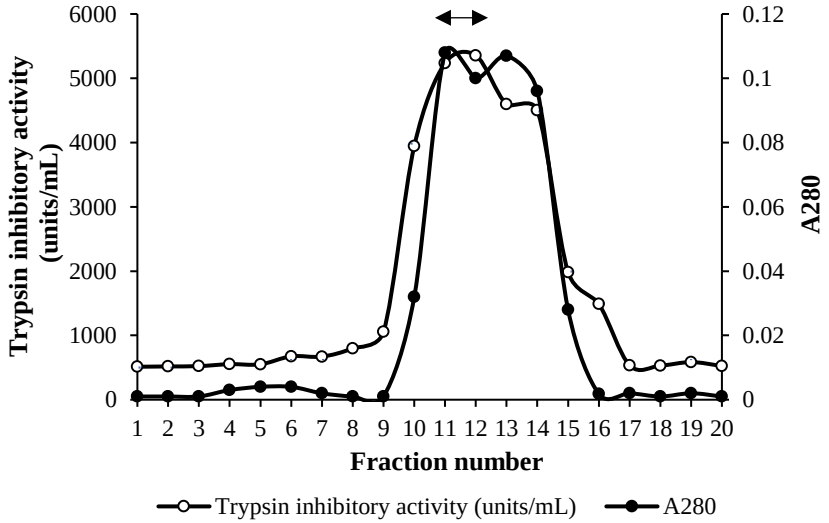

Figure 15. Elution profile of TI from duck egg albumen using trypsin-CNBr-activated Sepharose 4B column. AS (20%–40% saturation) fractions were loaded onto the column. Fractions (2 mL) were determined for TIA. ↔: pooled fractions.

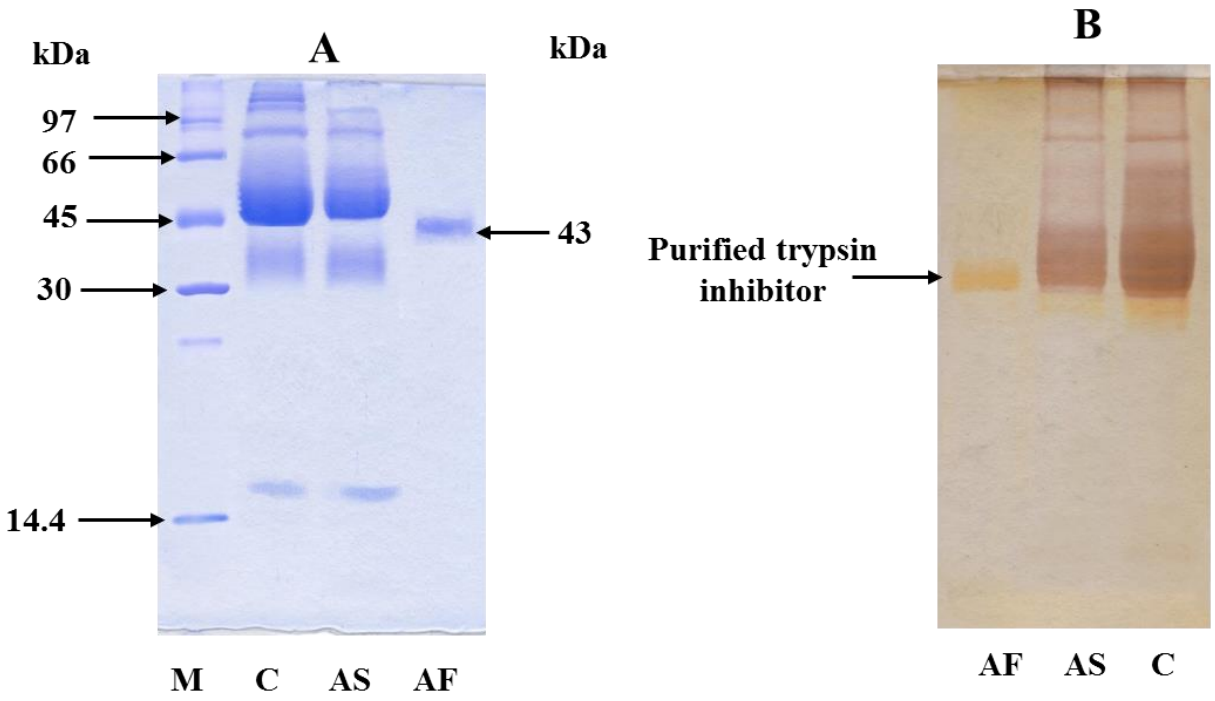

Figure 16. SDS-PAGE (A) patterns of purified TI from duck albumen under reducing condition and Native-PAGE (B). M: Low molecular weight standard; C: crude egg albumen; AS: AS fraction; AF: affinity column fraction.

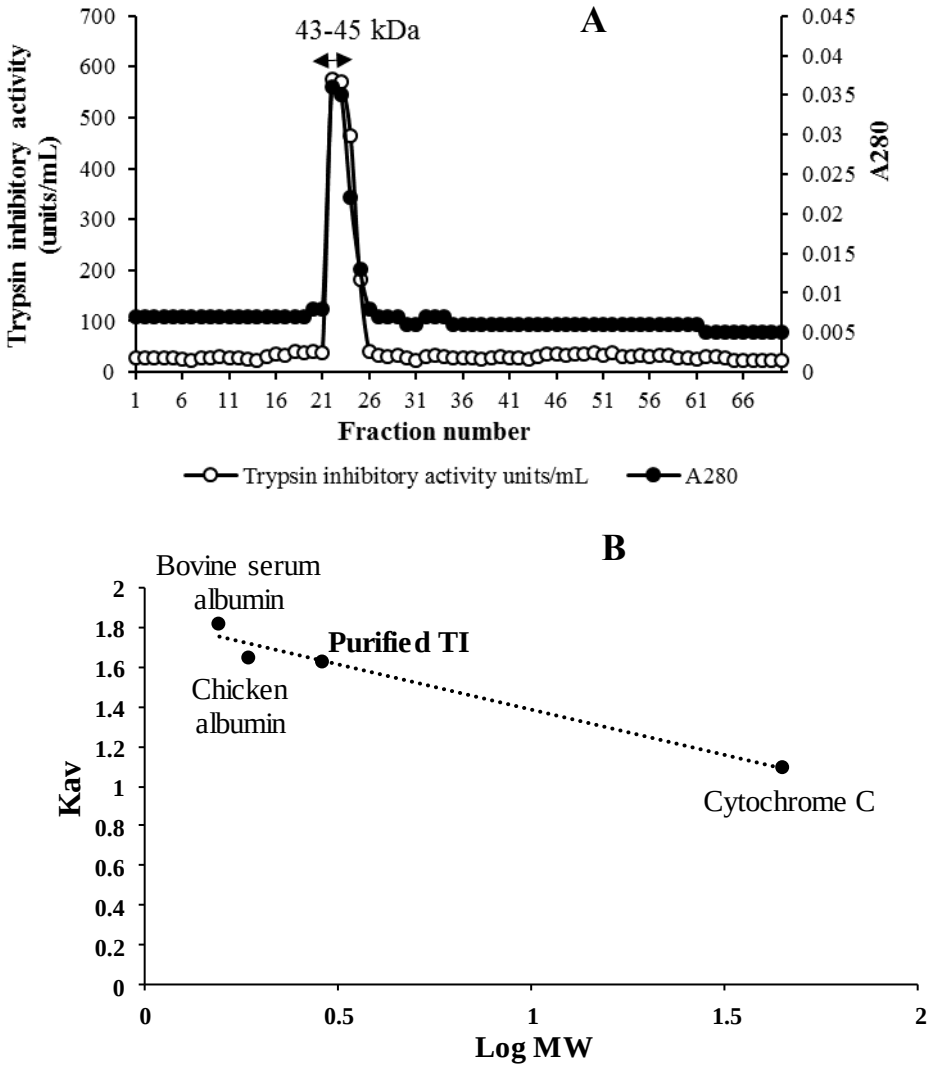

Figure 17. (A) Elution profile of TI from duck egg albumen using Sephadex G-50 gel filtration chromatography. (B) Calibration curve for the molecular weight determination of the purified TI on Sephadex G-50 gel filtration chromatography. Trypsin-CNBr-activated Sepharose 4B fractions were loaded onto the column. Fractions (3 mL) were determined for TIA. ↔: pooled fractions.

4.4.3 Stability of TI

4.4.3.1 Impacts of temperature and pH

Stability of purified TI subjected to heating at various temperatures is presented in Fig. 18A. The highest relative inhibitory activity (RIA) was recorded (about 98%) at 40 °C and the activity was still remained up to 50 °C (P>0.05). Above these temperatures, the RIA gradually decreased and reached the lowest value (around 50%) as purified TI was incubated at 90 °C (P<0.05). The loss in inhibitory activity of TI was probably related to the denaturation of TI induced by heat (Klomklao *et al.*, 2016a). It was known that the denaturation temperature of egg albumen proteins occurred a temperature ranging from 60 °C to 90 °C (Phillips and Williams, 2011). Moreover, TI isolated from soybean (Abd El-Latif, 2015), skipjack tuna roe (Choi *et al.*, 2002), yellowfin tuna roe (Klomklao *et al.*, 2016a), and glassfish roe (Ustadi *et al.*, 2005) were stable at temperatures lower than 60 °C. In general, muscle proteins underwent the proteolytic degradation at temperature range of 50–60 °C (Benjakul *et al.*, 2001). This result indicated that TI from duck egg albumen could be used to inhibit indigenous serine proteases localized in fish muscle or surimi, thus improving textural properties of corresponding gels. The stability of TI at pH levels (4 to 12) is presented in Fig. 18B. In the wide range of pH (pH 6.0–10.0), more than 90% inhibitory activity of TI were maintained (P<0.05). TI showed the highest RIA at pH 8.0. However, the activity of TI was lost about 30% at extreme pH values, either at pH 4.0 or pH 12.0 (P<0.05). Strong electrostatic repulsion force mediated by high net charge caused unfolding of TI molecules, as shown by loss of inhibitory activity (Klomklao *et al.*, 2016a).

4.4.3.2 Effect of salt concentrations

The impact of sodium chloride on the inhibitory activity of TI is shown in Fig. 18C. There was no significant change in RIA when TI was incubated in the presence of sodium chloride up to 3.5% (P>0.05). This was coincidental with the result of Klomklao *et al.* (2011) who documented that salt concentrations in the range of 1%–3% had no effect on the activity of the TI isolated from Thai mung bean. Nevertheless, a decreased inhibitory activity was recorded as TI was mixed with salt at concentrations

higher 5% (P<0.05). The RIA of TI after being incubated with salt at 5% and 6.5% was 78% and 74%, respectively, while the inhibitor was lost by 60% in the presence of 8% salt. Quan and Benjakul (2018b) found that TIA in duck albumen decreased about 50% after 30 days of salting, in which 8% salt was found in duck albumen. TI might be transformed in conformation under high salt concentration. As a result, the lower inhibitory activity was recorded (Huang *et al.*, 1999).

4.4.4 Inhibition kinetics

Inhibition kinetic of purified TI from albumen toward trypsin was studied by Lineweaver–Burk plots as demonstrated in Fig. 19A. The plot was attained using various concentrations of TI. With increasing concentrations of TI, both V_{max} and K_{max} were decreased, while K_{max}/V_{max} values (the slope) were not changed. This confirmed that the inhibitory action of duck albumen TI toward trypsin was uncompetitive type. For the uncompetitive mode, the inhibitor might bind to the enzyme-substrate complex but not the active site of free enzyme, thus generating its catalytical inactivity without interference to the affinity of substrate and enzyme (Sae-leaw *et al.*, 2017). However, some serine protease inhibitors of egg albumen such as ovomucoid, ovoinhibitor, or ovostatin possess some binding sites with proteases such as trypsin, chymotrypsin, subtilisin, elastase, and some enzymes (Saxena and Tayyab, 1997). To determine the inhibition constant (Ki), the concentrations of inhibitor [I] were plotted versus the values of the reciprocal maximum velocity (l/V_{max}) at every inhibitor concentration (a secondary plot). The Ki was calculated to be 508.67 nM (Fig. 19B). However, Zhou *et al.* (1989) reported that TI from bovine pancreas, ovomucoid, and soybean were the competitive inhibitors, in which the inhibition rate constants were 590, 1,300, and 6,100 nM, respectively, using benzoylarginine ethyl ester as a substrate. The Ki of an uncompetitive inhibition mode is the dissociation constant for the binding of inhibitor to the enzyme-substrate complex. The result suggested that TI from duck egg albumen could be considered as a potential inhibitor with strong inhibitory effect toward trypsin as evidenced by low Ki value.

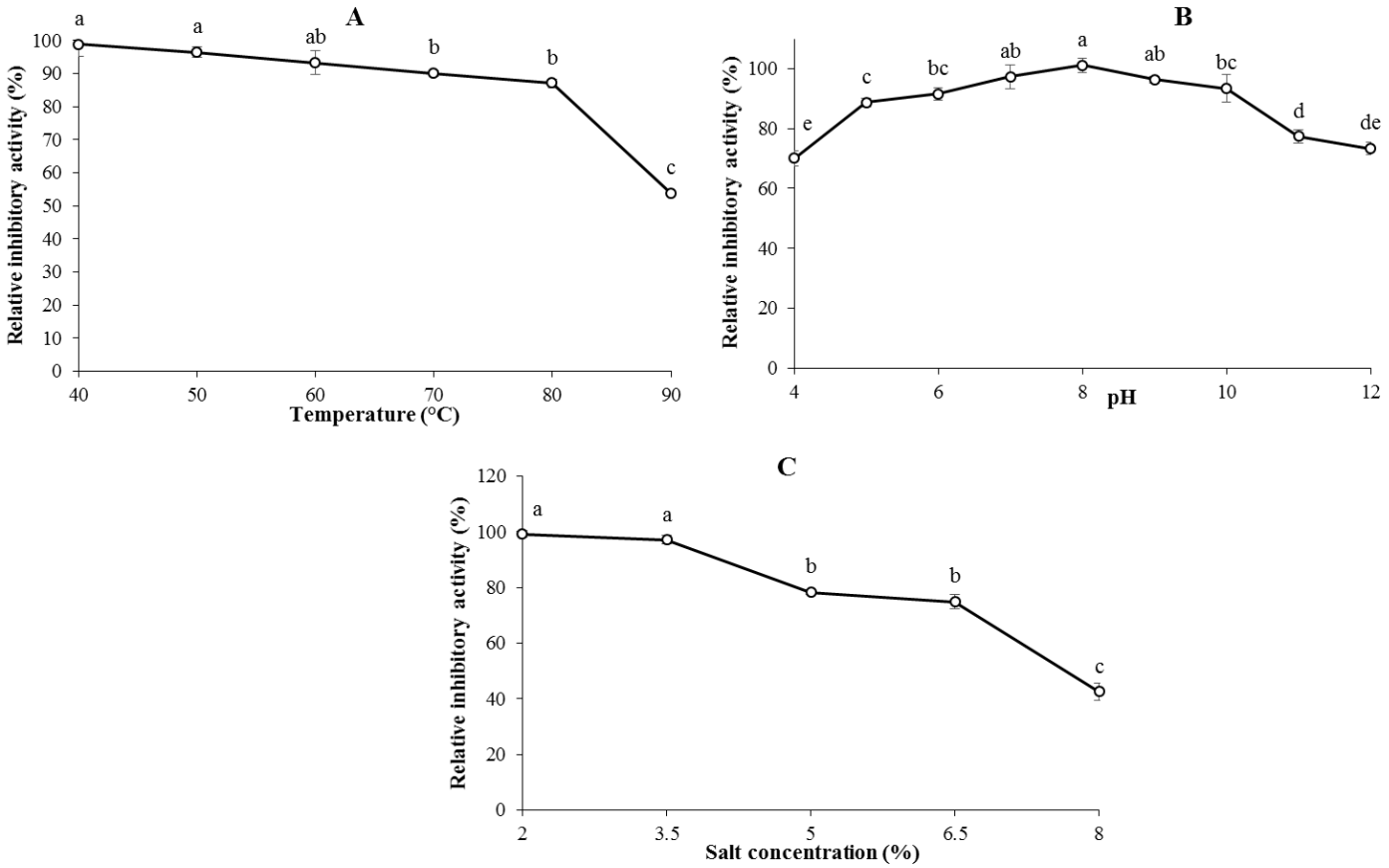

Figure 18. Thermal (A), pH (B), and salt (C) stability of purified TI from duck egg albumen. Different letters on the line in each figure indicate significant difference (P<0.05). Mean±SD (n=3).

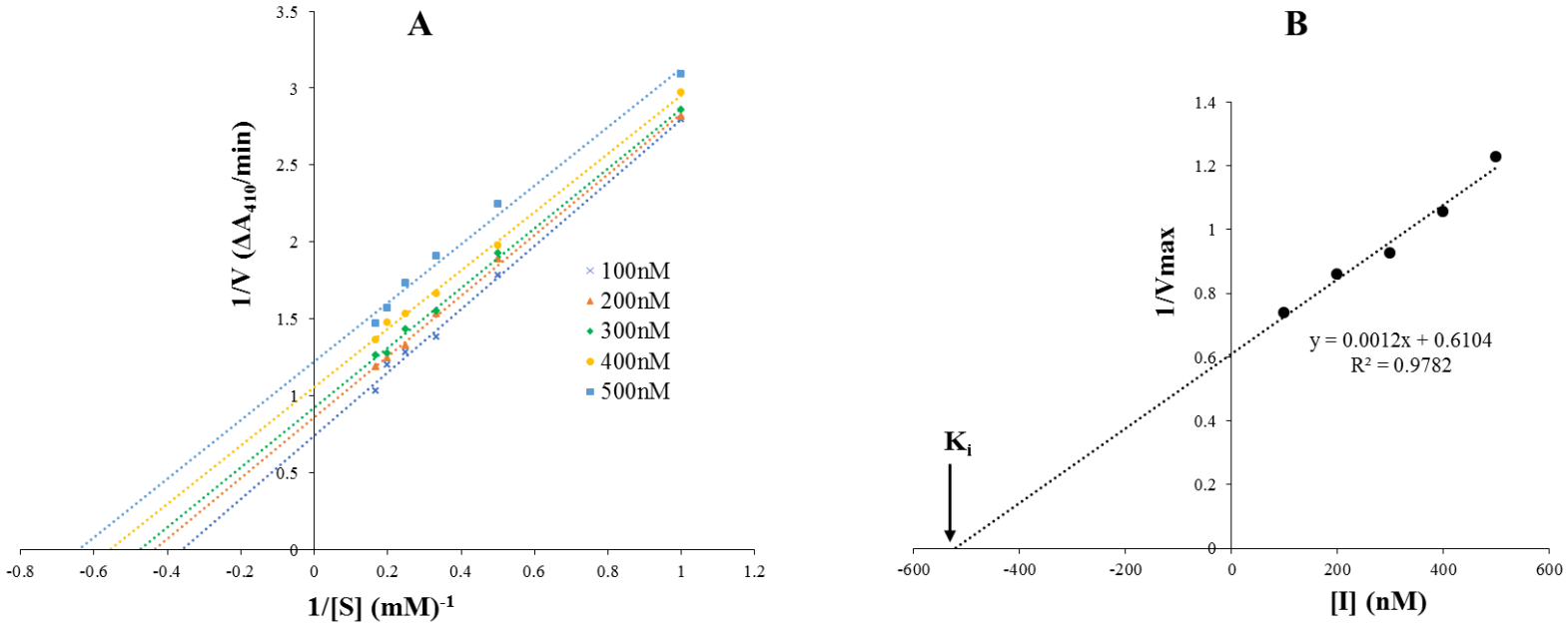

Figure 19. (A) The Lineweaver–Burk plot of 1/V (ΔA_{410}/min) versus different TI concentrations [I] (100, 200, 300, 400, and 500 nM). The concentration of trypsin was 1 μM. (B) The secondary plot of l/V-axis intercept versus [I] for Ki determination.

4.5 Conclusion

TI from duck egg albumen was isolated and purified using Trypsin-CNBr-activated Sepharose 4B-trysin affinity column with high purity. TI was stable within the temperature range of 40–60°C and pH of 7–9. The concentration of salt higher than 5% led to the decreases in inhibitory activity of TI. The inhibition mode of TI was classified as uncompetitive type with low Ki value. Thus, TI present in duck egg albumen could inhibit serine proteases localized in fish muscle or surimi, leading to the improved gel property.

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CHAPTER 5
COMPARATIVE STUDY ON THE EFFECT OF DUCK AND HEN EGG ALBUMENS ON PROTEOLYSIS AND GEL PROPERTY OF SARDINE SURIMI

5.1 Abstract

The effects of duck and hen albumens at different levels (1%–4%, w/w) on proteolysis and gel properties of sardine (*Sardinella albella*) surimi were studied. The addition of both albumens into surimi resulted in the increases in breaking force and deformation, especially with increasing levels added (P<0.05). Both albumens inhibited autolysis of surimi in a dose dependent manner as indicated by the lower TCA-peptide content and more retained myosin heavy chain and actin. Texture profile analysis showed that hardness, cohesiveness, springiness, gumminess and chewiness of surimi gel increased with increasing albumen levels. In general, breaking force, hardness, gumminess and chewiness of surimi gel added with duck albumen were higher than those added with hen albumen when the same level was used. Lightness and whiteness of gels added with albumen increased, particularly at higher amount of albumen. Surimi gel added with both albumens showed compact network with more connectivity and smaller voids than the control gel as visualised by scanning electron microscopy. Thus, duck albumen could be used to replace hen albumen for improvement of sardine surimi gel properties.

5.2 Introduction

Surimi is myofibrillar protein concentrate obtained by washing fish mince. It has a high commercial value with a wide range of applications, particularly for making a variety of gelling products, e.g. fish ball, imitation crab and/or lobster (Jitesh *et al.*, 2011; Zaghbib *et al.*, 2016). Surimi has commonly been produced from lean fish due to the white colour and superior gelling property. Nevertheless, sardine (*Sardinella albella*), a pelagic dark-fleshed fish, has been also used as a raw material for surimi production. In general, dark-fleshed fish contains high level of endogenous proteases,

which cause the gel softening. This limits the use of surimi from dark-fleshed fish (Buamard and Benjakul, 2015; Jitesh *et al.*, 2011). To conquer the gel weakening in surimi, several protein additives possessing protease inhibitory activity such as egg albumen, soy protein isolate, porcine and chicken plasma protein, etc. have been employed (Benjakul *et al.*, 2001; Benjakul *et al.*, 2004; Jafarpour *et al.*, 2012).

Egg albumen is well-known to contain protease inhibitors that inhibit cysteine, serine, aspartyl, and metalloproteases (Réhault, 2007). Five protease inhibitors have been reported in egg albumen. Those include ovostatin, ovomucoid, ovoinhibitor, cystatin C, and ovalbumin (Saxena and Tayyab, 1997). Due to its efficacy in lowering proteolysis in surimi, hen egg white or albumen has been used to improve the textural properties of surimi gel (Jafarpour *et al.*, 2012). Egg albumen has been employed in surimi of lizardfish, king weakfish, Pacific whiting, Alaska pollock, etc (Benjakul *et al.*, 2004; Hunt *et al.*, 2009; Kuhn *et al.*, 2004). Additionally, egg albumen is often used as an ingredient to enhance the water-holding capacity of food products, especially in surimi (Phillips and Williams, 2011).

Hen and duck eggs are among the most consumed avian eggs in Asian countries. Quail egg is also used for some particular cuisine (Miguel *et al.*, 2005). Egg albumen has been used in several foods and its compositions vary with species. Protein contents of lyophilised albumen from duck and quail eggs were higher than that of hen egg (Hu *et al.*, 2016; Miguel *et al.*, 2005). Moreover, ovomucoid from duck and hen albumen also showed different protease inhibitory activity in protection of salmon calcitonin breakdown by serine proteases (Shah and Khan, 2004). In general, hen albumen has been commonly manufactured into different forms, such as powder, pasteurised liquid albumen, etc (Trziszka *et al.*, 2013). Due to the excellent functional properties such as foaming and gelling properties, hen albumen has become the crucial ingredient in a wide range of food products (Abeyrathne *et al.*, 2013). Recently, duck albumen has been found to exhibit high protease inhibitory activity with superior gelling property (Quan and Benjakul, 2018). Thus, duck albumen could be employed as potential protein additive, which was able to improve gel property of muscle foods, especially surimi suffering from gel weakening. Nevertheless, no information regarding the use of duck

albumen in surimi exists. Therefore, this study aimed to comparatively investigate the impact of duck and hen albumens on proteolysis and gel properties of sardine surimi.

5.3 Materials and methods

5.3.1 Chemicals

All chemicals used in this study were of analytical grade. Bovine serum albumin (BSA) and trypsin from bovine pancreas (Type I, ~10,000 BAEE units/mg protein) were obtained from Sigma Chemical Co. (St. Louis, MO), and high molecular protein markers were purchased from GE healthcare UK Limited (Buckinghamshire, UK). Sodium dodecyl sulfate (SDS), β-mercaptoethanol (β-ME), trichloroacetic acid, Folin–Ciocalteu's phenol reagent, tyrosine, glutaraldehyde, ethanol and Coomassie blue R-250 were obtained from Merck (Darmstadt, Germany).

Frozen sardine surimi, AA grade, was purchased from Man A Frozen Co., Ltd. (Songkhla, Thailand) and kept at −20 °C until use, but not longer than two months.

5.3.2 Preparation of duck and hen albumens

Fresh duck and hen eggs within 24 h after laying were collected randomly at farm houses in Kantang, Trang province, Thailand and Faculty of Natural Resources, Prince of Songkla University, respectively. Eggs were broken and albumen was separated from egg yolk manually. Then, albumen was lyophilised using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The powders obtained were placed in polyethylene bag and kept at −20 °C until use but not longer than 2 months.

5.3.3 Study on the effect of hen and duck albumens on autolysis of sardine surimi

Autolysis assay was performed according to the method of Benjakul *et al.* (2001) Firstly, surimi (3g) was placed in 50 mL beaker. Thereafter, hen and duck albumens at various levels (0-4%, w/w) were added and thoroughly mixed. Then, the mixtures were spread at the bottom of beaker and covered with aluminum foil. Samples were incubated at 60 °C for 60 min. Subsequently, the reaction was terminated by adding 27 mL of cold 5% (w/v) trichloroacetic acid (TCA). The mixture was homogenised at 5000 rpm for 2 min using an IKA homogeniser Model T 25 D (IKA-

Werke GmbH & Co. KG, Staufen, Germany). The homogenate was centrifuged at 8000 × g for 10 min using a centrifuge (Allegra 25R centrifuge, Beckman Coulter, Palo Alto, CA, USA). TCA-soluble peptide content in the supernatant was determined by the Lowry method (Lowry *et al.*, 1951) and expressed as μmole of tyrosine equivalent/g sample.

Protein pattern of autolysed sardine surimi was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (Laemmli, 1970). Autolysis of surimi was performed in the same manner as previously described, except that 5% SDS solution (85 °C) was used instead of 5% TCA. The mixture was homogenised at a speed of 5000 rpm for 2 min. The mixture was centrifuged for 20 min at 12,000 ×*g*. The supernatant was subjected to SDS-PAGE analysis.

SDS-PAGE was carried out using 10% running gel and 4% stacking gel. The sample was mixed with sample buffer (50 mM Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, 10% β-ME). Sample (15 mg protein as determined by the method of Robinson and Hogden (1940) was loaded onto the gel and subjected to electrophoresis at constant current of 15 mA/gel using electrophoresis unit (Miniprotein III; Bio-Rad Laboratories, Richmond, CA, USA). The gels were stained with Coomassie Brilliant Blue R-125 (0.125%) in 25% methanol and 10% acetic acid. Destaining was performed using 40% methanol and 10% acetic acid. Molecular weight (MW) of protein bands was estimated from the plot of MW standards and Rf. Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems).

5.3.4 Determination of trypsin inhibitory activity

Trypsin inhibitory activity was measured as per the method of Benjakul *et al.* (2001). Albumen samples with an appropriate dilution (200 μL) were incubated with 200 μL of porcine pancreas trypsin (0.05 mg/mL) at 37 °C for 15 min. Then, 1000 μL of reaction buffer (50 mM Tris-HCl containing 20 mM $CaCl_2$, pH 8.2) were added. Thereafter, 200 μL of BAPNA (2 mg/mL) were added and the mixture was incubated

at 37 °C for 15 min. To terminate the reaction, 200 μL of 30% acetic acid (v/v) were added. The release of ρ-nitroalaniline was monitored by measuring the absorbance at 410 nm using a spectrophotometer (UV-16001, Shimadzu, Kyoto, Japan). One unit of trypsin activity was defined as the enzyme causing an increase of 0.01 absorbance unit/min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduced trypsin activity by one unit. Trypsin inhibitory activity of albumen was expressed as units/mg solid.

5.3.5 Study on the impact of hen and duck albumen on gel properties of sardine surimi

5.3.5.1 Preparation of sardine surimi gel

Surimi gel was prepared following the method of Buamard and Benjakul (2015). Firstly, surimi was thawed under running water (25–28 °C) until the core temperature reached 0–2 °C. The surimi was chopped into small pieces and mixed with 2.5% salt in a mixer (National Model MK-5080M, Selangor, Malaysia). During chopping, hen and duck albumen powders were added into surimi paste at different levels (0-4%, w/w). The temperature was maintained at 2–7 °C during chopping. The moisture content of surimi paste was adjusted to 80% with cold distilled water. The paste was stuffed into a polyvinylidene chloride casing with a diameter of 2.5 cm. Both ends were sealed tightly and incubated at 40 °C for 30 min, followed by heating at 90 °C for 20 min. Thereafter, all gels were cooled in iced water for 30 min and stored at 4 °C for 24 h before analyses.

5.3.5.2 Analyses

5.3.5.2.1 Breaking force and deformation

Breaking force and deformation of surimi gels added with hen or duck albumens at various levels were determined using a texture analyzer (Model TA-XT2, Stable MicroSystems, Surrey, UK) as described by Singh and Benjakul (2016). Five cylindrical samples were cut into 2.5 cm height and 2.5 cm diameter and equilibrated at room temperature (25–28 °C) before analyses. Sample was perpendicularly pressed by a spherical plunger of 5 mm diameter into the cut surface of gel at a constant

depression speed (60 mm/min). The force to puncture into the gel (breaking force) and the distance at which the plunger punctured into the gel (deformation) were both recorded.

5.3.5.2.2 Expressible moisture content

Expressible moisture content of surimi gel was measured according to the method of Buamard *et al.* (2017). Cylindrical gel samples were cut into a thickness of 5 mm, weighed accurately (*X*) and placed between three pieces of Whatman filter paper No.1 (Whatman International Ltd., Maidstone, UK) at the bottom and two pieces on the top of the sample. A standard weight of 5 kg was placed on the top of the sample for 2 min. The samples were then removed from the papers and weighed again (*Y*). Expressible moisture content was calculated with the following equation and expressed as percentage of sample weight:

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

5.3.5.2.3 Texture profile analysis

Texture profile analysis of surimi gels was also performed using a texture analyzer (Model TA-XT2i, Stable Micro System, Surrey, England). The samples were compressed twice to 50% of their original height with a compression cylindrical aluminum probe (50 mm diameter). Force-distance deformation curve was recorded at a cross head speed of 3 mm/s and the recording speed was 3 mm/s. Hardness, springiness, cohesiveness, chewiness, and gumminess were evaluated. These parameters were recorded using the MicroStable software version 6 (Surrey, England).

5.3.5.2.4 Determination of color

The color of surimi gel samples were determined by a colorimeter (ColorFlex, Hunter Lab Reston, USA) and reported in CIE system. L^*, a^*, b^* and ΔE^*, representing lightness, redness/greenness, yellowness/blueness and total difference of color, respectively, were recorded. The whiteness of gels was calculated (Singh and Benjakul, 2017) using the following equation:

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

The ΔE^* was also calculated by the following formulation:

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

where ΔL^*, Δa^*, Δb^* are the difference between color parameters of the samples and the color parameters of control gel (L^* = 59.60, a^* = - 1.92, b^* = 10.02).

5.3.5.2.5 Determination of microstructure

Microstructure of surimi gels added without and with duck and hen albumen at 3% was examined by a scanning electron microscopy as described by Buamard and Benjakul (2018) Surimi samples were cut into small pieces (1x1x1 mm^3). Samples were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. Then, fixed samples were rinsed with distilled water for 1 h. Subsequently, samples were dehydrated sequentially using a series of ethanol (25%, 50%, 70%, 80%, 90% and 100%) for 15 min at each concentration. The dehydrated samples were subjected to critical point drying (CPD). The samples were coated with 100% gold (sputter coater SPI-Module, West Chester, PA, USA). The gel microstructure was visualised by a scanning electron microscope (JEOL JSM-5800LV, Tokyo, Japan).

5.3.6 Statistical analysis

All the experiments were conducted in triplicate using three lots of samples. Data were presented as mean value with standard deviation. One way variance of analysis (ANOVA) was performed. For pair comparison, T-test was used. The Duncan's multiple range test was carried out to determine the significant difference between samples at $P<0.05$ level using the statistical program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

5.4 Results and discussion

5.4.1 Effect of hen and duck albumens at different levels on autolysis of sardine surimi

5.4.1.1 TCA-soluble peptide content

Autolysis of sardine surimi in the absence and presence of hen and duck albumens at various levels expressed as TCA-soluble peptide content is shown in Fig. 20. The highest TCA-soluble peptide content was observed in surimi without albumen addition ($P<0.05$). When surimi paste was added with hen or duck albumens, TCA-soluble peptide content decreased as the levels of albumen increased ($P<0.05$). Nevertheless, there were no differences in TCA-soluble peptide content in samples added with albumen at 3% and 4%, regardless of type of egg ($P>0.05$). TCA-soluble peptides indicate the degradation of muscle proteins during heat induced gelation of surimi as the result of indigenous proteases (Oujifard *et al.*, 2012). This result indicated that hen and duck albumens were able to inhibit the proteolysis of surimi in a dose-dependent manner. At the same level of albumen added, TCA-soluble peptide content of surimi incubated in the presence of hen albumen was higher than that of surimi incorporated with duck albumen ($P<0.05$). Higher efficacy of duck albumen in preventing autolysis was in accordance with its higher trypsin inhibitory activity, compared to hen albumen. In the present study, trypsin inhibitory activity of duck albumen was 5880 ± 27 units/mg solid, while hen albumen had the activity of 3925 ± 22 units/mg solid. Egg albumen contains several protease inhibitors, namely ovoinhibitor, ovamacroglobulin, etc. which were able to inhibit serine protease (Jitesh *et al.*, 2011). Hu *et al.* (2016) reported that duck ovostatin (ovamacroglobulin) is capable of inhibiting serine protease as well as metalloprotease, while chicken ovostatin inhibits only metalloprotease. Moreover, Shah and Khan (2004) proved that duck ovomucoid effectively stabilised salmon calcitonin against hydrolysis mediated by three proteases including trypsin, α-chymotrypsin and elastase. However, chicken ovomucoid could not prevent the degradation of salmon calcitonin. This result reconfirmed that duck albumen had higher inhibitory activity toward autolysis of sardine surimi. Inhibitory activity toward autolysis of both albumens was in a dose dependent manner.

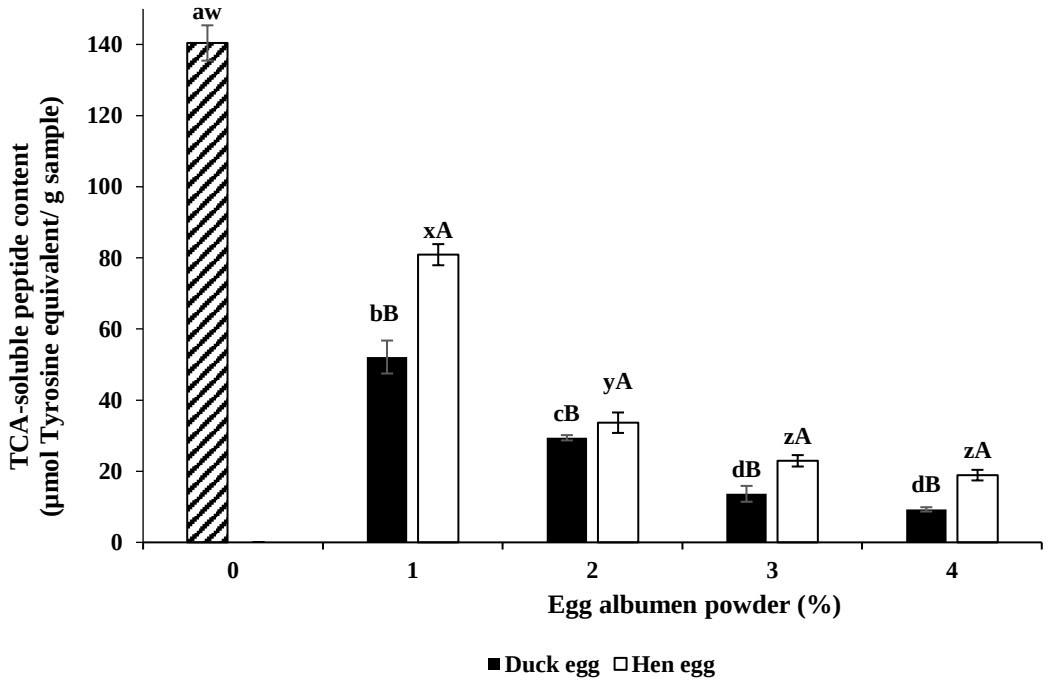

Figure 20. TCA-soluble peptide content of sardine surimi incubated at 60 °C for 60 min in the absence or presence of duck and hen albumens at different levels. Mean±SD (n=3). Different lowercase letters on the bar under the same egg albumen including the control (without albumen) indicate significant differences (P<0.05). Different uppercase letters on the bars under the same level of albumen indicate significant differences (P<0.05).

5.4.1.2 Protein pattern

Protein patterns of surimi added with hen or duck albumens at different levels and subjected to autolysis at 60 °C for 60 min are shown in Fig. 21. The lowest intensity of myosin heavy chain (MHC) and actin bands was observed in the samples without hen or duck albumen addition. The intensity of MHC bands increased with increasing albumen levels, indicating that autolysis was inhibited more effectively as the concentration of albumen increased. This result was in agreement with the higher decrease in TCA-soluble peptide content when albumen at higher concentrations was incorporated. Surimi gel added with hen egg albumen and duck egg albumen at 3% and 4% showed similar band intensity of MHC. With addition of 3% and 4% hen egg albumen, MHC band intensity was increased by 49.3% and 51.6%, respectively, compared to that of the control gel. Surimi gel added with 3% and 4% duck egg

albumen had the increase in MHC band intensity by 49.8% and 50.2%, respectively. Generally, actin and tropomyosin were resistant to proteolytic enzyme, while MHC was the main protein susceptible to degradation (Fowler and Park, 2015; Oujifard *et al.*, 2012). It was noted that band intensity of proteins with MW of 76 and 43–45 kDa were increased with increasing albumen levels. It was suggested that ovotransferrin and ovalbumin from egg albumen contributed to the increasing intensity of aforementioned protein bands. The increase was more pronounced when the amount of egg albumen was increased. Egg albumen contained ovalbumin (MW: 45 kDa) and ovotransferrin (MW: 76 kDa as determined under reducing condition) as the dominant proteins (Phillips and Williams, 2011). Ovalbumin contributes around 54%–58% of egg albumen proteins (Phillips and Williams, 2011). At the same level of albumen used, proteins including MHC were more retained when duck albumen was added, in comparison with hen albumen. This was in agreement with the higher trypsin inhibitory activity of duck albumen. As a consequence, lower degradation of muscle proteins in sardine surimi was attained when duck albumen was incorporated.

5.4.2 Effect of hen and duck albumen on properties of sardine surimi gel

5.4.2.1 Breaking force and deformation

Breaking force and deformation of sardine surimi gels added with hen or duck albumen at different levels are shown in Table 9. Breaking force (g) of control surimi gel (without albumen) was lowest (235.76 g) (P<0.05). Breaking force of gel increased as the concentrations of both albumens increased (P<0.05). At the same concentration of albumen added, the resulting gel containing duck albumen showed the higher breaking force, compared to that added with hen albumen (P<0.05). Similar trend was found for deformation. When duck and hen albumens at the level of 3% were added, breaking force of surimi gel increased by 55% and 46%, compared to that of control gel, respectively. The increases in breaking force by 96% and 54% were obtained as duck and hen albumens at a level of 4% was incorporated, respectively. The increases in breaking force and deformation were in agreement with the decrease in TCA-soluble peptide (Fig. 20). Duck albumen was more effective in inhibiting proteolysis in surimi than hen albumen. MHC, the main protein components for gel formation in surimi, was more retained when protease inhibitors were present (Oujifard

et al., 2012). Moreover, egg albumen is capable of water-holding with gel forming ability (Ren *et al.*, 2010). Quan and Benjakul (2018) reported that duck albumen showed the increased gel strength, especially after storage at 4°C for 6 days. Albumen proteins plausibly formed gel along with muscle proteins, especially MHC, in surimi. As a result, strong gel network could be developed. Jitesh *et al.* (2011) also reported that the highest gel strength was obtained in lizardfish (*Saurida tumbil*) surimi gel added with 3% egg albumen. The result suggested that duck albumen was more sufficient for strengthening sardine surimi than hen albumen. The gel strengthening effect of both albumens was in a dose dependent manner.

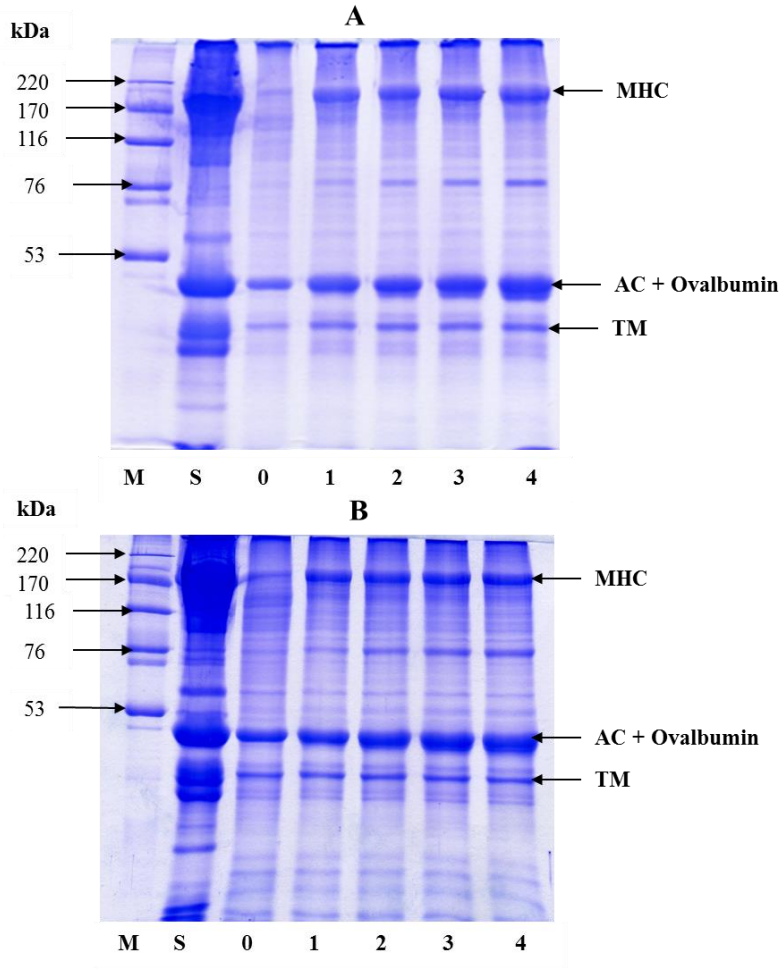

Figure 21. Protein pattern of sardine surimi incubated at 60°C for 60 min in the absence or presence of duck (A) and hen (B) albumens. Numbers denote the concentration of egg albumens (%, w/w). MHC: myosin heavy chain; AC: Actin; TM: Tropomyosin; H: High molecular weight marker; S: surimi paste.

Table 9. Breaking force, deformation, and expressible moisture content of sardine surimi gels added with hen or duck albumens at different levels

Albumen	Concentration (%)	Breaking force (g)	Deformation (mm)	Expressible Moisture content (%)
Control	0	235.76±3.65$^{\dagger ez}$	6.14±0.56^{cz}	6.60±0.66^{aw}
Hen egg	1	283.23±4.39^{dB}	6.56±0.18^{cB}	5.40±0.33^{bA}
	2	328.34±0.77^{cB}	7.07 ± 0.02^{bA}	4.92±0.10^{cA}
	3	344.67±2.86^{bB}	7.14 ± 0.02^{bB}	4.66±0.14^{cA}
	4	362.59±2.34^{aB}	7.69 ± 0.19^{aB}	3.63±0.11^{dA}
Duck egg	1	306.50±2.95^{yA}	7.08±0.08^{yA}	4.70±0.16^{xB}
	2	335.82±3.46^{xA}	7.54±0.32^{yA}	4.24±0.03^{xyB}
	3	365.37±1.97^{vA}	8.08±0.24^{xA}	4.03±0.04^{yB}
	4	463.23±2.32^{uA}	8.31±0.05^{xA}	3.10±0.29^{zA}

†Mean±SD (n=3). Different lowercase superscripts (a-e) and (u-z) in the same column under the same egg albumen including the control indicate significant differences (P<0.05). Different uppercase superscripts (A-B) in the same column under the same level of albumen indicate significant differences (P<0.05).

5.4.2.2 Texture profile

Texture profile of surimi gel added with duck or hen albumens at various concentrations is presented in Table 10. Hardness of surimi gel increased with increasing albumen concentrations (P<0.05). The lowest hardness was noticeable in control gel (58.90 N), whereas the highest values (97.25 and 87.22 N) were found in the samples added with 4% duck or 4% hen albumen, in which the increases by 60% and 48% were obtained, respectively. When comparing hardness of gel added with hen or duck albumens at the same concentrations, the latter had the higher hardness (P<0.05). Hardness is related to the strength of gel structure under compression and is the peak force during the first compression cycle (Chandra and Shamasundar, 2015). The addition of albumen more likely prevented the degradation of myosin in sardine muscle. Egg albumen has been known to contribute to gel strengthening via protease inhibition and its binding effect (Benjakul *et al.*, 2004). Jafarpour *et al.* (2012) also

reported that hardness, cohesiveness, adhesiveness and elasticity of common carp surimi gel increased as the level of egg albumen increased. Increases in hardness were in accordance with the increased breaking force of gel incorporated with albumens.

The slight increase in cohesiveness of surimi gel was observed when both hen and duck albumens were added (Table 10). At the same level, higher cohesiveness was found in the samples added with hen albumen, compared to those containing duck albumen. However, there was no difference in cohesiveness among the surimi gels added with albumen in the range of 1–4% ($P>0.05$). Cohesiveness is a parameter to measure of the difficult level in breaking down the internal structure of gel (Lau *et al.*, 2000). For springiness, no differences were obtained in all samples, regardless of albumen addition. Springiness is considered as "elasticity" or "rubberiness" of the gel in the mouth, and is a parameter to show how much the gel structure is broken down by the initial compression (Lau *et al.*, 2000). Gumminess and chewiness of surimi gels without and with duck or hen egg albumens at various levels are shown in Table 10. In general, these values increased continuously as albumen concentrations increased ($P<0.05$). The changes in gumminess and chewiness were in correlation with those of hardness. Gumminess and chewiness were calculated based on hardness, which suggests the resistance to compression force (Yilmaz *et al.*, 2012). At the same level of albumen, higher gumminess and chewiness were obtained in samples added with duck albumen than those containing hen albumen. The result suggested that duck albumen was found to be effective in improving the textural properties of sardine surimi gel.

Table 10. Texture profile of sardine surimi gels added with hen or duck albumens at different levels

Albumen	Concentration (%)	Hardness (N)	Cohesiveness	Springiness (cm)	Gumminess (N)	Chewiness (N.cm)
Control	0	58.90±2.59$^{\dagger dz}$	0.77±0.01^{bz}	0.91±0.02^{az}	44.62±0.32^{ez}	44.01±1.20^{ey}
Hen egg	1	70.08±1.62^{cB}	0.79±0.01^{aA}	0.91±0.01^{aA}	56.38±1.26^{dB}	52.01±1.80^{dA}
	2	72.28±0.21^{cB}	0.80±0.00^{aA}	0.92±0.01^{aA}	60.15±0.89^{cB}	55.48±1.65^{cB}
	3	76.90±0.10^{bB}	0.81±0.01^{aA}	0.92±0.00^{aA}	63.92±0.39^{bB}	58.96±0.22^{bB}
	4	87.22±1.28^{aB}	0.81±0.00^{aA}	0.93±0.00^{aA}	68.35±1.05^{aB}	63.81±0.33^{aB}
Duck egg	1	78.09±1.66^{yA}	0.78±0.00^{yA}	0.92±0.00^{zA}	61.14±1.16^{yA}	56.80±1.08^{xA}
	2	82.48±1.33^{xA}	0.79±0.01^{yB}	0.93±0.01^{zA}	65.29±0.48^{xA}	60.90±0.16^{wA}
	3	85.36±0.51^{wA}	0.79±0.00^{yA}	0.93±0.01^{zA}	67.39±0.44^{wA}	62.77±0.59^{wA}
	4	97.25±1.14^{vA}	0.79±0.01^{yB}	0.93±0.00^{zA}	75.39±0.79^{vA}	70.05±0.73^{vA}

†Mean±SD (n=3). Different lowercase superscripts (a-e) and (u-z) in the same column under the same egg albumen including the control indicate significant differences (P<0.05). Different uppercase superscripts (A-B) in the same column under the same level of albumen indicate significant differences (P<0.05).

5.4.2.3 Expressible moisture content

Expressible moisture contents of surimi gels added with hen and duck albumens at different levels are shown in Table 9. The highest expressible moisture content was found in the control surimi gel (6.60%). The expressible moisture content decreased as the levels of both albumens increased (P<0.05). When the same level of albumen was used, higher expressible moisture content was observed for surimi added with hen albumen, compared to that of surimi incorporated with duck egg (P<0.05). With the addition of 3% duck or hen albumens, the expressible moisture content decreased by approximately 39% and 30%, respectively. The expressible moisture content is indicative for the water binding properties. During thermal gelation, protein matrix was formed and water was imbibed throughout the network (Rawdkuen *et al.*, 2004). The decrease in expressible moisture content was coincidental with the increases in breaking force and deformation of gel added with albumens. The greater amount of water was imbibed in the gel network when the order and finer network was formed. This correlated with the increased gel strength as indicated by the higher breaking force and deformation. Jafarpour *et al.* (2012) also reported that water holding capacity of surimi gel from common carp (*Cyprinus carpio*) increased continuously when egg albumen ranging from 1% to 3% was added. This suggested that duck albumen could be used to replace hen albumen for improvement of the gelling properties of surimi via increasing water holding capacity.

5.4.2.4 Color and whiteness

Color and whiteness of sardine surimi gels added with duck or hen albumens at different levels are shown in Table 11. It was observed that surimi gels added with duck or hen albumen had slightly higher lightness (L*), compared to the control surimi gel (P<0.05). There was no difference in L*- value between surimi gels incorporated with duck and hen albumen (P>0.05) when the same level was used. Similar trend was noticeable for the whiteness, in which the addition of both albumens at 3% or 4% yielded the gels with the highest whiteness (P<0.05). The increase in lightness or whiteness was probably due to the white color of albumen gel. As a result, the sardine gel with grey color had the increased lightness and whiteness as albumen was

incorporated. According to Jafarpour *et al.* (2012) the addition of egg albumen up to 3% increased the lightness and whiteness of common carp surimi gel. Nevertheless, Benjakul *et al.* (2004) reported that the whiteness of lizardfish surimi gel was not affected by addition of egg albumen up to 3%. The slight increases in both *b**- and *a**-values of surimi gels were observed as the levels of both albumens increased ($P<0.05$). For *a**- value, surimi gels added with hen albumen showed higher value than those incorporated with duck albumen, especially at the concentration of 2%–3%. However, there were no differences in *b**- values between gels added with duck and hen albumens at the same levels. Nevertheless, Jafarpour *et al.* (2012) reported that increase in egg albumen concentration caused a decrease in the yellow color (*b**-value) of common carp surimi gel. For ΔE^*, the increase in value was obtained for surimi gel added with hen and duck albumens, compared to the control. The increase in ΔE^* was coincidental with the increase in whiteness. In general, egg albumen affected the color of sardine surimi gel to some degrees. Duck albumen could enhance the lightness and whiteness of gel from sardine surimi effectively.

5.4.2.5 Microstructure

Scanning electron micrographs of sardine surimi added with hen or duck albumens at 3% and the control surimi gel are illustrated in Fig. 22. Surimi gel without albumen was less compact with more opened structure. Its network contained larger voids with less connectivity. However, more compact and denser gel network was observed in surimi added with hen or duck albumens at 3%. The denser and more compact structure of gel coincided with the increased breaking force (Table 9) and hardness (Table 10). Albumen with protease inhibitory activity could inhibit indigenous proteases in surimi. As a result, MHC was retained and underwent aggregation, in which more ordered and finer network was developed. Furthermore, egg albumen protein also can form gel with good properties and high water holding capacity (Phillips and Williams, 2011; Quan and Benjakul, 2018). It was noted that albumens either from duck or hen eggs at a level of 3% was sufficient to control proteolysis in surimi. Similar MHC band intensity was observed when albumen at 3% and 4% was used (Fig. 21). It was assumed that the increase in breaking force or hardness of gel added with 4% albumen was more likely caused by gelling ability of albumen. However, the use of egg

white at high level might result in off odor, which was not desirable (Ahamed *et al.*, 2007). The level of albumen up to 3% has been widely used in surimi (Benjakul *et al.*, 2004; Jafarpour *et al.*, 2012; Jitesh *et al.*, 2011). When comparing the microstructure of surimi added with hen and duck albumens, gel added with hen albumen showed slightly coarser network with larger voids. The less compactness was related with the lower breaking force and hardness of hen albumen added surimi gel (Table 10). The result revealed that hen and duck albumen had the impact on gel structure of sardine surimi, contributing to gel property of surimi.

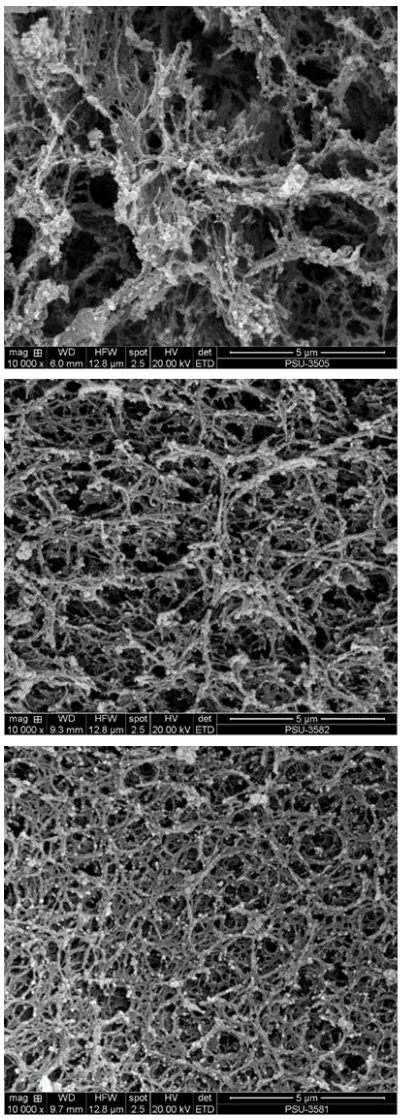

Figure 22. Scanning electron microscopic photograph of sardine surimi gels without and with the addition of 3% duck or hen albumens. Magnification: 10,000×. Scale bar = 5 μm.

Table 11. Color and whiteness values of sardine surimi gels added with hen or duck albumens at the different levels.

Albumen	Concentration (%)	L^*	a^*	b^*	ΔE^*	Whiteness
Control	0	$59.60\pm0.43^{\dagger bz}$	-1.92 ± 0.07^{dz}	10.02 ± 0.04^{cz}	00.00 ± 0.00^{ez}	58.33 ± 0.41^{bz}
Hen egg	1	60.02 ± 0.86^{bA}	-1.70 ± 0.02^{cA}	10.04 ± 0.04^{cA}	0.42 ± 0.05^{dB}	58.74 ± 0.83^{bA}
	2	60.49 ± 0.25^{bB}	-1.56 ± 0.03^{cA}	10.67 ± 0.06^{bA}	1.62 ± 0.06^{cB}	59.03 ± 0.25^{bB}
	3	62.12 ± 0.52^{aA}	-1.37 ± 0.02^{bA}	10.72 ± 0.17^{bA}	2.46 ± 0.29^{bA}	60.61 ± 0.49^{aA}
	4	62.66 ± 0.52^{aA}	-1.15 ± 0.05^{aA}	11.37 ± 0.56^{aA}	3.78 ± 0.04^{aA}	60.95 ± 0.49^{aA}
Duck egg	1	60.78 ± 0.74^{yA}	-1.70 ± 0.06^{yA}	10.24 ± 0.30^{yA}	1.42 ± 0.28^{yA}	59.86 ± 0.75^{yA}
	2	61.43 ± 0.31^{xyA}	-1.64 ± 0.03^{xB}	10.61 ± 0.03^{xA}	1.82 ± 0.14^{xA}	59.96 ± 0.30^{xyA}
	3	61.58 ± 0.35^{xA}	-1.55 ± 0.04^{xB}	10.67 ± 0.07^{xA}	2.25 ± 0.21^{wA}	60.10 ± 0.35^{xyA}
	4	61.90 ± 0.33^{xA}	-1.37 ± 0.06^{wA}	10.83 ± 0.09^{xA}	2.39 ± 0.19^{wB}	60.37 ± 0.32^{xA}

†Mean±SD (n=3). Different lowercase superscripts (a-f) and (u-y) in the same column under the same egg albumen including the control indicate significant differences (P<0.05). Different uppercase superscripts (A-B) in the same column under the same level of albumen indicate significant differences (P<0.05).

5.5 Conclusion

The addition of hen and duck albumens in sardine surimi gel could reduce the degradation of muscle proteins, especially MHC. Gel strength and whiteness of surimi gel were improved. Duck albumen showed higher efficiency in enhancing the gelling properties of sardine surimi than hen albumen. Thus, duck albumen was considered to replace hen albumen and could be used as protein additive in surimi gel, where protease inhibitors from duck albumen were able to alleviate gel weakening caused by endogenous proteases.

5.6 References

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CHAPTER 6
IMPACTS OF DESUGARIZATION AND DRYING METHODS ON PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF DUCK ALBUMEN POWDER

6.1 Abstract

Desugarization of duck albumen using glucose oxidase/catalase was optimized before drying. Optimum condition for desugarization using response surface methodology was as follows: glucose oxidase 31.24 units and catalase 781 units/mL albumen and incubation time of 6.55 h at 30 °C. Foaming capacity (FC) and foam stability (FS) were enhanced, while the solubility decreased after desugarization. This coincided with the increase in surface hydrophobicity (P<0.05). Higher trypsin inhibitory activity was found in freeze-dried albumen powder than spray-dried counterpart. Trypsin inhibitory activity was continuously decreased as the inlet temperature for spray-drying increased (P<0.05). Desugarization could increase FC and FS, but slightly decreased solubility of powders. No marked differences in protein patterns were observed in all the powders, regardless of desugarization and drying methods. L^* of albumen powder decreased but ΔE^*, a^*-, b^*-values and browning index increased as spray-drying temperatures increased (P<0.05). Therefore, prior desugarization could lower browning and increased foaming and gelling properties of duck albumen, particularly when spray dried with inlet temperature of 160 °C.

6.2 Introduction

Duck egg has become increasingly more important in Asian countries because of its nutritive value. Additionally, less capital input is required for its production (Huang and Lin, 2011). Egg albumen has been considered as an excellent source of protein and contains several bioactive compounds. Lysozyme possesses bacteriostatic, bacteriolytic, and bacteriocidal activity (Ren *et al.*, 2010; Wu, 2014). Ovomucoid and ovoinhibitor are capable of inhibiting trypsin, chymotrypsin, fungal, and bacterial proteases (Li-Chan and Kim, 2008; Wu, 2014). Recently, Quan and Benjakul (2018a) reported that duck albumen exhibiting high protease inhibitory activity could increase

gel strength of sardine surimi. Thus, duck albumen was suggested to replace hen albumen to alleviate gel weakening caused by endogenous proteases (Quan and Benjakul, 2018b).

Nowadays, egg albumen, especially in the powder form, is an important raw material for food industry. This is owing to low space required for transportation and storage, low susceptibility to microbial growth, and uniformity (Katekhong and Charoenrein, 2018). In general, the original liquid form of albumen contains glucose about 4 g/L. Glucose is involved in Maillard reaction of albumen during drying process. This contributes to undesirable brown color. Furthermore, the glucose–cephalin reaction (a reaction between a cephalin amino group and aldehydes of glucose) is responsible for off-flavor development during dehydration and storage (Lechevalier *et al.*, 2013; Wu, 2014). To conquer this drawback, desugarization is a crucial step before dehydration to obtain perfectly white powders. Desugarization can be conducted using bacterial or yeast fermentations. However, these processes pose some bacteriological problems. The use of commercial glucose oxidase and catalase is effectively converts glucose into gluconic acid (Lechevalier *et al.*, 2013). Enzymatic treatment for glucose removal before dehydration was the best method to prepare Egyptian egg albumen powder (Darwish and Sadek, 1978). Enzymatic glucose hydrolysis has been implemented in industrial scale prior to spray drying of hen egg albumen (Lechevalier *et al.*, 2007). Dried egg albumen can be manufactured by spray drying or freeze drying. Freeze drying is one of the best drying technologies for maintaining the quality of foods. This technology is suitable for heat sensitive food components. However, freeze drying takes a long period of time for operation and it has high capital and process costs (Chen *et al.*, 2012; Wang *et al.*, 2016; Zhou *et al.*, 2014). Spray-drying is the most popular drying technology used for production of hen egg albumen powder (Wu, 2014). Ma *et al.* (2013) reported optimum spray-drying conditions including spraying flow of 22 mL/min, feeding temperature of 39.8 °C, and inlet-air temperature of 178.2 °C, in which high quality and functional properties of hen egg albumen were obtained. Moderate spray-drying conditions yielded dried hen albumen and whole egg, which could be applied in baking, dressings, and confectionery products (Ayadi *et al.*, 2008).

However, no information on desugarization and spray-drying condition on the properties of duck egg albumen exists. Therefore, the objectives of this study were to optimize desugarization using response surface methodology (RSM) and to investigate the effects of spray-drying with different inlet temperatures and freeze-drying on physiochemical properties, trypsin inhibitory activity, and functional properties of duck albumen powders.

6.3 Materials and methods

6.3.1 Chemicals/enzymes

All chemicals were of analytical grade. Na-Benzoyl-$_{DL}$-arginine-ρ-nitroanilide (BAPNA), trypsin from bovine pancreas (Type I, ~10,000 BAEE units/mg protein), and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). High and low molecular protein markers were purchased from GE healthcare UK Limited (Buckinghamshire, UK). Sodium dodecyl sulfate (SDS), β-mercaptoethanol (β-ME), 8-anilino-1-naphthalenesulfonic acid (ANS), glutaraldehyde, ethanol, and Coomassie blue R-250 were produced from Merck (Darmstadt, Germany).

Mixed enzyme containing glucose oxidase (12,000 units) and catalase (300,000 units) was purchased from Megazyme U.C. (Wicklow, Ireland). Before use, 20 mL of 200 mM Tris-HCl buffer (pH 7.6) were added into the enzyme powder. Unit of enzymes used was expressed in terms of glucose oxidase/mL of albumen.

6.3.2 Optimization of desugarization in duck albumen using glucose oxidase/catalase

6.3.2.1 Optimization experiment

The optimization of desugarization conditions for duck albumen was conducted using a RSM. A central composite design (CCD) with two independent variables including mixed enzyme (glucose oxidase/catalase) concentration (X_1, units/mL albumen) and time (X_2, h) at five code levels was used. The central point of all variables was coded as zero. The minimum and maximum points of all variables were assigned. Full experimental plan with regards to their actual and code is provided in Table 12. The experimental plan containing 13 runs with five replicates at the centre

point (run order 9–13) were used to estimate a pure error sum of squares. Glucose content (mg/mL) was the dependent variable (*Y*). The experimental data were fitted to a polynomial model. A second-order model was applied in RSM as follows:

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

where β_0 is the intercept constant; β_1 and β_2 are the linear coefficients of X_1 and X_2, respectively, and β_{12} is the interaction coefficient. The optimum condition was determined by covering maximum areas of all responses representing all combinations of independent variables using the Design-Expert Statistical package version (Statease, Inc., Minneapolis, MN). The fitted polynomial equation was then expressed in the form of contour plot, in order to illustrate the relationship between dependent variable and the experimental variables tested.

6.3.2.2 Preparation of desugarized albumen

Fresh duck eggs within 24 h after laying were collected from a farm house in Kantang, Trang province, Thailand. The eggs were broken and albumen was separated from egg yolk manually. The chalazae was removed. In order to ensure a uniformity of samples, egg albumen (7.8 L) was homogenized at 4000 rpm for 3 min using an IKA homogenizer Model T 25D (IKAWerke GmbH & Co. KG, Staufen, Germany). Albumen with the initial pH of 8.66 (200 mL) was mixed with different levels of mixed enzyme. The mixture was adjusted to pH 7.6 using 0.1 M HCl and stirred for various time as described in Table 12 in the presence of 0.1mL of 30% H_2O_2. Thereafter, the resulting albumen samples were determined for glucose content.

Table 12. Central composite design (CCD) for desugarization of duck albumen using RSM and experimental data for glucose content of duck albumen treated with different enzyme concentrations for various time

Std.	Coded levels		Actual levels		Glucose content (μg/mL)
	Time (h)	Glucose oxidase/catalase concentration (units/mL albumen)	Time (h)	Glucose oxidase/catalase concentration (units/mL albumen)	
1	-1	-1	2.00	15.00/375.00	185.43 ± 6.18[†]
2	1	-1	8.00	15.00/375.00	86.24 ± 5.70
3	-1	1	2.00	35.00/875.00	146.07 ± 4.94
4	1	1	8.00	35.00/875.00	9.94 ± 0.91
5	-1.414	0	0.76	25.00/625.00	252.23 ± 2.91
6	1.414	0	9.24	25.00/625.00	77.83 ± 1.05
7	0	-1.414	5.00	10.86/271.50	90.86 ± 1.18
8	0	1.414	5.00	39.14/978.50	10.07 ± 3.40
9	0	0	5.00	25.00/625.50	43.45 ± 6.08
10	0	0	5.00	25.00/625.50	42.35 ± 3.29
11	0	0	5.00	25.00/625.50	44.87 ± 4.06
12	0	0	5.00	25.00/625.50	45.08 ± 2.36
13	0	0	5.00	25.00/625.50	43.83 ± 1.48

[†]Mean±SD (*n*=3).

6.3.2.3 Determination of glucose content

All duck albumen samples were determined for glucose content using Somogyi–Nelson method with a slight modification using glucose as a standard (Hatanaka and Kobara, 1980). Diluted albumen samples (0.25 mL) were mixed with 0.25 mL of alkaline copper reagent. The mixture was boiled in water bath for 20 min. Then, the boiled mixture was cooled with tap water. Thereafter, 1 mL of Folin–Ciocalteu phenol reagent was added into the cooled mixture. The mixture solution was centrifuged at 8,000 ×*g* for 15 min using a microcentrifuge (Hettich Mikro 20, Tuttlingen, Germany). The supernatant was measured for the absorbance at 660 nm

using a spectrophotometer (UV-16001, SHIMADZU, Kyoto, Japan). The glucose content was calculated from a glucose standard curve with the concentration range of 0–0.1 g/L. Albumen treated with glucose oxidase/catalase under the condition yielding the lowest glucose content was prepared and used for drying.

6.3.3 Effects of spray drying conditions on physicochemical properties, trypsin inhibitory activity and functional properties of duck albumen powders

6.3.3.1 Drying of desugarized and non-desugarized duck albumen

Desugarized and non-desugarized duck albumen (moisture contents of 87.45% ± 0.03 and 87.49% ± 0.11, respectively) were subjected to freeze-drying and spray-drying. For freeze-drying, both albumen samples were frozen at -40 °C for 8 h. The frozen samples were subjected to sublimation using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) at -50 °C for 96 h. Freeze-dried duck albumen powders obtained were ground using a blender (Panasonic, Model MX-898N, Berkshire, UK) and subjected to sieving (an 80-mesh sieve). Thereafter, the resulting powders were placed in polyethylene bag and kept at -20 °C until use but not longer than 2 months.

To prepare spray-dried duck albumen, both albumens were diluted in distilled water at a ratio of 1:2 (v/v) before drying to reduce viscosity of samples. Spray-drying was carried out using a spray dryer (LabPlant SD-06 Basic, North Yorkshire, England) equipped with a spray-drying chamber having the dimensions of 500 mm height and 210 mm diameter and a spray nozzle type of two-liquid nozzle (0.5 mm in size). A cyclone separator, a hot-air blower, and an exhaust blower were equipped. Albumen solutions were fed by a peristaltic pump at 10 mL/min into the chamber and atomized by hot air (air velocity of 2 m/s) from the blower in a downward current flow mode. Various inlet temperatures (140, 160, and 180 °C) were used and the atomizing pressure was 2.8 bars. Outlet temperature was about 85–95 °C. All dried albumen samples were subjected to analyses.

6.3.3.2 Analyses

6.3.3.2.1 Determination of moisture content and water activity

Moisture content of albumen samples was determined using an oven method (AOAC, 2000). Water activity (a_w) value of duck albumen powders was measured with a water activity analyzer (4TEV Aqualab, Decagon Devices,WA).

6.3.3.2.2 Determination of browning index

Browning index of duck albumen powders was determined according to the method of Chuaychan *et al.* (2017). Powders (60 mg) were mixed with distilled water (1 mL). A ten-fold dilution was made using the distilled water. The contents of intermediate and advanced Maillard reaction products were measured at 294 and 420 nm, respectively, using a UV/Vis spectrophotometer.

6.3.3.2.3 Determination of color

The color of albumen powder samples was determined by a colorimeter (ColorFlex, Hunter Lab, Reston, VA) and reported in CIE system. L^*, a^*, b^* and ΔE^*, represent lightness, redness/greenness, yellowness/blueness, and total difference of color, respectively. Total difference in color (ΔE^*) was calculated according to the following equation:

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

where ΔL^*, Δa^*, Δb^* are the difference between color parameters of the samples and those of the desugarized albumen produced by freeze drying (L^*=97.93, a^*=1.83, b^*=0.97).

6.3.3.2.4 Determination of surface hydrophobicity

Surface hydrophobicity of albumen powders was determined according to the method of Kaewmanee *et al.* (2011) using ANS as a probe. Albumen powder was added with 0.2 M sodium phosphate buffer pH 7.0 to obtain the final protein concentrations of 0.125, 0.25, 0.5, and 1 mg/mL as determined by the Biuret method. Prepared solution of 2 mL was mixed with 10 mL of 8mM ANS. The fluorescence

intensity of ANS-conjugates was measured using RF-1501 spectrofluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 374 nm and an emission wavelength of 458 nm. Surface hydrophobicity was calculated from the initial slope of the plot of fluorescence intensity against protein concentration using a linear regression analysis. The initial slope was referred to as surface hydrophobicity (S_0ANS).

6.3.3.2.5 Determination of foaming capacity (FC) and foam stability (FS)

FC and FS of duck albumen powders were determined by following the method of Singh *et al.* (2017). Albumen solution (1%, w/v) was adjusted to pH 7.0 by 4 N HCl or 4 N NaOH. Thereafter, 30 mL of albumen solution was placed in a 250-mL cylinder and whipped using a IKA homogenizer (IKA, Labortechnik homogenizer Selangor, Malaysia) at 13,400 rpm for 1 min. Volumes were recorded before and after whipping and the percentage of increase in foam volume was calculated according to the following equation:

$$\text{FC} = (A - B)/C * 100$$

where *A* is the total volume after whipping, *B* is the volume of liquid after whipping, and *C* is the volume of liquid before whipping. FS was calculated by the same equation of FC. However, after whipping, the cylinder containing foam was covered with a parafilm to avoid drying. The samples were allowed to stand for 60 min. Total volume and volume of liquid after 60 min were recorded. FS was calculated and reported.

6.3.3.2.6 Determination of solubility

Albumen powders were dissolved in deionized water to obtain a concentration of 1% (w/v). The pH was adjusted to pH 7 with either 4 N NaOH or 4 N HCl. Suspensions were centrifuged at 14,000 ×*g* for 15 min at room temperature (25 °C) using a centrifuge (Allegra 25R centrifuge, Beckman Coulter, Palo Alto, CA). Protein content in supernatant was measured using the Biuret method (Robinson and Hogden, 1940). For total protein, the samples were solubilized with 0.5 M NaOH. Solubility was reported as the percentage relative to total protein content of duck albumen following the method of Singh *et al.* (2018).

6.3.3.2.7 Determination of trypsin inhibitory activity

Trypsin inhibitory activity was measured as per the method of Benjakul *et al.* (2001). Solutions of albumen powders were firstly subjected to 50-fold dilution using distilled water. The prepared solutions (0.2 mL) were incubated with 0.2 mL of porcine pancreas trypsin (0.05 mg/mL) at 37 °C for 15 min. Then, 1 mL of reaction buffer (50 mM Tris-HCl containing 20 mM $CaCl_2$, pH 8.2) were added. Subsequently, 0.2 mL of BAPNA (2 mg/mL) were added and the mixture was incubated at 37 °C for 15 min. To terminate the reaction, 0.2 mL of 30% acetic acid (v/v) was added. The release of *ρ*-nitroaniline was monitored by measuring the absorbance at 410 nm using a spectrophotometer. One unit of trypsin activity was defined as the enzyme causing an increase of 0.01 absorbance unit/min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduced trypsin activity by one unit.

6.3.3.2.8 SDS-PAGE

Protein patterns of albumen powders were determined by SDS-PAGE according to the method of (Laemmli, 1970). To prepare protein sample, 2 mg of albumen powders were mixed with 6 mL of 5% SDS and boiled at 85 °C for 40 min. The prepared sample (15 mg protein as determined by the method of Robinson and Hogden (1940) was mixed with sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, with and without 10% β-ME) and loaded onto the gel (12% running gel and 4% stacking gel). Electrophoresis was performed at a constant current of 15 mA/gel using electrophoresis unit (Miniprotein III; Bio-Rad Laboratories, Richmond, CA). The gels were stained with Coomassie Brilliant Blue R-125 (0.125%) in 25% methanol and 10% acetic acid. Destaining was performed using 40% methanol and 10% acetic acid. Molecular weight (MW) of protein bands was estimated from the plot of MW standards and Rf.

6.3.3.2.9 Scanning electron micrograph

Microstructure of the selected albumen powders was visualized using a scanning electron microscope (Quanta400, FEI, Czech Republic) at an accelerating voltage of 20 kV. Prior to visualization, the samples were mounted on a bronze stub

and sputter-coated with gold (Sputter coater SPIModule, West Chester, PA) in order to make the sample conductive.

6.3.4 Statistical analysis

All the experiments were conducted in triplicate using three lots of samples. Data were presented as mean value ± standard deviation. One-way analysis of variance (ANOVA) was performed. The Duncan's multiple range test and paired-samples T-test were carried out to determine the significant difference between samples at $P<0.05$ level using the statistical program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL).

6.4 Results and discussion

6.4.1 Optimization of desugarization in duck albumen using RSM

Glucose content in duck albumen was 4294.87±47.17 µg/mL. The experimental glucose contents of duck albumen treated with mixed glucose oxidase/ catalase are given in Table 12, which ranged from 9.94 to 252.23 µg/mL. A polynomial model was regressed between glucose content and desugarization conditions and given as follows:

$$Y = 43.92 - 28.74X_1 - 60.24X_2 - 9.24X_1X_2 + 3.07X_1^2 + 60.35X_2^2$$

The closer coefficients of analysis (R^2) value to unity, the better the empirical model fits the actual data (Sharma *et al.*, 2012). In this study, R^2 and adjusted R^2 were 0.9996 and 0.9994, respectively for the regressed models, suggesting a good fit. The small value of coefficient of variation (CV) (2.17) and the non-significant lack of fit (0.0826) revealed that the experimental results were precise and reliable. The ANOVA result was used to indicate that at least one of the parameters of the model could explain the experimental variation for response variables as shown by significant *P* value for the model (Chotphruethipong *et al.*, 2017). Glucose content was highly affected by enzyme level (X_1) and incubation time (X_2) ($P<0.0001$). Interaction between the level of enzyme and incubation time (X_1X_2) was also found ($P<0.0001$). The 3D surface plot (Fig. 23) reflected the influence of various desugarization parameters on the glucose content of resulting treated duck albumen. It was noted that the increase in both variable values caused the decrease in glucose content. The lowest glucose content (9.94±0.91

µg/mL sample) was found when glucose oxidase (35 units) and catalase (875 units)/mL albumen with incubation time of 8 h were used. There is an inverse proportion between the level of enzymes and the time required to remove glucose. Incubation times of 4–6 h are generally practical (Reed and Underkofler, 1966). Sharma *et al.* (2012) reported that reducing sugar of liquid whole hen egg was completely depleted within 12 h by baker's yeast. The result indicated that the chosen range of time and enzyme level was sufficient for the depletion of glucose in duck albumen. After desugarization, the residual glucose concentration must be lower than 0.01 g/L or 10 µg/mL (Lechevalier *et al.*, 2013).

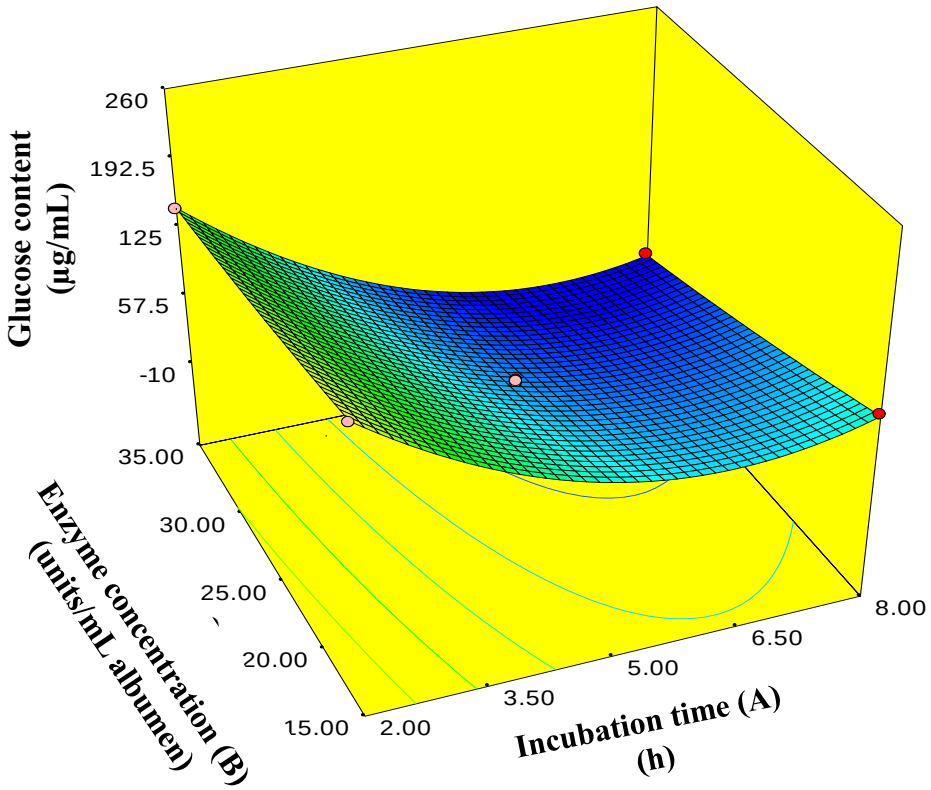

Figure 23. The 3D surface plot of residual glucose content of duck albumen as affected by incubation time (A) and glucose oxidase/catalase concentrations (B). Enzyme unit was expressed in term of glucose oxidase.

Validation of the statistical model and regression equation of optimal condition was mixed enzyme (glucose oxidase 31.24 units and catalase 781 units/mL)

and incubation time of 6.55 h. Under the optimized condition, the predicted and observed values of glucose content were 9.57 and 10.02±0.41 µg/mL, respectively. Similar predicted and observed values (error = 4.54%) demonstrated the acceptability and validity of the statistical model for the optimization of desugarization in duck albumen. Hence, the response surface modelling could be applied effectively to predict the desugarization of duck albumen before dehydration.

6.4.2 Effect of desugarization and drying methods on physical, chemical properties and trypsin inhibitory activity of duck albumen powders

6.4.2.1 Moisture content and water activity

Moisture contents (%) and a_w of duck albumen powders spray-dried with different inlet temperatures (140, 160, and 180 °C) and freeze-dried as affected by prior desugarization are given in Table 13. Overall, lower moisture content was found in the powder from albumen with prior desugarization compared to that of non-desugarized samples, regardless of drying condition. For non-desugarized samples, the remaining sugar was able to bind water. As a result, water had less mobility to evaporate from the powder (Chen and Mujumdar, 2008; Landfeld *et al.*, 2008). The highest moisture content (10.23%) was found in the powder obtained from spray-drying of albumen without prior desugarization with the lowest inlet temperature (140 °C) (140-W0) (P<0.05). The lowest content was found in the powder prepared by freeze-drying of desugarized albumen (P<0.05). Moisture content of duck albumen powder generally decreased with increasing inlet temperatures. Moisture removal rate was higher when the heat of evaporation was increased (Landfeld *et al.*, 2008). The high inlet temperature led to a greater temperature gradient between the atomized feed and the drying air, resulting in a greater driving force for water evaporation (Chuaychan *et al.*, 2017).

a_w values of freeze-dried and spray-dried powder prepared without and with prior desugarization are presented in Table 13. a_w values varied in the range of 0.20–0.48, in which the lowest value was found in freeze-dried powder (P<0.05). It was noted that the sample with prior desugarization had the lower a_w value than non-desugarized counterpart (P<0.05). The a_w decreased when albumen was dried by spray drying at

higher inlet temperature (P<0.05). a_w of powder decreased with increasing spray-drying inlet temperature (P<0.05). The lowest value (0.23) was obtained in spray-dried powder with inlet temperature of 180 °C (P<0.05). However, with inlet temperature of 180 °C, the resulting powder from albumen with and without prior desugarization had no differences in a_w (P>0.05). Koç *et al.* (2011) documented that moisture content and a_w of whole hen egg powder dried at inlet temperature of 180 °C were 2.5% and 0.125, respectively. Overall, high relationship between moisture content and a_w was observed since a_w is the driving force behind water transfer from/to food (Chirife *et al.*, 2006). It is generally accepted that the moisture content and a_w of whole egg powder should be below 5% and 0.40, respectively, in order to ensure stability (Koç *et al.*, 2011; Wu, 2014). The result suggested that higher inlet temperature used for spray-drying resulted in the lower moisture content and a_w of resulting powder. Also, desugarization was a major factor determining the moisture content and a_w of duck egg powder.

6.4.2.2 Surface hydrophobicity

Surface hydrophobicity of duck albumen powder with and without prior desugarization obtained from freeze-drying and spray-drying process under various conditions was monitored (Table 13). In general, freeze-dried powder showed the lower surface hydrophobicity than those obtained from spray-drying process (P<0.05). Sublimation under low pressure induces less heat denaturation than spray-drying (Chen and Mujumdar, 2008). The gradual increase in surface hydrophobicity of spray-dried powders was observed when inlet temperatures increased from 140 °C to 160 °C. The increase in S_0ANS indicated protein conformational change induced by heat. Hydrophobic domains originally buried in the protein core were exposed, thus more readily available for binding with ANS used as the probe (Van der Plancken *et al.*, 2005). After heat treatment of hen egg white protein, surface hydrophobicity was increased at temperatures above 55 °C. This was probably due to the unfolding of heat sensitive proteins (Van der Plancken *et al.*, 2006). Nevertheless, the decrease in S_0ANS was noticeable as albumen was spray-dried at 180 °C. At higher temperature, the interaction of hydrophobic domains via hydrophobic interaction might be enhanced. Consequently, those hydrophobic residues were embedded inside the aggregates as shown by the lowered S_0ANS (Lechevalier *et al.*, 2007). Desugarization showed the

significant influence on surface hydrophobicity. The higher S_0ANS values were found in desugarized powders (P<0.05), irrespective of drying methods or condition. When the conjugate of protein and sugar was formed, the concentration of free amino groups, especially hydrophobic amino acids, decreased as they reacted with reducing group of glucose (Van der Plancken *et al.*, 2006). Glucose with hydroxyl groups, particularly in the form of glycoproteins, could make protein more polar or hydrophilic in nature. Thus, desugarization was found an essential factor affecting surface hydrophobicity of duck albumen powders.

6.4.2.3 Protein pattern

SDS-PAGE patterns of desugarized and non-desugarized duck albumen powders produced by freeze-drying and spray-drying in the presence and absence of β-ME are illustrated in Fig. 24. The most dominant protein was ovalbumin with MW of 46–48 kDa. Under non-reducing condition, ovalbumin (MW of 48 kDa) was dominant protein. Additionally, proteins with MW of 80 and 15 kDa, which were more likely ovotransferrin and lysozyme, respectively, were detected. Ovalbumin (40%) and ovotransferrin (2%) are considered as the main proteins found in duck albumen (Huang and Lin, 2011). Under reducing condition, ovalbumin was also found as predominant. Proteins with MW of 77 and 14 kDa considered as ovotransferrin and lysozyme, respectively, were also observed. Proteins having MW of 185 and 208 kDa were also found. In general, the protein patterns of spray-dried powder and freeze-dried powder were similar. Therefore, drying process had no marked effect on degradation or polymerization of proteins in duck albumen. The unfolded proteins may then undergo aggregation by the interaction of the exposed hydrophobic regions with other unfolded molecules until precipitation occurs (Haque and Adhikari, 2015). However, those weak bonds were destroyed by the chemicals used for electrophoresis such as SDS, etc. Thus, the similar protein patterns were noticeable. Moreover, there was no difference in protein patterns between powder with and without prior desugarization. The result indicated that no dramatic degradation or polymerization of proteins took place in albumen after desugarization.

Table 13. Moisture content, water activity, surface hydrophobicity, solubility, FC, and FS of duck albumen powder as affected by prior desugarization and drying conditions

Drying/ desugarization	Moisture content (%)	Water activity (a_w)	Surface hydrophobicity (S_0ANS)	Solubility (%)	FC (%)	FS_{60} (%)
FD-DE††	1.14±0.01$^{\dagger Bg}$	0.20±0.00^{Bf}	528.77±4.80^{Ac}	96.76±0.63^{Bcd}	46.67±0.00^{*Ad}	46.67±0.00^{Abcd}
FD-W0	2.33±0.03^{Af}	0.22±0.00^{Ae}	464.95±9.97^{Be}	99.18±0.74^{Aa}	41.67±2.36^{Bef}	41.67±2.36^{Bde}
140-DE	5.93±0.40^{Bc}	0.34±0.00^{Bc}	571.90±4.41^{Aab}	96.57±0.20^{Bd}	51.67±2.36^{Ac}	51.67±2.36^{Ab}
140-W0	10.23±0.60^{Aa}	0.48±0.00^{Aa}	526.06±2.06^{Bc}	98.36±0.71^{Aab}	45.00±2.36^{Bde}	45.00±2.36^{Bcd}
160-DE	4.86±0.13^{Bd}	0.24±0.00^{Bd}	582.18±2.78^{Aa}	95.64±0.47^{Bd}	71.67±2.36^{Aa}	71.67±2.36^{Aa}
160-W0	8.42±0.09^{Ab}	0.45±0.00^{Ab}	530.33±4.9^{Bc}	97.86± 0.84^{Abc}	46.67±0.00^{Bd}	46.67±0.00^{Bbcd}
180-DE	2.51±0.06^{Af}	0.23±0.00^{Ae}	557.39±8.49^{Ab}	81.52±1.11^{Bf}	60.00±0.00^{Ab}	48.33±2.36^{Abc}
180-W0	3.69±0.27^{Ae}	0.23±0.00^{Ae}	500.04±13.49^{Bd}	94.26 ± 0.52^{Ae}	40.00±0.00^{Bf}	38.33±2.36^{Be}

†Mean±SD (n=3). Different lowercase letters in the same column indicate significant differences (P<0.05). Different uppercase letters within the same drying condition in the same column indicate significant differences (P<0.05).

††140, 160, 180: inlet temperatures for spray-drying; DE: desugarization; W0: without desugarization; FD: freeze-drying.

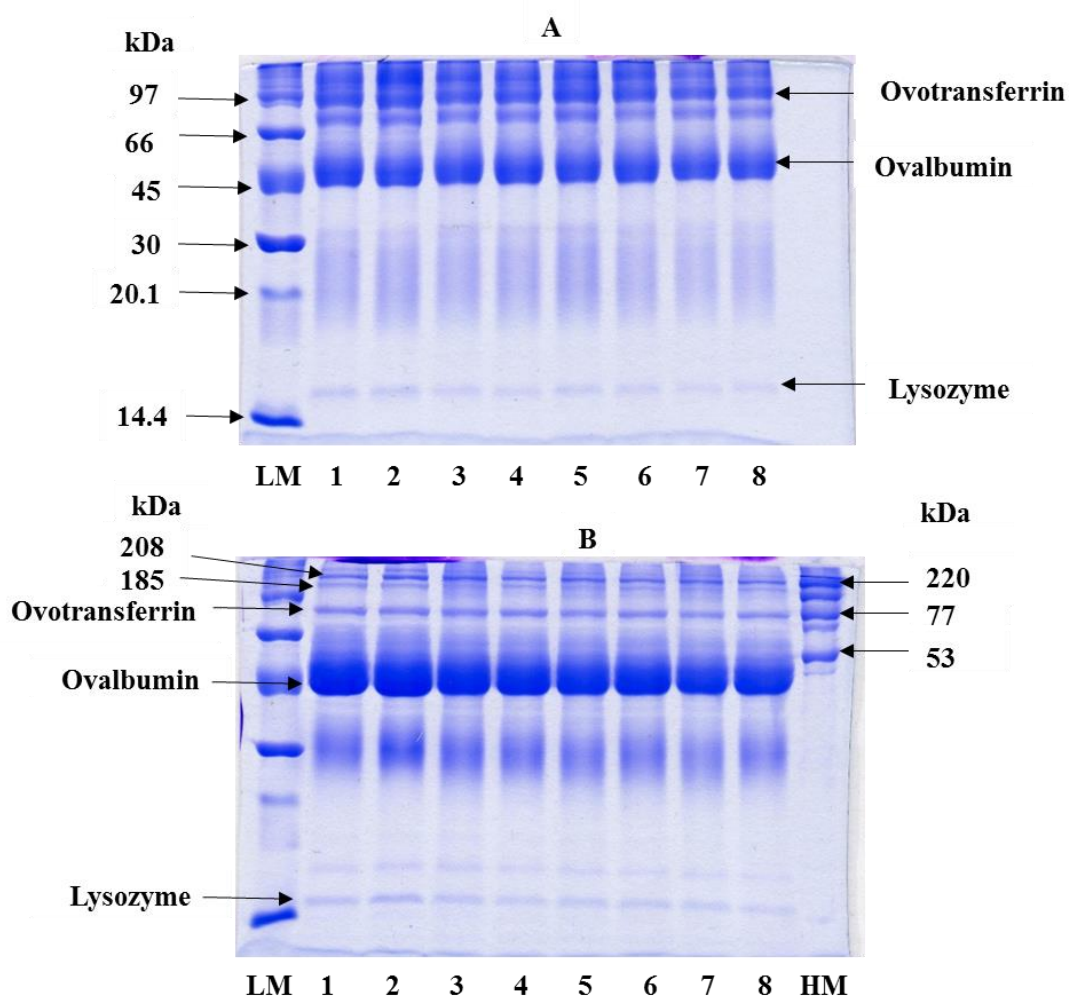

Figure 24. Protein patterns (SDS-PAGE) of duck albumen powder under non-reducing (A) and reducing (B) condition as affected by desugarization and drying conditions. LM: low molecular weight marker; 1: Freeze-drying without prior desugarization; 2: Freeze drying with prior desugarization; 3: spray-drying with inlet temperature of 140 °C without prior desugarization; 4: spray-drying with inlet temperature of 140 °C with prior desugarization; 5: spray-drying with inlet temperature of 160 °C without prior desugarization; 6: spray-drying with inlet temperature of 160 °C with prior desugarization; 7: spray-drying with inlet temperature of 180 °C without prior desugarization; 8: spray-drying with inlet temperature of 180 °C with prior desugarization; HM: high molecular weight marker.

6.4.2.4 Change in trypsin inhibitory activity

The changes in total trypsin inhibitory activity in albumen powders as a function of desugarization and drying conditions are depicted in Fig. 25. Trypsin inhibitory activity in freshly laid duck egg decreased after either freeze-drying or spray-drying (P<0.05), in which 76%, 73%, 71%, and 70% of activities were retained in albumen powders without prior desugarization obtained from freeze-drying and spray-drying at 140, 160, and 180 °C, respectively. The proteins, especially protease inhibitors, underwent partial denaturation when subjected to drying. Ovotransferrin, lysozyme, ovalbumin, and trypsin inhibitors lost their inhibitory activities when dried (Hammershøj *et al.*, 2004). It was noted that freeze-dried powder also showed the decreased trypsin inhibitory activity. However, the activity was slightly higher than those of samples subjected to spray-drying. Higher temperature used for spray-drying, even for a short time, more likely showed negative effect on trypsin inhibitor. The reduction in antiprotease activity correlates with the deterioration of some pivotal proteins such as clusterin, lysozyme, ovotransferrin, ovomucoid, and ovoinhibitor (Quan and Benjakul, 2018b). Thus, the higher trypsin inhibitory activity was observed in freeze-dried powers than spray-dried counterpart (P<0.05). When sublimation took place, water in albumen passed from the solid to the vapor phase, while leaving the other components unchanged. Freeze-dried albumen powder contained more thermo-sensitive compounds than those prepared by heat drying (Dang *et al.*, 2017; Jesús *et al.*, 2013). Additionally, prior desugarization also affected the trypsin inhibitory activity, in which lower value was attained (P<0.05). It was found that the remaining activities of 76%, 72%, 70%, and 69% were obtained in powders from desugarized albumen, subjected to freeze-drying and spray-drying at 140, 160, and 180 °C, respectively. For albumen without prior desugarization, protein–sugar conjugates produced via the Maillard reaction plausibly enhanced the stabilization of protein during drying process (Akhtar and Ding, 2017). The remaining trypsin inhibitor activity in duck albumen powder could play a role in controlling proteolysis in surimi or other meat mediated by endogenous protease. Therefore, drying methods and conditions as well as prior desugarization had the impact on trypsin inhibitors in duck albumen powders. This was

more likely related to the ability of controlling protein degradation in surimi or other products containing proteases.

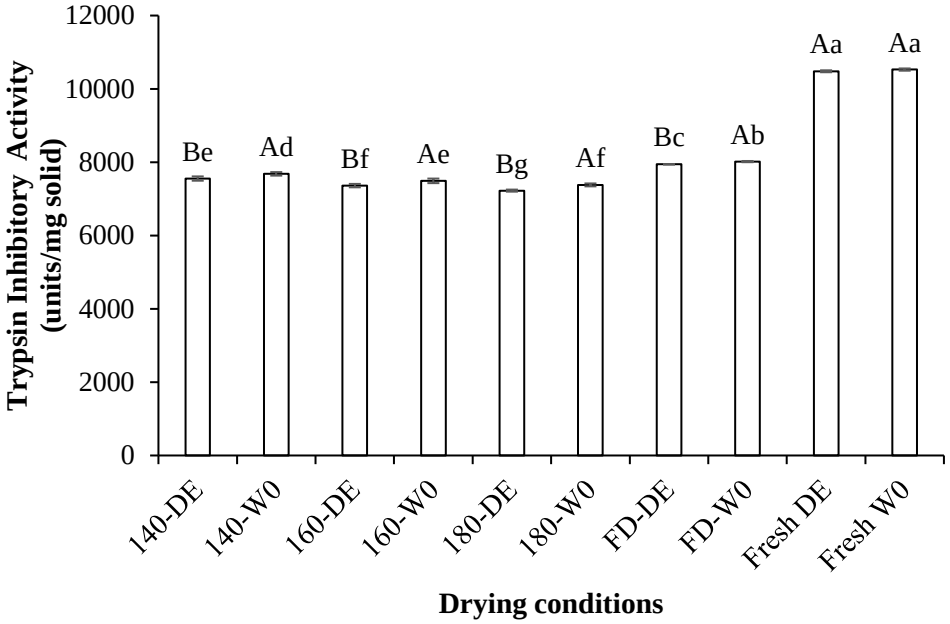

Figure 25. Effect of prior desugarization and drying conditions on trypsin inhibitory activity of duck albumen powder. Mean±SD (n=3). Different lowercase letters on the bars indicate significant differences (P<0.05). Different uppercase letters within the same drying condition on the bars indicate significant differences (P<0.05). 140, 160, 180: inlet temperatures for spray-drying; DE: desugarization; W0: without prior desugarization; FD: freeze-drying; Fresh: fresh albumen.

6.4.2.5 Color and browning index

Color of powders prepared from duck albumen by freeze-drying and spray-drying with and without prior desugarization is shown in Table 14. The highest lightness (L*) was found in freeze-dried albumen powder. L*-value was decreased in albumen subjected to spray drying (P<0.05). The lowest L*-value was obtained in powders produced by spray-drying with the inlet temperature of 180 °C (P<0.05). The decrease in lightness was more pronounced in albumen without prior desugarization. This was caused by the presence of glucose, which could undergo Maillard reaction during the spray-drying, particularly at high temperature. Duck albumen contained glucose (4294.87±47.17 µg/mL). Reducing sugar could react with amino group in the

egg white protein via Maillard reaction (Hammershøj *et al.*, 2004; Katekhong and Charoenrein, 2018). Nevertheless, desugarization could improve the lightness of powders produced by spray-drying process at all inlet temperatures used. The higher lightness of spray-dried powders was observed in desugarized albumen (P<0.05) at all inlet temperatures used. However, there was no difference in L*-value between freeze-dried powder without and with desugarization (P>0.05). The result suggested that prior desugarization could retard the browning reaction, especially when spray-drying was implemented. The increases in both a*- and b*-values of albumen powders were observed when inlet temperatures for spray-drying increased (P<0.05). The a*- and b*-values indicate redness/greenness and yellowness/blueness, respectively. The increase in redness and yellowness indicated that browning reaction took place in albumen. The lowest a*- and b*-values were found in freeze-dried powder. For ΔE*, the lower value was obtained for powders prepared from albumen with prior desugarization for all drying processes used. Increases in ΔE* values were obtained in the powder prepared by spray-drying with increasing inlet temperatures. The increase in ΔE* was coincidental with the increases in the browning index of albumen powder. The browning index of albumen powders prepared with different conditions as indicated by A_{294} and A_{420} is presented in Table 14. Among all the samples, those prepared at inlet temperature of 180 °C showed the highest A_{294} and A_{420}, especially those without prior desugarization. The lower A_{294} and A_{420} were noticeable in all desugarized powders, regardless of drying processes (P<0.05). However, there was no difference in A_{420} between freeze-dried powder without and with prior desugarization (P>0.05). Increases in a*, b*, and ΔE* were found when samples were spray-dried at higher temperature. The higher A_{294} of sample prepared by spray-drying, compared to freeze-drying, suggested the higher rate of intermediate product formation. Additionally, the increase in A_{420} was used as an indicator for development of brown products in the final stage of the browning reaction (Chuaychan *et al.*, 2017). Thus, desugarization and drying methods significantly affected the color of albumen powder. Desugarization could reduce the undesirable browning reaction taken place in duck albumen powder produced by spray-drying.

Table 14. Color values and browning index of duck albumen powder as affected by prior desugarization and drying conditions

Drying/ desugarization	L^*	a^*	b^*	ΔE^*	Browning index	
					294 nm	420 nm
FD-DE††	97.93±0.10$^{\dagger Aa}$	-1.83±0.05^{Bg}	0.97±0.06^{Bh}	0.00±0.00^{Bf}	12.92±0.04^{Bh}	0.68±0.00^{Af}
FD-W0	97.34±0.15^{Aab}	-1.31±0.03^{Af}	1.85±0.05^{Ag}	1.17±0.04^{Ae}	13.05±0.01^{Ag}	0.69±0.00^{Af}
140-DE	97.26±0.03^{Ab}	-0.87±0.01^{Be}	2.02±0.03^{Bf}	1.54±0.02^{Be}	13.10±0.01^{Bf}	0.70±0.01^{Bf}
140-W0	96.68±0.14^{Bc}	-0.64±0.03^{Ad}	2.64±0.02^{Ae}	2.40±0.06^{Ad}	13.25±0.01^{Ae}	0.78±0.00^{Ad}
160-DE	96.55±0.02^{Ac}	-0.65±0.04^{Bd}	2.75±0.04^{Bd}	2.55±0.02^{Bd}	13.29±0.00^{Bd}	0.73±0.00^{Be}
160-W0	95.24±0.05^{Bd}	-0.45±0.02^{Ab}	3.22±0.02^{Ac}	3.77±0.05^{Ab}	13.41±0.02^{Ac}	0.95±0.01^{Ac}
180-DE	96.52±0.02^{Ac}	-0.56±0.04^{Bc}	3.27±0.01^{Bb}	2.99±0.02^{Bc}	13.35±0.00^{Bb}	1.02±0.00^{Bb}
180-W0	93.37±0.89^{Be}	0.08±0.01^{Aa}	3.56±0.02^{Aa}	5.60±0.03^{Aa}	13.67±0.01^{Aa}	1.15±0.04^{Aa}

†Mean ± SD (n=3). Different lowercase letters in the same column indicate significant differences (P<0.05). Different uppercase letters within the same drying condition in the same column indicate significant differences (P<0.05).

††140, 160, 180: inlet temperatures for spray-drying; DE: desugarization; W0: without desugarization; FD: freeze-drying.

6.4.2.6 Microstructure

Scanning electron micrographs of albumen powder produced by freeze-drying and spray-drying at inlet temperature of 160 °C without and with prior desugarization are illustrated in Fig. 26. Freeze and spray dried powders were different in morphology and size. Freeze-dried powder showed broken glass-like or flake-like structure. On the other hand, spray-dried powder had round-shaped particles, in which some had smooth surface and some contained dents. In general, particles of freeze-dried powder were larger than those of spray-dried counterpart. During freeze drying, frozen liquid was not broken into droplets or the surface topology was not markedly altered during the sublimation. Conversely, atomization during spray-drying resulted in smaller droplets, and these atomized droplets were often spherical in shape (Chen *et al.*, 2012). Similar finding was reported by Chen *et al.* (2012) for morphology of freeze-dried and spray-dried hen albumen hydrolysate. There was no difference in microstructure of freeze-dried and spray-dried powders without and with prior desugarization. Therefore, drying methods played a profound role in microstructure, while desugarization had no impact on microstructure of duck albumen powder.

6.4.3 Effect of desugarization and drying methods on functional properties of duck albumen powders

6.4.3.1 Solubility

Solubility of duck albumen powder dried by different drying methods without and with prior desugarization is presented in Table 13. The higher solubility was found in freeze-dried albumen powder than spray-dried counterpart ($P<0.05$), irrespective of prior desugarization. The solubility of the spray-dried samples slightly decreased, particularly at high temperature (180 °C) ($P<0.05$), regardless of desugarization. This result was in accordance with the study of Van der Plancken *et al.* (2005). However, Katekhong and Charoenrein (2018) reported that there was no significant difference between the solubility of hen egg albumen dried at 140, 160, and 180 °C. The protein solubility progressively decreased with increasing drying temperatures. This was plausibly associated with a substantial increase of surface hydrophobicity up to 160 °C. The decrease at 180 °C was mostly related with the

formation of aggregate stabilized by hydrophobic–hydrophobic interaction. Moreover, the result showed that the solubility was significantly affected by desugarization (P<0.05). The loss in solubility was obtained in desugarized powders. With removal of glucose, the hydrophobic residues were proportionally increased and hydrophobic–hydrophobic interaction was enhanced. As a result, larger aggregates were formed, thus lowering the solubility. This was agreement with the result of Katekhong and Charoenrein (2017) who reported that lower glass transition temperature (T*g*) value was observed in freeze-dried powder than hot air drying. T*g* values increased with increasing sample molecular weight. Thus, this result suggested that drying sample at high temperature more likely induced the aggregation of proteins to a higher extent than at lower temperature. Solubility is one of the most important functional properties of proteins. It determines other functional properties, such as emulsifying and foaming properties (Chen *et al.*, 2012). Thus, drying methods or conditions as well as pre-desugarization before drying affected solubility of duck albumen powder.

6.4.3.2 FC and FS

FC and FS of duck albumen powder without and with prior desugarization prepared by freeze-drying and spray-drying are shown in Table 13. Desugarization intensively improved FC and FS of albumen powders dried by either freeze-drying or spray-drying (P<0.05). After desugarization, FC increased by 12%, 15%, 54%, and 50% in powder produced by freeze drying and spray-drying at 140, 160, and 180 °C, respectively. In the absence of sugar, proteins with increased surface hydrophobicity could migrate to air interface effectively. Proteins could adsorb more easily at the interface because of their higher flexibility (Lechevalier *et al.*, 2007). Spray-drying also improved foaming properties of duck albumen powders, especially when spray-dried with inlet temperature of 160 °C. Such an increase was more pronounced when pre-desugarization was employed before drying. This was plausibly associated with the exposed hydrophobic domains formed during drying at 160 °C, which was related with the increased surface activities (Liu *et al.*, 2015). However, both FS and FC were decreased gradually as albumen was subjected to spray-drying with inlet temperature of 180 °C. This result was in agreement with the decrease in surface hydrophobicity, mostly caused by the formation of aggregates. This also coincided with the decreased

solubility. Lechevalier *et al.* (2007) documented that prior desugarization improved foaming properties of spray dried hen albumen powder. During this step, protein interfacial unfolding was favored in the absence of sugar. Moreover, the FC of hen albumen dried at 120 °C was 4-times higher than that of the fresh sample. However, at high temperature (125 °C), a reduction in FC was observed (Ayadi *et al.*, 2008). The result indicated that foaming property of albumen powder, as indicated by both FC and FS, was affected by the method of drying and prior desugarization. Thus, desugarization prior to spray-drying at inlet temperature of 160 °C could improve both FC and FS of duck albumen powder.

6.5 Conclusion

Physiochemical and foaming properties as well as protease inhibitor were affected by desugarization and drying conditions. The optimization of desugarization for duck albumen was as follows: glucose oxidase/catalase concentration of 31.24/781 units/mL albumen and incubation time of 6.55 h. The whiteness and foaming property of powder could be improved by desugarization. Spray-drying of desugarized albumen with the inlet temperature of 160 °C yielded the powder with high foaming property, which could be used as foaming agent and had the moisture content and a_w of 4.86% and 0.24, respectively. Drying method was the major factor affecting the microstructure of albumen powder. Spray-drying reduced trypsin inhibitory activity in duck albumen to some extent. However, the remaining trypsin inhibitors of albumen powder could prevent proteolysis, thus strengthening the gel of surimi or other meat products.

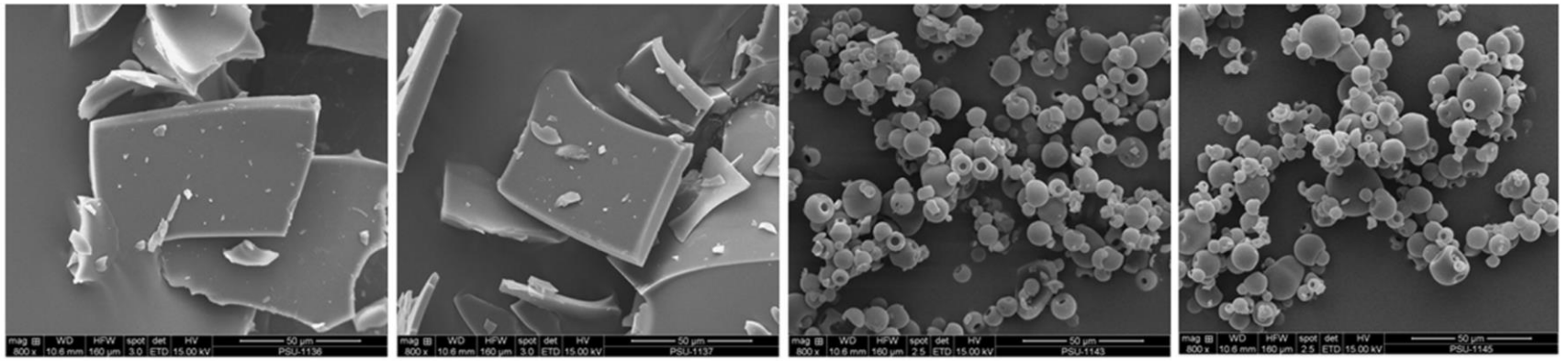

Figure 26. Scanning electron microscopic photograph of duck albumen powder prepared by spray- and freeze-drying without and with prior desugarization. 160: Spray-drying with the inlet temperatures of 160 °C; FD: Freeze-drying; DE: with prior desugarization; W0: without prior desugarization. Magnification: 800×. Scale bar = 50 μm.

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CHAPTER 7
GELLING PROPERTIES OF DUCK ALBUMEN POWDER AS AFFECTED BY DESUGARIZATION AND DRYING CONDITIONS

7.1 Abstract

The effects of desugarization using glucose oxidase/catalase and spray-drying conditions on gelling properties of duck albumen powder were studied. Gelling temperatures increased as spray drying inlet temperatures (140–180 °C) were increased (P<0.05). ΔE*, a*-, and b*- values of gel increased but L* and whiteness decreased when higher spray-drying temperatures were used (P<0.05). However, whiteness and lightness of albumen gel were drastically increased after desugarization (P<0.05). Texture profile analysis showed that hardness, springiness, gumminess, and chewiness of gel decreased with increasing spray-drying temperatures. Moreover, gel of freeze-dried desugarized albumen powder had higher hardness, springiness, gumminess, and chewiness than that of spray-dried non-desugarized counterpart (P<0.05). Albumen gel prepared from desugarized albumen powder showed the compact network with more connectivity and smaller voids than that from non-desugarized one as visualized by scanning electron microscopy, regardless of drying conditions. Prior desugarization could lower browning and increased gelling properties of duck albumen powder. Higher spray drying inlet temperature generally exhibited the adverse effect on properties of resulting albumen powder. Both desugarization and drying conditions had the profound influence on characteristics and textural property of duck egg albumen.

7.2 Introduction

Duck egg has been consumed in many Asian countries and serves as an important source of nutrients in many developing countries. Thailand is one of the top 10 countries for egg production in the world (Huang and Lin, 2011). Egg albumen has been widely utilized as a food ingredient with a diversity of bioactive components (Ren *et al.*, 2010). Heat-induced gelation is one of important functional properties of egg albumen, thus widening its uses in food systems (Matsudomi *et al.*, 2002). Recently, Quan and Benjakul (2018b) reported that duck albumen containing high protease

inhibitory activity could increase gel strength of sardine surimi. Thus, duck albumen was suggested to replace hen albumen to alleviate gel weakening caused by endogenous proteases (Quan and Benjakul, 2018a). In general, egg albumen has been commonly manufactured into different forms, such as powder, pasteurized liquid albumen, and so on. (Trziszka *et al.*, 2013). Nowadays, dried egg products are being used extensively instead of liquid forms in several food industries. This is due to higher storage stability, lower space, and cost for transportation and storage (Katekhong and Charoenrein, 2017). Technically, browning is the main drawback of spray-dried egg albumen. Such a discoloration, mediated by Maillard reaction in the presence of glucose (4 g/L) in albumen, could be triggered during high-temperature processing, especially spray drying. In addition, glucose–cephalin reaction (a reaction between a cephalin amino group and aldehydes of glucose) was responsible for off-flavor development during dehydration and storage (Lechevalier *et al.*, 2013; Wu, 2014). To conquer this problem, prior desugarization is a crucial step before dehydration to perfectly obtain white powder for albumen powder manufacture. Glucose oxidase and catalase have been widely used to convert glucose into gluconic acid. It was demonstrated as the best method to prepare dehydrated Egyptian egg albumen, compared to the use of bacteria and yeast (Darwish and Sadek, 1978; Lechevalier *et al.*, 2013). In addition, enzymatic method has been implemented in industrial scale prior to spray drying of hen egg albumen (Lechevalier *et al.*, 2007). Nevertheless, hen albumen powder desugarized by baker's yeast showed the lower gel firmness than non-desugarized counterpart (Lee and Chen, 1999). Spray drying is the most popular drying technology used for production of hen egg albumen powder (Wu, 2014). Ma *et al.* (2013) documented that the optimum spray-drying conditions included spraying flow of 22 mL/min, feeding temperature of 39.8 °C and inlet-air temperature of 178.2 °C. Spray drying generally affected solubility, interfacial properties, and gelling properties of spray-dried hen albumen powder (Ayadi *et al.*, 2008; Hammershøj *et al.*, 2004; Katekhong and Charoenrein, 2018). Due to the heat sensitivity of egg white proteins, the temperature control of all drying methods is crucial. It is necessary to preserve the native characteristics of egg white proteins, which includes the ability to gel with heat and the production of stable foams (Phillips and Williams, 2011).

However, no information on desugarization and spray-drying conditions on gelling properties of duck egg albumen powder prepared by different drying methods have been reported. Therefore, the objective of this study was to investigate the impact of prior desugarization and spray-drying inlet temperatures on color and properties of gels from duck albumen powders.

7.3 Materials and methods

7.3.1 Chemicals/enzymes

All chemicals used in this study were of analytical grade. Glutaraldehyde, sodium phosphate, and ethanol were obtained from Merck (Darmstadt, Germany). Mixed enzymes containing glucose oxidase (12,000 units) and catalase (300,000 units) were purchased from Megazyme U.C. (Wicklow, Ireland). Before use, 20 mL of 200 Mm Tris–HCl buffer (pH 7.6) were added into the enzyme powder, in which the resulting solution contained glucose oxidase and catalase of 12,000 and 300,000 units/mL, respectively.

7.3.2 Preparation of desugarized albumen

Fresh duck eggs within 24 h after laying were collected from a farm house in Kantang, Trang province. The eggs were broken and albumen was separated from egg yolk manually. The chalazae were removed. To ensure a uniformity of samples, egg albumen was homogenized at a speed of 4,000 rpm for 3 min using an IKA homogenizer Model T 25 D (IKA-Werke GmbH & Co. KG, Staufen, Germany). Albumen solution (300 mL) was mixed with glucose oxidase (31.24 units/mL) and catalase (781 units/mL). The mixture was incubated at 30 °C for 6.55 h at pH 7.6 in the presence of 0.1 mL of 30% H_2O_2. Such a condition was optimized for desugarization using response surface methodology, based on study from chapter 5.

7.3.3 Effects of spray-drying conditions on gelling properties of duck albumen powders

7.3.3.1 Drying of desugarized and non-desugarized duck albumen

Desugarized and non-desugarized fresh duck albumens (moisture content of 87.45% ± 0.03 and 87.49% ± 0.11, respectively) were subjected to freeze drying and

spray drying. For freeze drying, both albumen samples were frozen at −40 °C for 8 h. The frozen samples were subjected to sublimation using a freeze-dryer (CoolSafe 55, Scan-Laf A/S, Lynge, Denmark) at −50 °C for 96 h. Freeze-dried duck albumen powder obtained was placed in polyethylene bag and kept at −20 °C until use but not longer than 2 months.

To prepare spray-dried duck albumen, both desugarized and non-desugarized duck egg albumens were diluted with distilled water at a ratio of 1:2 (v/v) before drying to reduce viscosity of samples. Spray drying was carried out using a spray dryer (LabPlant SD-06 Basic, North Yorkshire, UK) equipped with a spry-drying chamber having the dimensions of 500 mm height and 210 mm diameter and a spray nozzle type of two-liquid nozzle (0.5 mm in size). A cyclone separator, a hot-air blower, and an exhaust blower were equipped. Albumen solutions were fed by a peristaltic pump at 10 mL/min into the chamber and atomized by hot air (air velocity of 2 m/s) from the blower in a downward current flow mode. Parameters tested included various inlet temperatures (140, 160, and 180 °C), while outlet temperature was maintained at about 85–95 °C. The atomizing pressure was 2.8 bars. All dried albumen samples obtained were subjected to analyses.

7.3.3.2 Dynamic rheological property and gelling temperature of albumen solution

Rheological behavior of the sol–gel transition of duck albumen solutions (10% solid content, w/v) was determined using a RheoStress RS 1 rheometer (HAAKE, Karlsruhe, Germany). The measuring geometry used was a stainless steel 60-mm-diameter parallel plate and the gap was set at 1.0 mm. The albumen solution (2.9 mL) was loaded on the Peltier plate and equilibrated to 25 °C before measurements. The sample was covered with a transparent sample hood to prevent water evaporation during the experiment. For temperature sweep, the albumen samples were heated to 90 °C at a rate of 3 °C/min according to the method of Eleya and Gunasekaran (2002). The measurements were conducted at a constant frequency of 1 Hz, and a constant applied stress of 3 Pa. The elastic modulus G′ and the loss (viscous) modulus G″ were recorded. The gelling temperatures were defined as the temperature where tan δ (G″/G′) was 1 or δ was 45°.

7.3.3.3 Textural property, color, and microstructure of albumen gels

7.3.3.3.1 Preparation of gel

Gels of albumen powders were prepared following the method of Mmadi *et al.* (2014). Albumen solutions (10% of solid content, w/v) were prepared using distilled water as a diluent. The pH of solutions was adjusted to pH 9.0 by either 1N NaOH or 1N HCl and stirred gently. Then, the solution was transferred into a casing (diameter of 25 mm). Both ends were sealed tightly, and gels were heated at 90 °C for 30 min. Thereafter, the gels were cooled immediately using iced water and kept at 4 °C overnight. Finally, gel samples were cut into cylinders (diameter 25 mm, height 30 mm) prior to analyses.

7.3.3.3.2 Texture profile analysis

Texture profile analysis of gels was performed using a texture analyzer (Model TA-XT2i, Stable Micro System, Surrey, UK). The samples were compressed twice to 40% of their original height with a compression cylindrical aluminum probe (50 mm diameter). Force-distance deformation curve was recorded at a cross head speed of 3 mm/s and the recording speed was 3 mm/s. Hardness, springiness, cohesiveness, chewiness, and gumminess were evaluated using the MicroStable software version 6 (Surrey, UK).

7.3.3.3.3 Determination of color

The color of gel samples was determined by a colorimeter (ColorFlex, HunterLab Reston, Virginia) and reported in CIE system. L^*, a^*, b^*, and ΔE^*, represent lightness, redness/greenness, yellowness/blueness, and total difference of color, respectively. The whiteness of gel was calculated (Kaewmanee *et al.*, 2011) using the following equation:

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

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

where ΔL^*, Δa^*, Δb^* are the difference between color parameters of the samples and those of the desugarized albumen produced by freeze drying (L^* = 73.84, a^* = - 5.23, b^* = -13.28).

7.3.3.3.4 Determination of microstructure

Microstructure of albumen gels was visualized by a scanning electron microscopy as described by Kaewmanee *et al.* (2011). Albumen gels were cut into small pieces (1×1×1 mm^3). Samples were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. Then, fixed samples were rinsed with distilled water for 1 h. Subsequently, samples were dehydrated using ethanol with various concentrations (25, 50, 70, 80, 90, and 100%) for 15 min at each concentration. The dehydrated samples were subjected to critical point drying. The samples were coated with 100% gold (Sputter Coater SPI-Module, West Chester, Pennsylvania). The gel microstructure was visualized by a scanning electron microscope (Quanta 400, FEI, Czech Republic).

7.3.4 Statistical analysis

All the experiments were conducted in triplicate using three lots of samples. Data were presented as mean value with ± SD. One-way analysis of variance was performed. The Duncan's multiple range test and paired-samples T-test were carried out to determine the significant difference between samples at $P<0.05$ level using the statistical program (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois).

7.4 Results and discussion

7.4.1 Rheology and gelling temperature of duck albumen as influenced by prior desugarization and drying conditions

Figure 27 shows the development of storage (or elastic) modulus (G′) and loss (or viscous) modulus (G″) as a function of temperature sweep for solutions of duck albumen powder as affected by desugarization and drying conditions. Upon heating, G′ remained unchanged until temperature reached 75 °C. Thereafter, G′ increased rapidly, indicating a typical transition from liquid-like to solid (gel)-like state at high temperature. It was observed that all the samples showed a gel-like behavior, in which G′ was higher than G″. Overall, duck albumen solutions underwent thermal gelation,

regardless of desugarization and drying conditions. Nevertheless, the higher G′ and G″ were found in the solution of freeze-dried albumen powder than those of spray dried counterpart, irrespective of desugarization. In general, G′ and G″ of the solutions of spray-dried albumen decreased as inlet temperatures were increased. The lowest G′ and G″ values were found in the sample spray-dried at high inlet temperature (180 °C). With increasing temperatures, the conformational changes of proteins took place, in which buried hydrophobic groups were exposed. Subsequent aggregation via hydrophobic–hydrophobic interaction was enhanced. In addition, other bonds, especially disulfide bond, were formed. Such a large aggregate lost their solubility, leading to the poor gelation. With the same drying method used, solutions of albumen with prior desugarization showed the higher G′ and G″ than those from non-desugarized powder. Sugar retained in albumen might undergo glycation with amino acid groups, especially at high temperature for spray drying. Temperature is one of the prime factors affecting Maillard reaction (Chuaychan *et al.*, 2017). Cross-linking via Maillard reaction induced aggregation, causing the loss in solubility as well as gelation. Thus, desugarization of albumen prior to drying could help improve gelling property as evidence by the increased G′ and G″ values.

Gelling temperatures of solutions from duck albumen powder prepared by spray drying at different inlet temperatures (140, 160, and 180 °C) and freeze drying as affected by prior desugarization are presented in Table 15. Gelling point or gelling time can be detected when G′ becomes greater than G″ (the crossover method) (Gunasekaran and Ak, 2000). Changes in the phase angle have been used to monitor the thermal transitions of albumen solution. The gelling temperature of all solutions of albumen powders increased as inlet temperature of spray drying increased (P<0.05). However, there was no difference in gelling temperature of powder dried without and with prior desugarization (P>0.05) when the same drying condition was implemented. The lowest gelling temperatures (69 °C) were found in the albumen powder prepared by freeze drying (P<0.05). Highest gelling temperature was obtained in sample spray-dried at inlet temperature of 180 °C (P<0.05). Nevertheless, there were no differences in gelling temperature between spray-dried powders with inlet temperatures of 140 and 160 °C (P<0.05). Gel formation of hen albumen started at 71–74 °C when the rates of heating

0.5 or 1 °C/min were used. The gel formation is measured as an increase in rigidity and a change from viscous to viscoelastic properties (Zayas, 1997). The increase in gelling temperature might be related to excessive protein denaturation during spray drying, particularly at high inlet temperature. Katekhong and Charoenrein (2018) reported partial loss of protein structure of hen egg albumen spray-dried at higher temperature. The unfolded proteins might undergo aggregation by the interaction of the exposed hydrophobic regions with other unfolded molecules until precipitation occurred (Haque and Adhikari, 2015). With non-ordered protein coagulation, heating at higher temperatures was required to dissociate the coagulate, in which solubilized proteins could form the initial network for gel development (Raikos *et al.*, 2007). For freeze drying, it basically utilizes the mechanism of ice sublimation under low pressure and induces less heat denaturation than spray drying (Chen and Mujumdar, 2008). Less denatured proteins required less heat for complete disassembly of aggregate prior to gelation. Raikos *et al.* (2007) reported that increased gelling temperature of hen egg protein added with salt and sugar was caused by the unordered aggregation of egg proteins. Therefore, drying condition showed the direct impact on gelling temperature of duck albumen, while prior desugarization had no effect on gelling temperature.

7.4.2 Texture, color, and microstructure of duck albumen gels as influenced by prior desugarization and drying conditions

7.4.2.1 Texture profile analysis

Texture profiles of duck albumen gel from freeze-dried and spray dried powder prepared without and with prior desugarization are presented in Table 15. Among all samples, gel from freeze-dried powder showed the highest hardness (P<0.05). It was noted that the sample with prior desugarization had the higher hardness than non-desugarized counterpart (P<0.05). Hardness of gel decreased with increasing spray-drying inlet temperature (P<0.05). The hardness decreased when albumen was dried by spray drying at higher inlet temperature (P<0.05). The lowest value (6.25 N) was obtained in spray-dried powder with inlet temperature of 180 °C (P<0.05). Nevertheless, prior desugarization could improve the hardness of gel produced by spray drying at all inlet temperatures. The hardness was decreased markedly in all albumen dried using spray drying but the decrease in hardness was lowered by prior

desugarization. Hardness is related to the strength of gel structure under compression and is measured from the peak force during the first compression cycle (Chandra and Shamasundar, 2015). The thermal gelation of egg white proteins is generally regulated by different types of intermolecular interactions such as hydrogen bonding, electrostatic and hydrophobic interactions, Vander Waals forces, and covalent bonds (disulfide cross-links) (Eleya and Gunasekaran, 2002). The excessive aggregation and coagulation induced during spray drying at high temperature could impede hydrogen bonding and hydrophobic interactions or other bonds during thermal gelation of albumen powder (Ayadi *et al.*, 2008). Moreover, desugarization had a beneficial effect on hardness of duck albumen by preventing the occurrence of Maillard reaction during drying. Therefore, protein cross-links mediated by covalent bonds or other bonds were limited (Katekhong and Charoenrein, 2017). The slight increase in cohesiveness of duck albumen gel was observed when inlet temperatures of spray drying were increased (Table 15). Lower cohesiveness was attained in gel prepared from freeze-dried powder than those of gel from spray-dried powders ($P<0.05$). Moreover, higher cohesiveness was found in the samples without prior desugarization, compared with those of desugarized samples, regardless of inlet temperatures used ($P<0.05$). Cohesiveness is a parameter to measure of the difficult level in breaking down the internal structure of gel (Singh and Benjakul, 2017). Springiness of albumen gel slightly decreased as spray-drying inlet temperatures were increased ($P<0.05$). However, no difference in springiness was obtained in all gels of albumen powders without and with prior desugarization ($P>0.05$). Springiness is related to the height that gel recovers during the end of the first bite and the start of the second bite. If springiness is high, it requires more mastication energy in the mouth (Chandra and Shamasundar, 2015). The decreased springiness was concomitant with the decreases in hardness.

Gumminess and chewiness of gels from albumen powders as influenced by prior desugarization and different drying conditions are shown in Table 15. The highest gumminess and chewiness were observed in gels from freeze-dried powders. Prior desugarization resulted in the increases in both attributes tested. In general, these values decreased continuously as spray-drying inlet temperatures increased ($P<0.05$). The higher gumminess and chewiness values were found in gels from desugarized powders

(P<0.05) under the same drying condition used. The changes in gumminess and chewiness were in correlation with those of hardness. Gumminess and chewiness were calculated based on hardness, which suggests the resistance to compression force (Yilmaz *et al.*, 2012). Those changes were mainly mediated by the protein conformational changes induced by heat. Therefore, textural properties of gels of duck albumen were affected by spray-drying inlet temperature as well as prior desugarization.

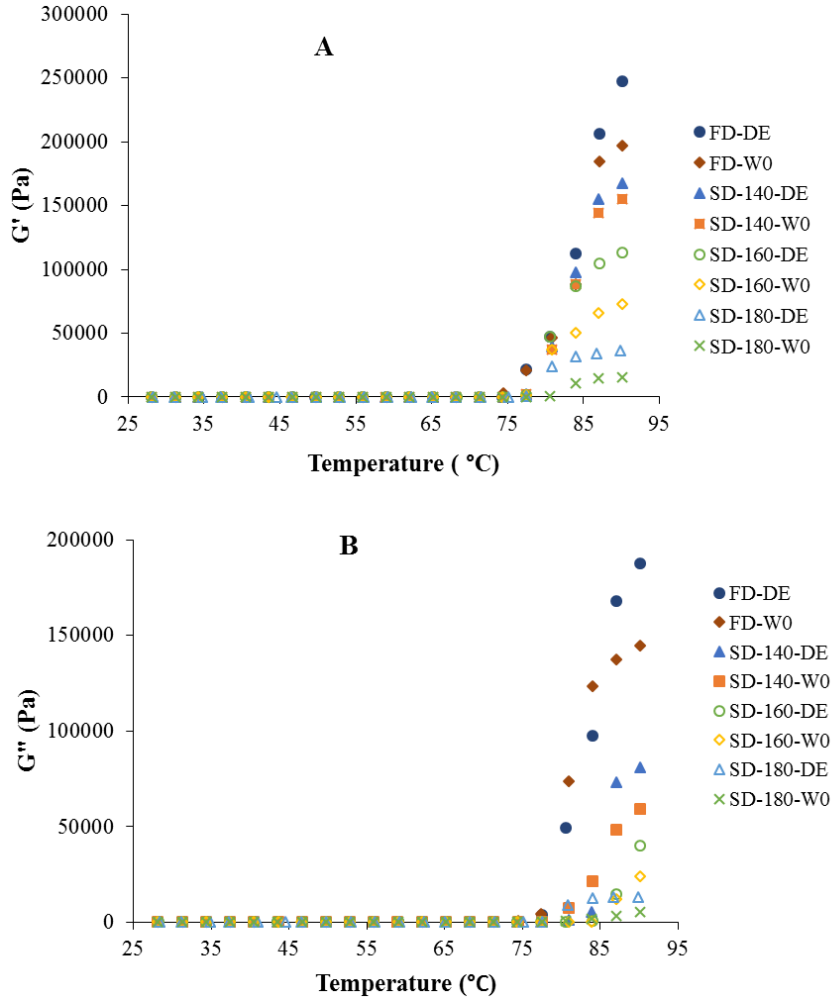

Figure 27. Effect of prior desugarization and drying conditions on G′ (A) and G″ (B) of solution of spray-dried and freeze-dried albumen powders. 140, 160, 180: inlet temperatures (°C) for spray drying; DE: with prior desugarization; W0: without prior desugarization; FD: freeze drying; SD: spray drying

Table 15. Gelling temperature and texture profile analysis of duck albumen gel as affected by prior desugarization and drying conditions

Drying conditions	Gelling temperature (°C)	Hardness (N)	Cohesiveness	Springiness (cm)	Gumminess (N)	Chewiness (N.cm)
FD-DE††	69.29±0.03$^{\dagger Ac}$	19.48±0.12^{Aa}	0.88±0.01^{Be}	1.02±0.02^{Aa}	16.49±0.10^{Aa}	16.74±0.23^{Aa}
FD-W0	69.40±0.17^{Ac}	17.49±0.10^{Bb}	0.91±0.00^{Ad}	1.00±0.02^{Aab}	15.79±0.17^{Bb}	15.70±0.53^{Bb}
SD-140-DE	70.51±0.20^{Ab}	15.23±0.12^{Ac}	0.93±0.02^{Bc}	0.99±0.00^{Aabc}	14.46±0.10^{Ac}	14.38±0.26^{Ac}
SD-140-W0	70.49±0.20^{Ab}	7.91±0.07^{Bf}	1.01±0.01^{Aa}	0.99±0.01^{Aabc}	7.67±0.03^{Bf}	7.32±0.04^{Bf}
SD-160-DE	70.58±0.01^{Ab}	14.61±0.18^{Ad}	0.94±0.00^{Bb}	0.98±0.01^{Abc}	12.98±0.07^{Ad}	12.77±0.12^{Ad}
SD-160-W0	70.61±0.39^{Ab}	7.18±0.09^{Bg}	1.01±0.14^{Aa}	0.98±0.01^{Ac}	6.25±0.08^{Bg}	6.13±0.09^{Bg}
SD-180-DE	72.27±0.30^{Aa}	12.18±0.10^{Ae}	0.94±0.00^{Bb}	0.98±0.01^{Abc}	11.68±0.33^{Ae}	11.49±0.34^{Ae}
SD-180-W0	72.36±0.21^{Aa}	6.25±0.11^{Bh}	1.00±0.01^{Aa}	0.98±0.00^{Ac}	6.19±0.17^{Bg}	6.04±0.14^{Bg}

†Mean±SD (n=3). Different lowercase superscripts (a-h) in the same column indicate significant differences (P<0.05). Different uppercase superscripts (A-B) within the same drying condition in the same column indicate significant differences (P<0.05).

††140, 160, 180: inlet temperatures (C) for spray drying; DE: prior desugarization; W0: without prior desugarization; FD: freeze drying; SD: spray drying.

7.4.2.2 Color

Color of duck albumen gels prepared from freeze-dried and spray dried powder without and with prior desugarization is illustrated in Table 16 and Fig. 28. The highest lightness (L^*) and whiteness were attained in gel from freeze-dried albumen powder. L^*- and whiteness value were gradually decreased after albumen was dried by spray drying, especially at higher inlet temperatures ($P<0.05$). The lowest L^*- and whiteness were obtained in gel from powders produced by spray drying with inlet temperature of 180 °C ($P<0.05$). The decreases in lightness and whiteness were more pronounced in gels from albumen without prior desugarization ($P<0.05$). This was mainly caused by the presence of glucose, which could undergo Maillard reaction during the spray drying, particularly at high temperature. Duck albumen contained glucose (4,295 ± 47 μg/mL). Reducing sugar could react with amino group albumen proteins via Maillard reaction (Hammershøj *et al.*, 2004; Katekhong and Charoenrein, 2018). Nevertheless, prior desugarization could improve the lightness and whiteness of gels from spray-dried powders prepared at all inlet temperatures. The result suggested that prior desugarization could retard the browning reaction, especially when spray drying was implemented at high temperatures. The increases in both a^*- and b^*- values, representing redness/greenness and yellowness/blueness, respectively, of albumen gels were observed when inlet temperatures of spray drying were increased ($P<0.05$). The increase in redness and yellowness indicated that browning reaction occurred in albumen. The lowest a^*- and b^*- values were found in gel from freeze-dried powders. For ΔE^*, the lower value was obtained for gels prepared from albumen powders with prior desugarization for all drying processes used ($P<0.05$). Increase in ΔE^* of gels was noticeable in the samples from powder prepared by spray drying with increasing inlet temperatures. The decrease in ΔE^* was coincidental with the increases in a^*- and b^*- values and the decrease in L^* and whiteness.

Figure 28 shows the photos of duck albumen gels prepared from freeze- and spray-dried powders without and with prior desugarization. It appears that the gel from freeze-dried powder with desugarization had the most whitish color. Gel became less whitish when powder was prepared from albumen without prior desugarization. Spray-drying process affected the gel color significantly. The higher inlet temperatures

adversely affected the color of gels, in which yellow color became more intense. This result was in accordance with study of Ayadi *et al.* (2008). These color changes could be because Maillard reaction products were formed. Therefore, the whiter color was obviously observed in gels from desugarized powders, when drying was carried out at low temperature. The depletion of glucose drastically lowered Maillard reaction products in dried albumen. These results indicated that prior desugarization could improve the color and appearance of albumen gel produced by spray drying.

Table 16. Color values and whiteness of duck albumen gel affected by desugarization and drying conditions

Drying conditions	*L**	*a**	*b**	ΔE^*	Whiteness
FD-DE[††]	73.84±1.30[†Aa]	-5.23±0.03[Bf]	-13.28±0.17[Bh]	0.00±0.00[Ah]	70.20±1.14[Aa]
FD-W0	70.53±0.65[Bb]	-5.08±0.01[Ae]	-11.96±0.30[Ag]	3.58±0.66[Bg]	67.78±0.56[Bb]
140-DE	53.07±0.69[Ac]	-5.03±0.01[Be]	-11.55±0.22[Bf]	20.85±0.70[Af]	51.40±0.62[Ac]
140-W0	31.95±0.57[Bf]	-4.43±0.05[Ad]	-0.68±0.03[Ac]	43.75±0.54[Bc]	31.80±0.57[Bf]
160-DE	51.27±0.41[Ad]	-4.43±0.03[Bd]	-10.57±0.19[Be]	22.75±0.41[Ae]	49.93±0.42[Ad]
160-W0	29.55±0.17[Bg]	-4.05±0.04[Ab]	0.66±0.10[Ab]	46.44±0.16[Bb]	29.43±0.17[Bg]
180-DE	44.48±0.30[Ae]	-4.23±0.05[Bc]	-9.58±0.15[Bd]	29.61±0.29[Ad]	43.50±0.30[Ae]
180-W0	27.87±0.79[Bh]	-3.84±0.07[Aa]	3.09±0.04[Aa]	48.82±0.75[Ba]	27.70±0.79[Bh]

[†]Mean±SD (n=3). Different lowercase superscripts (a-h) in the same column indicate significant differences (P<0.05). Different uppercase superscripts (A-B) within the same drying condition in the same column indicate significant differences (P<0.05).

[††]140, 160, and 180: inlet temperatures (°C) for spray drying; DE: prior desugarization; W0: without prior desugarization; FD: freeze drying; SD: spray drying.

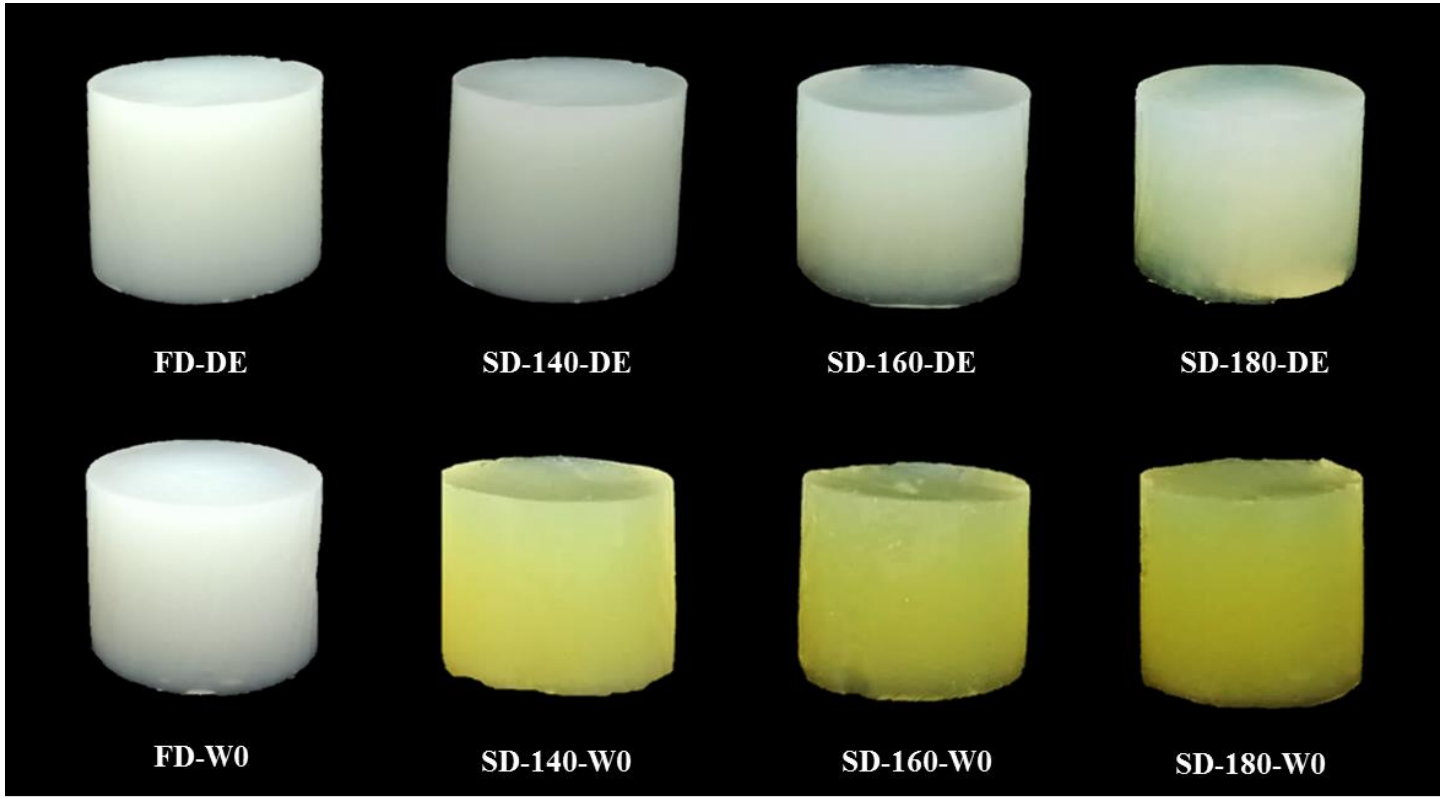

Figure 28. Photographs of duck albumen gels as affected by prior desugarization and drying conditions. 140, 160, and 180: inlet temperatures (°C) for spray drying; DE: prior desugarization; W0: without prior desugarization; FD: freeze drying; SD: spray drying

7.4.2.3 Microstructure

Scanning electron micrographs of albumen gel produced by freeze drying and spray drying without and with prior desugarization are illustrated in Fig. 29. Gel produced from freeze-dried powder had the denser network with the small voids, compared with those of spray-dried powders. For gel from spray-dried powder, the larger voids were observed when the inlet temperatures were increased. Gel network became much coarser with less connectivity when the inlet temperature of 180 °C was used for spray drying. Regardless of drying conditions, the marked difference in gel network was noted between the sample with and without prior desugarization. Gel from non-desugarized albumen had less compactness with more opened structure. The network contained larger voids with less connectivity. On the other hand, more compact and denser networks were observed in gel prepared from desugarized powder. The result reconfirmed that protein aggregation or coagulation during spray drying, especially at high inlet temperatures could not form the fine network. The unfolded protein underwent aggregation via hydrophobic–hydrophobic interaction or disulfide bond during drying at high temperature (Haque and Adhikari, 2015). As a consequence, non-ordered protein coagulation gel with less connectivity was developed. The loss of dense and less compact structure of gel coincided with the decreased gel strength of protein network (Table 15). Moreover, prior desugarization could enhance gel strength of albumen by preventing Maillard reaction. The resulting gels were more uniform in networks with minimal pore size. The result revealed that both prior desugarization and drying conditions had the significant impact on structure of gel from duck albumen powder.

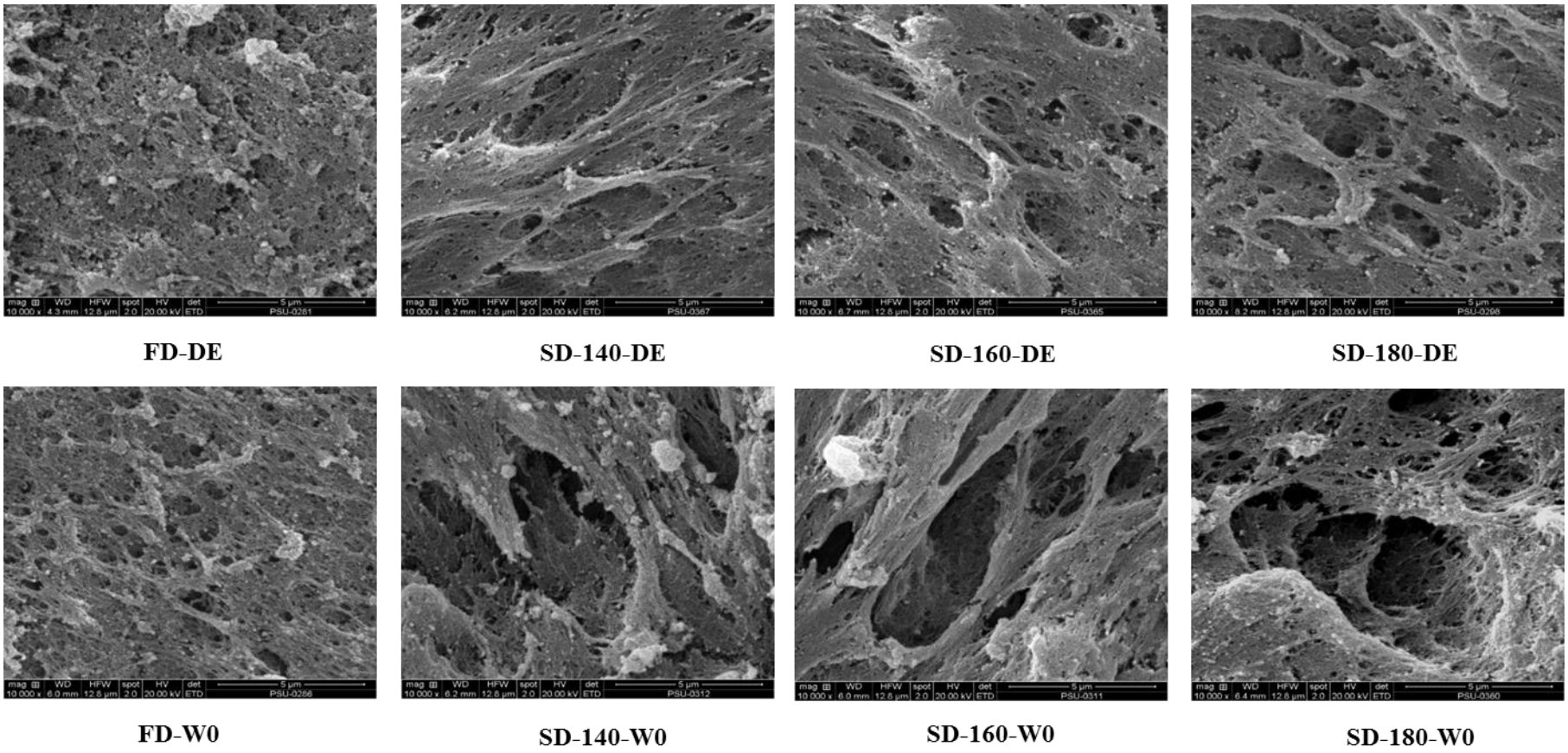

Figure 29. Scanning electron microscopic photograph of gel from duck albumen prepared by spray drying and freeze drying without and with prior desugarization. 140, 160, and 180: inlet temperatures (°C) for spray drying; DE: with prior desugarization; W0: without prior desugarization; FD: freeze drying; SD: spray drying. Magnification: ×10,000. Scale bar = 5 μm.

7.5 Conclusion

Desugarization and drying conditions significantly affected the gelling temperature, color and gelling properties of duck albumen powder. Gel strength, lightness, and whiteness of gel were reduced when albumen was subjected to spray drying. Prior desugarization could markedly improve the whiteness and gelling properties of powder, in which higher gel strength with coincidental increases in hardness, gumminess, and chewiness were attained. Prior desugarization could yield the gel with more compact and denser network. Moreover, freeze drying rendered the gel having the denser network with smaller voids than spray drying. Thus, prior desugarization was a crucial step for duck egg powder production to ensure the quality of powders. Inlet temperature of 160 °C was recommended for spray drying of desugarized duck albumen.

7.6 References

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CHAPTER 8
IMPACT OF SALTED DUCK EGG ALBUMEN POWDER ON PROTEOLYSIS AND GELLING PROPERTIES OF SARDINE SURIMI

8.1 Abstract

The influences of salted duck egg albumen powder (SDEAP) as salt replacer at various levels (0.5–2.5%) on autolysis and gelling properties of sardine surimi were investigated. SDEAP had high salt (33.67%) and protein contents (64.52%) with trypsin inhibitory activity of 5,975 units/mg solid. SDEAP was white in color with *L**-value of 96.72. It had low moisture content (3.98%) and water activity (0.38). Autolysis of sardine surimi was drastically inhibited when SDEAP was incorporated with increasing levels as indicated by the more retained myosin heavy chain and the reduced trichloroacetic acid-soluble peptide content. Breaking force and deformation of surimi gel increased, while expressible moisture content decreased as the levels of SDEAP added were increased ($P<0.05$). Gumminess, hardness, chewiness, springiness, and cohesiveness of surimi gels also increased as SDEAP levels increased ($P<0.05$). Lightness and whiteness were higher in all surimi gels incorporated with SDEAP than the control ($P<0.05$). For microstructure, surimi gels incorporated with SDEAP at all levels used had finer gel network with smaller voids and more connectivity than the control gel. Thus, SDEAP could be used as a salt replacer for sardine surimi gel preparation and it could improve the properties of resulting gel.

8.2 Introduction

Salted egg is a popular traditional product in China and some Asian countries. In general, salted egg yolk is higher demand than salted albumen, which is commonly discarded. Albumen of salted duck egg contained 4–7% sodium chloride and had the salty taste (Quan and Benjakul, 2018b). In several traditional Chinese foods, salted duck egg yolk is commonly used as a filling material, for instance glutinous rice dumplings and mooncakes. With increasing demand in aforementioned foods, an increasing amount of salted duck egg albumen considered as waste is produced inevitably (Tan *et al.*, 2016). Thus, salted duck albumen needs to be more exploited, especially as the food

additive. Salted duck albumen was rich in protein and still contained protease inhibitors (Quan and Benjakul, 2018b).

Surimi is a form of wet concentrated fish myofibrillar proteins. First, fish are mechanically deboned and the obtained mince is washed thoroughly to concentrate myofibrillar proteins and simultaneously remove water-soluble proteins (Nopianti *et al.*, 2011). Surimi has been known to exhibit the excellent gel forming ability. Gelation includes dissociation and unfolding of myofibrillar proteins. A three-dimensional gel network is subsequently developed when unfolded proteins undergo aggregation as induced by heat (Petcharat and Benjakul, 2017). Both dark and white fleshed fishes, including fresh water and marine fishes, have commonly been used in surimi production, such as lizardfish (Benjakul *et al.*, 2004), bigeye snapper (Julavittayanukul *et al.*, 2006), tilapia (Rawdkuen *et al.*, 2009), mackerel (Balange & Benjakul, 2009), croaker (Panpipat *et al.*, 2010), Alaska pollock (Yin and Park, 2014), silver carp (Majumdar *et al.*, 2013), and common carp (Ganesh *et al.*, 2006). Sardine (*Sardinella albella*) is one of the pelagic dark-muscled fishes, which has been used as an alternative raw material for surimi production. Generally, those pelagic fishes yield surimi with poor gelling properties because of the large amount of lipids, sarcoplasmic proteins as well as indigenous proteases (Buamard and Benjakul, 2015; Zaghbib *et al.*, 2016). To enhance the gel forming property of surimi, several food grade protease inhibitors including soy protein isolate, egg albumen, chicken and porcine plasma protein, and so on have been studied (Benjakul *et al.*, 2004; Jafarpour *et al.*, 2012; Rawdkuen *et al.*, 2004). Recently, Quan and Benjakul (2018c) reported that duck egg albumen showed high protease inhibitory activity. The addition of duck egg albumen up to 4% increased breaking force and deformation of sardine surimi in a dose-dependent manner. Therefore, duck albumen could be considered as an alternative to hen albumen for alleviating gel softening caused by indigenous proteases from sardine surimi (Quan and Benjakul, 2018a).

For making surimi gel, the certain amount of salt (sodium chloride) must be added in the surimi to solubilize myofibrillar proteins. Basically, salt at 2.5–3% total weight is used for surimi gel preparation (Tolano-Villaverde *et al.*, 2016). Since salted duck egg albumen had high salt content (33%; dry weight basis), it can be used to

replace the salt, solar salt, or earth salt used in surimi. Tan *et al.* (2016) utilized salted duck egg albumen in yellow alkaline noodle as a substitute of salt. Yellow alkaline noodles added with salted duck egg albumen showed higher protein content and had lighter color, compared to the control. The resulting noodles were sensorially acceptable by panelists. However, no study on the use of salted duck egg albumen in surimi was conducted. Thus, this study aimed to investigate the impact of salted duck egg albumen powder on autolysis and gel property of sardine surimi.

8.3 Materials and methods

8.3.1 Chemicals and materials

8.3.1.1 Chemicals

Analytical grade chemicals were used. Coomassie blue R-250, Folin–Ciocalteu's phenol reagent, sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), β-mercaptoethanol (β-ME), tyrosine, glutaraldehyde, and ethanol were purchased from Merck (Darmstadt, Germany). Molecular protein marker was obtained from GE healthcare UK Limited (Buckinghamshire, UK). Trypsin from bovine pancreas (Type I, ~10,000 BAEE units/mg protein) was purchased from Sigma Chemical Co. (St. Louis, MO).

8.3.1.2 Surimi and salted duck egg albumen

Frozen sardine surimi (AA grade) was procured from Chaicharoen Marine (2002) Co., Ltd. (Pattani, Thailand) and stored at −20 °C until needed for use. Salted duck egg albumen (15-20 days of salting) was obtained from Chao Sua Noi farm (Nakhon Si Thamarat province, Thailand).

8.3.2 Preparation of salted duck egg albumen powder

Salted duck egg albumen powder was prepared using a spray dryer (LabPlant SD-06 Basic, North Yorkshire, England) as outlined by Quan and Benjakul (2019). The inlet and outlet temperatures were set at 160 and 90 °C, respectively, while atomizing pressure was set at 2.80 bar. The powder was collected and kept into polyethylene bag. The salted duck egg albumen powder obtained was named as “SDEAP.”

8.3.3 Chemical compositions and trypsin inhibitory activity of SDEAP

8.3.3.1 Determination of moisture, salt, protein contents, and water activity

Moisture content of SDEAP was measured using an oven method. Salt content was determined by the AOAC method using 0.1M KSCN for titration. Protein content was analyzed using the Kjeldahl method, in which 5.74 was the conversion factor (AOAC, 2000). Water activity (a_w) was measured using a water activity analyzer (4TEV Aqualab, Decagon Devices, WA).

8.3.3.2 Determination of trypsin inhibitory activity

Trypsin inhibitory activity was analyzed as described by Benjakul *et al.* (2001). The activity was expressed as units/g solid.

8.3.4 Study on autolysis inhibition of sardine surimi by SDEAP

The procedure tailored by Benjakul *et al.* (2001) was adopted for autolysis study. First, SDEAP was added to surimi at different levels (Table 17) and mixed thoroughly. Thereafter, the mixtures (3 g) were placed in a beaker (50 mL) and covered with aluminum foil to prevent evaporation. Subsequently, the mixtures were incubated for 60 min at 60 °C by immersing the beaker in a water bath (Memmert WB 14, GmbH + Co. KG, Schwabach, Germany). After incubation, the mixtures were added with 27 mL of cold 5% TCA (2–4 °C) to terminate the reaction. Then, the mixtures were homogenized at 4,000 rpm for 3 min using an IKA homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). The homogenates were centrifuged at 8,000 ×*g* for 15 min using a centrifuge Model Allegra 25R (Beckman Coulter, Palo Alto, CA). TCA-soluble peptide content (TCA-SPC) expressed as μmole tyrosine equivalent/g sample was determined in the supernatant by the Lowry method (Lowry *et al.*, 1951).

For another set of samples, after being incubated as described above, 27 mL of 5% SDS solution (85 °C) were used to terminate the reaction and solubilize the proteins. Protein patterns of surimi paste and autolyzed surimi samples were determined as per the method of Laemmli (1970). Sodium dodecyl sulfate polyacrylamide electrophoresis was carried out using 10% running gel and 4% stacking gel. The sample

was mixed with sample buffer (50 mM Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol, 10% β-ME). Sample (15 mg protein) was loaded onto the gel and subjected to electrophoresis at constant current of 15 mA/gel using electrophoresis unit (Miniprotein III; Bio-Rad Laboratories, Richmond, CA). The gels were stained with Coomassie Brilliant Blue R-125 (0.125%) in 25% methanol and 10% acetic acid. Destaining was performed using 40% methanol and 10% acetic acid. Molecular weight (MW) of protein bands was estimated from the plot of MW standards and Rf.

Table 17. Levels of SDEAP and salt added in sardine surimi

Treatments	**Salt (%)**	**Amount of SDEAP (g)**
Control	2.5	0 (0, 0) †
SDEAP-1	2.0	1.72 (0.5, 0.95)
SDEAP-2	1.5	3.45 (1.0, 1.91)
SDEAP-3	1.0	5.17 (1.5, 3.33)
SDEAP-4	0.5	6.89 (2.0, 2.86)
SDEAP-5	0	8.61 (2.5, 4.77)

†Values in parenthesis represent the salt and protein content (%), respectively, obtained from SDEAP in 100 g of surimi paste (moisture content of 80%). SDEAP: salted duck egg albumen powder.

8.3.5 Study on the effect of SDEAP on properties of sardine surimi gels

8.3.5.1 Preparation of sardine surimi gel added with SDEAP at different levels

Surimi was added with salt and SDEAP at different levels (Table 17) to obtain the final salt content of 2.5% in the mixture. The procedure of Buamard and Benjakul (2015) was adopted for the preparation of sardine surimi gel, in which two-step heating was implemented. Surimi paste was set at 40 °C for 30 min, followed by heating at 90 °C for 20 min prior to cooling in iced water. All the resulting gels were analyzed.

8.3.5.2 Determination of properties of sardine surimi gels

8.3.5.2.1 Breaking force and deformation

Breaking force (BF) and deformation force (DF) of surimi gels were determined using a texture analyzer (Model TA-XT2, Stable MicroSystems, Surrey, UK) as described by Buamard and Benjakul (2017).

8.3.5.2.2 Texture profile analysis

Surimi gel was subjected to texture profile analysis using a texture analyzer (Model TA-XT2i, Stable Micro System, Surrey, England) following the method of Singh and Benjakul (2017). Five cylindrical samples were cut into 2.5 cm height and 2.5 cm diameter and equilibrated at room temperature (25–28 °C) before analyses. The samples were compressed twice to 50% of their original height with a compression cylindrical aluminum probe (50 mm diameter). Force distance deformation curve was recorded at a cross head speed of 3 mm/s and the recording speed was 3 mm/s. Hardness, cohesiveness, springiness, gumminess, and chewiness were evaluated using MicroStable software version 6 (Surrey, England).

8.3.5.2.3 Determination of expressible moisture content

Expressible moisture content (EMC) of surimi gels was examined as per the method of Singh and Benjakul (2017). It was expressed as percentage of sample weight.

8.3.5.2.4 Determination of color

The colors of SDEAP and surimi gels were measured following the method of Buamard and Benjakul (2015). L^*, a^*, and b^*, representing lightness, redness/greenness, and yellowness/blueness, respectively, were recorded.

For surimi gel, total difference of color (ΔE^*) was calculated using the following formula:

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

where ΔL^*, Δa^*, Δb^* are the difference between color parameters of the samples and those of control samples (without SDEAP; L^* = 70.26, a^* = −1.59, b^* = 10.19).

The whiteness of gels was calculated as described below:

Whiteness = 100 - $\sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$

8.3.5.2.5 Determination of microstructure

Microstructure of surimi gels was observed by a scanning electron microscopy as outlined by Buamard and Benjakul (2017).

8.3.5.2.6 Sensory evaluation

All the surimi gels were sliced into a bite size (2.50 cm diameter and 1 cm thickness). Samples were placed in plastic cup and encoded randomly (3-digit numbers). Fifty untrained panellists (aged between 23 and 35) who were familiar with surimi products and had no allergy to egg albumen were recruited for sensorial evaluation. The appearance, texture, color, odor, taste, and overall likeness of samples were evaluated using 9-point hedonic scale (Meilgaard *et al.*, 2010).

8.3.6 Statistical analysis

All the experiments were done in triplicate. Mean ± standard deviation was presented for all data. One-way variance of analysis (ANOVA) and the Duncan's multiple range test were performed to analyze the significant difference among the samples at a level of P<0.05 using SPSS version 11.0 (SPSS, Inc., Chicago, IL).

8.4 Results and discussion

8.4.1 Chemical compositions and physical properties of SDEAP

Moisture contents and a_w of SDEAP were 3.83% and 0.38, respectively (Table 18). Moisture contents and a_w of egg powder should be below 5 and 0.40%, respectively, to ensure its stability (Koç *et al.*, 2011). High salt content (33.67%, dry weight basis) of SDEAP indicated that it could be a promising source of salt for uses in some food products, especially surimi gels. SDEAP also possessed high protein content

(64.52%, dry weight basis). SDEAP also contained serine protease inhibitors (5,975 units/mg solid). SDEAP could therefore prevent proteolysis in surimi, thus alleviating gel softening induced by indigenous proteases (Klomklao *et al.*, 2016). SDEAP had white color. *L**, *a**-, and *b**- values were 96.72, −0.49, and 3.11, respectively. This might be due to low glucose content in liquid salted duck egg albumen (285.73 ± 12.80 μg/mL). As a result, Maillard reaction (a reaction between carbonyl group of glucose and cephalin amino group) during spray drying at high temperature was negligible (Quan and Benjakul, 2019). Thus, non-enzymatic browning reaction products were negligible in SDEAP. The result suggested that SDEAP with high salt and protein contents as well as trypsin inhibitory activity could be used as potential salt replacer and binder in surimi for improvement of gelling properties, particularly via inhibiting the proteolysis.

Table 18. Chemical compositions, trypsin inhibitory activity, and color of SDEAP

Chemical compositions and physical property	Values
Moisture content (%)	3.83±0.15[†]
Salt content (%, dry basis)	33.67±0.33
Protein content (%, dry basis)	64.52±0.95
Water activity (a_w)	0.38±0.01
Trypsin inhibitory activity (units/mg solid)	5,675±91.98
Color	
*L**	96.72±0.13
*a**	-0.49±0.02
*b**	3.11±0.04

[†]Mean±SD (*n*=3)

8.4.2 Effect of SDEAP at different levels on autolysis of sardine surimi

8.4.2.1 TCA-soluble peptide content

Autolysis of sardine surimi added without and with SDEAP at various levels as indicated by TCA-SPC is presented in Fig. 30. TCA-SPC has been used to represent degradation products liberated under heat setting and thermal gelation of surimi. The

phenomenon was mediated by indigenous proteases at high temperature used for heat induced gelation process (Klomklao *et al.*, 2016). In general, TCA-SPC was gradually decreased when the levels of SDEAP in surimi paste were increased (P<0.05). The sample without SDEAP (control) showed the highest TCA-SPC, while sample added with 8.61 g SDEAP/100 g surimi (SDEAP-5) had the lowest value (P<0.05). The decrease in TCA-SPC in surimi added with SDEAP was mainly due to inhibitory activity of SDEAP. Egg albumen generally consists of several protease inhibitors named ovoinhibitor, ovomucoid, ovomacroglobulin, and so on, which are proficient in inhibiting serine proteases (Jitesh *et al.*, 2011). However, duck egg ovostatin (ovomacroglobulin) is able to inhibit both serine protease and metalloprotease (Hu *et al.*, 2016). Quan and Benjakul (2018a) documented that duck albumen effectively inhibited autolysis in sardine surimi as compared to hen albumen. This result suggested that SDEAP could be used to prevent autolysis mediated by indigenous proteases.

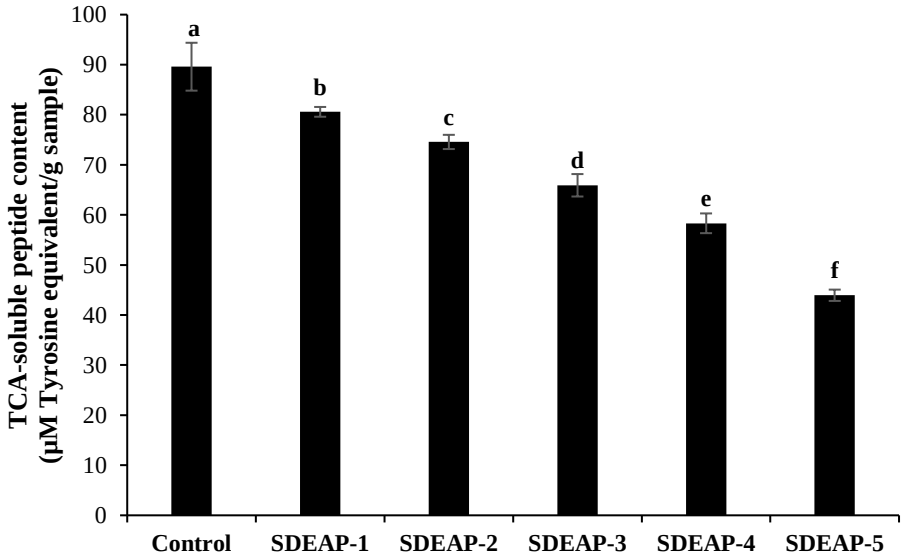

Figure 30. TCA-soluble peptide content of sardine surimi incubated at 60 °C for 60 min in the absence and presence of SDEAP at different levels. Mean±SD (n=3). Different lowercase letters on the bar indicate significant differences (P<0.05).

8.4.2.2 Protein patterns

Protein patterns of sardine surimi in the absence and presence of SDEAP at different levels autolyzed at 60 °C for 60 min are presented in Fig. 31. Myosin heavy chain (MHC), tropomyosin (TM), and actin (AC) were the main proteins in sardine surimi. MHC band intensity decreased notably after incubation at 60 °C in the control sample. The degradation of MHC was triggered by indigenous proteases, which were activated at 55–60 °C (Benjakul *et al.*, 2004). The band intensity of MHC was lowest in surimi without SDEAP. However, more increasingly retained band intensity of MHC was observed when SDEAP at higher amount was incorporated into surimi. This finding was in line with the lower TCA-SPC when SDEAP at higher levels was added. Egg albumen has been used widely for inhibition of autolysis of lizardfish surimi (Benjakul *et al.*, 2004; Yongsawatdigul and Piyadhammaviboon, 2004), red tilapia surimi (Duangmal and Taluengphol, 2010), Alaska pollock surimi and Pacific whiting surimi (Hunt *et al.*, 2009). Recently, Quan and Benjakul (2018a) documented that MHC band intensity of sardine surimi was more retained as duck egg albumen was incorporated. In general, AC and TM were found to be more resistant to proteolysis than MHC (Benjakul *et al.*, 2001). Nevertheless, the band intensity of AC with MW of 43–44 kDa was noticeably increased with increasing amount of SDEAP (SDEAP-4 and SDEAP-5 samples). It was reinforced that ovalbumin in duck egg albumen (MW: 44 kDa) contributed to the increase in the aforementioned band intensity (Quan and Benjakul, 2018a). Both AC and ovalbumin appeared as the same protein band.

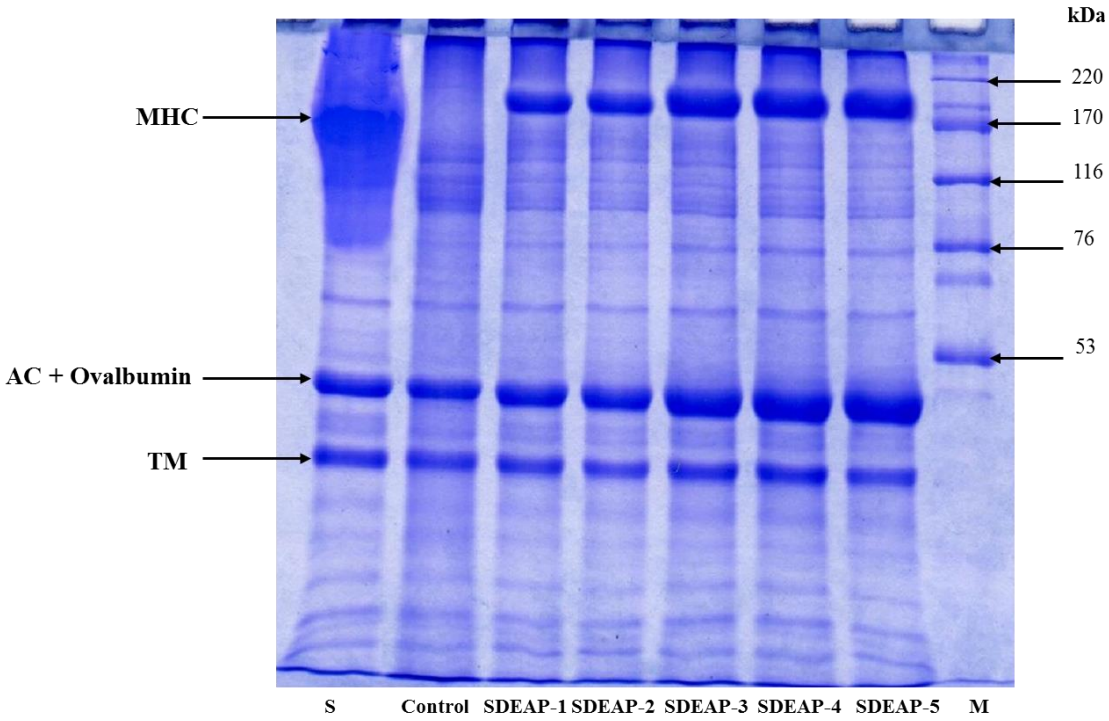

Figure 31. Protein pattern of sardine surimi incubated at 60 °C for 60 min in the absence and presence of SDEAP at different levels. MHC, myosin heavy chain; AC, Actin; TM, tropomyosin; M, high molecular weight marker; S, surimi paste; SDEAP, salted duck egg albumen powder.

8.4.3 Effect of SDEAP on gelling properties of sardine surimi

8.4.3.1 Breaking force and deformation

BF and DF of sardine surimi gels incorporated with SDEAP at different levels are shown in Table 19. These two parameters were lowest in the control gel (without SDEAP; $P<0.05$). The BF of gels continuously increased with increasing amount of SDEAP ($P<0.05$). When SDEAP was added at the highest amount (SDEAP-5), the highest BF (655.36 g) was obtained, in which the value was increased by 86.48%, compared to that of control gel. Similar result was noticeable for DF. However, the DFs between SDEAP-3 and SDEAP-4 samples were not different ($P>0.05$). The increases in BF and DF were in accordance with the lower protein degradation as shown by more retained MHC (Fig. 31) and the decreased TCA-SPC (Fig. 30). MHC is the main protein responsible for the gel formation of surimi, which are susceptible to autolysis by the action of indigenous proteases localized in muscle (Kuhn *et al.*, 2004).

Additionally, egg albumen can be used as a functional binder in fish protein gels (Hunt *et al.*, 2009). Thus, the improved BF and DF of surimi added with SDEAP could be attributed to trypsin inhibitors presenting in SDEAP as well as gelling capacity of albumen (Quan and Benjakul, 2018c). These results suggested that SDEAP had ability in enhancing gel strength and elasticity of sardine surimi.

8.4.3.2 Expressible moisture content

Table 19 shows EMC of surimi gels added with SDEAP at different levels. The EMC of surimi gel decreased as the amount of SDEAP incorporated increased ($P<0.05$). The highest EMC was found in the control (4.45%). The SDEAP-5 sample, in which salt was replaced totally by SDEAP to obtain 2.5% salt, showed the lowest EMC (2.88%). The EMC of SDEAP-5 sample was decreased by 54.51%, compared to that of control sample. However, there was no difference in the EMC between SDEAP-4 and SDEAP-5 samples ($P>0.05$). The reduction of EMC showed that water holding capacity (WHC) of surimi gel was improved with increasing amount of SDEAP used, more likely related to higher amount of water retained in the surimi gel network (Klomklao *et al.*, 2016). Due to the same level of salt among different gel samples, the improved WHC of surimi gel was more likely governed by proteins present in SDEAP. Surimi proteins along with albumen could form the strong gel matrix during heat-induced gelation, which could imbibe more water. Therefore, the higher amount of water was entrapped in the gel network. Egg albumen with high WHC might absorb water effectively, especially during gelation induced by heat (Ren *et al.*, 2010). This result was in line with the increased BF and DF. Quan and Benjakul (2018a) documented that EMC of sardine surimi gel continuously increased when duck albumen was added from 1 to 4%. Thus, SDEAP could be used to substitute salt used for surimi preparation. It also improved WHC of resulting gels.

Table 19. Breaking force, deformation, and expressible moisture content of sardine surimi gels added with SDEAP at different levels

Samples	Breaking force (g)	Deformation (mm)	Expressible moisture content (%)
Control	351.43±3.81$^{\dagger f}$	9.53±0.24^{e}	4.45±0.14^{a}
SDEAP-1	480.20±4.90^{e}	10.23±0.21^{d}	4.07±0.12^{b}
SDEAP-2	520.84±6.11^{d}	10.63±0.24^{c}	3.75±0.10^{c}
SDEAP-3	569.90±2.30^{c}	11.11±0.15^{b}	3.45±0.04^{d}
SDEAP-4	616.02±4.49^{b}	11.32±0.08^{b}	3.06±0.08^{e}
SDEAP-5	655.36±4.62^{a}	11.95±0.15^{a}	2.88±0.14^{e}

†Mean±SD (n=3). Different lowercase superscripts (a-f) in the same column indicate significant differences (P<0.05).

8.4.3.3 Texture profile analysis

The influence of SDEAP at varying amounts on texture profile of surimi gel is presented in Table 20. With increasing amount of SDEAP, surimi gels had the increased hardness (P<0.05). The lowest hardness was noted in the control (59.95 N), while the highest value (108.23 N) was attained in the SDEAP-5 sample (P<0.05), in which the increase by 80.53% was obtained, as compared to that of the control. Hardness is the force required to break the sample into several pieces during the first bite by the molars and is related to the strength of gel structure (Buamard and Benjakul, 2017). The increase in hardness coincided with the increased BF of surimi incorporated with SDEAP. Duck egg albumen rich in protease inhibitors could strengthen gel via preventing MHC from hydrolysis caused by proteases in surimi (Quan and Benjakul, 2018a). Cohesiveness and springiness of surimi gels slightly increased when SDEAP was incorporated (Table 20). Cohesiveness of control gel was 0.84, whereas it was increased to 0.92 in SDEAP-5 sample (P<0.05). For springiness, there was no difference among SDEAP-3,4, and 5 samples (P>0.05). Cohesiveness is related to the work required to overcome the internal bonding of the material (Yuan and Chang, 2007). Springiness is considered as "rubberiness" of gel in the mouth, and it also indicates how well a product physically springs back after deforming during the first compression (Lau *et al.*, 2000; Yilmaz *et al.*, 2012).

Chewiness and gumminess of surimi gels generally increased as the amount of SDEAP increased (P<0.05). The increases in both textural parameters were in line with the increases in hardness. The calculations of chewiness and gumminess were generally based on hardness. Chewiness is the energy for the chewing of the gel to the point that is able to swallow, and gumminess is the energy for the swallowing of semisolid food (Buamard and Benjakul, 2015). Moreover, Quan and Benjakul (2018a) also documented that gumminess, chewiness, hardness, cohesiveness, and springiness of sardine surimi gel increased as the levels of duck egg albumen added were increased up to 4%. This result reconfirmed that SDEAP could be considered as a potential protein additive for enhancing the textural properties of sardine surimi gel.

Table 20. Texture profile of sardine surimi gels added with SDEAP at different levels

Samples	Hardness (N)	Cohesiveness	Springiness (cm)	Gumminess (N)	Chewiness (N.cm)
Control	59.95±0.84$^{\dagger f}$	0.84±0.00^{e}	0.94±0.00^{c}	51.01±0.64^{f}	48.49±0.65^{f}
SDEAP-1	75.78±0.20^{e}	0.85±0.00^{d}	0.95±0.00^{b}	67.35±1.77^{e}	64.40±1.91^{e}
SDEAP-2	82.76±0.83^{d}	0.86±0.00^{c}	0.95±0.00^{b}	71.97±0.75^{d}	68.60±0.66^{d}
SDEAP-3	91.36±0.65^{c}	0.86±0.00^{c}	0.96±0.00^{a}	78.52±0.49^{c}	74.53±0.60^{c}
SDEAP-4	100.04±1.20^{b}	0.87±0.00^{b}	0.96±0.00^{a}	84.91±1.38^{b}	80.25±1.02^{b}
SDEAP-5	108.23±0.85^{a}	0.92±0.00^{a}	0.96±0.00^{a}	90.43±0.90^{a}	86.22±0.53^{a}

†Mean±SD (n=3). Different lowercase superscripts (a-f) in the same column indicate significant differences (P<0.05).

8.4.3.4 Color and whiteness

Lightness (L*) and whiteness of surimi gels slightly increased as the levels of SDEAP increased (P<0.05; Table 21). The increases in L*-value and whiteness of sardine surimi gels were most likely owing to the white color of SDEAP (L*-value = 96.72). The results suggested that the sardine gel possessing gray color became whiter as evidenced by the increased L*-value and whiteness when SDEAP was incorporated. The incorporation of egg albumen was reported to increase L*-value and whiteness of surimi gel from common carp (Jafarpour *et al.*, 2012). Nonetheless, the whiteness of lizardfish surimi was not changed as egg albumen was added (Benjakul *et al.*, 2004). The incorporation of SDEAP in sardine surimi slightly increased a*-value of resulting gel, as compared to control gel (P<0.05). However, no difference in a*-value between SDEAP-4 and SDEAP-5 surimi samples was observed (P>0.05). Conversely, b*-value of surimi gels was slightly decreased when levels of SDEAP added were increased (P<0.05). This finding was in agreement with Jafarpour *et al.* (2012) who documented that the b*-value of common carp surimi gel decreased as the egg albumen concentration increased. In general, surimi gels were more acceptable as they had high L* and whiteness but low b*-values (Duangmal and Taluengphol, 2010). Higher ΔE* values were obtained in the surimi added with SDEAP at higher levels (P<0.05). The increase in ΔE* was coincidental with the increases in the whiteness and L* of surimi

gels. Thus, the addition of SDEAP in sardine surimi played a role in improving the color of sardine surimi gel via increasing the whiteness.

Table 21. Color and whiteness values of sardine surimi gels added with SDEAP at the different levels

Samples	*L**	*a**	*b**	ΔE*	Whiteness
Control	70.26±0.03[†e]	-1.59±0.02[d]	10.19±0.09[a]	0.00±0.00[f]	68.52±0.06[f]
SDEAP-1	70.38±0.02[e]	-1.46±0.02[c]	10.05±0.10[b]	0.20±0.01[e]	68.69±0.04[e]
SDEAP-2	70.83±0.06[d]	-1.32±0.02[b]	9.82±0.02[c]	0.75±0.02[d]	69.19±0.06[d]
SDEAP-3	71.26±0.04[c]	-1.30±0.01[b]	9.74±0.02[d]	1.16±0.02[c]	69.63±0.04[c]
SDEAP-4	71.74±0.03[b]	-1.22± 0.01[a]	9.51±0.02[e]	1.69±0.02[b]	70.16±0.03[b]
SDEAP-5	72.21±0.33[a]	-1.22±0.02[a]	9.49±0.01[e]	2.32±0.02[a]	70.60±0.31[a]

[†]Mean±SD (*n*=3). Different lowercase superscripts (a-f) in the same column indicate significant differences ($P<0.05$).

8.4.3.5 Sensory property

Addition of SDEAP had the impact on sensorial attributes of sardine surimi gels. The addition of SDEAP increased color and appearance likeness of surimi gels ($P<0.05$; Table 22). No difference in appearance likeness score between SDEAP-4 and SDEAP-5 samples ($P>0.05$) was observed. Moreover, SDEAP higher than 1.72/100 g surimi did not affect the color likeness score of resulting gel samples ($P>0.05$). The increases in color and appearance likeness were plausibly due to the white color of SDEAP. The SDEAP also improved the odor likeness of sardine surimi gels ($P<0.05$). The highest score was recorded for SDEAP-3 sample ($P<0.05$). However, this value decreased as the levels of SDEAP increased ($P>0.05$), more likely due to the sulfur smell from SDEAP. It was noted that the gels incorporated with SDEAP at higher levels had the increases in texture and overall likeness scores ($P<0.05$). This was in agreement with the increases in textural properties (Table 20) and BF (Table 19) of gel added with SDEAP. It was noted that the use of SDEAP as a salt replacer in surimi gel did not affect the taste likeness score ($P>0.05$). This was because the same final salt content (2.5%) was used for all samples. Jafarpour *et al.* (2012) documented that hen egg albumen incorporated into common carp surimi gel at 2 and 3% had the improved

likeness score of all sensorial attributes. Thus, the addition of SDEAP, particularly at the level of 2.0/100 g (salt replacement), could improve the likeness of sardine surimi gels.

Table 22. Likeness score of sardine surimi gel added with SDEAP at different levels

Samples	Appearance	Color	Odor	Texture	Taste	Overall
Control	6.75±0.55$^{\dagger b}$	6.95±0.60^{b}	6.70±0.65^{b}	7.10±0.64^{b}	7.40±0.50^{a}	6.80±0.41^{c}
SDEAP-1	7.50±0.51^{b}	7.50±0.61^{b}	7.20±0.62^{b}	7.50±0.51^{b}	7.45±0.68^{a}	7.30±0.65^{c}
SDEAP-2	7.80±0.69^{b}	7.90±0.64^{ab}	7.35±0.49^{ab}	7.60±0.50^{b}	7.45±0.51^{a}	7.55±0.60^{bc}
SDEAP-3	7.80±0.61^{b}	7.85±0.67^{ab}	7.60±0.50^{a}	8.00±0.56^{a}	7.45±0.50^{a}	7.70±0.66^{ab}
SDEAP-4	8.30±0.65^{a}	8.05± 0.60^{a}	7.45±0.51^{ab}	8.05±0.60^{a}	7.45±0.49^{a}	8.05±0.60^{a}
SDEAP-5	8.35±0.67^{a}	8.10±0.67^{a}	7.25±0.44^{ab}	8.05±0.44^{a}	7.40±0.31^{a}	8.00±0.56^{a}

†Mean±SD (n=50). Different lowercase superscripts (a-e) in the same column indicate significant differences (P<0.05).

8.4.3.6 Microstructure

Scanning electron micrographs of sardine surimi gels added without and with SDEAP are depicted in Fig. 32. Control surimi gel had less dense structure. It was indicated by larger voids and cavities in gel network with less connectivity. This was consistent with the lowest BF (Table 19), hardness (Table 20), and the highest EMC (Table 19). However, the denser and finer structure of gels was observed when higher amount of SDEAP was added. The more ordered microstructure with higher interconnection containing less voids and cavities was found. Protease inhibitors could inhibit proteolysis occurring in surimi during heat-induced gelation (Klomklao *et al.*, 2016). As a result, more ordered and finer network mediated by connected protein strands was established. Additionally, albumen in SDEAP might act as an effective protein binder, which promoted the protein–protein interaction, thus strengthening network of resulting surimi gels.

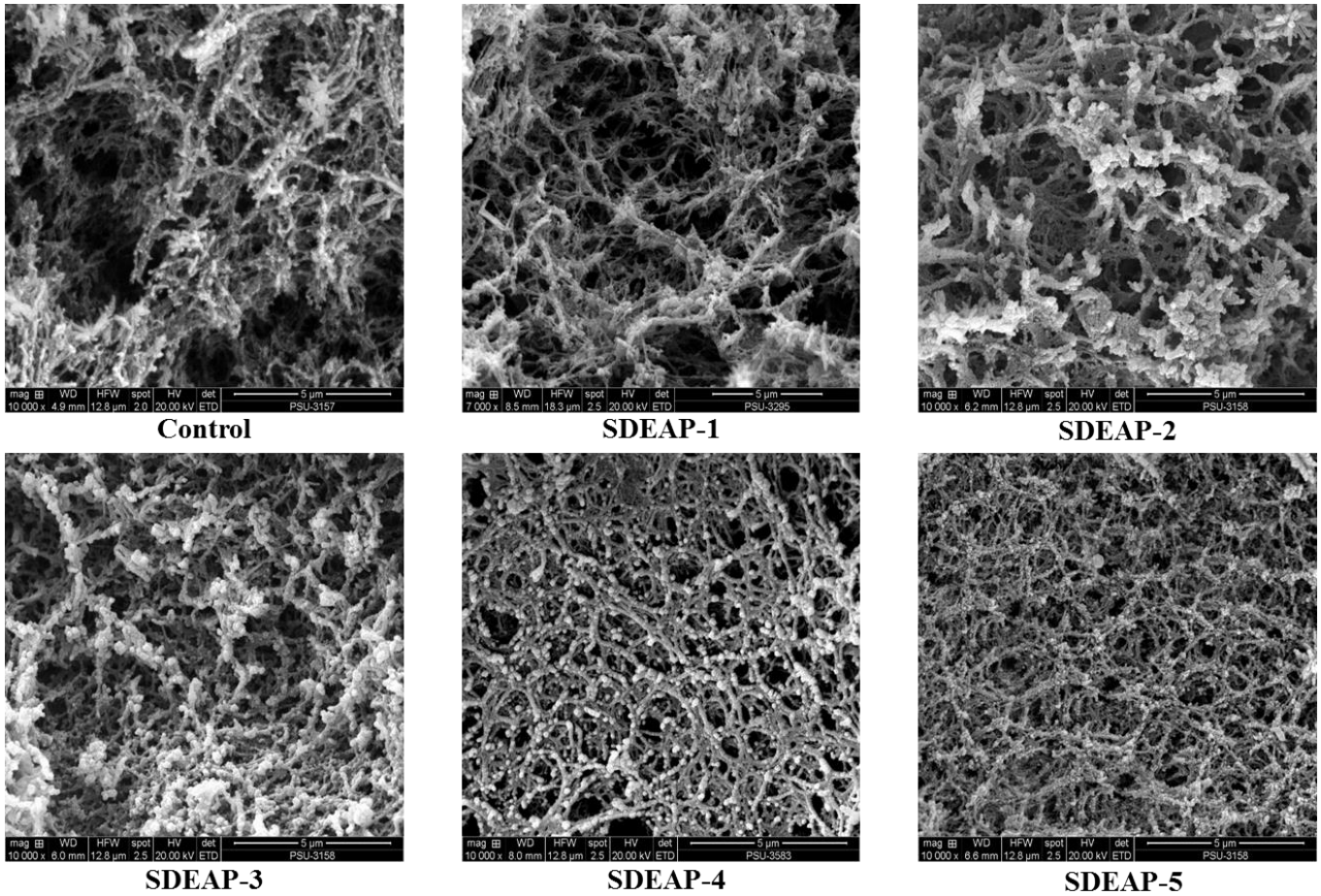

Figure 32. Scanning electron microscopic photographs of sardine surimi gels in the absence and presence of salted duck albumen powder at different levels. Magnification: ×10,000. Scale bar = 5 μm

8.5 Conclusion

SDEAP possessed high salt and protein contents as well as trypsin inhibitory activity, and could be used as a promising salt replacer or protein additive in surimi. Sardine surimi gels added with SDEAP had the lowered proteolysis, in which MHC was more retained. The addition of SDEAP could improve the gel strength and color of surimi. Gelling properties and sensorial characteristics of surimi were enhanced when SDEAP was used as salt replacer. Denser and finer gel structure was visualized in all surimi gels in the presence of SDEAP. Therefore, SDEAP could be used as a promising ingredient to replace salt used in surimi gel preparation. It mainly lowered gel weakening caused by indigenous proteases and strengthened gel network.

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CHAPTER 9

PRODUCTION AND CHARACTERIZATION OF DUCK ALBUMEN HYDROLYSATE USING ENZYMATIC PROCESS

9.1 Abstract

Influences of different ultrasound treatments combined with heat pre-treatment on enzymatic hydrolysis, emulsifying properties and antioxidant activities of hydrolysates from duck egg albumen were studied. Heat pre-treatment at 95 °C for 30 min inhibited both serine and cysteine protease inhibitors effectively. Ultrasonication of heated duck albumen at 60% amplitude for 10 min yielded the highest surface hydrophobicity. Coincidentally, aforementioned pre-treatment rendered the hydrolysate with highest degree of hydrolysis (DH) than other pre-treatments when Alcalase was used. The resulting hydrolysate showed the highest antioxidant activities including DPPH radical and ABTS radical cation scavenging activities and ferric reducing antioxidant power as well as emulsifying properties when hydrolysis time of 90 min was used. The hydrolysate possessed the peptides with molecular weight of 219–255 Da with the highest ABTS radical scavenging activity. Thus, heat pre-treatment, followed by ultrasonication of duck albumen under appropriate condition could increase DH, antioxidant activities and emulsifying properties of duck albumen hydrolysate.

9.2 Introduction

Over the last two decades, enzymatic process is a well-known biotechnological method for improvement of chemical, physical and functional properties of natural egg albumen protein (Garcés-Rimón *et al.*, 2016). Protein hydrolysate has higher digestibility and is easier to solubilize in water. Allergy can be reduced as compared with the native egg proteins (Jovanović *et al.*, 2016). As a nutritious and inexpensive source of proteins, duck eggs have been commonly consumed in Asian countries. While the yolk is preferably employed in food industries, a vast amount of duck albumen has been leaving as by-product, which could be utilized to produce novel bioactive peptides (Ren *et al.*, 2014). The peptides from ovalbumin and ovotransferrin hydrolysates have

been proven to have strong copper and iron-binding capacities, antioxidant properties, anti-inflammatory, anti-angiotensin converting enzyme and anticancer activity (Jahandideh *et al.*, 2016; Lee *et al.*, 2017). To produce bioactive peptides from egg albumen, proteases from animal pancreas, microbial and plant sources have been employed (Liu *et al.*, 2017; Stefanović *et al.*, 2014b). However, protease inhibitors present in fresh egg albumen may greatly reduce the efficiency of the enzymatic reaction. Therefore, heat pretreatment was implemented prior to enzymatic hydrolysis to inactivate protease inhibitors present in egg albumen and to denature protein molecules (Adjonu *et al.*, 2013). As a result, heat treated egg albumen became more susceptible to enzymatic hydrolysis (Ren *et al.*, 2014). Nevertheless, thermal pretreatment might induce coagulation of proteins, leading to less exposure or availability of cleavage sites towards proteases. Thus, further treatment could be introduced to conquer such a problem by increasing the surface area of heat-treated proteins.

Ultrasound is considered as a non-thermal processing technique, which has a frequency of sound wave higher than the level detection of human audition (Huang *et al.*, 2014). Ultrasound was reported to modify the functional properties of food via cavitation, which enhances efficacy and reduces processing time, compared to conventional method (Sae-leaw and Benjakul, 2018). Jovanović *et al.* (2016) documented that ultrasound with high-intensity not only improved enzymatic hydrolysis, but also decreased the loss in food nutrients and the generation of off-flavors. Ultrasound could accelerate the enzymatic hydrolysis of rice protein, whey protein and milk protein with the high yield of soluble protein and increase the antioxidant activity of the released peptides (Abadía-García *et al.*, 2016; Uluko *et al.*, 2015). For hen egg albumen protein hydrolysis, the use of ultrasound prior to hydrolysis at frequency of 40 kHz yielded the hydrolysate with higher degree of hydrolysis than that with heat pretreatment (Jovanović *et al.*, 2016). In addition, Stefanović *et al.* (2014b) documented that the ultrasound pretreatment at frequency of 40 kHz and calorimetric power of 21.3 W for 15 min at 25 °C yielded the increases in initial rate and degree of hydrolysis of hen egg albumen. However, less information on the enzymatic hydrolysis of duck egg albumen, particularly after heat treatment for inactivation of indigenous protease inhibitors with the aid of ultrasound pretreatment,

has been available. Therefore, the purpose of current study was to elucidate the influences of thermal pretreatment in combination with ultrasonication of duck egg albumen on the degree of enzymatic hydrolysis, emulsifying properties and antioxidant activities of resulting hydrolysates.

9.3 Materials and methods

9.3.1 Chemicals

Alcalase from Bacillus licheniformis (2.4 L enzyme, 20 units/g dry matter) and papain from the latex of Carica papaya were purchased from Novozymes (Bagsvaerd, Denmark). N-α-benzoyl-$_{DL}$-arginine-β-naphthylamide (BANA), bovine pancreas trypsin (Type I, ~10 000 BAEE units/mg protein), N-α-benzoyl-$_{DL}$-arginine-*p*-nitroanilide (BAPNA), 2,4,6-tritrobenzenesulphonic acid (TNBS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiaziline-6-sulphonic acid) (ABTS), 2,4,6-tripyridyltriazin (TPTZ) and 8-anilo-1-naphthalenesulfonic acid (ANS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

9.3.2 Preparation of duck albumen

Duck eggs laid within 24 h were obtained from a farmhouse in Kantang, Trang province, Thailand. The eggs were broken and albumens were manually collected. Chalaza was carefully removed. Subsequently, albumen was stirred at 3000 rpm for 5 min using a homogenizer (T 25 D; IKA-Werke GmbH & Co. KG, Staufen, Germany). Thereafter, homogenised albumen was subjected to pre-treatments.

9.3.3 Heat and ultrasound pre-treatment

Heat pre-treatment was performed by heating duck albumen solution (40 mg/mL) at 95 °C for 15, 30 and 45 min. The solutions were immediately cooled using iced water. Thereafter, treated albumen samples were tested for inhibitory activity towards papain and trypsin using BANA and BAPNA as the substrates, respectively, following the procedure of Benjakul *et al.* (2001). The heat treated duck albumen with the lowest relative trypsin and papain inhibitory activities was selected for further ultrasonication.

Heat treated duck albumen solution was subjected to ultrasonic processor (Sonics, VC750; Sonic & Materials, Inc., Newtown, CT, USA). The operating intensity power of 750 W and frequency of 20 kHz ± 50 Hz with various amplitudes (40%, 60% and 80%) and time (10 and 20 min) were applied. The samples (300 mL) prepared in a 500 mL-beaker were subjected to different ultrasound treatments. During operation, the temperature was maintained at 25–28 °C using an iced box. Fresh albumen and all the pre-treated samples were determined for surface hydrophobicity (SHP).

9.3.4 Enzymatic hydrolysis of duck albumen

Hydrolysis of duck albumen, fresh and pre-treated with various conditions, was conducted as per the method of Ren *et al.* (2014). Briefly, duck albumen solutions (40 mg/mL of protein, pH 8.0) were incubated at 50 °C for 30 min in a shaking water bath (WNE14; Memmert, Schwabach, Germany). The hydrolysis reaction was started by adding Alcalase at 2% of protein concentration. During 4 h of hydrolysis, 5 mL of samples were taken randomly and heated at 90 °C for 15 min to stop the enzymatic reaction. The final hydrolysate as supernatant was collected by centrifuging at 10,000 ×*g* for 10 min at 4 °C using a refrigerated centrifuge (CR22N; Hitachi Koki Co., Ltd., Tokyo, Japan). The obtained hydrolysates were subjected to analyses.

9.3.5 Analyses

9.3.5.1 Determination of surface hydrophobicity

Surface hydrophobicity (SHP) of albumen samples was determined as per the method of Kaewmanee *et al.* (2011) using ANS as a probe. The samples were diluted to obtain 0.125, 0.25, 0.5 and 1 mg/ mL protein content, using 0.2 M sodium phosphate buffer (pH 7.0). Two millilitres of prepared solutions were mixed with 10 µL of 10 mM ANS. Fluorescence intensity of the mixture was measured using spectrofluorometer RF-1501 (Shimadzu, Kyoto, Japan) at excitation and emission wavelengths of 374 and 485 nm, respectively. SHP was calculated from the initial slope of the plot of fluorescence intensity against protein concentrations determined by the Biuret method using a linear regression analysis. The initial slope was referred to as SHP (S_0ANS).

9.3.5.2 Degree of hydrolysis

Degree of hydrolysis (DH) was measured as per the protocol of Benjakul *et al.* (1997). To the diluted samples (0.125 mL), 2.0 mL of 0.2125 M phosphate buffer (pH 8.2) were added and thoroughly mixed. Thereafter, the mixture was added with 1.0 mL of 0.01% TNBS, followed by incubation at 50 °C for 30 min in a water bath. Then, 0.1 M Na_2SO_3 (2.0 mL) were added to stop the reaction. The absorbance at 420 nm was recorded using a spectrophotometer (UV-160; Shimadzu) and L-leucine (0–5 mM) was used as standards. The DH was calculated as follows:

$$DH = [(L_H-L_0)/(L_m-L_0)]\times 100$$

where L_H is the amount of α-amino acid (α-AA) in hydrolysate. L_0 is the amount of α-AA in original duck albumen. L_m is the maximum amount of α-AA in duck albumen obtained after acid hydrolysis.

For acid hydrolysis, duck albumen sample (0.5 mL) was mixed with 6 N HCl (4.5 mL) in a screw-cap tube. The mixtures were flashed with nitrogen gas and closed tightly. The reaction was conducted at 105 °C for 24 h. The acid-hydrolysed samples were filtrated using filter papers (Whatman paper no. 1; Whatman™, Buckinghamshire, UK) to eliminate the non-hydrolysed debris. Before a-AA determination, the supernatant was neutralised using 6 N NaOH.

9.3.5.3 Antioxidant activities

9.3.5.3.1 ABTS radical scavenging activity

The protocol of Re *et al.* (1999) was implemented for ABTS free radical scavenging assay. Potassium persulfate (2.6 mM) and ABTS (7.4 mM) were mixed at a ratio of 1:1 (v/v) for 12 h in dark and room temperature to generate ABTS free radical. Thereafter, ABTS radical solution ($ABTS^{\bullet+}$) (0.5 mL) was mixed with distilled water (25 mL) to reach an A_{734} of 1.1 ± 0.02 units. To 0.15 mL of sample, 2.85 mL of $ABTS^{\bullet+}$ were added and thoroughly vortexed. The mixture was then incubated in dark for 45 min at room temperature, and its A_{734} was read. The standard curve of Trolox (0–500 µM) was prepared in a similar way. The activity was represented in µmol Trolox equivalent/mL (µmol TE/mL).

9.3.5.3.2 DPPH radical scavenging activity

DPPH radical scavenging activity was measured as tailored by Binsan *et al.* (2008). Diluted samples (1.5 mL) were mixed with 0.1 mM DPPH (1.5 mL). Then, the mixtures were vigorously stirred and kept in the dark for 30 min at room temperature. The resulting solutions were read for A_{517}. Preparation of standard curve of Trolox (10–60 µM) was done in the same way. The activity was represented in µmol Trolox equivalent/mL (µmol TE/mL).

9.3.5.3.3 Ferric reducing antioxidant power

The protocol of Benzie and Strain (1996) was adopted for ferric reducing antioxidant power (FRAP) assay. Briefly, 2.85 mL of FRAP solution were mixed well with 0.15 mL of samples. The mixture was incubated in dark and room temperature for 30 min. A_{593} was then recorded using a UV–vis spectrophotometer. The standard curve of Trolox (0–400 µM) was prepared in a similar way. FRAP was represented in µmol Trolox equivalent/mL (µmol TE/mL).

9.3.5.2 Emulsifying properties

Measurements of emulsifying activity index (EAI) and emulsion stability index (ESI) were carried out following the procedure of Cho *et al.* (2014). Summarily, samples were diluted to obtain a final protein concentration of 10 mg/mL using phosphate buffer (50 mM, pH 7.6). To prepare an emulsion, 12 mL of the diluted samples and corn oil (4 mL) were homogenized using an IKA homogenizer at 12 000 rpm for 1 min. Fifty microlitres of the emulsions were taken from the bottom of beaker at 0 and 10 min after homogenization and diluted with 0.1% SDS (5 mL). The absorbance of diluted emulsion was read at 500 nm. EAI and ESI were calculated according to the following equations:

$$\text{EAI } (m^2/g) = \frac{4.606 \times A_0 \times D}{\phi \times C \times L \times 10^4}$$

$$\text{ESI (min)} = 10 \times A_0/(A_0 - A_{10})$$

where D is the dilution factor, ϕ is the volume fraction of the dispersed phase (oil), C is protein concentration (g/m^3) of aqueous phase, A_0 and A_{10} are A_{500} at 0 min and 10 min, respectively, and L is the path length of the cuvette (m).

9.3.5.3 Size exclusion chromatography

Duck albumen hydrolysate with the highest antioxidant activities and emulsifying properties was subjected to gel filtration chromatography using a Sephadex G-25 GF column (2.5 × 50 cm) (GE Healthcare, Bio-Science AB, Uppsala, Sweden). Hydrolysate sample (1 mL) was loaded onto the column at room temperature (25–28 °C). For elution, distilled water at a flow rate of 0.5 mL/min was used. Three millilitres of fractions were collected and their A_{280} and A_{220} were recorded. ABTS radical scavenging activity was tested for all fractions. Void volume was measured using Blue dextran (2 000 000 Da). Molecular weight (MW) of hydrolysates was determined using MW protein standards including tyrosine (181 Da), glycine-tyrosine (238 Da), vitamin B_{12} (1355 Da) and insulin chain B (3496 Da). The plot between the logarithm of the MW of protein standards and the available partition coefficient (Kav) was prepared.

9.3.6 Statistical analysis

All the experiments and analysis were done in triplicate. The one-way analysis of variance (ANOVA) and the Duncan's multiple range test were performed to analyze the significant difference among the samples at a level of $P<0.05$ using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

9.4 Results and discussion

9.4.1 Effect of heat pre-treatment on inhibitory activity towards trypsin and papain of duck egg albumen

Trypsin and papain inhibitory activities of duck albumen affected by heat pretreatment (95 °C) as a function of time (0–45 min) are illustrated in Fig. 33. Duck albumen showed inhibition towards both trypsin and papain, indicating the presence of both serine and cysteine protease inhibitors in albumen, respectively. Heat treatment of duck albumen at 95 °C caused the loss of trypsin and papain inhibitory activities to a high degree, as heating time increased ($P<0.05$). Prolonged heating resulted in the

greater energy for denaturation of inhibitors. The lowest relative trypsin and papain inhibitory activities were around 35% and 10% (P<0.05), respectively when duck albumen was heated at 95 °C for 45 min. Thus, loss in protease inhibitory activity of duck albumen was attributed to the denaturation of protease inhibitors induced by heat (Klomklao *et al.*, 2016). It has been known that ovalbumin in duck albumen belongs to the serpins, antitryptic proteins family, while cystatin has a strong inhibitory activity towards cathepsins, papain and ficin (Réhault, 2007). Recently, Quan and Benjakul (2019) reported that purified trypsin inhibitor from duck albumen lost its activity by 50% after incubating at 90 °C for 30 min. Ren *et al.* (2014) documented that Alcalase used for hydrolysis had 72% activity retained when fresh duck albumen was heated at 95 °C for 30 min. This finding indicated that thermal pretreatment of duck albumen at 95 °C for 30 and 45 min could greatly reduce inhibitors against trypsin and papain, respectively. As a consequence, the indigenous protease inhibitors in albumen could be destroyed via heat treatment. This could facilitate the hydrolysis of proteins in albumen when Alcalase was applied for production of hydrolysate.

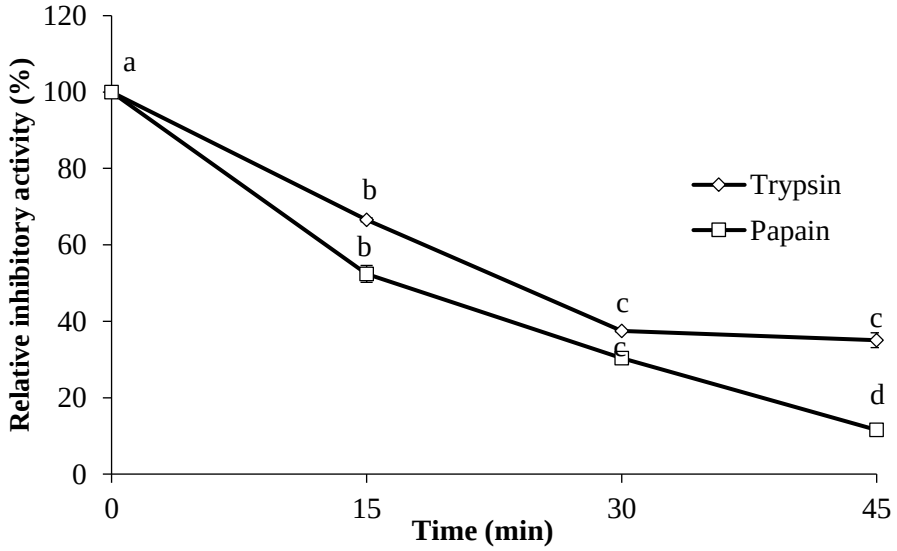

Figure 33. Relative inhibitory activity towards trypsin and papain of duck egg albumen after heat treatment at 95 °C for various times (min). Mean±SD (n=3). The letters on lines within the same enzyme tested denote the significant differences (P<0.05).

9.4.2 Effect of ultrasound pretreatment on SHP of duck egg albumen

Surface hydrophobicity is a useful parameter for predicting the conformation changes of protein via the exposure of hydrophobic domains (Sheng *et al.*, 2018). Fig. 34 represents SHP of duck albumen treated with heat only and heat followed by ultrasonication at various amplitudes and times. SHP of fresh duck egg albumen increased rapidly after heating at 95 °C for 30 min (P<0.05). The increase in SHP indicated the conformational change of duck albumen proteins induced by heat. When hydrophobic groups localized inside protein molecules were exposed, they readily bound with ANS used as the probe (Kaewmanee *et al.*, 2011). SHP of heated duck albumen continuously increased after being ultrasonicated. Increasing amplitude of ultrasonication typically induced the increase in SHP. The highest value of SHP was obtained at amplitude of 60% and 10 min (P<0.05). However, SHP decreased significantly as the amplitude of 80% was used for a longer time (20 min) (P<0.05). During acoustic cavitation, the more exposure of hydrophobic domains in protein molecules mediated by ultrasonication could increase SHP of proteins. Moreover, an extreme ultrasonication more likely enhanced protein aggregation via hydrophobic-hydrophobic interactions (Sheng *et al.*, 2018). Sheng *et al.* (2018) also reported that SHP of hen egg albumen increased at ultrasonic intensity of 260 W and decreased when ultrasonic power reached 480 W. This result suggested that ultrasound treatment of preheated albumen at an amplitude of 60% for 10 min might help in hydrolysis efficiency of proteins in albumen via exposing more cleavage sites for enzymatic reaction by Alcalase.

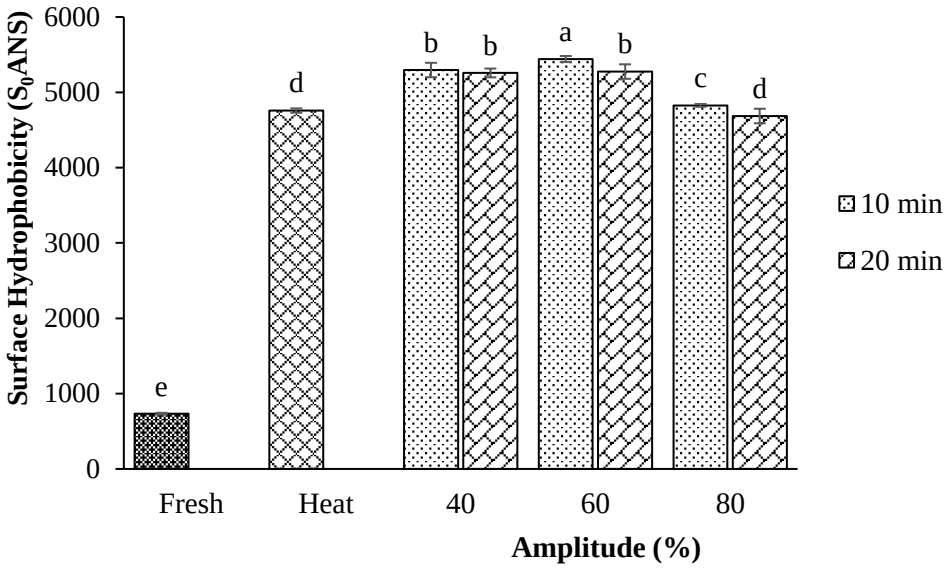

Figure 34. Surface hydrophobicity of duck egg albumen without and with heat treatment alone or heat in combination with ultrasonic pretreatments at various amplitudes for different times. Mean±SD (n=3). The letters on bars denote the significant differences (P<0.05).

9.4.3 Effects of heat and ultrasound pretreatments on degree of hydrolysis of duck egg albumen

As depicted in Fig. 35, degree of hydrolysis (DH) of hydrolysate varied, depending on pretreatment conditions. In general, the DH of all samples increased rapidly in initial phase, especially within 90 min (P<0.05), and reached the plateau in the latter stage up to 240 min (P>0.05). The lowest DH was noted for untreated (fresh) duck albumen, which reached the plateau at 60 min with the DH of 7.0%. Heat pretreatment more likely increased the DH of hydrolysate, in comparison with that produced from untreated counterpart. Basically, heat pretreatment could increase the rate of hydrolysis. Unfolded proteins induced by heat could provide more hydrolysis sites for enzymatic reaction (Uluko *et al.*, 2015). Coincidentally, the indigenous inhibitor activities of duck albumen towards trypsin and papain were mostly lost after heat treatment (Fig. 33). As a result, hydrolytic activity of Alcalase could proceed effectively. Additionally, hydrolysates from preheated albumen with subsequent ultrasound treatment showed the remarkably higher DH than that of sample treated with

heat alone at the same hydrolysis time (P<0.05). Generally, no marked difference in initial rate of hydrolysis between preheated samples followed by ultrasound at different amplitudes was observed (P>0.05). After 150 min of hydrolysis, the sample ultrasonicated with amplitude of 60% showed the highest DH (31%), followed by that subjected to 40% amplitude (27%) (P<0.05). Nevertheless, higher amplitude of ultrasound (80%) resulted in the decreased DH (P<0.05). Therefore, ultrasound treatment around 60% amplitude induced the changes in conformation of duck albumen protein and promoted enzymatic hydrolysis. Nevertheless, excessive ultrasound amplitude seemed to have adverse effect. When thermal treatment was used in combination with ultrasound prior to hydrolysis, the more porosity or looser protein conformation induced by cavitation effect was generated, thus increasing accessibility of the proteins to Alcalase hydrolysis (Sae-leaw and Benjakul, 2018). As ultrasound at high amplitude was introduced, aggregation took place, in which dimers or polymers could be formed via hydrophobic-hydrophobic interaction (Sheng *et al.*, 2018). This result agreed with the decreased SHP of albumen when the ultrasound amplitude was increased (Fig. 34). Stefanović *et al.* (2014b) also revealed that ultrasound power of 21.3 W could improve the DH of hen egg albumen, while higher power (65.7 W) rendered the lower rate of hydrolysis. This result suggested that heat treatment in combination with ultrasound at 60% amplitude prior to enzymatic hydrolysis by Alcalase could enhance the DH of hydrolysate from egg duck albumen.

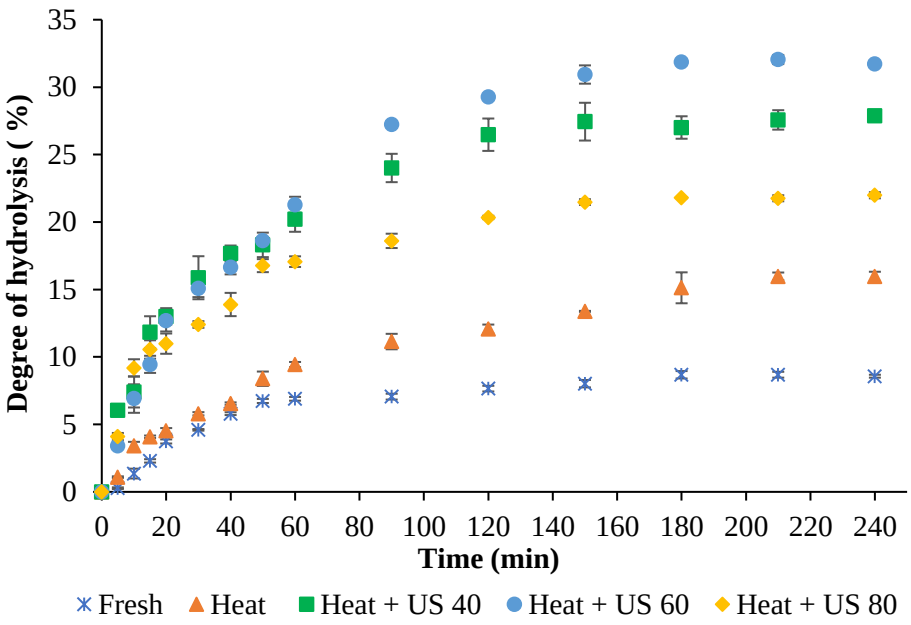

Figure 35. Degree of hydrolysis of duck albumen with different pretreatments. Fresh: without pretreatment, Heat: Heat at 95 °C for 30 min, Heat + US 40: Heat at 95 °C for 30 min followed by ultrasound (40% amplitude for 10 min), Heat + US 60: Heat at 95 °C for 30 min and followed by ultrasound (60% amplitude for 10 min), Heat + US 80: Heat at 95 °C for 30 min and followed by ultrasound (80% amplitude for 10 min). Mean±SD (n=3).

9.4.4 Effects of heat and ultrasound pretreatments on antioxidant activities and emulsifying properties of duck albumen hydrolysate

9.4.4.1 Antioxidant activities

Antioxidant activities (AA) of duck albumen hydrolysates including ABTS and DPPH radical scavenging activities (RSA), and ferric reducing antioxidant power (FRAP) as affected by heat and ultrasound pretreatments during hydrolysis are shown in Fig. 36. Heat and ultrasound pretreatments could increase the AA of duck albumen hydrolysate as indicated by higher AA, compared to hydrolysate from fresh albumen at all hydrolysis time (P<0.05). Approximately, three-time higher ABTS-RSA and FRAP and two-time increase in DPPH-RSA were noted for hydrolysate when duck albumen treated with heat in combination with ultrasound (60% amplitude) was used as substrate, as compared to untreated albumen. Among all hydrolysates, that prepared

from preheated albumen followed by ultrasound at 60% amplitude exhibited the highest AA (P<0.05). The AA of proteins basically depends on multiple factors, including amino acid residues and the degree of hydroxylation (Ashokkumar *et al.*, 2008). AA of proteins could be improved by partial denaturation mediated by heat and ultrasound, which can expose the buried amino acid residues having antioxidant potential in protein core (Singh and Ramaswamy, 2014). This phenomenon was also documented by Stefanović *et al.* (2014a) who found that antioxidant properties of hen albumen were enhanced after being ultrasonicated with frequency of 20 kHz for 15 min.

After hydrolysis, AA of all samples increased significantly as hydrolysis time increased up to 90 min (P<0.05). The rapid increase in AA was recorded in the initial stage of hydrolysis (within the first 90 min) (P<0.05). This agreed with the increase in DH of hydrolysate, reflecting the generation of free amino acids and smaller peptides. The DH of hydrolysates plays a major role in determining redox reaction or the degree of accessibility of the potential amino acids or peptides responsible for AA (Davalos *et al.*, 2004). Hydrolysates from duck albumen appeared to be more effective in ABTS-RSA, DPPH-RSA and FRAP when albumen was preheated followed by ultrasonication at 60% amplitude at all hydrolysis times used (P<0.05). Those activities decreased as amplitude of ultrasound used for treatment of heated albumen was increased to 80% (P<0.05). ABTS-RSA is one of the common assays used to measure the capacity of antioxidant in donating a hydrogen atom or an electron, which convert radicals into the non-radical species for either hydrophilic or hydrophobic compounds (Cho *et al.*, 2014; Sae-leaw and Benjakul, 2018). Similarly, hydrolysates prepared from preheated albumen subjected to ultrasound with amplitude of 60% showed the highest DPPH-RSA and FRAP at the hydrolysis time of 90 min, compared to those pretreated with heat alone or heating in combination with ultrasound at amplitude of 80% (P<0.05). DPPH-RSA assay determines the ability of antioxidants to reduce DPPH radical, while FRAP assay determines the ability of antioxidants to reduce the ferric iron to ferrous iron (Uluko *et al.*, 2015). These findings were also in line with the former studies on AA of hen egg albumen and milk protein hydrolysates, in which thermal and ultrasound pretreatments could increase AA of resulting hydrolysates (Uluko *et al.*, 2015). The results suggested that the hydrolysate obtained from duck albumen pretreated with heat

in combination with ultrasound at the amplitude of 60% and the hydrolysis time of 90 min could serve as natural antioxidant.

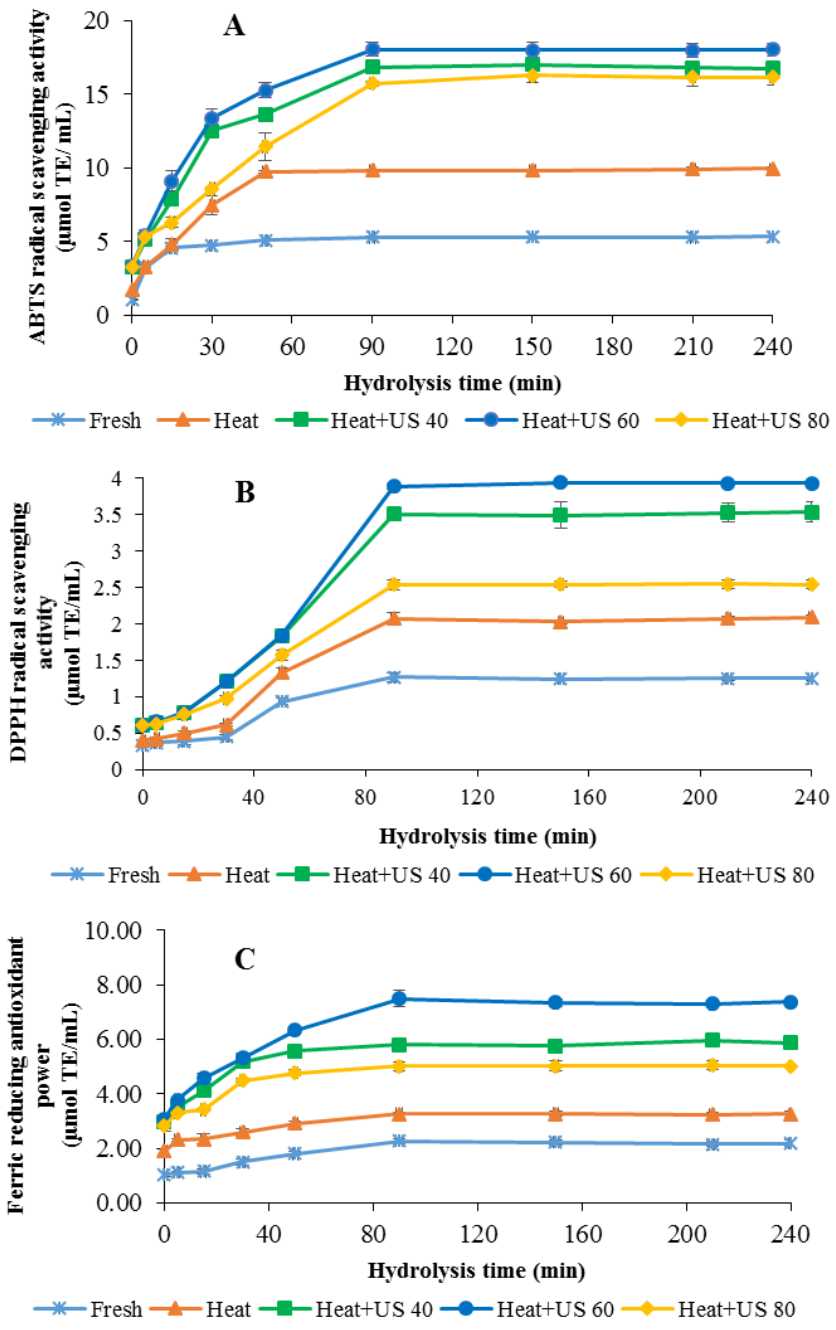

Figure 36. ABTS radical scavenging activity (A), DPPH radicals scavenging activity (B) and ferric reducing antioxidant power (C) of hydrolysate from duck albumen with different pretreatments. Caption: See Figure 35. Mean±SD (*n*=3).

9.4.4.2 Emulsifying properties

Emulsion activity index (EAI) and ESI of duck albumen hydrolysate as affected by heat and ultrasound pre-treatments are depicted in Fig. 37. Higher EAI of hydrolysate was obtained from pre-treated albumen with heat or/and ultrasound as compared to that of non-pretreated albumen (P<0.05). EAI of hydrolysate from untreated albumen was 45.4 m^2/g, which was two-fold or three-fold lower than those prepared from albumen subjected to heat (93.5 m^2/g) or heat combined with ultrasound (60% amplitude) pre-treatments (146 m^2/g), respectively. Pre-treatment of albumen using ultrasound at amplitude of 80% lowered EAI of the resulting duck albumen hydrolysate (P<0.05). ESI of hydrolysate from preheated duck albumen also showed tendency to increase as heat and ultrasound of albumen substrate were applied before hydrolysis. However, the increase in ultrasound amplitude (80%) yielded hydrolysate with decreased ESI of resulting hydrolysate (P<0.05). The increase in EAI indicated the better potential adsorption of duck egg albumen peptides at the water-oil interface, while ESI has been used to estimate the ability of proteins for localization at water-oil interface after emulsification (Stefanović *et al.*, 2014b). The increased EAI and ESI of hydrolysates from treated duck albumen were related to the increases in SHP (Fig. 34). Peptides with unfolded structure and more exposed hydrophobic groups were able to migrate and locate at the water-oil interface. Consequently, those peptides could rapidly adsorb on films surround the oil droplets and reduce the interfacial tension at the water-oil interface (Arzeni *et al.*, 2012). This result suggested that heat and ultrasound pre-treatments under an appropriate condition of duck egg albumen could enhance EAI and ESI of resulting hydrolysate. In general, heat in conjunction with ultrasound yielded hydrolysates with higher EAI and ESI, compared with heat alone (P<0.05). However, the increase in ultrasound amplitude (80%) caused the decreases in EAI and ESI of hydrolysate (P<0.05). Overall, the highest EAI of hydrolysate was noted when albumen pre-treated with heat, followed by ultrasonication at amplitude of 60% and hydrolysed by Alcalase for 15 min (192.4 m^2/g). Nevertheless, ESI reached the highest value after 90 min of hydrolysis (88 min). The increases in both EAI and ESI were in agreement with the increased DH of hydrolysate, in which small peptides with high solubility could immediately migrate and adsorb at the interface. Even though small peptides

migrate promptly towards the water-oil interface, they are less efficiency to stabilise the emulsion stabilisers, because they may not agglomerate to form a thick layer at oil droplet membrane because of charge repulsions (Cho *et al.*, 2014). Thus, hydrolysate produced from duck albumen pre-treated by the combination of heat and ultrasound at 60% amplitude with hydrolysis time of 90 min by Alcalase could be considered as an emulsifying agent.

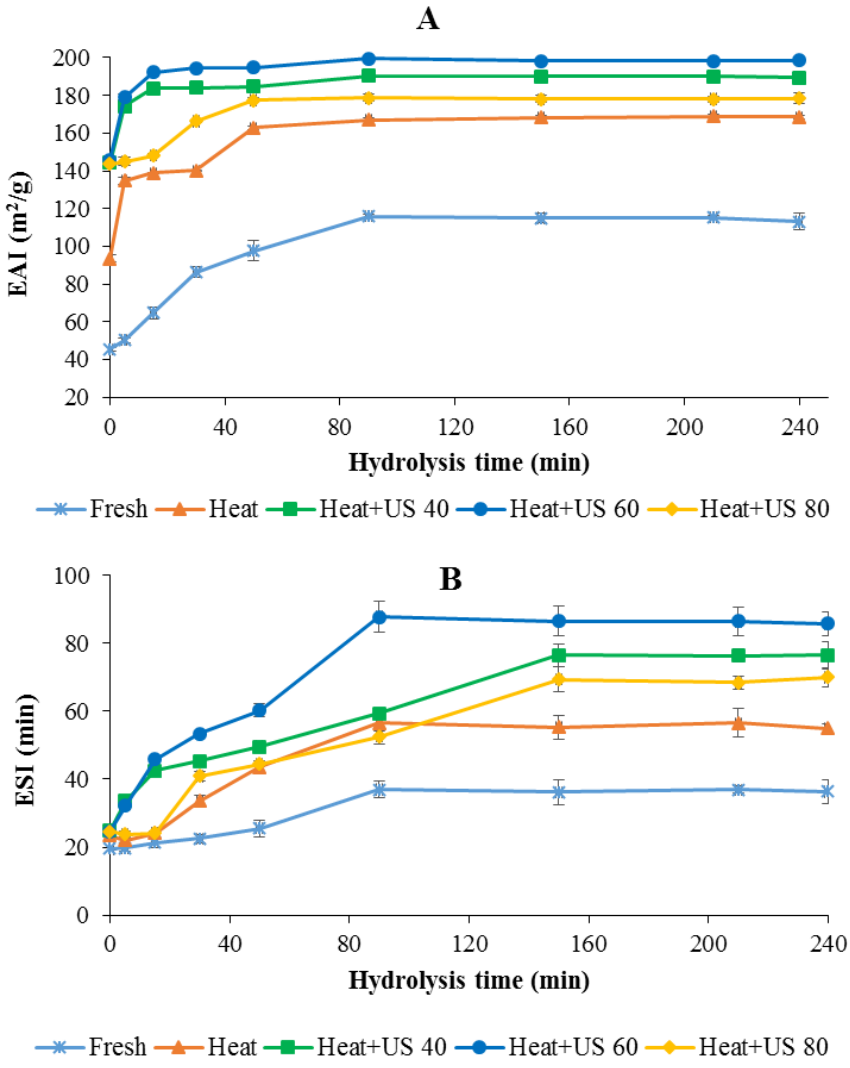

Figure 37. Emulsifying activity index (EAI) (A) and emulsion stability index (ESI) (B) of hydrolysate from duck albumen with different pre-treatments. Caption: See Figure 35. Mean±SD (*n*=3).

9.4.4.3 Molecular weight distribution of duck albumen hydrolysate

Hydrolysate produced from duck albumen treated by heat in combination with ultrasound at amplitude of 60% using Alcalase for 90 min contained peptides with MWs ranging from 138 to 4041 Da (Fig. 38A). However, three major peaks were observed for both A_{280} and A_{220}, representing peptides with MWs of 4041, 871 and 255 Da as calculated from the plot (Fig. 38C). A_{280} was commonly used to monitor proteins, peptides, or amino acids with aromatic domains, while A_{220} was used to determine peptide bonds (Sae-leaw and Benjakul, 2018). The peptides of duck albumen hydrolysates exhibited the ABTS-RSA. The smaller peptides exhibited the greater AA. The highest ABTS-RSA was recorded for the peptides with MWs of 219-255 Da (Fig. 38B). Low MW peptides mostly had the more exposed amino acids, which provided the available electrons to free radicals easily. Consequently, the reaction between free radicals and low MW peptides was more potent (Sae-leaw and Benjakul, 2018). Stefanović *et al.* (2014b) revealed that hen albumen peptides with MW<1000 Da showed the highest ABTS-RSA when hen albumen treated with ultrasound at frequency of 40 kHz and 21.3 W for 15 min prior to hydrolysis. Duck albumen hydrolysate produced by two-step of enzymatic hydrolysis yielded the peptides with MWs ranging from 202 to 514 Da, exhibiting the highest AA (Ren *et al.*, 2014). This indicated that small peptides from duck albumen hydrolysate contributed to AA of the hydrolysate prepared from albumen after heating in combination with ultrasound pre-treatment.

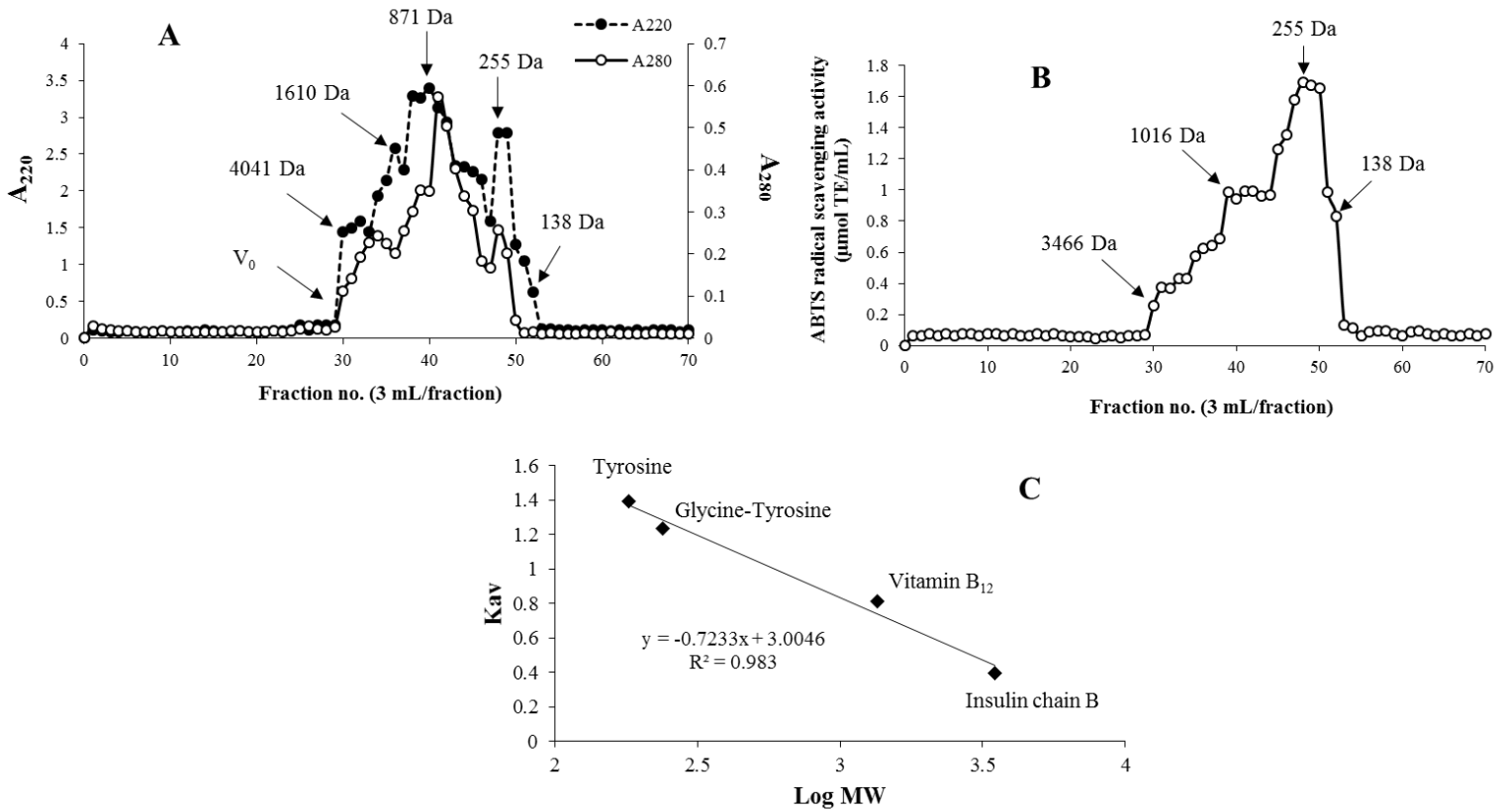

Figure 38. Elution profiles of hydrolysate produced from preheated duck albumen, followed by ultrasound at amplitude of 60% and hydrolysed by Alcalase for 90 min. SephadexTM G-25 gel filtration chromatography was used and A_{220} and A_{280} (A) and ABTS radical scavenging activity of fractions (B) were monitored. The plot between the logarithm of the MW of protein standards and the available partition coefficient (Kav) was prepared for determination of MW of peptides (C).

9.5 Conclusion

Heat treatment of duck egg albumen at 95 °C for 30 min with subsequent ultrasound pretreatment at 60% amplitude with intensity power of 750 W and frequency of 20 kHz for 10 min could effectively increase the DH of duck albumen. The highest antioxidant activities as well as emulsifying properties of duck albumen hydrolysate were found after 90 min of hydrolysis. Hydrolysate contained the peptides with low MWs (219–255 Da), exhibiting antioxidative activity.

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CHAPTER 10

DUCK EGG ALBUMEN HYDROLYSATE-EPIGALLOCATECHIN GALLATE CONJUGATES: ANTIOXIDANT, EMULSIFYING PROPERTIES AND THEIR USE IN FISH OIL EMULSION

10.1 Abstract

Duck albumen hydrolysates (DAH) conjugated with epigallocatechin gallate (EGCG) at various levels (2–5%, w/w) were prepared and characterized. FTIR analysis showed that the conjugation between DAH and EGCG induced the change of secondary structure and modified functional groups of DAH. Furthermore, surface hydrophobicity of DAH was increased, while total sulfhydryl group and total carbonyl contents were decreased when EGCG was conjugated, especially when EGCG at higher levels was used (P<0.05). Antioxidant activities and emulsifying properties of DAH-EGCG conjugates were enhanced, particularly when DAH conjugated with 4% EGCG (P<0.05). Storage stability of fish oil emulsion (FOE) stabilized by DAH or DAH-EGCG conjugate was also investigated up to 15 days at 26–28 °C. Emulsion stabilized by DAH-EGCG conjugate had higher magnitude of ζ-potential (negative charge), smaller oil droplets size (d_{43}), as well as lower flocculation and coalescence of oil droplets as compared to those of emulsion prepared using DAH throughout the storage. Moreover, oxidative stability of FOE was improved when DAH-EGCG conjugate was incorporated as evidenced by lowered thiobarbituric acid-reactive substances and peroxide value. Thus, the conjugate between DAH and 4% EGCG could serve as an antioxidant emulsifier for enhancement of physical and oxidative stability of FOE.

10.2 Introduction

Fish lipids commonly comprise high level of long-chain n-3 fatty acids, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which promote human health by enhancing brain function and lowering the risk of cardiovascular diseases (Sae-leaw and Benjakul, 2017). However, the applications of fish oils in food products are limited due to their susceptibility to oxidation associated with fishy odor or off-odor (Feng *et al.*, 2018). The oxidative degradation could also

induce the deterioration of nutrients and color (Fan *et al.*, 2018). Emulsion-type products have gained popularity due to their excellent mouthfeel and smooth body (Takeungwongtrakul and Benjakul, 2013). Because of the larger surface of oil droplets (OD) in emulsion, the oil is susceptible to oxidation. Thus, retardation or prevention of these deteriorative reactions of fish oils, particularly emulsion enriched with fish oil, is required during processing and storage (Li *et al.*, 2019). The rate and pathway of lipid oxidation (LO) mainly depend on the characteristics of the water-oil interface, where the oxidative reactions are normally initiated and propagated (Gu *et al.*, 2017a). Physiochemical properties of the water-oil interface are primarily affected by the employed emulsifier as well as antioxidants added. To maximize the preventive potential toward oxidation, the use of emulsifier with antioxidant activity could stabilize emulsion, while exhibiting its antioxidative property simultaneously at the interface (Cheetangdee and Benjakul, 2017). Nowadays, many types of food proteins such as gelatin, milk, and whey proteins have been reported to act as emulsifiers because of their amphiphilic molecules, which could rapidly adsorb to water-oil interface and impede the coalescence and flocculation of emulsion (Fan *et al.*, 2018; Feng *et al.*, 2018). Additionally, protein hydrolysates from plant and animal produced by enzymatic process exhibited superior antioxidant activities and emulsifying properties (Chen *et al.*, 2019). Quan and Benjakul (2019a) recently documented that duck egg albumen hydrolysate produced using Alcalase showed high ABTS and DPPH radical scavenging activities, ferric reducing antioxidant power (FRAP), as well as emulsifying properties. Since stabilities of emulsion are depending on interfacial properties of emulsifiers including protein hydrolysate such as chemical composition, thickness, charge, and polarity (Fan *et al.*, 2018), the appropriate modifications of protein hydrolysates to enhance the antioxidant activities and emulsifying properties could be implemented, in which the stability of emulsion enriched with fish oil could be increased.

Covalent grafting of proteins with phenolic compounds has been reported to be a potential technique to enhance those aforementioned properties of proteins by providing a large number of hydroxyl groups and hydrophobic domains (Feng *et al.*, 2018). Epigallocatechin gallate (EGCG) is one of the most active flavanols of tea

polyphenols, exhibiting the excellent antioxidant activity and has a key role in inhibition of LO (Nilsuwan *et al.*, 2019). The protein-polyphenol conjugates with higher antioxidant activities and emulsifying properties could dramatically improve the oxidative and emulsion stabilities of FOE as compared to that with proteins alone. Protein-EGCG conjugates have been prepared as multi-function additive. Whey protein-EGCG (Fan *et al.*, 2018), ovalbumin-EGCG (Feng *et al.*, 2018), and ovotransferrin-catechin conjugates (You *et al.*, 2014) have been synthesized. Nonetheless, no information regarding the grafting of duck albumen hydrolysate and EGCG as well as its impact on the stability of FOE has been documented. Thus, this work aimed to study on the characteristics and functionalities of duck albumen hydrolysate-EGCG conjugate and its effects on the stability of FOE.

10.3 Materials and methods

10.3.1 Chemicals

2,4,6-Tripyridyltriazin (TPTZ), 2,2-diphenyl-1picrylhydrazyl (DPPH), 8-anilo-1 naphthalenesulfonic acid (ANS), and 2,2-azino-bis(3-ethylbenzothiaziline-6-sulphonic acid) (ABTS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). EGCG was purchased from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China).

10.3.2 Preparation of duck albumen hydrolysate

Duck albumen hydrolysate (DAH) was prepared as per the procedure of Quan and Benjakul (2019a). Fresh DAH solution (40 mg protein/mL) was heated at 95 °C for 30 min followed by ultrasonication at 60% amplitude, 750W intensity power and 20 kHz ± 50 Hz frequency for 10 min using a ultrasonic processor (Sonics, VC750, Sonic & Materials, Inc., CT, USA). To initiate hydrolysis reaction, Alcalase at 2% concentration (based on protein content) was added into pre-treated albumen solution (50 °C). After 90 min of hydrolysis, the solution was heated at 95 °C for 10 min to inactivate the enzymatic reaction. The final hydrolysate was lyophilized using a freeze dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark), placed in aluminium bag laminated with PE, vacuum sealed, and stored at −20 °C for further studies.

10.3.3 Preparation of duck albumen hydrolysate (DAH)-EGCG conjugates

The protocol of Feng *et al.* (2018) was adopted for DAH-EGCG conjugate preparation. Firstly, 100 mL of DAH solution (1%, w/v) was subjected to oxidation by mixing with 0.15 g of ascorbic acid and 0.5 mL of 10M H_2O_2. The mixture was stirred for 2 h at room temperature (26–28 °C) and named as "oxidized DAH, ODAH". Thereafter, EGCG was added at various levels (0, 2, 3, 4, and 5%, based on DAH weight) into ODAH solution and stirred at 4 °C for 24 h. Then, the samples were lyophilized. The obtained powers referred to as "DAH-EGCG conjugate" were analyzed. Some portions were packed and stored at −20 °C as mentioned above.

10.3.4 Analyses

10.3.4.1 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) was measured as per the procedure of Thanonkaew *et al.* (2006), in which ANS was used as a probe.

10.3.4.2 Total sulfhydryl group and total carbonyl contents

The protocol of Singh *et al.* (2018) was adopted for determination of total sulfhydryl group content. Total carbonyl content was determined as per the procedure of Chanarat *et al.* (2015).

10.3.4.3 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of DAH and DAH-EGCG conjugates were obtained using a FTIR spectrometer Model EQUINOX 55 (Bruker, Ettlingen, Germany) as tailored by Aewsiri *et al.* (2009).

10.3.4.4 Emulsifying properties

Measurements of emulsifying activity index (EAI) and emulsion stability index (ESI) were conducted following the protocol of Cho *et al.* (2014). Firstly, DAH-EGCG conjugates and DAH were diluted to obtain a final protein concentration of 10 mg/mL using phosphate buffer (50 mM, pH 7.6). To prepare an emulsion, 12 mL of the diluted samples and corn oil (4 mL) were homogenized using an IKA homogenizer at 12,000 rpm for 1 min. Fifty μL of the emulsions were taken from the bottom of beaker

at 0 and 10 min after homogenization and diluted with 0.1% SDS (5 mL). The absorbance of diluted emulsion was read at 500 nm. EAI and ESI were calculated according to the following equations:

$$\text{EAI } (m^2/g) = \frac{4.606 \times A_0 \times D}{\phi \times C \times L \times 10^4}$$

$$\text{ESI (min)} = 10 \times A_0/(A_0 - A_{10})$$

where D is the dilution factor, ϕ is the volume fraction of the dispersed phase (oil), C is protein concentration (g/m^3) of aqueous phase, A_0 and A_{10} are A_{500} at 0 min and 10 min, respectively, and L is the path length of the cuvette (m).

10.3.4.5 Antioxidant activities

The procedure of Re *et al.* (1999) was adopted for ABTS free radical scavenging assay. DPPH radical scavenging activity was determined as described by Binsan *et al.* (2008). For FRAP assay, the protocol of Benzie and Strain (1996) was adopted. The standard curve of Trolox (0–600 μM) was prepared in the same manner. Antioxidant activities were expressed in term of μmol Trolox equivalent/mg sample (μmol TE/mg sample).

10.3.4.6 Size exclusion chromatography

EGCG, oxidized DAH, and DAH-5% EGCG conjugate were subjected to gel filtration chromatography using a Sephadex G-25 GF column (GE Healthcare, Bio-Science AB, Uppsala, Sweden). Samples (1 mL) were loaded onto the column at room temperature (25–28 °C). For elution, distilled water at a flow rate of 0.5 mL/min was used. Fractions (3 mL) were collected and their A_{280} and A_{220} were recorded. All fractions were tested for total phenolic compound, following the protocol of Chotphruethipong *et al.* (2019) and expressed as mg EGCG equivalent (EGCGE)/mL fraction. Molecular weight (MW) of hydrolysate or conjugate was calculated based on MW markers (Sae-leaw and Benjakul, 2018).

10.3.5 Stability and lipid oxidation of fish oil emulsions (FOE) as affected by DAH-EGCG conjugates

10.3.5.1 Preparation of FOE

Firstly, oil from Asian sea bass visceral depot fat was extracted following the protocol of Sae-leaw and Benjakul (2017). One hundred mL of DAH and DAH-EGCG conjugate (3%, w/v) were added with 10 mL of fish oil. Then, the mixtures were homogenized at 12,000 rpm for 2 min using a homogenizer model T 25D (IKA-Werke GmbH & Co. KG, Staufen, Germany). Thereafter, the coarse emulsions were subjected to ultrasonic processor at 60% amplitude and 20 kHz frequency for 5 min with pulse duration of 5 s (Gani and Benjakul, 2018). During operation, the temperature was maintained at 25–28 °C using an iced box. To inhibit the microbial growth, 0.02% NaN_3 was added to the emulsions. The oil-in-water emulsions were stored at room temperature (26–28 °C). FOE was placed in amber bottles covered with lids and analyzed for peroxide value (PV) and thiobarbituric acid-reactive substances (TBARS) at day 0, 3, 6, 9, 12, 15 of storage. For ζ-potential, particle size distribution, and microstructure, the samples were analyzed at day 0 and 15.

10.3.5.2 Analyses

10.3.5.2.1 Particle size distribution

Particle size distribution of FOE was measured using a liquid particle size analyzer (Model LS 230, Beckman Coulter, Fullerton, CA, USA) as tailored by Intarasirisawat *et al.* (2014). Before analysis, 5 mL of emulsion were diluted with 20 mL of distilled water or 1% SDS to dissociate flocculated droplets. The volume weighted mean particle diameters (d_{43}) of emulsion droplets in the absence and presence of 1% SDS were determined at day 0 and day 15 of the storage.

10.3.5.2.2 Flocculation and coalescence

The flocculation and coalescence of FOE were calculated using the equations as documented by Benjakul *et al.* (2016).

$$\text{Flocculation} = \frac{d_{43-SDS}}{d_{43+SDS}}$$

$$\text{Coalescence} = \frac{\left(d_{43+SDS,t} - d_{43+SDS,in}\right)\times 100}{d_{43+SDS,in}}$$

where $d_{43\text{-SDS}}$ and $d_{43+\text{SDS}}$ are the volume-weighted mean particle diameters of the emulsion droplets in the absence and presence of 1% SDS, respectively; $d_{43+\text{SDS,t}}$ and $d_{43+\text{SDS,in}}$ are the volume-weighted mean particle diameters of the emulsion droplets in the presence of 1% SDS at day 15 and day 0, respectively.

10.3.5.2.3 ζ-potential

ζ-potential of OD in the FOE was measured using a ZetaPlus zeta potential analyzer (Model ZetaPALS, Brookhaven Instrument Corp., Holtsville, NY, USA).

10.3.5.2.4 Confocal laser scanning microscopy

Microstructures of FOE samples were visualized with a confocal laser scanning microscopy (CLSM) (FV300, Olympus, Tokyo, Japan) following the procedure of Intarasirisawat *et al.* (2014). To the samples, Nile blue A solution was added at a ratio of 1:10 (v/v). The mixture was stirred to obtain uniformity. The prepared mixture (20 μL) was smeared on a microscopy slide. The CLSM was conducted using the fluorescence mode (excitation and emission wavelength of 533 and 630 nm, respectively).

10.3.5.2.5 Peroxide value (PV) and thiobarbituric acid-reactive substances (TBARS)

Emulsion samples were analyzed for PV and TBARS. Peroxide value (PV) was determined as per the protocol of Takeungwongtrakul and Benjakul (2013). Cumene hydroperoxide (0–10 ppm) was used for standard curve preparation. PV was expressed as mg cumene hydroperoxide/L of emulsion.

TBARS were determined as tailored by Buege and Aust (1978), in which malondialdehyde (MDA) at concentrations of 0–10 ppm was used for the standard curve preparation. TBARS was expressed as mg MDA/L emulsion.

10.3.6 Statistical analysis

All the experiments and analyses were done in triplicate. The one-way analysis of variance (ANOVA) was carried out. For pair comparison between samples, T-test was used. The Duncan's multiple range test was performed to analyze the significant difference among the samples at a level of $P<0.05$ using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

10.4 Results and discussion

10.4.1 Characterization of DAH-EGCG conjugates prepared using EGCG at different levels

10.4.1.1 Fourier transform infrared spectra (FTIR)

FTIR spectra (4000–400 cm^{-1}) of DAH and DAH conjugated with EGCG at different levels are demonstrated in Fig. 39. The FTIR spectrum of DAH exhibited the major bands at 3290, 1650, 1541, and 1240 cm^{-1} representing amide A (associated with NH-stretching, coupled with hydrogen bonding), amide I (representing C=O stretching/hydrogen bonding coupled with C-N stretch and CCN deformation), amide II (corresponding to NH bending coupled with C-N stretching), and amide III (relating to the stretching of C-N combined with N-H deformation) (Chen *et al.*, 2019; Nilsuwan *et al.*, 2018). The spectrum of DAH was typical for peptides or proteins. Ovalbumin is the major protein in albumen (Quan and Benjakul, 2019b). However, some peptide bonds were cleaved when Alcalase was used for preparation of DAH. Modification of DAH using EGCG resulted in some changes in spectra, suggesting interaction between DAH and EGCG. Compared with DAH, DAH-EGCG conjugates showed the slight shift in wavelength of amide A (from 3292 cm^{-1} to 3290 cm^{-1}), especially when 5% EGCG was used. The result indicated the interaction of EGCG and hydrolysate via H bond (Nilsuwan *et al.*, 2018). Moreover, amide B absorbance detected at wavenumber of 3083 cm^{-1}, corresponding to asymmetric stretching vibration of NH_3^+ and =C-H, was increased when DAH was conjugated with EGCG in a dose-dependent fashion. Similarly, the higher intensity of peaks at wavenumbers of 2924–2935 cm^{-1} and 2875–2878 cm^{-1} were recorded in DAH-EGCG conjugates, compared to that of DAH, indicating the symmetrical and asymmetrical stretching vibrations of the aliphatic C-H

in CH_3 and CH_2 groups, respectively (Ali *et al.*, 2018; Nilsuwan *et al.*, 2018). The results reconfirmed that the incorporation of EGCG into DAH induced the conformational changes of DAH.

For DAH-EGCG conjugates, the wavenumbers of amide I shifted from 1650 to 1655 cm^{-1}, while amide II decreased from 1541 to 1536 cm^{-1}, compared with that of DAH. Basically, amide I spectrum demonstrates the secondary structure of proteins. β-antiparallel sheet can be observed at wavenumber of 1680–1692 cm^{-1}, while β-sheet, α- helix, β-turn, and random coil are detected at wavenumber of 1610–1637 cm^{-1}, 1649–1660 cm^{-1}, 1660–1680 cm^{-1}, and 1638–1648 cm^{-1}, respectively (Czubinski and Dwiecki, 2017). The interaction between proteins and polyphenols caused the decreases in β-antiparallel, β-turn, and α-helix fractions of proteins (Feng *et al.*, 2018). These changes were governed by covalent conjugation of hydrolysate and EGCG. The finding was in line with those of Fan *et al.* (2018) who documented that an increase in amide I wavenumber and a decrease in amide II wavenumber were found after whey protein isolate conjugated with EGCG mediated by the C=O stretching of EGCG (Fan *et al.*, 2018). Moreover, the increased amplitude at wavenumbers of 1075 and 1400 cm^{-1}, related to the stretching vibration of the C-O ester group (commonly found in polyphenols) and the bending of O-H bonds, respectively, were observed when DAH was conjugated with EGCG (Aewsiri *et al.*, 2009; Nilsuwan *et al.*, 2018). The higher amplitude indicated higher incorporation of EGCG into DAH. It indicated that EGCG could provide the OH group into DAH. Thus, the conjugation of DAH and EGCG was able to modify and affect the secondary structure and functional groups of resulting conjugate. Additionally, levels of EGCG were also proven to have the impact on modification of DAH.

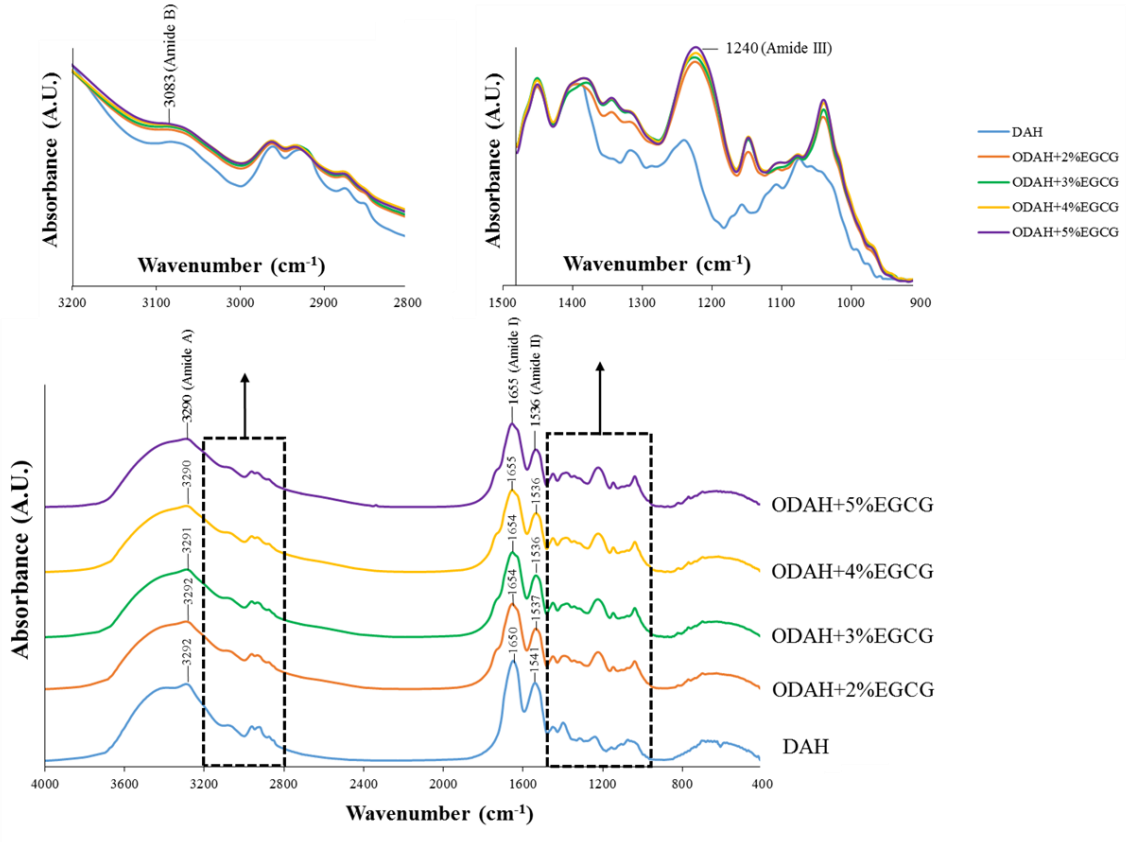

Figure 39. Fourier transform infrared spectra (FTIR) of DAH-EGCG conjugates prepared using EGCG at different levels.

10.4.1.2 Surface hydrophobicity, total sulfhydryl group and total carbonyl contents

Surface hydrophobicity (SHP), total sulfhydryl group (TSG), and total carbonyl content (TCT) of DAH and DAH conjugated with EGCG at various levels are presented in Table 23. SHP of DAH was markedly increased when DAH was oxidized (6013±43). SHP of DAH-EGCG conjugates increased, especially when EGCG at higher levels was used (P<0.05). The increase in SHP of conjugates indicated the attachment of nonpolar groups of EGCG on the surface of protein molecule (Liu *et al.*, 2017). Moreover, the covalent conjugation of DAH and EGCG using radical grafting resulted in the conformational change of protein, in which internal hydrophobic domains turned to localize on protein surface, thus increasing its SHP (Feng *et al.*, 2018). However, SHP was significantly decreased as the concentration of EGCG was higher than 4% (P<0.05). The higher levels of EGCG might cause the excessive

polymerization or aggregation of protein molecules via covalent interaction or hydrophobic–hydrophobic interaction, which resulted in lower SHP (Liu *et al.*, 2015). Feng *et al.* (2018) found that SHP of ovalbumin-catechin conjugate was two-time higher than that of native ovalbumin. In addition, the hydrophobic groups of bovine serum albumin were more exposed after conjugated with caffeic acid (Fan *et al.*, 2018). The conjugation of egg albumen protein and EGCG could enhance SHP by exposing more hydrophobic groups of native protein (Yin *et al.*, 2014). Therefore, covalent interaction between DAH and 4% EGCG increased surface hydrophobicity of DAH.

Additionally, total sulfhydryl group (TSG) content of DAH was drastically decreased after being oxidized (3.02±0.21 nmol/g protein). TSG content of oxidized DAH continuously decreased when EGCG was conjugated (P<0.05). The higher levels of EGCG used caused the lower TSG content, in which DAH incorporated with 5% EGCG showed the lowest TSG content (approximately 2.5-time lower than that of DAH) (Table 23). Nevertheless, total carbonyl content (TCT) of oxidized DAH drastically increased (99.13±3.25 nmol/g protein) (P<0.05). After being oxidized by oxidative agents, in which hydrogen peroxide and ascorbic acid were used as a redox pair of initiator system, peptides or proteins in DAH underwent oxidation. Li *et al.* (2018) revealed that the oxidation of ovalbumin mediated by hydroxyl radical method induced the decrease in TSG and the increase in TCT. Nevertheless, the covalent conjugation between DAH and EGCG could lower the TCT of conjugates (P<0.05), indicating the prevention effect of EGCG toward protein oxidation. The lowest TCTs were recorded when 4% and 5% EGCG were used (Table 23). The decreases in TSG and TCT of DAH-EGCG conjugates were related with the covalent cross-links (C-N or C-S) between DAH and EGCG (Liu *et al.*, 2017; Spizzirri *et al.*, 2009). To form DAH-EGCG conjugate, reactive side chains of peptides or proteins were firstly oxidized by hydroxyl radicals initially generated by the reaction of redox pair. The radicals localized on protein molecules then reacted with EGCG via covalent bonds (Liu *et al.*, 2017; Rawel *et al.*, 2001). This finding coincided with Liu *et al.* (2015) who revealed that covalent bonding between lactoferrin and polyphenols decreased the TSG and free amino group, compared to native lactoferrin. The results suggested that the covalent

conjugation between DAH and EGCG could modify the conformation of protein, especially via enhanced exposure of hydrophobic domains of resulting conjugates.

Table 23. Surface hydrophobicity, total sulfhydryl group and total carbonyl contents of DAH-EGCG conjugates prepared using EGCG at different levels.

Samples	Surface hydrophobicity (S_0ANS)	Total sulfhydryl group (nmol/g protein)	Total carbonyl content (nmol/g protein)
DAH	5882±34[†d]	3.69±0.15[a]	10.81±1.47[d]
ODAH	6013±43[d]	3.02±0.21[b]	99.13±3.25[a]
ODAH+2% EGCG	6240±23[c]	2.66±0.12[c]	94.73±4.73[a]
ODAH+3% EGCG	6323±52[bc]	2.24±0.14[d]	87.00±2.50[b]
ODAH+4% EGCG	6736±68[a]	2.02± 0.10[d]	78.13±1.48[c]
ODAH+5% EGCG	6475±95[b]	1.44±0.15[e]	74.78±2.87[c]

[†]Mean±SD (n=3). Different lowercase superscripts (a–d) in the same column indicate significant differences (P<0.05). DAH: duck albumen hydrolysate; ODAH: oxidized DAH.

10.4.1.3 Antioxidant activities

Antioxidant activities (AA) of DAH and DAH-EGCG conjugates prepared using EGCG at different levels including ABTS and DPPH radical scavenging activities (RSA), and FRAP are shown in Table 24. There was no significant different between the FRAP of native DAH and ODAH (P>0.05), while DPPH and ABTS radical scavenging activities were slightly increased (P<0.05) after oxidation, which may be because when ascorbic acid was oxidized by hydrogen peroxide, some reductive products formed with the generation of hydroxyl free radicals. The interactions between these products and DAH may be responsible for quenching some of the radicals formed (Gu *et al.*, 2017a). Conjugation of DAH and EGCG could increase AA of DAH, compared to those found in DAH and ODAH (P<0.05). DPPH-RSA of DAH was 3.22 μmole TE/mg sample. After being conjugated with EGCG, this value dramatically increased, particularly with increasing levels of EGCG used (P<0.05). The highest DPPH-RSA was recorded in conjugates formed between DAH and 5% EGCG (5.38 μmole TE/mg sample). DPPH-RSA assay determines the ability of antioxidants to reduce DPPH radicals (Sae-leaw and Benjakul, 2018). It indicated that DAH-EGCG

conjugates exhibited higher proton-donating ability than that of DAH alone. Similarly, DAH-EGCG conjugates showed higher ABTS-RSA and FRAP than those of DAH (Table 24) (P<0.05). These values were increased approximately two-time when DAH conjugated with 4% and 5% EGCG, as compared to those of DAH. FRAP and ABTS-RSA are the common assays used to determine AA of hydrolysates as well as phenolic compounds. FRAP assay determines the capacity of antioxidants to reduce the ferric iron to ferrous iron, while ABTS-RSA measures the hydrogen-donating ability of antioxidant to convert radicals into non-radicals (Chen *et al.*, 2019; You *et al.*, 2014). Among all conjugates, that prepared using 5% EGCG exhibited the highest AA (P<0.05). The AA of DAH-EGCG conjugates was increased, mainly because of the introduction of hydroxyl groups from EGCG into DAH (Czubinski and Dwiecki, 2017; Yildirim-Elikoglu and Erdem, 2018). Jiang *et al.* (2018) found that radical scavenging capacity was significantly increased in a dose-dependent manner when whey protein and casein were conjugated with chlorogenic acid. Similarly, antioxidant activities of EGCG-α-lactalbumin, α-casein, β-lactoglobulin, and β-casein conjugates were higher than those of native proteins (Almajano *et al.*, 2007). Moreover, Feng *et al.* (2018) revealed that conjugates of EGCG, catechin, or epigallocatechin with ovalbumin synthesized by free-radical grafting approach showed the stronger ORAC, DPPH and ABTS radical scavenging activities than ovalbumin. Among all the conjugates, ovalbumin-EGCG conjugate possessed the highest antioxidant activities. The results suggested that conjugates between DAH and 4% or 5% EGCG could be used as potential antioxidants to enhance the oxidative stability of several lipid-based foods.

Table 24. Antioxidant activities of DAH-EGCG conjugates prepared using EGCG at different levels.

Samples	DPPH radical scavenging activity (μmol TE/mg sample)	ABTS radical scavenging activity (μmol TE/mg sample)	Ferric reducing antioxidant power (μmol TE/mg sample)
DAH	3.22±0.15[†f]	12.92±1.53[f]	5.95±0.11[e]
ODAH	4.18±0.04[e]	16.38±0.11[e]	6.20±0.13[e]
ODAH+2% EGCG	4.45 ± 0.08[d]	20.22 ± 0.5[d]	7.17 ± 0.15[d]
ODAH+3% EGCG	4.77 ± 0.07[c]	22.11 ± 0.18[c]	9.57 ± 0.13[c]
ODAH+4% EGCG	5.00 ± 0.14[b]	24.27 ± 0.55[b]	10.87 ± 0.38[b]
ODAH+5% EGCG	5.38 ± 0.09[a]	30.16 ± 0.18[a]	11.71 ± 0.10[a]

[†]Mean±SD (n=3). Different lowercase superscripts (a–f) in the same column indicate significant differences (P<0.05).

10.4.1.4 Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of DAH modified by EGCG at various levels are shown in Table 25. EAI of conjugates was increased with increasing levels of EGCG up to 4%, followed by slight decrease when 5% EGCG was used. EAI of unmodified DAH was 194 m^2/g, which was increased by 42% after DAH was conjugated with 4% EGCG (275 m^2/g). However, conjugation between DAH and 5% EGCG lowered EAI of the resulting conjugate (P<0.05). This result indicated that the increased EAI was mainly attributed to the increased SHP of conjugates after incorporation with EGCG (Table 23). Similarly, ESI of DAH also showed tendency to increase when the concentration of EGCG increased (P<0.05), especially in the range of 2–4%. However, the high level of EGCG used (5%) yielded the conjugate with lowered ESI, compared to that using 4% EGCG (P<0.05). The higher EAI indicated the better potential adsorption of DAH-EGCG conjugate at the water-oil interface, whereas ESI has been used to estimate the ability of proteins for localization at water-oil interface after emulsification (Arzeni *et al.*, 2012). The covalent attachment of EGCG moieties could alter DAH conformation to form the conjugate with higher exposed hydrophobic domains, which were able to migrate and

localize at water-oil interface (Czubinski and Dwiecki, 2017; Gu *et al.*, 2017b). As a consequence, the conjugate could rapidly adsorb at oil interface, forming the film surrounding oil droplets and reduced the interfacial tension at the water-oil interface (Arzeni *et al.*, 2012). However, the conjugate prepared using 5% EGCG had plausibly higher aggregation with less hydrophobic domains. The rigid structure with less flexibility could not form a thick layer at OD film, thus causing the adverse effect on emulsifying properties (Chen *et al.*, 2019). These results coincided with those of Liu *et al.* (2015) who synthesized lactoferrin–polyphenols (EGCG, chlorogenic acid and gallic acid) conjugates using free-radical grafting method, which had better emulsifying properties than unmodified lactoferrin. Recently, Abd El-Maksoud *et al.* (2018) covalently coupled caffeic acid to β-lactoglobulin using alkaline method. The resulting conjugates showed better emulsifying properties than native β-lactoglobulin. Moreover, porcine plasma protein hydrolysate-oxidized chlorogenic acid conjugate could rapidly adsorb and make a thicker interfacial film around OD, thus enhancing the emulsifying properties (Chen *et al.*, 2018). This result suggested that the conjugation between DAH and 4% EGCG could enhance EAI and ESI of resulting conjugate.

Table 25. Emulsifying activity index (EAI) and emulsifying stability index (ESI) of DAH-EGCG conjugates prepared using EGCG at different levels.

Samples	Emulsifying activity index (m^2/g)	Emulsifying stability index (min)
DAH	$194.99\pm1.41^{\dagger f}$	86.29 ± 3.30^{d}
ODAH	213.92 ± 0.64^{e}	93.56 ± 1.27^{cd}
ODAH+2% EGCG	227.23 ± 2.93^{d}	93.31 ± 2.95^{cd}
ODAH+3% EGCG	243.09 ± 3.86^{c}	100.22 ± 1.32^{bc}
ODAH+4% EGCG	275.23 ± 2.22^{a}	114.58 ± 4.74^{a}
ODAH+5% EGCG	263.39 ± 0.42^{b}	105.99 ± 5.89^{b}

†Mean±SD (n=3). Different lowercase superscripts (a-f) in the same column indicate significant differences ($P<0.05$).

10.4.1.5 Molecular weight distribution of DAH-EGCG conjugate

Molecular weight (MW) distribution of oxidized DAH, DAH-EGCG conjugate, and EGCG monitored using A_{280} is illustrated in Fig. 40. A_{280} was widely used to monitor amino acids, peptides, proteins with aromatic regions or phenolics (Jia *et al.*, 2016; Sae-leaw and Benjakul, 2018). There were three major peaks observed in elution profile of oxidized DAH with the peaks at 621, 1590, and 5471 Da. After being conjugated, two dominant peaks, having MWs of 4690 and 2530 Da were recorded in DAH-ECGC conjugate. Peptides with low MW were absent in DAH-EGCG conjugate fractions. Furthermore, the peak with higher MW was also noted. This indicated that EGCG was successfully bound with low MW peptides of DAH, thereby increasing MWs of conjugate. Total phenolic content was also determined in DAH-EGCG conjugate fractions (Fig. 40B). The highest total phenolic compound was recorded in the fraction of DAH-EGCG conjugate having MW of 4690 Da, which was not found in DAH fractions. Moreover, the peak of EGCG was not detected in DAH-EGCG conjugate, indicating no free form of EGCG present in the resulting conjugate. MW of EGCG was commonly found at 450-458 Da (Fig. 40A). This result reconfirmed that EGCG was totally conjugated with peptides of DAH. Yi *et al.* (2015) documented that MW of β-Lactoglobulin was increased after being conjugated with catechin. This result also coincided with that of Yi *et al.* (2016) who found that MW of α-lactalbumin-catechin conjugate was higher than that of α-lactalbumin after covalent grafting. Modification with EGCG by radical grafting method caused the increase in MW of native whey protein (Jia *et al.*, 2016). The increase in MW might affect the physiochemical and functional properties of DAH-EGCG conjugate.

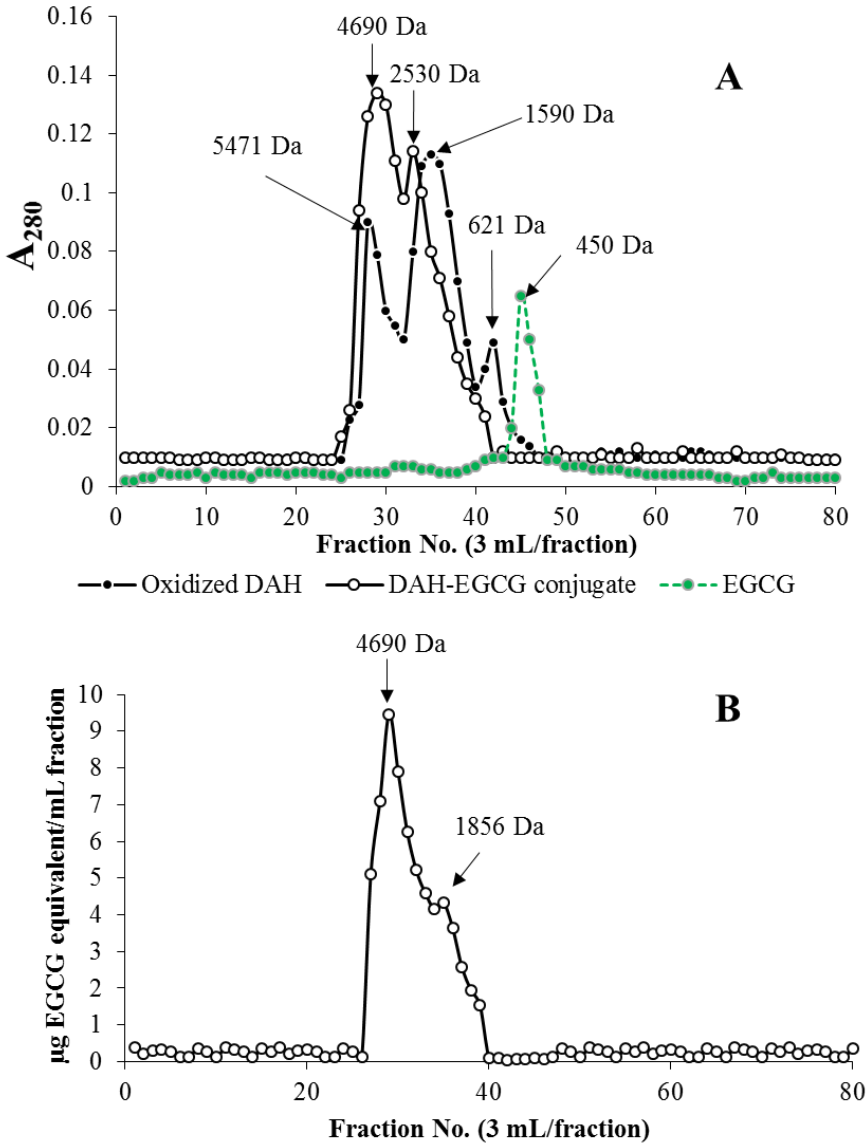

Figure 40. Elution profiles of oxidized DAH, DAH-EGCG conjugate, and EGCG using SephadexTM G-25 gel filtration chromatography (A) and total phenolic content in all fractions of DAH-EGCG conjugate (B).

10.4.2 Effects of DAH-EGCG conjugate on stability of FOE

10.4.2.1 Electrical charge and particle size of oil droplets

Electrical charge (ζ-potential) and particle size (d_{43}) of OD in FOE systems stabilized by DAH or DAH-EGCG conjugate are presented in Table 26. ζ-potential of OD in emulsion containing either DAH or DAH-EGCG conjugate, had the negative charge. Higher negative charge was found in the latter (P<0.05). The emulsion

stabilized by DAH had ζ-potential about -29.01 mV, while that of emulsion stabilized by DAH-EGCG conjugate was -34.20 mV at day 0 of storage. The electrical charge of OD became less negatively charged after emulsion was stored for 15 days (P<0.05). The lower ζ-potential of OD in emulsion stabilized by DAH-EGCG might be due to the masking of positive charge on peptides by bulky groups of EGCG at a neutral pH (pH 7.0). After storage, the aggregation of OD (coalescence or flocculation) via ionic interaction mostly occurred during the storage (Wang *et al.*, 2015) as indicated by the decrease in negative charge. Intarasirisawat *et al.* (2014) documented that emulsion having the ζ-potential lower than −30 mV or higher than +30 mV tended to be electrostatically stable, while that exhibiting the electrical charge in range of +30 to −30 mV tended to flocculate or coagulate. In the present study, the emulsion stabilized by DAH-EGCG conjugate still had ζ-potential of −30.57 mV at day 15. Yi *et al.* (2016) documented that β-carotene emulsion stabilized by α-lactalbumin-catechin conjugate had more negative charge than that of emulsion added with α-lactalbumin. Intarasirisawat *et al.* (2014) also documented that oxidized tannic acid incorporated with skipjack roe protein hydrolysate could decrease ζ-potential of OD, reflecting higher negative charge. For OD size, the emulsion stabilized by DAH had OD with higher d_{43}, compared to that of emulsion stabilized by DAH-EGCG conjugate (P<0.05). At the beginning of the storage, the d_{43} of OD in DAHEGCG based emulsion was 2.09±0.10 μm, while higher d_{43} value was found in emulsion stabilized by DAH (3.89±0.10 μm). The d_{43} of OD was significantly increased after both emulsion samples were stored for 15 days (P<0.05), in which approximately two-time increase in droplet diameter of oil was recorded in emulsion added with DAH. However, smaller OD was still remained in emulsion stabilized with DAH-EGCG conjugate (3.04±0.04 μm). This indicated that DAH-EGCG conjugate effectively produced the emulsion with small OD size or prevented coalescence of the droplets. Basically, an increase in OD size during storage might be mediated by coalescence or flocculation, which related to the decrease in the magnitude of ζ-potential on the OD (Staszewski *et al.*, 2014). With decreased electrostatic or steric repulsions, associated with the lower negative charge, hydrophobic–hydrophobic interaction or van der Waals force became dominant, thus increasing the OD diameter (Liu *et al.*, 2016). Fan *et al.* (2018) documented that conjugation between EGCG and whey protein isolate yielded the emulsion with

decreased d_{43} with coincidentally increased magnitude of surface charge of OD. This finding suggested that DAH-EGCG conjugate could be used as a potential emulsifier for enhancing the physical stability of FOE.

Table 26. ζ-potential, d_{43}, flocculation and coalescence of oil droplets in emulsion incorporated with DAH or DAH-EGCG conjugate during storage.

Samples	Storage time (days)	ζ-potential (mV)	d_{43} (μm)	Flocculation	Coalescence
DAH based emulsion	0	-29.01±0.43$^{\dagger bA}$	3.86±0.14^{bA}	2.13±0.08^{bA}	-
	15	-23.76±1.53^{aA}	6.94±0.14^{aA}	3.56±0.03^{aA}	7.73±0.96^{A}
DAH-EGCG conjugate based emulsion	0	-34.20±1.19^{bB}	2.09±0.10^{bB}	0.97±0.03^{bB}	-
	15	-30.57±1.07^{aB}	3.04±0.04^{aB}	1.36±0.04^{aB}	3.54±0.23^{B}

†Mean±SD (n=3). Different lowercase superscripts (a–b) in the same column within the same samples indicate significant differences (P<0.05). Different uppercase superscripts (A–B) in the same column within the same storage time indicate significant differences (P<0.05).

10.4.2.2 Flocculation and coalescence

Flocculation and coalescence of FOE stabilized by DAH or DAH-EGCG conjugate during storage are presented in Table 26. Higher flocculation of emulsion freshly prepared by DAH was recorded (2.13±0.08) as compared to that of emulsion prepared using DAH-EGCG conjugate (0.97±0.03) (P<0.05). The lower flocculation of DAH-EGCG conjugate based emulsion might be attributed to the higher magnitude of ζ-potential on OD, thus enhancing the repulsion force between OD and preventing the flocculation of OD (Staszewski *et al.*, 2014). After 15 days of storage, the flocculation of OD was significantly increased in both emulsion samples (P<0.05). Nevertheless, FOE stabilized by DAH-EGCG conjugate had the lower flocculation than that prepared by DAH (P<0.05). Similarly, lower coalescence value was recorded in conjugate based emulsion, compared to that found in emulsion stabilized using DAH (P<0.05). Flocculation and coalescence are the crucial parameters for indicating the instability of the emulsion. The increase in flocculation or/and coalescence contributes to the collapse of the OD and caused an increase in OD diameter (Intarasirisawat *et al.*,

2014). Chen *et al.* (2018) documented that porcine plasma protein hydrolysate-oxidized chlorogenic acid conjugate could enhance the stability of rapeseed oil emulsion during storage by reducing the flocculation and coalescence values. Thus, conjugation between DAH and EGCG could promote physical stability of fish oil emulsion by preventing the flocculation and coalescence of OD.

10.4.2.3 Confocal laser scanning microscopic (CLSM) images

Distribution of OD in the FOE systems incorporated with DAH or DAH-EGCG conjugate visualized by CLSM at day 0 and day 15 is depicted in Fig. 41. At day 0 of storage, the small OD uniformly dispersed in an aqueous phase of emulsions stabilized by DAH or DAH-EGCG conjugate and showed the mono-disperse emulsions (Fig. 42A). However, more uniform shape and smaller size of OD were observed in DAH-EGCG conjugate based emulsion, compared to those of DAH counterpart. An emulsion possessing the similar OD size is considered as a mono-disperse emulsion, while that having various sizes of OD is considered as a poly-disperse emulsion (Patil and Benjakul, 2017). After 15 days of storage, the larger size and non-uniform of OD were observed for emulsions stabilized by both DAH and DAH-EGCG conjugate, as evidenced by the formation of poly-disperse emulsions (Fig. 42B). Nevertheless, the smaller sizes of OD were still abundant in emulsion containing DAH-EGCG conjugate. The difference in OD sizes was mostly related to the difference in emulsifying properties between DAH and DAH-EGCG conjugate. Higher emulsifying properties of DAH-EGCG more likely yielded the FOE with smaller size of OD and higher magnitude of ζ- potential on OD, as compared to that prepared using DAH (Table 26). The DAH-EGCG conjugate with higher surface hydrophobicity could rapidly localize and adsorb in the oil-water interface, thus reducing the coalescence and flocculation of OD. Intarasirisawat *et al.* (2014) documented that skipjack roe protein hydrolysate incorporated with oxidized tannic acid could stabilize FOE, rendering the smaller size of OD and more uniform distribution, as compared to that stabilized by hydrolysate alone. Abd El-Maksoud *et al.* (2018) also found that β-lactoglobulin-caffeic acid conjugate could stabilize FOE with homogenous distribution of OD. These results suggested that DAH-EGCG conjugate could augment the stability of FOE.

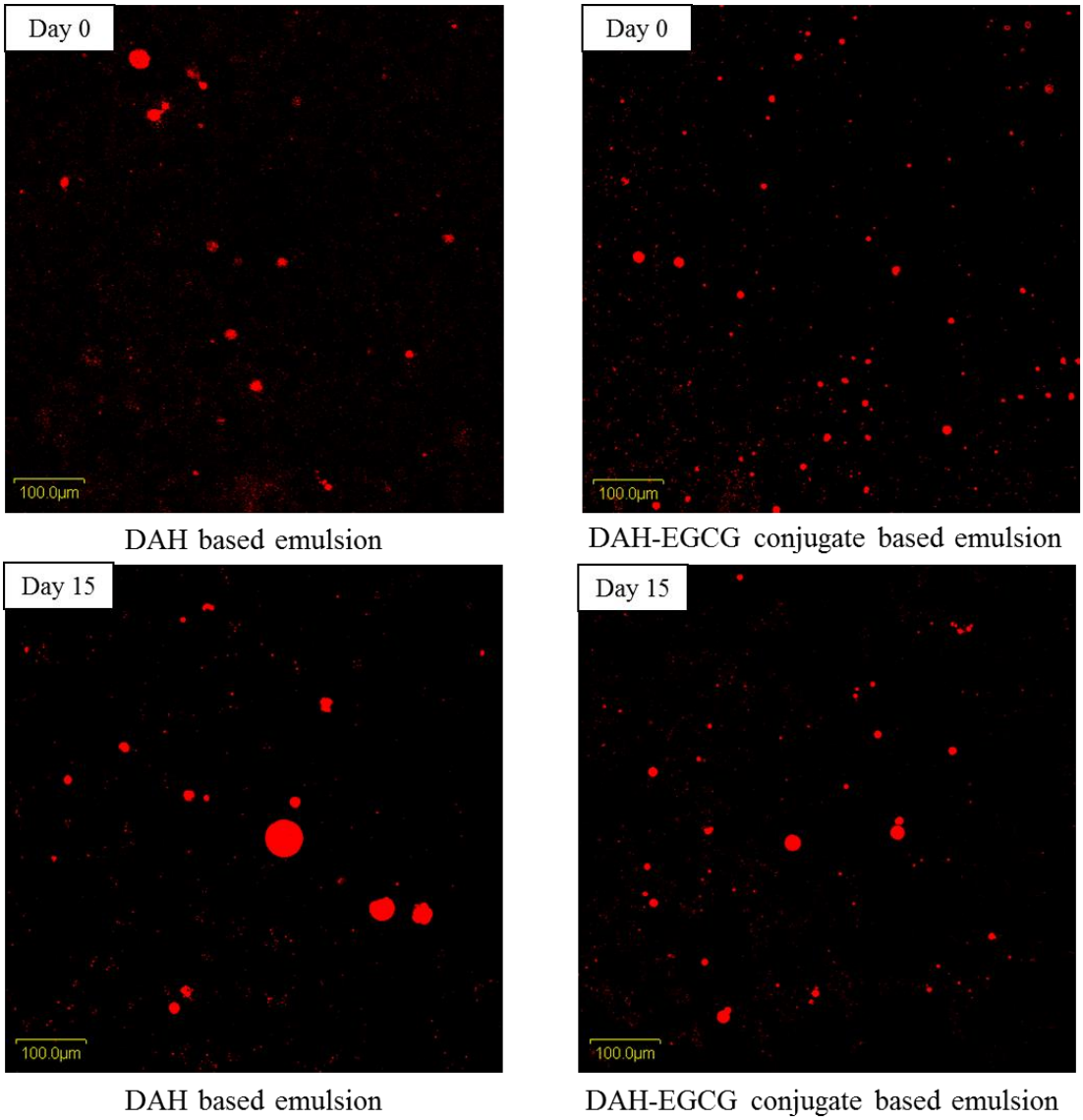

Figure 41. CLSM micrograph of fish oil emulsions stabilized by DAH or DAH-EGCG conjugate at day 0 and day 15 of storage at 26–28 °C. Magnification: 200×

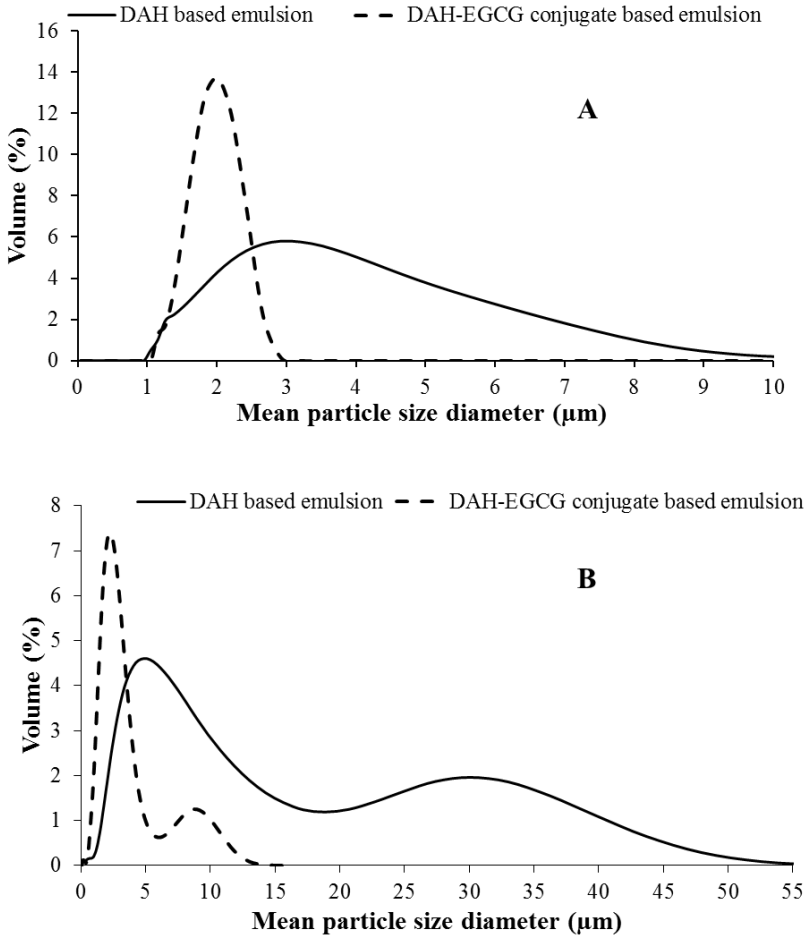

Figure 42. Particle size distribution of fish oil emulsion stabilized by DAH or DAH-EGCG conjugate at day 0 (A) and day 15 (B) of storage at.26–28 °C.

10.4.2.4 Lipid oxidation (LO)

Oxidation of FOE stabilized by DAH or DAH-EGCG conjugate during the storage was monitored in terms of PV and TBARS (Fig. 43). No marked change in PV of FOE stabilized by both DAH and DAH-EGCG conjugate (14.46–16.28 mg cumene hydroperoxide/L emulsion) (P>0.05) during the first 3 days of storage was recorded. The drastically increased PV was recorded in the FOE added with DAH after 3 days of storage (P<0.05) (Fig. 43A). Generally, PV is commonly used to measure the concentration of hydroperoxides (ROOH) formed in the initial phase of LO, indicating that emulsions were in propagation phase of the oxidative process with a lower rate of the decomposition of ROOH (Sae-leaw and Benjakul, 2017). However, the FOE containing DAH-EGCG conjugate had the lower rate of LO, compared to that stabilized

with DAH throughout the storage of 15 days. This result coincided with the higher antioxidant activities of DAH-EGCG conjugate than those of DAH (Table 24). EGCG conjugated with DAH could inactivate the pro-oxidants or/and scavenge the free radicals at interface of oil-water, thereby preventing the decomposition of ROOH into alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals (Feng *et al.*, 2018). Staszewski *et al.* (2014) documented the lower concentration of ROOH in FOE incorporated with polyphenol-β-lactoglobulin conjugate than that of emulsion added with β-lactoglobulin alone during storage at 25 °C for 30 days.

The oxidation of FOE was also evaluated with TBA assay, which measures the secondary products of LO formed by the decomposition of ROOH (Fan *et al.*, 2018). In general, TBARS of FOE containing DAH or DAH-EGCG conjugate increased with increasing storage time ($P<0.05$) (Fig. 43B). The TBARS values of FOE were 0.72–0.73 mg MDA/L emulsion at day 0 of storage. After 3 days, TBARS values were significantly increased (1.01 mg MDA/L emulsion) and reached the highest value at day 15 of storage (1.53 mg MDA/L emulsion) in FOE stabilized by DAH ($P<0.05$). Nevertheless, emulsion added with DAH-EGCG conjugate showed lower TBARS than that of emulsion incorporated with DAH at all the storage time ($P<0.05$). The increased TBARS value indicated the formation of secondary oxidative products including aldehydes in the later phases of LO (Takeungwongtrakul and Benjakul, 2013). This suggested that DAH conjugated with EGCG exhibited the higher free radicals scavenging activity than DAH alone. As a consequence, the oxidative stability of FOE could be enhanced by using DAH-EGCG conjugate. Apart from hydrogen and electron donating abilities of EGCG in conjugate, DAH-EGCG conjugate could form strong viscoelastic films around oil droplets and provide them with physical-chemical barrier against oxidation, which is commonly initiated at the oil-water interface. The conjugation of proteins and nonpolar EGCG could increase surface hydrophobicity of the modified proteins, thereby enhancing the emulsifying properties of the native proteins. Thus, the conjugate has been considered as the effective antioxidant emulsifier, which could localize and function at the oil-water interface and prevent the oxidation in fish oil emulsion (Tan *et al.*, 2018; Tan *et al.*, 2016).

These results coincided with those of Chen *et al.* (2019) who documented that LO of rapeseed oil-in-water emulsion was reduced when porcine plasma protein hydrolysate-chlorogenic acid conjugate was incorporated, as shown by lower TBARS and PV values, compared to that of emulsion added with hydrolysate alone. Feng *et al.* (2018) also revealed that PV and TBARS in FOE stabilized by ovalbumin-catechin conjugate were lower than those from emulsion stabilized by native ovalbumin. Additionally, Intarasirisawat *et al.* (2014) documented that skipjack roe protein hydrolysate modified by oxidized tannic acid could inhibit the LO of menhaden oil emulsion. The results suggested that DAH-EGCG conjugate could be used as an effective antioxidant for improving the oxidative stability of FOE.

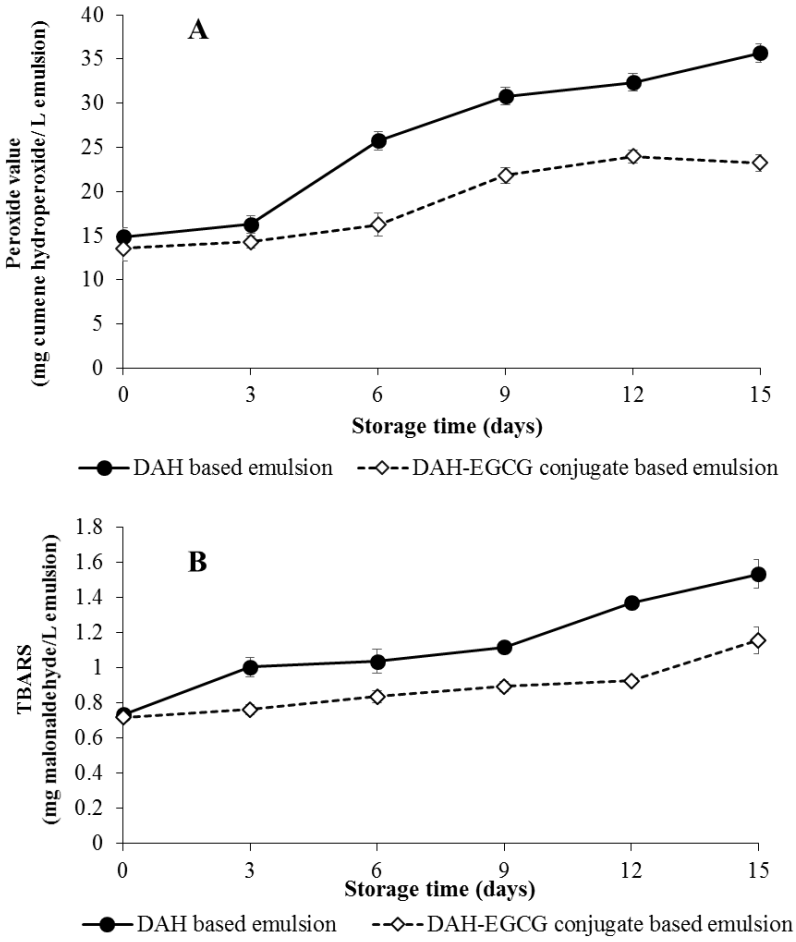

Figure 43. Peroxide value (PV) (A) and thiobarbituric acid reactive substances (TBARS) (B) of fish oil emulsion stabilized by DAH or DAH-EGCG conjugate during storage of 15 days at 26–28 °C. Mean±SD (n=3).

10.5 Conclusion

Conjugation between DAH and EGCG yielded the conjugate with increased AA and better emulsifying properties, especially when 4% EGCG was used. DAH-EGCG conjugate significantly improved physical stability FOE by lowering d_{43}, reducing flocculation and coalescence, but increasing magnitude of electrical charge of OD. Furthermore, DAH-EGCG conjugate improved oxidative stability of FOE during 15 days of storage more effectively than DAH alone. Thus, DAH-EGCG conjugate could be served as a potential natural antioxidant emulsifier used in food emulsion products.

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CHAPTER 11

QUALITY AND STORAGE STABILITY OF FISH TOFU AS AFFECTED BY DUCK ALBUMEN HYDROLYSATE-EPIGALOCATECHIN GALLATE CONJUGATE

11.1 Abstract

The impacts of duck albumen hydrolysate-epigalocatechin gallate (DAH-EGCG) conjugate at different levels (0.5-2.0%) on gel properties, sensory characteristics and storage stability of fish tofu during 21 days of refrigerated storage were evaluated. The incorporation of DAH-EGCG conjugate increased breaking force, deformation, hardness, chewiness, cohesiveness, gumminess, and springiness of fish tofu as the levels of DAH-EGCG conjugate were augmented (P<0.05). However, fish tofu containing DAH-EGCG conjugate showed lowered whiteness and L*, while ΔE*, a*, and b* values were augmented (P<0.05). Fish tofu containing 1.5-2.0% DAH-EGCG conjugate showed higher likeness scores than the control (P<0.05). Additionally, DAH-EGCG conjugate could effectively retard lipid oxidation (LO) of fish tofu during the storage ascertained by lowered increases in peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). Fish tofu incorporated with 1.5-2.0% DAH-EGCG conjugate had lower psychrophilic bacterial count (PBC) and total viable count (TVC) throughout the storage than those containing 0.5-1.0% DAH-EGCG conjugate and the control. Thus, DAH-EGCG conjugate could be considered as a potential protein cross-linker, antimicrobial agent, and antioxidant to assure quality and extend shelf-life of fish emulsion gels.

11.2 Introduction

Surimi-based seafood products have been widely consumed worldwide, especially in Asian countries, because of their unique textural property (Sae-Leaw *et al.*, 2018). Nevertheless, surimi raw materials have a low amount of desired ω-3 polyunsaturated fatty acids (ω-3, PUFA) associated with the removal of some lipids during washing process (Buamard and Benjakul, 2019; Pérez-Mateos *et al.*, 2006). In general, vegetable oils are commonly fortified in surimi-based products as an ingredient to improve whiteness as well as to modify the texture of resulting products (Sae-leaw

et al., 2017; Shi *et al.*, 2014). Fish tofu is a formulated fish meat based emulsion from surimi, in which gel is induced via heating process (Ketnawa *et al.*, 2016). Basically, fish tofu contains vegetable oil at a level in range of 5.0–15%, depending on producers (Yamsaengsung *et al.*, 2017). To enhance the level of PUFA, especially ω-3 fatty acids, fish oils such as seabass oil could be fortified in conjunction with vegetable oil in fish tofu. To make fish tofu, deep frying of prepared gels is a crucial process implemented at high temperature (170-180 °C). At such temperatures, fish tofu enriched with fish oil easily undergo LO, resulting in rancidity, or off-flavors, changed in nutritive quality, particularly during storage, thus reducing the shelf-life of the products (Pérez-Mateos *et al.*, 2004). Therefore, retardation or inhibition of LO of fish oil enriched emulsion using antioxidants, is necessary during processing and storage. Several natural antioxidants have been used in meat based emulsion products such as skipjack roe protein hydrolysate (Intarasirisawat *et al.*, 2014), shrimp hydrolysate (Ketnawa *et al.*, 2016), and snakehead (*Channa striata*) protein hydrolysate (Zakaria and Sarbon, 2018). Polyphenols have also known as the excellent antioxidants and have a profound role in inhibition of LO (Maqsood *et al.*, 2013). Buamard and Benjakul (2019) found that the fortification of coconut husk extract into sardine surimi gel emulsified with seabass oil could enhance oxidative stability and gelling properties of sardine surimi gels during refrigerated storage.

Modification of protein molecules by covalent grafting with polyphenol is an interesting technique for enhancing emulsifying property and antioxidant activities by attaching hydrophobic domains and hydroxyl groups into the protein molecule (Fan *et al.*, 2018). Recently, Quan and Benjakul (2019a) documented that emulsifying properties and antioxidant activities of DAH-EGCG conjugates were augmented, especially when 4% EGCG was used for conjugation with duck albumen hydrolysate. Furthermore, fish oil emulsion containing DAH-EGCG conjugate had lower LO than the control as proven by lowered PV and TBARS detected (Quan and Benjakul, 2019a). Nonetheless, no information concerning the impacts of DAH-EGCG conjugate on quality and storage stability of fish tofu has been documented. Therefore, this work aimed to elucidate gelling properties and storage stability of fish tofu enriched with seabass oil as affected by DAH-EGCG conjugate at different levels.

11.3 Materials and methods

11.3.1 Chemicals and surimi

Malonaldehyde (MDA), cumene hydroperoxide, and 2-thiobarbituric acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Frozen threadfin bream surimi (grade B), purchased from Chaichareon Marine Co., Ltd. (Pattani, Thailand) was kept in polyethylene bag and stored at −20 °C until used.

11.3.2 Preparation of DAH-EGCG conjugate

The procedure of Quan and Benjakul (2019b) was adopted for production of duck albumen hydrolysate (DAH) with degree of hydrolysis of 27%. DAH-EGCG conjugate was prepared as tailored by Quan and Benjakul (2019a).

11.3.3 Preparation of fish tofu

Fish tofu added with and without DAH-EGCG conjugate were prepared as per the procedure of Ketnawa *et al.* (2016). Fish tofu formulation (w/w) was as follows: 72.0% surimi from threadfin bream, 14.6% iced water, 12.0% seabass oil, and 1.46% salt. Surimi was blended with salt at a moderate speed using a mixer for 3 min at 4 °C. Temperature was maintained at 4 °C by adding iced water. DAH-EGCG conjugate at various levels (0.5, 1.0, 1.5, and 2.0%) was pre-emulsified with seabass oil by firstly dissolving DAH-EGCG conjugate in iced water and subsequently homogenizing with 12 mL of seabass oil at 13000 rpm for 3 min. Pre-emulsified oil was then added into fish tofu paste and blending was conducted for 3 min. All samples were adjusted to moisture contents of 70%. After stuffing into a 2.5 cm diameter casing, the samples were then steamed for 20 min, before cooling for 30 min in iced water. Thereafter, all the resulting gels were cut into 2.5 cm cylinders and subjected to frying in 10 volumes of palm oil for 3 min at 180 °C using a basket deep fryer (TEF-7 L, Samutprakan, Thailand). The fried fish tofu was equilibrated to room temperature prior to analyses.

11.3.4 Characterization of fish tofu enriched with seabass oil in the presence of DAH-EGCG conjugate at different levels of DAH-EGCG conjugate

Fish tofu added with DAH-EGCG conjugate at various levels were subjected to analyses.

11.3.4.1 Texture profile analysis (TPA)

Fish tofu samples were subjected to TPA using a texture analyzer (Model TA-XT2i, Stable Micro System, Surrey, England) as described by Vate and Benjakul (2016).

11.3.4.2 Breaking force (BKF) and deformation (DFM)

The protocol of Singh and Benjakul (2016) was adopted for determination of BKF and DFM of fish tofu using a texture analyzer (Model TA-XT2, Stable MicroSystems, Surrey, UK). Crush of fish tofu was removed using a sharp knife manually prior to analysis.

11.3.4.3 Color and whiteness

*L**, *a**, *b** of crust and inner portion of fish tofu were measured by a colorimeter (ColorFlex, HunterLab Reston, Virginia) (Buamard and Benjakul, 2018). Total difference of color (Δ*E**) was calculated using the following equation:

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

where Δ*L**, Δ*b**, and Δ*a** are the difference between color parameters of the samples and those of the control (without DAH-EGCG conjugate) having color parameters of crust (*L** = 55.34, *a** = 3.45, and *b** = 20.94) and those of inner portion (*L** = 73.37, *a** = 0.27, and *b** = 7.36).

The whiteness of fish tofu samples was calculated as followed equation:

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

11.3.4.4 Scanning electron microscopic (SEM) images

Microstructures of fish tofu samples were visualised using a scanning electron microscope (Quanta 400, FEI, Czech Republic), in which the protocol of Intarasirisawat *et al.* (2014) was adopted. Samples with a thickness of 2-3 mm were fixed with 25 mM glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). Fixed samples were washed with 0.1 M phosphate buffer (pH 7.2) for 10 min and then post-fixed in 0.2 M phosphate buffer (pH 7.2) containing 10 g/L osmium tetraoxide for 1 h. The fixed samples were then rinsed in 0.1 M phosphate buffer for 10 min and rinsed with distilled water for 10 min before being dehydrated in ethanol with serial concentrations of 50%, 70%, 80%, 90% and 100%. 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 at an acceleration voltage of 10 kV.

11.3.4.5 Sensory evaluation

Fifty untrained panelists were recruited for sensory evaluation. Panelists were requested to assess for odor, color, appearance, and overall likeness of fish tofu samples using a 9-point hedonic scale (Meilgaard *et al.*, 2010).

11.3.5 Storage stability of fish tofu enriched with seabass oil in the presence of DAH-EGCG conjugate at different levels

For storage stability study, fish tofu containing DAH-EGCG conjugate at different levels were packed in PE-LD zipper bags and stored at 4 °C. Analyses were made every 3 days for totally 21 days for PV, TBARS, and microbiological counts.

11.3.5.1 Peroxide value (PV) and thiobarbituric acid-reactive substances (TBARS)

PV was measured as per the procedure of Buamard and Benjakul (2019). TBARS value was examined as tailored by Egan *et al.* (1981).

11.3.5.2 Microbial load

Psychrophilic bacterial count (PBC) and total viable count (TVC) were enumerated as per the protocol of Sae-Leaw *et al.* (2018), in which log cfu/g was reported.

11.3.6 Statistical analysis

All the analyses and experiments were conducted in triplicate. The one-way analysis of variance (ANOVA) was done. The Duncan's multiple range test was carried out to analyze the significant difference among the samples at a level of $P<0.05$ using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

11.4 Results and discussion

11.4.1 Effects of DAH-EGCG conjugate at different levels on textural properties of fish tofu

11.4.1.1 Breaking force (BKF) and deformation (DFM)

BKF and DFM of fish tofu added with DAH-EGCG conjugate at various levels are presented in Fig. 44. BKF and DFM of fish tofu increased when the conjugate was incorporated ($P<0.05$). BKF of all samples was in the ranges of 129.3 – 438.7 g, in which the lowest BKF was found in the control sample (without addition of DAH-EGCG conjugate). Fish tofu added with 2.0% DAH-EGCG conjugate had the dramatical increase in BKF by 4 times, compared to that of control ($P<0.05$). However, the slight increase in DFM, the distance from the gel surface to the point of fracture, of fish tofu fortified with the DAH-EGCG conjugate was attained ($P<0.05$). No difference in DFM between the samples incorporated with 0.5% DAH-EGCG conjugate and the control was observed ($P>0.05$). Thus, the highest BKF and DFM were recorded in fish tofu incorporated with 2.0% DAH-EGCG conjugate. The results were in line with those of Zhou *et al.* (2019) who found that egg albumen protein modified by tea polyphenols could significantly increase BKF and DFM of gurnard surimi gels. Plant polyphenols, especially EGCG, have been known as the protein cross-linkers, since polyphenols are rich in hydroxyl groups, which can strengthen protein gel network via hydrogen bonding, hydrophobic-hydrophobic interaction, or other interactions (Zhao and Sun,

2017; Zhou *et al.*, 2019). Balange and Benjakul (2009) documented that BKF and DFM of surimi from mackerel were remarkably increased when oxidized polyphenols had been incorporated. Additionally, fish protein hydrolysates were also reported to enhance textural properties of fish emulsion sausage (Intarasirisawat *et al.*, 2014; Zakaria and Sarbon, 2018). Therefore, DAH-EGCG conjugate plausibly served as a cross-linker and induced polymerization between surimi proteins, thus resulting in the strengthened gel network. The results suggested that DAH-EGCG conjugate could improve BKF and DFM of fish tofu in a dose dependent manner.

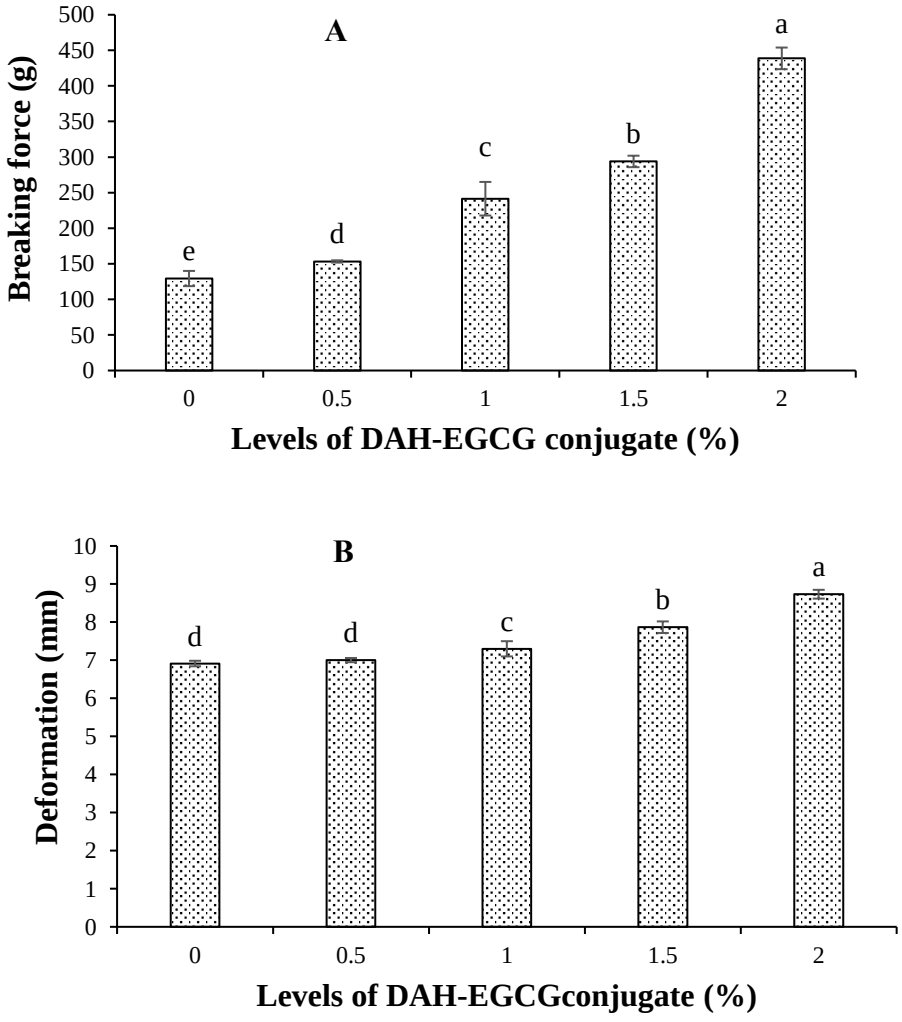

Figure 44. Breaking force (g) and deformation (mm) of fish tofu as affected by DAH-EGCG conjugate at different levels. Mean±SD (n=3). Different letters on bars indicate significant difference (P<0.05).

11.4.1.2 Texture profile

Table 27 shows texture profile of fish tofu in the absence and presence of DAH-EGCG conjugate at various concentrations. The incorporation of DAH-EGCG conjugate at all levels increased the cohesiveness, hardness, gumminess, springiness as well as chewiness of fish tofu (P<0.05). The augmented hardness, presenting as strength of gel structure under compression, of fish tofu was found when the levels of DAH-EGCG conjugate were increased. This result was associated with the increases in BKF and DFM (Figure 44). EGCG has been reported to have high binding affinity to proteins via non-covalent interactions (Chanphai *et al.*, 2018). DAH-EGCG conjugate more likely enhanced proteins cross-linking in fish tofu gel network. Moreover, DAH-EGCG conjugate possessing antioxidant activity could protect and maintain the integrity of muscle membrane against LO as well as reduce moisture loss during deep drying. This might help retain textural properties of fish tofu subjected to deep frying (Quan and Benjakul, 2019a; Zakaria and Sarbon, 2018).

Table 27. Texture profile of fish tofu added with DAH-EGCG conjugate at different levels

Levels of DAH-EGCG conjugate (%)	Hardness (N)	Cohesiveness	Springiness (cm)	Gumminess (N)	Chewiness (N.cm)
0	11.95±0.90$^{\dagger d}$	0.50±0.01^{d}	0.83±0.01^{c}	6.07±0.39^{d}	5.02±0.29^{d}
0.5	13.95±0.20^{c}	0.52±0.02^{d}	0.84±0.01^{c}	7.00±0.08^{cd}	5.90±0.13^{cd}
1.0	14.80±0.37^{c}	0.55±0.02^{c}	0.86±0.00^{b}	7.84±0.06^{c}	6.74±0.05^{c}
1.5	16.84±0.12^{b}	0.62±0.02^{b}	0.87±0.01^{b}	9.46±0.11^{b}	8.26±0.05^{b}
2.0	19.83±0.93^{a}	0.70±0.01^{a}	0.90±0.01^{a}	12.24±1.10^{a}	11.43±1.09^{a}

†Mean±SD (n=3). Different lowercase superscripts (a-f) in the same column indicate significant differences (P<0.05).

Springiness and cohesiveness of fish tofu were in ranges of 0.83-0.90 cm and 0.50-0.70, respectively (Table 27). The increased springiness and cohesiveness of fish tofu were gained as DAH-EGCG conjugate was added (P<0.05). Nonetheless, cohesiveness and springiness between the control and the sample added with 0.5% DAH-EGCG conjugate were similar (P>0.05). Springiness reflects how well the gel

springs back after deformation at the first compression, whereas cohesiveness measures the required force to overcome the internal bonds of gel network (Singh and Benjakul, 2017). Chewiness and gumminess of fish tofu were also increased continuously when the concentrations of DAH-EGCG conjugate were augmented (P<0.05). The highest values were recorded for the sample incorporated with 2.0% DAH-EGCG conjugate (P<0.05). The increases in chewiness and gumminess were related to the increased hardness. The results suggested that DAH-EGCG conjugate had ability to improve textural properties of fish tofu effectively.

11.4.1.3 Microstructure

Microstructures of fish tofu without and with DAH-EGCG conjugate addition at different concentrations are depicted in Fig. 45. Generally, fish tofu fortified with DAH-EGCG conjugate had less voids and more compact structure than that prepared without DAH-EGCG conjugate. High connectivity and denser gel network of fish tofu were line with the increased BKF, DFM, and hardness of fish tofu when high levels of DAH-EGCG conjugate were incorporated. Moreover, the size of oil droplets in the control fish tofu was larger as compared to that of droplets found in samples added with DAH-EGCG conjugate. DAH-EGCG conjugate at higher levels contributed to the formation of smaller oil droplet in the resulting fish tofu. This was more likely due to emulsifying properties of DAH-EGCG conjugate. Quan and Benjakul (2019a) documented that DAH-EGCG conjugate yielded fish oil emulsion with lower coalescence and flocculation of oil droplets as well as smaller oil droplets, compared to duck albumen hydrolysate alone. Zakaria and Sarbon (2018) also found that fish emulsion sausage incorporated with snakehead protein hydrolysate had smaller size of fat globules in the sausage as the levels of hydrolysate were increased. This finding also coincided with the result of Ghelichi *et al.* (2018) who revealed that fish sausages added with common carp (*Cyprinus carpio*) defatted roe protein hydrolysate had better gel network with smaller oil droplets and fewer voids. This result reconfirmed that DAH-EGCG conjugate could potentially improve gelling properties of fish tofu.

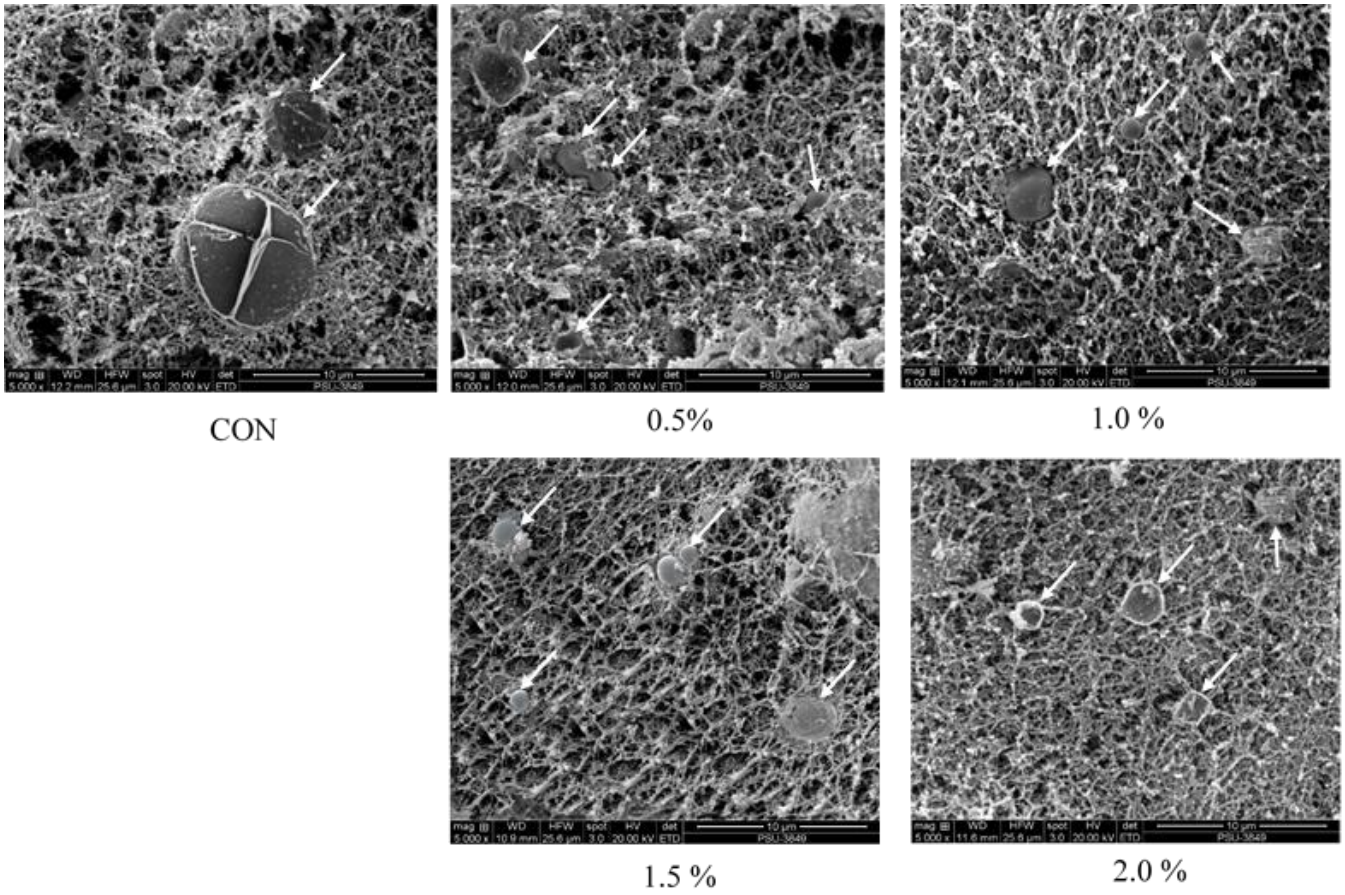

Figure 45. Scanning electron microscopic images of fish tofu without and with DAH-EGCG conjugate at different levels. Magnification: 5,000×. Scale bar = 10 μm. CON: without DAH-EGCG conjugate addition. Arrow signs indicate oil droplets.

11.4.2 Effects of DAH-EGCG conjugate at different levels on color of fish tofu

Color of fish tofu in the presence and absences of DAH-EGCG conjugate expressed as lightness (L^*), blueness/yellowness (b^*), greenness/redness (a^*), total difference of color (ΔE^*), and whiteness is presented in Table 28. The addition of DAH-EGCG conjugate slightly decreased the L^* and whiteness of both crust and inner portion of fish tofu ($P<0.05$). However, the addition of the conjugate at 0.5-1.5% had no effect on L^* and whiteness of crust (ranging from 55.34 – 53.75, and 49.70 – 47.25, respectively), while there was no difference in L^* and whiteness of inner part between samples incorporated with the conjugate ranging from 0.5 to 1.5% ($P>0.05$). The lowest whiteness and L^* values were recorded in fish tofu fortified with 2.0% DAH-EGCG conjugate ($P<0.05$). Nevertheless, the slight increases in both b^*- and a^*- values of both crust and inner part of fish tofu were found as higher concentrations of the conjugate were used ($P<0.05$). Redness and yellowness of inner part of resulting fish tofu were commonly from the pale yellow color of EGCG, especially its oxidation product generated during drying at high temperature (Nilsuwan *et al.*, 2018; Zhou *et al.*, 2019). The decreases in L^* and whiteness and the increases in b^*- and a^*- values of fish tofu, which finally resulted in an increase in ΔE^*, were noticeable as the concentration of DAH-EGCG conjugate increased for both crust and inner part. For the crust, caramelization or Maillard reaction might take place since surimi mostly contains sugar as cryoprotectant, which could undergo aforementioned reactions at high temperature during deep frying. Zhou *et al.* (2019) also documented that addition of egg albumen protein modified with tea polyphenols decreased L^* and whiteness of surimi gels. Additionally, the incorporation of 0.3% tannic acid resulted in the lowered whiteness of the resulting surimi gel from bigeye snapper (Balange and Benjakul, 2009). Maqsood *et al.* (2012) also revealed that there was an increase in ΔE^* value after fish emulsion sausages were added with tannic acid. The results indicated that the use of DAH-EGCG conjugate had an impact on the color of fish tofu to some extent.

Table 28. Color and whiteness values of fish tofu added with DAH-EGCG conjugate at different levels

	Levels of DAH-EGCG conjugate (%)	*L**	*a**	*b**	ΔE^*	Whiteness
Crust	0	55.34±1.26$^{\dagger a}$	3.45±0.46^{d}	20.94±1.00^{d}	0.36±0.09^{d}	50.55±1.20^{a}
	0.5	54.86±0.69^{a}	4.21±0.22^{c}	21.78±0.96^{cd}	1.60±0.25^{d}	49.70±0.97^{ab}
	1.0	53.86±0.59^{ab}	4.47±0.14^{bc}	23.33±0.47^{bc}	3.02±0.55^{c}	48.10±0.60^{bc}
	1.5	53.75±1.07^{ab}	4.79±0.13^{b}	24.89±0.73^{ab}	4.52±1.00^{b}	47.25±1.28^{c}
	2.0	52.53±0.03^{b}	5.41± 0.45^{a}	26.68±2.18^{a}	7.06±1.02^{a}	45.23±0.91^{d}
Inner portion	0	73.37±2.43^{a}	0.26±0.03^{c}	7.36±0.09^{b}	0.04±0.00^{c}	72.36±2.31^{a}
	0.5	69.17±1.07^{b}	0.27±0.02^{c}	7.40±0.09^{b}	4.19±0.58^{b}	68.30±0.58^{b}
	1.0	69.06±0.92^{b}	0.54±0.06^{b}	7.48±0.06^{ab}	4.32±0.92^{b}	68.16±0.88^{b}
	1.5	67.52±0.42^{bc}	0.63±0.01^{a}	7.55±0.12^{ab}	5.86±0.42^{ab}	66.65±0.39^{bc}
	2.0	67.31±0.84^{c}	0.64± 0.02^{a}	7.85±0.32^{a}	7.09±0.82^{a}	66.40±0.78^{c}

†Mean±SD (n=3). Different lowercase superscripts (a-f) in the same column under the same portion indicate significant differences (P<0.05).

11.4.3 Effects of DAH-EGCG conjugate at various levels on sensorial properties of fish tofu

No differences in odor, color, and taste likeness of fish tofu after DAH-EGCG conjugate was added at all levels (P>0.05) (Table 29). However, slightly higher scores for appearance likeness was noticeable for fish tofu incorporated with DAH-EGCG conjugate at concentrations higher than 1.0% (P<0.05), while no difference in appearance likeness between the control and the sample added with 0.5% DAH-EGCG conjugate existed (P>0.05). Fish tofu containing DAH-EGCG conjugate had higher texture likeness score than the control (P<0.05). This finding was in line with the increases in BKF, DFM (Fig. 44), and hardness (Table 27) of fish tofu incorporated with DAH-EGCG conjugate. Among fish tofu samples added with DAH-EGCG conjugate at various levels, that containing 2.0% DAH-EGCG conjugate had the highest texture likeness score (P<0.05). Nevertheless, there was no difference in texture likeness score between the samples incorporated with 1.5% and 2.0% DAH-EGCG

conjugate (P>0.05). The increase in texture likeness score probably resulted in higher score of overall likeness of fish tofu added with DAH-EGCG conjugate (P<0.05), indicating that DAH-EGCG conjugate had positive impact on the overall acceptability of fish tofu. Balange and Benjakul (2009) found that oxidized polyphenols had no adverse influence on the taste and color of the resulting surimi gels. The fortification of other natural antioxidants including skipjack roe protein hydrolysate (Intarasirisawat *et al.*, 2014), tannic acid (Maqsood *et al.*, 2012), or green tea extract (Jongberg *et al.*, 2013) into fish emulsion sausages did not had negative influence on sensorial attributes. Therefore, the addition of 1.5% or 2.0% DAH-EGCG conjugate more likely contributed to the improved organoleptic quality of fish tofu as indicated by increased texture and overall likeness scores.

Table 29. Likeness score of fish tofu added with DAH-EGCG conjugate at different levels

Levels of DAH-EGCG conjugate (%)	Appearance	Color	Odor	Texture	Taste	Overall
0	6.67±0.49$^{\dagger d}$	7.10±0.46^{a}	7.43±0.98^{a}	5.67±0.62^{b}	7.60±0.74^{a}	6.13±0.73^{b}
0.5	6.93±0.80^{cd}	7.13±0.64^{a}	7.47±0.83^{a}	5.93±0.76^{b}	7.63±0.96^{a}	6.60±0.75^{b}
1.0	7.23±0.70^{bc}	7.33±0.98^{a}	7.50±0.83^{a}	6.13±0.73^{b}	7.73±0.70^{a}	6.93±0.80^{ab}
1.5	7.53±0.83^{ab}	7.40±0.74^{a}	7.45±0.80^{a}	7.73±0.68^{a}	7.77±0.62^{a}	7.65±0.83^{a}
2.0	7.87±0.64^{a}	7.73± 0.70^{a}	7.55±0.56^{a}	8.27±0.70^{a}	7.83±0.74^{a}	7.93±0.72^{a}

†Mean±SD (n=50). Different lowercase superscripts (a-e) in the same column indicate significant differences (P<0.05).

11.4.4 Effects of DAH-EGCG conjugate at different levels on stability of fish tofu during refrigerated storage

11.4.4.1 Lipid oxidation (LO)

Figure 46 shows LO of fish tofu incorporated with DAH-EGCG conjugate at varying levels during the refrigerated storage of 21 days, in which PV and TBARS were monitored. At day 0, PV of control sample was higher than that of those containing DAH-EGCG conjugate (P<0.05). Generally, PV of all the samples augmented after 6

days of storage (P<0.05). Subsequently, the decreased PV was found as storage time was extended up to 21 days. This was plausibly because of the decomposition of hydroperoxide (ROOH), indicating that the oxidative process of fish tofu was in propagation stage. Nevertheless, the rate of LO was lower in the samples incorporated with DAH-EGCG conjugate than that of the control (P<0.05). The lowest PV was found in fish tofu fortified with 1.5% and 2.0% DAH-EGCG conjugate (P<0.05). It indicated that DAH-EGCG conjugate could delay the generation of primary products of LO in fish tofu. The same trend was also recorded in TBARS value, representing the secondary products of LO generated from the disintegration of ROOH. The initial TBARS values at day 0 were 3.12-3.19 mg MDA/kg sample, which continuously increased after 3 days of storage (P<0.05). TBARS values were lower in samples incorporated with 1.0-2.0% DAH-EGCG conjugate (P<0.05) than that of the control throughout the storage. The reduction of PV and TBARS might be due to inhibition of LO by DAH-EGCG conjugate, which had antioxidant activity (Quan and Benjakul, 2019a). DAH-EGCG conjugate could scavenge the free radicals and/or inactivate the pro-oxidant at the oil-water interface, thus lowering the decomposition of ROOH into variety of secondary oxidative products including ketone, acid, aldehydes, etc. (Sae-leaw and Benjakul, 2017). This finding was in line with that reported by Alirezalu *et al.* (2017) who documented that frankfurter type sausage incorporated with 500 ppm green tea extract possessed the lowest TBARS values during the refrigerated storage, compared to those of the control. Moreover, plant polyphenol extracts including green tea, grape seed (Li *et al.*, 2013; Wenjiao *et al.*, 2014), drumstick (*Moringa oleifera*) leave (Jayawardana *et al.*, 2015), Marcela extract (*Achyroclines satureiodes*) (de Lima *et al.*, 2017) have been documented to lower the LO of meat based emulsion products during the refrigerated storage. Additionally, protein hydrolysate such as skipjack roe protein hydrolysate (Intarasirisawat *et al.*, 2014) or shrimp hydrolysate (Ketnawa *et al.*, 2016) could prevent LO of sausage or fish tofu during the extended storage as reflected by the lowered TBARS and PV. The results indicated that DAH-EGCG conjugate could inhibit LO of fish tofu to some degree during storage at 4 °C.

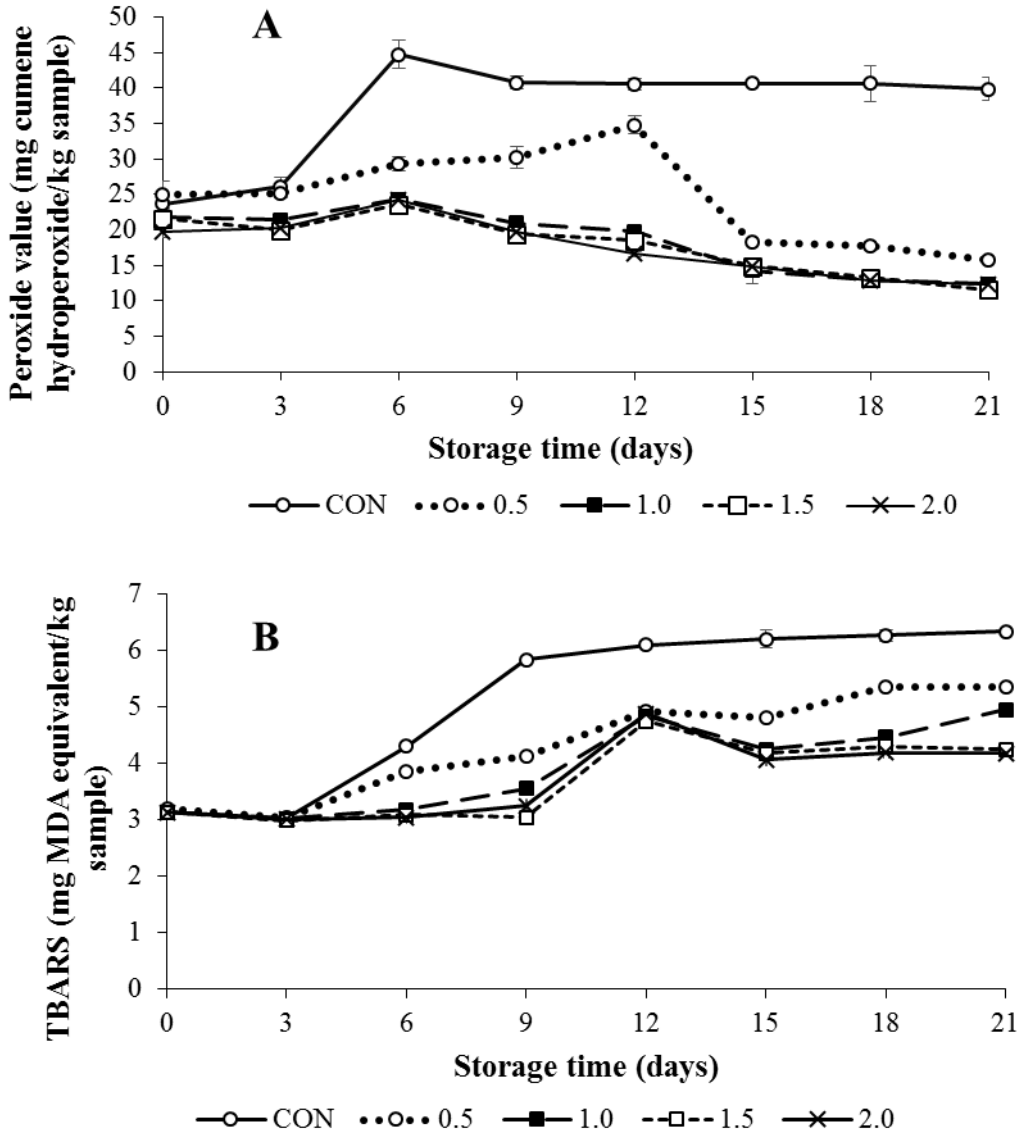

Figure 46. Effects of DAH-EGCG conjugate at different levels on (A) peroxide value (PV) and (B) thiobarbituric acid-reactive substance (TBARS) of fish tofu during 21 days of refrigerated storage at 4°C. Mean±SD (n=3). CON: without DAH-EGCG conjugate addition

11.4.4.2 Microbial load

PBC and TVC of fish tofu incorporated with DAH-EGCG conjugate throughout refrigerated storage are illustrated in Fig. 47. At day 0, TVC and PBC were not found, which might be due to the deep drying process used for preparation of fish

tofu. TVC and PBC significantly increased during storage (P<0.05). Nevertheless, there were no differences in TVC and PBC among all the samples after 6 days of storage, which were in ranges of 2.08-2.33 log cfu/g and 2.11-2.20 log cfu/g, respectively (P>0.05). TVC and PBC of the control reached 5.43 and 5.60 log cfu/g after 18 days storage, respectively, which were higher than those of fish tofu samples incorporated with 1.5% and 2.0% DAH-EGCG conjugate ranging from 4.30-4.32 log cfu/g and 4.90-4.92 log cfu/g, respectively. Bacterial count higher than 5.0 log cfu/g indicates the spoilage and deterioration of meat based products (Intarasirisawat *et al.*, 2014). At day 21, TVC and PBC of all the samples were higher than 5.0 log cfu/g. Nevertheless, lower TVC and PBC were noted in fish tofu fortified with 1.5% and 2.0% DAH-EGCG conjugate (P<0.05). Therefore, the incorporation of DAH-EGCG conjugate at high level impacted microbial load and shelf-life of fish tofu. The reduction of both TVC and PBC in fish tofu added with DAH-EGCG conjugate during storage might relate to antibacterial ability of EGCG in the conjugate. Papuc *et al.* (2017) documented that the galloyl moiety in EGCG can decompose the outer membrane and increase the permeability of cytoplasmic membrane of bacteria, thus altering ion transport processes and affecting respiration. Maqsood *et al.* (2016) found that TVC and PBC of camel meat sausage added with catechin and green tea extract were lower than those of control sample. This finding was also in line with that of Wenjiao *et al.* (2014) who revealed that pork sausage treated with tea polyphenol had the decreased TVC during refrigerated storage. Moreover, fish tofu added with 2.0% shrimp hydrolysate possessed the lowered mold, TVC, and yeast during 14 days of storage at 4 °C (Ketnawa *et al.*, 2016). The result indicated that DAH-EGCG conjugate at levels of 1.5% or 2.0% could be used as antimicrobial agent for extending shelf-life of fish tofu.

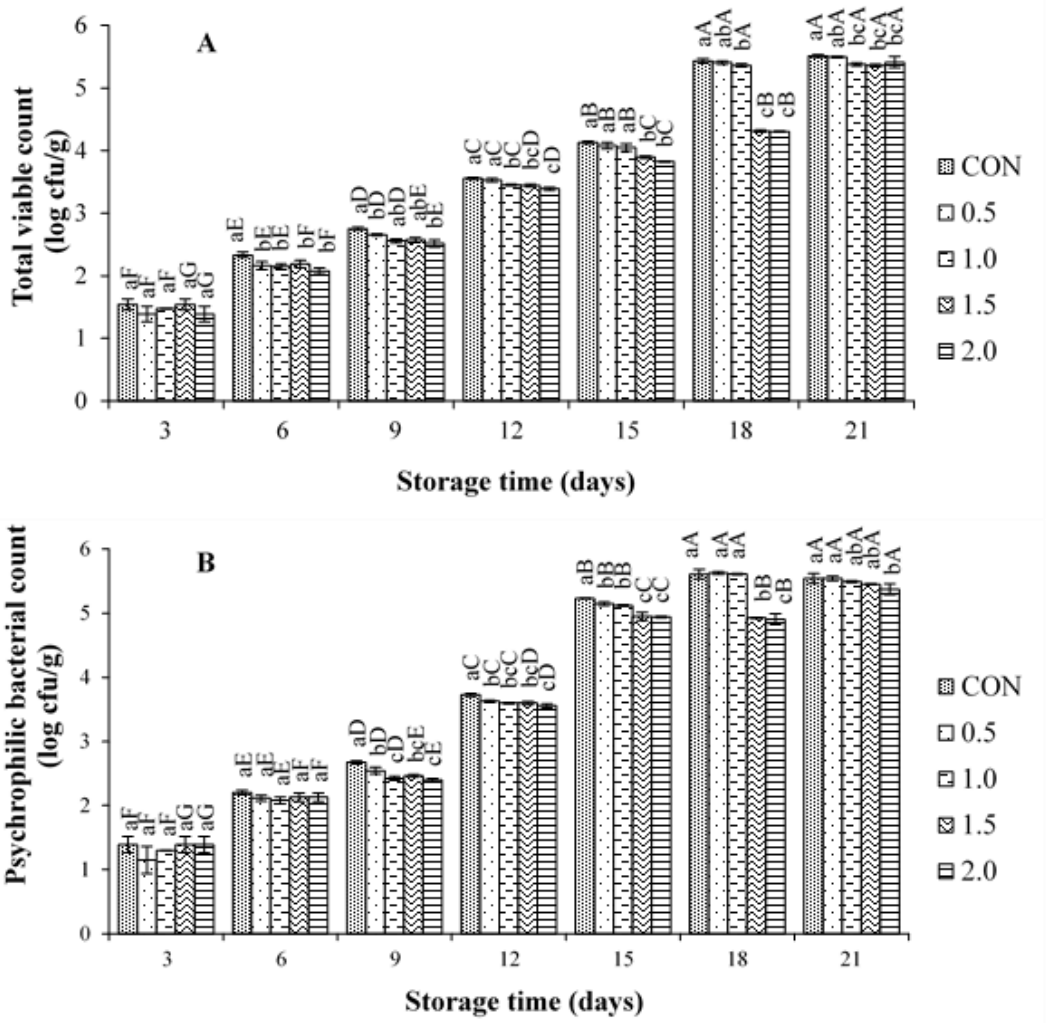

Figure 47. Changes in total viable count (TVC) (A) and psychrophilic bacterial count (PBC) (B) of fish tofu added with DAH-EGCG conjugate at different levels during 21 days of refrigerated storage at 4°C. Mean±SD (n=3). Different lowercase letters (a-c) on the bars within the same storage time indicate significant differences (P<0.05). Different uppercase letters (A-G) on the bars within the same sample indicate significant difference (P<0.05). CON: without DAH-EGCG conjugate addition.

11.5 Conclusion

The incorporation of DAH-EGCG conjugate could improve the textural properties of fish tofu by increasing BKF, DFM, hardness, cohesiveness, springiness, gumminess and chewiness, especially when 1.5% or 2.0% DAH-EGCG conjugate was used. Although the slight decreases in L^* and whiteness of fish tofu were recorded with

increasing levels of DAH-EGCG conjugate, the addition of DAH-EGCG conjugate improved the sensory properties of fish tofu with higher likeness scores. In addition, DAH-EGCG conjugate could extend shelf-life of fish tofu by lowering PV and TBARS values and retarding microbial growth during the extended refrigerated storage. Therefore, DAH-EGCG conjugate could be used as an effective protein cross-linker, antioxidant and antimicrobial agent in fish meat based emulsion products.

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CHAPTER 12

CONCLUSION AND SUGGESTION

12.1 Conclusion

1. Duck eggs stored at 4 °C could maintain quality and bioactive component, especially trypsin inhibitors during storage. Properties of albumen gel could be improved when eggs were kept for at least 3 days.

2. Salting process could reduce trypsin inhibitory activity in duck albumen to some degree. Protein with MW of 44 kDa acted as trypsin inhibitor in duck egg albumen. Gel became weaker with the increased whiteness as salting time increased.

3. Trypsin inhibitor (TI) from duck egg albumen was isolated and purified using Trypsin-CNBr-activated Sepharose 4B affinity column with high purity. TI was stable within the temperature range of 40–60 °C, pH of 7–9, and salt concentration lower than 5%. The inhibition mode of TI was classified as uncompetitive type with low Ki value.

4. The addition of hen and duck albumens in sardine surimi gel could reduce the degradation of muscle proteins, especially MHC. Duck albumen showed higher efficiency in enhancing the gelling properties of sardine surimi than hen albumen.

5. The optimization of desugarization for duck albumen was as follows: glucose oxidase/catalase concentration of 31.24/781 units/mL albumen and incubation time of 6.55 h. The whiteness and foaming property of powder could be improved by desugarization. Spray-drying of desugarized albumen with the inlet temperature of 160 °C yielded the powder with high foaming property.

6. Prior desugarization could markedly improve the whiteness and gelling properties of powder, in which higher gel strength with coincidental increases in hardness, gumminess, and chewiness were attained. Inlet temperature of 160 °C was recommended for spray drying of desugarized duck albumen.

7. The addition of salted duck albumen powder could improve the gel strength and color of surimi. Gelling properties and sensorial characteristics of surimi were enhanced when 3% salted duck egg albumen powder was used as salt replacer.

8. Heat treatment of duck egg albumen at 95 °C for 30 min with subsequent ultrasound pretreatment at 60% amplitude for 10 min could effectively increase the degree of hydrolysis (DH) of duck albumen. The highest antioxidant activities as well as emulsifying properties of duck albumen hydrolysate were found after 90 min of hydrolysis.

9. Conjugation between DAH and EGCG yielded the conjugate with increased antioxidant activity and better emulsifying properties, especially when 4% EGCG was used. DAH-EGCG conjugate significantly improved physical and oxidative stability of fish oil emulsion during 15 days of storage more effectively than DAH alone.

10. The addition of DAH-EGCG conjugate could improve textural and sensorial properties of fish tofu, especially when 1.5% or 2% DAH-EGCG conjugate was incorporated. Moreover, DAH-EGCG conjugate extended shelf-life of fish tofu by retarding microbial growth and lowering lipid oxidation during the extended refrigerated storage.

12.2 Suggestion

1. Impact of non-thermal pre-treatments (ultrasound, pulse electric field, and cold plasma) on quality, physicochemical, and functional properties of duck egg albumen during storage should be studied.

2. Bioactive peptides from duck albumen hydrolysate should be further isolated and identified.

3. Amino acid sequence of trypsin inhibitor from duck egg albumen should be determined.

4. DAH-EGCG conjugate should be studied as a nano-vehicle for the delivery of the selected nutraceuticals.

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