Chemical Compositions and Properties of Alkali Pickled Egg (Pidan) as Affected by Cations and Selected Pickling Ingredients

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ABSTRACT

Changes in chemical composition, physical property and microstructure of pidan white and yolk were monitored during pickling in the presence of different divalent ($CaCl_2$, $MgCl_2$) and monovalent (KCl) cations at different levels (0.2 and 0.5%) up to 3 weeks, followed by ageing for another 3 weeks. Pidan prepared following the commercial process, in which 0.2% PbO_2 or 0.2% $ZnCl_2$ was incorporated, was also tested. Hardness, cohesiveness and adhesiveness of pidan white gradually increased during pickling, but these parameters decreased during ageing time ($P<0.05$), regardless of cations used. Nevertheless, pidan white treated with 0.2% PbO_2 retained hardness and cohesiveness but had a slight decrease in adhesiveness, when pickling/ageing time increased up to week 6 ($P<0.05$). Yolk of pidan treated with 0.2% PbO_2 was semisolid with lower hardening ratio, hardness, cohesiveness and adhesiveness, compared with those from other treatments. Those treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ yielded higher hardening ratio, hardness and cohesiveness but lower adhesiveness than others. Transmission electron microscopic (TEM) studies of pidan white indicated that the aggregation of egg proteins took place in pidan white gels treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ during pickling. However the degree of aggregation varied with cations used. Confocal laser scanning microscope of pidan yolk indicated that the greater dehydration and release of lipids during pickling/ageing of 6 weeks.

Effects of selected cations (0.2% PbO_2, 0.2% $ZnCl_2$, 0.2% $CaCl_2$ or 0.5% $CaCl_2$) on chemical composition and microstructure of pidan white and yolk during pickling and ageing were investigated. During 3 weeks of pickling and further

3 weeks of ageing, ammonia and ash contents were increased but varied with the types of cations used. Lower protein degradation of pidan white was observed in pidan treated with 0.2% PbO_2, compared with other treatments. Scanning electron microscopic (SEM) studies indicated that the greater aggregation of egg proteins took place in pidan white treated with PbO_2. Therefore, various cations had varying impact on composition and microstructure of resulting pidan white and yolk.

Changes in physical properties of pidan white were monitored during pickling in the absence and presence of Chinese tea at levels of 2 and 5% together with selected cations, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$, up to 3 weeks, followed by ageing for another 3 weeks. Hardness and cohesiveness of pidan white treated with 0.2% $ZnCl_2$ (without Chinese tea) were more retained but showed a decrease in adhesiveness as pickling and ageing time increased up to week 6 ($P<0.05$). Higher browning intensity and a*-value were noticeable in the pidan white treated with Chinese tea at both levels at week 1, irrespective of cations used ($P<0.05$). Thereafter, the impact of tea on color was negligible. Thus, Chinese tea had no pronounced effect on physical properties of pidan white, whereas divalents showed the varying impact on textural property of pidan white. When acetic acid pretreated egg were used for pidan production in conjunction with the green tea and Chinese tea at levels of 2 and 5%, hardness, cohesiveness and adhesiveness of pidan white gradually increased during pickling, but subsequently decreased during ageing ($P<0.05$), regardless of types and amounts of tea used. Nevertheless, hardness and cohesiveness of pidan white treated without tea were more retained during ageing ($P<0.05$). Browning intensity (A_{420}), A_{294} and pH of pidan white increased with increasing pickling and ageing, while fluorescence intensity decreased during ageing ($P<0.05$), irrespective of treatments. Furthermore, higher browning intensity and a*-value were noticeable in the pidan white treated with either green tea or Chinese tea at higher level (5%), compared with the lower level (2%) ($P<0.05$). Browning of all pidans increased during pickling and reached the plateau at the first week of ageing. Acetic acid pretreatment along with the sufficient amount of tea generally accelerated the pidan formation and enhanced brown color development of pidan white.

Changes in texture and color of pidan white as influenced by glucose treatment at levels of 0, 2 and 5% were determined after pickling (week 3) and during the storage up to 12 weeks. Hardness and cohesiveness of pidan white without glucose treatment were more retained but showed a decrease in adhesiveness as storage time increased up to week 12 ($P<0.05$). Higher browning intensity and a*-value were noticeable in the pidan white treated with glucose at both levels as the storage time increased ($P<0.05$). Thus, glucose could enhance the development of brown color, mainly via the Maillard reaction with free amino groups of pidan white at alkaline pH.

Ion induced gelation under alkaline condition of pidan white was elucidated. Turbidity and particle size of 2 % egg white protein in 1% NaCl solution containing $CaCl_2$, PbO_2 or $ZnCl_2$ at a level of 0.1% increased as time increased up to 1 h, followed by a decrease ($p<0.05$). Nevertheless, the turbidity was more retained in samples added with PbO_2, suggesting high stability of aggregate formed. Zeta potential study revealed that the lower negative charge of the aggregates treated with PbO_2 was obtained, compared with other samples. Light microscopic studies indicated that the aggregation of egg white proteins was induced by ions but varied with the types of ions and incubation time. Therefore, PbO_2 exhibited the highest stabilizing effect on egg white protein under alkaline condition.

Effects of different cations on the characteristics of pidan white and yolk were investigated. Fourier transform infrared (FTIR) study of 0.2% PbO_2 and 0.2% $ZnCl_2$ treated pidan white and yolk had similar spectra to those of fresh egg. Mineral composition of white and yolk varied with the type of cations used in pickling solution. Scanning electron microscopic study showed that the more ordered network was found in 0.2% PbO_2 treated pidan white, compared with 0.2% $ZnCl_2$ treated counterpart. Confocal laser scanning microscopic study indicated that the lower release of yolk lipid was obtained in 0.2% $ZnCl_2$ treated pidan, compared with 0.2% PbO_2 treated counterpart. Thus cations in the pickling solution affected the characteristics of pidan white and yolk.

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CHAPTER 1

INTRODUCTION AND REVEIW OF LITERATURE

1.1 Introduction

Pidan or century egg also known as preserved egg, hundred-year egg, and thousand-year-old egg is a Chinese cuisine ingredient made by preserving duck, chicken or quail eggs in a mixture of clay, ash, salt, lime, and rice straw for several weeks to several months, depending on the method of processing. Egg curing in traditional methods is accomplished by introducing alkali hydroxide ions and sodium into the egg (Yang, 1994). The formation of pidan is caused by the penetration of alkali through the egg shell and membrane, leading to chemical changes in the egg components. This results in gelation of the albumen during pickling or mud coating at the alkaline condition. It has been believed that the use of tea, wood ashes and local clay gives the century eggs a more detectable taste and a characteristic terroir. Li and Hsieh (2004) reported that the color may be due to the Malliard reactions between the glucose of the albumen and amino acids. Additionally the pigment mainly from phenols of the tea contributes to the development of the brown-colored albumen gel. Cysteine and cysteine produced by protein hydrolysis can be continuously decomposed into ammonia and hydrogen sulfide, which contributes to the unique flavor of pidan. Hydrogen sulfide produced from the decomposed protein reacts with the iron in the yolk, which gives pidan its typical dark-green-colored yolk. The pine-floral-like structures formed between the yolk and albumen of pidan come from the degraded protein products such as alkalinized amino acids. In general, high-quality pidan has more pine-floral crystals.

In the traditional processes, some heavy metals are usually added to improve pidan quality. As a result, the products have high levels of lead and copper residues. Due to the safety concerns, most consumers typically request "lead-free" pidan. Alternatively, zinc is used to produce pidan with no black spots on the eggshell, and the color of the pidan's albumen and yolk was more stable (Chen and

Su, 2004). Nevertheless, no basic information on the quality of pidan produced from various cations has been reported. Additionally, the development of texture induced by different cations and phenolic compounds has not been clarified. Therefore, the study on the role of selected cations and tea phenolic compounds in the development of pidan with desirable quality attributes would be an approach to understand the gelation mechanism of pidan as well as factors governing the unique characterestics of pidan.

1.2 Review of Literature

1.2.1 Egg compositions

Egg is composed of three main parts; shell, albumen (egg white), and yolk. The yolk is surrounded by an albumen layer, and this structure is covered by a hard eggshell (Yamamoto *et al*., 1996). The weight of an egg and the weight distribution of three parts differ considerably, depending on the kind of egg and their age (Okubo *et al.*, 1997). An egg shell comprises a thin film of cuticle, a calcium carbonate layer, and two shell membranes. There are funnel-shaped small holes called pore canals on the surface of the shell for gas exchange. The pore canals are scatteringly located between the palisade layers of the shell, directed to the exterior. The diameter of the pore canal ranges from 10 to 30 μm. An egg has about 10,000 pore canals on the shell surface. The pore canal allows air and moisture to pass through, but does not allow liquid or water. The cuticle, the most external layer of eggs, is about 10 μm thick and covers the pore canals. It protects the egg from moisture and invasion of microorganisms (Board and Hall, 1973). The cuticle permits the exchange of gas in the egg. The cuticle is removed from the shell easily by soaking eggs in either weak acid solutions or metal chelator containing solutions or by washing with water (Belyavin and Boorman, 1980). Egg shell contains 95% inorganic substance, 3.3% protein, and 1.6% moisture (Okubo *et al.*, 1997). Calcium carbonate is the major component of the inorganic substances. The egg shell (palisade layer) is very dense and hard. Its crystalline structure is formed by calcification of calcium

carbonate and contains a small amount of magnesium, which constructs a spongy matrix together with collagen (Okubo *et al.*, 1997). The egg shell membrane is composed of inner and outer membranes. Their structure looks like entangled threads or randomly knitted net. This structure is important in obstructing the invasion of microorganisms by catching them in the meshwork (Okubo *et al.*, 1997).

The principal parts of the egg are the yolk and the white or albumen. The relative proportions of each of these constituents can vary considerably. The average values applicable to a hen's egg are: white 61.5%, yolk 29.0% and shell 9.5% (Linden and Lorient, 2000). A whole egg contains approximately 66% water, 11% mineral substances and 23% organic substances (12% proteins; 11% lipids) (Linden and Lorient, 2000). Albumen or egg white is made up of outer thin white, thick white, inner thin white and chalaziferous layer (inner thick white). The proportion of the layer varies, dependent on the breed, environmental condition, size of the egg and rate production (Romanoff and Romanoff, 1949). Egg white contains 9.7 to 12% proteins. Feeney (1964) characterized the proteins in albumen as follows: ovalbumin 54%; conalbumin 13%; ovomucoid 11%; lysozyme 3.5%; ovomucin 1.5%; flavoprotein-apoprotein 0.8%; proteinase inhibitor 0.1%; avidin 0.05%, and non-identified proteins 8%. The protein patterns of those components are shown in Figure 1.

Egg white consists of several proteins with the different characterestics and properties. Those are shown as follows:

1.2.1.1 Ovalbumin

Ovalbumin constitutes 54% of egg white's total protein (Zabik, 1992). Vadehra and Nath (1973) reported the existence of three ovalbumin fractions (A1, A2 and A3), detected by electrophoretic techniques. These fractions differ regarding the phosphorus content of their molecules. Ovalbumin A_1 has two phosphate groups per molecule; A_2 has one group, and A_3 has no phosphate group (Vadehra and Nath, 1973; Zabik, 1992). Ovalbumin is a monomer, globular phospoglycoprotein with molecular weight of 44.5 kDa, isoelectric point of 4.5, and has 385 amino acid residues (Nisbet *et al.*, 1981) with 50% hydrophobic residues (Powrie and Nakai, 1985; Zabik, 1992.). Ovalbumin contains 3.5% carbohydrates and has four free sulphydrilic groups and a disulfide bond. It can be denatured by heat exposure, by

surface absorption in films through agitation, or by the action of several denaturant agents (Vadehra and Nath, 1973).

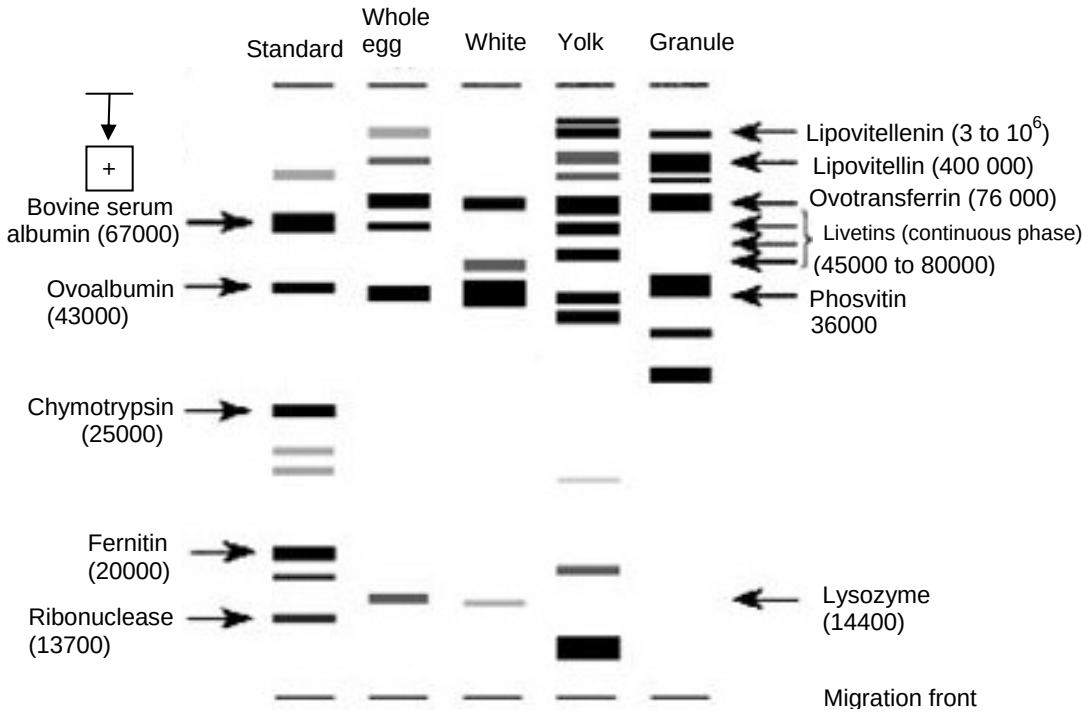


Figure 1. Electrophoregram of egg proteins. Migration carried out in 7.5% polyacrylamide gel

Source: Linden and Lorient (2000)

During storage, ovalbumin is altered to s-ovalbumin, an extra heat-stable form (denaturation at 92.5°C) in comparison to ovalbumin (denaturation at 84.0°C), as determined by thermogram of the differential scanning calorimeter (DSC) (Donovan and Mapes, 1976). S-ovalbumin has a slightly lighter molecular weight than ovalbumin and its relative quantity in the egg white can increase during the storage period, from 5% in fresh eggs to 81% after six months of refrigerated storage. Both pH and temperature also affect the s-ovoalbumin formation (Vadehra and Nath, 1973). The s-ovalbumin dependency on pH indicates a step of initial ionization involving a sulphydrilic group and an amino group, or even the dependency on the

concentration of hydroxyl ion, similar to an alkaline hydrolysis (Smith and Back, 1965). The conversion of ovalbumin to the extra stable form, s-ovalbumin, results from differences in the structure of covalent bonding (Zabik, 1992). When eggs are covered with oil, coated with whey concentrate protein (Alleoni and Antunes, 2004), or stored under refrigeration, this conversion is delayed as a result of the lower carbon dioxide loss by the pores of the egg shell. Alleoni and Antunes (1997) evaluated effects of storage periods and two temperatures on poultry eggs. S-ovalbumin contents were 18, 26 and 24% in eggs stored at 8°C for 7, 14 and 21 days, respectively. When stored at 25°C, eggs contained 56 and 69% s-ovalbumin at 7 and 14 days, respectively. Smith and Back (1965) showed that 95% of ovalbumin is converted to s-ovalbumin when an ovalbumin solution is kept at pH 10 and 55°C for 20 h. The maximum production of s-ovalbumin occurs at pH 9.2 and the minimum at pH 7.9 at 20°C (Nguyen and Smith, 1984).

1.2.1.2 Conalbumin or Ovotransferin

Conalbumin or ovotransferin is glycoprotein that represents 13% of egg white; its molecular weight varies from 60 to 95 kDa, with isoelectric point between 6.0 and 6.6 (Vadehera and Nath, 1973). Conalbumin can bind to metal ions and form a protein-metal complex resistant to denaturation by heat, pressure, proteolytic enzymes, and denaturants (Azary and Feeney, 1958). This protein is complexed with two moles of metallic ion per molecule with relative stability, and its ability to bind to Fe is related to its antimicrobial activity. Conalbumin has about 15 disulfide bonds and about 55% reactive residues (Zabik, 1992).

1.2.1.3 Ovomucoid

Ovomucoid is a glycoprotein bearing from 20 to 25% carbohydrates (Osuga and Feeney, 1977). It is resistant to heat denaturation in acid solutions, and present inhibitory activity to trypsin (Stadelman and Coterril, 1973). Osuga and Feeney (1977) observed that ovomucoid from poultry eggs inhibits the bovine trypsin but not the human trypsin. Ovomucoid's molecular weight varies from 26,100 to 28,300 Da, and its isoelectric point lies between 3.9 and 4.3 (Vadehra and Nath, 1973). This protein is stabilized by hydrophobic interaction and its high resistance to heat results from its high cystine contents and, consequently, to the high number of disulfide linkages. (Vadehra and Nath, 1973).

1.2.1.4 Ovomucin

Ovomucin is a glycosulphiprotein that contributes to the gel-like structure of the egg white thick layer. Ovomucin presents from one to 2% of egg white's total proteins (Vadehra and Nath, 1973), and differs from other proteins because its molecule is extremely large and contains sulphate ester, large amounts of cystine (interconnected through intermolecular linkages), and 50% of the total egg white sialic acid contents (Stadelman and Coterril, 1973). Ovomucin's molecular weight varies from 5.5 to 8.3 KDa. Ovomucin also has a substantial amount of disulfide bonds, and up to 33% carbohydrates (Vadehra and Nath, 1973).

1.2.1.5 Lysozyme

Lysozyme is a glycoprotein, a single polypeptide chain with 129 residues of amino acids linked by four disulfide bonds, representing 3.5% of the egg white; its molecular weight ranges from 14,300 to 14,600 Da, and its isoelectric point is 10.7 (Stadelman and Coterril, 1973). Lysozyme was previously placed in the ovoglobulin group and referred to as G_1. Lysozyme's acid and basic lateral chains and terminal groups are distributed in the molecular surface. The localization of polar chain also seems to be in the surface, although most non-polar chains (hydrophobic) are inside the molecule. One of the hydrophobic groups is localized in the protein's surface, where the protein's active site is located (Li-Chan and Nakai, 1989).

1.2.1.6 Ovoglobulins G_2 and G_3

Ovoglobulins encompass altogether 0.4% of the total egg white's proteins. Their molecular weights range from 30,000 to 45,000 Da, and their isoelectric points are 5.5 and 5.8 for G2 and G3, respectively. Ovoglobulins have 3.2 to 3.7% hexoses and 2.4 to 2.5% hexose amines (Vadehra and Nath, 1973).

Yolk is a dispersion of particles (granules) in a continuous aqueous phase (plasma). The composition of granules and plasma was reported by Anton and Gandemer (1997). Granules contain mainly 70% high-density lipoprotein (HDL), 16% phosvitin and 12% low-density lipoproteins (LDL). Plasma is composed of 85% LDL and 15% livitin (McCully *et al*., 1962). The yolk is actually a source of lipids, which are easily dispersed in water, thus permitting emulsification of other substances. These properties are due to their high content in phospholipids and to the

fact that all the lipids (including the triglycerides) are associated with at least two proteins, vitellin and vitellenin (Linden and Lorient, 2000).

Chemical hydrolysis of protein is achieved by cleaving peptides bonds with either acid or base. It is relatively inexpensive and quite simple to conduct. There are, however, many limitations to produce food ingredients. Protein hydrolysis with strong chemicals and solvents is performed at extreme temperatures and pHs and generally yields product with reduced nutritional qualities, poor functionality and restricted to use as flavor enhancer.

1.2.2 Gelation of egg proteins

Egg proteins possess physical-chemical functional properties that contribute to the desirable characteristics of a food and its physical behavior during preparation, transformation and storage. Egg proteins undergo gelation under the appropriate condition. Gel is an intermediary form between solid and liquid. It is the cross-linking among polymeric molecules which make an intermolecular network within a liquid medium. In food systems, this liquid is water, a solvent which affects the nature and the magnitude of intermolecular strengths that keep the integrity of the polymeric network. This network retains water, avoiding losses (Oakenfull, 1987). Food processing and the development of new products require ingredients such as the gelling agents, which build up a structural matrix that supplies food's desirable texture (Phillips *et al*., 1994). Protein gel formation can be primarily described as a two-step process. The first step involves changes in the conformation usually induced by heat or partial denaturation of the protein molecule by chemicals. With denaturation, the dispersion velocity increases as a result of increasing molecular dimensions caused by unfolding of the protein molecule (Ferry, 1948). In the second step, a gradual association or molecule aggregations of denatured proteins leads to an exponential increase of viscosity, and to the formation of a continuous network. Process formation in this step is slower, in comparison to the first step, and ends when an organized network is formed. If the 2^{nd} step occurred any faster, a disorganized protein cluster (a coagulum) would be formed; this coagulum would not be able to hold water, consequently resulting in syneresis (Phillips *et al.,* 1994).

The establishment of gel networks at 85 to 90°C is attributed to the formation of covalent linkages, to the changes of thiol group to disulfide linkages, and to hydrophobic interactions (Phillips *et al*., 1994). These interactions between nonpolar segments of adjacent polypeptides occur only if these polypeptides are opened, induced by heating. The first step of thermal coagulation comprises the formation of disulfide linkages which exposes hydrophobic groups (Shimada and Matsuhita, 1981). During heating, egg white is polymerized by intermolecular exchange linkages from sulphydrilic groups to disulfide linkages, which makes a network. Thermocoagulation requires balance of electrostatic attractions between protein molecules and hydrophobic interactions during the gel formation (Ma and Holme, 1982). The physical integrity of gels depends on the balance between attractive and repulsive strengths of the protein molecules involved in the system (Hermanssom, 1979; Schmidt, 1981; Kinsella, 1984; Mulvihill and Kinsella, 1987; Ziegler and Foegeding, 1990). If the attractive strengths predominate, a coagulum is formed, and water is driven off the network matrix. If the repulsive strengths dominate, a three-dimensional network can not be formed (Kinsella, 1984).

The nature and properties of gels are influenced by several factors, such as protein concentration, solution pH, nature, and concentration of the electrolyte (Mulvihill and Kinsella, 1988). Gelation can occur during heating or cooling and depends on the nature of the protein and on the process itself. The heat-induced formation of translucent gel network involves the ordinate association of unfolded chains of polypeptides through non-covalent bonds (e.g. hydrogen bonds, ionic and hydrophobic interactions) and in some cases, through covalent bonds (disulfide linkages) (Hermanssom, 1979; Xiong and Kinsella, 1990). Differences in gel-forming ability among globular proteins generally reflect the variety of degrees of protein-protein interactions and the number and extension of interactive sites available within the opened molecule (Phillips *et al*., 1994). The intermolecular disulfide linkages increase the stability of the gel matrix. The increased size of polypeptide chains can delay the rupture of non-covalent interactions, and favor the gel network stability. The inaccessible thiol groups become exposed and can be activated by the unfolding of protein molecule during heating, or by alterations in the solvent conditions (Kella and Kinsella, 1988). Consequently, the reactive thiol groups are exposed and can yield

intermolecular disulfide bond (Schmidt *et al*., 1979). The reactions of intermolecular changes of thiol-disulfide groups can promote an increase of crossed disulfide linkages within the gel matrix. The dimerous molecule formed by this reaction can keep on reacting with sulphydrylic groups in other protein molecules, producing the necessary cross-linking to gelation (Phillips *et al*., 1994).

1.2.2.1 Heat induced gelation

a) Egg white

Egg white proteins undergo thermal gelation or coagulation differentially. Only ovomucoid and ovomucin are not coagulable by heat (Johnson and Zabik, 1981); except when complexed with Fe or Al. Conalbumin is especially sensitive to heat (Cunningham and Lineweaver, 1965). The denaturation temperatures of conalbumin, globulin, ovalbumin and lysozyme are 57.3, 72.0, 71.5, and 81.5°C, respectively (Yang and Baldwin, 1995). Montejano *et al.* (1984) reported that the rigidity of the egg white started at 71°C and increased at 83°C, and the elasticity developed between 70 and 74°C. Denaturation of ovalbumin occurred from 79° to 84°C. The increase of temperature and the heating period improve the linkages with water molecules and increases cross-linking in the gel structure (Yang and Baldwin, 1995). The stability of s-ovalbumin regarding the denaturation by heating can affect the gelation properties of egg white. Shitamori *et al*. (1984) reported that even though the profiles of pH in gel formation was similar between ovalbumin and s-ovalbumin, the resistance of heat-induced s-ovalbumin gel was lower than that of ovalbumin at several temperatures. Alleoni and Antunes (2005) reported that higher hardness in egg white gels was obtained when pH varied from 9.0 to 9.45 than when pH varied from 7.7 and 8.1. S-ovalbumin can, along with other proteins, increase hardness of albumen gel. When pH is around 9.0, there is an increase in the percentage of this protein in the egg white. However there is a minimum transformation from ovalbumin to s-ovalbumin when pH ranges from 7.0 to 8.0. Alleoni and Antunes (2005) observed an increased expressible moisture in egg white gels at pH between 7 and 8, in comparison to those with pH around 9. Heating is needed to achieve the optimum hardness, but can also bring on undesirable syneresis (Lowe, 1955). The syneresis may have resulted from supercoagulation, when the whole egg and the egg white were cooked in microwaves (Chen and Hsu, 1981).

b) Egg yolk

Yolk proteins have been known to possess gel forming ability. Gel network formation of yolk is attributed to the denaturation of its proteins, leading to molecular interactions and development of a hard and rubbery structure (Kiosseoglou, 2003). Yolk is not a pure protein solution, but rather a dispersion of particle (LDL micelles and HDL granules), where the neutral triglycerides are buried in the particle interior, while the protein dominate the particle surface (Paraskevopoulo and Kiosseoglou, 1997). The gelation of yolk, therefore, can be envisaged as a process of yolk particle destabilization brought about by denaturation, upon heating of particle-stabilizing yolk protein molecules, leading to an interparticle network formation (Kiosseoglou *et al.*, 2005). Anton *et al*. (2003) reported that yolk gelation process is dominated by LDL apolipoprotein molecular interactions. Granules were less effective in gel network development. This difference in behavior was attributed to lower sensitivity of granular protein due to their globular structure, compared to that of LDL apolipoprotein, which denature at a relatively lower temperature (Le Denmet *et al*., 1999). Heat treatment of yolk may lead to gels exhibiting elasticity (Woodward and Cotterill 1987). According to Woodward and Cotterill (1987), raw yolk is made up of "spheres" in adjoining polyhedrons (grains) ranging in size from 40 to 100 μm and capable of gelling. Gentle stirring disrupts 90 to 95% of these grains releasing protein into the solution and allowing the formation of a three-dimentional protein network with a hard, cohesive and rubbery texture during heating. Raikos *et al*. (2007) reported that the gelation of whole egg, white and yolk protein was induced by heat. When heated, yolk proteins unfolded and interacted to form high-molecular weight aggregates.

Guerrero *et al*. (2004) studied the thermally-induced transitions of egg yolk protein and found that gelation was affected by the pH, ionic strength and salt type. Kiosseoglou and Paraskevopoulou (2005) studied that molecular interaction in gel of egg yolk and its plasma and granules fraction upon heating at 90°C for 30 min in the presence of D, L-dithiothreitol (DTT), N-ethylmaleimide (NEM) or Tween 40 to establish the role of disulfide covalent bonds and hydrophobic interactions between yolk protein constituents in the formation of gel network structure. It was found that yolk and its plasma fraction exhibit a similar behaviour. The involvement of disulfide

bonds between their protein constituents in gel structure development was elucidated. Granule fraction gels, on the other hand, were not markedly affected by disulfide bond-splitting or sulfhydryl group-blocking reagent.

1.2.2.2 Ion induced gelation

Ion induced gel network depends mainly on the physicochemical conditions of the medium (specifically pH and type of salts) (Croguennec *et al*., 2002). Cunningham and Cotterill (1962) reported the phenomenon of alklained gelled albumen. Time required for gelation depends upon the strength of the alkali, the rate of pH changes as well as temperature. Lin and Liao (1973) observed that gelation of the duck egg albumen at pH levels above 11.8 resulted in a translucent gel. Also the time required for the gelation decreased as the pH was increased. However the gelled albumen became viscous liquid when the pH was above 13.6. If held at the room temperature with pH values between 12.0 to12.8, the liquefaction did not occur.

Salt has been known to have impact on gelation of egg white proteins. Bull and Breeze (1970) found that several salts decreased the amount of water bound to the albumen. Egg albumen contained many hydrophobic amino acids and easily formed turbid coagulum even at an alkaline pH when salts are added. Removal of naturally occurring salts from egg white impaired its ability to coagulate. At pH values sufficiently far from the isoelectric point of the proteins and at low ion concentrations, the unfolded proteins tend to remain separate due to the electrostatic repulsive forces between molecules. Upon the addition of salt, these repulsive forces are screened, and the protein molecules can aggregate and form a gel (Barbut and Foegeding, 1993; Ju and Kilara, 1998). Monovalent and divalent ions are able to screen electrostatic interactions between charged protein molecules (Yasuda *et al*., 1986). Divalents salts tend to be more effective in aggregation than the univalent ions, while the influence of polyvalent salts is moderate (Nakamura *et al.,* 1984). The resulting size, shape and spatial arrangement of the protein aggregates and their response to deformation can therefore vary widely and have an impact on gel. The concentration of salt used to form a gel is of the major determinants of the structure and spatial organization of the protein aggregates (Hongsprabhas *et al*., 1999). Low-salt concentrations produce filamentous type gels, while higher concentrations induce the formation of particulate gel (Hongsprabhas and Barbut, 1997). Salt at very low

concentration aids in protein solubilization prior to heating and provides a cross link in the network (Mulvihill and Kensilla 1988). Nevertheless, further addition of salts simply promotes aggregation. Shimada and matsushita (1981) observed that critical pH for coagulation formation of egg white shifted to a more alkaline pH as salts concentrations increased. The effect of ions on egg albumen coagulation indicated an order of sulphate>chloride>bromide for anions and calcium>lithium>sodium for cations.

1.2.3 Pidan

Pidan has been known as an alkaline-fermented ethnic food for many generations in China. Wang and Fung (1996) prepared pidan using pickling solution (4.2% NaOH and 7.0% NaCl) at room temperature (20° C) for 11 days, and pidan white gel had pH of 11.18 and yolk had pH of 9.16. Su and Lin (1992) showed that duck eggs preserved in solution (4.2% NaOH and 5.0% NaCl) at room temperature (20-25°C) for 12 days had the egg yolk pH of 9.16 and the addition of alkaline zinc salt plays a catalytic role in pidan formation. However, the gelation was not significant. Hydrogen sulfide formed during the pidan formation interacts with the zinc, forming zinc sulfide compounds (Wang and Fung, 1996). Manufacturers often add heavy metals (such as lead, copper) in preserved eggs, pidan. When duck egg was immersed with different impregnating solutions containing $ZnCl_2$ or $ZnSO_4$ for some time, there was no black spots and decoloration during extensive storage of eggs. The dark greenish pidan is formed by the gelation of egg white and yolk protein in alkalized condition and sophisticate flavor of this product has resulted in it being known as the “thousand-year-old egg” in Western society. Depending on the processing methods, several types of pidan are available, such as the pine-floral pidan, soft yolk pidan and hard-yolk pidan (Wang and Fung, 1996). In traditional pidan processing, different methods can be found among different places.

1.2.3.1 Classification of pidan

Processing methods and chemical changes involved in making pidan can be used to classify the pidan (Wang and Fung, 1996).

a) Processing methods

1. Rolling powder method

Traditionally, fresh duck eggs are used. The eggs are coated with a thin layer of mud paste and rolled in the rolling powder, in which all the ingredients have been included, before being packed and sealed in the jar. The powder-rolled eggs are allowed to ferment for 20-30 days at room temperature. The ingredients used for the rolling powder method vary slightly according to the season. The rolling powder method produces hard yolk pidan. The advantages of this method are its low cost and ease in handling.

Table 1. Ingredients for rolling, coating and immersion method

Ingredient	Rolling		Coating	Immersion
	Amount (Kg)			
	Spring and Fall	Summer	Summer	Summer
Na_2CO_3	1.5	1.5	10	3.6
CaO	3.8-4.2	4.5-5.5	25	14
PbO	-	-	0.45	0.37
Salt	1.4	1.4	4	2
Tea	0.1	0.1	-	1.5
Water	3	3	50	50
Yellow earth	2	2	-	-
Dry mud	-	-	25	0.5
Wood ash	-	-	25	1
Pine needle	-	-	-	0.25

Source: Wang and Fung. (1996) and Li and Hsieh (2004)

2. Coating method

A muddy paste containing the coating ingredients is required for pidan production. The following ingredient shown in (Table 1) was reported by Wang and Fung (1996). Muddy paste can be used for 1000 eggs. Fresh duck eggs are completely coated with the prepared muddy paste and rolled in rice hulls. The rice hulls prevent the coated eggs from sticking together. They are then placed into jars, sealed with mud and allowed to ferment for 40 days in the summer and 50 days in the winter.

The sealing step is very important for producing a high quality product. A well sealed jar prevents the mud coating from becoming dry. This coating method

prevents soft yolk pidan. Another coating method as reported by Lin (1973) is described as follows: For 100 eggs, 125 g of sodium carbonate, 625 g of wood ash, 1000 gm of calcium oxide, 100 g of salt and 500 g of water were mixed to form a paste. The surface of the eggs was coated with 1 cm thickness of paste and then rolled in rice hulls. Coated eggs are then packed in wooden jar and then sealed. After 5-6 months of pickling, the product is ready for sale**.**

3. Immersion method

In this method, all of the ingredients are mixed into a curing solution. Eggs are immersed in the solution for 45 days at 20-25°C. After the curing process is completed, the eggs are removed, washed with water and allowed to air dry. The eggs are further coated with liquid paraffin before packaging and marketing. The following pickling composition was reported by Wang and Fung (1996) (Table1).

According to Wang and Fung (1996), the pickling ingredients are unevenly distributed in the brine and there may be three different layers of pickling brine existing in jar. For an improved method, the brine is allowed to flow during the fermentation for an even development of aroma and color. In the Immersion method, ginger and cinnamon are added to the pickling solution to reduce the pungent taste. Lin (1973) suggested immersing 1000 eggs for 2 weeks in a mixture of 5.63 Kg of sodium carbonate, 5.63 Kg of salt, 0.38 Kg of charcoal, 3.6 litres of tea and 36 litres of water at temperature of 30°C. Sodium hydroxide can be used instead of sodium carbonate and calcium oxide in the pickling or mud coating. The alkali penetrates the egg shell and membrane and causes chemical changes in the egg components, which results in gelation of the albumen (Wang and Fung, 1996).

b) Appearance of pidan egg yolk

Pidan can be classified into two categories, according to whether the yolk is semi-solid or hard (Wang and Fung, 1996).

1. Semi-solid yolk pidan

The semi-solid yolk has a pleasant, fragrant taste without any pungent lime flavor and with no after-taste. In processing of eggs to obtain a semisolid yolk, the paste or coating fluid has less table salt and lower alkalinity and contains a small amount of lead oxide (Wang and Fung, 1996).

2. Hard yolk pidan

Pidan with hard yolks has a slightly pungent, somewhat salty taste and a longer-lasting after taste. The amounts of the ingredients in the coating mixture used for hard-yolked pidan are somewhat different, and lead oxide is omitted.

1.2.3.2 Chemical changes of pidan during processing

After the ingredients to make pidan are mixed, the following chemical reactions take place (Blunt and Wang, 1918):

$CaO + H_2O \rightarrow Ca(OH)_2$

$Ca(OH)_2 + Na_2CO_3 \rightarrow 2NaOH + CaCO_3$

$Na_2O_3 + H_2O \rightarrow 2NaOH + O_2$

$K_2O + H_2O \rightarrow 2KOH$

(Na_2CO_3 and K_2O are from the plant ash.)

Because of the porosity of the egg shell, NaOH is first adsorbed to the surface. Owing to a change in the osmotic pressure, NaOH enters the egg through the pores and subsequently penetrates the semi-permeable membrane, coming into contact with the egg protein, causing it to become denaturized and hydrolyzed into polypeptides and finally into amino acids (Blunt and Wang, 1918). The best pidan product can be obtained when the NaOH concentration is maintained at 3.6 to 4.6 %. A concentration of 3.6 % is able to coagulate the egg protein within 4-8 days, while a 4.6 % concentration coagulates the protein within 2-4 days. With the former concentration, the pidan ripens in 55 to 65 days: For the latter, it takes 35 to 40 days. When NaOH concentration is lower than 1.6 %, the egg protein does not coagulate, and thus the end-product is not pidan. The most suitable processing temperature is between 20 and 30°C. The higher temperature accelerates processing. The presence of tea leaf tannin, lead oxide, and calcium oxide also influences the coagulation process as well as osmosis, and thus it influences the ripening (Blunt and Wang, 1918). Mailliard reactions between the glucose of the albumen and amino acids, combined with the pigment of the tea contribute to the development of the brown-colored albumen gel (Wang and Fung, 1996). The decomposition of proteins produces polypeptide and amino acids. Cysteine and cysteine produced by protein hydrolysis

can be continually decomposed into ammonia and hydrogen sulfide, which contributes to the unique flavor of pidan. Hydrogen sulfide produced from the denatured protein reacts with the iron in the yolk, which gives pidan its typical dark-green-colored yolk (Wang and Fung, 1996).

1.2.3.4 Lysinoalanine formation in pidan

Proteins have been alkali-treated to improve the extractability, solubilization, gelation and dispersability for preparing texturized products (Chang *et al.,* 1999a). However, it generally causes the formation of lysinoalanyl (LAL) and lanthionyl residues, racemization and degradation of amino acid residues, and Maillard reactions, especially when the protein-containing foods are thermally treated during the processing (Masters and Friedman, 1979; Achor *et al.,* 1981; Friedman and Masters, 1982; Liardon and Hurrel, 1983). Moreover, most of these reactions are the main causes of nutritional losses (Friedman *et al.,* 1984). However, alkaline treatment is effective in destroying toxins, such as aflatoxin and protein inhibitors (Ma *et al.,* 1990). LAL and related cross-linked amino acids may be derived from the reaction of lysine with dehydroalanine (DHA) residues formed from substituted serines, cystine, and cysteine residues in proteins through nucleophilic reactions (Fletcher *et al.,* 1963). However, the cross-linked products obtained are indigestible by proteases. The decline in digestibility of proteins and the loss of lysine are the main causes of nutritional losses of proteins. In addition, nephrocytomegalia was found in rats fed with LAL-containing diets (De Groot *et al.,* 1976).

Friedman *et al*. (1984) pointed out that the factors affecting LAL formation are pH, alkaline treating time and heating temperature as well as sources of protein. Cysteine was found to be most unstable to alkaline and could mostly be destroyed when casein was alkali-treated at pH 9.0, 75°C for 3 h (Friedman and Masters, 1982). Chemical modifications of proteins, addition of metal ions, use of dimethyl sulfoxide and reductants have been used to control the formation of LAL (Finley *et al*., 1978). Acylation of ε-amino group of lysine, complex formation of metal ions with the imidazol group of histidine and ε -amino group of lysine were effective in reducing the formation of LAL (Henkin, 1974). About 65 and 35% of cysteine in the albumen and yolk of alkali-pickled duck eggs, respectively, were degraded (Chang *et al.,* 1999b). The decline in lysine was enhanced with increasing

pH and alkaline treating time, accompanied by LAL formation (Chang *et al.,* 1999a). However LAL formation in both pidan albumen and yolk was markedly reduced by the addition of certain additives in pickling solution such as $ZnSO_4$, PbO and L - cysteine or reduced glutathione (Chang *et al.,* 1999a).

1.2.3.5 Heavy metals in pidan

Heavy metals become toxic when they are not metabolized by the body and accumulate in the soft tissues. Some well-known toxic metallic elements with a specific gravity that is 5 or more times that of water are arsenic, 5.7; cadmium, 8.65; iron, 7.9; lead, 11.34; and mercury, 13.546. In small quantities, certain heavy metals are nutritionally essential for a healthy life. Some of these are referred to as the trace elements (e.g., iron, copper, manganese, and zinc) (International Occupational Safety and Health Information Centre 1999). The Department of Health and Human Services (DHHS) has determined that lead acetate and lead phosphate may reasonably be anticipated to be carcinogens based on studies in animals. In the central nervous system, lead causes edema, and its effects are often irreversible. Reduced IQ, learning and behavioral difficulties, have been reported in children even with low blood lead levels (Kosobucki *et al*., 2004). Neurological and behavioral effects have been reported after occupational exposure to lead, but peripheral neuropathy, (leading to weakness and palsy with wrist drop) is seen with decreasing frequency. Environmental Protection Agency (EPA) limits the heavy metal in air in which the level does not exceed 1.5 micrograms/m³ averaged over 3 months. For lead, EPA limit in drinking water and food is 15 micrograms/L and that in blood is 40 micrograms/dL. Lead (2 ppm) and Cu (8 ppm) are allowed in pidan (Wang and Fung, 1996). However, in order to ensure the pidan formation, the uses of lead oxide or copper oxide and other substances, are overdose. This makes pidan contains too high lead or copper content (Wang and Fung, 1996). So health authorities for many years have been given to pidan. Harmful health effects of zinc generally begin at levels from 10-15 times the Recommended Dietary Allowance (in the 100 to 250 mg/day range). Su and Lin (1992) used other additives like zinc compound to produce pidan which has no black spot on the egg shell and the color of the pidan albumen and yolk were more stable. Eating large amounts of zinc, even for a short time, can cause stomach cramps, nausea, and vomiting. Taken longer, it can cause anemia, pancreas

damage, and lower levels of high-density lipoprotein cholesterol (HDL - the good form of cholesterol). The addition of zinc salt in the egg protein led to the aggregate formation (Samontha *et al.,* 2008). This may be due to the fact that hydrogen sulfide formed in the eggs during alkaline treatment may combine with zinc to form zinc sulfide compounds. This combining state of zinc sulfide may lead to instability and easy decomposition of egg protein (Chen and Su, 2004). Pidan consumption with lead leads to various toxicological effects in human body, since lead has high electronegativity and flexible coordination number that facilitates its interactions with oxygen and sulfur atoms of proteins to form stable complexes (Godwin, 2001). The toxic effects of lead depend on the duration and magnitude of its exposure and also age and nutritional status (Meyer *et al.,* 2008). About 75–90% of absorbed lead is stored in bones and teeth with the remainder in red blood cells and soft tissues including liver (Meyer *et al*., 2008). Lead can influence any part of the brain; it preferentially affects prefrontal cerebral cortex, cerebellum and hippocampus, leading to cognitive defects, motor function and memory disturbances. Suggested mechanisms of action include induction of oxidative stress and interference with calcium and other signalling pathways but the exact molecular targets for lead have not been clearly established (Patrick, 2006).

1.2.4 Phenolic compounds

Plant phenolic compounds, also denoted polyphenols, are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s). These compounds are derived from the secondary metabolism of plants (Parr and Bolwell, 2000; Robards *et al.*, 1999). In plants, phenolic compounds play a role in numerous processes, such as plant growth and reactions to stress and pathogen attack (Parr and Bolwell, 2000). Plant phenolic compounds are present in products ranging from food to sunblockers and paper. Yellowing of paper over the years is caused by photochemical reactions of phenolic compounds (Zhu and Gray, 1995). Phenolic compounds can be found in many foods and drinks from plant origin, e.g. fruits, vegetables, coffee (Clifford, 1999), tea (Lakenbrink *et al.*, 2000), beer, wine and chocolate (Arts *et al.*, 1999). Red wine has a total content of phenolic compounds of 1-4 g/l (Shahidi and Naczk, 1995). These high amounts have led to the hypothesis that

phenolic compounds would be one of the responsible factors for the beneficial effect of wine consumption on cardiovascular diseases (Wallerath *et al.,* 2005). Dark chocolate contains approximately 1.6 g/kg of oligomeric phenolic compounds, called proanthocyanidins (USDA database, 2004), while a member of the proanthocyanidin sub-class, the procyanidins, is present in particularly high concentrations in apples and cider (2-3 g/L) (Shahidi and Naczk, 1995). The content of phenolic compounds in foods may change during storage as induced by light and temperature (Friedman, 1997). Apart from being naturally present in the raw materials used for foods, phenolic compounds are also added to some foods for their coloring properties and for their antioxidant effects (O'Connell and Fox, 2001; Richelle *et al.*, 2001). Aewsiri *et al.* (2009) reported that cuttlefish skin gelatin modified with oxidised tannic acid can be used as emulsifier possessing antioxidative activity in emulsion systems. Further (Balange and Benjakul, 2009) reported that oxidised phenolic compounds can be used to improve the gel strength of surimi manufactured from dark flesh fishes.

The presence of phenolic compounds may be easily observable due to the chromophoric groups that some phenolic compounds bear, e.g. the red-purple anthocyanins (Bakowska *et al.*, 2003), or by the brown and green reaction products of phenolic compounds with themselves or with proteins (Montavon *et al.,* 2003). The presence of phenolic compounds can also affect the taste of food. Low concentrations of phenolic compounds may be responsible for desirable sweet, smoky or caramel flavors in foods e.g. dairy products (O'Connell and Fox, 2001). High concentrations of phenolic compounds in tea and wine provide the astringent sensation. The latter results from the precipitation of saliva proteins on the tongue by interactions with specific phenolic compounds (Baxter *et al.*, 1997; Charlton *et al.*, 2002). If milk is added to tea, the proteins present in milk will bind most of the present phenolic compounds, leaving the saliva proteins unaffected. On the other hand, the interactions between phenolic compounds and proteins may lead to a decrease of protein digestibility by blocking the substrate and/or inhibiting certain proteases (Kroll *et al.,* 2003).

1.2.4.1 Tea and active component

Tea essentially signifies two or three leaves and the terminal apical buds of the tropical shrub *Camellia sinensis, Camellia assamica* and other varieties.

The plant was originally discovered and grown in south-east Asia 1000 of years ago and according to the Chinese mythology, the emperor Shen Nung discovered tea for the first time in 2737 (Harbowy and Balentine, 1997) Tea is the most widely consumed and cheapest non-alcoholic drink next to water. It can be categorized into three types, depending on the level of fermentation, i.e., green (unfermented), oolong (partially fermented) and black (fermented) tea (Gupta, 2002). Although this process is often assumed incorrectly to be fermentation, the more correct term should be oxidation which means exposure to air while drying. In general, green tea has been found to be superior to black tea in terms of antioxidant activity owing to the higher content of (−)-epigallocatechin gallate (Cheng, 2004). The processes used in the manufacture of black tea are known to decrease levels of the monometric catechins to a much greater extent than the less severe conditions applied to other teas (Cheng, 2004). The production and consumption of the partially fermented oolong tea are confined to China, whereas green tea and Chinese tea are consumed worldwide. Catechins are the major biochemical constituents of tea, during fermentation, the simple substrates, i.e., catechins, are acted upon by the oxidative enzymes, polyphenol oxidase and peroxidase, to form theaflavins and thearubigins (Lakshminarayanan and Ramaswamy, 1978). Theaflavins contain benzotropolene rings with dihydroxy or trihydroxy substitution systems and exists as catechin dimmers, while the other polymeric polyphenols often called thearubigins are even more extensively oxidized and polymerized. They are of more complex, mainly unknown composition. Catechins and their oxidation products are mainly responsible for the taste and astringent character of black tea. Apart from quality characters, catechins are also found to possess properties of benefit to human health. The major catechins present in tea leaves are catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG).

1.2.4.2 Catechin

Catechins are polyphenolic antioxidant plant metabolites, specifically flavonoids called flavan-3-ols. Although present in numerous plant species, the largest source in the human diet is from various teas derived from the tea-plant (*Camellia sinensis*) such as green tea. The major phenolic compounds of green tea are catechins and derivatives (Figure 2).Depending on the pH of the solution, catechin may exist

undissociated or in any of its anionic forms. The hydrogen or electron abstraction reactions of catechin may lead to the formation of various radicals: where, up to the physiological pH of the media, the parent molecules are catechin and/or phenolate monanions (Lemańska *et al.*, 2001).

$$R(OH)_2 \rightarrow R(OH)_2^{\bullet +} + e^- \text{ (electron abstraction)}$$
$$R(OH)_2 \rightarrow ROHO^{\bullet} + H^+ + e^- \text{ (Hydrogen abstraction)}$$
$$ROHO^- \rightarrow ROHO^{\bullet} + e^- \text{ (electron abstraction)}$$
$$ROHO^- \rightarrow ROO_2^{\bullet -} + H^+ + e^- \text{ (Hydrogen abstraction)}$$

The phenoxyl radical ($ROHO^{\bullet}$) formed in the first oxidation step is most likely to undergo a second oxidation step and form a more stable catechin *o*-quinone (RO_2). However, at physiological pH, the chemical and electrochemical oxidation pathway of catechin is complicated by the subsequent dimerization reactions. Both, enzymatic and non-enzymatic chemical oxidation of catechin produce *o*-quinone species that are prone to a nucleophilic attack by a catechin unit on the B-ring in a Michael-type addition whereby a dimeric product is formed (Janeiro and Oliveira Brett, 2004) It has also been found that radicals formed by the one-electron abstraction from phenolate anions at an electrode, at physiological pH, may enter subsequent polymerization reactions rather than be further oxidized by a second-electron abstraction to more stable quinonic forms. Dimerization reaction is thought to proceed as an irreversible coupling of the two radicals or as an irreversible coupling of the phenoxyl radicals with the excess phenolate anions that yield a dimer radical anion. Dimer radicals can be immediately oxidized at the electrode surface and/or by electron exchange with a radical phenoxyl. In acidic solutions at low concentrations, the coupling reactions are significantly suppressed and *o*-quinone is the most abundant oxidation product.

There are numerous studies on *in vitro* antioxidative activity of tea catechins against various radicals such as hydroxyl, superoxide, peroxyl and DPPH. Radical scavenging activities of major tea catechins including catechin, epicatechin and gallates of epicatechin have been reported (Yilmaz, 2006). Nakao *et al*. (1998) found that epicatechingallate, epicatechin and catechin have a higher peroxyl radical

scavenging activity than *L*-ascorbate and β-carotene. Nanjo *et al*. (1996) reported that DPPH radical scavenging activity of catechin and epicatechin is less than epigallocatechin, epicatechingallate, and epigallocatechin-3-gallate. Tang *et al*. (2001) found that the addition of tea catechins at a level of 300 mg/kg inhibited lipid oxidation significantly in red meat and poultry patties. The antioxidant activity of green tea polyphenol in several edible oils and fried noodle was investigated by Koketsu and Satoh (1997). Green tea polyphenols showed stronger antioxidative activities as compared to tocopherol in both lard and soybean oil. The antioxidant effect of green tea polyphenols in lard and soybean oil was dose-dependent (Koketsu and Satoh, 1997). The antioxidative activity of the green tea polyphenols in fish oil was also investigated. The green tea polyphenols showed stronger antioxidative activity, compared to BHA and tocopherol. The antioxidative effect of green tea polyphenols was also dose-dependent (He and Shahidi, 1997). Noodles were fried in lard containing green tea polyphenols at several concentrations. The oxidative stability of the noodle was proportional to the green tea polyphenol concentration in lard (Koketsu and Satoh, 1997).

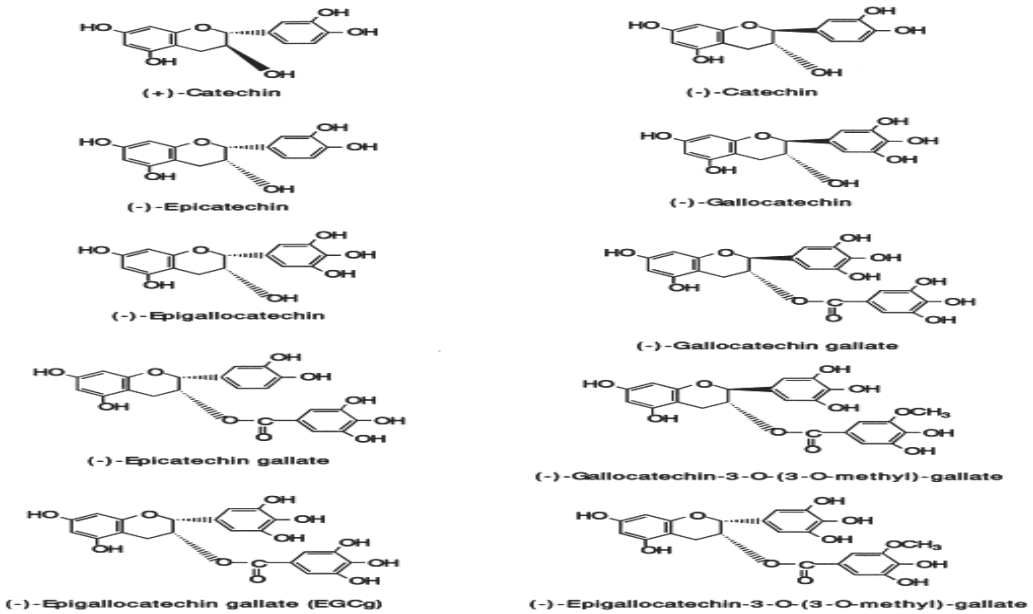


Figure 2. Chemical structures of catechin compounds

Source: Saeki *et al.* (2000)

1.2.4.3 Chinese black tea in pidan production

Chinese black tea is consumed throughout the world for its unique taste, briskness and flavor. It is not only consumed as a black tea extract, it is also used as an ingredients in different food systems. Among the various ingredients used in the pidan production, Chinese tea also serves as an ingredient in the pidan production. However the concentration of tea varies with the method involved in the pidan production (Table 1). It has been believed that the use of tea, wood ashes and local clay gives the century eggs a more detectable taste and a characteristic terroir. Li and Hsieh (2004) reported that the color developed in pidan may be due to the Malliard reactions between the glucose of the albumen and amino acids. Additionally the pigment mainly from phenols of the tea contributes to the development of the brown-colored albumen gel. During the black tea fermentation, an enzymatic oxidation of tea polyphenols, especially tea catechins takes place, leading to formation of a series of colored chemical compounds, such as theaflavins (TFs) and thearubigins (TRs), which are responsible for the characteristics of the black tea liquors (Biswas and Biswas, 1971). Liang and Xu (2001) showed that theaflavin makes a greater contribution to the brightness of black tea infusion than theaflavin gallates but theaflavin gallates have stronger ability to form tea cream than theaflavin. Traditionally people believe that the addition of tea contributes to color of pidan.

1.2.4.4 Protein-phenolic interactions

Phenolic compounds can interact with proteins in two different ways: via non-covalent (reversible) interactions and via covalent interactions, which in most cases are irreversible. Two types of complexation mechanisms can be distinguished: a monodentate and a multidentate mechanism (Haslam, 1989). “Monodentate” means that a phenolic compound interact with only one protein site. At a high phenolic compound to protein ratio, phenolics can form a layer around a protein molecule, thereby more or less covering its surface, via a monodentate mechanism (Figure 3). The layer at the surface of the protein makes it less hydrophilic, which may lead to aggregation. The other mechanism, the multidentate mechanism, applies only to phenolic compounds with sufficient size to be able to interact with more than one site, thus being able to form cross-links between proteins (Figure 3). Both complexation mechanisms may lead to aggregation and precipitation (Charlton *et al.*, 2002; Haslam,

1989). The multidentate mechanism requires a much lower phenolic compound / protein molar ratio and thus a lower phenolic compound concentration than the monodentate mechanism. Proanthocyanidins, therefore, would decrease protein solubility at much lower ratios than monomeric phenolic compounds. Phenolic compounds-protein interaction can be summarized in Figure 4.

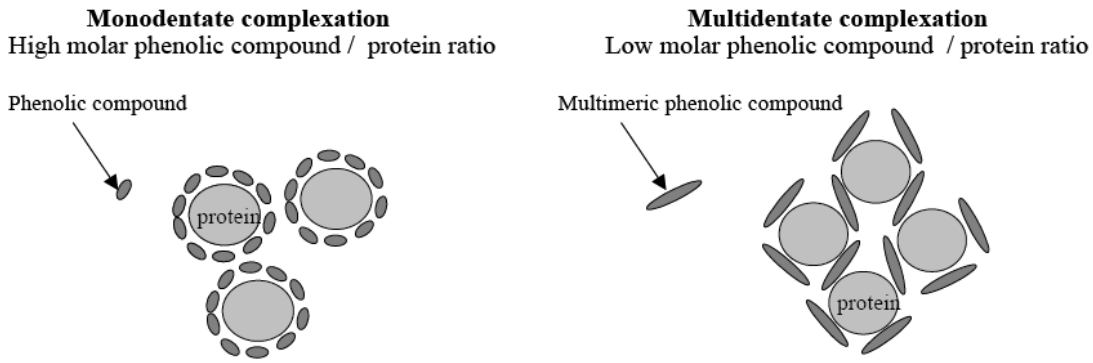


Figure 3. Monodentate and multidentate mechanism
Source: Haslam (1989)

The non-covalent and covalent interactions between phenolic compounds and proteins do not only depend on the phenolic compound / protein ratio but also on factors such as steric hindrance and the polarity of both the protein and the phenolic compound involved (Prigent, 2005). Therefore, the nature and the sequence of amino acid residues in the protein chain are of particular importance (Prigent, 2005). The interactions between phenolic compounds and proteins have consequences for the production of plant protein ingredients, as these interactions may hinder protein extraction. Removing phenolic compounds is one of the main issues for the production of protein products from sunflower (Gonzalez-Perez *et al.,* 2002) and phenolic compounds may be responsible for the low solubility of some potato protein preparations (Van Koningsveld *et al.,* 2002). For phenolic compound-protein interactions, hydrogen bonding and hydrophobic interactions are recognized as the main driving forces. Hydrogen bonds may involve the interactions between hydroxyl groups of phenolic compounds and the nitrogen or oxygen of lysine, arginine, histidine, asparagine, glutamine, serine, threonine, aspartic acid, glutamic acid, tyrosine, cysteine and tryptophan (Prigent, 2005). Hydrophobic interactions may

occur between phenolic compounds and amino acids such as alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine residues (Prigent, 2005).

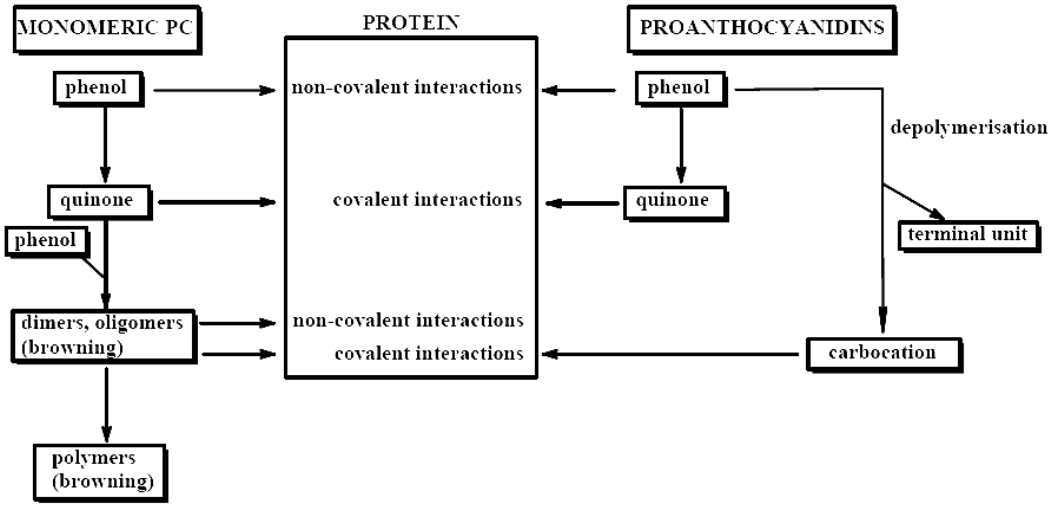


Figure 4. Overview of the phenolic compound – protein interactions
Source: Prigent (2005)

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

Considering the types of phenolic compounds, not only the polarity of the phenolic compound influences the binding, but also the size and the flexibility of the phenolic compound. The larger the phenolic compound, or more exactly the more binding sites the phenolic compound possesses, the stronger the association is expected (Hagerman *et al.*, 1998), i.e. proanthocyanidin trimers bind more tightly to BSA than proanthocyanidin dimers (Artz *et al.*, 1999). Less protein is precipitated with procyanidins > 3.5 kDa than with smaller procyanidins (De Freitas and Mateus, 2001). The lower solubility of large phenolic compounds causes the difficulty to interact with proteins (De Freitas and Mateus, 2001). In addition, the size of the phenolic compound can decrease its conformational flexibility, which is observed to be an important parameter in protein-phenolic compound interactions. With respect to the type of protein, it has been shown that globular proteins, which are small and compact, have a lower affinity for phenolic compound than proteins with a more open conformation, e.g. proline-rich proteins (PRP), such as collagen (Prigent, 2005). When a proline residue interacts with a phenolic compound, a specific interaction takes place. This binding is controlled by weak forces occurring at short distances between the aromatic groups of phenolic compounds and proline (Bianco *et al.*, 1997). Such interactions may also occur between aromatic groups of two phenolics (Baxter *et al.*, 1997).

1.2.5 Maillard reactions

Maillard reaction is a non-enzymatic browning which links the carbonyl group of reducing carbohydrates and the amino group of free amino acids as well as of lysyl residues in proteins (Ajandouz, *et al*., 2001). The Maillard reaction is also called as non-enzymatic browning reactions and has been associated with the formation of compounds with strong radical scavenging activity. The Maillard reaction takes place in three major stages including (a) early stage (b) advanced stage and (c) final stage (Figure 6) (Ajandouz, *et al*., 2001).

a. Early stage

Early Maillard reaction takes place through a condensation of the reducing sugar, like glucose, and the compound possessing a free amino group (of an

amino acid or in proteins mainly the ε-amino group of lysine, but also the α-amino groups of terminal amino acids) to give a condensation product N-substituted glycosylamine, which rearranges to form the Amadori rearrangement product (ARP). The Amadori or Heyns rearrangement are reversible and the reaction products, aldosamine or ketosamine, are still colorless (Berk, 1976**).** The radical scavenging activity derived from the uncolored reaction products is smaller than the brightly colored pigments. An alternative view on the Maillard reaction has been given by Yaylayan (1997) who considers the initial stage as three primary fragmentation pools arising from sugars, amino acids and Amadori / Heyns products. The Maillard reaction then propagates by interactions between the different pools to generate low and high molecular weight end products.

b. Advanced stage

The intermediate stage starts from the Amadori/Heyns product, leading to sugar fragmentation products and release of the amino group. The subsequent degradation of the Amadori product is dependent on the pH of the system. At pH 7 or below, it undergoes mainly 1,2-enolisation with the formation of furfural (when pentoses are involved) or hydroxymethylfurfural (HMF) (when hexoses are involved). At pH >7 the degradation of the Amadori compound is thought to involve mainly 2,3 enolisation, where reductones, such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one (HMF^{one}), and a variety of fission products, including acetol, pyruvaldehyde and diacetyl are formed (Martins, 2003). All these compounds are highly reactive and take part in further reactions. Carbonyl groups can condense with free amino groups, which results in the incorporation of nitrogen into the formation of aldehydes and α-amino ketones. This reaction is called as the Strecker degration (Martins *et al*., 2001).

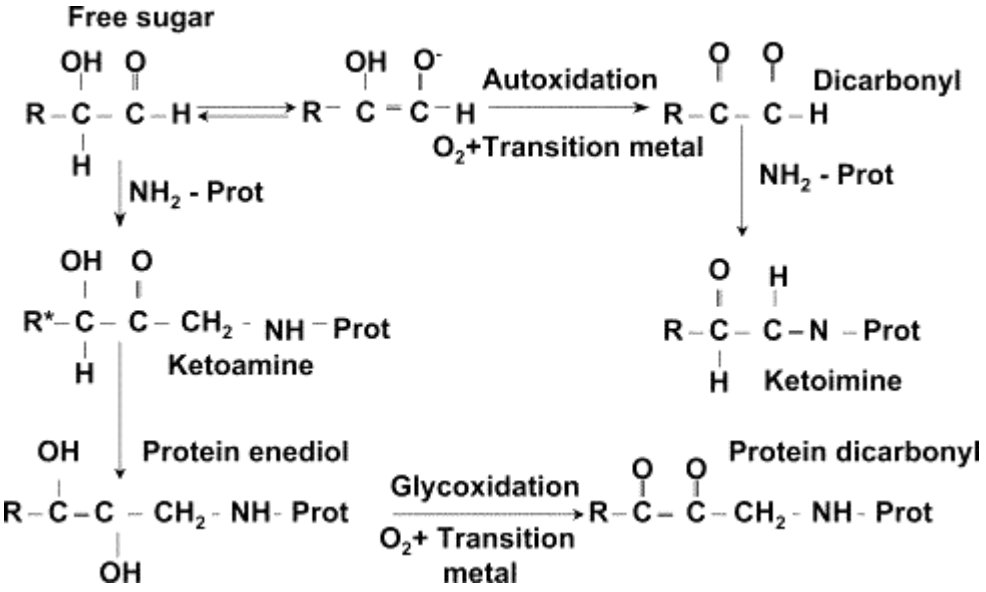


Figure 5. Reaction schemes for glucose autoxidation and glycoxidation

Source: Baynes and Thorpe (1999)

c. Final stage

At the final stage, the reactive dicarbonyl again react with free amino groups and, through oxidation, dehydration and cyclization reactions, form yellow-brown, often fluorescent, insoluble, irreversible compounds, usually called Advanced Glycation End-Products (AGEs), which accumulate on long-lived proteins and cause damage (Martins, 2003). Although the chemical nature of these compounds is not yet well-defined, recent investigations indicate that they include post-Amadori products derived from oxidation and further structural rearrangements, so that compounds which are neither cross-linked nor fluorescent have been considered to belong to the AGE group. In this context, it should be emphasized that oxidation processes are important in the formation of many AGEs (Baynes and Thorpe, 1999). There are two mechanisms through which these processes take place, both catalyzed by metals such as copper and iron. The first involves auto-oxidation of free sugar in the presence of oxygen and free metals, leading to more reactive dicarbonyl compounds, which react with proteins to form highly reactive ketoamines. The second mechanism involves protein-bound products of the Amadori pattern which, in the presence of oxygen and free metals, are oxidized and give origin to highly reactive protein-enediols and protein-dicarbonyls which can generate AGEs (Figure 5) (Baynes and Thorpe, 1999).

1.2.5.1 Formation of color and flavor in the Maillard reaction

The degree of browning (usually measured via absorbance at 420 nm) is often used analytically to assess the extent to which the Maillard reaction has taken place in foods. Nevertheless, it has been stated that fluorescent compounds are formed prior to brown compounds (Baisier and Labuza 1992). The isolation and identification of colored Maillard products has so far been achieved only with model systems, mostly for low molecular weight (<500 Da) products. Hashiba (1982) concluded that browning was directly proportional to the reducing power of the sugar and to the amounts of glycine consumed, by comparing different sugars with one single amino acid. However, Rizzi (1997) stated that many colored products appear to be (retro) aldolization/dehydration products of sugars which may or may not be attached to proteins or other sources of amino nitrogen. Also Hofmann (1999) using dosage/activity relationship combined with chemical/instrumental techniques and visual/sensory measurments, identified carbohydrate degradation products as browning precursors. Those include deoxyosones, glyoxal, methylglyoxal, hydroxy-2-propanone, 3-hydroxy-2-butanon and glycoaldehyde.

Flavor compound formation in the Maillard reaction depends on (1) the type of sugars and amino acids involved, and (2) on reaction temperature, time, pH and water content (Jousse *et al.*, 2002). In general, the first factor determines the type of flavor compounds formed (Table 2), while the second factor influences the kinetics. Some examples of the first factor are that meat-related flavor compounds are mainly sulphur-containing compounds, derived from cysteine and ribose (coming from nucleotides), while the amino acid proline gives rise to typical bread, rice and popcorn flavors. Lane and Nursten (1983) reported a thorough study on odours produced in Maillard reaction systems. They identified 12 amino acids, five to seven of which they thought to produce bread, crusty biscuits, cake or toast aroma at each of the four temperatures studied, and using single amino acid/glucose combinations at different temperatures. Also, Fors (1983) published a literature review of the sensory properties of volatile Maillard products and related compounds. It includes qualitative aroma and flavor descriptions and sensory threshold values for various compounds, classified according to the chemical structure. The origin of some volatile compounds

responsible for flavor is still relatively difficult to determine, due to their multiple origin.

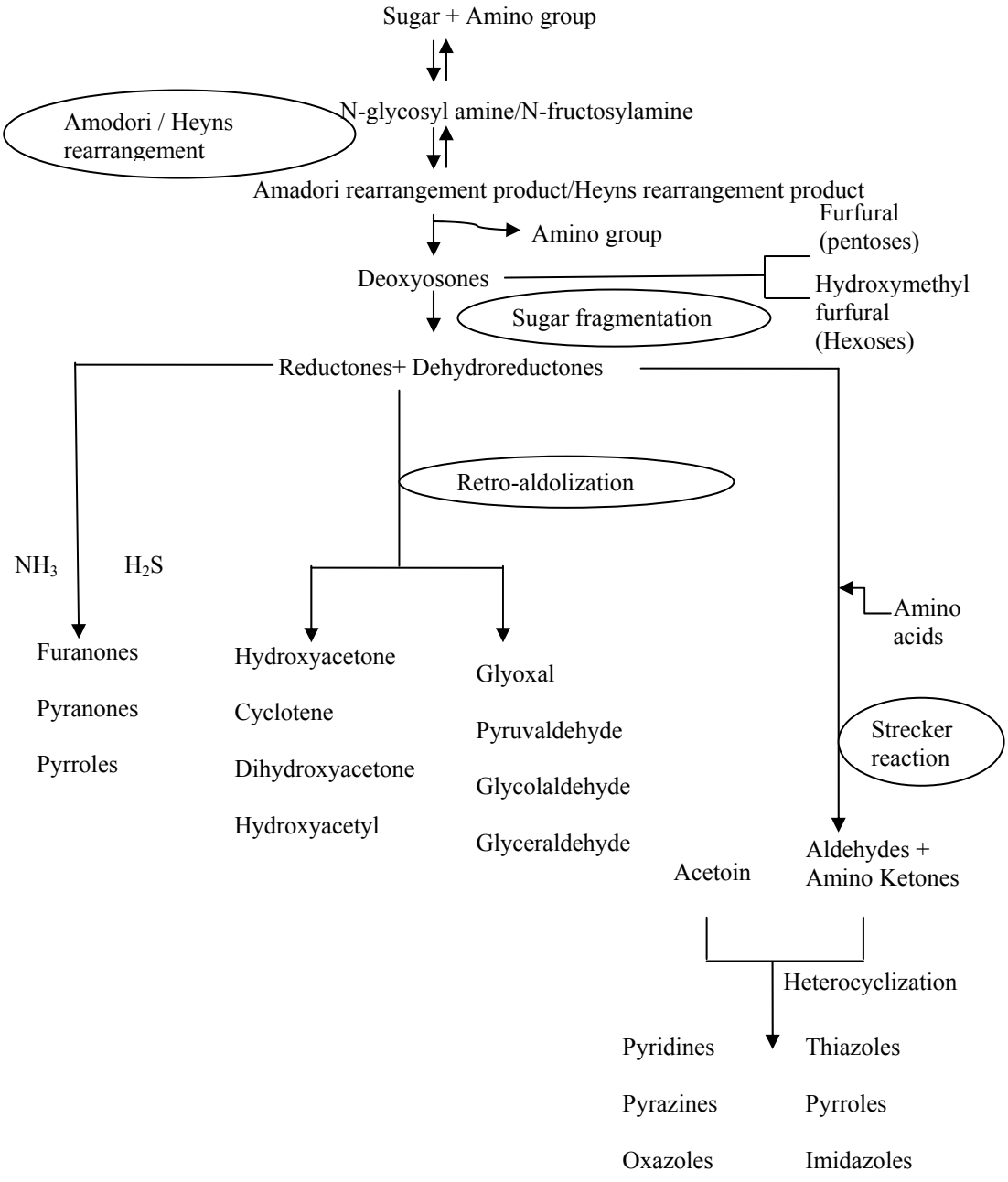


Figure 6. General overview of the Maillard reaction showing flavor compounds as end products

Source: Ho (1996)

Table 2. Overview of some classes of Maillard-derived flavor compounds

Compound class	Associated flavor/aroma	Food examples	Remarks
Pyrazines	Cooked, roasted, toasted, baked cereals	Heated foods in general	
Alkylpyrazines	Nutty, roasted	Coffee	
Alkylpyridines	Green, bitter, astringent, burnt	Coffee, barley, malt	Generally regarded as unpleasant
Acylpyridines	Cracker-like	Cereal products	
Pyrroles	Cereal-like	Cereal coffee	
Furans, furanones, pyranones	Sweet, burnt, pungent, caramel-like	Heated foods in general	
Oxazoles	Green, nutty, sweet	Cocoa, coffee, meat	
Thiofenes	Meaty	Heated meat	Typical for heated meat, formed from ribose and cysteine

Source: Teranishi *et al*. (1999)

1.2.5.2 Factors affecting Maillard reaction

The formation of brown color and flavors generally depends on the precursors, pH, and quantitative ratio of amino nitrogen to reducing sugar (Martins, 2003). Therefore, many factors involve in the development of brown color mediated by Maillard reaction.

a) Sugars

Reducing sugars are essential ingredients in Maillard reaction, as they provide the carbonyl groups for interaction with the free amino groups of aminoacids, peptides and proteins. The initial kinetics of glycation are dependent on the propotion of the reducing sugar existing in the acyclic or active form under the reaction condition (Yaylayan *et al.,* 1993) and on the electrophilicty of the sugar carbonyl group (Bunn and Higgins, 1981). Starch and non-reducing sugars such as sucrose may be hydrolyzed to form reducing sugars that can participate in the Maillard reaction (Camire *et al.,* 1990). The reactivity of reducing sugars was reported to decrease in the following order: aldopentoses> aldohexoses> aldoketoses> disacchrides (Spark, 1969). Pentoses yield stronger color intensity than hexoses (Lingnert, 1990). Kim *et al.* (2009) also reported that MRPs from ribose showed the higher browning than other sugar. Among hexoses examined, the reactivity decreased in the order of D-galactose>D-mannose>D-glucose, corresponding to the decreasing rate of ring opening (Eskin, 1990). Brands and Van Boekel (2002) revealed large differences between the reaction behavior of glucose and fructose when heated in the presence of a protein. These differences are mainly due to the differences in the reaction mechanism between aldose and ketose sugars which result in altered browning. Type and concentration of sugars also contributes to the flavor of MRPs. Inulin, a fructo polysaccharide, enhances the formation of volatiles which is responsible for the flavor of breads during baking (Poinot *et al.,* 2010). Meinert *et al.* (2007) reported that hexose contributes to flavors to a greater extent than ribose in the aroma volatiles of pork.

b) Amino acid and protein

Amino acids participating in the generation of melanoidins is more influence on the melanoidin formation than carbonyl compound (glucose or L-(+)-ascorbic acid) (Eskin, 1990). The reactivity of the aminoacids to form Maillard reaction products is different. The reactivity decreases in the order of lysine>glycine>alanine (Morales and Jimenez-Perez, 2001). Additionally, the increase in reaction rate was observed with increasing amino acid concentration (Toribio and Lozano, 1986). Kwak and Lim (2004) found that the reactivity of lysine to form Maillard reaction products was 2-3 times higher than other amino acid. The

higher reactivity of lysine is attributed to the two α- and ε- amino groups. Cysteine was found to have the lowest contributory effect to browning. It is known that sulfur amino acids and peptides such as cysteine and glutathione are generally effective for inhibiting non enzymatic browning (Kwak and Lim, 2004). The rate of Maillard reaction is also altered based on the chain length of peptide or protein. Kim and Lee (2009) reported that MRPs derived from the Glu-Triglycine model system were most prominent than Glu-diglycine and Glu-glycine model system. Maillard flavor compounds generation is enhanced by the addition of some aminoacids and peptides. Addition of glycine increased the quantities of several alkylpyrazines in potato model systems (Mei *et al.,* 2007). Some dipeptide derived MRPs can generate more flavor compounds than amino acids. Pyrazines flavor derived from the lysine-containing dipeptides were generally higher than those derived from amino acids (Van Lancker *et al.,* 2010).

c) pH

The carbonyl amino reaction can takes place in acidic or alkaline media, although it is favored under alkaline conditions. The active form of amine groups in aminoacid, peptide and protein at alkaline pH is basic (Van Boekel, 2001). Increasing the pH also ensures that sugars are in the open chain or reducing form (Van Boekel, 2001). Ajandouz *et al.* (2001) reported that increase in Maillard reaction rate was observed as the initial pH of heated fructose-lysine model system at 100°C increased. Higher pH favors the reductone formation over furfural production from the Amadori products, leading to color development. Gu *et al.* (2009) also reported that browning and intermediate products increased with increasing heating time at initial higher pH in a casein-glucose model system. Increase heating time also causes the decerase in pH. The decrease in pH of sugar-amino system after heating might be due to the formation of organic acids. Formic and acetic acids were generated from the degradation of 1,2-enediol and 2,3-enediol, the intermediate products from the heated casein-sugar model system (Van Boekel, 1996). MRPs derived from aqueous model systems with different peptide chain lengths showed decrease in pH when heating time increased to 4 h (Kim and Lee, 2009). Yu and Zhang (2010) showed that furans, such as furfural, 2-furanmethanol, benzofuran, 2,5-furandicarboxaldehyde and

2-furfurylfuran were formed mainly at acidic pH. In contrast, higher pH values could promote the production of pyrazines.

d) Temperature

Generally, the rate of chemical reaction increases with increasing temperature. Since the Maillard reaction consisits of several reaction steps, each with possible different temperature sensitivity, it strongly depends on tempeartaure (Brands and Van Boekel, 2002). At low temperature (20-60°C), the reaction rate is lower than that of high temperature (100-150°C). Furthermore, temperature affects the activities of the reactants. The active form of the sugar is considered to be the open chain, which is formed markedly with increasing temperature (Van Boekel, 2001). The percentage of fructose in its acylic form at neutral pH is about 0.7% at room temperature and 13.1% at 80°C (Yaylayan *et al.,* 1993). The Maillard reaction rate increased four-fold for each 10°C rise in temperature. Brands and Van Boekel (2002) reported that an increase of heating temperature in monosaccharide-casein model system leads to the higher loss of the reactants and an increased formation of the reactant products. Maillard reaction was enhanced at the higher rate in the aqueous model system when the temperature increased from 60 to 100°C (Ajandouz *et al*., 2008). Temperature is also important parameters that affect the aroma characteristics of Maillard foods (Labuza and Baisier, 1992). Maillard reactions in foods can produce many MRPs including volatile compounds of low molecular mass, non-volatile colored compounds of intermediate molecular mass and brown substances of high molecular weight (Lana *et al.,* 2010). Meinert *et al.* (2007) identified 31 volatiles from fried pork chops at 150°C and 250°C. Among them, lipid-derived aldehydes dominated at 150°C, while Maillard-related aldehydes and ketones dominated at 250°C. Thus temperature also contributes to Maillard flavor mediated by MRPs.

e) Other factors

The formation of metal complexes with amino acids can influence the Maillard reaction. The Maillard reaction is catalyzed by copper and iron, while manganese and tin can inhibit this reaction. Kato *et al.* (1981) found that Cu^{2+} and Fe^{2+} increased the reaction rate more effectively than Fe^{2+}, while Na^{+} showed no effect on the reaction rate. The Maillard reaction time has influence on browning, fluorescent development and also Maillard products generation. Jing and Kitts (2002)

found that an increase in heating time of casein –sugar model system at 55°C and pH 7 enhanced the generation of fluorescent compound and brown products. Futhermore, the role of buffers in non enzymatic reactions has been shown to determine the rate of browning for sugar-amino acid systems as a result of their influence on the ionic environment in which the reaction takes place (Eskin, 1990).

1.2.5.3 Influence of Maillard reaction on food properties

One of the most obvious negative consequences of the Maillard reaction in food is the loss of nutritive value of proteins involved, with a loss of quality and a possible decrease of food safety. This reaction is also attributed to decrease of digestibility, destruction and/or biological inactivation of amino acids, including essential amino acids like lysine and tryptophan, inhibition of proteolytic and glycolitic enzymes, and interaction with metal ions (Namiki,1988; Friedman,1996.). Also, protein molecules can be crosslinked by Maillard reaction products (Chuyen *et al*., 1991). The nutritional value of foods may be depleted by the Maillard reaction via the destruction of amino acids and the production of toxic compounds. Amadori compounds, formed by the reaction of reducing sugars with amino acids, have no nutritional value (Chang *et al.,* 1999a). The modification of lysine is particularly significant because it is one of eight amino acids that the body cannot manufacture itself. Depletion of food nutrition also may result from the formation of sugar-derived cross-links between proteins that reduce their ability to be digested (Moughan *et al.,* 1996). Moreover, some Maillard reaction products may inhibit the activity of digestive enzymes, such as trypsin (O'Brien and Morrissey, 1989). Moreover, the loss of nutritive value has also been associated with the formation of mutagenic compounds. The reaction mechanism seems to have a major influence in the mutagenicity of the reaction products. For instance, ketose sugars showed a higher mutagenic activity than the corresponding aldose sugars.

Nevertheless, Maillard reaction has been shown to produce antioxidative components. Maillard reaction can produce many Maillard reaction products with different antioxidant activity. Reaction products from various amino acids and sugars were studied with regard to antioxidative properties (Chuyen *et al*., 1998). Maillard reaction products derived from the glycated egg white proteins with Psi (a rare ketohexose, obtained from the hydrolysis of sucrose) exhibited a higher

free radical scavenging effect and antioxidant activity than those of the glycated proteins with alimentary sugars (Sun *et al.*, 2004). Maillard reaction products (MRPs) derived from porcine plasma protein-sugar system possessed the antioxidative activity (Lertittikul *et al*., 2007). Lingnert and Eriksson (1980) examined the effect of time, initial pH and molar ratio of arginine to xylose on antioxidant activity. The pH of 5.0 appeared to be optimal for antioxidant activity. However, neutral or slightly basic condition favored the formation of antioxidant products from histidine-glucose model system (Lingnert and Eriksson, 1980).

At pH values close to those found in most foods, melanoidins have a net negative charge and are able to bind metallic ions. Binsan *et al.* (2008) also reported that traditional Thai products called Mungoong, shrimp extract paste containing Maillard compounds exhibited antioxidant activity. Alfawaz *et al.* (1994) reported the antioxidative activity of MRPs, obtained by autoclaving glucose with acid or enzymatic protein hydroyslates of egg albumin or soy protein isolate, in cooked ground beef during refrigerated storage for 8 days. The antioxidative activity of MRPs was found to be influenced by the heating time of protein hydrosylates-glucose mixtue and by the MRP level added. Alfawaz *et al.* (1994) also found that MRPs obtained by autoclaving egg albumin acid hydrosylate and glucose for 1 h rendered the antioxidative activity in cooked ground beef stored at 4°C for 8 days. Volatile products in Maillard reaction, such as dihydrofuran, dihydropyidine or dihydropyrazine derivatives, are further oxidized to substituted furan pyridines and pyrazines, respectively. Oxygen present in the system is consumed by MRPs and the oxidation of lipid is prevented (Pokorny and Schmidt, 2001). Yoshimura *et al*. (1997) reported that the glucose-glycine MRPs heated over boiling water bath for 1 h could scavenge more than 90% of active oxygen species. The reducing materials produced from Maillard reaction have a scavenging activity towards active oxygen. MRPs obtained from the cysteine showed antioxidant activity under alkaline condition (Phonpala *et al.,* 2009). Cheriot *et al*. (2007) also reported that MRPs obtained from the cysteine and glucose showed antioxidant activity.

The stability of antioxidative activity of MRPs has been reported to depend on many factors. Lingnert and Waller (1983) examined the antioxidant activity of product generated from histidine–glucose system. The loss of antioxidant

activity was evident in the presence of air compared to storage at 25°C under nitrogen. Moreover, the loss of antioxidant activity was less at pH (2.0), compared to high pH (8-10). Mastrocola and Munari (2000) studied the antioxidant activity of MRPs derived from different model systems and found that the antioxidant activity of MRPs stored at 25°C decreased after 40 days of storage.

1.3 Objectives of study

1. To investigate the changes in chemical compositions, physical properties and microstructure of pidan white and yolk obtained with the aid of different cations during pickling and ageing.
2. To investigate the role of Chinese tea in combination of selected cations on the properties of pidan.
3. To study the effect of acetic acid pretreatment and glucose incorporation on the properties of pidan.
4. To elucidate gelling characteristic of egg white protein in pidan model systems induced by different cations.
5. To comparatively characterize the pidan treated with different cations.

CHAPTER 2

CHEMICAL COMPOSITION, PHYSICAL PROPERTIES AND MICROSTRUCTURE OF PIDAN WHITE AS AFFECTED BY DIFFERENT DIVALENT AND MONOVALENT CATIONS

2.1 Abstract

Changes in chemical composition, physical property and microstructure of pidan white were monitored during pickling in the presence of different divalent ($CaCl_2$, $MgCl_2$) and monovalent (KCl) cations at different levels (0.2 and 0.5%) up to 3 weeks, followed by ageing for another 3 weeks. Pidan prepared following the commercial process, in which 0.2% PbO_2 or 0.2% $ZnCl_2$ was incorporated, was also tested. Hardness, cohesiveness and adhesiveness of pidan white gradually increased during pickling, but these parameters decreased during ageing time ($P<0.05$), regardless of cations used. Nevertheless, pidan white treated with 0.2% PbO_2 retained hardness and cohesiveness but had a slight decrease in adhesiveness, when pickling/ageing time increased up to week 6 ($P<0.05$). Transmission electron microscopic (TEM) studies indicated that the aggregation of egg proteins took place in pidan white gels treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ during pickling. However the degree of aggregation varied with cations used.

2.2 Introduction

Pickling is one of the oldest techniques in the food preservation as it not only extends the shelf-life but also enhances the flavor and acceptability of the product. Pidan is a Chinese-style pickled egg with an extremely long shelf-life. Its

pleasant and fragrant taste is preferred by most of the people in Thailand, South Korea and other South East Asian countries. Pidan is normally made by pickling the eggs in 4.2% NaOH/5.0% NaCl solution at room temperature (30°C) for 20 days (Su and Lin, 1994). Ageing of pidan generally takes 15 -18 days at room temperature after pickling process. The formation of pidan is caused by the penetration of alkali through the egg shell and membrane, leading to chemical changes and gelation of egg proteins. Generally, albumen and yolk gradually become solidified and hardened during pidan production.

Gelation is an aggregation of denatured molecules with a certain degree of order, resulting in the formation of a continuous network (Wong, 1989). Gelation is basically a two-step process: denaturation and aggregation (Matsumura and Mori, 1996). Under alkaline pH used for pidan production, electrostatic repulsion extensively opposes protein-protein interactions, thereby preventing gel formation. The addition of selected ions to protein solution diminishes the repulsive forces, and protein–protein association occurs, forming a self-supporting gel. Electrostatic repulsive forces are reduced by increasing ionic strength (Mulvihill and Kinsella, 1988). Monovalent and divalent salt ions both screen electrostatic interactions between the charged protein molecules (Yasudha *et al.*, 1986). Zhu and Damodaran (1994) showed that Ca^{2+} caused the aggregation of whey protein isolate and the aggregation was a slow time-dependent process. Physical properties of gels containing salt were strongly related to salt concentration (Ju and Kilara, 1998). Kuhn and Foegeding (1991) reported that increasing salt concentrations resulted in the initial increases in the gel strength of whey protein, followed by decreases. The amount of Ca^{2+} that was necessary to induce the aggregation of whey protein was equivalent to the net negative charge of the protein (De Wit, 1981).

Lead has been used in pidan for gel stabilisation but it is toxic for consumption (Chen and Su, 2004). Alternatively, Zn^{2+} can be used to produce pidan with no black spots on the egg shell, and the color of the pidan's albumen and yolk was more stable (Chen and Su, 2004). To gain the better benefit and safety for consumers, the development of new process using the alternative cations should be a means to replace the toxic lead, in which the safe pidan can be obtained and becomes more marketable. In general, pidan white characteristic including elastic texture with

amber/brown color is a quality index for consumers. However, a little information regarding the changes of pidan white during pickling and ageing process, particularly those pickled in the presence of alternative divalent or monovalent, has been reported. Therefore, the objectives of this study were to investigate the changes in chemical composition, physical properties and microstructure of pidan white produced with the aid of different divalent and monovalent cations during pickling and ageing for up to 6 weeks.

2.3 Materials and Methods

Chemicals

Lead oxide (PbO_2), zinc chloride ($ZnCl_2$), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium hydroxide, nitric acid and sodium chloride were purchased from Lab-Scan (Bangkok, Thailand). Glutaraldehyde, ethanol and silver nitrate were obtained from Merck (Darmstadt, Germany). Purity of all salts used was greater than 99%.

Duck egg collection

Fresh eggs of duck (*Anas platyrhucus*) with the weight range of 65–75 g were obtained within 1 day of laying from a farm in Rathabhum, Songhkla Province, Thailand. Duck eggs were cleaned and checked for any crack prior to pickling.

Preparation of pidan

Clean duck eggs were soaked in a pickling solution containing 4.2% NaOH, 5% NaCl and 2% Chinese tea without and with the addition of different divalent and monovalent cations. Divalent cations used included $ZnCl_2$, $CaCl_2$ $MgCl_2$ and monovalent cation was KCl. $CaCl_2$, $MgCl_2$ and KCl was added in the pickling

solution at the concentrations of 0.2% and 0.5%. For PbO_2 and $ZnCl_2$, a level of 0.2% was used. Eggs (10 eggs) were soaked in different pickling solutions (1L) at room temperature (30-32°C) for 3 weeks. Pickled eggs were removed and coated with white clay paste (clay: water, 4:1 w/v) to obtain a thickness of 2-3 mm. Coated eggs were left at room temperature for another three weeks for ageing. During pickling and ageing, the samples were taken for analyses every week.

Determination of pH, moisture and salt contents

At week 3 and 6, pidan white samples with different treatments were determined for pH in comparison with fresh egg white according to the method of Benjakul *et al.* (1997). Moisture and salt contents in the pidan white samples were measured as per the method of AOAC (2000) with the analytical No. of 925.10 and 939.10, respectively. To determine salt content, sample (1 g) was added with 20 ml of 0.1 N $AgNO_3$ and 10 ml of HNO_3. The mixture was boiled gently on a hot plate until all solids except $AgCl_2$ were dissolved (usually 10 min). The mixture was cooled using running water. Five ml of 5 % ferric alum indicator ($FeNH_4 (SO_4)_2 \cdot 12 H_2O$) were added. The mixture was titrated with the standardised 0.1 N KSCN until the solution became permanently light brown. The percentage of salt was then calculated as follows:

$$\text{Salt content (\%)} = 5.8 \times [(V1 \times N1) - (V2 \times N2)]/W$$

where V 1 = volume of $AgNO_3$ (ml); N 1 = concentration of $AgNO_3$ (N);
V 2 = volume of KSCN (ml); N 2 = concentration of KSCN (N); and
W = weight of sample (g).

SDS-polyacrylamide gel electrophoresis

Protein patterns of fresh egg white, pidan white samples treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ obtained at week 3 and 6 were determined according to the method of Laemmli (1970) using 4% stacking gel and 12% separating gel. Samples (3 g) were homogenised with 27 ml of 5% SDS using a

homogeniser (Polytron, PT 2100, Kinematica AG, Luzern, Switzerland) at a speed of 12,000 rpm for 1 min. The homogenate was heated at 85C for 1 h, followed by centrifugation at 7,500*x*g for 10 min at room temperature using a centrifuge (Sorvall, Model RC-B Plus, Newtown, CT, USA). The protein concentration of supernatant was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin (BSA) as standard. The prepared sample (20 μg protein) was loaded onto the gel. Electrophoresis was performed using a vertical gel electrophoresis unit (Mini-protein II; Bio-Rad Laboratories, Richmond, CA, USA) at the constant voltage of 200 V/plate. The gels were stained with 0.125% Coomassie Brilliant Blue R-125 in 25% methanol and 10 % acetic acid. Destaining was performed using 40% methanol and 10% acetic acid. Protein markers including myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and α-lactalbumin (14 kDa) were used for estimation of molecular mass.

Texture profile analysis (TPA)

Pidan white samples with different treatments obtained at week 1, 2, 3, 4, 5 and 6 were subjected to TPA. TPA was performed as described by Bourne (1978) with a TA-XT2i texture analyser (Stable Micro Systems, Surrey, England). Prior to analysis, pidan white samples of various treatments were cut into a cube (1 x 1 x 1 cm^3). The samples were compressed twice to 50% of their original height with a compression cylindrical aluminum probe (15 mm diameter). Textural analyses were performed at room temperature. Force-distance deformation curves were recorded at cross-head speed of 5 mm/s and the recording speed was 5 mm/s. Hardness (g), adhesiveness (g s), and cohesiveness were evaluated using the Micro Stable software (Stable Micro Systems, Surrey, England).

Transmission electron microscopy (TEM)

Pidan white samples were fixed at room temperature in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. Fixed samples were rinsed with 0.2 M phosphate buffer three times (pH 7.2) to remove glutaraldehyde. The sample was embedded in Epone resin and polymerised for 24 h at 70°C. Thin sections were cut with a diamond knife in a LKB Ultra microtome. The sections were 80 nm thick, and were deposited on copper grids, stained with 1% uranyl acetate, and examined using a transmission electron microscope (TEM) (JEOL JEM 2010, Tokyo, Japan) at 160 kV.

Color measurement

The color of pidan white obtained at week 3 and 6 was measured using a Hunter Lab Labscan II colourimeter (Hunter Associates Laboratory Inc., Reston, VA, USA) and expressed as L* (lightness), a* (redness/greeness) and b* (yellowness/blueness). The samples with the dimension of 1 x 1 x 1 cm^3 were used for measurement.

Measurement of browning intensity

Browning intensity of pidan white samples of all treatments obtained at week 1, 3 and 6 was measured at a wavelength of 420 nm (Benjakul *et al.,* 2005). Sample was homogenised using an IKA Labortechnik homogeniser (Selangor, Malaysia) with 5 volumes of deionised water (w/v), followed by centrifugation at a speed of 10,000 g for 10 min. Appropriate dilution (approximately 20-fold) of supernatant was made using distilled water to obtain the protein content of 10 mg/ml determined by the Biuret method (Robinson and Hodgen, 1940). The absorbance of prepared solution was measured at 420 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan).

Statistical analysis

Completely randomized design was used throughout the study. The experiments were run in triplicate. Data were presented as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out and means comparisons were run by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analyses were performed with the statistical program (SPSS for windows (Version 10), SPSS Inc, Chicago, IL, USA).

2.4 Results and Discussion

Changes in chemical composition of pidan white during pickling and ageing

Changes in moisture and salt contents as well as pH of pidan white treated with different divalent and monovalent cations were monitored during pickling and ageing (Table 3). As the pickling time increased, the decreases in moisture content with coincidental increases in salt content and pH in pidan white were observed ($P<0.05$), regardless of type of ions used. The lower changes were noticeable during ageing, in comparison with pickling period. At the same pickling or ageing time (week 3 or 6), similar moisture content of pidan white was obtained in all treatments except that treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$, which showed the lower moisture content ($P<0.05$). With increasing pickling and ageing time, the greater loss of water from the pidan white to the outside was most likely caused by osmosis process. Chi and Tseng (1998) reported that water could be migrated from egg white and egg yolk to the environment through the egg shell. This resulted in the reduction of moisture content. The lowest moisture content found in the white of pidan treated with 0.2% PbO_2 after ageing (week 6) was probably resulted from a greater interaction of white proteins induced by lead ion. The enhanced interaction between protein molecules might be associated with the lesser hydrophilic or charged domains, which were capable of binding water. Additionally, ion might undergo electrostatic interactions with the charged groups of proteins. At alkaline pH, protein molecules have negative net charges (COO^-) on their surfaces

and the repulsive force increases, decreasing the protein–protein interactions and increasing the protein–water interactions. In the presence of cations, especially divalent cations, the salt bridges could be formed and the gel-like structure could be obtained.

At week 3 of the pickling, the pidan white from all treatments had the marked increase in the salt content and pH (Table 3). Chang *et al.* (1999a) reported that the final pH of pidan white pickled for 20 days were 11.12. The increase in pH and salt content indicated the migration of alkali and salt from pickling solution into egg white, respectively. Alkali and salt might have the impact on the aggregation of white proteins, resulting in the formation of gel-like structure. For pickling solution containing KCl, pidan white contained the higher salt (NaCl) than those from other treatments ($P<0.05$). KCl might facilitate the migration of NaCl through the shell egg, possibly owing to the lower molecular weight of both salts. After ageing (week 6), the higher salt content was found in pidan white ($P<0.05$), suggesting the dehydration of pidan white accompanied with more concentrated salt content in pidan white. The decrease in moisture content of pidan white during pickling and ageing also resulted in the increased concentrations of proteins. As a consequence, gelation could be enhanced. Concentrations of divalent and monovalent had no marked influence on the chemical composition of white ($P>0.05$).

Table 3. Moisture content, pH and salt content of pidan white during pickling and ageing in the presence or absence of different divalent and monovalent cations

Parameters	Treatments	Pickling/ageing time (weeks)		
		0	3	6
Moisture content (%)	No cations	87.72±1.06‡ C	26.53±1.37 b†, B††	14.86±1.54 c, A
	0.2% $Pb0_2$		16.51±1.63 a, B	10.01±1.78 a, A
	0.2% $ZnCl_2$		16.50±1.73 a, B	12.03±1.38 b, A
	0.2% $CaCl_2$		16.47±1.35 a, B	12.05±1.45 b, A
	0.5% $CaCl_2$		26.56±1.94 b, B	14.84±1.40 c, A
	0.2% $MgCl_2$		26.62±1.81 b, B	14.78±1.32 c, A
	0.5% $MgCl_2$		26.52±1.37 b, B	14.79±1.45 c, A
	0.2% KCl		26.58±1.68 b, B	14.98±1.54 c, A
	0.5% KCl		26.54±1.69 b, B	14.81±1.73 c, A
pH	No cations	8.95±1.34 A	11.05±0.01 a, B	12.64±0.03 a, C
	0.2% $Pb0_2$		11.05±0.01 a, B	12.64±0.03 a, C
	0.2% $ZnCl_2$		11.05±0.01 a, B	12.64±0.03 a, C
	0.2% $CaCl_2$		11.05±0.01 a, B	12.64±0.03 a, C
	0.5% $CaCl_2$		11.05±0.01 a, B	12.64±0.03 a, C
	0.2% $MgCl_2$		11.05±0.01 a, B	12.64±0.03 a, C
	0.5% $MgCl_2$		11.05±0.01 a, B	12.64±0.03 a, C
	0.2% KCl		11.05±0.01 a, B	12.64±0.03 a, C
	0.5% KCl		11.05±0.01 a, B	12.64±0.03 a, C
NaCl content (%)	No cations	0.38±1.25 A	1.03±0.01 a, B	1.11±0.01 a, C
	0.2% $Pb0_2$		1.03±0.01 a, B	1.10±0.02 a, C
	0.2% $ZnCl_2$		1.03±0.01 a, B	1.11±0.01 a, C
	0.2% $CaCl_2$		1.03±0.01 a, B	1.11±0.01 a, C
	0.5% $CaCl_2$		1.03±0.01 a, B	1.11±0.01 a, C
	0.2% $MgCl_2$		1.03±0.01 a, B	1.11±0.01 a, C
	0.5% $MgCl_2$		1.03±0.01 a, B	1.11±0.01 a, C
	0.2% KCl		1.16±0.02 b, B	1.21±0.02 b, C
	0.5% KCl		1.17±0.01 b, B	1.22±0.01 b, C

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

†† Different capital letters in the same row including the control (no cations) for each parameter indicate the significant differences ($P<0.05$). † Different letters in the same column within the same parameter indicate the significant differences ($P<0.05$)

Changes in protein patterns of pidan white during pickling and ageing

Protein patterns of fresh egg white and pidan white with treatments of 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ during pickling and ageing are shown in Figure 7. Degradation of white proteins occurred in pidan as a result of alkali penetration during pickling and ageing (Wang and Fung, 1996). Degradation was more pronounced during ageing period. During pickling and ageing, ovoalbumin in pidan treated with 0.2% PbO_2 was retained to a higher extent, in comparision with that of pidan treated with $ZnCl_2$ or $CaCl_2$. Additionally, the polymerized protein with molecular mass greater than 205 kDa was found at a higher extent in pidan white treated with PbO_2. The remaining albumin and cross-linked proteins might result in more stable gel of pidan treated with PbO_2, compared with other gels. The result suggested that lead ion probably cross-linked protein more effectively and the resulting cross-links were more resistant to degradation. Zn^{2+} and Ca^{2+} ions most likely involved in shielding of net negative charge of proteins and induced cross-links stabilized by ionic interaction which was not stable under alkaline condition.

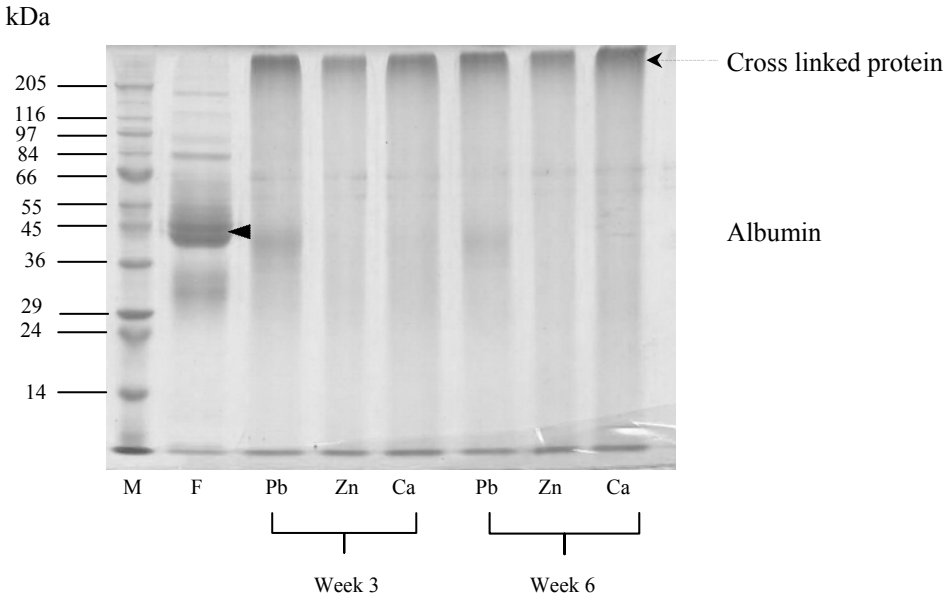


Figure 7. Protein patterns of fresh egg white and pidan white treated with different divalent cations after pickling (week 3) and ageing (week 6). M: molecular weight standard; F: fresh egg white; Pb: 0.2% PbO_2; Zn: 0.2% $ZnCl_2$; Ca: 0.2% $CaCl_2$

Changes in textural properties of pidan white during pickling and ageing

Hardness of pidan white of all treatments except that treated with Pb increased continuously and reached the maximum at week 3 ($P<0.05$) and gradually decreased up to week 6 (Figure 8A). However TPA at week 6 cannot be performed for treatments without cations, Mg^{2+} and K at the level of 0.5%, due to the complete liquefaction of pidan white. The result suggested that the hardness of pidan white obtained from all treatments become more resistance to compression, most likely due to the aggregation of egg white proteins in the presence of monovalent or divalent cations. This was coincidental with the development of gel-like structure of egg white. Proteins with negative charge under alkaline condition could interact each other in the presence of cations via lowering repulsive force between protein molecules. Cations including lead, zinc and calcium were more effective in increasing hardness than was monovalent cation such as K. However, no difference in hardness was found between pidan white treated with Mg^{2+} and K ($P>0.05$). Divalent cations tended to be more effective in protein aggregation than the univalent ions, while the influence of polyvalent salts is moderate (Nakamura *et al.,* 1984). Except for pidan treated with lead, hardness gradually decreased from week 4 to week 6, irrespective of treatments. The increase in pH of white proteins might lead to the repulsion between protein molecules. This might result in the loosen structure of protein aggregates and liquefaction of pidan white. However, in the presence of lead, Zn^{2+} and Ca^{2+} at a level of 0.2%, the liquefaction was retarded. Those divalents might stabilise the protein network, thereby lowering the dissociation of protein network previously formed, though slightly higher alkaline pH was obtained during ageing. Stabilising effect varied with type of divalents used. Monovalent and divalent ions both screen electrostatic interactions between charged protein molecules (Yasuda *et al.*, 1986). Electrostatic attraction between the positively charged Zn^{2+}–water complex and the carboxylic groups of the negatively charged protein forms an aggregate (Shi *et al.,* 2008). Divalent cations such as Ca^{2+} induced cross-linking via negatively charged carboxylic acid groups of protein molecules (Hongsprabhas and Barbut, 1997). Bryant and McClements (2000) reported that Ca^{2+} ion was more capable of inducing protein aggregation than was sodium ions because of the salt bridge effect. Based on the

hardness, lead ion yielded the pidan white gel without liquefaction throughout pickling and ageing period. Nevertheless, Ca^{2+} at higher concentration (0.5%) showed the lower ability in strengthening the gel than did the lower concentration as indicated by the lower hardness. The excessive cations might provide the positive charge of the protein molecules, leading to increased repulsion or dissociation between protein molecules. Monovalent cation was not able to stabilise the protein gel network at alkaline pH during ageing. The decreasing hardness of pidan white treated with monovalent cation at both concentrations used might be due to the screening effect on the charge of protein, thereby decreasing the cross-links between the protein chains. This resulted in the softening or liquefaction of gel formed. In this study, the same concentration (0.2%) of different salts has varying molarity and ionic strength (1.93 of 0.01 M PbO_2, 1.95 of 0.01 M $ZnCl_2$, 1.96 of 0.02 M $CaCl_2$, 1.97 of 0.02 M $MgCl_2$ and 1.94 of 0.03 M KCl). This also might contribute to the varying charge distribution of salt and their protein cross-linking ability.

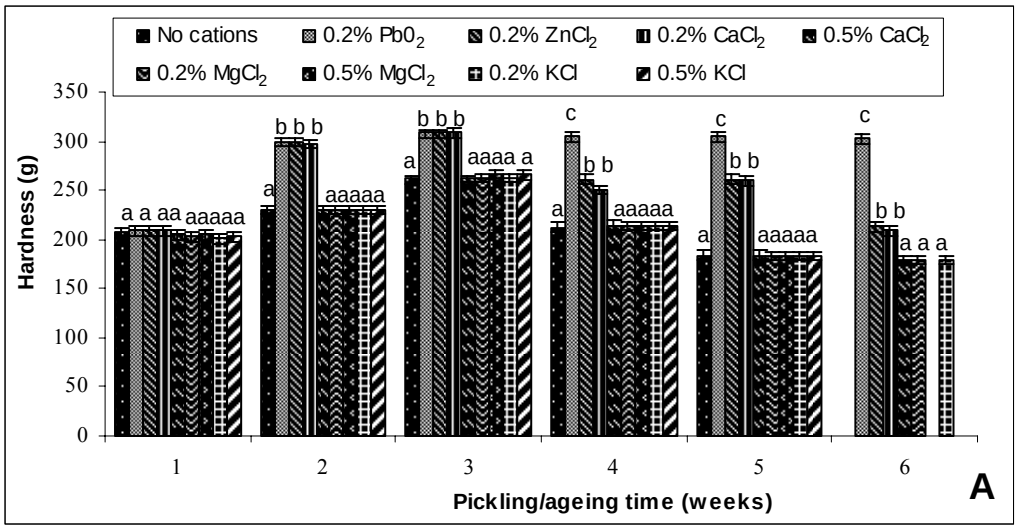


Figure 8. Changes in texture profile analysis (TPA) of pidan white during pickling and ageing in the presence or absence of different divalent and monovalent cations. Bars represent the standard deviations from triplicate determinations. Different letters on the bars within the same pickling/ageing time indicate significant differences ($P<0.05$)

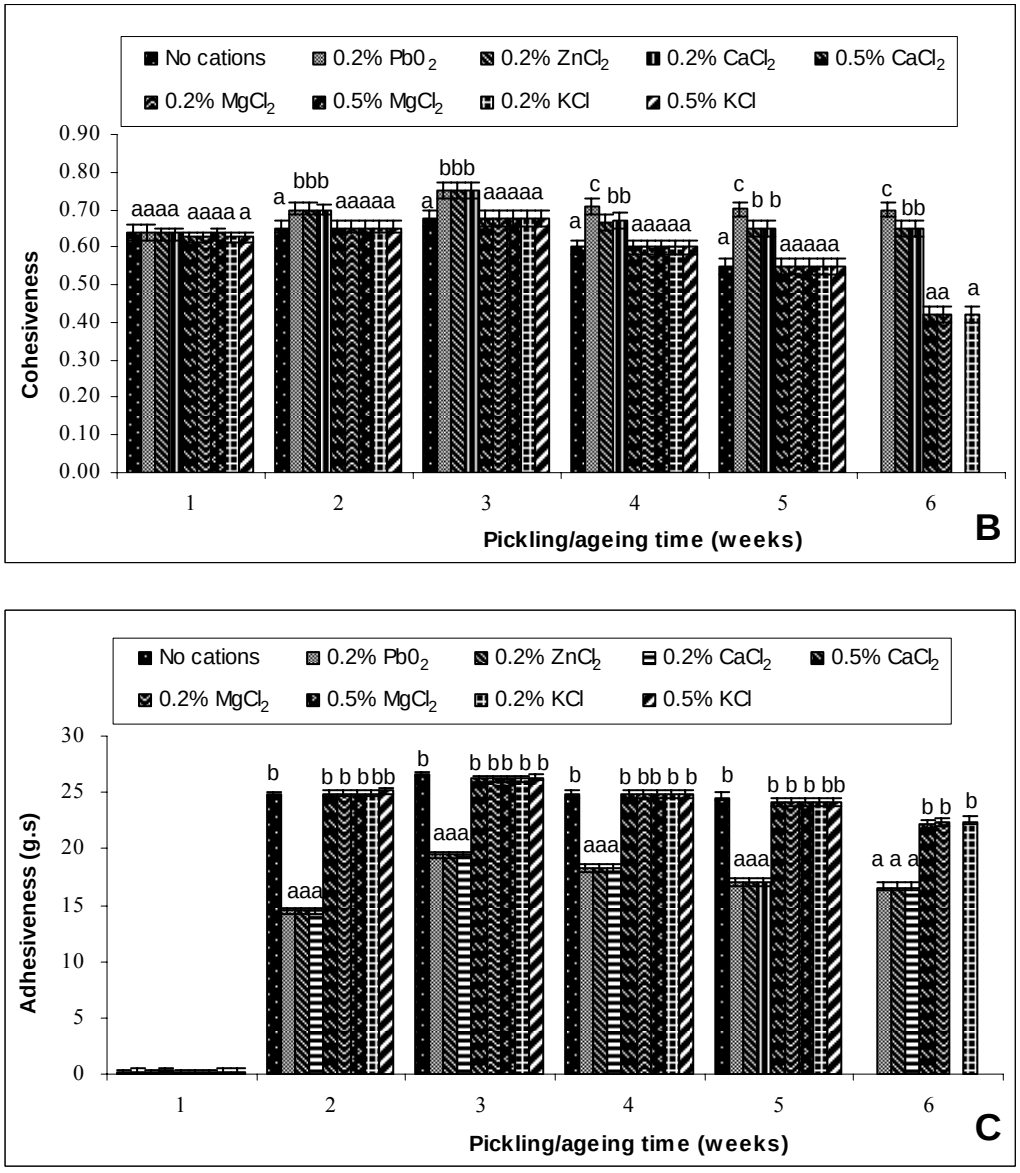


Figure 8 (Cont). Changes in texture profile analysis (TPA) of pidan white during pickling and ageing in the presence or absence of different divalent and monovalent cations. Bars represent the standard deviations from triplicate determinations. Different letters on the bars within the same pickling/ageing time indicate significant differences ($P<0.05$)

Cohesiveness is often used as an indice of the ability of gel to maintain an intact network structure. Higher values of cohesiveness indicate how well the product withstands intact network structure (Fernandez-Lopez *et al.*, 2006). Cohesiveness of pidan white increased continuously and reached the maximum at

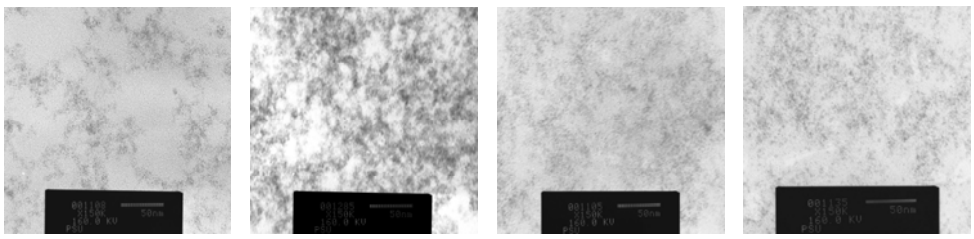
week 3 and gradually decreased up to week 6 ($P<0.05$) (Figure 8B). This change was in accordance with the change in hardness of pidan white. Alkali presented in pidan probably caused the weakening of protein gel formed with increasing ageing time. Cohesiveness of pidan white treated with 0.2% PbO_2, 0.2% $CaCl_2$ or 0.2% $ZnCl_2$ was higher than those from other treatments at all pickling and ageing times ($P<0.05$). Those divalents could maintain an intact gel network effectively along with the continuous dehydration of pidan white. It was noted that the decrease in cohesiveness of all treatments except those treated with lead was observed as pickling and ageing time increased up to week 6 ($P<0.05$). The results were in good agreement with Kaewmanee *et al.* (2009) who reported that cohesiveness was slightly decreased when the salting time increased in the salted egg.

Adhesiveness is defined as the work necessary to overcome the attractive forces between the product and a specific surface (Raikos *et al.*, 2007). A remarkable increase in adhesiveness was observed at week 2 of pickling (Figure 8C). Among all samples, those treated with PbO_2, $ZnCl_2$ or $CaCl_2$ at a level of 0.2% showed the lower adhesiveness, indicating the less stickiness of pidan white. Thereafter a slight increase in adhesiveness of those three samples was found at week 3 and the gradual decrease in adhesiveness was observed after ageing (week 6). At week 6, the higher adhesiveness of pidan white was obtained in those treated with 0.2% $MgCl_2$ or 0.2% KCl or 0.5% $CaCl_2$. Gradual decrease in adhesiveness after 4 weeks of pickling and ageing in all treatments might be attributed to the lowered moisture content of pidan white. As a result, pidan white had the less stickiness. From the result, the concentrations of $MgCl_2$ and KCl had no effect on adhesiveness of resulting pidan white. However, the concentrations of $CaCl_2$ had the effect on adhesiveness of pidan white, in which 0.5% $CaCl_2$ yielded pidan egg white with the higher adhesiveness ($P<0.05$).

Changes in microstructure of pidan white after pickling

Microstructures of pidan after pickling for 3 weeks visualised by TEM are shown in Figure 9. Aggregation of protein molecules was noticeable in pidan white treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ obtained after 3 weeks of

pickling (Figure 9). Pidan white treated with 0.2% PbO_2 showed the closer aggregates with denser network, whereas the pidan white treated with 0.2% $CaCl_2$ or 0.2% $ZnCl_2$ exhibited the looser network with the larger irregularly shaped voids. More compact structure of protein aggregates in pidan treated with 0.2% Pb might be more stable under the increasing alkaline condition, especially as the pickling or ageing time increased. For the control (without cation), the lower aggregation took place. At alkaline pH, repulsive electrostatic forces among protein molecules dominate and aggregation of linear molecules becomes favored, leading to the formation of homogeneous network (Aymard *et al.*, 1999). In the presence of divalents, the higher aggregates were formed via the salt bridges but the degree and pattern of aggregates were determined by the type of cations. From the image, the speck was observed, suggestion the aggregate of hydrolyzed proteins with the short chain.



A) No cations B) 0.2% PbO_2 C) 0.2% $ZnCl_2$ D) 0.2% $CaCl_2$

Figure 9. Transmission electron microscopic photograph of pidan white after 3 weeks of pickling**.** Magnification: 150X

Changes in the color and browning intensity of pidan white during pickling and ageing

The color of pidan white obtained during pickling and ageing is shown in Table 4. L* value of pidan white decreased as the pickling and ageing time increased up to week 6. However pidan white treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ showed higher L* values, compared to other treatments ($P<0.05$). This was probably due to the higher aggregation of proteins, which exhibited higher light scattering effect. Chantrapornchai and McClements (2002) reported that whey protein gels increased its lightness with increasing protein size. Increased b* values were

found in pidan white after pickling (week 3), possibly owing to the formation of yellow or brown pigments. However pidan treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ showed the lower b* values, compared to other treatments ($P<0.05$). At week 6, the lower b*-value with increase in a*-values were found in pidan white, irrespective of treatments. Among all samples, pidan white treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ had the lower b*-value but higher L* and a* values after ageing, compared to other samples ($P<0.05$). Increased a* values were possibly owing to the formation of brown pigments, which might derive from Mailliard reaction of egg white.

Browning intensity of pidan white with different treatments during pickling and ageing is shown in Figure 10. Maillard reaction is a non-enzymatic browning reaction which links the carbonyl group of reducing carbohydrates and the amino group of free amino acids as well as of lysine residues in proteins (Ajandouz *et al.*, 2001; Kato *et al.,* 1978). Maillard reaction is considered important in pidan white because of the significant amount of glucose naturally present in the egg white proteins. Egg white based films underwent yellow coloration due to the glucose present in egg white (Powrie, 1977). The browning intensity increased with increasing pickling and ageing times, irrespective of the treatments. However pidan treated with 0.2% PbO_2, 0.2% $CaCl_2$ or 0.2% $ZnCl_2$ had the lower browning intensity, compared with other treatments ($P<0.05$). This was in agreement with the lower a* and b*-values of those three treatments (Table 4). Higher browning intensity of other treatments obtained with increasing pickling and ageing time was most likely due to less cross-linking of proteins. As a result, amino groups were more available for Maillard reaction. Furthermore, tea in pickling solution might enhance the brown color in the pidan white gels due to oxidation of flavonols in the alkaline environment. Wang *et al.* (2000) studied the sensory properties of tea and hypothesised that the oxidation of flavanols during processing and storage resulted in color development. Mizooku *et al.* (2003) reported that the oxidation of flavanols was determined by pH. Flavanols was colorless in aqueous solution at pH 7.6, however it turned yellow at pH 10.6. Therefore, divalent at a particular concentration had an impact on color of pidan white.

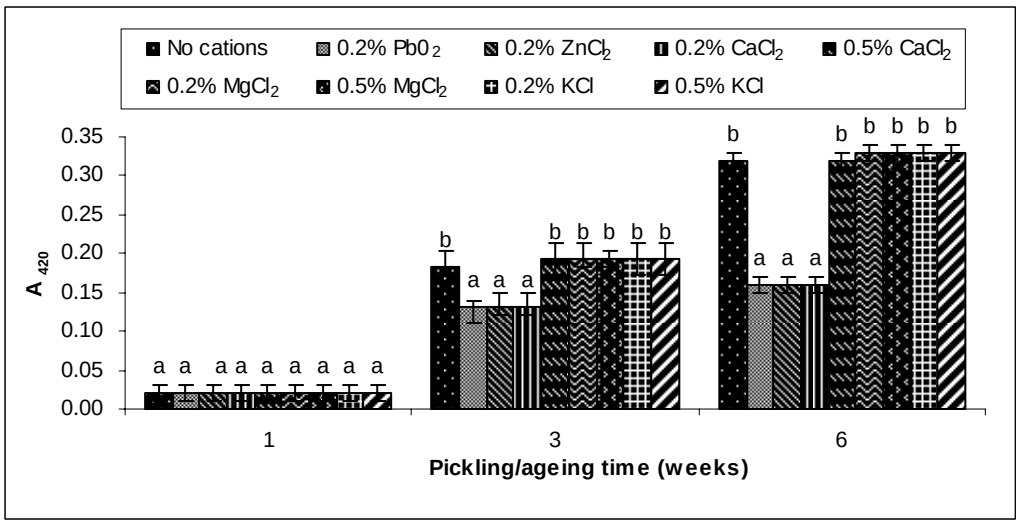


Figure 10. Browning intensity of pidan white during pickling and ageing in the presence or absence of different divalent and monovalent cations. Bars represent the standard deviations from triplicate determinations. Different letters on the bars within the same pickling/ageing time indicate significant differences ($P<0.05$)

Table 4. L*, a* and b*-values of pidan white during pickling and ageing in the presence or absence of different divalent and monovalent cations

Parameters	Treatments	Pickling/ageing time (weeks)		
		0	3	6
L*	No cations	95.85±1.20‡ C	17.15±1.54 a†, B††	11.24±1.78 a, A
	0.2% $Pb0_2$		25.20±1.17 b, B	15.25±1.78 b, A
	0.2% $ZnCl_2$		25.65±1.47 b, B	15.55±1.71 b, A
	0.2% $CaCl_2$		25.83±1.57 b, B	15.65±1.69 b, A
	0.5% $CaCl_2$		17.11±1.54 a, B	11.23±1.85 a, A
	0.2% $MgCl_2$		17.10±1.52 a, B	11.26±1.81 a, A
	0.5% $MgCl_2$		17.13±1.54 a, B	11.25±1.80 a, A
	0.2% KCl		17.15±1.57 a, B	11.28±1.82 a, A
	0.5% KCl		17.13±1.58 a, B	11.23±1.85 a, A
a*	No cations	0.23±1.20 A	6.62±1.65 b, B	11.33±1.47 b, C
	0.2% $Pb0_2$		3.45±1.51 a, B	7.79±1.57 a, C
	0.2% $ZnCl_2$		3.47±1.54 a, B	7.87±1.55 a, C
	0.2% $CaCl_2$		3.75±1.52 a, B	7.49±1.61 a, C
	0.5% $CaCl_2$		6.62±1.63 b, B	11.24±1.37 b, C
	0.2% $MgCl_2$		6.72±1.62 b, B	11.34±1.42 b, C
	0.5% $MgCl_2$		6.79±1.59 b, B	11.39±1.38 b, C
	0.2% KCl		6.76±1.61 b, B	11.38±1.41 b, C
	0.5% KCl		6.75±1.63 b, B	11.35±1.43 b, C
b*	No cations	8.2±1.25 B	13.30±0.65 b, B	5.42±1.76 a, A
	0.2% $Pb0_2$		9.20±0.54 a, B	1.85±1.75 b, A
	0.2% $ZnCl_2$		9.02±0.56 a, B	1.92±1.69 b, A
	0.2% $CaCl_2$		9.79±0.50 a, B	1.96±1.71 b, A
	0.5% $CaCl_2$		13.37±0.50 b, B	5.37±1.74 a, A
	0.2% $MgCl_2$		13.32±0.59 b, B	5.30±1.82 a, A
	0.5% $MgCl_2$		13.29±0.55 b, B	5.31±1.78 a, A
	0.2% KCl		13.36±0.65 b, B	5.39±1.79 a, A
	0.5% KCl		13.28±0.55 b, B	5.34±1.75 a, A

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

†† Different capital letters in the same row including the control (no cations) for each parameter indicate the significant differences ($P<0.05$). † Different letters in the same column within the same parameter indicate the significant differences ($P<0.05$)

2.5 Conclusions

Type and concentration of cations had varying influences on the characteristics of pidan white. PbO_2, $ZnCl_2$ or $CaCl_2$ at a low concentration (0.2%) had the significant influence on texture and color of the pidan white and yielded the hard gel during pickling. However, only PbO_2 showed the stabilising effect on gel formed during ageing. Pickling generally resulted in significant changes in TPA, whereas ageing helped in improvement of color. Therefore, the use of divalent cations such as $ZnCl_2$ or $CaCl_2$ may be an alternative divalent for pidan production. However further studies will be focused to increase the gel stability during ageing or storage.

CHAPTER 3

PHYSICAL PROPERTIES AND MICROSTRUCTURE OF PIDAN YOLK AS AFFECTED BY DIFFERENT DIVALENT AND MONOVALENT CATIONS

3.1 Abstract

Changes in physical property and microstructure of pidan yolk were monitored during pickling in the presence of different divalent ($CaCl_2$, $MgCl_2$) and monovalent (KCl) cations at different levels (0.2 and 0.5%) up to 3 weeks, followed by ageing for another 3 weeks. Pidan prepared following the commercial process, in which 0.2% PbO_2 or 0.2% $ZnCl_2$ was incorporated, was also tested. Hardness, hardening ratio, NaCl content and pH of yolk gradually increased, whereas moisture content, cohesiveness and adhesiveness decreased as the pickling and ageing time increased up to 6 weeks, regardless of cations used. During pickling and ageing, L* and b* values of interior yolk and a* of exterior yolk decreased, while L* and b* values of exterior yolk and a* value of interior yolk increased ($P<0.05$). Yolk of pidan treated with 0.2% PbO_2 was semisolid with lower hardening ratio, hardness, cohesiveness and adhesiveness, compared with those from other treatments. Those treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ yielded higher hardening ratio, hardness and cohesiveness but lower adhesiveness than others. Confocal laser scanning microscope indicated that the greater dehydration and release of lipids took place in pidan yolk during pickling and ageing of 6 weeks.

3.2 Introduction

Pidan or century egg also known as preserved egg, hundred-year egg, and thousand-year-old egg is one of the most traditional and popular preserved egg products in Thailand, China and South East Asian countries. Generally, pidan can be

made by pickling the eggs in 4.2% NaOH/5.0% NaCl solution at room temperature (30°C) for 20 days (Su and Lin, 1994). The formation of pidan is caused by the penetration of alkali through the egg shell and membrane, leading to chemical changes in the egg components. Generally, albumen and yolk gradually become solidified and hardened (Blunt and Wang, 1918). All changes occurring during the pickling of pidan possibly determine the preferential characteristics of pidan or thousand year old egg.

Network formation via salt-mediated interactions of the soluble proteins can take place at a low temperature depending on the types of protein used and gelation time required (Hongsprabhas and Barbut, 1997). Action of the different chloride salts was dependent on protein and varied with the kind of cation involved (Telis and Kieckbusch, 1997). Monovalent and divalent salt ions both screen electrostatic interactions between the charged protein molecules (Yasudha *et al.,* 1986). Nevertheless, divalent cations such as Ca^{2+} have the cross-linking effect towards negatively charged carboxylic acid groups (Hongsprabhas and Barbut, 1997). Gordan and Barbut (1990) observed that the matrix of meat batters added with $MgCl_2$ had the larger pores with the fewer interconnecting strands, when compared to those added with NaCl or KCl. Causeret *et al.* (1991) postulated that the Ca^{2+} ions could cause the development of ionic bridges between phosphate groups in phosphovitin, lipovitelinin and low density lipoprotein in egg yolk. Grizzuti and Perlmann (1973) confirmed that phosphovitin present in the egg yolk had the capacity of binding calcium and magnesium.

In the traditional process, some heavy metals, especially lead, are usually added to improve pidan quality. As a result, the products have high levels of lead residue. Due to the safety concerns, most consumers typically request "lead-free" pidan. Alternatively, Zn^{2+} has been used to produce pidan with no black spots on the eggshell, and the color of the pidan's albumen and yolk was more stable (Chen and Su, 2004). Apart from pidan white, the properties of yolk have been taken into consideration by the consumers. Different ions used in the pickling solution might contribute to the development of pidan yolk differently, leading to the varying characteristics of pidan yolk. To exploit the better benefit for consumers, the development of new process using the alternative divalent cations should be a means

to replace the toxic lead, in which the safe pidan can be obtained and becomes more marketable. However, little information regarding the texture, microstructure and color of pidan yolk during the pickling and ageing process of pidan, particularly those pickled in the presence of alternative divalent or monovalent cations, have been reported. Therefore, the objective of this study was to investigate the changes in physical properties and microstructure of pidan yolk obtained with the aid of different divalent and monovalent cations during pickling and ageing for up to 6 weeks.

3.3 Materials and Methods

Chemicals

Lead oxide (PbO_2), zinc chloride ($ZnCl_2$), calcium chloride ($CaCl_2$) magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium hydroxide, nitric acid and sodium chloride were purchased from Lab-Scan (Bangkok, Thailand). Silver nitrate was obtained from Merck (Darmstadt, Germany). Nile blue A was procured from Merck (Darmstadt, Germany). All salts used had the purity greater than 99%.

Duck egg collection

Fresh eggs of duck (*Anas platyrhucus*) with the weight range of 65–75 g were obtained within 1 day of laying from a farm in Rathabhum, Songhkla Province, Thailand. Duck eggs were cleaned with tap water and checked for any crack prior to pickling. Three different lots of egg were used for the study and three eggs were used for each run.

Preparation of pidan

Clean duck eggs were soaked in a pickling solution containing 4.2% NaOH, 5% NaCl and 2% Chinese tea without and with the addition of different divalent and monovalent cations. Divalent cations used included $ZnCl_2$, $CaCl_2$ $MgCl_2$

and monovalent cation was KCl. $CaCl_2$, $MgCl_2$ and KCl was added in the pickling solution at the concentrations of 0.2% and 0.5%. For PbO_2 and $ZnCl_2$, a level of 0.2% was used. Eggs (10 eggs) were soaked in different pickling solutions (1L) at room temperature (30-32°C) for 3 weeks. Pickled eggs were removed and coated with white clay paste (clay: water, 4:1 w/v) to obtain a thickness of 2-3 mm. Coated eggs were left at room temperature for another three weeks for ageing. During pickling and ageing, the samples were taken for analyses every week.

Determination of pH, moisture and NaCl contents

At week 3 and 6, pidan yolk samples with different treatments were determined for pH in comparison with fresh egg white according to the method of Benjakul *et al.* (1997). Moisture and salt contents in the pidan white samples were measured as per the method of AOAC (2000) with the analytical No. of 925.10 and 939.10, respectively. To determine salt content, sample (1 g) was added with 20 ml of 0.1 N $AgNO_3$ and 10 ml of HNO_3. The mixture was boiled gently on a hot plate until all solids except $AgCl_2$ were dissolved (usually 10 min). The mixture was cooled using running water. Five ml of 5 % ferric alum indicator ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$) were added. The mixture was titrated with the standardised 0.1 N KSCN until the solution became permanently light brown. The percentage of salt was then calculated as follows:

$$\text{Salt content (\%)} = 5.8 \times [(V1 \times N1) - (V2 \times N2)]/W$$

where V 1 = volume of $AgNO_3$ (ml); N 1 = concentration of $AgNO_3$ (N);
V 2 = volume of KSCN (ml); N 2 = concentration of KSCN (N); and
W = weight of sample (g).

Texture profile analysis (TPA)

Yolks of pidan with different treatments obtained at week 1, 2, 3, 4, 5 and 6 were subjected to TPA. TPA was performed as described by Bourne (1978) with a TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England). Prior to

analysis, pidan yolks were rolled on a filter paper (Whatman No. 1) to remove pidan white. The samples were compressed twice to 50% of their original height with a compression cylindrical aluminum probe (50 mm diameter). Textural analyses were performed at room temperature. Force-distance deformation curves were recorded at cross head speed of 5 mm/s and the recording speed was 5 mm/s. Hardness (g), adhesiveness (g.s), and cohesiveness were evaluated. These parameters were obtained using the Micro Stable software (Stable Micro Systems, Surrey, England).

Measurement of hardening ratio

Hardening ratio of pidan yolk was determined following the method of Chi and Tseng (1998). The pidan yolk was rolled on a filter paper (Whatman No. 1) to remove pidan white. The weight of pidan yolk was measured (W_o). The pidan yolk was cut with a knife and the removable interior yolk (soft or liquid) was scraped out using a teaspoon. The weight of exterior yolk (W_{ex}) was measured. The hardening ratio of the pidan yolk was calculated as follows:

$$\text{Hardening ratio} = (W_{ex}/ W_o) \times 100$$

Color measurement

The color of the pidan yolk, both exterior and interior, obtained at week 3 and 6 was measured using a Hunter Lab Labscan II colorimeter (Hunter Associates Laboratory Inc., Reston, VA) and expressed as L* (lightness), a* (redness/greeness) and b* (yellowness/blueness).

Determination of microstructure using confocal laser scanning microscopy

Microstructures of yolks of pidan treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ obtained at week 6 after pickling and ageing were examined with a confocal laser scanning microscopy (CLSM) (Olympus, FV300, Tokyo, Japan) following the modified method of Mineki and Kobayashi (1997). Yolk of all pidans

was separated into three parts: outer layer, middle gelled layer and inner liquid layer. Each portion was suspended in 0.1 g kg^{-1} Nile blue A solution at a ratio of 1:10 (w/v) and manually stirred until the uniformity was obtained. Fifty µl of suspension was smeared on the microscopy slide. The CLSM was operated in the fluorescence mode at the excitation wavelength of 533 nm and the emission wavelength of 630 nm using a Helium Neon Red laser (HeNe-R) for lipid analysis and at the excitation wavelength of 488 nm and the emission wavelength of 540 nm using a Helium Neon Green laser (HeNe-G) for protein analysis.

Statistical analysis

Completely randomized design was used throughout the study. The experiments were run in triplicate. Data were presented as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out and means comparisons were run by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analyses were performed with the statistical program (SPSS for windows, SPSS Inc, Chicago, IL, USA).

3.4 Results and Discussion

Changes in the chemical composition of pidan yolk during pickling and ageing

Changes in pH, moisture and NaCl contents of yolk of pidan treated with different divalent and monovalent cations were monitored during pickling and ageing (Table 5). Decreases in moisture content with coincidental increases in NaCl content and pH in pidan yolk were observed during pickling and ageing, regardless of type of ions used (Table 5). At the same pickling time (week 3), similar moisture content of pidan yolk was obtained in all treatments except that treated with 0.2% PbO_2, which showed the higher moisture content ($P<0.05$). During ageing, the moisture content decreased slightly and was more pronounced in yolk treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$. Again, the highest moisture content was found in yolk of

pidan that had been treated with 0.2% PbO_2. This was most likely due to the loss of water from the pidan yolk to the outside, caused by osmosis process during pickling and ageing. The higher moisture content found in the yolk of pidan treated with 0.2% PbO_2 was probably associated with the higher cross- linking of yolk proteins by Pb^{2+} ion. Cross linked proteins might prevent the dehydration of yolk to some extent. Blunt and Wang (1918) reported that the moisture loss in the pidan yolk was due to loss of water to albumen of pidan and through the pores of egg shell.

At week 3 of the pickling, the pidan yolk from all treatments had the marked increase in NaCl content and pH, regardless of treatments (Table 5). Chang *et al.* (1999a) reported that the final pH of pidan yolk pickled for 20 days were 10.12. The increase in pH and NaCl content indicated the migration of alkali from pickling solution and NaCl penetration, respectively. Alkali and NaCl might be associated with aggregation of yolk proteins, resulting in the formation of gel-like structure. Chi and Tseng (1998) reported that during salting, water could be migrated from egg yolk to egg white and subsequently to the environment through the egg shell. This resulted in the reduction of moisture content. For treatment with KCl, yolk contained the higher salt (NaCl), compared with those from other treatments ($P<0.05$). After ageing (week 6), the higher NaCl content was found in pidan yolk ($P<0.05$), irrespective of the treatments. This result indicated that the dehydration of pidan yolk was accompanied with a rise in NaCl content in pidan yolk. The reduction of moisture of pidan yolk during pickling and ageing was mostly associated with the hardening. Higher concentration (0.5%) of divalent and monovalent had no marked influence on the pH, moisture and NaCl contents of yolk, in comparison with the lower concentrations (0.2%) ($P>0.05$). Changes in pH, moisture and NaCl contents were more pronounced during pickling (week 0-3) than during ageing, regardless of treatments.

Table 5. pH, Moisture and NaCl contents of yolk from the pidan during pickling and ageing in the presence or absence of different divalent and monovalent cations

Parameters	Treatments	Pickling and ageing time (weeks)		
		0	3	6
pH	No cations	5.75±0.01$^{a\dagger, A}$	10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
	0.2% $Pb0_2$		10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
	0.2% $ZnCl_2$		10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
	0.2% $CaCl_2$		10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
	0.5% $CaCl_2$		10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
	0.2% $MgCl_2$		10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
	0.5% $MgCl_2$		10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
	0.2% KCl		10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
	0.5% KCl		10.16±0.01 $^{a, B}$	10.26±0.01 $^{a, C}$
Moisture content (%)	No cations	43.51±0.02$^{a, C\dagger\dagger}$	40.82±0.47 $^{a, B}$	38.06±0.20 $^{b, A}$
	0.2% $Pb0_2$		42.09±0.26 $^{b, B}$	41.03±0.45 $^{c, A}$
	0.2% $ZnCl_2$		40.18±0.34 $^{a, B}$	36.05±0.50 $^{a, A}$
	0.2% $CaCl_2$		40.03±0.44 $^{a, B}$	36.06±0.23 $^{a, A}$
	0.5% $CaCl_2$		40.17±0.40 $^{a, B}$	38.04±0.40 $^{b, A}$
	0.2% $MgCl_2$		40.86±0.44 $^{a, B}$	38.00±0.50 $^{b, A}$
	0.5% $MgCl_2$		40.45±0.49 $^{a, B}$	38.00±0.40 $^{b, A}$
	0.2% KCl		40.82±0.42 $^{a, B}$	38.05±0.20 $^{b, A}$
	0.5% KCl		40.38±0.35 $^{a, B}$	38.00±0.40 $^{b, A}$
NaCl content (%)	No cations	0.45±0.02$^{a, A}$	0.61±0.02 $^{a, B}$	0.98±0.02 $^{c, C}$
	0.2% $Pb0_2$		0.61±0.02 $^{a, B}$	0.82±0.02 $^{a, C}$
	0.2% $ZnCl_2$		0.61±0.02 $^{a, B}$	0.82±0.02 $^{a, C}$
	0.2% $CaCl_2$		0.61±0.02 $^{a, B}$	0.83±0.02 $^{a, C}$
	0.5% $CaCl_2$		0.61±0.02 $^{a, B}$	0.94±0.02 $^{b, C}$
	0.2% $MgCl_2$		0.61±0.02 $^{a, B}$	0.98±0.02 $^{c, C}$
	0.5% $MgCl_2$		0.61±0.02 $^{a, B}$	0.98±0.02 $^{c, C}$
	0.2% KCl		0.82±0.02 $^{b, B}$	1.06±0.02 $^{d, C}$
	0.5% KCl		0.84±0.02 $^{c, B}$	1.10±0.02 $^{e, C}$

†† Different capital letters in the same row including the control (no cations) for each parameter indicate the significant differences ($P<0.05$).† Different letters in the same column within the same parameter indicate the significant differences ($P<0.05$). Values are mean $\pm$ standard deviation (n=3)

Changes in textural properties of pidan yolk during pickling and ageing

Hardening ratio of pidan yolk was defined as the weight percent of hard exterior yolk and used as an index for the completeness of pickling (Chi and Tseng, 1998). At week 1 of pickling, the yolk became harden, especially at the surface, named "exterior egg yolk"**,** while viscous liquid yolk was found inside, termed "interior egg yolk". Hardening ratio increased gradually during pickling and ageing irrespective of treatments (Figure 11A). It indicated that alkali penetration along with salt and cations during pickling caused the major changes in the yolk proteins, particularly protein aggregation. Yang and Baldwin (1995) reported that the addition of salt to the protein suspensions promoted protein aggregation, which might result in network formation of yolk. The thickness of exterior yolk layer of pidan increased with increasing pickling and ageing. As determined by texture profile analysis (TPA), hardness of pidan yolk increased continuously and reached the maximum at week 4 and gradually decreased up to week 6 (Figure 11B). TPA of pidan yolk could not be performed at week 1 due to weak aggregates of yolk, which could not withstand the compression of analyzer. The decrease in hardness of yolk aggregates during ageing was probably due to the increase in pH of yolk, which might cause the repulsion between protein molecules. This might result in the loosen structure of protein aggregates. Hardening ratio and hardness of yolk obtained from pidan treated with 0.2% PbO_2 had remarkably lower value than those obtained from other treatments at all pickling and ageing times used ($P<0.05$). The softer texture of pidan yolk treated with PbO_2 was coincidental with the higher moisture content of this sample (Table 5). Weak gel of yolk protein developed upon dehydration was attributed to hydrophobic interaction and hydrogen bond (Paraskevopoulou *et al.*, 2000). Hydrophobic interactions of yolk proteins induced by 0.2% PbO_2 possibly lowered the dehydration of yolk. The highest hardness and hardening ratio was found in yolk from pidan treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$. Zn^{2+} has higher binding capacity to lipovitellin, which account for 90% binding of Zn^{2+} in egg yolk (Tupper *et al*., 1954). Furthermore Ca^{2+} ions can form ion bridges between negatively charged protein molecules. Bryant and McClements (2000) reported that Ca^{2+} ion could induce protein aggregations more effectively than sodium ions because of the salt

bridge effect. When the alkali penetrated through albumen to yolk, the yolk proteins might undergo unfolding and salt most likely induced the interaction of those unfolded proteins. The mechanisms of salt-induced gelation of globular proteins have been described elsewhere (Doi, 1993). When the pickling and ageing proceeded to week 6, the interior yolk was further dehydrated and became hardened, regardless of treatments.

Cohesiveness is often used as an indice of the ability of gel to maintain an intact network structure. It indicates how well the product withstands a second deformation relative to its behavior during the first deformation. Cohesiveness of the pidan yolk gradually decreased with increasing pickling and ageing time ($P<0.05$) (Figure 11C). This was possibly because of the continuous penetration of alkali, which probably caused the weakening of aggregates formed. Cohesiveness of yolk obtained from pidan treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ was higher than those from other treatments. Protein aggregate could maintain an intact network structure associated with the dehydration of pidan yolk. Among all yolk samples, that of pidan treated with 0.2% PbO_2 exhibited the lower cohesiveness during week 3-5 of pickling and ageing ($P<0.05$). In general, cohesiveness of the other treatments was not significantly different from that of control ($P>0.05$). The result revealed that salt-induced aggregate with different divalent and monovalent cations showed the decrease in cohesiveness as pickling and ageing time increased to week 6. The results were in good agreement with Kaewmanee *et al.* (2009) who reported that cohesiveness was slightly decreased when the salting time increased in the salted egg.

Adhesiveness is defined as the work necessary to overcome the attractive forces between the product and a specific surface. A remarkable increase in adhesiveness was observed at 2^{nd} weeks of pickling (Figure. 11D). Thereafter the decrease in adhesiveness was found at week 3 and negligible adhesiveness was observed after 3 weeks of pickling. Free lipid released from the lipoprotein together with the co-released water and proteins could make yolk sticky and more adhesive. Salt at a level of 2% caused the major release of lipids by rupturing of yolk granules (Burley and Cook, 1961). The lower adhesiveness of yolk obtained from the from the pidan treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ was possibly due to the saponification of free lipid by alkali penetrated into the yolk. Yolk from other

treatments showed significantly higher adhesiveness ($P<0.05$). Negligible adhesiveness after 4 weeks of pickling and ageing in all treatments might be associated with the complete saponification of yolk lipid. As a result, pidan yolk had the less stickiness.

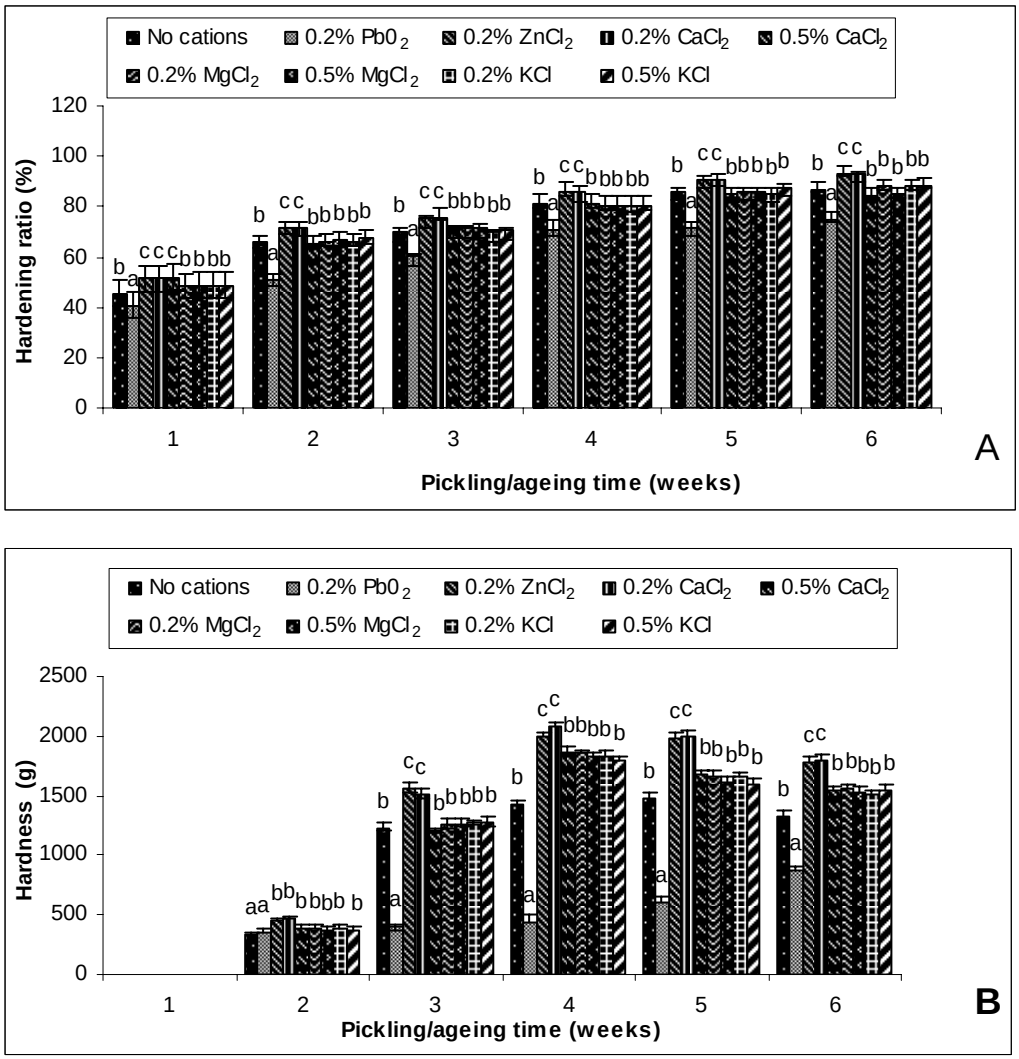


Figure 11. Changes in hardening ratio and TPA of yolk from pidan during pickling and ageing in the presence or absence of different divalent and monovalent cations. Bars represent the standard deviation (n=3). Different letters on the bars within the same pickling and ageing time indicate significant differences ($P<0.05$)

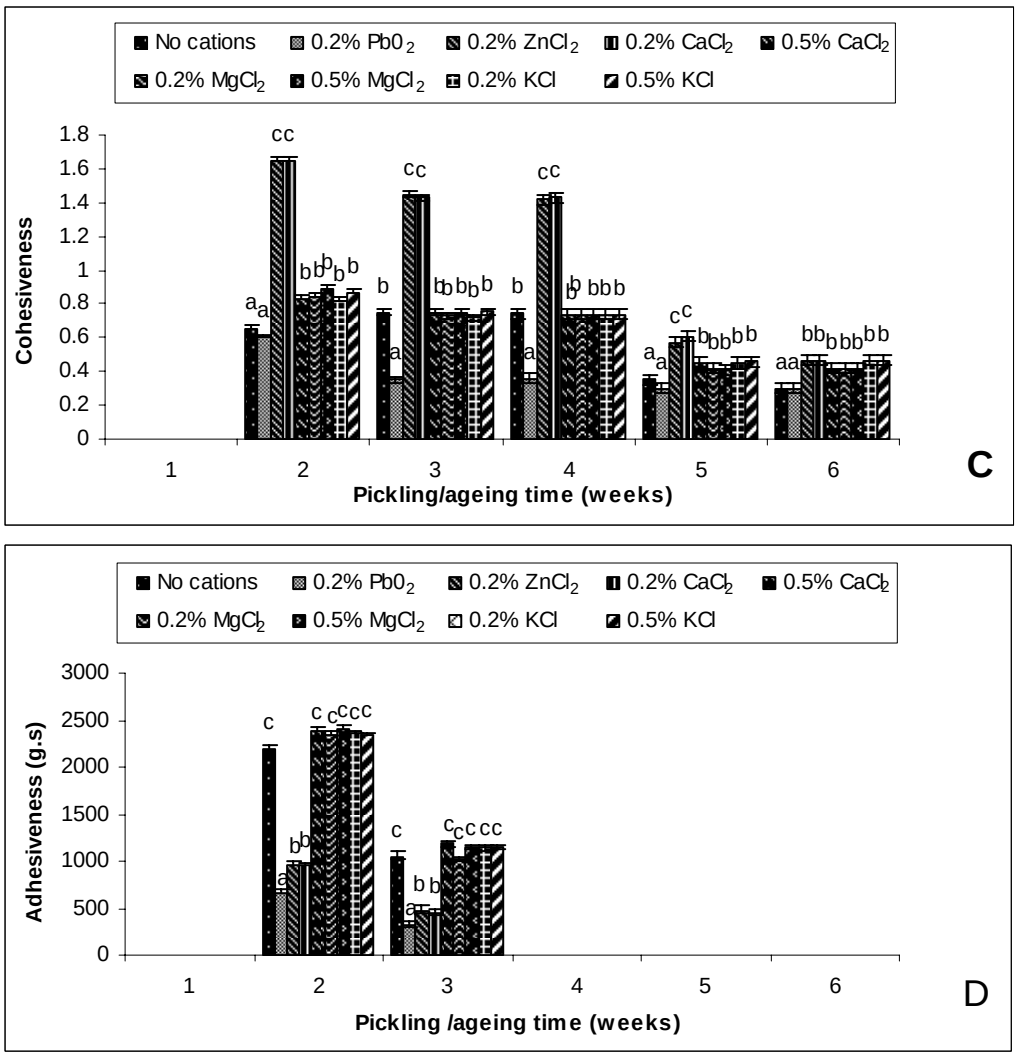


Figure 11 (Cont). Changes in TPA of yolk from pidan during pickling and ageing in the presence or absence of different divalent and monovalent cations. Bars represent the standard deviation (n=3). Different letters on the bars within the same pickling and ageing time indicate significant differences ($P<0.05$)

During pickling (week 2-3), the decrease in adhesiveness was more pronounced in the pidan yolk. For ageing (week 4-6), pidan yolk showed no adhesiveness. The result suggested that alkali penetration during pickling was associated with the major release of free lipids, which subsequently underwent saponification. Higher concentrations of ions (0.5%) had no effect on adhesiveness of resulting yolk.

Changes in the color of the pidan yolk during pickling and ageing

The color of pidan yolk during pickling and ageing is shown in Table 6. For both interior and exterior portions, yolk from pidan treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ had the lower L* and b* values than others after 6 weeks of pickling/ ageing ($P<0.05$). For exterior portion, L* and b* values increased after ageing (week 6). This was probably due to the more amounts of released free lipids, which exhibited the light scattering effect. Aydin *et al.* (2001) suggested that discoloration of albumen and yolk may be associated with the increased ratio of saturated fatty acid to monounsaturated fatty acid in egg yolk. Free lipid might be released from low density lipoprotein micelles, due to the structural changes induced by dehydration. Increased b* values were found in exterior pidan yolk after ageing (week 6), possibly owing to the formation of pyrrole pigments which might derive from phospholipids oxidation on the surface. Pyrroles formed in the reaction of oxidized lipids with protein are important precursors of fluorescing yellow compounds (Zamora *et al.*, 2000). At week 3, the lower a* values were found in interior part of yolk from pidan treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$, but the higher values were obtained after ageing (week 6). This suggested the more redness of interior yolk. For exterior portion, the lower a* values were observed in yolk from pidan treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$. Kikugawa *et al.* (1984) reported that the interaction between primary amines and malonaldehyde or/and monofunctional aldehydes produced yellow pigments. The amines in phospholipids, such as phosphatidylethanolamine, have been shown to participate in non-enzymatic browning reactions *in vitro* (Zamora *et al.*, 2005). In general, the changes in color were more pronounced in yolk of pidan treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$. These changes were coincidental with the increased dehydration. The egg yolk stored at lower moisture content for a long time becomes darker yellow color, due to removal of moisture (Lin, 1983). It was noted that the higher a* value of interior pidan yolk with the treatment of 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ might be due to the increase in concentration of pigments. The yellow orange color of yolk has been attributed to the presence of fat-soluble carotenoids in the lipid portion of lipoproteins (Shenstone, 1968). For pidan treated with 0.2% PbO_2, the darker color

of interior portions as indicated by the lowest L* values was most likely because PbO_2, which has the black color, stained the egg yolk.

Table 6. Color of interior and exterior yolk from the pidan during pickling and ageing in the presence or absence of different divalent and monovalent cations

	Pickling and ageing time (weeks)			
	Interior pidan yolk		Exterior pidan yolk	
Treatments	3	6	3	6
L*				
No cations	49.00±2.35 [b†]	22.68±1.45 [b]	45.27±2.32 [b]	59.18±2.15 [b]
0.2% $Pb0_2$	42.42±1.50 [a]	17.00±1.24 [a]	34.54±2.24 [a]	48.72±2.35 [a]
0.2% $ZnCl_2$	42.00±1.50 [a]	18.78±1.34 [a]	35.25±2.21 [a]	49.76±2.75 [a]
0.2% $CaCl_2$	42.00±1.24 [a]	18.17±1.35 [a]	34.32±1.25 [a]	49.49±2.50 [a]
0.5% $CaCl_2$	49.98±2.14 [b]	21.36±1.42 [b]	45.83±2.24 [b]	59.12±2.43 [b]
0.2% $MgCl_2$	49.00±2.25 [b]	22.57±1.27 [b]	44.30±2.21 [b]	60.24±2.25 [b]
0.5% $MgCl_2$	49.02±2.41 [b]	21.00±1.56 [b]	46.87±2.35 [b]	59.21±2.35 [b]
0.2% KCl	48.80±2.15 [b]	21.51±1.65 [b]	44.35±2.45 [b]	59.12±2.46 [b]
0.5% KCl	48.57±2.20 [b]	22.41±1.87 [b]	43.55±2.35 [b]	58.75±2.45 [b]
a*				
No cations	6.00±0.35 [b]	14.00±1.10 [a]	13.38±2.02 [b]	5.02±0.14 [b]
0.2% $Pb0_2$	4.03±0.50 [a]	19.24±1.20 [b]	6.36±0.46 [a]	3.45±0.43 [a]
0.2% $ZnCl_2$	4.05±0.50 [a]	19.00±0.65 [b]	7.91±0.25 [a]	3.45±0.42 [a]
0.2% $CaCl_2$	4.01±0.42 [a]	19.85±1.08 [b]	8.20±0.45 [a]	3.11±0.46 [a]
0.5% $CaCl_2$	5.76±0.65 [b]	14.06±1.35 [a]	13.63±0.25 [b]	5.17±0.46 [b]
0.2% $MgCl_2$	6.12±0.45 [b]	14.00±1.46 [a]	13.04±0.89 [b]	5.44±0.24 [b]
0.5% $MgCl_2$	6.00±0.42 [b]	14.31±1.06 [a]	13.32±0.42 [b]	5.47±0.25 [b]
0.2% KCl	6.04±0.46 [b]	14.05±1.45 [a]	13.45±0.45 [b]	6.68±0.48 [c]
0.5% KCl	6.02±0.38 [b]	14.02±1.35 [a]	13.35±0.42 [b]	6.60±0.24 [c]
b*				
No cations	38.51±1.23 [b]	21.00±1.56 [b]	31.85±1.23 [b]	38.05±1.10 [b]
0.2% $Pb0_2$	32.57±1.13 [a]	13.90±1.25 [a]	23.96±1.65 [a]	32.84±1.17 [a]
0.2% $ZnCl_2$	32.49±1.16 [a]	14.64±1.32 [a]	23.01±1.75 [a]	32.63±1.34 [a]
0.2% $CaCl_2$	32.31±1.32 [a]	14.66±1.35 [a]	23.14±1.14 [a]	32.10±1.24 [a]
0.5% $CaCl_2$	39.76±1.42 [b]	21.03±1.25 [b]	29.78±1.35 [c]	38.35±1.12 [b]
0.2% $MgCl_2$	38.52±1.25 [b]	21.83±1.42 [b]	27.91±1.15 [b]	39.23±1.35 [b]
0.5% $MgCl_2$	38.46±1.24 [b]	20.61±1.35 [b]	26.21±1.10 [b]	39.08±1.26 [b]
0.2% KCl	39.22±1.32 [b]	21.33±1.23 [b]	26.19±1.15 [b]	38.99±1.45 [b]
0.5% KCl	38.49±1.42 [b]	21.00±1.65 [b]	26.21±1.65 [b]	38.14±1.75 [b]

† Different small letters in the same column within the same color parameter indicate the significant differences ($P<0.05$). Values are mean ± standard deviation (n=3)

Changes in microstructure of pidan yolk during pickling and ageing

The confocal laser scanning microscope (CLSM) micrographs of pidan yolk using a two channel technique, in which both protein and lipid were stained, are illustrated in Figures 12-14. A combined image is also presented. The protein and lipid distributed uniformly in fresh egg yolk. Proteins in yolk were organized into micellar and granular structures together with polar and non-polar lipid molecules (Kiosseoglou, 2003). When the penetration of alkali proceeded to interior yolk, 3 layers of pidan yolk were formed at week 6. The dehydration together with the release of lipid in pidan yolk might be associated with such a change. In general, the distribution of both protein and lipid of interior portion was similar to that of fresh yolk. Viscous continuous phase in the interior yolk might promote the stability of emulsions because it impeded the movement and coalescence of the dispersed lipids. The result suggests that a little change in the structure of protein and lipid in the interior yolk (Figure 12-In-P, Figure 13-In-Z and Figure 14-In-C) took place, compared to outer exterior layer (Figure 12-Ex-P, Figure 13-Ex -Z and Figure 14-Ex-C) and middle layer (Figure 12-Mi-P, Figure 13-Mi-Z and Figure 14-Mi-C) of pidan yolk, regardless of treatments. This was probably due to the less penetration of alkali into the interior yolk. When the pickling and ageing proceeded, the greater dehydration with release of lipid in pidan yolk might reduce emulsion capacity of protein portion, causing the change in the structure of lipid and protein in exterior yolk. Irregular shapes of both lipid and protein were found in both middle and outer pidan yolk, irrespective of treatments. Nevertheless, the amount of lipid released and alteration of lipid shape were more pronounced in exterior layer of pidan yolk. The greater release of free lipid from lipoprotein of pidan yolk was obtained when 0.2% PbO_2 was used as indicated by the denser lipid granules appeared in the combined image (Figure 12-Ex-P [C]). In the presence of alkali, saponified lipid was postulated to bind protein, leading to the formation of shielding surface. As a result, the dehydration was lowered and soft yolk pidan was obtained. This yolk differed from hard yolks from other pidans. For yolk from pidan treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$, less release of free lipids might be associated with hard pidan yolk. Kiosseoglou (2003) reported that yolk lipid molecules are somehow involved in gel

structure formation. CLSM from pidan treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ confirmed that binding of lipid to protein yielded the hard aggregated yolk, whereas 0.2% PbO_2 yielded soft yolk containing more lipids with less association with proteins.

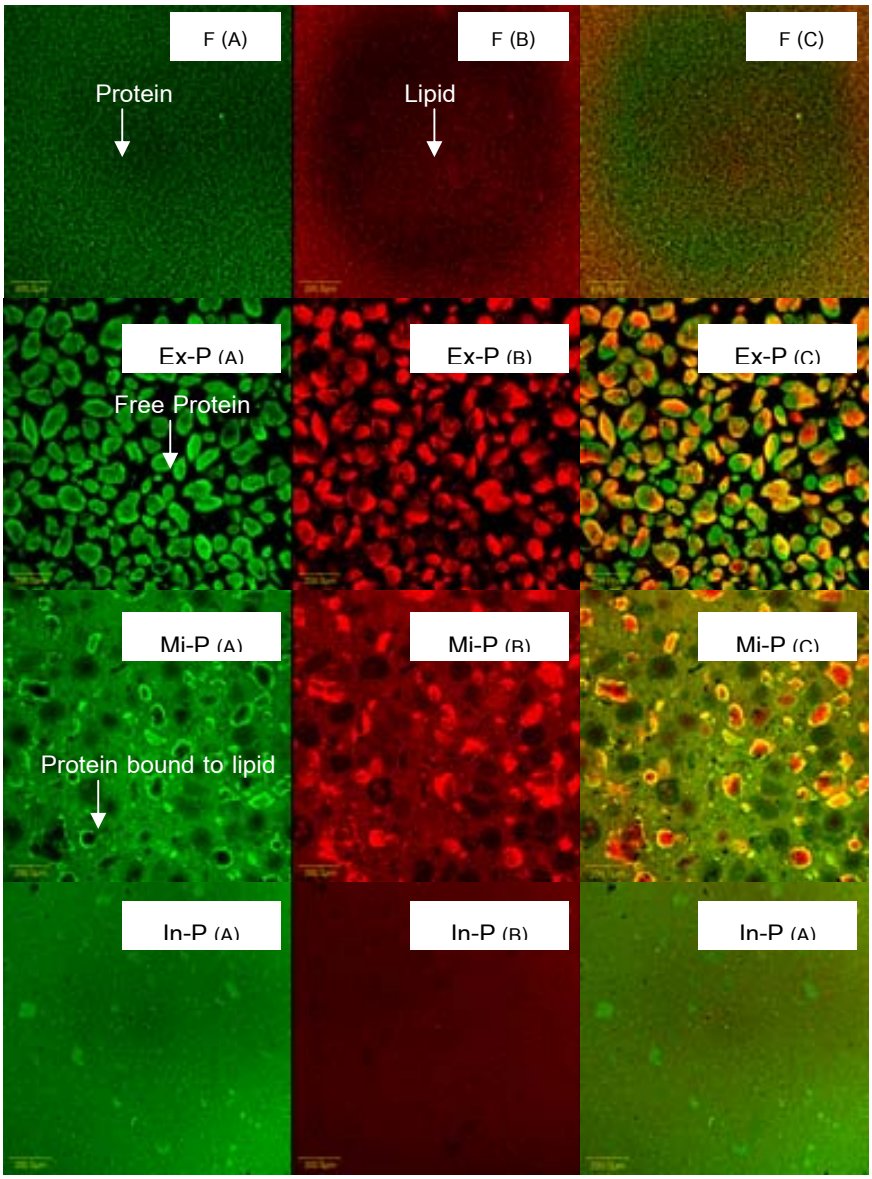


Figure 12. Confocal laser scanning microscope (CLSM) micrographs of duck egg yolks from pidan treated with 0.2% PbO_2 after pickling and ageing for 6 weeks. F; Fresh yolk Ex-P; Exterior yolk Mi-P; Middle layer yolk In-P; Interior yolk Magnification: 200X (zoom X2.5) protein distribution (A) and lipid distribution (B) and combined image of protein and lipid (C)

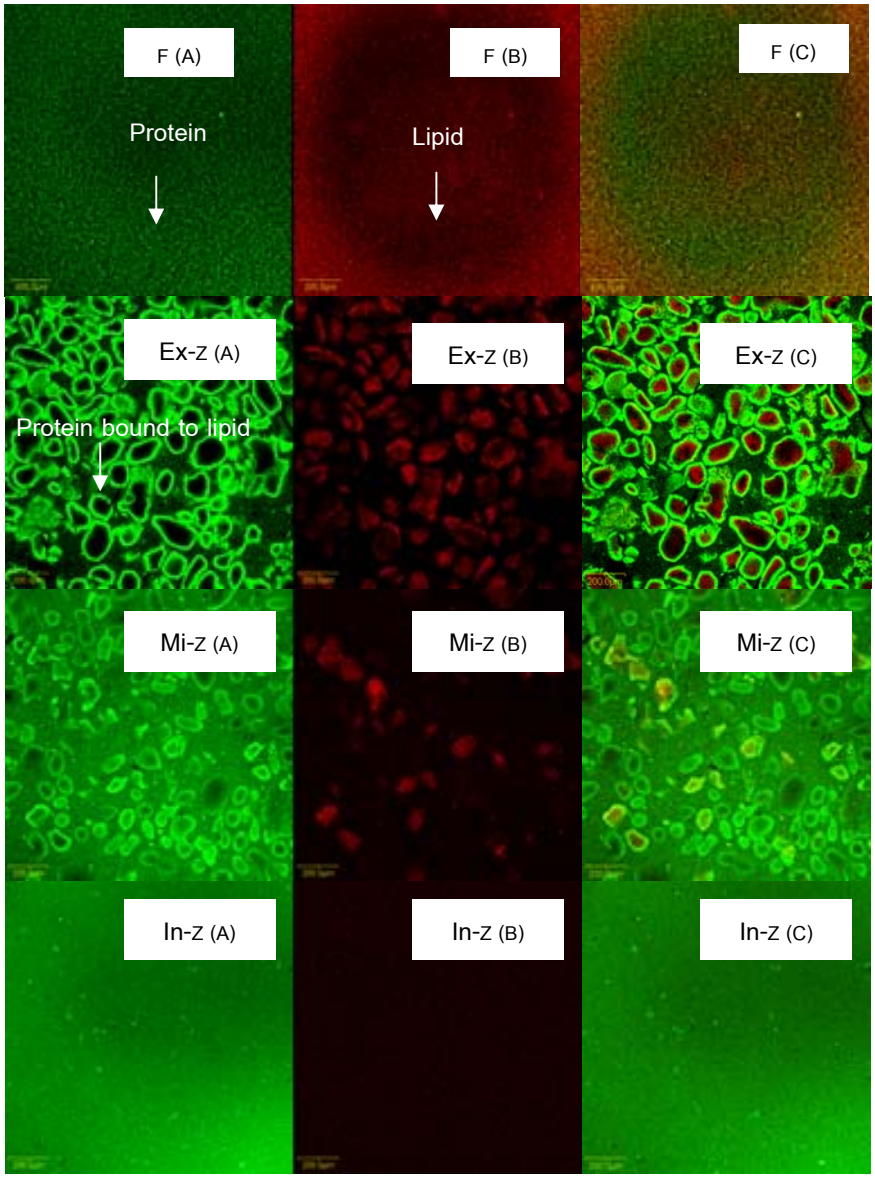


Figure 13. Confocal laser scanning microscope (CLSM) micrographs of duck egg yolks from pidan treated with 0.2% $ZnCl_2$ after pickling and ageing for 6 weeks. F; Fresh yolk Ex-Z; Exterior yolk Mi-Z; Middle layer yolk In-Z; Interior yolk Magnification: 200X (zoom X2.5) protein distribution (A) and lipid distribution (B) and combined image of protein and lipid (C)

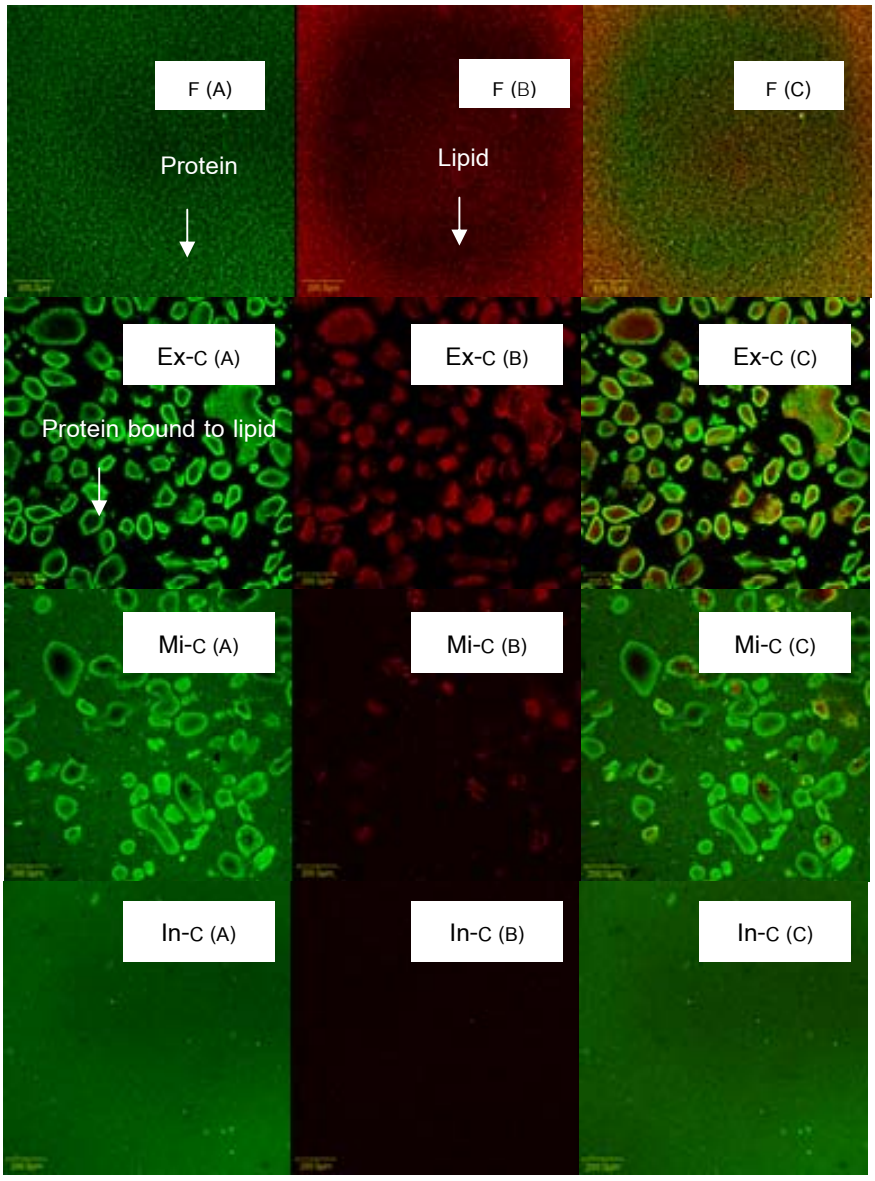


Figure 14. Confocal laser scanning microscope (CLSM) micrographs of duck egg yolks from pidan treated with 0.2% $CaCl_2$ after pickling and ageing for 6 weeks. F; Fresh yolk Ex-C; Exterior yolk Mi-C; Middle layer yolk In-C; Interior yolk Magnification: 200X (zoom X2.5) protein distribution (A) and lipid distribution (B) and combined image of protein and lipid (C)

3.5 Conclusions

Type and concentration of divalent cations had varying influences on the characteristics of pidan yolk. $ZnCl_2$ or $CaCl_2$ at a low concentration (0.2%) had the influence on texture and color of pidan yolk and yielded hard aggregates, whereas PbO_2 at the same level rendered the soft yolk aggregates with darker color. Pickling resulted in significant changes in TPA, whereas ageing helped in improvement of color, irrespective of treatments. Therefore, the use of divalent cations depends upon the consumer requirement. On the basis of health aspects, calcium can be served as an alternative mineral for the preparation of pidan yolk, which has less side effects in consumption.

CHAPTER 4

INFLUENCE OF DIFFERENT CATIONS ON CHEMICAL COMPOSITION AND MICROSTRUCTURE OF PIDAN WHITE AND YOLK DURING PICKLING AND AGEING

4.1 Abstract

Effects of different cations (0.2% PbO_2, 0.2% $ZnCl_2$, 0.2% $CaCl_2$ or 0.5% $CaCl_2$) on chemical composition and microstructure of pidan white and yolk during pickling and ageing were investigated. During 3 weeks of pickling and further 3 weeks of ageing, ammonia and ash contents were increased but varied with the types of cations used. Lower protein degradation of pidan white was observed in pidan treated with 0.2% PbO_2, compared with other treatments. Scanning electron microscopic (SEM) studies indicated that the greater aggregation of egg proteins took place in pidan white treated with PbO_2. Yolk of pidan treated with 0.2% PbO_2 had more release of free lipid as visualised by confocal laser scanning microscope (CLSM). Therefore, cations had the impact on composition and microstructure of resulting pidan white and yolk.

4.2 Introduction

Alkaline pickling of eggs has been used for centuries as a method to preserve egg and is widely practiced in China (Hou, 1981). Pidan is alternatively called as alkali pickled egg which is widely adopted into the local cuisine as an appetizer in Thailand and other South East Asian countries. Pidan is generally produced as a home-based practice, in which the pickling techniques have been

passed from one generation to another. Instead of using microorganisms, pidan is made using an alkali-treated pickling (Wang and Fung, 1996). The appearance of pidan differs from fresh eggs in that white becomes a semitransparent tea-brown color, and the yolk is solid or semisolid with a dark-green color. The nutritional value of pidan is slightly decreased compared with fresh eggs, but pidan has an extremely long shelf-life and a pleasant fragrant taste that is preferred by most people in Southeast Asian countries (Hou, 1981).

Salt has been known to have impact on gelation of egg white proteins. Several salts decreased the amount of water bound to the albumen (Bull and Breeze, 1970). Egg albumen contained many hydrophobic amino acids and easily formed turbid coagulum even at an alkaline pH when salts are added (Bull and Breeze, 1970). Salt at very low concentration aids in protein solubilization prior to heating and provides a cross-link in the network (Mullvihill and Kinsella, 1988). Nevertheless, further addition of salts simply promotes aggregation. Divalents salts tend to be more effective in aggregation than the monovalent ions, while the influence of polyvalent salts is moderate (Nakamura *et al.*, 1984). Alkaline denaturation and aggregation mainly contributes to gelation as well as characteristic color, aroma and taste of pidan (Hou, 1981).

Traditionally, pidan pickling mainly relies on Pb^{2+} cation for its gelation. As a result, the products have high levels of lead residue. Due to the safety concerns, most consumers typically request "lead-free" pidan. Alternatively, zinc is used to produce pidan with no black spots on the eggshell, and the color of the pidan's albumen and yolk was more stable (Chen and Su, 2004). Thus other cations can be an alternative gel enhancer, especially via salt bridge mechanism. Nevertheless, the basic information related to chemical and microstructural changes of pidan as affected by cations during pickling and ageing has not yet been reported. The objective of this study was to monitor the changes in chemical composition and microstructure of pidan white and yolk treated with different cations during pickling and ageing.

4.3 Materials and Methods

Chemicals

Lead oxide (PbO_2), zinc chloride ($ZnCl_2$), calcium chloride ($CaCl_2$), sodium hydroxide, and sodium chloride were purchased from Lab-Scan (Bangkok, Thailand). Nile blue A was obtained from Merck (Darmstadt, Germany). Purity of all salts used was greater than 99%.

Duck egg collection

Fresh eggs of duck (*Anas platyrhucus*) with the weight range of 65–75 g were obtained within 1 day of laying from a farm in Rathabhum, Songhkla Province, Thailand. Duck eggs were cleaned and checked for any crack prior to pickling. Eggs were used for pickling within 1-2 days after collection.

Preparation of pidan

Clean duck eggs (10 eggs) were soaked in 1 L of pickling solution containing 4.2% NaOH, 5% NaCl and 2% Chinese tea (Chinese tea No.3, Tesco Lotus, Hat Yai, Thailand) without and with the addition of different cations including PbO_2, $ZnCl_2$ and $CaCl_2$. $CaCl_2$ was added in the pickling solution at the concentrations of 0.2% and 0.5% (w/v). For PbO_2 and $ZnCl_2$, a level of 0.2% was used. Eggs (10 eggs) were soaked in different pickling solutions (1 L) at room temperature 30 to 32°C for 3 weeks. Pickled eggs were then removed and coated with white clay paste (Sunrise, Matsuoaka, Japan) using a clay/water ratio of 4:1 (w/v) to obtain a thickness of 2-3 mm. The thickness of clay layer was determined by a micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp, Kawasaki-shi, Japan). Coated eggs were left at room temperature (30-32°C) for another three weeks for ageing. During pickling (week 1 and 3) and ageing (week 6), the pidan samples were taken and separated into pidan white and yolk manually. Both pidan white and yolk were subjected to analyses.

Determination of ash content of pidan white and yolk

Pidan white and yolk with different treatments were determined for the ash according to the method of AOAC (2000) with the analytical No. of. 920.153.

Determination of ammonia content of pidan white and yolk

Ammonia content of pidan white and yolk obtained from different treatments was determined by distillation method as described by Parris and Foglia (1983) with a slight modification. Fifty ml of 10-fold diluted samples were placed in a Kjeldahl flask containing 100 ml of distilled water and 3 g of MgO. The mixture was distilled and the distillate was collected in 50 ml of 4% boric acid before titration with 0.05 M H_2SO_4 using methyl red-bromocresol green as an indicator. Ammonia content was calculated and expressed as percentage of sample

Determination of TCA-soluble peptide content of pidan white

TCA soluble peptide content of pidan white samples treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ was determined. To 2 g of finely ground pidan white samples, 18 ml of 5% TCA were added and the mixture was homogenised using an IKA Labortechnik homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. The homogenate was allowed to stand at 4°C for 1 h and centrifuged at 8000 × g for 5 min at 25°C using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). TCA-soluble peptide content in the supernatant was measured according to Lowry method (Lowry *et al.*, 1951) and expressed as µ mol tyrosine/g sample.

Scanning electron microscopy (SEM) of pidan white

Pidan white treated without and with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ at week 3 was cut into a piece of 0.5 × 0.5 cm and fixed at room temperature in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h. Fixed samples were rinsed with distilled water three times to remove salt. The samples were dehydrated in

graded series of ethanol (50, 70, 80, 90 and 100 %) and then were mounted on SEM stubs using a double backed cellophane tape. The samples were coated with gold and examined using a scanning electron microscope (JEOL JSM-5800LV, Tokyo, Japan).

Confocal laser scanning microscopy (CLSM) of pidan yolk

Microstructures of pidan yolk treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ at week 3 and 6 of pickling and ageing were examined with a confocal laser scanning microscopy (Olympus, FV300, Tokyo, Japan) following the modified method of Mineki and Kobayashi (1997). Yolk of all pidans was suspended in 0.1 % Nile blue A solution at a ratio of 1:10 (w/v) and manually stirred until the uniformity was obtained. Fifty µl of suspension was smeared on the microscopy slide. CLSM was operated in the fluorescence mode at the excitation wavelength of 533 nm and the emission wavelength of 630 nm using a Helium Neon Red laser (HeNe-R) for lipid analysis and at the excitation wavelength of 488 nm and the emission wavelength of 540 nm using a Helium Neon Green laser (HeNe-G) for protein analysis.

Statistical analysis

Completely randomized design was used throughout the study. The experiments were run in triplicate. Data were presented as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out and means comparisons were performed by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analyses were carried out with the statistical program (SPSS for windows (Version 10), SPSS Inc, Chicago, IL, USA).

4.4 Results and Discussion

Changes in chemical composition of pidan white during pickling and ageing

Ash content of pidan white treated with different cations was monitored during pickling and ageing (Figure 15). Continuous increases in ash

content were observed in pidan white during pickling and ageing ($P<0.05$). The increase in ash content indicated the migration of alkali, NaCl and cations from pickling solution. At week 3 and 6, the maximum ash content was found in pidan white treated with 0.5% $CaCl_2$ ($P<0.05$) and the minimum ash content was obtained in the sample control (without cation) ($P<0.05$). However, no differences in ash content were noticeable between pidan white treated with different cations at a level of 0.2% ($P>0.05$). Thus cations in the pickling solution partially contributed to the level of ash in the resulting pidan white. Cations at the higher level most likely penetrated into the egg more effectively as indicated by the greater ash content After ageing (week 6), the higher ash content was found in pidan white ($P<0.05$), compared with that found after pickling (week 3). This result indicated that the dehydration of pidan white during ageing was accompanied with the increases in ash content in pidan white (Ganesan and Benjakul, 2010a). After 3 weeks of pickling, eggs were removed from pickling solution containing cations. Therefore, no further migration of cations from pickling solution to egg white took place.

Ammonia content increased sharply after 3 weeks of pickling ($P<0.05$). The further increase in ammonia content was found after ageing (week 6) (Figure 15) Ammonia was formed in the pidan white, possibly by deamidation process (Hou, 1981). Deamidation of proteins occurs at pH above 8.0, dependent upon the H^+ or OH^- concentration and adjacent amino acid residue (Riha *et al.*, 1996). Lower ammonia content was found in pidan white treated with 0.2% PbO_2 after pickling and ageing ($P<0.05$), suggesting the lower deamidation of pidan white. On the other hand, the deamidation was more pronounced in pidan white treated with 0.5% $CaCl_2$ and the control. In the presence of calcium at a higher level (0.5%), the lesser cross-links might be formed due to the enhanced repulsive force.

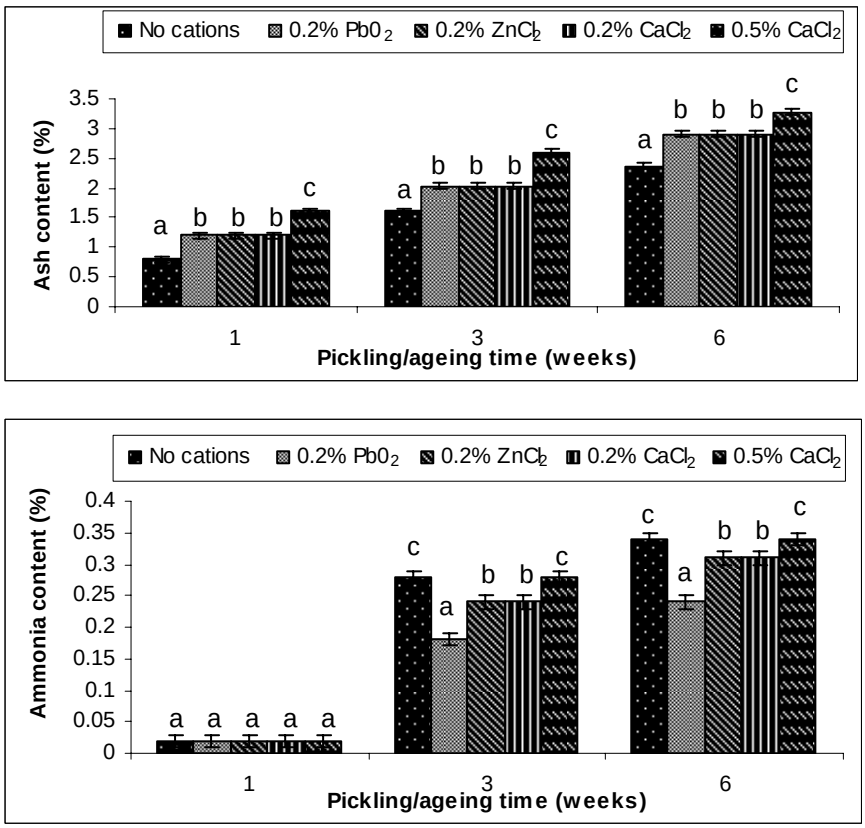


Figure 15. Ash and ammonia contents of pidan white during pickling and ageing in the presence or absence of different cations. Bars represent the standard deviations (n=3). Different letters on the bars within the same pickling/ageing time indicate significant differences ($P<0.05$)

As a result, the deamidation could occur easily with the looser protein aggregates. For the control, no salt bridges were formed and deamidation occured more effectively. Ammonia contributes to the unique odour and flavor of pidan. However, different ammonia contents found in pidan white with different treatments had no impact on the pH of pidan white as reported in our previous study (Ganesan and Benjakul, 2010a). In general, changes in ash and ammonia contents were more pronounced during pickling (week 1-3) than during ageing (week 6).

Changes in chemical composition of pidan yolk during pickling and ageing

Changes in ash and ammonia contents of pidan yolk treated with different cations were monitored during pickling and ageing (Figure 16). Continuous increases in ash and ammonia contents in pidan yolk were observed during pickling and ageing, regardless of type of cations used ($P<0.05$). At the same pickling time (week 1 and 3) and ageing time (week 6), ash and ammonia contents of pidan yolk varied with the treatments. The increase in ash content in pidan yolk indicated the migration of alkali, NaCl and cations from pickling solution through pidan white The continuous increase in the salt content of pidan yolk was reported previously (Ganesan and Benjakul, 2010b). It was noted that the highest ash content was obtained in pidan yolk treated with 0.5% $CaCl_2$. Yolk from pidan treated without different cations showed the lowest ash content ($P<0.05$). At the same level of cations used, similar ash content was found in all samples, regardless of type of cations ($P>0.05$). After ageing (week 6), slight increase in ash content was obtained in pidan yolk ($P<0.05$), irrespective of the treatments. This result indicated that the migration of inorganic matter from egg albumin to yolk still occurred during ageing.

Ammonia content increased sharply after 3 weeks of pickling ($P<0.05$). Only slight increase was noticeable after ageing (week 6). Similar result was observed in comparison with that of pidan white, in which the lowest ammonia content was found in pidan with 0.2% PbO_2 treatment. Pidan yolk treated with 0.5% $CaCl_2$ or without cation contained the highest ammonia content ($P<0.05$). No difference in ammonia content was observed between pidan yolks treated with 0.2% $ZnCl_2$ and 0.2% $CaCl_2$ ($P<0.05$). The result suggested that deamidation also occurred in pidan yolk at different degrees, depending on the treatments. The deamidation of pidan yolk gradually proceeded with increasing pickling and ageing time. Different cations exhibited the different impact on deamidation. Association of cations to proteins, particularly via amino groups, more likely impeded the deamidation process. Therefore, the chemical compositions of yolk were governed by cations to some degree. This might be associated with different characterstics of resulting pidan yolk.

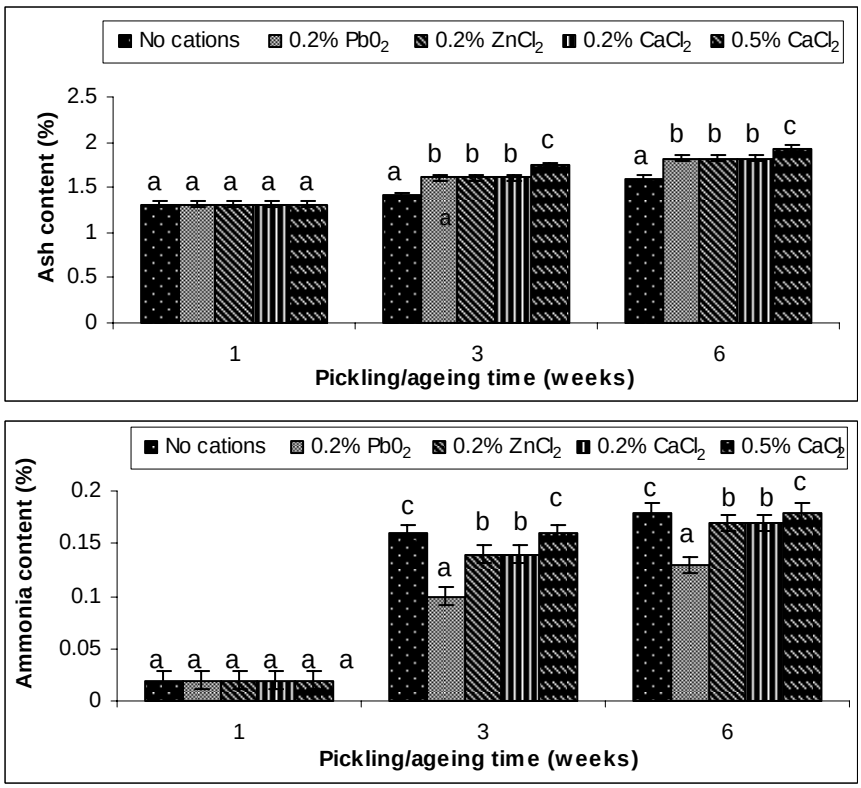


Figure 16. Ash and ammonia contents of pidan yolk during pickling and ageing in the presence or absence of different cations. Bars represent the standard deviations (n=3). Different letters on the bars within the same pickling/ageing time indicate significant differences ($P<0.05$)

Degradation of pidan white proteins during pickling and ageing

An increase in TCA-soluble peptide content was observed in pidan white during pickling and ageing, irrespective of treatments (Figure 17). Degradation of ovoalbumin and other egg white proteins resulted in the increases in free amino acid and small peptides, which partially contributed to development of flavor and aroma of pidan. Among all samples, pidan white treated with 0.2% PbO_2 showed the lowest TCA-soluble peptide content during pickling and ageing ($P<0.05$). This revealed that Pb^{2+} induced the formation of cross-links in pidan white, which were more resistant to degradation at alkaline pH when compared to the control (without cations) and other samples. TCA-soluble peptide contents of pidan white treated with

0.2% $ZnCl_2$ or 0.2% $CaCl_2$ were lower than that found in pidan treated with 0.5% $CaCl_2$ ($P<0.05$). The excess amount of calcium might result in the increased repulsion between egg white proteins, leading to the ease of hydrolysis under alkaline condition. Compounds such as peptides and amino acids may contribute to the taste in a complex manner, exceeding the taste properties of the pure compounds due to synergistic interactions (Solms, 1969). This chemical process causes an "inorganic version" of fermentation, which breaks down some of the complex flavorless proteins into simpler flavorful counterparts (Harold, 2004). Free amino acids released from hydrolysis react with carbonyl group of sugar which may also contributes to volatile Mailliard flavor compounds (Pripis-Nicolau *et al.*, 2000) as Maillard reaction indicators (Herrera-Jimenez *et al.,* 2007) and different sulphur flavor compounds may arise from methionine or cysteine under alkaline condition (Zhang and Ho, 1991) which contribute to the cysteine flavor to pidan. The degree of degradation of egg white protein was associated with the hardness of pidan white. PbO_2 treated pidan with the lowest degradation exhibited the higher hardness during pickling and ageing (Ganesan and Benjakul, 2010a). Thus, the type of cations played a role in protein aggregation of pidan white, determining the textural property and characteristics of resulting pidan.

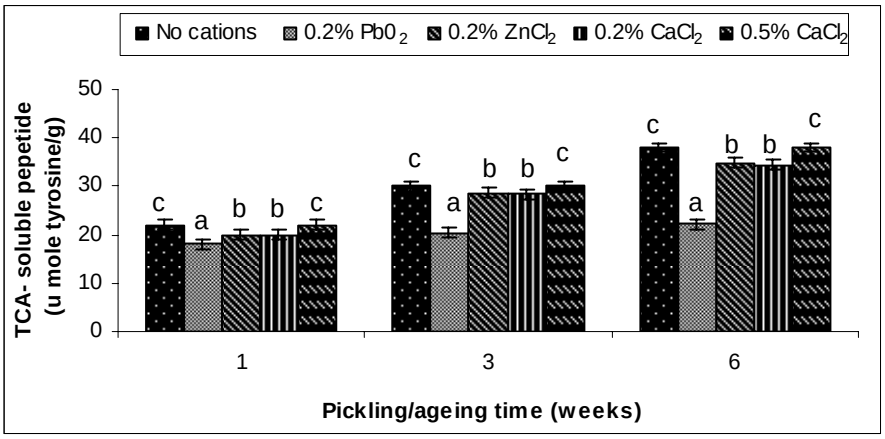
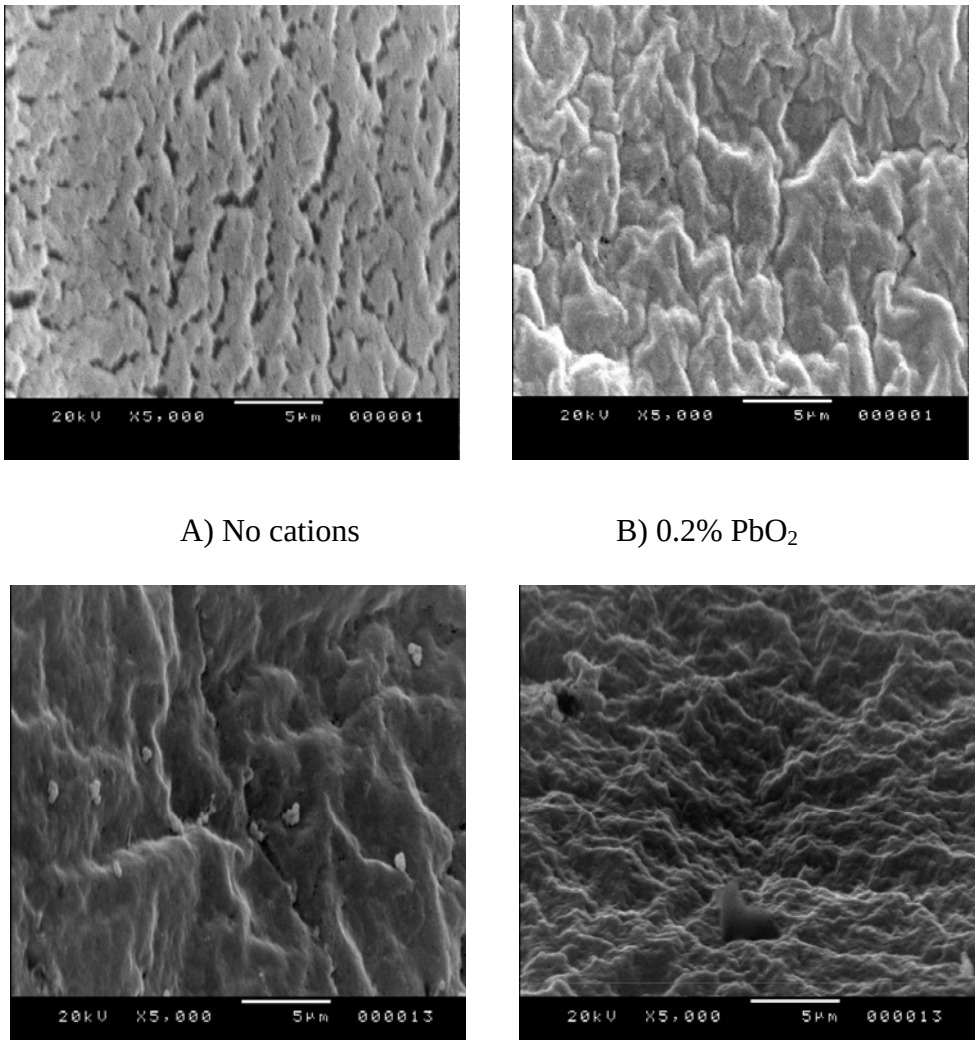


Figure 17. Changes in TCA-soluble peptides contents of pidan white treated without and with different cations during pickling and ageing. Bars represent the standard deviations (n=3). Different letters on the bars within the same pickling/ageing time indicate significant differences ($P<0.05$)

Changes in microstructure of pidan white during pickling and ageing

Microstructures of pidan white treated with 0.2% PbO_2, 0.2% $CaCl_2$ or 0.2% $ZnCl_2$ and the control (without cations) obtained after 3 weeks of pickling, visualised by SEM, are shown in Figure 18. Pidan white samples from most treatments including the control, 0.2% $ZnCl_2$, 0.2% $CaCl_2$ and 0.5% $CaCl_2$ were partially liquified after ageing (week 6). Thus, pidan white samples obtained after 3 weeks of pickling were used for microstructure study. Heterogeneous aggregates with the cracks were observed in pidan white of the control. Pidan gels had more compact structure without the gap or void in the network when treated with 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$. Smoother surface of pidan was observed in pidan treated with 0.2% PbO_2 or 0.2% $ZnCl_2$ whereas different surface was found in pidan white treated with 0.2% $CaCl_2$. Woodward and Cotterill (1987) reported that egg white gel examined with SEM was very coarse with large irregularly shaped voids. Nevertheless, ovalbumin gels showed the homogeneous microstructure (Heertje and Van Kleef, 1986). Alkaline conditions are known to unfold protein molecules (Creighton, 1993). Those unfolded proteins could be cross-linked to form protein networks, particularly in the presence of cation via salt bridge mechanism. Thus, the appropriate cations were most likely involved in ion-induced gelation of pidan white.



A) No cations B) 0.2% PbO_2

C) 0.2% $ZnCl_2$ D) 0.2% $CaCl_2$

Figure 18. Scanning electron microscopic photograph of pidan white after 3 weeks of pickling. Magnification: 5X

Changes in microstructure of pidan yolk during pickling and ageing

The confocal laser scanning microscope (CLSM) micrographs of pidan yolk after pickling (week 3) and ageing (week 6) using a two-channel technique, in which both protein and lipid were stained, are illustrated in Figures 19 and 20, respectively. Pidan yolk samples from some treatments including the control and

0.5% $CaCl_2$ treated sample were very sticky and adhesive with pidan white after pickling and ageing (week 3 and 6). This characteristic was not desirable for consumers. Thus, pidan yolk samples obtained from treatment of 0.2% PbO_2, 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ were used for microstructure study. A combined image is also presented. Proteins in yolk were organized into micellar and granular structures together with polar and non-polar lipid molecules (Kiosseoglou, 2003). In general, the lipids in egg yolk are organized in lipoproteins and are dispersed in aqueous phase (Schreiner, 2006). After 3 weeks of pickling, alkali more likely penetrated into yolk and pidan yolks became hardened. The dehydration together with the release of lipids in pidan yolk might be associated with such a change. Irregular shapes of both lipid and protein were found in pidan yolk, irrespective of treatments. Nevertheless, the release of lipids and alteration of lipid shape were more pronounced in pidan yolk with 0.2% PbO_2 treatment. In general, the distribution of both protein and lipid of yolk treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ was similar. Viscous continuous phase of yolk treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ might promote the stability of emulsions because it impeded the movement and coalescence of the dispersed lipids.

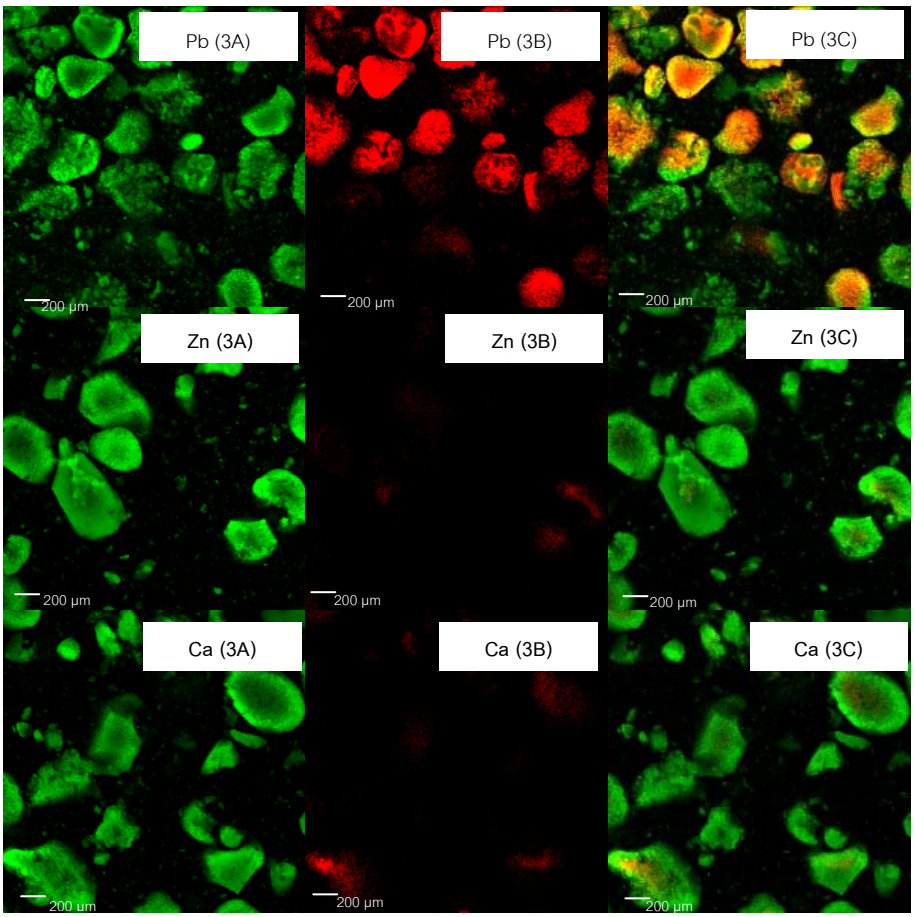


Figure 19. Confocal laser scanning microscope (CLSM) micrographs of exterior yolk for protein distribution (3A), lipid distribution (3B) and combined image of protein and lipid (3C) of pidan treated with different cations after pickling (week 3). Pb: 0.2% PbO_2; Zn: 0.2% $ZnCl_2$; Ca: 0.2% $CaCl_2$. Magnification: 200X (zoom X2.5)

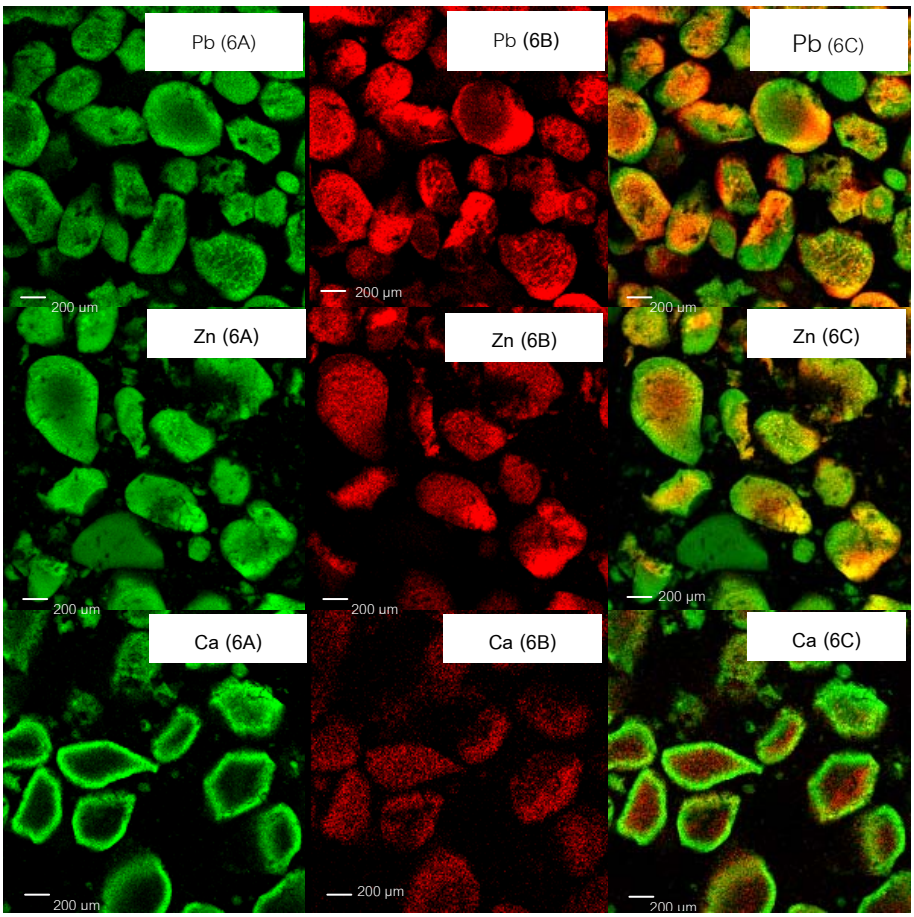


Figure 20. Confocal laser scanning microscope (CLSM) micrographs of exterior yolk for protein distribution (3A), lipid distribution (3B) and combined image of protein and lipid (3C) of pidan treated with different cations after pickling (week 3). Pb: 0.2% PbO_2; Zn: 0.2% $ZnCl_2$; Ca: 0.2% $CaCl_2$. Magnification: 200X (zoom X2.5)

4.5 Conclusions

During pickling and ageing, degradation and aggregation of proteins took place and were governed by cations introduced. Treatment with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ yielded pidan white with higher protein degradation and ammonia formation. With the treatment of 0.2% PbO_2, the lower degradation and ammonia content were observed in pidan white and the softer yolk with more released lipids was obtained. Thus, the type and level of cations used for pidan production had the influence on chemical composition and microstructure of both pidan white and yolk.

CHAPTER 5

INFLUENCE OF CHINESE TEA AND DIFFERENT DIVALENT CATIONS ON PHYSICAL PROPERTIES OF PIDAN WHITE

5.1 Abstract

Changes in physical properties of pidan white were monitored during pickling in the absence and presence of Chinese tea at levels of 2 and 5% together with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ up to 3 weeks, followed by ageing for another 3 weeks. Hardness and cohesiveness of pidan white treated with 0.2% $ZnCl_2$ (without Chinese tea) were more retained but showed a decrease in adhesiveness as pickling and ageing time increased up to week 6 ($P<0.05$). Higher browning intensity and a*-value were noticeable in the pidan white treated with Chinese tea at both levels at week 1, irrespective of cations used ($P<0.05$). Thereafter, the impact of tea on color was negligible. Thus, Chinese tea had no pronounced effect on physical properties of pidan white, whereas divalents showed the varying impact on textural property of pidan white.

5.2 Introduction

Pidan is normally made by pickling the eggs in 4.2% NaOH/5.0% NaCl solution at room temperature (30°C) for at least 20 days (Su and Lin, 1994). The formation of pidan is caused by the penetration of alkali through the egg shell and membrane, leading to chemical changes in the egg components. This results in gelation of the albumen during pickling at the alkaline condition. To stabilize egg white gel, Pb^{2+} has been used. Due to its high toxicity, other divalents such as Zn^{2+} has been considered as the promising alternative. Additionally, Zn^{2+} causes no black spots on the egg shell, and the color of the pidan's albumen and yolk was more stable (Chen and Su, 2004). Alternatively calcium could strengthen the egg yolk gel of pidan but it did not provide the stable gel during ageing (Ganesan and Benjakul, 2010c).

Generally, people have believed that tea in pickling solution yields pidan with desirable color and a characteristic pidan flavor. Li and Hsieh (2004) reported that the color may be due to the Malliard reactions between the glucose and amino acid in egg white. Glycosylation or glycation is an important reaction, which induces the covalent attachment of sugars to α- or ε-NH_2 groups of amino acids and protein to form glycated proteins (Friedman, 1996). The Maillard reaction produces a variety of intermediate products and finally brown pigments (melanoidins) are formed (Van Boekel, 1998). The Maillard reaction is influenced by many factors, including reactant concentration, temperature, time, initial pH and water activity (Ashoor and Zent, 1984; Baxter, 1995; Naranjo *et al.*, 1998; Tanaka *et al.*, 1994; Wijewickreme and Kitts, 1997). Maillard reaction is enhanced at alkaline pH found in pidan (Wang and Fung, 1996)

In general, pidan white characteristic including elastic texture with amber/brown color is a quality index for consumers. However, a little information regarding the changes of color mediated by Maillard reaction in pidan white during pickling and ageing process, and the role of Chinese tea and selected divalents on the physical properties exists. Therefore, the objectives of this study were to investigate the changes in physical properties of pidan white treated with divalent cations in the absence and presence of Chinese tea during pickling and ageing for up to 6 weeks.

5.3 Materials and Methods

Chemicals

Zinc chloride ($ZnCl_2$), calcium chloride ($CaCl_2$), sodium hydroxide and sodium chloride were purchased from Lab-Scan (Bangkok, Thailand). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and L-leucine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Glucose and other chemicals were purchased from Merck (Damstadt, Germany).

Duck egg collection

Fresh eggs of duck (*Anas platyrhucus*) with the weight range of 65–75 g were obtained within 1 day of laying from a farm in Rathabhum, Songhkla province, Thailand. Duck eggs were cleaned and checked for any crack prior to pickling.

Preparation of pidan

Clean duck eggs were soaked in a pickling solution containing 4.2% NaOH, 5% NaCl and divalents (0.2% $ZnCl_2$ or 0.2% $CaCl_2$). Chinese tea was added in the pickling solution at the concentrations of 0, 2 and 5% (w/v). Eggs (60 eggs) were soaked in different pickling solutions (6 l) at room temperature (30-32C) for 3 weeks. Pickled eggs were removed and coated with white clay paste (clay: water, 4:1 (w/v)) to obtain a thickness of 2-3 mm. Coated eggs were left at room temperature for another three weeks for ageing. During pickling and ageing, the samples were taken for analyses every week.

Texture profile analysis (TPA)

Pidan white samples with different treatments obtained at week 1, 2, 3, 4, 5 and 6 were subjected to TPA. TPA was performed as described by Bourne (1978) with a TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England). Prior to analysis, pidan white samples of various treatments were cut into a cube (1.0×1.0×1.0 cm^3). The samples were compressed twice to 50% of their original height with a compression cylindrical aluminum probe (15 mm diameter). Textural analyses were performed at room temperature. Force-distance deformation curves were recorded at cross-head speed of 5 mm/s and the recording speed was 5 mm/s. Hardness, adhesiveness, and cohesiveness were evaluated using the Micro Stable software (Stable Micro Systems, Surrey, England).

Determination of pH

At week 1, 3 and 6, pidan white samples with different treatments were determined for pH according to the method of Benjakul *et al.* (1997).

Measurement of UV-absorbance

UV-absorbance of pidan white samples was measured according to the method of Ajandouz *et al.* (2001). Prior to measurement, the samples were mixed with 5 volumes of deionized water (w/v). The mixtures were homogenized at a speed of 5,000 rpm for 10 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia) followed by centrifugation at a speed of 10,000×*g* for 10 min at 27°C using a refrigerated centrifuge (model J-E Avanti, Beckman Coulter, Inc., Palo Alto, CA, USA). The dilution of 20-fold was made. The absorbance was measured at 294 using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

Measurement of fluorescence intensity

Fluorescence intensity of pidan white samples was determined as described by Morales and Jimenez-Perez (2001). Twenty-fold diluted samples were prepared as previously described. The fluorescence intensity was measured at an excitation wavelength of 347 nm and an emission wavelength of 415 nm using a RF-1501 Fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

Measurement of browning intensity

Browning intensity of pidan white samples of all treatments was determined as described by Benjakul *et al.* (2005). The samples were prepared and 20-fold diluted as described previously. The absorbance was measured at 420 nm using a spectrophotometer.

Determination of free amino group content

Free amino group content was determined according to the method of Benjakul and Morrissey (1997). Pidan white samples (100-fold dilution) (125 µl) were mixed with 2.0 ml of 0.20 M phosphate buffer, pH 8.2, and 1.0 ml of 0.01% TNBS solution was then added. The solutions were mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Bavaria, Germany) at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The blank was prepared in the same manner as the samples except that distilled water was used instead of 0.01% TNBS. The absorbance was measured at 420 nm. Free amino group content was expressed in terms of L-leucine.

Determination of reducing sugar content

Reducing sugar content was determined according to the method of Chaplin (1994). All reagents were prepared as described by Chaplin (1994). One ml of pidan white samples (100-fold dilution) was mixed with 1.0 ml of reagent C in screw-sealed tubes. The mixtures were heated in boiling water for 15 min and then cooled with tap water. One ml of reagent D was added and mixed well. Finally, 3 ml of deionized water was added to the mixtures. The absorbance was measured at 520 nm. The reducing sugar content was calculated from the standard curve of glucose ranging from 10 to 100 mM.

Color measurement

The color of pidan white obtained at week 3 and 6 was measured using a Hunter Lab Labscan II colorimeter (Hunter Asociates Laboratory Inc., Reston, VA, USA) and expressed as L* (lightness), a* (redness/greenness) and b* (yellowness/blueness).

Statistical analysis

Completely randomized design was used throughout the study. The experiments were run in triplicate using three lots of eggs. Data were presented as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out and means comparisons were run by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analyses were performed with the statistical program (SPSS for windows (Version 10), SPSS Inc, Chicago, IL, USA).

5.4 Results and Discussion

Changes in textural properties of pidan white during pickling and ageing

Hardness of pidan white of all treatments increased continuously and reached the maximum at week 3 ($P<0.05$) and gradually decreased up to week 6 (Figure 21A). The result suggested that the hardness of pidan white obtained from all treatments became more resistance to compression within the first 3 weeks, most likely due to the aggregation of egg white proteins in the presence of divalent cations, irrespective of tea incorporated. This was evidenced by the gradual development of gel-like structure of egg white within the first three weeks. Proteins with negative charge under alkaline condition could interact each other in the presence of divalent cations, thereby lowering the repulsive force between protein molecules. Divalent cations alone including Zn^{2+} and Ca^{2+} (without Chinese tea) were more effective in increasing hardness than those in conjunction with Chinese tea during pickling (Figure 21A). In presence of tea, the penetration of divalents through pores of egg shell and membrane was more likely retarded by polymerized Chinese tea at the outer layers of membrane. As a result, the divalent bridges formed between protein molecules were formed at a lower extent. During ageing, the decrease in hardness was found in pidan white of all treatments, irrespective of cations and Chinese tea added. The increase in pH of white proteins might lead to the repulsion between protein molecules. This might result in the loosen structure of protein aggregates previously

formed during pickling and the liquefaction of pidan white was obtained. However, in the presence of Zn^{2+} (without Chinese tea), the liquefaction was slightly retarded. Those divalents might stabilize the protein network, thereby lowering the dissociation of protein network previously formed, though slightly higher alkaline pH was obtained during ageing. Electrostatic attraction between the positively charged Zn^{2+}–water complex and the carboxylic groups of the negatively charged protein forms an aggregate, resulting in the formation of gel-like structure (Shi *et al*., 2008).

Cohesiveness is often used as an indice of the ability of gel to maintain an intact network structure. Higher values of cohesiveness indicate how well the product withstands intact network structure (Fernandez-Lopez *et al*., 2006). Cohesiveness of pidan white increased continuously and reached the maximum at week 3 and gradually decreased up to week 6, irrespective of Chinese tea incorporated and divalent used ($P<0.05$) (Figure 21B). This change was in accordance with the change in hardness of pidan white. The continuous penetration of alkali probably caused the weakening of protein gel formed. Cohesiveness of pidan white treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ (without Chinese tea) was slightly higher than that of pidan white obtained from other treatments at all pickling times ($P<0.05$). However cohesiveness of pidan white treated with 0.2% $CaCl_2$ (without Chinese tea) were lower than 0.2% $ZnCl_2$ (without Chinese tea) during the ageing period ($P>0.05$). Those divalents could maintain an intact gel network effectively along with the continuous dehydration of pidan white.

Adhesiveness is defined as the work necessary to overcome the attractive forces between the product and a specific surface (Raikos *et al*., 2007). A remarkable increase in adhesiveness was observed at week 2 of pickling (Figure 21C). Among all samples, those treated with 0.2% $ZnCl_2$ or 0.2%$CaCl_2$ (without Chinese tea) showed the lower adhesiveness, indicating the less stickiness of pidan white during pickling. High polarity or hydrophilicity of egg proteins at alkaline pH more likely contributed to the stickiness of pidan white.

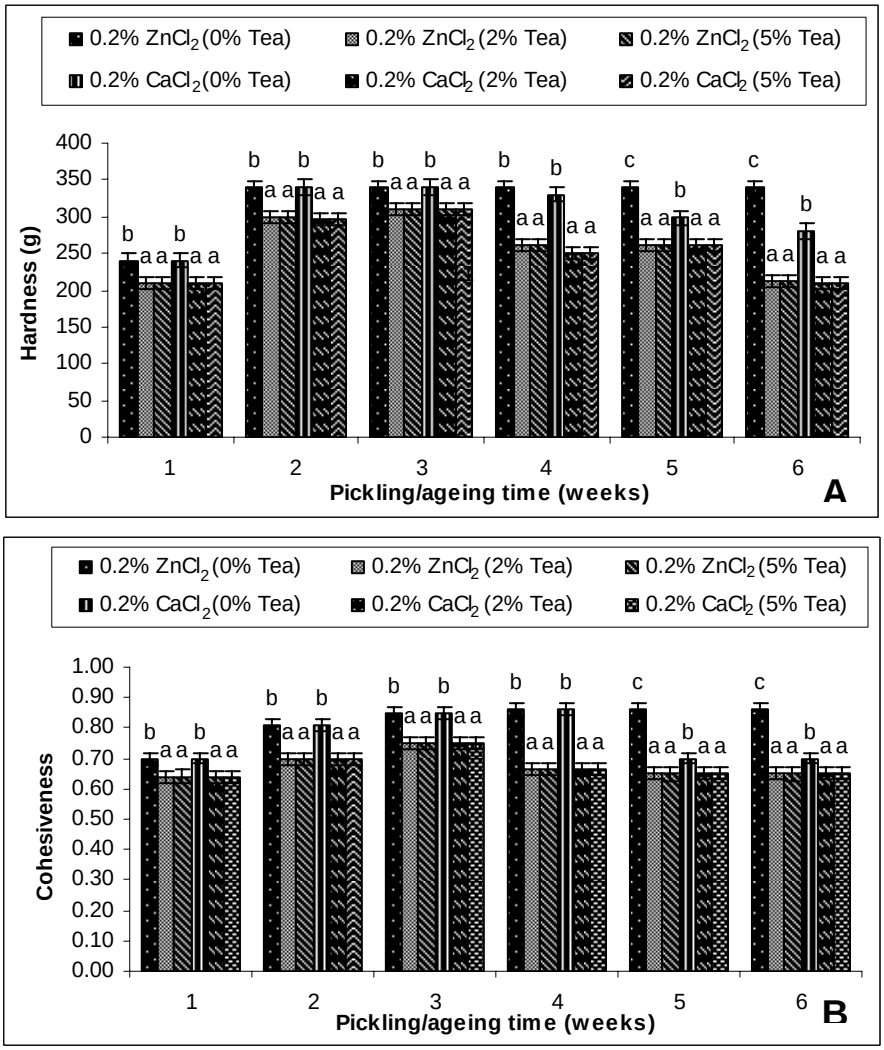


Figure 21. Changes in texture profile analysis (TPA) of pidan white pickled in the presence of different divalents without and with Chinese tea during pickling and ageing. Bars represent the standard deviations (n = 3)

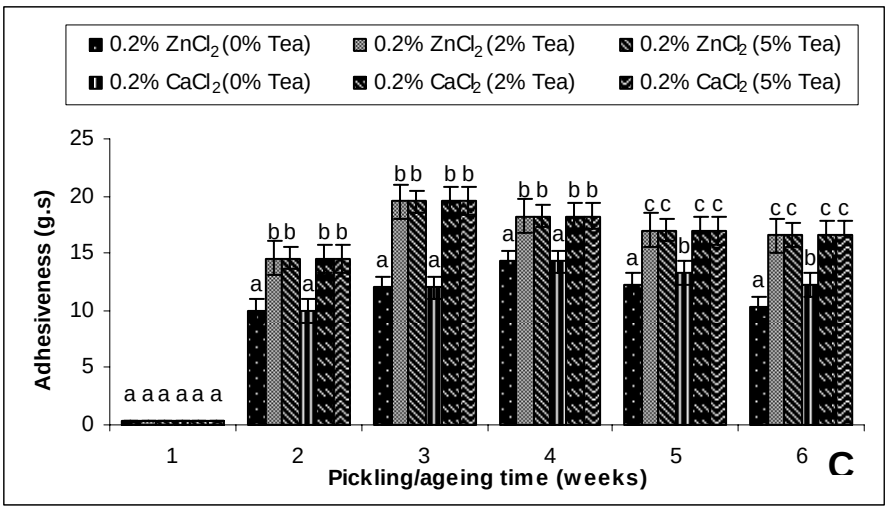


Figure 22 (Cont). Changes in texture profile analysis (TPA) of pidan white pickled in the presence of different divalents without and with Chinese tea during pickling and ageing. Bars represent the standard deviations (n = 3)

The highest increase in adhesiveness of all samples was found at week 3 and the gradual decrease in adhesiveness was observed after the ageing (week 6) ($P<0.05$). Gradual decrease in adhesiveness after 4 weeks of pickling and during ageing in all treatments might be attributed to the moisture loss of pidan white during ageing. As a result, pidan white had the less stickiness. However, the adhesiveness of pidan white treated with 0.2% $ZnCl_2$ (without Chinese tea) and 0.2% $CaCl_2$ (without Chinese tea) were lower than those treated with Chinese tea during pickling and ageing time ($P>0.05$). Thus Chinese tea components might contribute to increased water binding of egg white, which was associated with the greater adhesiveness of pidan white during pickling and ageing.

Changes in pH, A_{294}, fluorescence intensity and A_{420} of pidan white during pickling and ageing

Changes in pH of pidan white pickled in solution of divalent cations with and without Chinese tea were monitored during pickling and ageing. Increases in pH of pidan white were observed (10.40-10.50 at week 1, 11.20-11.30 at week 3 and 12.05-12.14 at week 6) during pickling and ageing ($P<0.05$), regardless of type of

ions and Chinese tea incorporated. Chang *et al.* (1999a) reported that the final pH of pidan white pickled for 20 days were 11.12. The increase in pH indicated the migration of alkali from pickling solution into egg white. Alkaline pH of pidan white had the influence on color as well as textural development of pidan. Nevertheless, type of divalents and levels of Chinese tea had no effect on the pH of pidan white ($P>0.05$).

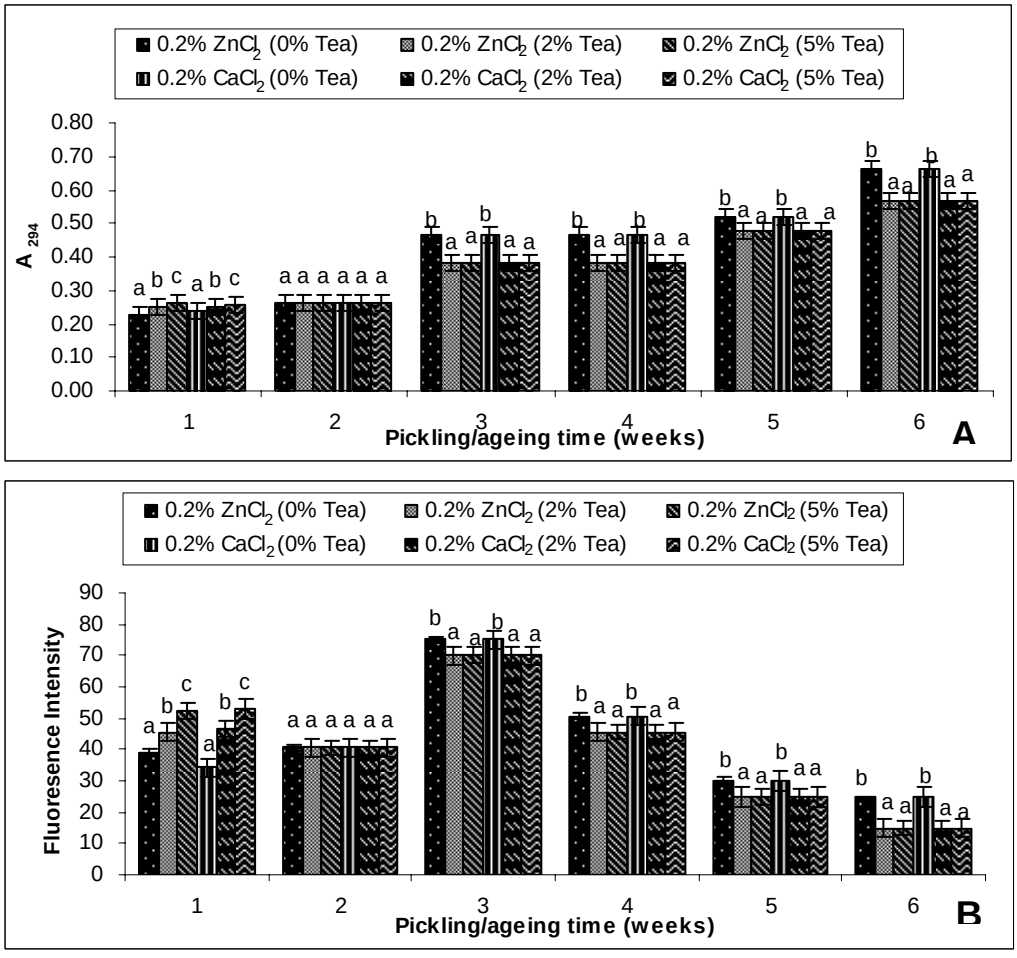


Figure 22. Changes in A_{294} (A), Fluorescence intensity (B) of pidan white pickled in the presence of different divalents without and with Chinese tea during pickling and ageing. Bars represent the standard deviations (n = 3)

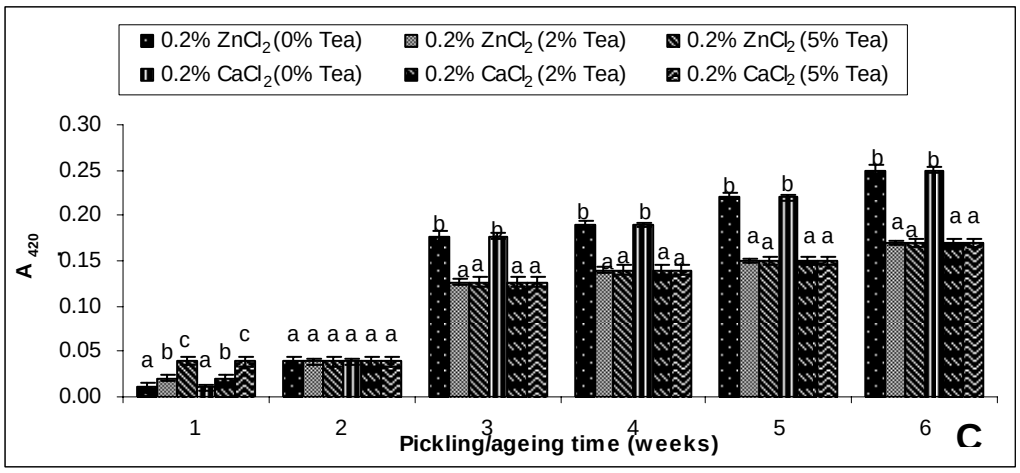


Figure 22 (Cont). Changes in A_{420} (C) of pidan white pickled in the presence of different divalents without and with Chinese tea during pickling and ageing. Bars represent the standard deviations (n = 3)

Continuous increases in A_{294} of pidan white samples were observed during pickling and ageing, irrespective of treatments (Figure 22A). Pidan white treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ (without Chinese tea) showed the higher increase in $A_{294,}$ compared with those pickled in solution containing Chinese tea, irrespective of cations used ($P<0.05$). Absorbance at 294 nm was used to determine the intermediate compounds of the Maillard reaction (Ajandouz *et al.*, 2001; Lerici *et al.*, 1990; Lertittikul *et al.*, 2007). The increase in absorbance at 294 nm suggested the formation of an uncolored compound, which could be the precursor of the Maillard reaction (Ajandouz *et al.,* 2001). This was more likely due to the higher formation of intermediate generated during a very high alkaline pH. However the presence of Chinese tea prevents the formation of colorless intermediate to some extent. Under alkaline condition, phenolic compound, most likely oxidized form, could interact with nucleophilic amino group of egg proteins. As a consequence, amino groups were less available for Maillard reaction. Furthermore the bulky structure of tea components anchored with proteins might prevent glycation process. Different intermediate products are formed, either fluorescent or non-fluorescent compounds, during the Maillard reaction (Benjakul *et al.,* 2005). Some intermediate products might undergo conversion to the final brown compounds, while some intermediates were still generated.

Fluorescence intensity of all pidan white samples increased gradually during pickling (Figure 22B). Subsequently, a gradual decrease was observed up during ageing, irrespective of treatments ($P < 0.05$). Generally, an increase in pH of the system influenced the rate of Maillard reaction and alkaline condition favored the reaction (Martins *et al.,* 2003). The Maillard reaction is associated with the development of fluorescent compounds formed prior to the generation of brown pigments (Baisier and Labuza, 1992; Morales *et al.,* 1996). This fluorescent compounds may be precursors of brown pigments (Labuza and Baisier, 1992; Morales and Van Boekel, 1997). The lower fluorescence intensity of pidan white during ageing was probably caused by the rapid transformation of the intermediates to brown compounds. This led to less remaining fluorescent intermediate products, as shown by the lower fluorescence intensity. However, the pidan white treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ (without Chinese tea) showed slightly higher fluorescent intensity than those pickled with cations in the presence of Chinese tea. This result was in agreement with that of A_{294}.

Continuous increases in A_{420} of pidan white samples were observed during pickling and ageing, irrespective of treatments (Figure 22C). Maillard reaction is a non-enzymatic browning reaction which links the carbonyl group of reducing carbohydrates and the amino group of free amino acids, especially lysine residues in proteins (Ajandouz *et al.*, 2001; Kato *et al.*, 1978). Maillard reaction is considered important in pidan white because of the significant amount of glucose naturally present in the egg white proteins (Powrie, 1977). The browning intensity increased sharply after 2 weeks of pickling. Thereafter, the gradual increase in A_{420} was found at week 3 of pickling and during the ageing. At week 1, pidan white treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ along with Chinese tea at both levels showed the higher browning intensity of pidan white, compared with other samples ($P<0.05$). This was most likely due to the staining effect of Chinese tea components used in the pickling solution. Tea component in alkaline pickling solution might develop the brown color in the pidan white gels due to the oxidation of phenolic components in the alkaline environment. Mizooku *et al.* (2003) reported that flavanols was colorless in aqueous solution at pH 7.6; however it turned yellow at pH 10.6. However, at week 6, pidan white treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ along with Chinese tea at various levels

showed the lower browning intensity, compared with other treatments ($P<0.05$). The increases in browning intensity of all samples with increasing pickling and ageing time was most likely due to the production and conversion of fluorescent or non-color compounds into brown pigment. Therefore, the incorporation of Chinese tea in the pickling solution most likely retarded the development of brown color of pidan white.

Changes in free amino group and reducing sugar contents of pidan white during pickling and ageing

Changes in free amino group and reducing sugar contents of pidan white samples of various treatments are shown in (Figure 23A) and (Figure 23B)**,** respectively**.** Continuous decreases in amino group and reducing sugar content of all pidan white samples were noticeable when the pickling and ageing time increased to week 6 ($P < 0.05$). This result suggested that α- or ε-NH_2 groups of amino acids or proteins, which were hydrolyzed at very high alkaline pH, covalently attached to a sugar to form glycated proteins to a greater extent, particularly when the pickling and ageing time increased to week 6. The first glycation product, or Schiff base, rearranges to a more stable ketoamine or Amadori product. The Amadori products can then form cross-links between adjacent proteins or with other amino groups, resulting in polymeric aggregates called advanced glycation end-products (Friedman, 1996). The decreases in free amino group were in accordance with the increase in browning (Figure 22C) and A_{294} (Figure 22A) and the decrease in fluorescence intensity (Figure 22B). This indicated that increased pickling and ageing time enhanced the interaction between free amino groups of proteins or peptides and sugar via glycation process.

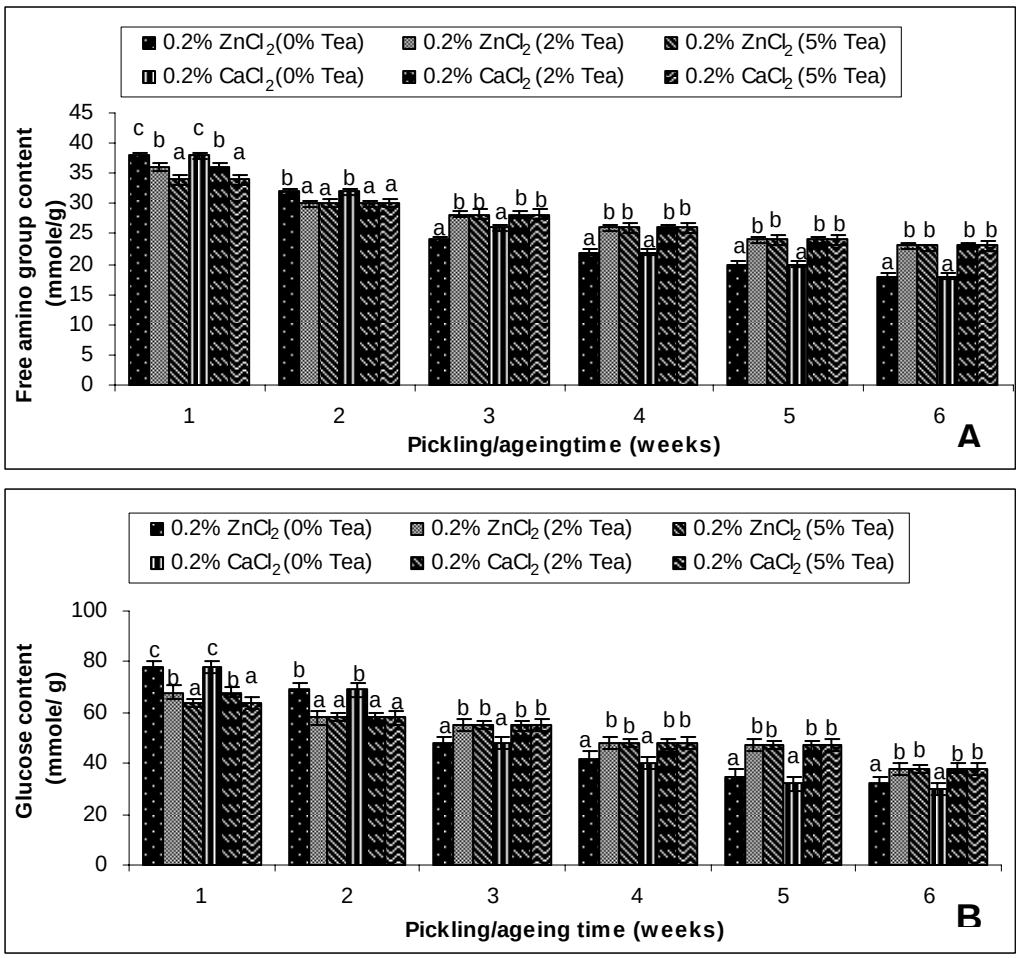


Figure 23. Changes in free amino groups (A) and reducing sugar (B) of pidan white pickled in the presence of different divalents without and with Chinese tea during pickling and ageing. Bars represent the standard deviations (n = 3)

As a result, intermediate products were formed and further converted to brown pigments, as observed by the increased A_{420}. In general, pidan white treated without Chinese tea was more reactive in forming the glycated product than that treated with Chinese tea, irrespective of cations. This was evidenced by the greatest decrease in free amino group and reducing sugar contents with the concomitant increase in browning. The reaction rate of glycation between casein and sugars depended on the percentage of the acyclic form and the electrophilicity of the carbonyl groups (Naranjo *et al.*, 1998; Bunn and Higgins, 1981). The difference in

reaction rate of sugar in glycation process was possibly governed by the conformation of protein. The result indicated that tea components might impede Maillard reaction by functioning as the bulky groups preventing the reaction between amino group and reducing sugar.

Changes in the color of pidan white during pickling and ageing

The color of pidan white of different treatments during pickling and ageing is shown in Table 7. L* and b*-values of pidan white generally decreased, whereas a*-values increased with increasing pickling and ageing, regardless of Chinese tea and cations incorporated. However, pidan white treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ (without Chinese tea) showed the higher L* value values, compared to other treatments ($P<0.05$). This was probably due to the higher aggregation of proteins, which exhibited higher light scattering effect. Chantrapornchai and McClements (2002) reported that whey protein gels increased its lightness with increasing protein size. Higher b* and a* values were found in pidan white treated with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ (without Chinese tea) at week 3 and 6, more likely owing to the formation of yellow or brown pigments. Increased a* values were possibly due to the formation of brown pigments, which might derive from Mailliard reaction of egg white. This was in accordance with higher browning intensity of pidan white (Figure 22C). Furthermore, higher a*-values were noticeable in the pidan white treated with Chinese tea at both levels at week 1, which was coincidental with the increasing $A_{420.}$ However, Chinese tea at various concentrations had no marked impact on color of pidan white developed after ageing.

Table 7. L*, a* and b*-values of pidan white pickled in the presence of different divalents without and with Chinese tea during pickling and ageing

Parameters	Treatments	Pickling/ageing time (weeks)		
		1	3	6
L*	0.2% $ZnCl_2$ (0% Tea)	34.01±0.33‡ c†, C††	27.25±0.38 b, B	16.34±0.22 b, A
	0.2% $ZnCl_2$ (2% Tea)	30.36±0.43 b, C	25.55±0.47 b, B	15.05±0.15 b, A
	0.2% $ZnCl_2$ (5% Tea)	27.36±0.32 a, C	25.59±0.77 b, B	15.07±0.16 b, A
	0.2% $CaCl_2$ (0% Tea)	34.10±0.21 c, C	27.25±0.32 b, B	16.28±0.32 b, A
	0.2% $CaCl_2$ (2% Tea)	30.46±0.32 b, C	25.63±0.40 a, B	15.10±0.10 a, A
	0.2% $CaCl_2$ (5% Tea)	27.47±0.23 a, C	25.73±0.32 a, B	15.16±0.05 a, A
a*	0.2% $ZnCl_2$ (0% Tea)	-2.49±0.80 a, A	5.24±0.12 b, B	9.70±0.15 b, C
	0.2% $ZnCl_2$ (2% Tea)	-0.69±0.40 b, A	3.40±0.54 a, B	7.79±0.45 a, C
	0.2% $ZnCl_2$ (5% Tea)	2.46±0.32 c, A	3.42±0.34 a, B	7.72±0.32 a, C
	0.2% $CaCl_2$ (0% Tea)	-2.15±0.30 a, A	5.27±0.38 b, B	9.67±0.08 b, C
	0.2% $CaCl_2$ (2% Tea)	-1.78±0.42 b, A	3.45±0.52 a, B	7.80±0.16 a, C
	0.2% $CaCl_2$ (5% Tea)	2.14±0.32 c, A	3.43±0.54 a, B	7.82±0.23 a, C
b*	0.2% $ZnCl_2$ (0% Tea)	20.20±0.47 c, C	12.01±0.36 b, B	4.57±0.56 b, A
	0.2% $ZnCl_2$ (2% Tea)	16.29±0.17 b, C	9.05±0.25 a, B	2.02±0.29 a, A
	0.2% $ZnCl_2$ (5% Tea)	12.34±0.21 a, C	9.04±0.23 a, B	2.15±0.19 a, A
	0.2% $CaCl_2$ (0% Tea)	20.67±0.24 c, C	12.12±0.28 b, B	4.37±0.36 b, A
	0.2% $CaCl_2$ (2% Tea)	16.97±0.53 b, C	9.09±0.24 a, B	2.09±0.21 a, A
	0.2% $CaCl_2$ (5% Tea)	12.37±0.22 a, C	9.29±0.03 a, B	2.14±0.10 a, A

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

†† Different capital letters in the same row indicate the significant differences ($P< 0.05$). † Different letters in the same column within the same parameter indicate the significant differences ($P<0.05$)

5.5 Conclusions

Chinese tea had no pronounced effect on physical properties of pidan white, whereas divalent ions showed the varying impact on textural property of pidan white in the absence of Chinese tea. Browning intensity and brown color were significantly developed in pidan white in the absence of Chinese tea, irrespective of cations used. Therefore, brown color of pidan white was more likely mediated by Maillard reaction, not Chinese tea**.**

CHAPTER 6

EFFECTS OF GREEN TEA AND CHINESE TEA ON THE COMPOSITION AND PHYSICAL PROPERTIES OF PIDAN WHITE

6.1 Abstract

Changes in compositions and physical properties of pidan white prepared from acetic acid pretreated egg were monitored during pickling in the presence and absence of green tea and Chinese tea at levels of 2 and 5% up to 3 weeks, followed by ageing for another 3 weeks. Hardness, cohesiveness and adhesiveness of pidan white gradually increased during pickling, but subsequently decreased during ageing ($P<0.05$), regardless of types and amounts of tea used. Nevertheless, hardness and cohesiveness of pidan white treated without tea were more retained during ageing ($P<0.05$). Browning intensity (A_{420}), A_{294} and pH of pidan white increased with increasing pickling and ageing, while fluorescence intensity decreased during ageing ($P<0.05$), irrespective of treatments. Furthermore, higher browning intensity and a*-value were noticeable in the pidan white treated with either green tea or Chinese tea at higher level (5%), compared with the lower level (2%) ($P<0.05$). Browning of all pidans increased during pickling and reached the plateau at the first week of ageing. Acetic acid pretreatment along with the sufficient amount of tea generally accelerated the pidan formation and enhanced brown color development of pidan white.

6.2 Introduction

Pidan or century egg is one of the most traditional and popular preserved egg products in Thailand, China and South East Asian countries. Generally, pidan can be made by pickling the eggs in 4.2% NaOH and 5.0% NaCl solution at

ambient temperature for 20 days (Su and Lin, 1994). The formation of pidan is caused by the penetration of pickling ingredients through the egg shell and membrane, leading to chemical changes in the egg components (Ganesan and Benjakul, 2010b). Generally, albumen and yolk of pidan egg gradually become and hardened. All changes occurring during the pickling of pidan more likely determine the preferential characteristics of pidan.

Color and texture are associated with the quality of pidan and it relies on the ingredients used in the pidan production. Among the ingredients used in the pidan production, Chinese tea also serves as ingredient in pidan production in the traditional process. The addition of Chinese tea at various concentrations in pickling solutions, yielded the pidan with loosen texture and lighter color, which might be due to the block of tea flavonoids on the pores of egg shell during pickling (unpublished data). Flavanoids in tea is known to interact with proteins to form complexes whose properties depend on the structure of both flavanoids and protein (Yuksel *et al.*, 2010). Green tea contains mainly catechins and black tea is rich in thearubigins and theaflavins, which are dimers of catechins (Majchrzak *et al.*, 2004). To tackle the problem related with membrane binding with tea compounds, the enhancement of tea components through the egg shell and membrane should be focused. Acid pretreatment has been used to shorten the salting time of duck egg. The salt penetration into egg was enhanced 2-10 folds, when duck eggs were treated with 0.1 N HCl. (Lai *et al.*, 1997). However, pretreatment of egg using inorganic acid might not be acceptable for consumer and organic acids may be a promising alternative with the safety concern. Along with the thinner shell or membrane, the use of tea at sufficient amount could enhance the penetration of tea component, in which pidan can be formed at a shorter time. Generally, Chinese tea has been used and no information regarding the use of green tea for pidan production has been reported. Therefore, the objective of this study was to investigate the effect of acetic acid pretreatment together with the use of green tea and Chinese tea on composition and physical properties of pidan white during pickling and ageing for up to 6 weeks.

6.3 Materials and Methods

Chemicals

Zinc chloride ($ZnCl_2$), sodium hydroxide and sodium chloride were purchased from Lab-Scan (Bangkok, Thailand). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and L-leucine, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glucose and other chemicals were purchased from Merck (Damstadt, Germany).

Duck egg collection

Fresh eggs of duck (Anas platyrhucus) with the weight range of 65–75 g were obtained within 1 day of laying from a farm in Rathabhum, Songhkla Province, Thailand. Duck eggs were cleaned and checked for any crack prior to pickling.

Preparation of pidan

Shell eggs were soaked in 5% acetic acid for 30 min at room temperature (28-30°C) and washed with running water to remove acid. The eggs were then air-dried for 1 h. Acid treated duck eggs were soaked in a pickling solution containing 4.2% NaOH, 5% NaCl and 0.2% $ZnCl_2$. Green tea or Chinese tea was added in the pickling solution at the concentrations of 2% and 5%. The treatment without the addition of green tea and Chinese tea were used as the control. Eggs (10 eggs) were soaked in different pickling solutions (1 L) at room temperature (28-30°C) for 3 weeks. Pickled eggs were removed and coated with white clay paste (clay: water, 4:1 w/v) to obtain a thickness of 2-3 mm. Coated eggs were left at room temperature for another three weeks for ageing. During pickling and ageing, the samples were taken for analyses every week.

Measurement of thickness of egg shell and egg shell membrane

The egg shell membrane was manually stripped from the shell. The thickness of egg shell and shell membrane were measured using a micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp, Kawasaki-shi, Japan). Five random locations around each egg shell and shell membrane sample were used for thickness determination.

Determination of microstructure of egg shell and shell membrane

Microstructure of shell and shell membrane was observed by scanning electron microscope (SEM). Membrane was manually removed after cleaning. Shell membranes were dried with a series of ethanol (50-100%). The samples were mounted on SEM stubs using a double backed cellophane tape. The stub and sample were coated with gold (Sputter coater SPI-module, West Chester, PA, USA) and examined using a scanning electron microscopy (JEOL JSM-5800LV, Tokyo, Japan).

Texture profile analysis (TPA)

Pidan white samples with different treatments obtained at week 1, 2, 3, 4, 5 and 6 were subjected to TPA. TPA was performed as described by Bourne (1978) with a TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England). Prior to analysis, pidan white samples of various treatments were cut into a cube (1.0×1.0×1.0 cm^3). The samples were compressed twice to 50% of their original height with a compression cylindrical aluminum probe (15 mm diameter). Textural analyses were performed at room temperature. Force-distance deformation curves were recorded at cross-head speed of 5 mm/s and the recording speed was 5 mm/s. Hardness, adhesiveness, and cohesiveness were evaluated using the Micro Stable software (Stable Micro Systems, Surrey, England).

Determination of pH

At week 1, 3 and 6, pidan white samples with different treatments were determined for pH according to the method of Benjakul *et al.* (1997).

Measurement of UV-absorbance

UV-absorbance of pidan white samples was measured according to the method of Ajandouz *et al.* (2001). Prior to measurement, the samples were mixed with 5 volumes of deionized water (w/v). The mixtures were homogenized at a speed of 5,000 rpm for 10 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia) followed by centrifugation at a speed of 10,000×*g* for 10 min at 27°C using a refrigerated centrifuge (model J-E Avanti, Beckman Coulter, Inc., Palo Alto, CA, USA). The dilution of 20-fold was made. The absorbance was measured at 294 using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

Measurement of fluorescence intensity

Fluorescence intensity of pidan white samples was determined as described by Morales and Jimenez-Perez (2001). Twenty-fold diluted samples were prepared as previously described. The fluorescence intensity was measured at an excitation wavelength of 347 nm and an emission wavelength of 415 nm using a RF-1501 Fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

Measurement of browning intensity

Browning intensity of pidan white samples of all treatments was determined as described by Benjakul *et al.* (2005). The samples were prepared and 20-fold diluted as described previously. The absorbance was measured at 420 nm using a spectrophotometer.

Determination of free amino group content

Free amino group content was determined according to the method of Benjakul and Morrissey (1997). Pidan white samples (100-fold dilution) (125 µl) were mixed with 2.0 ml of 0.20 M phosphate buffer, pH 8.2, and 1.0 ml of 0.01% TNBS solution was then added. The solutions were mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Bavaria, Germany) at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The blank was prepared in the same manner as the samples except that distilled water was used instead of 0.01% TNBS. The absorbance was measured at 420 nm. Free amino group content was expressed in terms of L-leucine.

Determination of reducing sugar content

Reducing sugar content was determined according to the method of Chaplin (1994). All reagents were prepared as described by Chaplin (1994). One ml of pidan white samples (100-fold dilution) was mixed with 1.0 ml of reagent C in screw-sealed tubes. The mixtures were heated in boiling water for 15 min and then cooled with tap water. One ml of reagent D was added and mixed well. Finally, 3 ml of deionized water was added to the mixtures. The absorbance was measured at 520 nm. The reducing sugar content was calculated from the standard curve of glucose ranging from 10 to 100 mM.

Color measurement

The color of pidan white obtained at week 3 and 6 was measured using a Hunter Lab Labscan II colorimeter (Hunter Asociates Laboratory Inc., Reston, VA, USA) and expressed as L* (lightness), a* (redness/greenness) and b* (yellowness/blueness).

Statistical analysis

Completely randomized design was used throughout the study. The experiments were run in triplicate using three lots of eggs. Data were presented as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out and means comparisons were run by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analyses were performed with the statistical program (SPSS for windows (Version 10), SPSS Inc, Chicago, IL, USA).

6.4 Results and Discussion

Effect of acetic acid pretreatment on shell and shell membrane of duck egg before pickling of pidan

Egg treated with 5% acetic acid for 30 min had the thickness of 0.39±0.02, while the shell of egg without the treatement had the thickness of 0.44±0.03 mm. The rough surfaces of egg shell were observed in egg, which was pretreated with 5% acetic acid. Egg shells consists of mineral component, namely the trigonal phase of calcium carbonate ($CaCO_3$), known as calcite, which is the more stable polymorph at room temperature (Stadelman, 2000). Mineral can be removed from the egg shell by decalcification with EDTA or acetic acid (Nys *et al.,* 2004). Acetic acid can break apart the solid calcium carbonate crystals that make up the egg shell into their calcium and carbonate parts. The calcium ions float free, while the carbonate turn to be carbon dioxide bubbles. This reaction causes the decrease in shell thickness (Nys *et al.,* 2004). Shell thickness of less than 0.2 mm was observed when soaking the egg in 0.1 N HCl for 30 min (Lai *et al.,* 1997). The pore sizes of egg shell became larger after pretreatment with acetic acid when examined under SEM (data not shown). However, there was no marked difference in pore size and thickness for shell membrane. Generally, the outer surface of the egg shell is covered with a mucin protein that acts as a soluble plug for the pores in the shell. The removal of cuticle and

partial part of spongy layer opened the pores. As consequence, pickling solution containing tea components could be penetrated into the egg more effectively. The pretreated eggs were further used for pidan production and the shell of obtained egg was not too thin and fragile for further processes.

Effect of green tea and Chinese tea at different levels on textural properties of pidan white

Hardness of pidan white of all treatments increased continuously and reached the maximum at week 3 ($P<0.05$) and gradually decreased up to week 6 (Figure 24A). However pidan treated with both teas showed the higher hardness, compared with the control ($P<0.05$). The hardness of pidan white increased with increasing concentrations of tea during pickling ($P<0.05$). This was most likely due to the higher aggregation of egg white protein mediated by tea components, which could penetrate through acetic acid pretreated shell and shell membrane. Decreased hardness during ageing suggested that aggregate of egg white protein induced by tea phenolics might be reversible (Wu *et al.,* 2007). However the hardness of pidan white obtained from the control (without tea) became more resistant to compression. In the absence of tea, ionic interaction caused by zinc ion as the bridges could be more enhanced, while aggregate induced by tea polyphenol might have the lower binding site for salt bridges. Proteins with negative charge under alkaline condition could interact each other in the presence of cations via lowering repulsive force between protein molecules. Furthermore, the less sealing effect of tea component through pores of egg shell and membrane was negligible for the control. As a result, zinc could pass through the shell and shell membrane to a higher content. During ageing, the higher decrease in hardness was found in pidan white treated with green tea, compared with that treated with Chinese tea ($P<0.05$). The different types of phenolic compounds between green tea and Chinese tea might contribute to varying stability of aggregates formed in alkali condition. This might result in the loosen structure of protein aggregates and liquefaction of pidan white during ageing.

Cohesiveness is often used as an indice of the ability of gel to maintain an intact network structure. Higher values of cohesiveness indicate how well the product withstands intact network structure (Fernandez-Lopez *et al.*, 2006). Cohesiveness of pidan white increased continuously and reached the maximum at week 3 and gradually decreased up to week 6, irrespective of tea used ($P<0.05$) (Figure 24B). This change was in accordance with the change in hardness of pidan white. The continuous penetration of alkali probably caused the weakening of protein gel formed. Cohesiveness of pidan white treated without tea was slightly higher than those from other treatments during ageing period ($P<0.05$). This was in accordance with the hardness of pidan white. The divalent could maintain an intact gel network effectively along with the continuous dehydration of pidan with the aid of acetic acid pretreatment.

Adhesiveness is defined as the work necessary to overcome the attractive forces between the product and a specific surface (Raikos *et al.*, 2007). A gradual increase in adhesiveness was observed during pickling (Figure 24C). Among all samples, those treated with Chinese tea showed the lowest adhesiveness, indicating the less stickiness of pidan white during pickling. The decrease in adhesiveness was more pronounced as the tea at higher level was used. When the hydrophobic protein underwent aggregation induced by both cation and tea phenolic compounds, the availability of reactive group for interaction with other surface became less. Nevertheless, adhesiveness increased up to 3 weeks of pickling. Thereafter, a gradual decrease in adhesiveness was observed after ageing (week 6). Gradual decrease in adhesiveness during ageing in all treatments might be attributed to the moisture loss of pidan white during ageing. As a result, pidan white had the less stickiness. However, the pidan treated with green tea showed the higher adhesiveness, indicating the higher stickiness of pidan white during pickling at week 6. This correlated with the lower hardness of pidan white treated with green tea. Weak aggregation of proteins was more likely associated with the looser water bound with the gel network, resulting in the higher adhesiveness of those pidan white during ageing ($P>0.05$).

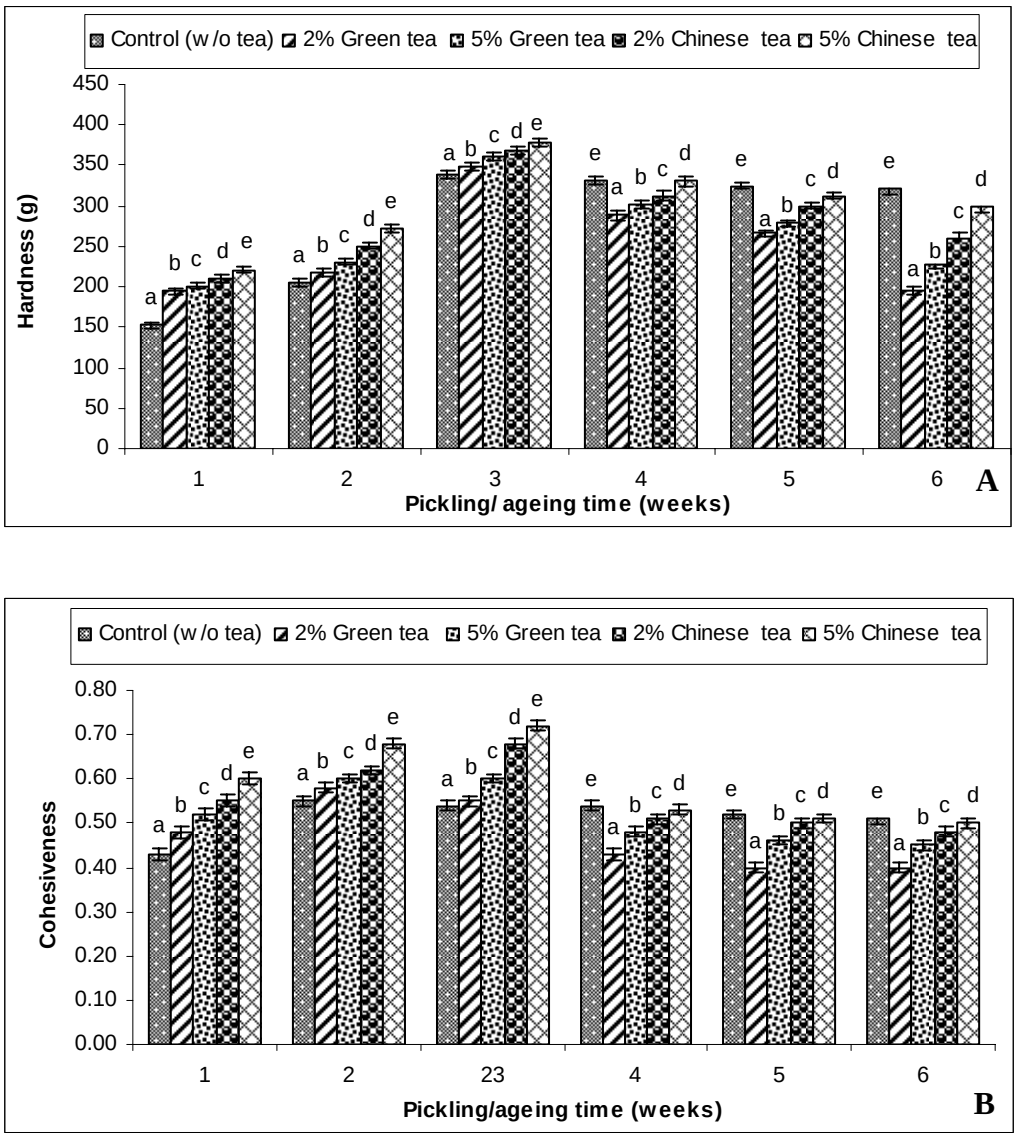


Figure 24. Changes in texture profile analysis (TPA) of pidan white prepared from acetic acid treated duck egg as affected by green tea and Chinese tea at different levels. Bars represent the standard deviations (n = 3)

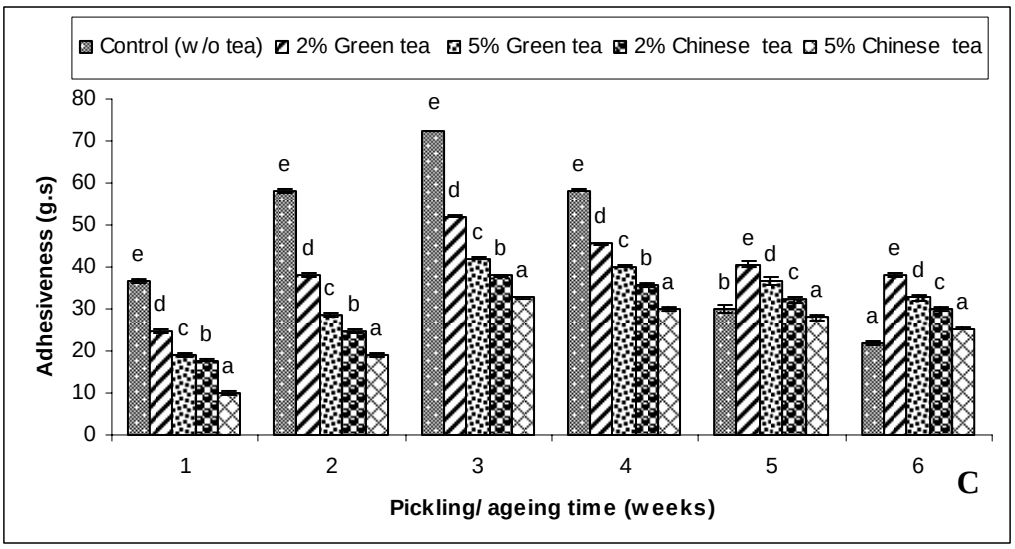


Figure 24 (Cont). Changes in texture profile analysis (TPA) of pidan white prepared from acetic acid treated duck egg as affected by green tea and Chinese tea at different levels. Bars represent the standard deviations (n = 3)

Effect of green tea and Chinese tea at different levels on pH and browning of pidan white

Changes in pH of pidan white pickled with and without different tea at different levels were monitored during pickling and ageing (Figure 25). The pH of pidan white was increased from 10.64-10.82 at week 1 to 11.55-11.75 and 12.10-12.18 at week 3 and 6, respectively. The lower changes in pH were noticeable during ageing, in comparison with pickling period. Ganesan and Benjakul (2010a) reported that pH of pidan white pickled for 18 days was 11.05. The greater increase in pH during pickling indicated that acetic acid pretreatment enhanced the migration of alkali from pickling solution into egg white. Alkali might have the impact on the aggregation of white proteins, by inducing the unfolding of egg white proteins, in which the reactive groups of proteins could undergo aggregation mediated by divalent (Zn^{2+}) more effectively. Type and concentrations of tea used for pidan production had no marked influence on the pH of pidan white ($P>0.05$). Thus acetic acid pretreatment

was proved to increase the penetration of alkali as well as divalent into egg white, irrespective of tea used in the pickling solution.

Continuous increases in A_{294} of pidan white pickled with and without different tea at various levels were monitored during pickling and ageing (Figure 25A). Pidan white treated with both green tea and Chinese tea showed the higher A_{294}, compared with the control ($P<0.05$). Absorbance at 294 nm was used to determine the intermediate compounds of the Maillard reaction (Ajandouz *et al.,* 2001 and Lerici *et al.*, 1990). The increase in absorbance at 294 nm suggested the formation of an uncolored compound, which could be the precursor of the Maillard reaction (Ajandouz *et al.,* 2001). This was more likely due to the higher formation of intermediate generated during a very high alkaline pH. Higher formation of intermediate compounds of pidan white treated with tea confirmed that tea compound involved in the generation of some intermediate compound. However, tea had no impact on browning of pidan white produced from egg without acetic acid pretreatment (Ganesan and Benjakul, 2010d). The interaction of egg white with tea phenolic compounds might favor the conformational changes of egg white proteins in the way which the amino groups could undergo schiff base reaction (glycation). Different intermediate products are formed, either fluorescent or non-fluorescent compounds, during the Maillard reaction (Benjakul *et al.,* 2005). From the result, some intermediate products might undergo convert to the final brown compounds, while some intermediates were still being generated.

Fluorescence intensity of all pidan white samples pickled with and without different tea reached the maximum at week 3 ($P < 0.05$) (Figure 25B). Subsequently, a gradual decrease was observed up to week 6 ($P < 0.05$). Generally, the increase in pH of the system influenced the Maillard reaction rate (Martins *et al.,* 2003). The Maillard reaction is associated with the development of fluorescent compounds formed prior to the generation of brown pigments (Baiser and Labuza, 1992; Morales *et al.,* 1996). This fluorescent compounds may be precursors of brown pigments (Labuza and Baisier, 1992; Morales and Van Boekel, 1997). The lower fluorescence intensity of pidan white during ageing was probably caused by the rapid transformation of the intermediates to brown compounds. This led to less remaining

fluorescent intermediate products, as shown by the lowered fluorescence intensity. However, the pidan white treated with different teas showed the higher fluorescence intensity than the control, indicating the synergistic effect of tea components on the formation of Maillard reaction intermediate products. Additionally tea components also contributed to the formation of fluorescent compounds. Mizooku *et al.* (2003) reported that flavanols was colorless in aqueous solution at pH 7.6; however it turned to yellow and fluorescent compounds at pH 10.6. It was postulated that fluorescent compounds might undergo the polymerization to form brown pigment much faster than those with the UV absorbing property during ageing.

Browning intensity of pidan white with different treatments during pickling and ageing is shown in Figure 25C. Maillard reaction is a non-enzymatic browning reaction which links the carbonyl group of reducing carbohydrates and the amino group of free amino acids as well as of ε- amino groups of lysine residues in proteins (Ajandouz *et al.,* 2001; Kato *et al.*, 1978). Maillard reaction is considered important in pidan white. The significant amount of glucose is naturally present in the egg white. The browning intensity of all samples increased with increasing pickling times ($P < 0.05$). At the same time of pickling, pidan white treated with both teas at high levels (5%) showed the higher browning intensity of pidan white, most likely due to the staining effect of tea used in the pickling solution. Tea in pickling solution might enhance the brown color in the pidan white gels due to oxidation of flavonols under the alkaline condition. Mizooku *et al.* (2003) reported that the oxidation of flavanols was determined by pH. However, the ageing time had no impact on browning intensity of pidan white. Therefore, the development of brown color was more pronounced up to week 4 of processing time. Moreover, higher browning intensity of pidan treated with tea with increasing picking time was most likely due to the conversion of fluorescent compounds to brown pigment at a higher and faster rate.

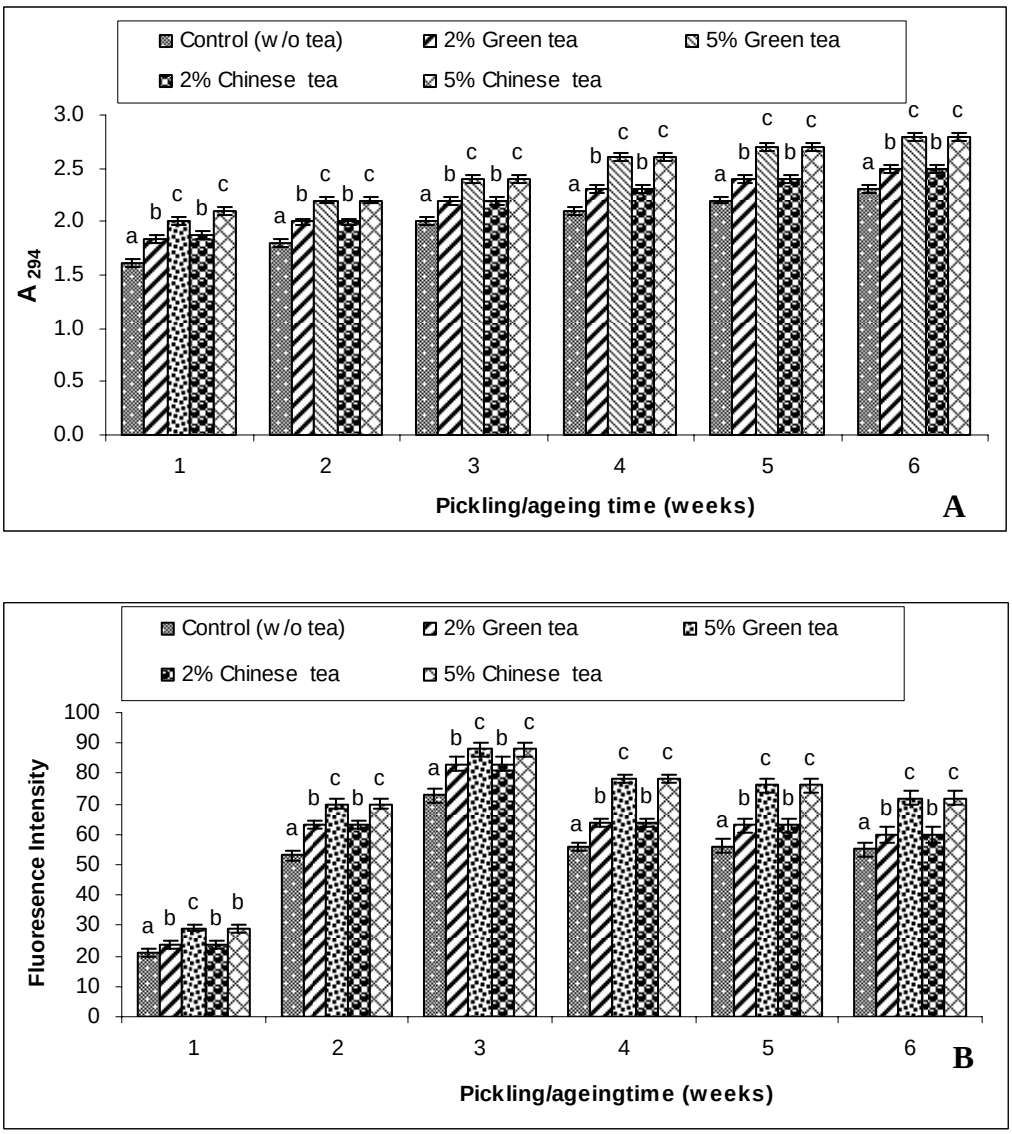


Figure 25. Changes in A_{294} (A), fluorescence intensity (B), and browning intensity (C) of pidan white prepared from acetic acid treated duck egg as affected by green tea and Chinese tea at different levels. Bars represent the standard deviations (n = 3)

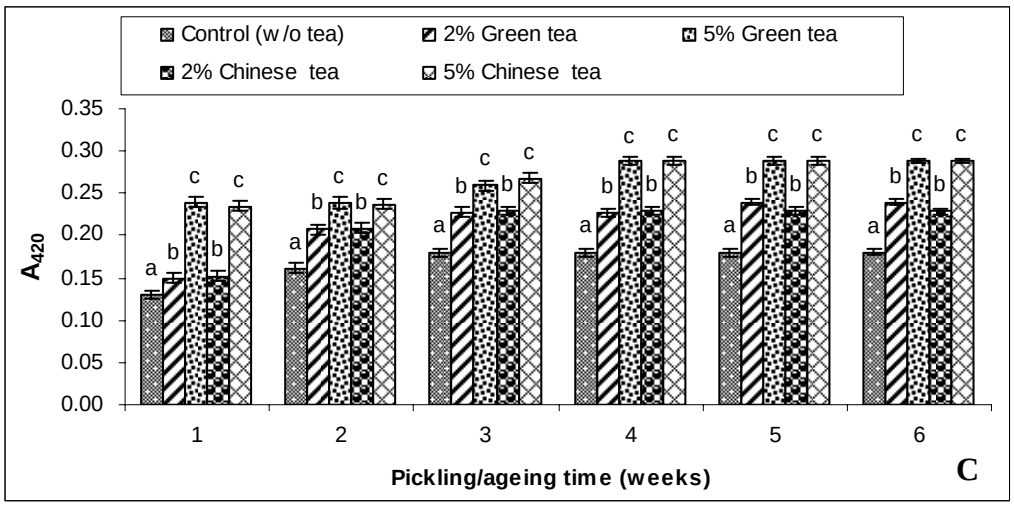


Figure 25 (Cont). Changes in browning intensity (C) of pidan white prepared from acetic acid treated duck egg as affected by green tea and Chinese tea at different levels. Bars represent the standard deviations (n = 3)

Effect of green tea and Chinese tea at different levels on free amino group and reducing sugar contents of pidan white

Changes in free amino group and reducing sugar contents of pidan white samples of various treatments during pickling and ageing are shown in Figure 26A and Figure 26B**,** respectively**.** Continuous decreases in amino group content of all pidan white samples were noticeable when the pickling increased to week 3 ($P < 0.05$). Nevertheless, no changes in amino group content were noticeable during ageing. This result suggested that α- or ε-NH_2 groups of amino acids or proteins, covalently attached to a sugar to form glycated proteins to a greater extent, particularly during the pickling period (Ganesan and Benjakul, 2010a). The first glycation product, or Schiff base, rearranges to a more stable ketoamine or Amadori product. The Amadori products can then form cross-links between adjacent proteins or with other amino groups, resulting in polymeric aggregates called advanced glycation end-products (Friedman, 1996). The decreases in free amino group were in

accordance with the increase in browning (Figure 25C) and A_{294} (Figure 25A) and the decrease in fluorescence intensity (Figure 25B).

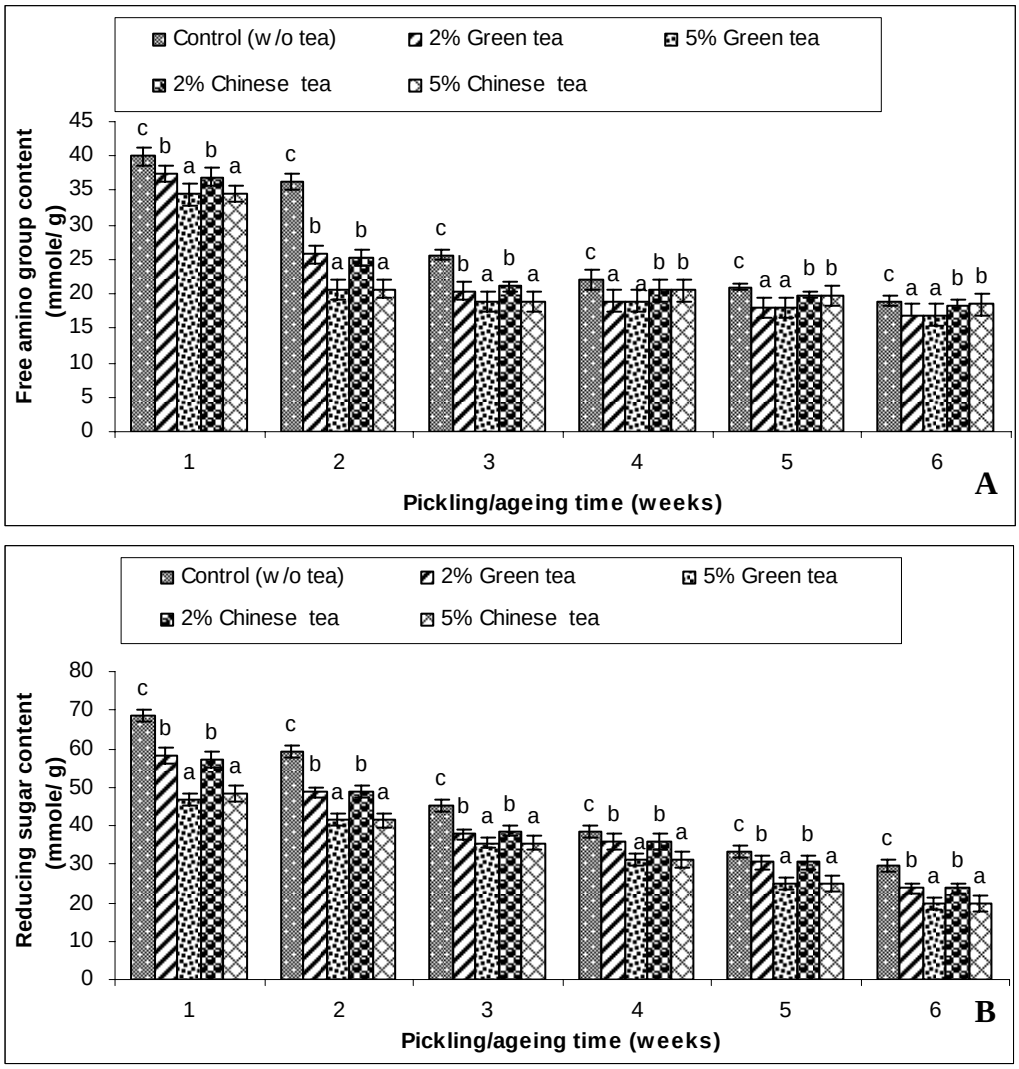


Figure 26. Changes in free amino group (A) and reducing sugar (B) contents of pidan white prepared from acetic acid treated duck egg as affected by green tea and Chinese tea at different levels. Bars represent the standard deviations (n = 3)

During ageing, when the higher alkali pH was obtained, alkali hydrolysis might take place to the similar rate with glycation via fee amino groups. As a result, no changes in free amino group were found during ageing. For reducing sugar content, the continuous decrease was observed during pickling and ageing. This indicated that glycation or Maillard occurred continuously during pidan processing. In general, pidan white treated without tea (Control) was less reactive in forming the glycated product than those treated with tea, as indicated by the lower reducing sugar content in pidan treated with tea. Thus, acetic acid pretreatment enhanced the penetration of both divalent and tea components, which significantly contributed to the enhancement of aggregation and brown color development of pidan.

Effect of green tea and Chinese tea at different levels on color of pidan white

Changes in the color of pidan white with different treatments are shown in Table 8. L* and b*-values of all pidan white generally decreased, whereas a*-values increased with increasing pickling and ageing. However pidan white treated with both teas at the level of 5% showed the higher b* and a* values at week 1, 3 and 6, possibly owing to the increased content of yellow or brown pigments. Increased a* values were more likely due to the formation of brown pigments, which might derive from Mailliard reaction of egg white or due to the color of tea at alkaline pH. It was coincidental with the lower level of L* of pidan white treated with both tea. Acetic acid aided pretreatment might involve in the enhanced penetration of tea components which might reduce the lightness of pidan white when pickling and ageing time increased. Higher b* values were found in the treatment of both tea at the level of 5%. This was most likely due to the oxidation of tea at very high alkaline pH, which could generate yellow color fluorescent compounds. The color of pidan white treated without tea (control) was lower, compared with reported in our previous study (Ganesan and Benjakul, 2010d), in which higher browning intensity and high a* and b*- values were obtained. Enhanced divalent penetration due the acetic acid pretreatment might cause the higher binding of zinc with protein, thereby lowering the

rate of Maillard reaction Therefore, acetic acid pretreatment and tea incorporation had an impact on color of pidan white.

Table 8. L*, a* and b*-values of pidan white prepared from acetic acid treated duck egg as affected by green tea and Chinese tea at different levels

Parameters	Treatments	Pickling/ageing time (weeks)		
		1	3	6
L*	Control (w/o tea)	32.11±1.17‡ c†, C††	26.86±0.07 c, B	23.46±0.50 c, A
	2% Green tea	28.85±0.33 b, C	25.13±0.09 b, B	21.03±0.18 b, A
	5% Green tea	26.55±0.19 a, C	24.18±0.26 a, B	17.47±0.78 a, A
	2% Chinese tea	29.15±0.03 b, C	25.23±0.10 b, B	21.49±0.50 b, A
	5% Chinese tea	26.36±0.42 a, C	24.22±0.19 a, B	17.97±0.38 a, A
a*	Control (w/o tea)	0.40±0.20 a, A	3.03±0.15 a, B	4.27±0.31 a, C
	2% Green tea	0.95±0.06 b, A	4.23±0.25 b, B	5.24±0.32 b, C
	5% Green tea	1.52±0.23 c, A	5.87±0.31 c, B	7.67±0.42 c, C
	2% Chinese tea	2.07±0.21 d, A	4.43±0.40 b, B	5.31±0.26 b, C
	5% Chinese tea	3.33±0.42 e, A	5.67±0.42 c, B	8.00±0.20 c, C
b*	Control (w/o tea)	8.03±0.16 a, C	5.28±0.35 a, B	2.26±0.13 a, A
	2% Green tea	10.04±0.68 b, C	7.80±0.16 b, B	3.40±0.11 b, A
	5% Green tea	14.81±0.38 c, C	10.37±0.53 c, B	7.30±0.23 c, A
	2% Chinese tea	10.18±0.66 b, C	7.46±0.41 b, B	3.49±0.07 b, A
	5% Chinese tea	15.02±0.47 c, C	10.29±0.33 c, B	7.25±0.32 c, A

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

†† Different capital letters in the same row indicate the significant differences ($P< 0.05$). † Different letters in the same column within the same parameter indicate the significant differences ($P<0.05$)

6.5 Conclusions

The reduction in shell thickness could be achieved by acetic acid pretreatment, thereby enhancing the penetration of tea and divalent into egg white. Tea components at higher content might undergo polymerization, leading to the browner color in pidan. Different tea has different impact on the gel and color of pidan white. Thus an acetic acid pretreatment aid along with tea incorporated at sufficient level could maneuver the characteristics of pidan white.

CHAPTER 7

EFFECT OF GLUCOSE TREATMENT ON TEXTURE AND COLOR OF PIDAN WHITE DURING STORAGE

7.1 Abstract

Changes in texture and color of pidan white as influenced by glucose treatment at levels of 0, 2 and 5% were determined after pickling (week 3) and during the storage up to 12 weeks. Hardness and cohesiveness of pidan white without glucose treatment were more retained but showed a decrease in adhesiveness as storage time increased up to week 12 ($P<0.05$). Higher browning intensity and a*-value were noticeable in the pidan white treated with glucose at both levels as the storage time increased ($P<0.05$). Thus, glucose could enhance the development of brown color, mainly via the Maillard reaction with free amino groups of pidan white at alkaline pH. Nevertheless, it showed the varying impact on textural property of pidan white during storage.

7.2 Introduction

Alkaline process has been used in food industry to destroy toxins (Ma, 1983) and to obtain products with a prolonged shelf-life. Pidan is one of the typical examples of such products, which are produced by soaking duck eggs in 4.2% NaOH/5.0% NaCl with 0.2% $ZnCl_2$ solution at room temperature (30°C) for 3 weeks and ageing for another 3 weeks (Ganesan and Benjakul, 2010a). Alkaline treatment can induce the degradation of proteins as well as Maillard reactions during the processing of pidan. Apart from brown color, gel-like texture of pidan is desirable and has been governed by cations used (Ganesan and Benjakul, 2010a). Texture of pidan white pickled with zinc cation was quite similar with those traditionally prepared

pidan pickled with lead. However the color of pidan white treated with zinc cation is amber brown color, compared to lead treated pidan white, with brown in color. However, lead has been toxic and prohibited for food processing.

Color of pidan may be due to the Malliard reactions between the glucose and amino acid in egg white (Li and Hsieh, 2004). Sankaran *et al.* (1989) reported that glucose in egg white accounts for 0.4%. Reducing sugars are essential ingredients in Maillard reaction, as they provide the carbonyl groups for interaction with the free amino groups of amino acids, peptides and proteins. Glycosylation or glycation induces the covalent attachment of sugars to α- or ε-NH_2 groups of amino acids and protein to form glycated proteins (Friedman, 1996). The Maillard reaction produces a variety of intermediate products and finally brown pigments (melanoidins) are formed (Van Boekel, 1998). Ageing of pidan aids in the development of brown color during processing of pidan. (Ganesan and Benjakul, 2010b). In order to enhance the color development during ageing of pidan pickled with Zinc cations further development process should be focused. The addition of glucose, a precursor for Maillard reaction, would be a means to enhance the development of brown color, in which the desirable color can be obtained.

Although egg white contains glucose at some level, it might not be sufficient to accelerate the Maillard reaction. Furthermore, the enhanced Maillard reaction might play a role in textural properties of pidan white. Therefore, the objectives of this study were to investigate the changes in color and texture of pidan white treated with zinc cations in the absence and presence of glucose at different levels after pickling and during storage for up to 12 weeks.

7.3 Materials and Methods

Chemicals

Zinc chloride ($ZnCl_2$), sodium hydroxide and sodium chloride were purchased from Lab-Scan (Bangkok, Thailand). 2,4,6-Trinitrobenzenesulfonic acid

(TNBS) and L-leucine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Glucose and other chemicals were purchased from Merck (Damstadt, Germany).

Duck egg collection

Fresh eggs of duck (*Anas platyrhucus*) with the weight range of 65–75 g were obtained within 1 day of laying from a farm in Rathabhum, Songhkla province, Thailand. Duck eggs were cleaned and checked for any crack prior to pickling.

Preparation of pidan

Clean duck eggs were soaked in a pickling solution containing 4.2% NaOH, 5% NaCl and divalent (0.2% $ZnCl_2$). Traditional prepared pidans using lead cation were taken as the control. Eggs (60 eggs) were soaked in different pickling solutions (6 L) at room temperature (30-32°C) for 3 weeks. Pickled pidan was soaked in the glucose solution at the concentrations of 0, 2 and 5% (w/v) for 48 h. Thereafter, all samples were removed and coated with white clay paste (clay: water, 4:1 (w/v)) to obtain a thickness of 2-3 mm. Coated eggs were left at room temperature and stored up to 12 weeks. During storage, the samples were taken for analyses every two week.

Texture profile analysis (TPA)

Pidan white samples with different treatments obtained at week 3, 6, 8, 10 and 12 were subjected to TPA. TPA was performed as described by Bourne (1978) with a TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England). Prior to analysis, pidan white samples of various treatments were cut into a cube (1.0×1.0×1.0 cm^3). The samples were compressed twice to 50% of their original height with a compression cylindrical aluminum probe (15 mm diameter). Textural analyses were performed at room temperature. Force-distance deformation curves were recorded at cross-head speed of 5 mm/s and the recording speed was 5 mm/s. Hardness,

adhesiveness, and cohesiveness were evaluated using the Micro Stable software (Stable Micro Systems, Surrey, England).

Determination of pH

At week 3, 6 and 12, pidan white samples with different treatments were determined for pH according to the method of Benjakul *et al.* (1997).

Measurement of UV-absorbance

UV-absorbance of pidan white samples was measured according to the method of Ajandouz *et al.* (2001). Prior to measurement, the samples were mixed with 5 volumes of deionized water (w/v). The mixtures were homogenized at a speed of 5,000 rpm for 10 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia) followed by centrifugation at a speed of 10,000×*g* for 10 min at 27°C using a refrigerated centrifuge (model J-E Avanti, Beckman Coulter, Inc., Palo Alto, CA, USA). The dilution of 20-fold was made. The absorbance was measured at 294 using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

Measurement of fluorescence intensity

Fluorescence intensity of pidan white samples was determined as described by Morales and Jimenez-Perez (2001). Twenty-fold diluted samples were prepared as previously described. The fluorescence intensity was measured at an excitation wavelength of 347 nm and an emission wavelength of 415 nm using a RF-1501 Fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

Measurement of browning intensity

Browning intensity of pidan white samples of all treatments was determined as described by Benjakul *et al.* (2005). The samples were prepared and

20-fold diluted as described previously. The absorbance was measured at 420 nm using a spectrophotometer.

Determination of free amino group content

Free amino group content was determined according to the method of Benjakul and Morrissey (1997). Pidan white samples (100-fold dilution) (125 µl) were mixed with 2.0 ml of 0.20 M phosphate buffer, pH 8.2, and 1.0 ml of 0.01% TNBS solution was then added. The solutions were mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Bavaria, Germany) at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The blank was prepared in the same manner as the samples except that distilled water was used instead of 0.01% TNBS. The absorbance was measured at 420 nm. Free amino group content was expressed in terms of L-leucine.

Determination of reducing sugar content

Reducing sugar content was determined according to the method of Chaplin (1994). All reagents were prepared as described by Chaplin (1994). One ml of pidan white samples (100-fold dilution) was mixed with 1.0 ml of reagent C in screw-sealed tubes. The mixtures were heated in boiling water for 15 min and then cooled with tap water. One ml of reagent D was added and mixed well. Finally, 3 ml of deionized water was added to the mixtures. The absorbance was measured at 520 nm. The reducing sugar content was calculated from the standard curve of glucose ranging from 10 to 100 mM.

Color measurement

The color of pidan white obtained at week 3 and 6 was measured using a Hunter Lab Labscan II colorimeter (Hunter Asociates Laboratory Inc., Reston, VA,

USA) and expressed as L* (lightness), a* (redness/greenness) and b* (yellowness/blueness).

Statistical analysis

Completely randomized design was used throughout the study. The experiments were run in triplicate using three lots of eggs. Data were presented as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out and means comparisons were run by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analyses were performed with the statistical program (SPSS for windows (Version 10), SPSS Inc, Chicago, IL, USA).

7.4 Results and Discussion

Effect of glucose treatment on textural properties of pidan white during storage

Hardness of pidan white of all treatments gradually decreased up to week 12 of storage ($P<0.05$) (Figure 27A). Hardness of pidan white obtained from all treatments was more resistant to compression at week 3 of pickling, most likely due to the aggregation of egg white proteins in the presence of cations. This was evidenced by the gel-like structure of egg white after 3 weeks of pickling. Proteins with negative charge under alkaline condition could interact each other in the presence of cations, thereby lowering the repulsive force between protein molecules (Ganesan and Benjakul, 2010b). During storage, the decrease in hardness was found in pidan white of all treatments, irrespective of cations and glucose added. However the decrease in hardness of pidan white was more pronounced with samples treated with glucose at both levels ($P<0.05$). Ageing of pidan resulted in the weakening of aggregate formed during pickling. Furthermore, glucose penetrated into egg white might enhance Maillard reaction at alkaline pH, competing with cations in forming the bridges between protein molecules. As a result, the gel network formed previously through

cations was weakened. However, in the presence lead or zinc cations (without glucose), the liquefaction was slightly retarded during storage ($P<0.05$). Cations might stabilize the protein network, thereby lowering the dissociation of protein network previously formed, though slightly higher alkaline pH was obtained during storage (Ganesan and Benjakul, 2010b). Electrostatic attraction between the positively charged Zn^{2+}–water complex and the carboxylic groups of the negatively charged protein played a role in protein aggregation. As a result, gel-like structure was formed (Shi *et al.,* 2008).

Cohesiveness is often used as an indice of the ability of gel to maintain an intact network structure. Higher values of cohesiveness indicate how well the product withstands intact network structure (Fernandez-Lopez *et al.,* 2006). Cohesiveness of pidan white gradually decreased up to week 12, irrespective of glucose used ($P<0.05$) (Figure 27B). This change was in accordance with that found for hardness of pidan white. The increase in pH of white proteins might lead to the repulsion between protein molecules to some degree (Ganesan and Benjakul, 2010b). Glucose soaking can enhance the Maillard reaction during storage, causing partial destabilization of ion -induced gel. Cohesiveness of pidan white treated with lead and zinc ions (without glucose treatment) was slightly higher than that of pidan white obtained from other treatments during storage ($P<0.05$). Those cations could maintain an intact gel network effectively along with the continuous dehydration of pidan white (Ganesan and Benjakul, 2010b).

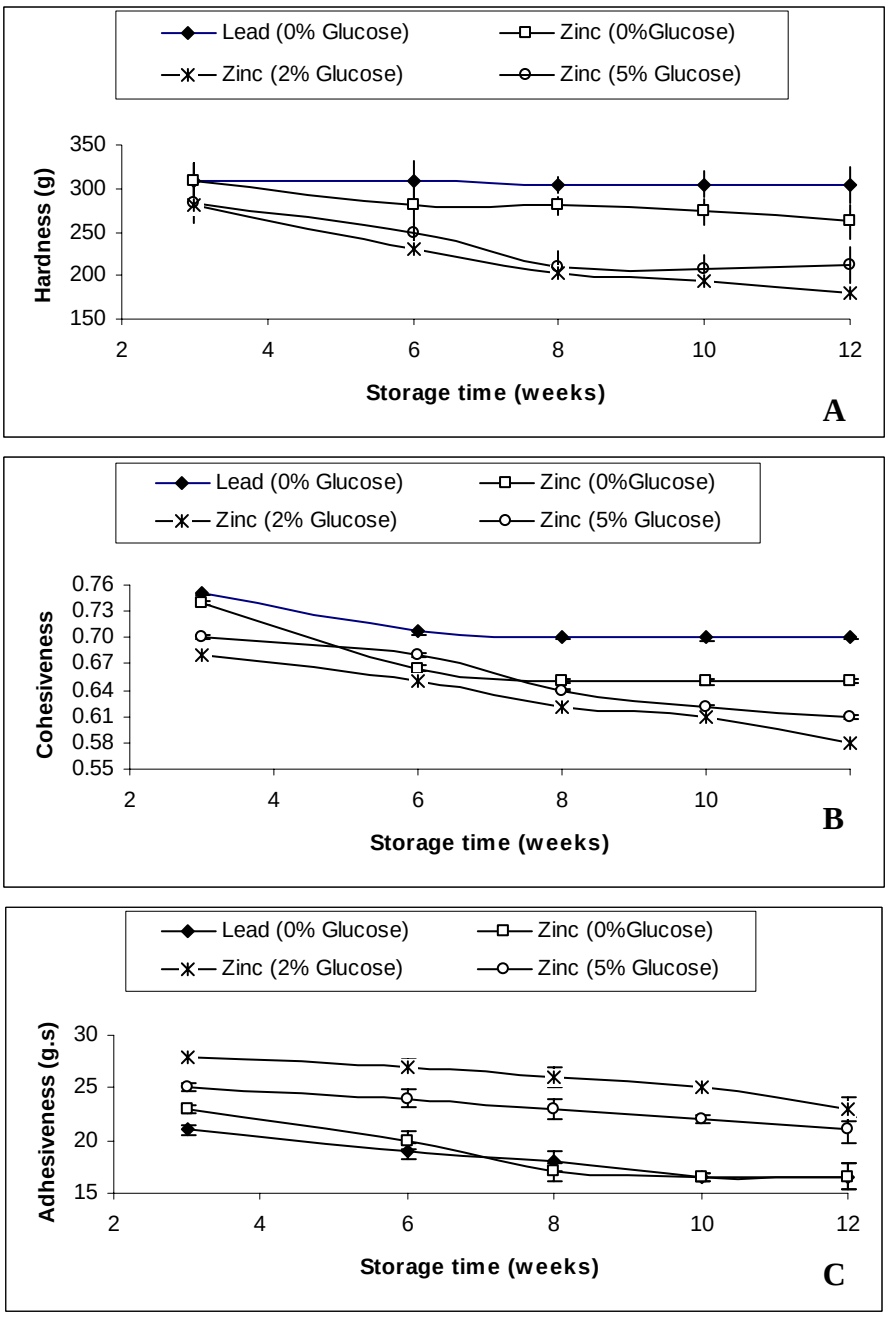


Figure 27. Changes in texture profile analysis (TPA) of pidan white treated with and without glucose during storage. Bars represent the standard deviations (n = 3)

Adhesiveness is defined as the work necessary to overcome the attractive forces between the product and a specific surface (Raikos *et al.,* 2007). The gradual decrease in adhesiveness was observed during storage up to week 12 ($P<0.05$) (Figure 27C). High polarity or hydrophilicity of egg proteins at alkaline pH more likely contributed to the stickiness of pidan white (Ganesan and Benjakul, 2010b). Among all samples, those treated with lead and zinc ions (without glucose treatment) showed the lower adhesiveness, indicating the less stickiness of pidan white during storage. Gradual decrease in adhesiveness during storage in all treatments might be attributed to the moisture loss of pidan white during prolonged storage (Ganesan and Benjakul, 2010b). As a result, pidan white had the less stickiness. However, the adhesiveness of pidan white treated with glucose, irrespective of amount used, was higher during storage ($P>0.05$). Glucose treatment probably resulted in the increase in hydrophilicity, in which proteins could bind more water, leading to stickiness of pidan white. Thus glucose treatment caused a slight decrease in the strength of pidan white gel during the extended storage.

Changes in pH, A_{294}, fluorescence intensity and A_{420} of pidan white during storage

Changes in pH of pidan with different treatments were monitored during pickling and storage. Increases in pH of pidan white were 11.20-11.30 at week 3, 12.05-12.14 at week 6 and 12.34-12.45 at week 12. Ganesan and Benjakul (2010b) reported that the final pH of pidan white pickled and aged for 6 weeks were 12.02. The increase in pH indicated the migration of alkali from pickling solution into egg white. Alkaline pH of pidan white had the influence on color as well as textural development of pidan (Ganesan and Benjakul, 2010b). Nevertheless, type of divalents and levels of glucose had the negligible effect on the pH of pidan white during storage ($P>0.05$).

Continuous increases in A_{294} of pidan white samples were observed during pickling and storage, irrespective of treatments (Figure 28A). Pidan white treated with zinc cation together with 2% and 5% glucose showed the higher increase

in $A_{294,}$ compared with those pickled in solution without glucose (P<0.05). Glucose could enhance the Maillard reaction at a very high alkaline pH (Li and Hsieh, 2004). Absorbance at 294 nm was used to determine the intermediate compounds of the Maillard reaction (Ajandouz *et al.,* 2001; Lerici *et al.,* 1990; Lertittikul *et al.*, 2007). The increase in absorbance at 294 nm suggested the formation of an uncoloured compound, which could be the precursor of the Maillard reaction (Ajandouz *et al.,* 2001). This was more likely due to the higher formation of intermediate generated during a very high alkaline pH. However the presence of lead cation prevented the formation of colorless intermediate to some extent. Lead has a strong affinity for some ligands, including the epsilon amino group of lysine, the carboxyl group of glutamic and aspartic acids, the sulfhydryl group of cysteine, and the phenoxy group of tyrosine and imidazole residues (Dunham, 1972). As a consequence, amino groups were less available for Maillard reaction. Different intermediate products are formed, either fluorescent or non-fluorescent compounds, during the Maillard reaction (Benjakul *et al.,* 2005). Some intermediate products might undergo conversion to the final brown compounds, while some intermediates were still generated.

Fluorescence intensity of all pidan white samples gradually decreased during storage, irrespective of treatments ($P < 0.05$). Generally, an increase in pH of the system influenced the rate of Maillard reaction and alkaline condition favored the reaction (Martins *et al.,* 2003). The Maillard reaction is associated with the development of fluorescent compounds formed prior to the generation of brown pigments (Baisier and Labuza, 1992; Morales *et al.,* 1996). This fluorescent compounds may be precursors of brown pigments (Labuza and Baisier, 1992; Morales and Van Boekel, 1997). The lower fluorescence intensity of pidan white during storage was probably caused by the rapid transformation of the intermediates to brown compounds. This led to less remaining fluorescent intermediate products, as shown by the lower fluorescence intensity. However, the pidan white treated with glucose at the level of 2% and 5% showed slightly higher fluorescent intensity than those pickled with cations in the absence of glucose. This result was in agreement with that of A_{294}. Addition of glucose therefore enhanced the Maillard reaction at a very high alkaline pH, mainly due to the higher carbonyl group involved in glycation.

Continuous increases in A_{420} of pidan white samples were observed during storage, irrespective of treatments (Figure 28C). Maillard reaction is a non-enzymatic browning reaction which links the carbonyl group of reducing carbohydrates and the amino group of free amino acids, especially lysine residues in proteins (Ajandouz *et al.,* 2001; Kato *et al.,* 1978). Maillard reaction is considered important in pidan white because of the significant amount of glucose naturally present in the egg white proteins (Powrie, 1977). Pidan white treated with zinc cation along with glucose at levels of 2 and 5% showed the higher browning intensity of pidan white, compared with other samples during storage up to 12 weeks ($P<0.05$). Maillard reaction took place rapidly at a very high alkaline pH. The increases in browning intensity of all samples with increasing storage was most likely due to the production and conversion of fluorescent or non-color compounds into brown pigment. Therefore, the incorporation of glucose in the pickling solution most likely enhanced the development of brown color of pidan white.

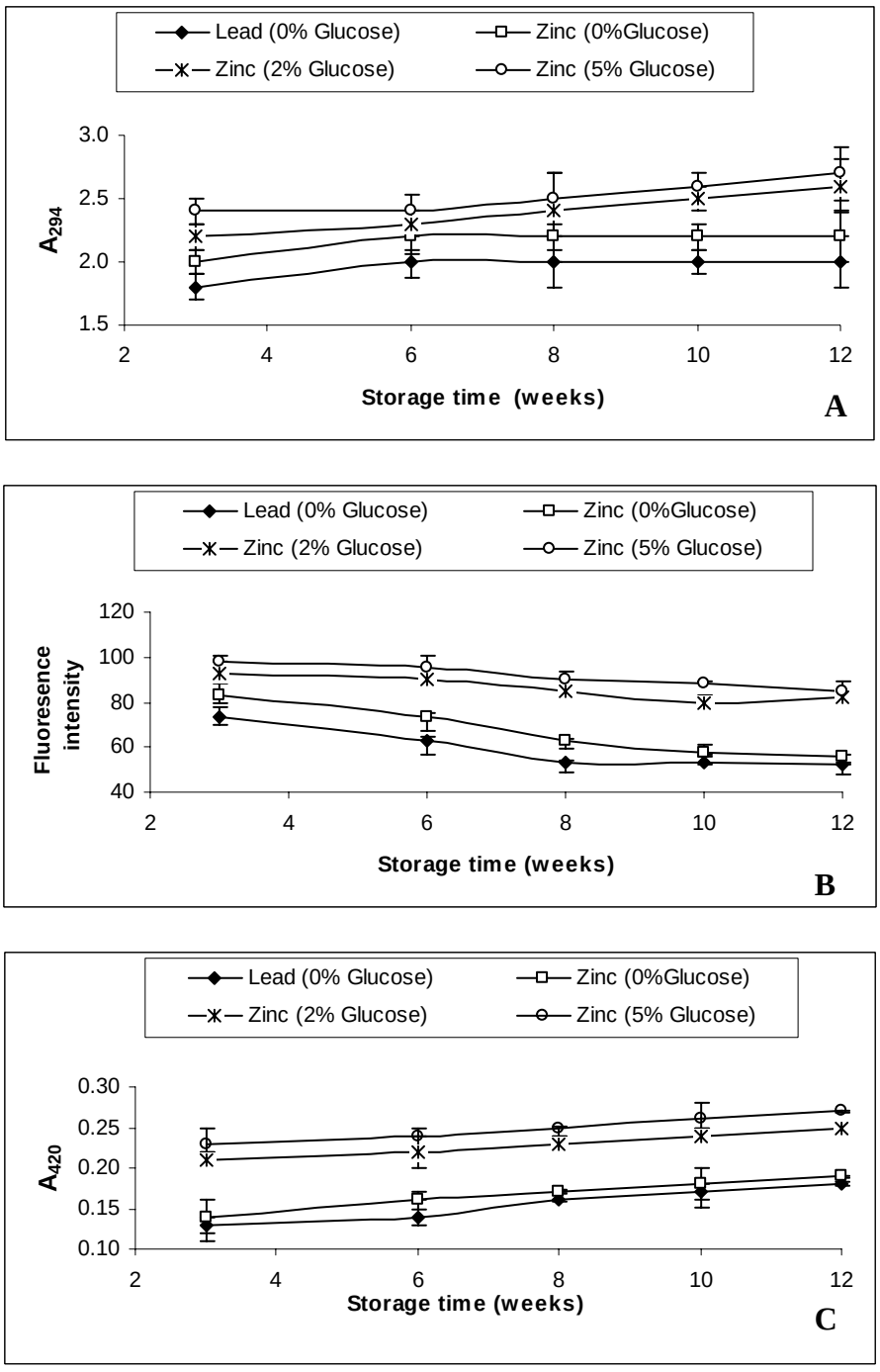


Figure 28. Changes in A_{294} (A), fluorescence intensity (B) and browning intensity (C) of pidan white treated with and without glucose during storage. Bars represent the standard deviations (n = 3)

Changes in free amino group and reducing sugar contents of pidan white during storage

Changes in free amino group and reducing sugar contents of pidan white samples with various treatments are shown in Figure 29A and 29B**,** respectively**.** Continuous decreases in amino group and reducing sugar content of all pidan white samples were noticeable when the storage time increased up to week 12 ($P < 0.05$). This result suggested that α- or ε-NH_2 groups of amino acids or proteins, which were hydrolyzed at very high alkaline pH, covalently attached to a sugar to form glycated proteins to a greater extent, particularly when the storage time increased to week 12. The first glycation product, or Schiff base, rearranges to a more stable ketoamine or Amadori product. The Amadori products can then form cross-links between adjacent proteins or with other amino groups, resulting in polymeric aggregates called advanced glycation end-products (Friedman, 1996). The decreases in free amino group were in accordance with the increase in browning (Figure 28C) and A_{294} (Figure 28A) and the decrease in fluorescence intensity (Figure 28B). This indicated that the increased storage time enhanced the interaction between free amino groups of proteins or peptides and glucose via glycation process (Ganesan and Benjakul, 2010). As a result, intermediate products were formed and further converted to brown pigments, as observed by the increased A_{420}. In general, pidan treated only with glucose at various levels (2 and 5%) was more reactive in forming the glycated product than that treated with cations (without glucose). This was evidenced by the greatest decrease in free amino group and reducing sugar contents with the concomitant increase in browning. The reaction rate of glycation between casein and sugars depended on the percentage of the acyclic form and the electrophilicity of the carbonyl groups (Naranjo *et al.,* 1998; Bunn and Higgins, 1981). The difference in reaction rate of sugar in glycation process was possibly governed by the conformation of protein. Under alkaline condition, sugar had the open structure, more favorable for glycation. Nevertheless, lead cation might impede Maillard reaction by higher cross linking and preventing the reaction between amino groups and reducing sugar.

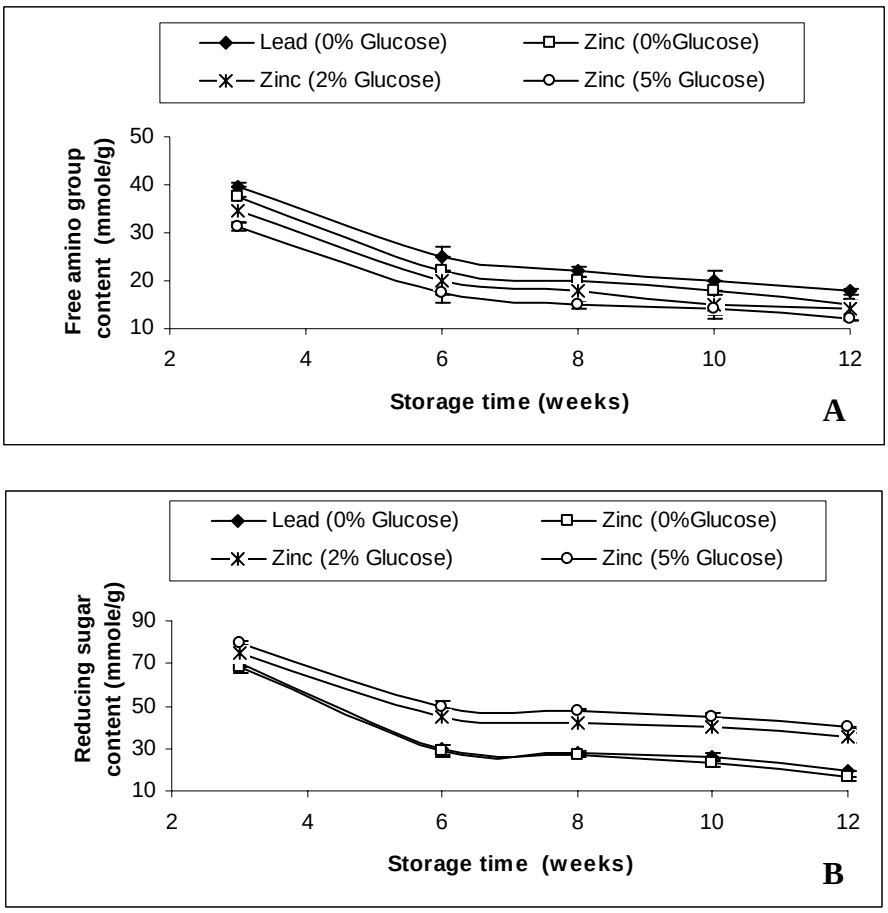


Figure 29. Changes in free amino groups (A) and reducing sugar (B) contents of pidan white treated with and without glucose during storage. Bars represent the standard deviations (n = 3)

Changes in the color of pidan white during storage

The color of pidan white of different treatments during storage is shown in Figure 30. L* and b*-values of pidan white generally decreased, whereas a*-values increased with increasing storage, regardless of glucose treatment. However, pidan white treated with lead ion showed the higher L* value values, compared to other treatments ($P<0.05$). This was probably due to the higher aggregation of proteins, which exhibited the higher light scattering effect.

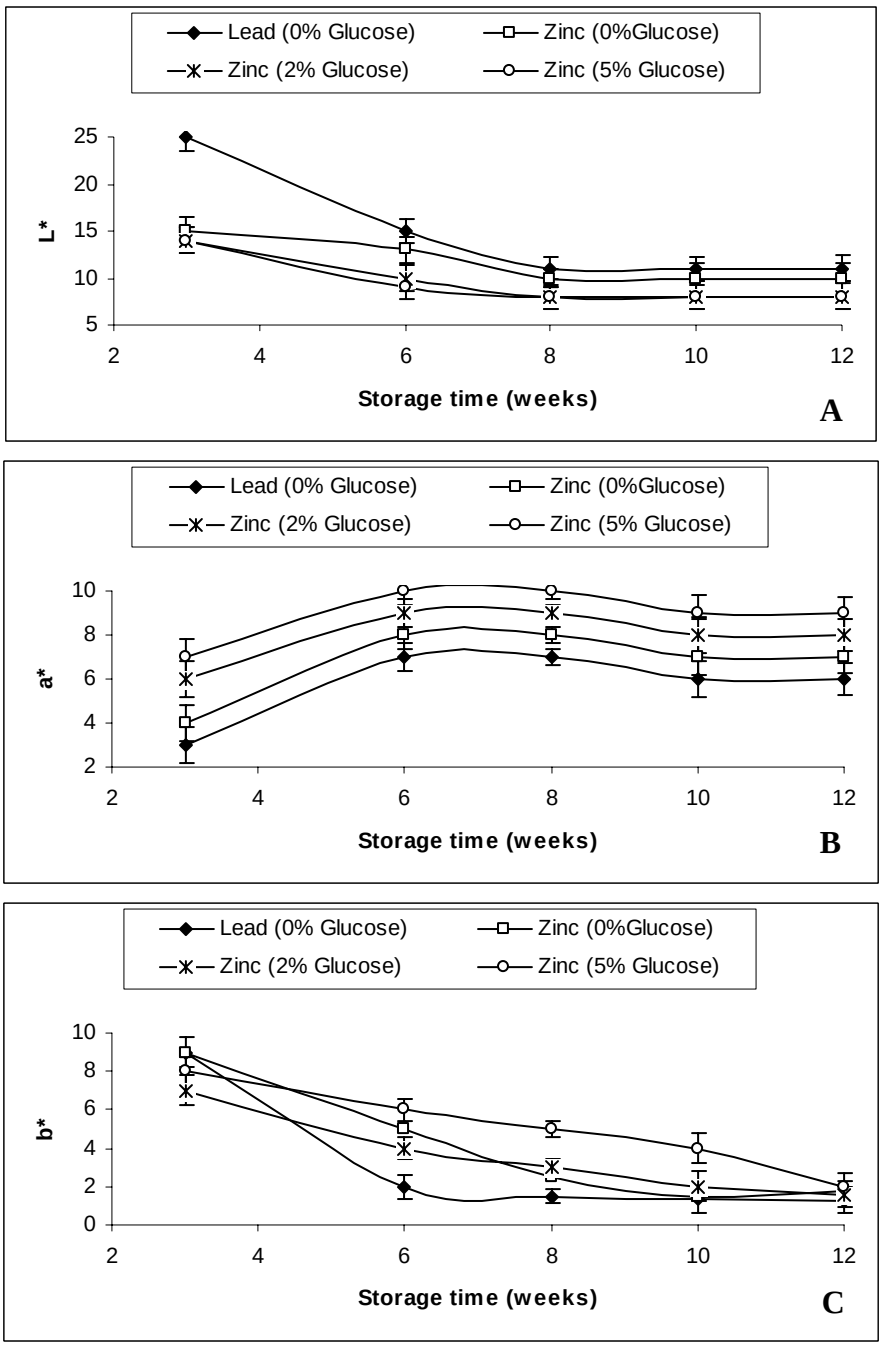


Figure 30. Changes in color of pidan white treated with and without glucose during storage. Bars represent the standard deviations (n = 3)

Chantrapornchai and McClements (2002) reported that whey protein gels increased its lightness with increasing protein size. Higher b* and a* values were found in pidan white treated with glucose at the level of 2 and 5% during storage, more likely owing to the formation of yellow or brown pigments. Increased a* values were possibly due to the formation of brown pigments, which might derive from Mailliard reaction of egg white (Ganesan and Benjakul, 2010b). This was in accordance with higher browning intensity of pidan white (Figure 28C). Furthermore, higher a*-values were noticeable in the pidan white treated with glucose at both levels which and was coincidental with the increasing $A_{420.}$ Thus glucose treatment had the marked impact on color of pidan white developed during storage.

7.5 Conclusions

Glucose had slight effect on brown color development of pidan white, whereas it showed the varying impact on textural property of pidan white during storage. Therefore, browning intensity was significantly developed in pidan white by the treatment of the pickled pidan in glucose solution before further storage.

CHAPTER 8

EFFECT OF THREE CATIONS ON THE STABILITY AND MICROSTRUCTURE OF PROTEIN AGGREGATE FROM DUCK EGG WHITE UNDER ALKALINE CONDITION

8.1 Abstract

Pidan has been consumed widely in oriental countries and lead, a toxic element, has been used traditionally to yield the desirable characteristics. For safety concerns, alternative cations can be used for the production of pidan with the comparable properties to the traditionally prepared pidan. Therefore, turbidity measured as absorbance at 400 nm and microstructure of duck egg white proteins at pH 12 as influenced by three cations at various levels were investigated. Turbidity and particle size of 2 % egg white protein in 1% NaCl sample added with $CaCl_2$, PbO_2 or $ZnCl_2$ at a level of 0.1% increased with time increased up to 1 h, followed by a decrease (p<0.05). Nevertheless, the turbidity was more retained in samples added with PbO_2, suggesting high stability of aggregate formed. Zeta potential study revealed that the lower negative charge of the aggregates treated with PbO_2 was obtained, compared with other samples. Light microscopic studies indicated that the aggregation of egg white proteins was induced by ions but varied with the types of ions and incubation time. Therefore, PbO_2 exhibited the highest stabilizing effect on egg white protein under alkaline condition. However, $ZnCl_2$ can be used as the alternative compound, though it showed slightly lower impact on stability of aggregate of duck egg white protein.

8.2 Introduction

Egg white is extensively utilised as a functional food material in food processing. Egg white gels consist of polymers connected to each other in order to form a 3-dimensional network. Protein gelation is thought to consist of multiphase reactions involving the initial protein structure unfolding (denaturation), followed by the aggregation of polypeptides, which gradually proceeds to form a technologically functional gel network (Ziegler and Foegeding, 1990). The network depends mainly on the physicochemical conditions of the medium (specifically pH, ionic strength, and type of salts) (Croguennec *et al.,* 2002). At pH values sufficiently far from the isoelectric point of the proteins and at low ion concentrations, the unfolded proteins tend to remain separate due to the electrostatic repulsive forces between molecules. Upon the addition of salt, these repulsive forces are lowered, and the protein molecules can aggregate and form a gel (Barbut and Foegeding, 1993; Ju and Kilara, 1998). Furthermore, egg white proteins spontaneously formed the soluble oligomers at pH 12.2. Those oligomers were stabilized by intermolecular disulphide bonds (Kumar *et al.,* 2008). Monovalent and divalent ions are able to screen electrostatic interactions between charged protein molecules (Yasuda *et al.,* 1986). Nevertheless divalent ions such as Ca^{2+} have the effect on protein cross-linking via the salt bridges between negatively charged carboxyl groups (Hongsprabhas and Barbut, 1997). The resulting size, shape and spatial arrangement of the protein aggregates and their response to deformation can therefore vary widely and have an impact on gel. The concentration of salt used to form a gel is of the major determinants of the structure and spatial organization of the protein aggregates (Hongsprabhas *et al.,* 1999). Low-salt concentrations produce filamentous type gels, while higher concentrations induce the formation of particulate gel (Hongsprabhas and Barbut, 1997).

Typical characteristics of pidan (Alkaline egg) are determined by the properties of duck egg white protein gels formed during the preparation (Ganesan and Benjakul, 2010a). Lead has been used traditionally to prepare the pidan gels but it is toxic and caused black spots on the pidan shell (Chen and Su, 2004). Due to safety

concerns, alternative cations particularly zinc and calcium ion, have been used for pidan production. However the egg white gel showed the less stability after ageing in comparison with those treated with lead (Ganesan and Benjakul, 2010a). The basic information related to cation induced aggregation of duck egg white protein and microstructure changes at alkaline pH have not yet been reported. The objective of this study was to monitor the aggregation and microstructure of egg white protein aggregates treated with three cations under alkaline condition.

8.3 Materials and Methods

Chemicals

Lead oxide (PbO_2), zinc chloride ($ZnCl_2$), calcium chloride ($CaCl_2$), sodium hydroxide and sodium chloride were purchased from Lab-Scan (Bangkok, Thailand). Purity of all salts used was greater than 99%. Mercury bromo phenol blue was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Sample preparation

Fresh eggs of duck (Anas platyrhucus) with the weight range of 65–75 g were obtained within 1 day of laying from a farm in Rathabhum, Songhkla province, Thailand. Egg white and yolk were separated manually with yolk separator. Egg white was pooled and homogenised at a speed of 11,000 rpm for 2 min using a homogeniser (IKA Labortechnik, Selangor, Malaysia).

Preparation of egg white protein aggregates

Egg white protein (2%) in 1% NaCl sample was prepared and adjusted to pH 12. The sample was added with and without (control) addition of different ions including PbO_2, $ZnCl_2$ or $CaCl_2$ at different levels (0.02, 0.05 and 0.1 %). To completely solubilise PbO_2, the distilled water was mixed with PbO_2, followed by

boiling using a hot plate for 15 min. After sudden cooling, the volume was adjusted to obtain the designated final concentration using NaCl solution (1% NaCl), pH 12. Sodium azide (0.1%) was added to the sample to prevent microbial growth. Various samples were stirred continuously at room temperature (26-28°C) for up to 90 min. The samples were taken every 15 min for aggregate analysis. For particle size analysis, samples added with cations at a level of 0.1% were taken every 30 min. To study the stability of aggregate formed, the turbidity of sample containing 0.1% cations was monitored up to 72 h.

Measurement of protein aggregates

Samples with various treatments were placed in the cuvette (light path length of 1 cm). Degree of protein aggregation was estimated by measuring the absorbance at 400 nm (Barbut and Foegeding, 1993) using a UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

Determination of particle size

Particle-size of sample was measured using a Beckman-Coulter particle-size analyser (Model LS230) with a fluid module, 750 nm laser and the companion software version 3.29 (Beckman Coulter, Inc., Miami, Florida, USA.). Each sample was circulated through and analysed by the LS230 for 90 s and the sample vessel was rinsed to background levels. The analyser was cleaned on a regular basis by disassembling the optical module and thoroughly cleaning all surfaces and also by circulating cleaning solution through the machine. In addition, Beckman-Coulter particle-size standards (Latron^{TM} 300 LS series) of 0.2, 50 and 500 μm were run on a regular basis for quality control assessments. The analysis was performed at room temperature (25-27°C).

Measurement of zeta potential

Zeta potential of proteins in different samples added with cations and the control (without the addition of cations) was determined with ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Hodifltsville, NY, USA) at room temperature.

Determination of microstructure

Microstructures of egg protein aggregates in different samples containing different cations at a level of 0.1% and the control sample at time 1 and 72 h were visualized using an Olympus DP 50 light microscope (Olympus Optical Co., Tokyo, Japan). The samples were placed along with tissue freezing medium on the stub and frozen at -20°C. The samples were cut into a thickness of 20 µm in a Reichert-Jung cryostat (Leica Instruments GmbH, Nussloch, Germany) at -20°C. The samples were then mounted in the frozen state to microscope slides and air dried. Thin sections (20 µm thick) were stained according to mercury bromophenol method (Pearse, 1972) with a slight modification (distilled water for 2 min, 0.05 % mercury bromophenol blue for 5 min, 0.5 % acetic acid for 2 min). Before examining the samples under the microscope, they were covered by a droplet of glycerol/water sample (1:1 v/v) and a cover glass. The samples were visualised using a light microscope with a magnification of 10X.

Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard.

Statistical analysis

Completely randomized design was used throughout the study. The experiments were run in triplicate using three lots of eggs. Data were presented as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out and means comparisons were run by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analyses were performed with the statistical program (SPSS for windows (Version 10), SPSS Inc, Chicago, IL, USA).

8.4 Results and Discussion

Effect of types and concentrations of cations on turbidity of egg white protein under alkaline condition

Turbidity of duck egg white protein sample added with and without (control) addition of cations including $CaCl_2$, PbO_2 and $ZnCl_2$ at the levels of 0.02, 0.05 and 0.1% was monitored up to 90 min (Figure 31). At cation concentration of 0.1%, turbidity of egg white protein sample increased continuously up to 60 min ($p<0.05$) and remained constant thereafter, indicating the formation of protein aggregates especially within the first 60 min. However the turbidity of aggregates formed varied with the type of cations used. At 60 min of incubation, the highest turbidity was found in the sample added with 0.1% of $CaCl_2$ ($p<0.05$), whereas the lower turbidity was noticeable in samples treated with 0.1% of PbO_2 or $ZnCl_2$. Nevertheless, no differences in turbidity were noticeable between the samples added with PbO_2 and $ZnCl_2$. At the lower level of cations, no changes in turbidity were noticeable within the first 60 min ($p>0.05$), but a slight increase in turbidity was found during 60-90 min of incubation ($p<0.05$). It was noted that the control had no change in turbidity during the incubation time of 90 min ($p>0.05$). Thus, cation at higher concentrations could induce the formation of aggregate of egg white protein

more effectively. In the presence of cations at the sufficient amount, the cross-linking via salt bridges could take place. As a result, the formation of aggregate was noticeable. Among all cations used, calcium ion caused a greater aggregation than other cations. This was more likely due to the greater screening effect on the negatively charged carboxyl groups of proteins (Twomey *et al.,* 1997). Zn and Pb showed the similar effect on protein aggregation. Thus, the type and concentration of cations played a role in protein aggregation of duck egg white under alkaline condition.

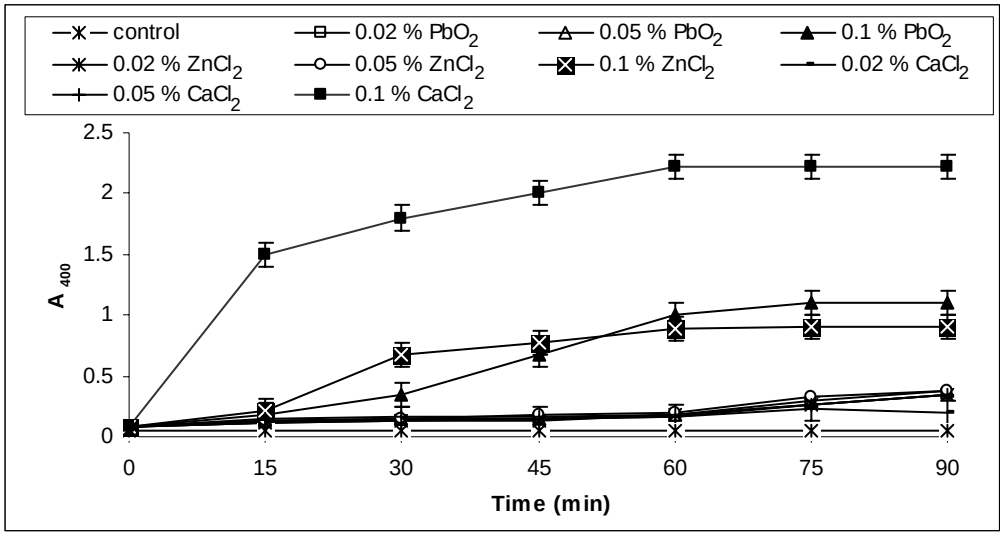


Figure 31. Turbidity of duck egg white protein samples (pH 12) as influenced by three types and concentrations of cations during incubation. Bars represent the standard deviation (n=3)

Effect of cations on particle size of egg white protein under alkaline condition

Particle size of duck egg white protein in alkaline sample added with and without (control) addition of cations including $CaCl_2$, PbO_2 and $ZnCl_2$ at a level of 0.1%, which was the concentration rendering the highest turbidity or aggregation, was monitored at various times up to 90 min (Figure 32). A negligible change in particle size was noticeable within the first 30 min (p>0.05). Thereafter, particle size of egg

white protein sample increased continuously up to 60 min (p<0.05). After 90 min of incubation, particle size remained constant. This result was in accordance with the turbidity of egg white proteins with different cations at alkaline condition (Figure 31). However, calcium ion yielded the higher particle size than other cations, especially when the incubation time of 60 min and 90 min was used. This was most likely due to the non specific binding of calcium to the protein molecules and salt bridging effect of calcium (Hongsprabhas and Barbut, 1997). Lower particle sizes were found in the control (without addition of cations). Difference in the extent of aggregation and particle size of duck egg white protein added with different cations at the same level (0.1%) suggested the different interactions between metal ions and protein molecules in terms of bonding involved, including hydrophobic interaction, ionic interaction, hydrogen bond and metal bridging.

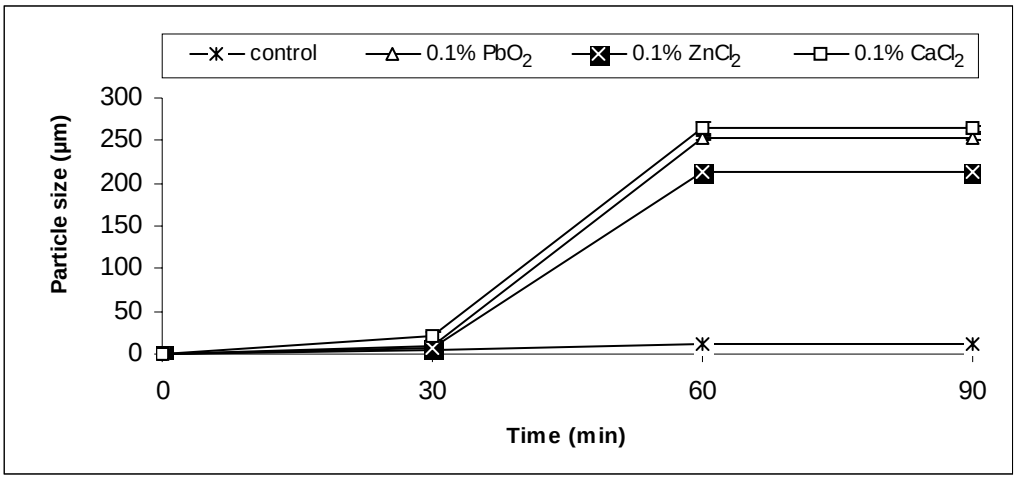


Figure 32. Particle size of duck egg white protein samples (pH 12) as influenced by three cations at a level of 0.1 % during incubation. Bars represent the standard deviation (n=3)

Effect of types of cations on stability of duck egg white protein under alkaline condition

Turbidity of duck egg white protein added with and without (control) addition of cations including $CaCl_2$, PbO_2 and $ZnCl_2$ at a level of 0.1% was monitored up to 72 h (Figure 33). For all samples, except that added with PbO_2, turbidity of duck egg white protein sample increased continuously up to 1.5 h and gradually decreased up to 72 h, irrespective of cation types. For sample added with PbO_2, the negligible decrease in turbidity was obtained after 3 h of incubation. These results indicated that the stability of protein aggregates formed varied with cations used. Changes in turbidity demonstrated that irreversible or slow reversible changes in protein structure were governed by the different initial formation of protein aggregates mediated by ions (Barbut and Foegeding, 1993). Hermansson (1986) reported that turbidity has been used to roughly estimate the degree of aggregation, which is affected by environmental conditions (pH and/or ionic strength). After 72 h, the higher turbidity was found in sample added with PbO_2 at a level of 0.1% and the lowest turbidity was obtained in the control (without addition of cations) ($p<0.05$). With increasing time, protein molecules of duck egg white aggregated with the aid of cation might not be stable under the high alkaline condition. Salt bridges might be disrupted, caused by the higher repulsive force mediated by very high alkaline pH. Additionally, alkaline hydrolysis might take place, cuasing the decrease in size of peptide (Larre *et al*., 2006). However aggregate induced by PbO_2 showed a higher stability than the other samples stabilized by other cations. This was most likely due to the stronger complex between lead and protein (Fowler, 1998). The lead can bind with the cysteine residues of protein more tightly by tris-thiol ligand (Godwin, 2001). This protein complex was more stable against hydroxyl ion attack. Thus, the type of cations played a role in stability of aggregate from duck egg white protein at alkaline pH.

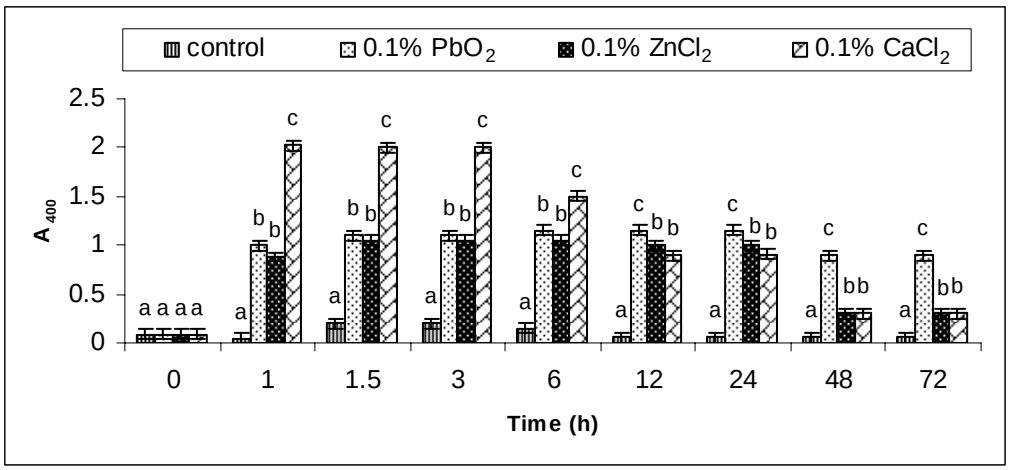


Figure 33. Turbidity of duck egg white protein samples (pH 12) as influenced by three cations at a level of 0.1 % during incubation. Bars represent the standard deviation (n=3). Different letters on the bars within the same incubation time indicate significant differences ($P<0.05$)

Effect of types of cations on zeta potential of duck egg white protein under alkaline condition

Zeta potential of duck egg protein sample added with and without (control) addition of cations including $CaCl_2$, PbO_2 and $ZnCl_2$ at a level of 0.1% was monitored at 1 and 72 h of incubation is shown in Table 9. The negative charge of the protein increased as the incubation time increased to 72 h, irrespective of treatments ($p<0.05$). The negative charge of the proteins might be due to the negatively charged amino acid in the egg white protein, mainly acidic amino acid, at a very high alkaline pH. At a pH far from isoelectric point, the carboxyl groups are negatively charged ($-COO^-$), and the net negative charge increased (Ma and Holme, 1982). With increasing incubation time, proteins were more unfolded, exposing the charged amino acids. During Pidan ageing, degradation of ovoalbumin and other egg white proteins resulted in the increases in small peptides and free amino acid at room temperature (25-27°C) (Ganesan and Benjakul, 2010b). Also, alkaline pH could promote slow hydrolysis of peptide bonds, resulting in the formation of peptides and amino acids.

This more likely caused the increases in number of negative charges in the peptides (Larre *et al*., 2006). The lower negative charge was observed in the samples added with cations at 1 and 72 h of incubation. It was suggested that neutralisation of negative charge took place in the presence of positive charge of cations. As a consequence, the lower negatively charged complex was obtained. After incubation for 72 h, the increase in negative charge was found in the samples added with $CaCl_2$ or $ZnCl_2$ (p<0.05). This was due to the instability of ionic interaction between proteins and Ca^{2+} or Zn^{2+} under alkaline condition. As a result, negative charge of protein was still available. However, the sample added with PbO_2 at a level of 0.1% had no increase in the negative charge of protein (Table 9). It reconfirmed that lead had the higher binding capacity with egg protein at a very high alkaline pH and the aggregate formed was stable most likely due to the tris-thiol ligand binding with cysteine residues (Godwin, 2001). This result was in accordance with the turbidity study (Figure 33).

Table 9. Zeta potential of duck egg white protein solutions (pH 12) added without and with different divalent cations at a level of 0.1 % after incubation for 1 and 72 h

Treatments	Zeta potential (mV)	
	1 h	72 h
Control	-16.67±1.21$^{c, A}$*	-21.57±1.21$^{c, B}$
0.1 % PbO_2	-9.96±0.57$^{a, A}$	-10.54±0.21$^{a, A}$
0.1 % $ZnCl_2$	-12.97±0.53$^{b, A}$	-15.37±0.75$^{b, B}$
0.1 % $CaCl_2$	-8.57±1.51$^{a, A}$	-15.65±0.40$^{b, B}$

Mean ± SD (n:3).

* Different superscripts (small letters) in the same column indicate significant differences (p < 0.05)

Effect of types of cations on microstructure of duck egg white aggregates under alkaline condition

Microstructures of duck egg protein aggregates added with and without (control) addition of cations including $CaCl_2$, PbO_2 and $ZnCl_2$ at a level of 0.1% visualized by light microscope are shown in Figure 34. Aggregation of protein molecules was noticeable in duck egg white added with all cations and the control at 1 and 72 h of incubation (Figure 34a and 34b). Van den Berg *et al.* (2009) reported that aggregated protein constituents were engaged in network formation. For the globular proteins at pH 7.0, small elongated aggregates are formed at low ionic strength, while at high ionic strength, larger aggregates appear to be formed by random association of the small aggregates (Durand *et al.*, 2002). Aggregates induced by $CaCl_2$, and PbO_2 at the level of 0.1% showed the denser network. Divalent calcium ion might engage in calcium bridging between negatively charged groups under alkaline condition on adjacent unfolded protein molecules (Matsudomi *et al.*, 1991). Barbut (1995b) reported that calcium induces the formation of fine and thick protein strands, varying with ion concentrations. $ZnCl_2$ at a level of 0.1% and the control exhibited the looser network with the irregularly shaped voids after 1 h of incubation. Barbut (1995a) reported that sodium induces the formation of fine strands at low ion concentration. After 72 h of incubation, the interaction and network varied with cations used. More void and looser network was found in all treatments (Figure 34b). Alkaline conditions are known to unfold protein molecules (Creighton, 1993) and might break down the network previously formed. However more compact structure of protein aggregates added with PbO_2 was obtained after 72 h of incubation. The result confirmed that network of egg white protein aggregate induced by Pb was stable under the alkaline condition.

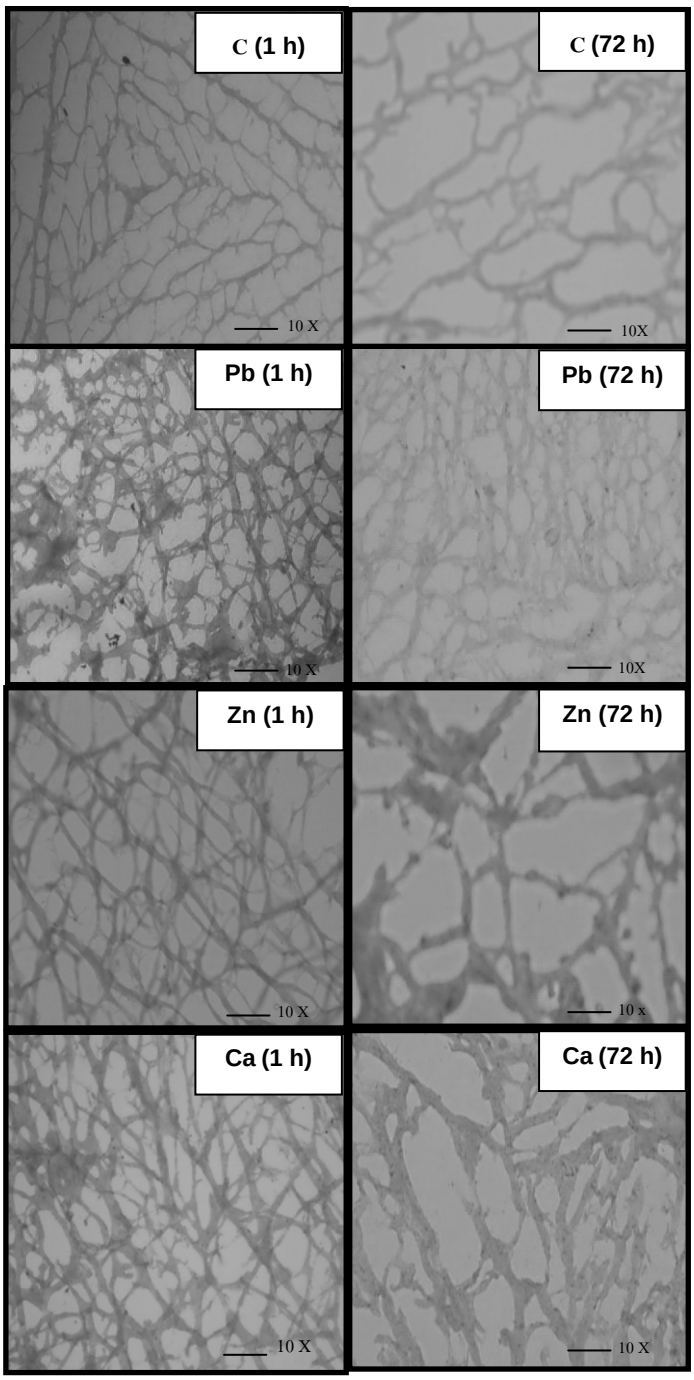


Figure 34. Light microscopic (LM) micrographs of duck egg white protein aggregates induced by three cations at a level of 0.1 % after 1 and 72 h of incubation. C; Control (without addition of cations) Pb; 0.1 % PbO_2 Zn; 0.1 % $ZnCl_2$ Ca; 0.1 % $CaCl_2$. Magnification: 10X

8.5 Conclusions

Stability and microstructure of duck egg protein aggregates varied with the type and concentrations of cations used. $CaCl_2$ and PbO_2 at a level of 0.1% induced the formation of protein aggregate effectively under alkaline condition. Nevertheless, PbO_2 yielded the aggregate with the high stability, in which gel network was stabilized for a longer time. $ZnCl_2$ showed the slightly lower stabilising effect on egg white protein under the alkaline condition. For the safety concern, $ZnCl_2$ can be recommended as an alternative compound for pidan production instead of PbO_2, which is toxic for consumption.

CHAPTER 9

COMPARATIVE STUDY ON CHARACTERISTICS OF PIDAN WHITE AND YOLK PRODUCED WITH THE AID OF CATIONS

9.1 Abstract

Effects of different cations on the characteristics of pidan white and yolk were investigated. Fourier transform infrared (FTIR) study of 0.2% PbO_2 and 0.2% $ZnCl_2$ treated pidan white and yolk had similar spectra to those of fresh egg. Mineral composition of white and yolk varied with the type of cations used in pickling solution. Scanning electron microscopic study showed that the more ordered network was found in 0.2% PbO_2 treated pidan white, compared with 0.2% $ZnCl_2$ treated counterpart. Confocal laser scanning microscopic study indicated that the lower release of yolk lipid was obtained in 0.2% $ZnCl_2$ treated pidan, compared with 0.2% PbO_2 treated counterpart. Thus cations in the pickling solution affected the characteristics of pidan white and yolk.

9.2 Introduction

Pidan or alkaline treated egg have been known as preserved egg, consumed widely in south-east Asia. Pidan white is generally amber brown in color with the gelly texture, whereas pidan yolk is solidified with greenish brown color. Under alkaline pH used for pidan production, electrostatic repulsion extensively opposes protein-protein interactions. The addition of cations in pickling solution enhances the penetration of those cations into egg, thereby diminishing the repulsive forces, and protein–protein association occurs, forming a self-supporting gel (Ganesan and Benjakul, 2010a). Lead has been used in pidan for gel stabilisation but it is toxic for consumption (Chen and Su, 2004). Alternatively pidan is produced by soaking duck eggs in 4.2% NaOH/5.0% NaCl with 0.2% $ZnCl_2$ or 0.2% $CaCl_2$ solution at

room temperature (30°C) for 3 weeks and ageing for another 3 weeks (Ganesan and Benjakul, 2010b).

Alkaline treatment also causes the exceptional formations of lysinoalanine, racemisation of amino acids, aminoacids degradation and Maillard reactions during the processing of pidan (Chang *et al.*, 1999a). The formation of certain compounds in the Maillard reaction of pidan white is very complicated and the classes of compounds are more or less known. However, the characteristic texture and brown color of pidan white makes the product more consumable. Although the impact of cation types on the properties of pidan white and yolk has been elucidated (Ganesan and Benjakul, 2010 a, b). A little information regarding the chemical composition and microstructure of pidan white and yolk induced by selected cations has been reported. Therefore, the objective of this study was to characterize of pidan white and yolk pickled with the aid of two different cations.

9.3 Materials and Methods

Chemicals

Lead oxide (PbO_2), zinc chloride ($ZnCl_2$), sodium hydroxide and sodium chloride were purchased from Lab-Scan (Bangkok, Thailand). Glutaraldehyde, ethanol and silver nitrate were obtained from Merck (Darmstadt, Germany). Nile blue A was procured from Merck (Darmstadt, Germany). Purity of all chemicals used was greater than 99%.

Duck egg collection

Fresh eggs of duck (*Anas platyrhucus*) with the weight range of 65–75 g were obtained within 1 day of laying from a farm in Rathabhum, Songhkla province, Thailand. Duck eggs were cleaned and checked for any crack prior to pickling.

Preparation of pidan

Clean duck eggs were soaked in a pickling solution containing 4.2% NaOH, 5% NaCl and divalent (0.2% $ZnCl_2$). Traditionally prepared pidan using lead cation (0.2% PbO_2) was used as the control. Eggs (60 eggs) were soaked in different pickling solutions (6 L) at room temperature (30-32°C) for 3 weeks. Pidans were removed and coated with white clay paste (clay: water, 4:1 (w/v)) to obtain a thickness of 2-3 mm. Coated eggs were left at room temperature for another three weeks for ageing. The samples were then taken for analyses.

Determination of pH, moisture and NaCl contents of pidan white and yolk

Pidan white and yolk samples with different treatments were determined for pH in comparison with fresh egg white and yolk according to the method of Benjakul *et al.* (1997). Moisture and NaCl contents in the pidan white samples were measured as per the method of AOAC (2000) with the analytical No. of 925.10 and 939.10, respectively.

To determine salt content, sample (1 g) was added with 20 ml of 0.1 N $AgNO_3$ and 10 ml of HNO_3. The mixture was boiled gently on a hot plate until all solids except AgCl2 were dissolved (usually 10 min). The mixture was cooled using running water. Five ml of 5 % ferric alum indicator ($FeNH_4 (SO_4)_2 \bullet 12 H_2O$) were added. The mixture was titrated with the standardised 0.1 N KSCN until the solution became permanently light brown. The percentage of salt was then calculated as follows:

$$\text{NaCl content (\%)} = 5.8 \times [(V1 \times N1) - (V2 \times N2)]/W$$

where V 1 = volume of $AgNO_3$ (ml); N 1 = concentration of $AgNO_3$ (N);
V 2 = volume of KSCN (ml); N 2 = concentration of KSCN (N); and
W = weight of sample (g).

Determination of ammonia content of pidan white and yolk

Ammonia content of pidan white and yolk at week 6 in comparison with fresh egg white and yolk obtained from different treatments was determined by distillation method as described by Parris and Foglia (1983) with a slight modification. Fifty ml of 10-fold diluted samples were placed in a Kjeldahl flask containing 100 ml of distilled water and 3 g of MgO. The mixture was distilled and the distillate was collected in 50 ml of 4% boric acid before titration with 0.05 M H_2SO_4 using methyl red-bromocresol green as an indicator. Ammonia content was calculated and expressed as percentage of sample.

Determination of mineral contents

Analyses of calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), zinc (Zn), lead (Pb), iron (Fe) and copper (Cu) of freeze-dried fresh and treated pidan white and yolk were carried out using the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Model 4300 DV, Perkin Elmer, Shelton, CT, USA) according to the method of AOAC (1999). Sample (4 g) was mixed well with 4 ml of 70% nitric acid. The mixture was heated on the hot plate until digestion was completed. The digested sample was transferred to a volumetric flask and the volume was made up to 10 ml with deionised water. The solution was then subjected to analysis. Flow rates of argon to plasma, auxiliary and nebuliser were maintained at 15, 0.2, and 0.8 l/min, respectively. Sample flow rate was set at 1.5 ml/min. The concentration of mineral was calculated and expressed as mg/kg sample.

Determination of Nitogen (N) and Sulfur (S) contents

N and S content was determined using CHNS-O analyzer (Model Flash 1112 EA Series, CE Instruments, Milan, Italy). The sample (2-3 mg dry matter) housed in a tin capsule was dropped into a quartz tube at 900°C with constant helium flow (carrier gas, flow rate 130 mL/min). Before the sample was dropped into the combustion tube, the stream was enriched with a measured amount of high purity

oxygen (250ml/min) to achieve a strong oxidizing environment which guaranteed almost complete combustion/oxidation even of thermally resistant substances. The component of the combustion mixture was detected by a thermal conductivity detector (Thermoquest, CE Instruments, Milan, Italy).

Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were obtained from pellets containing 2 mg freeze dried samples and approximately 100 mg potassium bromide (KBr). All spectra were recorded using Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany) from 4000 to 400 cm^{-1} at a data acquisition rate of 4 cm^{-1} per point. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker Co., Ettlingen, Germany).

Scanning electron microscopy (SEM) of pidan white

Pidan white treated with 0.2% PbO_2 or 0.2% $ZnCl_2$ was broken in liquid nitrogen into an approximate size of 0.5 × 0.5 cm and fixed at room temperature in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h. Fixed samples were rinsed with distilled water three times to remove salt. The samples were dehydrated in graded series of ethanol (50, 70, 80, 90 and 100 %) and then were mounted on SEM stubs using a double backed cellophane tape. The samples were coated with gold and examined using a scanning electron microscope (JEOL JSM-5800LV, Tokyo, Japan).

Confocal laser scanning microscopy (CLSM) of pidan yolk

Microstructures of pidan yolk treated with 0.2% PbO_2 or 0.2% $ZnCl_2$ and fresh egg yolk were examined with a confocal laser scanning microscopy (Olympus, FV300, Tokyo, Japan) following the modified method of Mineki and Kobayashi (1997). Yolk of fresh and all pidans was suspended in 0.1 % Nile blue A solution at a ratio of 1:10 (w/v) and manually stirred until the uniformity was obtained. Fifty μl of suspension was smeared on the microscopy slide. CLSM was operated in the fluorescence mode at the excitation wavelength of 533 nm and the

emission wavelength of 630 nm using a Helium Neon Red laser (HeNe-R) for lipid analysis and at the excitation wavelength of 488 nm and the emission wavelength of 540 nm using a Helium Neon Green laser (HeNe-G) for protein analysis.

Sensory analysis

The sensory evaluation was performed by 30 untrained panelists, who were the graduate students in Food Science and Technology programme with the age of 25–33 years and were familiar with pidan consumption. The assessment was conducted for the shell appearance, color, appearance, odor flavor, texture and overall of pidan white and yolk samples using a 9-point hedonic scale (Mailgaad *et al.*, 1999): 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much; 9, like extremely. The pidan samples were peeled and separated into white and yolk. The separated white and yolk were cut into small slices (2 x 2 x 2 cm^3) before analysis.

Statistical analysis

Completely randomized design was used throughout the study. The experiments were run in triplicate using three lots of eggs. Data were presented as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out and means comparisons were run by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analyses were performed with the statistical program (SPSS for windows (Version 10), SPSS Inc, Chicago, IL, USA).

9.4 Results and Discussion

Chemical compositions of white and yolk of fresh egg and pidan

pH, moisture, NaCl as well as ammonia contents of pidan white and yolk treated with different cations were determined in comparison with fresh counterparts (Table 10). Pidan white and yolk had the decreases in moisture content with coincidental increases in salt content and pH, compared with fresh egg ($P<0.05$),

regardless of type of ions used in pickling solution. Ammonia content of 0.25-0.31 and 0.18-0.21 % was found in pidan white and yolk, respectively. Nevertheless no ammonia content was found in both fresh white and yolk. The increase in pH and NaCl content indicated the migration of alkali and NaCl from pickling solution into egg white and yolk, respectively. Ganesan and Benjakul (2010 a,c) reported that pH and NaCl of pidan white and yolk increased during ageing, irrespective of cations used in pickling solution. The loss in moisture in white and yolk of pidan was mostly due to the migration of water from yolk to white and from white through the shell during increasing processing time (Ganesan and Benjakul, 2010a,c). Chi and Tseng (1998) reported that water could be migrated from egg white and egg yolk to the environment through the egg shell. This resulted in the reduction of moisture content. The lowest moisture content found in the white of pidan treated with 0.2% PbO_2 probably resulted from a greater interaction of white proteins induced by lead ion. Ammonia was formed in the pidan white and yolk, possibly by deamidation process (Hou, 1981). Deamidation of proteins occurs at pH above 8.0, dependent upon the H^+ or OH^- concentration and adjacent amino acid residues (Riha *et al.,* 1996). Higher ammonia content was found in pidan white and yolk treated with 0.2% $ZnCl_2$ treated pidan, compared with that of pidan treated with 0.2% PbO_2. Thus cations had the influence on the chemical composition of pidan white and yolk.

Table 10. Chemical composition of white and yolk of fresh egg and pidan

Parameters	Treatments	White	Yolk
pH	Fresh	8.56 ± 0.45 [a]	5.90 ± 0.25 [a]
	Pidan-Pb	12.68 ± 0.03 [b]	10.30 ± 0.04 [b]
	Pidan-Zn	12.63 ± 0.05 [b]	10.35 ±0.02 [b]
Moisture content (%)	Fresh	86.52 ± 0.51 [a]	44.51 ± 0.02 [a]
	Pidan-Pb	10.35 ± 0.23 [b]	41.22 ± 0.20 [b]
	Pidan-Zn	12.03 ± 0.42 [c]	37.05 ± 0.50 [c]
NaCl content (%)	Fresh	0.39 ± 0.04 [a]	0.47 ± 0.03 [a]
	Pidan-Pb	1.11 ± 0.03 [b]	0.85 ± 0.02 [b]
	Pidan-Zn	1.10 ± 0.04 [b]	0.80 ± 0.04 [b]
Ammonia content (%)	Fresh	ND	ND
	Pidan-Pb	0.25 ± 0.02	0.18 ± 0.02
	Pidan-Zn	0.31 ± 0.01	0.21 ± 0.01

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

† Different letters in the same column within the same parameter indicate the significant differences ($P<0.05$). ND = Not detectable. Pidan-Pb: PbO_2 treated pidan, Pidan-Zn: $ZnCl_2$ treated pidan

Mineral, nitrogen and sulfur contents of white and yolk of fresh egg and pidan

Egg white and yolk of fresh egg and pidan consisted of different minerals, nitrogen and sulfur at various levels as shown in Table 11. Higher contents of Na, K and Fe were found in pidan white, whereas Ca, Mg, Na and K were higher in pidan yolk, in comparison with those found in fresh counterparts. Pb was found in

only white and yolk of pidan treated with PbO_2. A higher content of Zn was obtained in either white or yolk of pidan treated with $ZnCl_2$. The increase in mineral content of pidan white and yolk indicated the migration of minerals from pickling solution into egg white and yolk, respectively. It was coincidental with the increase in pH and of pidan white and yolk. Shimada and matsushita (1981) observed that critical pH of egg white shifted to a more alkaline pH as salt concentrations increased. The high content of Na (2.1 to 2.2%) of pidan white and yolk indicated the greater migration of sodium ion from pickling solution through the egg shell. Addition of cations such as Pb and Zn ions in the pickling solution also provided those cations in pidan white and yolk. In general, the rate of migration varied with the type of cations used in pickling solution. Higher amount of minerals present in the pidan white than yolk confirmed that minerals were more retained in egg white during pickling of pidan. Since egg white was located outside, where ions were more absorbed. The decrease in some minerals indicated the dilution effect of major minerals from pickling solution migrated through the shell to white or yolk.

No differences in nitrogen content were found between egg white of fresh egg and pidan, regardless of cations used (Table 11). However, a higher nitrogen content was noticeable in pidan yolk, compared with that of fresh counterpart ($P<0.05$). This was mainly due to the increased mineral content in pidan yolk, thereby diluting the nitrogen content of dry matter of pidan yolk. For sulfur content of egg white, it was noted that pidan had the lower content of sulfur content than the fresh egg ($P<0.05$). Sulfur content was found to be retained in 0.2% PbO_2 treated pidan white than 0.2% $ZnCl_2$ treated pidan (Table 11). Pb possibly involved in the interaction with sulfur containing residues. Dunham (1972) reported that lead has a strong affinity for some ligands, including the ε- amino group of lysine, the carboxyl group of glutamic and aspartic acids and the sulfhydryl group of cysteine. Nevertheless, no differences in sulfur contents in yolk between fresh egg and both pidans were observed ($P >0.05$). Thus, the decomposition more likely took place in egg white rather than egg yolk of pidan, particularly during pickling and ageing.

Table 11. Mineral, nitrogen and sulfur contents of white and yolk of fresh egg and pidan

	White (mg/100g dry weight)			Yolk (mg/100g dry weight)		
Parameters	Fresh	Pidan-Pb	Pidan-Zn	Fresh	Pidan-Pb	Pidan-Zn
Ca	23.12	19.40	29.20	158.22	186.88	307.84
Mg	79.89	19.94	28.89	18.23	27.18	25.22
Na	754.00	2192.90	2250.90	72.30	711.20	1516.81
K	604.40	878.15	748.60	131.42	212.87	246.67
Zn	ND	0.56	2.63	3.95	3.03	3.75
Pb	ND	<0.05[b]	ND	ND	<0.05[b]	ND
Cu	0.79	0.52	0.35	0.14	0.16	0.34
Fe	1.11	2.33	2.19	8.51	7.38	6.63
N	12.86	13.00	12.68	4.91	5.36	5.32
S	1.70	1.40	1.17	0.28	0.29	0.29

a ND: Not detectable

b Expressed as mg/Kg

Pidan-Pb: PbO_2 treated pidan, Pidan-Zn: $ZnCl_2$ treated pidan

FTIR spectra of white and yolk of fresh egg and pidan

FTIR spectra of egg white from fresh egg and pidan are depicted in Figure 35A. The absorption bands in the spectra of all white samples were situated in the amide band region such as amide I (1600–1700 $cm^{-1)}$, amide II (1500–1600 cm^{-1}),

amide III (1200-1300 cm^{-1}), amide A (3293–3306 cm^{-1}), amide B (2920–2922 cm^{-1}) and S-S (400-500 cm^{-1}). For the spectrum of fresh egg white, the characteristic absorption bands at wavenumbers of 3303.35, 3082.83, 1651.04, 1542.10, 1249.99 and 457.36 cm^{-1} were observed, whereas pidan treated with 0.2% PbO_2 and 0.2% $ZnCl_2$ showed the main absorption bands at wavenumbers of 3304.97, 3084.26, 1651.45, 1542.15, 1238.57 and 464.84 cm^{-1} and 1652.10, 1547.80, and 1242.19 cm^{-1}. The amide I bands are originated from CO stretching vibrations coupled to N–H bending vibrations, CN stretch and CCN deformation. For amide I band, fresh egg white had the lowest wave number, indicating the interaction of CO with the adjacent chains; while 0.2% $ZnCl_2$ treated pidan white had the highest wave numbers. The amide II bands of all samples were obtained at different wavenumbers, representing N–H bending vibrations coupled to C–N stretching vibrations. Generally, the lower wavenumber showed the existence of hydrogen bonds, which were found in fresh egg white and 0.2% PbO_2 treated pidan white. The other bands, arising from the stretching vibrations of N–H group, appeared at 3303.35, and 3304.97 cm^{-1}, corresponding to amide A, which occurs commonly in the range of 3280–3300 cm^{-1}. The amide B with wavenumbers of 3082.83 cm^{-1} and 3084.26 cm^{-1} was observed for fresh and 0.2% PbO_2 treated pidan white. Higher wavenumber of 3084.26 cm^{-1} was noticeable for 0.2% PbO_2 treated pidan white. Amide B corresponds to asymmetric stretch vibration of C–H as well as $–NH_3$. The S-S with wavenumber of 400 - 500 cm^{-1} was observed for fresh and 0.2% PbO_2 treated pidan white. This coincided with the higher sulfur content in 0.2% PbO_2 treated pidan white. Thus, alkali penetration and cation binding of proteins in egg resulted in significant changes in the structure of protein.

FTIR spectra of fresh egg yolk, 0.2% PbO_2 and 0.2% $ZnCl_2$ treated pidan yolk extracted are illustrated in Figure 35B. FTIR spectra of different samples consisted of several peaks with different wavenumbers. Decrease in the absorbance band at 3600–3200 cm^{-1} was observed in 0.2% $ZnCl_2$ treated pidan yolk, indicating the loss of hydroperoxide, compared with that of fresh yolk. The result suggested that the decomposition of hydroperoxide took place, yielding the secondary lipid oxidation products during pidan production. Van de Voort *et al.* (1994) reported that the absorbance at 3800–3100 cm^{-1} in the ATR/FTIR spectra, was referred to as the OH

stretching region. Hydroperoxide moieties exhibit characteristic absorption bands between 3600 and 3400 cm^{-1} due to their –OO–H stretching vibrations (Van de Voort *et al.,* 1994). Pidan treated with 0.2% PbO_2 had no marked changes in the region, suggesting that lipid oxidation occurred at a low extent. Two stretched bands at wavenumbers of 2854.04 cm^{-1} and 2924.90 cm^{-1} were due to the methylene asymmetrical and symmetrical stretching vibration, respectively (Guillen and Cabo, 1997). Both the methylene asymmetrical stretching bands at approximately 2924.90 cm^{-1} and the methylene symmetrical stretching band near 2854.04 cm^{-1} were obviously present in most of the lipid samples (Guillen *et al.,* 2004). The bands associated with the fingerprint region observed between 1500 and 1000 cm^{-1} were not different between the fresh and pidan yolk. Carbonyl absorption of the triglyceride ester linkage was observed at 1746 cm^{-1} (Setiowaty *et al.,* 2000). Yolk lipid mainly contained triglycerides with very low amounts of free fatty acids. This might lead to less susceptibility toward oxidationas as evidenced by the low content of oxidation products.

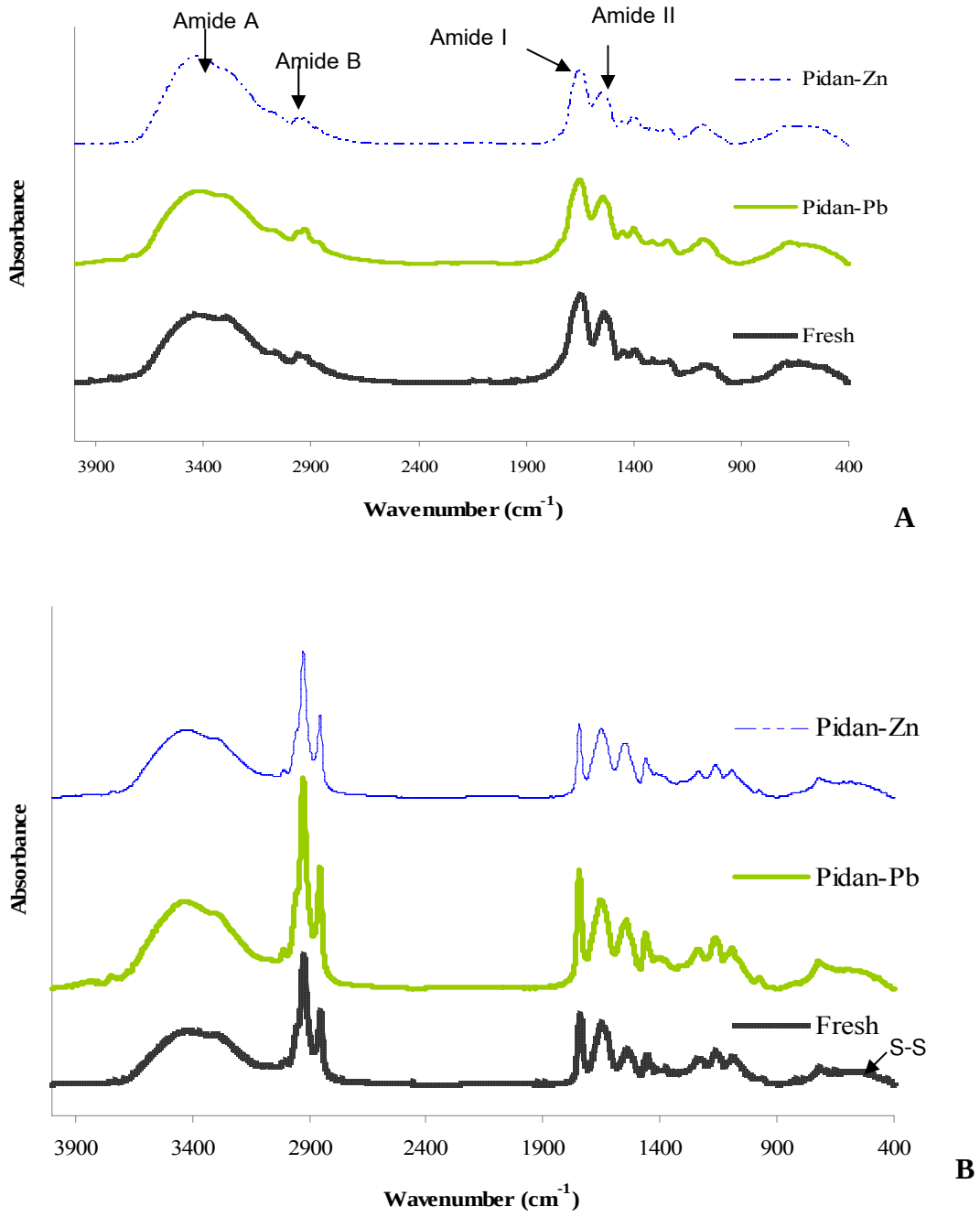


Figure 35. Fourier-transform infrared spectra of white and yolk of fresh egg and pidan Fresh: Fresh egg yolk, Pidan-Pb: PbO_2 treated pidan, Pidan-Zn: $ZnCl_2$ treated pidan, pidan white (A) and pidan yolk (B)

Microstructure of white and yolk of pidan

Microstructures of pidan white treated with 0.2% PbO_2 and 0.2% $ZnCl_2$ visualised by SEM are shown in Figure 36. Heterogeneous aggregates with the cracks were observed in pidan treated with 0.2% $ZnCl_2$. Pidan gels had more compact structure without the gap or void in the network when treated with 0.2% PbO_2. Rough surface of pidan was observed in pidan treated with 0.2% $ZnCl_2$ (Figure 36B), whereas highly cross linked network with fine strands was found in pidan white treated with 0.2% PbO_2 (Figure 36A). Woodward and Cotterill (1987) reported that egg white gel examined with SEM was very coarse with large irregularly shaped voids. Nevertheless, ovalbumin gels showed the homogeneous microstructure (Heertje and Van Kleef, 1986). The ordered structure of 0.2% PbO_2 treated pidan white indicated that Pb cation somehow involved in the formation of gel network with high stability (Figure 36A). Alkaline conditions are known to unfold protein molecules (Creighton, 1993). Those unfolded proteins could be cross-linked to form protein networks, particularly in the presence of cation via salt bridge mechanism. Thus, the appropriate cations most likely played an essential role in ion-induced gelation of pidan white.

The confocal laser scanning microscope (CLSM) micrographs of pidan yolk using a two channel technique, in which both protein and lipid were stained, are illustrated in Figure 37. A combined image is also presented. The protein and lipid distributed uniformly in fresh egg yolk. Proteins in yolk were organized into micellar and granular structures together with polar and non-polar lipid molecules (Kiosseoglou, 2003). When the penetration of alkali proceeded to interior yolk, 3 layers of pidan yolk were formed. Irregular shapes of both lipid and protein were found in both pidan yolk, irrespective of treatments. Nevertheless, the amount of lipid released and alteration of lipid shape varied with the types of cations used. The greater release of free lipid from lipoprotein of pidan yolk was obtained when 0.2% PbO_2 was used as indicated by the denser lipid granules appeared in the combined image (Figure 37-Pb (C)). In the presence of alkali, saponified lipid was postulated to bind protein, leading to the formation of shielding surface. As a result, the dehydration was lowered and soft yolk pidan was obtained. CLSM micrograph of

pidan treated with 0.2% $ZnCl_2$ suggested that binding of lipid to protein yielded the hard aggregated yolk, whereas 0.2% PbO_2 treatment rendered the soft yolk containing more free lipids with less association with proteins.

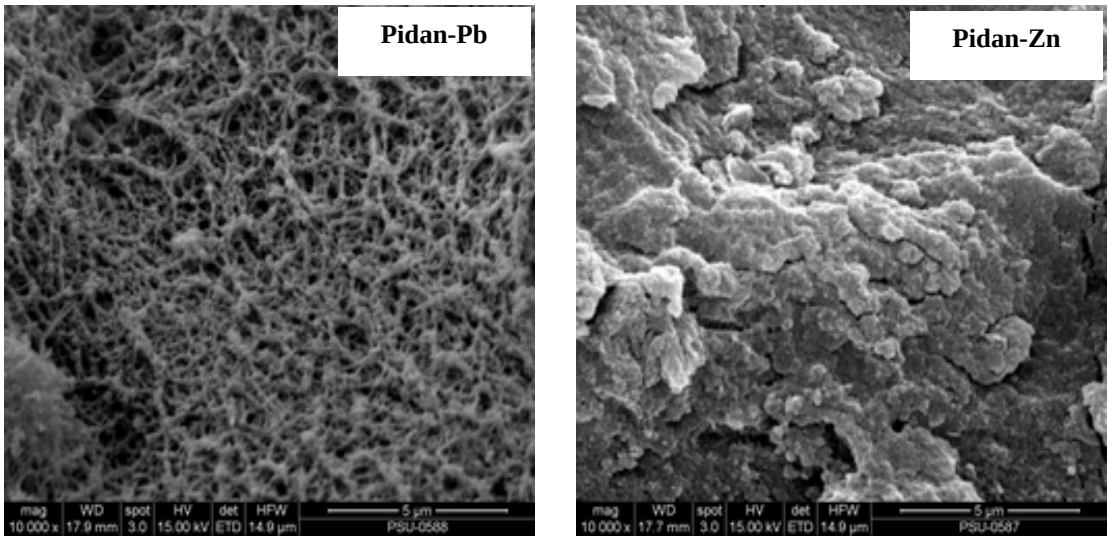


Figure 36. Scanning electron microscopic photograph of pidan white. Pidan-Pb: PbO_2 treated pidan yolk, Pidan-Zn: $ZnCl_2$ treated pidan yolk. Magnification: 10000X

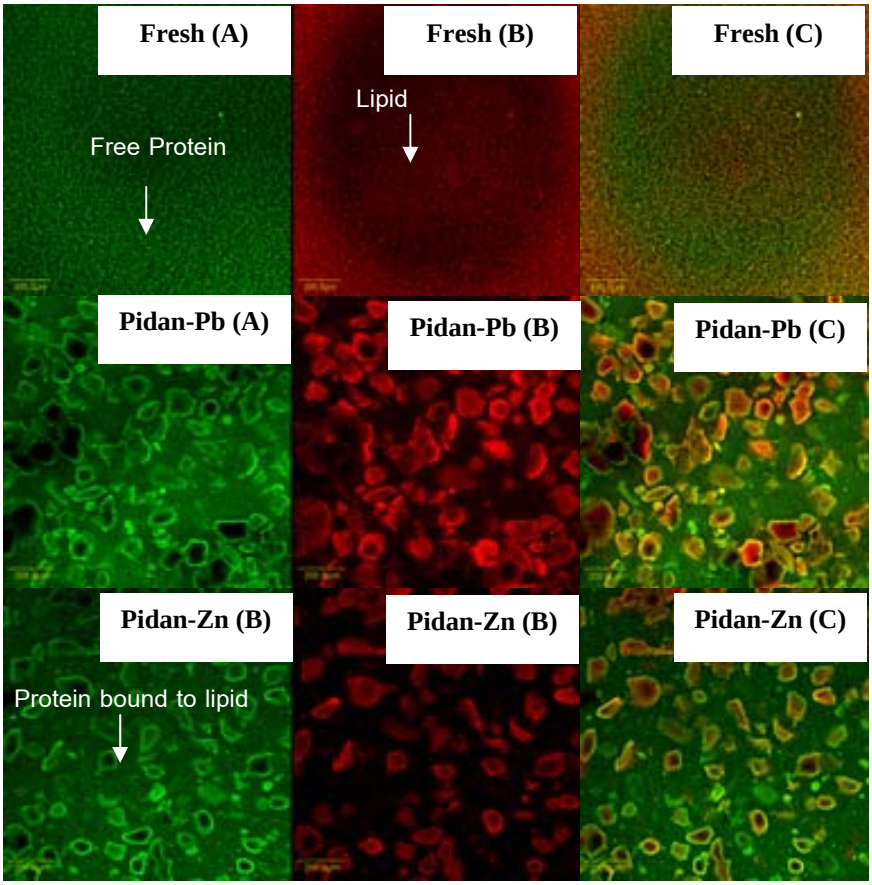


Figure 37. Confocal laser scanning microscope (CLSM) micrographs of pidan yolk. Fresh: Fresh egg yolk, Pidan-Pb: PbO_2 treated pidan yolk, Pidan-Zn: $ZnCl_2$ treated pidan yolk Magnification: 200X (zoom X2.5) protein distribution (A) and lipid distribution (B) and combined image of protein and lipid (C)

Sensory properties of white and yolk of pidan

Likeness scores of pidan white and yolk treated with 0.2% PbO_2 and 0.2% $ZnCl_2$ were compared with the commercial pidan as shown in Figure 38A and 38B, respectively. Different treatments resulted in the different sensory score of both pidan white and yolk. Shell appearance of pidan treated with 0.2% PbO_2 showed the lower score ($P<0.05$). This was mostly due to the presence of black spots on the shell. Wang and Fung (1996) reported that addition of zinc caused no black spots in egg shell and membrane. Color, appearance and flavor of treated pidan white was significantly higher than those pidan obtained from market ($P<0.05$). However texture of pidan white treated with 0.2% $ZnCl_2$ showed significantly lower than other pidan. This was in agreement with SEM micrograph which showed rough surface of pidan white. In pidan yolk treated with 0.2% $ZnCl_2$, color, appearance, flavor, texture and overall likeness were generally higher compared to commercially available pidan and 0.2% PbO_2 treated pidan yolk ($P<0.05$). In overall, likeness score of cations treated pidan yolk were higher that of commercial pidan except for odor, in which the higher score was obtained in commercial pidan ($P<0.05$).

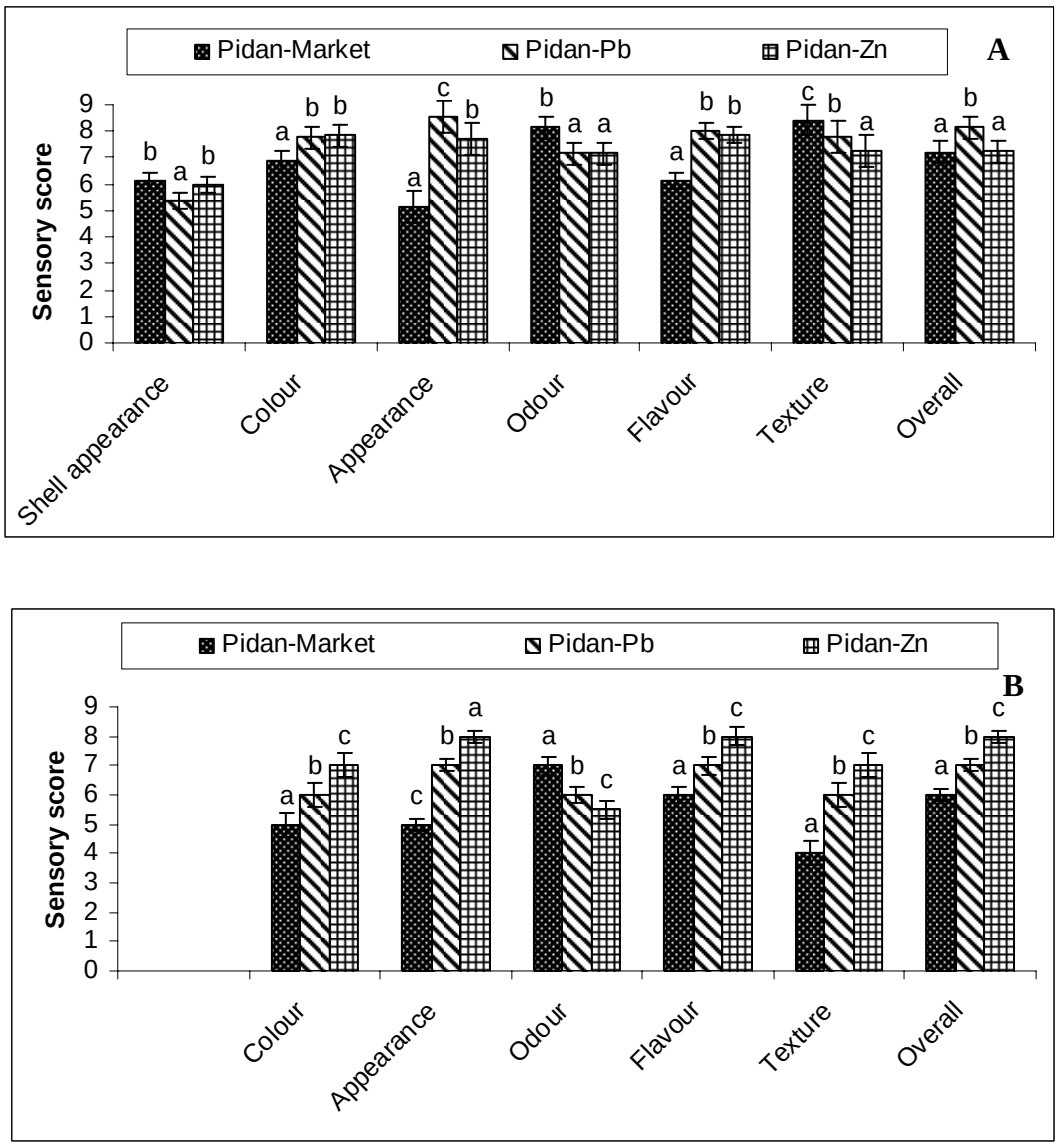


Figure 38. Likeness score of pidan white and yolk Pidan-Market: Commercial pidan. Pidan-Pb: PbO_2 treated pidan, Pidan-Zn: $ZnCl_2$ treated pidan, pidan white (A) and pidan yolk (B)

9.5 Conclusions

Chemical compositions and characteristics of pidan were governed by cations used. PbO_2 treated pidan white contained higher cross linking of protein than that produced with 0.2% $ZnCl_2$ treatment. Thus cations in the pickling solution affected compositions, characteristics and microstructure of both pidan white and yolk.

CHAPTER 10

SUMMARY AND FUTURE WORKS

10.1 Summary

1. Type and concentration of cations had varying influences on the characteristics of pidan white and yolk. PbO_2, $ZnCl_2$ or $CaCl_2$ at a low concentration (0.2%) had the significant influence on texture and color of the pidan white and yolk and yielded the hard gel during pickling. However, only PbO_2 showed the stabilizing effect on gel of piadn white formed during ageing. Pickling generally resulted in significant changes in TPA, whereas ageing helped in improvement of color. Therefore, the use of divalent cations such as $ZnCl_2$ or $CaCl_2$ could be an alternative divalents for pidan production.

2. Chinese tea had no pronounced effect on physical properties of pidan white, whereas divalent ions showed the varying impact on textural property of pidan white in the absence of Chinese tea. Therefore, brown color of pidan white was more likely mediated by Maillard reaction, not Chinese tea**.**

3. The reduction in shell thickness could be achieved by acetic acid pretreatment, thereby enhancing the penetration of tea and divalent into egg white. Different tea has different impact on the gel and color of pidan white. Thus an acetic acid pretreatment aid along with tea incorporated at sufficient level could maneuver the characteristics of pidan white.

4. Glucose soaking of pidan had slight effect on color of pidan white, whereas it showed the varying impact on textural property of pidan white during storage. Maillard reaction could be enhanced during storage when pre-soaking of pidan in glucose solution at appropriate levels was implemented.

5. Stability and microstructure of duck egg protein aggregates varied with the type and concentrations of cations used. $CaCl_2$ and PbO_2 at a level of 0.1% induced the formation of protein aggregate effectively under alkaline condition.

Nevertheless, PbO_2 yielded the aggregate with the high stability, in which gel network was stabilized for a longer time. $ZnCl_2$ showed the slightly lower stabilising effect on egg white protein under the alkaline condition. For the safety concern, $ZnCl_2$ can be recommended as an alternative compound for pidan production instead of PbO_2, which is toxic for consumption.

6. Chemical compositions and characteristics of pidan were governed by cations used. PbO_2 treated pidan white contained higher cross linking of protein than that produced with 0.2% $ZnCl_2$ treatment. Thus cations in the pickling solution affected compositions, characteristics and microstructure of both pidan white and yolk.

10.2 Future works

1. Use of chicken egg and quail egg for the pidan production with different cation should be studied.

2. Bioactivities of pidan white protein should be further evaluated.

3. Model study on the effect of different cations on gelation and color of pidan yolk should be further elucidated.

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List of Publication and Proceedings

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1. Ganesan, P. and Benjakul, S. 2010. Chemical composition, physical properties and microstructure of pidan Yolk as affected by different divalent and monovalent cations. LWT-Food Sci. Technol. 43: 77-85.
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1. Ganesan, P. and Benjakul, S. 2009. Influence of different divalent dations on chemical composition and microstructure of pidan white and yolk during pickling and ageing. International conference on innovations in agricultural, food and renewable energy productions for mankind. Nakhon Ratchasima, Thailand.
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