Article

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Effect of three cations on the stability and microstructure of protein aggregate from duck egg white under alkaline condition

P. Ganasen and S. Benjakul

Abstract

Pidan (alkaline egg) 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 comparable properties to traditionally prepared pidan. 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 egg white protein (20 g/kg) in 10 g/kg NaCl sample with $CaCl_2$, PbO_2 or $ZnCl_2$ added at a level of 1 g/kg increased with time up to 1 h, followed by a decrease ($p < 0.05$). Nevertheless, the turbidity was retained more in samples added with PbO_2, suggesting high stability of the aggregate formed. Zeta potential showed that the aggregates treated with PbO_2 had a comparatively lower negative charge. 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 an alternative compound even if it had lower impact on stability of aggregate of duck egg white protein.

Keywords

Protein aggregates, cation, zeta potential, microstructure, egg white

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INTRODUCTION

Egg white is extensively utilized as a functional food material in food processing. Egg white gels consist of polymers connected to each other in order to form a three-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, egg white formed soluble oligomers 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 effect

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Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.

Corresponding author:
S. Benjakul, Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
Email: soottawat.b@psu.ac.th

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 (Ganasen and Benjakul, 2011). Lead has traditionally been used 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 aging in comparison with those treated with lead (Ganasen and Benjakul, 2011). 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.

MATERIALS AND METHODS

Materials

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 bromophenol blue was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Sample preparation. Fresh eggs of duck (*Anas platyrhucus*) with weight range 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 homogenized at a speed of 11 000 rpm for 2 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia).

Preparation of egg white protein aggregates. Egg white protein (20 g/kg) in 10 g/kg NaCl sample was prepared and adjusted to pH 12. The samples were prepared with and without (control) addition of different ions including PbO_2, $ZnCl_2$ or $CaCl_2$ at different levels (0.2, 0.5 and 1 g/kg). To completely solubilize PbO_2, the mixture was boiled using a hot plate for 15 min. After sudden cooling, the volume was adjusted to obtain the designated final concentration using NaCl solution (10 g/kg NaCl), pH 12. Sodium azide (1 g/kg) was added to the sample to prevent microbial growth. Samples were stirred continuously at room temperature (26–28 °C) for up to 90 min with sampling every 15 min for aggregate analysis. For particle size analysis, samples with cations at a level of 1 g/kg were taken every 30 min. To study the stability of aggregate formed, the turbidity of the sample containing cations (1 g/kg) 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 using a UV-visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

Determination of particle size. Particle size was measured using a Beckman–Coulter particle size analyzer (Model LS230) with a fluid module, 750 nm laser and the companion software version 3.29 (Beckman Coulter, Inc., Miami, FL, USA.). Each sample was circulated through and analyzed by the LS230 for 90 s and the sample vessel rinsed to background levels. The analyzer was cleaned on a regular basis by disassembling the optical module and thoroughly cleaning all surfaces and also by circulating cleaning sample through the machine. In addition, Beckman–Coulter particle size standards 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 potentials of proteins in different samples added with cations and the control (without the addition of cations) were 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 1 g/kg and the control sample at times 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, 0.5 g/kg mercury bromophenol blue for 5 min, 5 g/kg 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 visualized using a light microscope with a magnification of 10×.

Protein determination. Proteins in sample and control were determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard.

Statistical analysis. Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). For pair comparison, *t*-test was used. Analysis of data was performed using a SPSS package (SPSS 11.0 for Windows, SPSS Inc, Chicago, IL, USA).

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 with and without (control) addition of cations ($CaCl_2$, PbO_2 and $ZnCl_2$) at levels 0.2, 0.5 and 1 g/kg was monitored up to 90 min (Figure 1). At cation concentration of 1 g/kg, 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 with 1 g/kg of $CaCl_2$ ($p < 0.05$), whereas the lower turbidity was found in samples with 1 g/kg of PbO_2 or $ZnCl_2$. Nevertheless, no differences in turbidity were found 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, higher concentrations of cations could more effectively induce formation of aggregate of egg white protein. In the presence of cations at the sufficient amount, cross-linking via salt bridges could take place. As a result, the formation of aggregate was noticeable. Among all the 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 a 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.

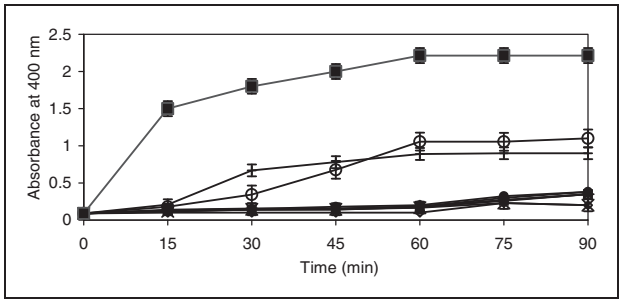

Figure 1. Turbidity of duck egg white protein samples (pH 12) as influenced by three cations at different concentrations during incubation. Bars represent the standard deviation ($n = 3$). (△) Control (without addition of cations); (•) PbO_2 (0.1 g/kg); (–) PbO_2 (0.5 g/kg); (x) PbO_2 (1 g/kg); (*) $ZnCl_2$ (0.1 g/kg); (◆) $ZnCl_2$ (0.5 g/kg); (▲) $ZnCl_2$ (1 g/kg); (–) $CaCl_2$ (0.1 g/kg); (+) $CaCl_2$ (0.5 g/kg); (■) $CaCl_2$ (1 g/kg).

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

Particle size of duck egg white protein in alkaline sample with and without (control) addition of cations ($CaCl_2$, PbO_2 and $ZnCl_2$) at a level of 1 g/kg, which was the concentration giving the highest turbidity or aggregation, was monitored at various times up to 90 min (Figure 2). A negligible change in particle size was noticeable within the first 30 min ($p > 0.05$). Thereafter, particle size of egg white protein sample

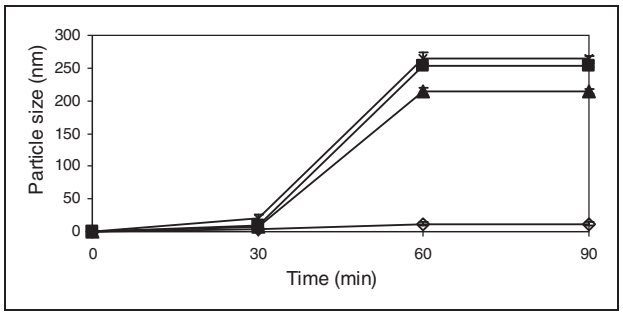

Figure 2. Particle size of duck egg white protein samples (pH 12) as influenced by three cations at a level of 1 g/kg during incubation. Bars represent the standard deviation ($n = 3$). (△) Control (without addition of cations); (x) PbO_2 (1 g/kg); (▲) $ZnCl_2$ (1 g/kg); (■) $CaCl_2$ (1 g/kg).

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 1). However, calcium ion yielded a higher particle size than that of the other cations, especially for incubation times 60 and 90 min. 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 with different cations added at the same level (1 g/kg) suggested different interactions between metal ions and protein molecules in terms of bonding involved, including hydrophobic interaction, ionic interaction, hydrogen bond and metal bridging.

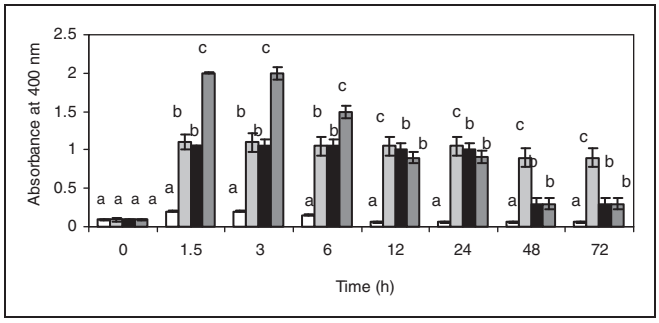

Figure 3. Turbidity of duck egg white protein samples (pH 12) as influenced by three cations at a level of 1 g/kg during incubation. Bars represent the standard deviation ($n = 3$). Different letters on the bars within the same incubation time indicate significant differences ($p < 0.05$). (■) Control (without addition of cations); (□) PbO_2 (1 g/kg); (■) $ZnCl_2$ (1 g/kg); (■) $CaCl_2$ (1 g/kg).

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

Turbidity of duck egg white protein with and without (control) addition of cations ($CaCl_2$, PbO_2 and $ZnCl_2$) at a level of 1 g/kg was monitored up to 72 h (Figure 3). For all samples, except with added 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 the sample added with PbO_2, a 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 formations 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 added at a level of 1 g/kg and the lowest turbidity 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, causing a decrease in peptide size (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 the stability of aggregate from duck egg white protein at alkaline pH.

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

	Zeta potential* (mV)	
Treatments	1 h	72 h
Control	−16.67 ± 1.21 c, A**	−21.57 ± 1.21 c, B
PbO_2 (1 g/kg)	−9.96 ± 0.57 a, A	−10.54 ± 0.21 a, A
$ZnCl_2$ (1 g/kg)	−12.97 ± 0.53 b, A	−15.37 ± 0.75 b, B
$CaCl_2$ (1 g/kg)	−8.57 ± 1.51 a, A	−15.65 ± 0.40 b, B

*Mean ± SD ($n = 3$). **Different lower case letters in the same column indicate significant differences ($p < 0.05$). Different upper case letters in the same row indicate significant differences ($p < 0.05$).

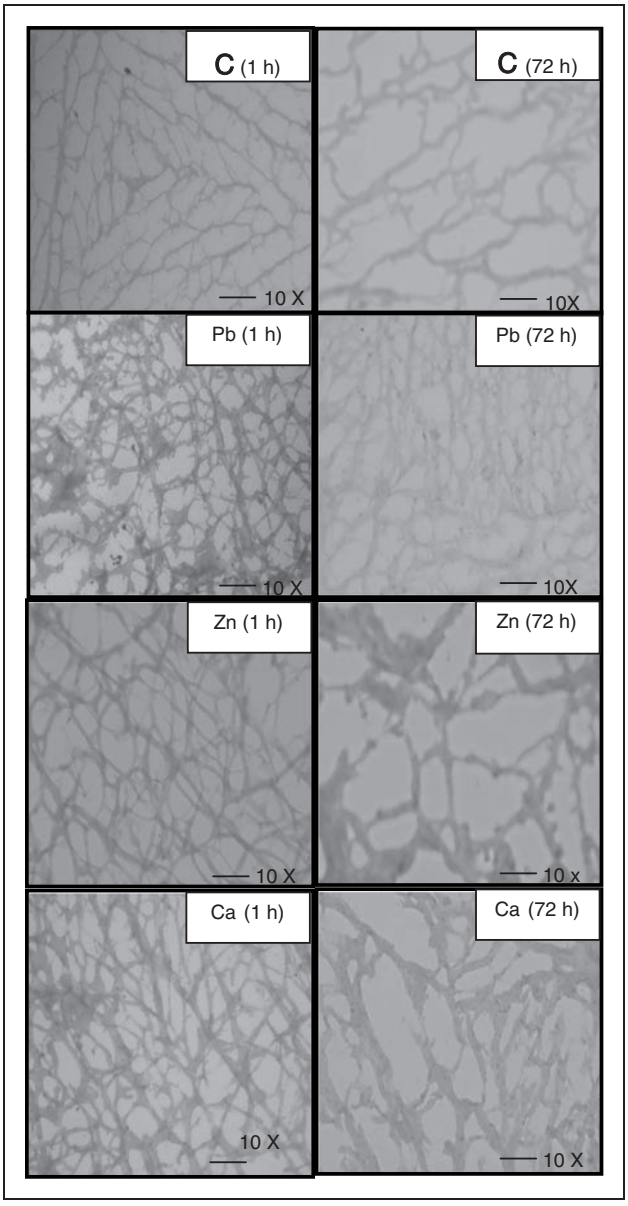

Figure 4. Light microscopic (LM) micrographs of duck egg white protein aggregates induced by three cations at a level of 1 g/kg after 1 and 72 h of incubation. C, Control (without addition of cations); Pb, PbO_2 (1 g/kg); Zn, $ZnCl_2$ (1 g/kg); Ca, $CaCl_2$ (1 g/kg); Magnification: 10×.

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

Zeta potentials of duck egg protein sample with and without (control) addition of cations ($CaCl_2$, PbO_2 and $ZnCl_2$) at a level of 1 g/kg were monitored at 1 and 72 h of incubation as given in Table 1. 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 was due to the acidic amino acids in the egg white protein, 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 aging, degradation of ovalbumin and other egg white proteins resulted in the increases in small peptides and free amino acid at room temperature (25–27°C) (Ganasen and Benjakul, 2010). Also, alkaline pH could promote slow hydrolysis of peptide bonds, resulting in the formation of peptides and amino acids. This was 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 neutralization 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 1 g/kg had no increase in the negative charge of protein (Table 1). 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 3).

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

Microstructures of duck egg protein aggregates with and without (control) addition of cations including ($CaCl_2$, PbO_2 and $ZnCl_2$) at a level of 1 g/kg visualized by light microscope are shown in Figure 4. 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 4a and b). 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). Aggregate induced by $CaCl_2$ and PbO_2 at a level of 1 g/kg showed a 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 1 g/kg 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 4b). 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.

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 1 g/kg 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 a slightly lower stabilizing effect on egg white protein under the alkaline condition. For safety reasons, $ZnCl_2$ can be recommended as an alternative compound for pidan production instead of PbO_2, which is toxic.

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