

## Olive Leaf Water Extract Protects Chicken Breast Sausages Against Quality Deterioration Induced by Frozen Storage

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This study aimed to determine the effect of olive leaf water extract (OEx) on the physical properties of chicken breast sausage (CBS) and the preventive effect of OEx against lipid oxidation in CBS during frozen storage. CBSs, to which 0.1 and 0.5% (w/w) OEx were added to minced meat, were stored frozen at  $-20^{\circ}\text{C}$  for 60 days. The thawing weight loss of control CBS without OEx increased with the frozen storage period, while OEx-CBSs did not change, from 15 to 60 days in storage. The water-holding capacity, breaking strength, elasticity, and viscosity of control CBS decreased upon frozen storage, while those of OEx-CBSs did not change. The observation of CBSs using scanning electron microscopy showed that OEx-CBSs that were stored frozen, unlike control CBS, maintained a structure similar to their unfrozen counterparts. These results indicate that OEx confers resistance to CBS upon freezing. Furthermore, the application of OEx to CBS suppressed lipid oxidation, decrease in pH and discoloration induced by frozen storage. Thus, this natural OEx is useful in improving the physical and chemical qualities of frozen processed poultry foods.

**Key words:** Cross-linking reaction, Frozen food, Lipid oxidation, Polyphenol, Syneresis

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### Introduction

Chicken breast sausage (CBS) is a gel product prepared from ground chicken breast and minor ingredients including seasoning. It is widely consumed in many countries, especially in developing countries. CBS has been recognized as a highly beneficial food because it contains a comparatively higher level of protein and a lower amount of fat (Marangoni *et al.*, 2015). Chicken meat and processed chicken meat can be alternatives to meat products made from red meat, and they can reduce the risk of cardiovascular diseases and breast cancer (Hu, 2005; Thompson, 2019). Accordingly, there is an increasing demand for CBS in developed countries. The global CBS market grew by 5.14% in the two years from

2016 until 2018 (Rajput and Manepalli, 2019).

Today's busy lifestyle calls for an increased demand for ready-to-eat food. Long-term storage of ready-to-eat gel products requires the water in the products to be frozen to avoid bacterial growth. However, thawing the gel products that were stored frozen induces syneresis, which results in texture deterioration, with the degree of deterioration depending on the use of food additives and the frozen storage conditions, such as cooling rate and storage temperature. Frozen storage of sausage is no exception. Thawing results in a soft or mushy texture that has low acceptance among consumers (Rodríguez *et al.*, 2015).

Some ingredients prevent the deterioration of texture of meat products induced by frozen storage. Starch can diminish the loss of binding properties of bologna sausage caused by freezing–thawing (Colmenero *et al.*, 1996). The use of phosphate and sodium chloride in frozen pork sausages results in higher tensile strength (Matlock *et al.*, 1984). Adding carrageenan enhances hardness and reduces cooking loss of frozen pork sausages (DeFreitas *et al.*, 1997). Transglutaminase that catalyzes the formation of the  $\epsilon$ -,  $\gamma$ -glutamyl lysine bond is useful in improving the frozen stability of a muscle proteinaceous gel product. Inserting cross-links in a protein gel network using microbial trans-

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glutaminase (MTGase) can maintain the breaking strength and binding ability of restructured fish gel in a frozen storage condition (Moreno *et al.*, 2010).

Recently, we reported that olive leaf water extract (OEx) enhances the water-holding capacity (WHC) of CBS (Rachman *et al.*, 2020), which we attribute to a more compact protein network via cross-links between the meat proteins formed by OEx. The improved WHC of CBS opens the possibility of suppressing cryosynthesis of CBS by OEx. Several studies have reported the beneficial effects of olive leaf extract, such as antioxidant activity and anticancer activity (El and Karakaya, 2009; Boss *et al.*, 2016). Thus, OEx could be expected to be an ingredient that is beneficial to human health. In this study, we investigated the effect of OEx on the physical properties of CBS that is stored frozen, and we also examined the preventive effect of OEx against lipid oxidation of CBS.

## Materials and Methods

### Materials

Fresh olive leaves were collected from olive trees (*Olea europaea* L.) grown at the Faculty of Agriculture, Kagawa University, Japan. Breast fillet used in this study were obtained from 43-day old ROSS 308 broiler chickens which were given feed mixtures dedicated for broiler chickens and were stored at 4°C after slaughter. Sodium polyphosphate was obtained from Kirin Kyowa Foods Co. Ltd. (Tokyo, Japan). Thiobarbituric acid (TBA) malondialdehyde standard was purchased from Cayman Chemical (Michigan, USA). Folin-Ciocalteu reagent was purchased from Nacalai Tesque Inc (Kyoto, Japan). All other chemicals used were of analytical grade.

### Preparation of Olive Leaf Water Extract Powder

The olive leaves were dried in an oven using a cold-air drying machine (Cool Dry Machinery Co. Ltd., Kagawa, Japan) at 40°C for 48 h and milled using a grinder to a grain diameter of less than 0.11 mm. The dried and ground olive leaf powder (50 g) was macerated with 500 mL of pure water and agitated using a magnetic stirrer for 1 h at 4°C. The extract was centrifuged at 8000×g for 20 min at 4°C. The supernatant was lyophilized. The yield of olive leaf water extract was 9.72±0.23%. The lyophilized powder was stored at -20°C until further use. The powder of the olive leaf water extract was named OEx.

### Protein Content of Chicken Breast Fillets

The protein content of chicken breast fillets was solubilized according to the method of Owusu-Apenten (2002). The partially dispersed chicken breast fillet samples (100 g) in 500 mL of 5% Sodium Dodecyl Sulfate (SDS) were solubilized by mixing in a Waring Blender (TK-551; Tescom Co., Ltd., Tokyo, Japan) at medium speed at 4°C for 5 min. All mixtures were centrifuged at 6100×g for 10 min at 4°C. The protein content of the supernatant was analyzed according to the biuret method (Torten and Whitaker, 1963).

### Lipid Content of Chicken Breast Fillets

The lipid content of chicken breast fillets was determined using a method described by Bligh and Dyer (1959). One

hundred gram of the sample was homogenized in 300 ml of chloroform: methanol (1:2, v/v) and the mixture was homogenized for another 2 min, followed by vacuum-filtration through Whatman No. 1 filter paper. The extraction procedure for the residue was repeated and the supernatants were combined. The chloroform layer containing the extracted lipid was transferred to a pre-dried and pre-weighed aluminium dish. The sample was heated at 60°C for 45 min, followed by further heating at 105±1°C for 15 min. The sample was cooled in a desiccator and weighed.

### Sausage Preparation and Frozen Storage

Six hundred grams of chicken breast (24 pieces of fillets) were cut into 1×1×1 cm cubes and ground at stir speed 2 (51 rpm) in a meat grinder (Kitchen Aid, Model KSM150; Whirlpool Corporation, Michigan, USA) at 4°C for 5 min. The grinding process was repeated twice. Then, 3.6 g sodium chloride, 1.0 g sodium polyphosphate, 5.0 g refined sugar, 40 mL cold water, and 0.0, 0.25 or 1.25 g OEx (for control, 0.1 or 0.5% OEx sausage, respectively) were added to 200 g of the ground meat and kneaded by hand for 3 min. Generally, nitrite is used for the development of cured color and flavor, suppression of microbial growth, and inhibition of oxidation in meat products. The kneaded meat product was homogenized for 30 s using a food processor. The resultant meat batter was stuffed into a Krehalon® PVDC casing (flat width 17.5 mm; Kureha Corp., Tokyo, Japan) using a handmade sausage stuffer at 4°C. Both ends of the cased meat batter were tied off with a kite string. The cased meat batter was heated in a water bath at 70±1°C for 30 min. Immediately after heating, the sausage gel formed was cooled in crushed ice for 30 min. After removing the casing, the gel was cut into small pieces (10 mm cylindrical) using a cutter knife before freezing. The resultant cylindrical gels (diameter 17.5 mm, height 10 mm) were weighed using an electronic balance. Some of the cut cylindrical gel samples were subjected to physical and chemical analyses after being left at room temperature for 3 h, and the remaining were stored frozen as follows: each gel sample was individually placed in a polypropylene plastic box with a lid and stored in a freezer at -20°C for 15, 30, 45, or 60 days. To completely thaw the sausage gel during the thawing process, the frozen gel samples in polyethylene bags were kept in a refrigerator at 4°C for 24 h. The gel samples were then taken out of the bags and reweighed using the balance. The thawed gel samples were subjected to physical and chemical analyses after being left at room temperature for 3 h.

### Thawing Loss of the Sausage

The thawing loss of CBS was evaluated according to the method described by DeFreitas *et al.* (1997) with slight modifications. The thawing loss of the frozen cylindrical gel samples was calculated using the following equation:

$$\text{Thawing loss (\%)} = (W_i - W_f) / W_i \times 100$$

where  $W_i$  is the weight of a cylindrical gel before freezing and  $W_f$  is the weight of the gel thawed after frozen storage. Seven pieces each of the treated samples (0.1% and 0.5% OEx-CBS) and untreated sample (control CBS) were mea-

sured.

#### WHC of the Sausage

The WHC was evaluated by measuring the expressible moisture as described by Uresti *et al.* (2003). The CBS (diameter 17.5 mm, height 10 mm) was sandwiched between two layers of No. 2 qualitative filter paper. The gel sample with filter paper was placed at the bottom of a 50 mL centrifugal tube and centrifuged at  $1000 \times g$  and  $15^\circ\text{C}$  for 15 min, and the pressed gel was weighed immediately after. Expressible moisture was calculated using the following equation:

$$\text{Expressible moisture (\%)} = (W_i - W_c) / W_i \times 100$$

where  $W_i$  is the weight of a cylindrical gel before compression and  $W_c$  is the weight of the gel after compression. Seven pieces each of the treated samples (0.1% and 0.5% OEx-CBS) and untreated sample (control CBS) were measured.

#### Mechanical and Rheological Properties of the Sausage

The mechanical (breaking test) and rheological properties (creep-recovery test) of the sausage gel were measured using a Rheoner II creep meter (RE 2-3305; Yamaden Co. Ltd., Tokyo, Japan). For the breaking test, the breaking stress and breaking strain were determined according to the method of Hadipernata *et al.* (2016) with slight modifications. A cylindrical gel (diameter 17.5 mm, height 10 mm) was placed on the stage of the creep meter equipped with a cylindrical plunger (diameter 3 mm), and the plunger penetrated the gel at a speed of 1.0 mm/s. The breaking stress and strain were represented as the stress ( $\text{N/m}^2$ ) and strain (%) at the top of the first peak of the stress versus strain curve.

The creep-recovery test was conducted using a uniaxial compression method using the creep meter equipped with a 4 cm diameter plate plunger. A cylindrical gel (diameter 17.5 mm, height 10 mm) was put on the stage of the creep meter. An instantaneous stress (120 gf) was applied to the sample for 60 s, and the resulting deformation was measured as a function of time. After a creep time of 60 s, the stress was discontinued, and deformation recovery was monitored for 60 s.

According to the method of Dzadz *et al.* (2015), the creep curve obtained was analyzed using the four-element mechanical model as described in the following equation:

$$J(t) = \gamma(t) / \sigma$$

$$J(t) = \frac{1}{E_0} + \frac{1}{E_1} \left( 1 - \exp\left(-\frac{E_1}{\eta_1} t\right) \right) + \frac{1}{\eta_N}$$

where  $J(t)$  is creep compliance ( $\text{m}^2/\text{N}$ ),  $t$  is time (s),  $\gamma(t)$  is shear deformation,  $\sigma$  is constant stress (Pa),  $E_0$  is the instantaneous elasticity modulus (MPa),  $E_1$  is the retarded elastic modulus (MPa),  $\eta_N$  is the viscosity of the dashpot component of the Maxwell component (MPa s), and  $\eta_1$  is the viscosity of the dashpot component of the Kelvin-Voigt part (MPa s).

The recovery rate, R (%), was calculated using the following formula (Herranz *et al.*, 2012):

$$R(\%) = \frac{\gamma_T - \gamma_P}{\gamma_T} \times 100$$

where  $\gamma_T$  and  $\gamma_P$  represent the maximum compliance and recovery compliance ( $\text{m}^2/\text{N}$ ), respectively. Seven pieces each of the treated samples (0.1% and 0.5% OEx-CBS) and untreated sample (control CBS) were measured.

#### pH Measurement

The pH values of CBS were measured with a pH meter (HM-30G, TOA-DKK corporation, Japan). The pH was measured after homogenizing 3.0 g sausage gel with 27 mL distilled water using an ACE Homogenizer AM-8 (Nissei Corp., Tokyo, Japan) at 4500 rpm for 30 s at room temperature. Seven pieces each of the treated samples (0.1% and 0.5% OEx-CBS) and untreated sample (control CBS) were measured.

#### Determination of Sausage Whiteness

The whiteness of the inner part of the CBS was measured using a color meter.  $L^*$  (lightness),  $a^*$  (redness), and  $b^*$  (yellowness) of 10 mm cylindrical sections of the sausage were measured using the reflection method using a TES-135A color meter (Tes Electrical Corp., Taipei, Taiwan). Seven pieces each of the treated samples (0.1% and 0.5% OEx-CBS) and untreated sample (control CBS) were measured. The whiteness of the sample was calculated as follows (León *et al.*, 2006):

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{0.5}$$

#### Microstructure of the Sausage

The microstructure of the CBS was observed using scanning electron microscopy (SEM) according to the method described by Hashemi and Jafarpour (2016) with slight modifications. A piece of gel 2-3 mm thick was immersed in 2.5% glutaraldehyde solution in 100 mM sodium phosphate buffer (pH 7.4), incubated at  $4^\circ\text{C}$  for 2 h, and rinsed with 100 mM sodium phosphate buffer (pH 7.4) four times within 24 h. The sample was fixed again with 2% osmium in 100 mM sodium phosphate buffer (pH 7.4) for 2 h and then rinsed with 100 mM sodium phosphate buffer (pH 7.4) six times. A fixed specimen was dehydrated in ethanol with serial concentrations of 50, 70, 80, 90, 95, and 100% (v/v) for 10 min. The dehydrated sample was freeze-dried and mounted on a bronze stub and sputter-coated with a gold layer (DII-29010SCTR; JEOL Ltd., Tokyo, Japan). The prepared sample was observed with a scanning electron microscope (JCM-6000; JEOL Ltd.) at an acceleration voltage of 15 kV.

#### Determination of Lipid Oxidation of the Sausage

Lipid oxidation of the CBS was evaluated using the thiobarbituric acid reactive substances (TBARS) method as described by Benjakul and Bauer (2001). Chopped CBS samples (5.0 g) were weighed in a 50 mL test tube and homogenized with 15 mL of distilled water using an ACE Homogenizer AM-8 (Nissei Corp., Tokyo, Japan) at 4500 rpm for 10 s. The sample was filtered using a nylon mesh filter (108  $\mu\text{m}$  mesh diameter). Then, 1.0 mL of the filtrate was put into a screw cap tube, and 2.0 mL of a stock solution containing 0.0375% TBA, 15% trichloroacetic acid, and 0.25 N HCl were added. The mixture was heated in boiling water for 10 min, followed by cooling under running tap water. The mixture was centrifuged at  $3600 \times g$  for 20 min at room

temperature, and the absorbance of the supernatant was measured with a spectrophotometer at 532 nm. TBARS was calculated from the standard curve of malondialdehyde (MDA) and expressed as milligrams of MDA per kilogram of dry weight of sample.

#### Determination of Polyphenol in the Sausage

The amount of polyphenol in the CBS was determined following the procedure described by Roby *et al.* (2013) with slight modifications. A CBS sample (1.0 g) was homogenized in 10.0 mL of 75% ethanol using the homogenizer at 4500 rpm for 1 min, and the homogenate was then shaken at room temperature for 20 min before it was centrifuged at  $4500 \times g$  for 20 min. The 75% ethanol extract (2.5 mL) was mixed with 0.5 mL of 2-times diluted Folin-Ciocalteu reagent and left to stand at room temperature for 5 min, and then 0.5 mL of 10% sodium carbonate solution was added. After incubation at room temperature in the dark for 30 min, the absorbance was measured at 760 nm. The amount of polyphenol was expressed as milligrams of gallic acid equivalents (GAE) by comparison with the absorbance of the corresponding gallic acid standards.

#### Statistical Analysis

Statistical analysis was performed using one-way analysis of variance using SPSS 15.0 (SPSS Inc., Chicago, IL, USA), and the statistical difference of the means was determined using Duncan's multiple range test with a significance level of 95%. The results were presented as the mean and standard deviation of at least three independent replicates for each treatment.

### Results and Discussion

Chicken breast fillets used to make sausage had a protein content of  $20.17 \pm 0.45$  g/100 g meat (w/w) and lipid content of  $2.73 \pm 0.21$  g/100 g meat (w/w). The protein and lipid contents of chicken breast fillets agreed with those reported in the main food composition database (USDA, 2013) varying from 19.70 to 21.50 g/100 g for protein content and 1.80 to 3.03 g/100 g for lipid content.

#### Effect of OEx on the Physical Deterioration of Sausage by Frozen Storage

##### 1. Behaviour of Water in the CBS

The drip loss that occurs after thawing frozen food is undesirable because it causes a deterioration in the texture and taste of food. The drip loss rate, that is, the thawing loss of the CBSs that were stored frozen is shown in Fig. 1A. In the sausage samples stored for 15 days, the thawing loss (1.67%) of the CBS to which 0.1% OEx was added (0.1% OEx-CBS) was much lower than that (1.82%) of the control CBS without OEx. The thawing loss (1.59%) of the CBS with 0.5% OEx (0.5% OEx-CBS) was the lowest of the three tested groups. In the samples stored for a longer period, the OEx-CBS showed a different pattern of thawing loss from the control CBS. The thawing loss of the control CBS increased with storage time. After 60 days of storage, the thawing loss was 2.31%, revealing that extending the storage period from 15 to 60 days caused a 26.92% increase in thawing loss. On the other hand, the thawing loss of the

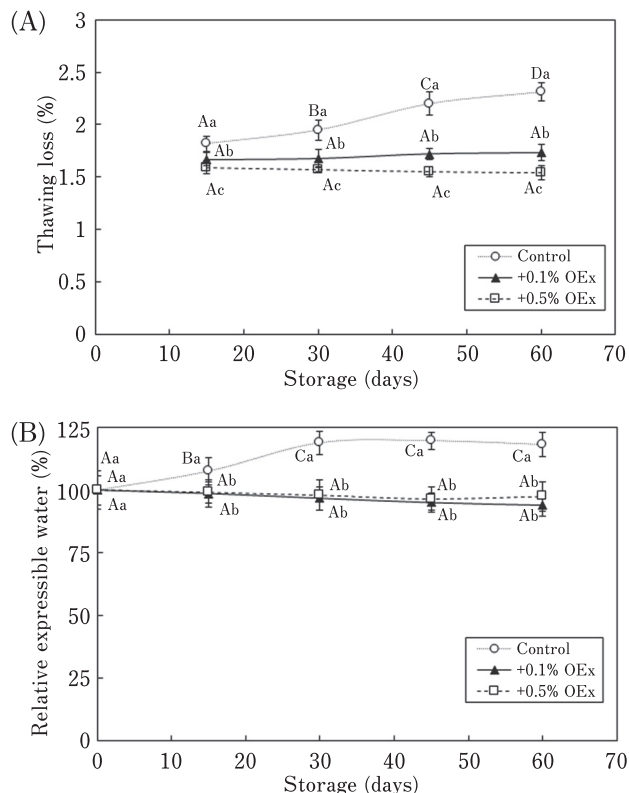
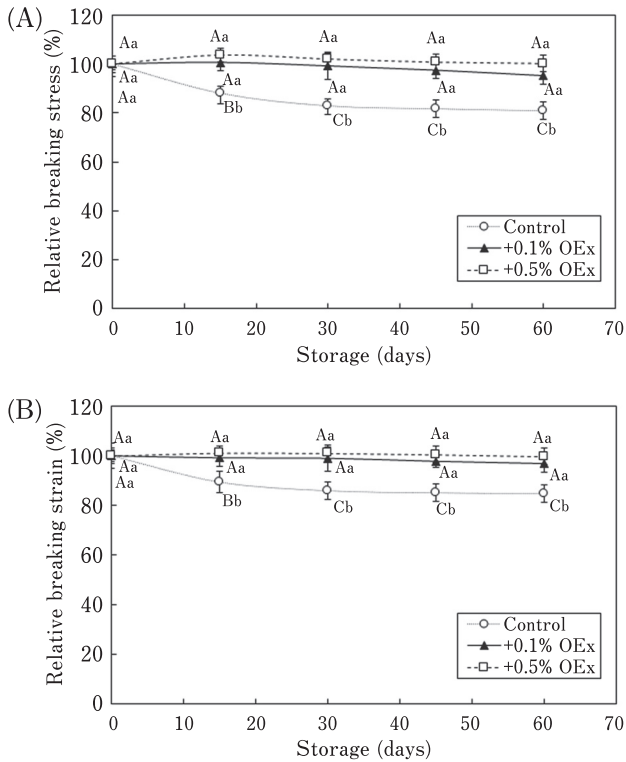


Fig. 1. Thawing loss (A) and expressible water (B) of OEx-CBS after frozen storage. Expressible water is expressed as a percent of the value of frozen-stored CBS relative to the value of unfrozen CBS. Data are presented as mean  $\pm$  SD ( $n=7$ ). Different superscript letters show statistically significant differences ( $P<0.05$ ) between different storage periods of the same sample (capital letters) or between samples at the same storage period (small letters).

0.1% OEx- and 0.5% OEx-CBS did not change upon extending the storage period from 15 to 60 days, suggesting that OEx protects against drip loss caused by thawing of CBS that were stored frozen. DeFreitas *et al.* (1997) reported similar results that showed a decrease in thaw drip in the frozen storage of cooked sausages with added carrageenan.

Figure 1B shows changes in the expressible water of the OEx-CBS during frozen storage. In control CBS, the expressible water increased significantly from day 0 until 60 days of frozen storage. On the other hand, in the 0.1% OEx- and 0.5% OEx-CBS, the expressible water did not change significantly over the 60 day storage period, indicating that the 0.1% or higher OEx suppresses the decrease in WHC. Thus, using OEx as an ingredient prevents drip loss of CBS and a fall in WHC during frozen storage. Oxidized products of phenolic compounds (ferulic acid, tannic acid, catechin, and caffeic acid) enhance the WHC of surimi gel (Balange & Benjakul, 2009). Rachman *et al.* (2020) showed that olive leaf water extract improved the WHC of chicken breast sausage and the oxidized polyphenols of the extract induced the cross-linking of myofibril proteins of chicken breast sau-

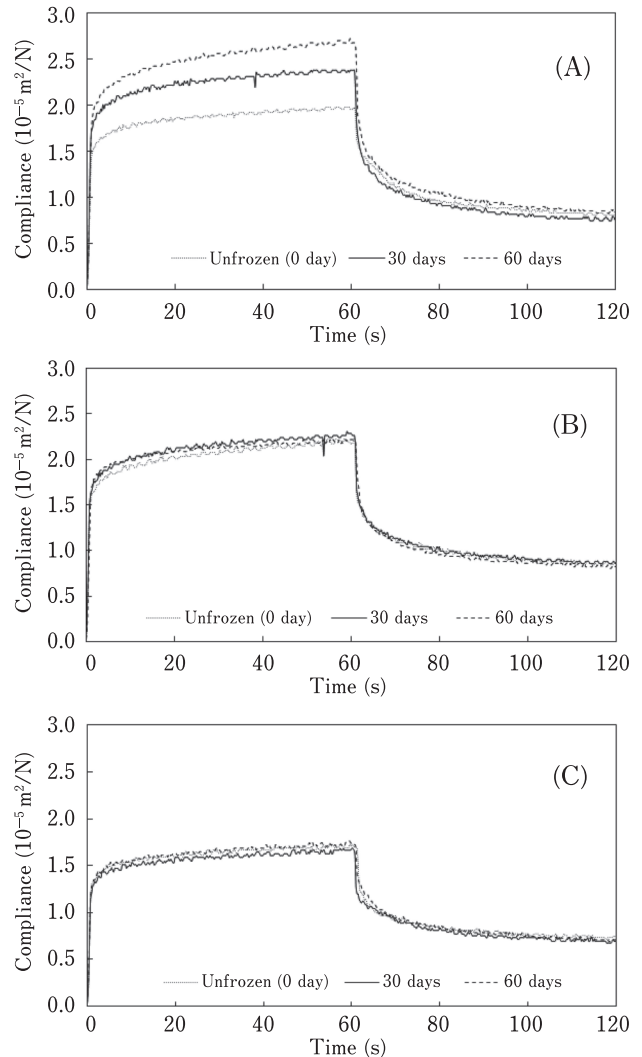


**Fig. 2. Changes in breaking stress (A) and breaking strain (B) of OEx-CBS during frozen storage.** Breaking stress and breaking strain are expressed as a percent of the value of the CBS that was stored frozen relative to the value of unfrozen CBS. Data are presented as mean  $\pm$  SD ( $n=7$ ). Different superscript letters show statistically significant differences ( $P<0.05$ ) between different storage periods of the same sample (capital letters) or between samples at the same storage period (small letters).

sage. Thus, WHC did not decrease after frozen storage because muscle fibers bind strongly with polyphenol, thus preventing the formation of ice crystals during freezing in the frozen storage. These results are consistent with the findings of Moreno *et al.* (2010), indicating that the treatment with MTGase and alginate suppressed the decrease of WHC of restructured gel caused by frozen storage. This suggests that inserting cross-links in a muscle proteinaceous gel results in the suppression of drip loss caused by frozen storage.

## 2. Mechanical and Viscoelastic Properties

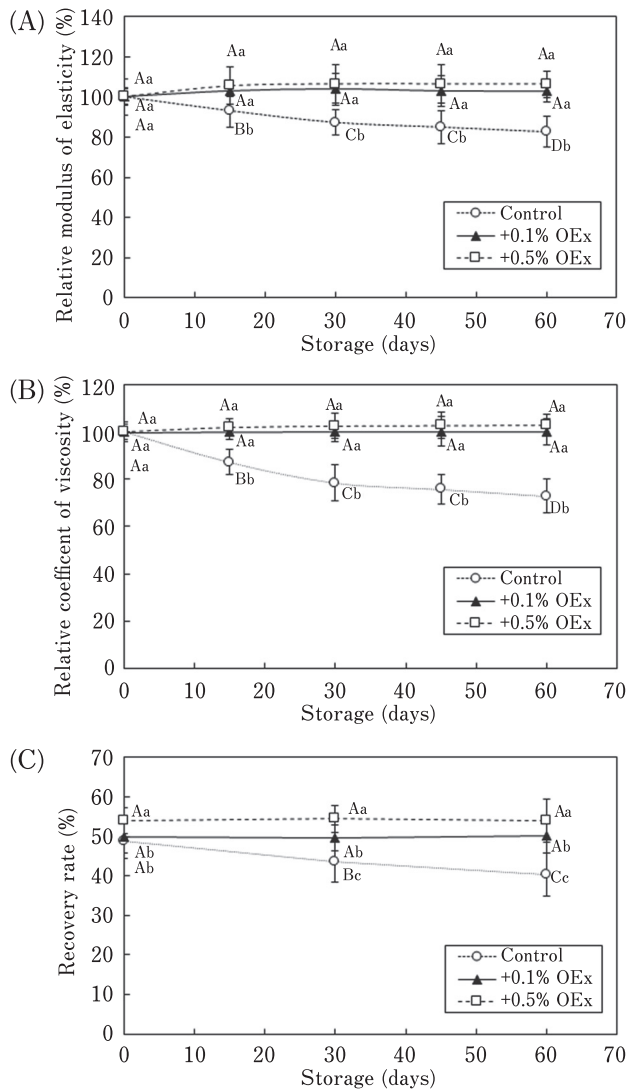
Changes in the breaking strength of the OEx-CBS during frozen storage are shown in Fig. 2. In the control CBS, the breaking stress decreased significantly in the first 30 days after freezing but did not fall in the long storage period beyond 30 days (30 to 60 days). The reduction in breaking stress during the first 30 day period was 17.01%. In contrast, the breaking stress of the 0.1% OEx- and 0.5% OEx-CBS remained unchanged during the entire storage period. A similar trend was seen in the breaking strain (Fig. 2B). The breaking strain of the control CBS decreased by 15% during the first 30 days of frozen storage, but there was no signifi-



**Fig. 3. Creep-recovery curves of OEx-CBS that were stored frozen.** (A) Control CBS; (B) 0.1% OEx-CBS; and (C) 0.5% OEx-CBS.

cant reduction during the storage period from 30 to 60 days, while the breaking strain of the 0.1% OEx- and 0.5% OEx-CBS remained unchanged during the entire storage period. This result indicates that adding OEx to the sausage ingredients prevents the deterioration of the mechanical properties of CBS with storage time. This preventative effect of OEx is similar to the effect of starch in preventing rheological change (shear force) during frozen storage (Prabpree and Pongsawatmanit, 2011).

The viscoelastic properties of the OEx-CBS that was stored frozen was evaluated using a creep and recovery test. Fig. 3 shows the creep-recovery curves of the control, 0.1% OEx-, and 0.5% OEx-CBS. The creep-phase curve of the control CBS shifted upward during frozen storage (Fig. 3A), while the curves of the 0.1% OEx- and 0.5% OEx-CBS hardly changed (Fig. 3B and 3C), suggesting that adding OEx suppressed the viscoelastic change in the CBS induced



**Fig. 4. Changes in elasticity (A), viscosity (B), and recovery value (C) of OEx-CBS during frozen storage.** Modulus of elasticity and coefficient of viscosity are expressed as a percent of the value of the CBS that was stored frozen relative to the value of unfrozen CBS. Data are presented as mean  $\pm$  SD ( $n=7$ ). Different superscript letters show statistically significant differences ( $P < 0.05$ ) between different storage periods of the same sample (capital letters) or between samples at the same storage period (small letters).

by cryopreservation. The modulus of elasticity, coefficient of viscosity, and the recovery rate of compliance, calculated from the creep–recovery curves, were plotted against storage period. Changes in those properties during the storage period are shown in Fig. 4. The modulus of elasticity of the control CBS decreased with the storage period (Fig. 4A). The decrease in the elasticity modulus value after 60 days of storage was 17.25% while that of the 0.1% OEx- and 0.5% OEx-CBS remained unchanged over the 60 day period of

frozen storage. Frozen storage of the control CBS produced a considerable decrease (22%) in the coefficient of viscosity, while that of the 0.1% OEx-CBS did not decrease (Fig. 4B). The recovery rate of the unfrozen CBS was 4% higher in the 0.5% OEx-CBS compared with control and the 0.1% OEx-CBSs. The high recovery rate of the 0.5% OEx-CBS reflects our previous result that using 0.5% OEx significantly changes the rheological properties of sausage (Rachman *et al.*, 2020). The recovery rate of the control CBS decreased with the storage period, and a 60 day storage period resulted in a 10% decrease (Fig. 4C). On the other hand, the recovery rate of the 0.1% OEx- and 0.5% OEx-CBS remained unchanged over the 60 day period of frozen storage.

The above results showed that adding OEx to the chicken sausage ingredients suppressed deterioration of the mechanical and rheological properties of CBS caused by frozen storage. Using SEM, we observed the microstructure of the OEx-CBS that had increased resistance to frozen storage. In the control CBS, a microstructure image of unfrozen sausage showed a fine network (Fig. 5A), while the sausage that was stored frozen had a coarse texture with large cavities (5 to 30  $\mu\text{m}$ ), which was clearly different from that of the unfrozen sausage. In the 0.1% OEx- and 0.5% OEx-CBS, the unfrozen gels had a fine network and had finer and smoother surface structure than the control gel. The 0.1% OEx- and 0.5% OEx-CBS that were stored frozen, although being slightly coarser than the unfrozen OEx-CBS, had a fine texture similar to that of the unfrozen control CBS (Fig. 5B and C). These minimal morphological changes in the OEx-CBS reflect the physical resistance to frozen storage, consistent with the results of the breaking strength and creep analyses.

The large cavities were seen only in the SEM images of the control CBS that were stored frozen. Hansen *et al.* (2003) reported that during the formation of ice crystals, pressure is produced only in a single direction. Free water contained in the gel network of CBS forms ice crystals during frozen storage. When ice crystals form inside the gel network, they produce pressure that separates the protein frameworks and the ice crystals become larger. This sequence also occurred in the control CBS that was stored frozen, resulting in the formation of large cavities. In contrast, such cavities were not observed in the OEx-CBS that was stored frozen, revealing that ice crystal formation is inhibited in the OEx-CBS. We recently reported that some compounds in OEx make the protein framework of CBS more rigid via protein–protein cross-links (Rachman *et al.*, 2020). In particular, the addition of OEx promotes the cross-linking of myosin heavy chain which is the main subunit involved in gelation. Ice crystal formation is observed even in the OEx-CBS that is stored frozen, but the framework reinforcement via cross-links is assumed to inhibit ice crystals from becoming larger.

#### **Effect of OEx on Lipid Oxidation of the Sausages that are Stored Frozen**

OEx had a high polyphenol content of  $138.5 \pm 0.30$  mg GAE/g dry weight (DW) powder. The polyphenol content of

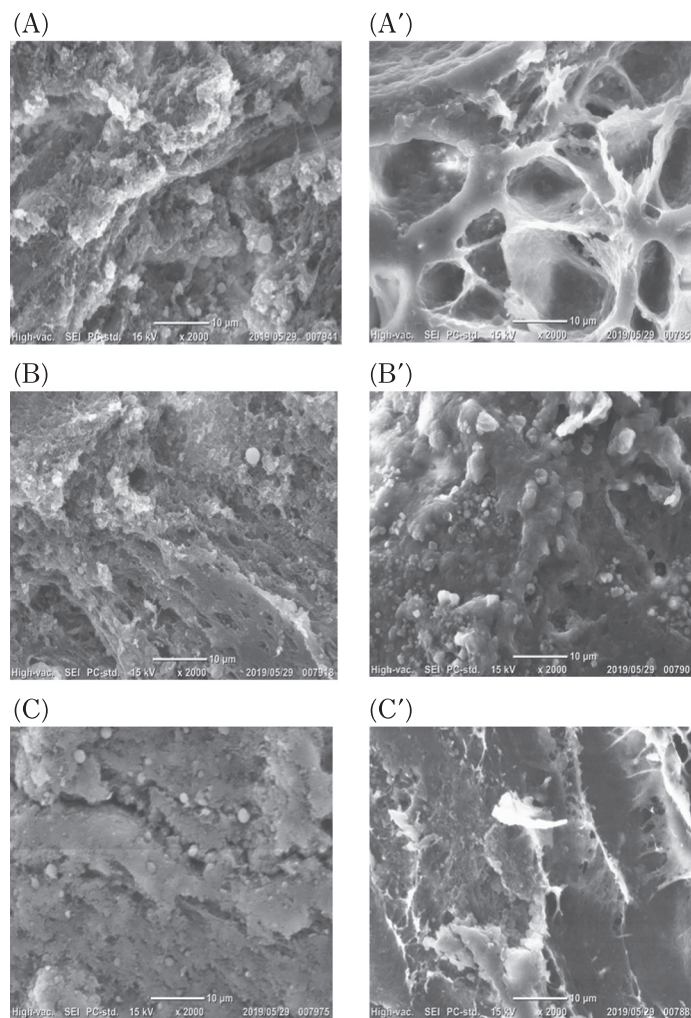
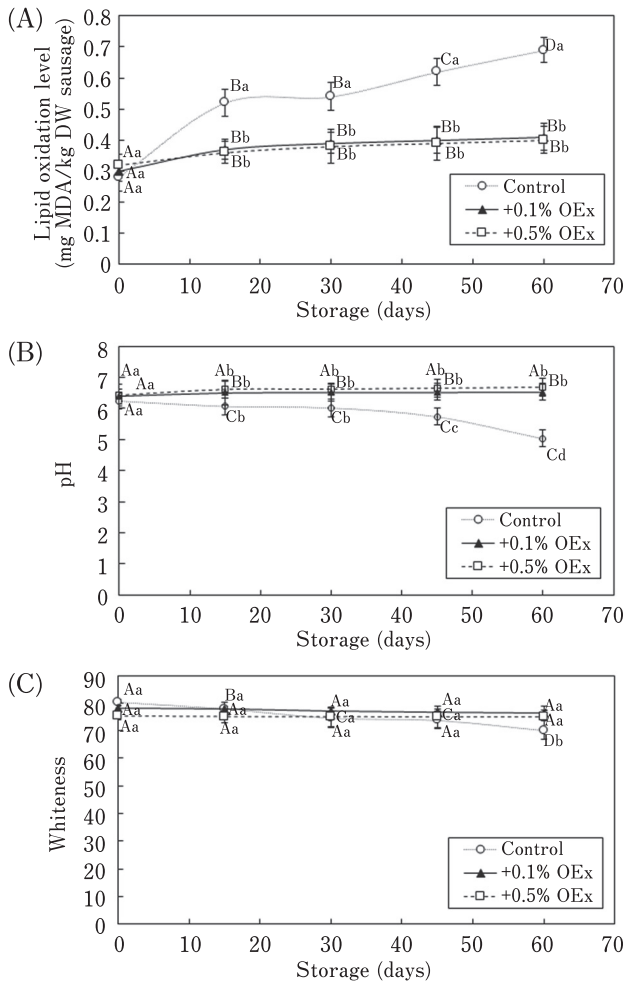


Fig. 5. **Microstructure of OEx-CBS that was stored frozen.** (A) and (A'), control CBS; (B) and (B'), 0.1% OEx-CBS; and (C) and (C'), 0.5% OEx-CBS. (A), (B), and (C) show micrographs of unfrozen sausages, and (A'), (B'), and (C') show micrographs of sausages thawed after 60 days of frozen storage. Scale bar, 10  $\mu$ m.

the 0.1% OEx-CBS was  $0.58 \pm 0.03$  mg GAE/g DW sausage, which was 11.6-times higher than that of the control CBS ( $0.05 \pm 0.04$  mg GAE/g DW sausage). The 0.5% OEx-CBS had 2-times the polyphenol content of the 0.1% OEx-CBS.

Frozen storage of food is prone to lipid oxidation. Olive leaf polyphenol has a strong antioxidant activity (Kontogianni and Gerothanassis, 2012). Therefore, we evaluated the effect of adding OEx on the lipid oxidation of CBS during frozen storage. Fig. 6A shows changes in the lipid oxidation level during frozen storage. The oxidation level of the unfrozen sausages was approximately 0.3 mg MDA/DW sausage for all three test samples and did not significantly differ among the three. The unfrozen OEx-CBSs having almost the same oxidation level as the unfrozen control CBS imply that OEx does not suppress lipid oxidation caused by the cooking process of chicken sausage. For each of the three CBSs, the

lipid oxidation level of the sausages that were stored frozen was higher than that of the unfrozen sausage. In the control CBS, the lipid oxidation level doubled by storing at  $-20^{\circ}\text{C}$  for 15 days. An increasing rate of the oxidation level from day 15 to day 60 was more gradual than that in the first 15 days. Similar to the control CBS, the 0.1% OEx-CBS showed an increase in the lipid oxidation level in the first 15 days of storage. However, the degree of oxidation was much smaller than that of the control CBS. One more difference between the control and 0.1% OEx-CBS was the change in lipid oxidation during the storage period after 15 days. The 0.1% OEx-CBS showed little increase in oxidation during the storage period from day 15 to day 60, suggesting that in the 0.1% OEx-CBS, lipid oxidation does not progress even if stored for a long time. These results indicate that the lipid oxidation of the OEx-CBS was mainly due to the thawing



**Fig. 6. Changes in lipid oxidation (A), pH (B), and whiteness (C) of OEx-CBS during frozen storage.** Data are presented as mean  $\pm$  SD ( $n=7$ ). Different superscript letters show statistically significant differences ( $P<0.05$ ) between different storage periods of the same sample (capital letters) or between samples at the same storage period (small letters).

process. The lipid oxidation curve (behaviour) of the 0.5% OEx-CBS was almost identical to that of the 0.1% OEx-CBS, implying that adding OEx at the concentration of 0.1% is sufficient to prevent CBS lipids from being oxidized during frozen storage. These results suggest that the shelf life of OEx-CBS may be longer than that of the control sausage. However, the microbiological test and sensory evaluation have not been conducted and hence the expiry date of the sausages has not been identified. Thus, determination of the expiry date of the sausages is an aspect to be considered in the future.

Several plant leaf extracts, such as *Ginkgo biloba*, green tea, and rosemary, are known to prevent lipid oxidation of meat products during cooking and refrigerated storage (Cisowska *et al.*, 2010; Chinprahast *et al.*, 2012; Jongberg *et al.*, 2014). However, there are few reports about the effect

of plant leaf extract on inhibiting lipid oxidation during frozen storage. To the best of our knowledge, our study is the first to show that a plant leaf extract can prevent lipid oxidation of meat products during frozen storage.

The inhibition of lipid oxidation of CBS by adding OEx is likely due to the antioxidative effect of phenolic compounds contained in olive leaves. The most abundant phenolic compound is oleuropein (ca 70% of total phenolics), which has high antioxidative activity (Goldsmith *et al.*, 2014). However, the most abundant phenolic compound in OEx is one form of oleuropein aglycone, 3,4-DHPEA-EDA, and not oleuropein (Rachman *et al.*, 2020). OEx has an antioxidative (DPPH radical scavenging) activity equivalent to the hot water extract of olive leaf where oleuropein is the main component (Rachman *et al.*, 2020). Accordingly, 3,4-DHPEA-EDA and other antioxidant compounds contained in OEx scavenge oxygen-centered radicals that could cause lipid oxidation (Umeno *et al.*, 2015), resulting in restricting lipid oxidation of CBS during frozen storage.

Figure 6B shows the pH value of CBSs after frozen storage. The pH value of the unfrozen control CBS was 6.24, while those of 0.1% and 0.5% OEx-CBS were 6.39 and 6.42, respectively. The result that OEx-CBSs had almost the same pH value as the unfrozen control CBS implies that the addition of OEx does not affect the pH of chicken sausage. After frozen storage for 60 days, the pH value of control CBS was decreased to 5.04. This pH reduction was probably due to some existing oxygen inside the package that triggered fat oxidation, thus resulting in a decrease in the pH value (Liu *et al.*, 2009). It has been reported that a reduction in the pH in meat products during refrigerated storage could be related to an increase in lactic acid, specifically caused by lactic acid bacteria (Flores *et al.*, 2015). Thus, the pH reduction in control-CBS may also be due to the development of lactic acid bacteria during the thawing process. On the other hand, the pH values of OEx-CBSs did not decrease. In each storage period, the pH values of OEx-CBSs were significantly higher than that of the pH of control CBS. Seo *et al.* (2019) reported that pH had a profound effect on the physical properties such as the emulsion stability, tenderness and color of cooked sausage in frozen storage. A high pH is closely related to high shear force and gel strength in meat products (Hur *et al.*, 2009). Thus, maintaining high pH values during frozen storage would also have contributed to the high frozen stability in the physical properties of OEx-CBSs. The decline in pH facilitates the oxidation of the muscle components including lipid as  $H^+$  may promote the redox cycle of myoglobin and its pro-oxidant action (Zhang *et al.*, 2011).

Frozen storage sometimes causes undesirable color changes. The color changes in the OEx-CBS during frozen storage were investigated by monitoring the whiteness inside the sausage and were compared with that of the control CBS (Fig. 6C). The whiteness of the control CBS decreased by 12.54% after 60 days of storage, whereas the whiteness of the 0.1% OEx- and 0.5% OEx-CBS hardly changed during the same period of 60 days. A decrease in the whiteness value could be related to lipid oxidation and the production of



malondialdehyde (Park *et al.*, 2006). Thus, OEx is useful in preventing discoloration of CBS during frozen storage. Gahruie *et al.* (2017) reported that shirazi thyme, cinnamon, and rosemary extract prevent color change in frozen beef burger. This prevention of color change is due to the suppression of lipid oxidation by polyphenols with antioxidative action. Thus, the color change of CBS induced by frozen storage is likely due to lipid oxidation. As mentioned previously, OEx has an antioxidative activity against lipid oxidation. The suppression of lipid oxidation by OEx should result in the prevention of discoloration of CBS during frozen storage.

In conclusion, this study showed that the application of OEx to CBS was found to be effective in suppressing physical property changes (syneresis and texture deterioration) and chemical property changes (lipid oxidation and discoloration) due to frozen storage. The physical property changes are suppressed by the cross-links between CBS protein molecules induced by 3,4-DHPEA-EDA, a major phenolic compound, in OEx. The chemical property changes are suppressed by some antioxidants (phenolic compounds) contained in OEx. The above results suggest that this natural olive leaf water extract is useful in improving the quality of frozen processed poultry foods.

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#### Conflict of Interest

The authors declare no conflict of interest.

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