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Article Title	Improved Physical Properties of Frozen Chicken Egg Gels with Olive Leaf Extract Fortification
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Abstract

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11 The research focused on evaluating the impact of olive leaf water extract (OEx4) on the
12 microbiological and physical properties of egg gels, as well as its ability to protect the
13 rheological properties of gel throughout freeze-thaw cycles. Egg gels with added OEx4 at
14 concentrations of 0.03% and 0.1% (w/w) a to minced whole egg (WE) and egg white (EW)
15 were frozen at -20°C for five days, undergoing three freeze-thaw cycles. The weight of the
16 OEx4-egg gels was constant throughout the cycles, in contrast to the control egg gels without
17 OEx4, which displayed accelerated thawing weight loss. The OEx4-egg gels maintained their
18 water-holding capacity, breaking strength, elasticity, and viscosity, but the control egg gels saw
19 a decrease. Using scanning electron microscopy, it was discovered that the OEx4-egg gels even
20 after freezing retained a structure similar to their non-frozen condition, in contrast to the control
21 egg gels. These findings suggest that OEx4 imparts freeze-resistance to egg gels. Additionally,
22 OEx4 application improved the interaction between non-polar groups and water molecules, in
23 egg gels leading to a rise in pH. Then, OEx4 has been found to effectively hinder the
24 proliferation of bacteria while also minimizing the occurrence of gel contamination in eggs
25 subjected to the freeze-thaw process. Therefore, OEx4 proves to be beneficial in enhancing the
26 physical, chemical and microbiological properties of frozen processed poultry products.

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28 **Keywords: Olive Leaf Extract, Frozen Food, Egg Gel**

Introduction

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The global egg industry produces more than a million tons of eggs each year, making a significant contribution to the world's protein supply and supporting global nutrition and food security. Egg white proteins are commonly used in various processed foods (Herawati et al., 2021; Zhang et al., 2014). Enhancing the natural gelling properties of egg proteins could lead to an improved quality of these processed foods (Li et al., 2021). Freezing egg products for preservation is a popular practice, but it can lead to texture deterioration due to syneresis, which depends on storage conditions and ingredients (Lucia et al., 2000; Sen et al., 2021; Eregama et al., 2023).

The leaves of olive trees (*Olea europaea* L., Oleaceae) contain high levels of phenolic compounds, with the primary phenolic compound being oleuropein at 10.9-12.6 g/100 g dry weight of olive leaf (Oyama et al., 2016). Oleuropein is specific to plants in the Oleaceae family and is not found in plants from other families. In addition to oleuropein, olive leaves also contain other major phenolics such as hydroxytyrosol and verbascoside, which are structurally different from the flavonoids found in the leaves of plants from other families, such as theaceae and vitaceae. Olive leaves also contain oxidative enzymes, including peroxidase and polyphenol oxidase, which play a role in converting phenolic compounds into quinone forms (Motamed et al., 2007; Ortega-García et al., 2008; De Leonardis et al., 2015).

The enhancement of the gel properties can be attributed to the promotion of intermolecular cross-linking of proteins by phenolic compounds. This process leads to a more stable and structured gel network, resulting in improved performance and functionality. Phenolic compounds can interact with proteins through both non-covalent and covalent interactions (Prigent et al., 2003). Covalent interactions, in particular, have been extensively studied for their role in modifying the physical and chemical properties of protein gels. When phenolic compounds with an ortho-diphenol structure are oxidized, they form a covalent bond with

55 proteins, leading to the formation of cross-linked protein polymers (Strauss & Gibson, 2004).
56 This process enhances the strength of protein gels by creating crosslinks among protein chains.

57 Recent studies have shown that adding plant extracts or phenolic compounds to food protein
58 hydrogels can alter the physical properties of egg gels. For instance, the addition of tea extract
59 containing catechins has been found to enhance the breaking strength of egg white gel (Wu et
60 al., 2007; Hatanaka et al., 2009). Akazawa et al. (2021) have suggested that using olive leaf
61 extract, prepared with cold water from olive leaves without blanching treatment, can improve
62 the viscoelasticity, breaking stress, and water-holding capacity of egg white gel. This additive
63 helps to maintain the texture of egg gel products during frozen storage. Additionally, according
64 to Rachman et al. (2021), olive leaf extract can significantly reduce the deterioration of the
65 rheological properties of sausages during frozen storage, thereby improving the preservation
66 stability of meat products in frozen foods.

67 As the frozen food market expands, many gel-like processed foods must be equipped with
68 high frozen resistance, particularly the anti-syneresis effect. This is because defrosting frozen
69 foods causes syneresis, resulting in the deterioration of texture. There is no data on the anti-
70 syneresis effect or frozen resistance of egg gels treated by chemical modifications such as
71 glycation and the addition of polyphenols. In our research, we examined the properties of egg
72 gels following freeze-thaw cycles to evaluate the freeze resistance of egg gels that have been
73 fortified with an extract from olive leaves.

74

75 **Materials and Methods**

76 **Materials**

77 The materials used in the study included fresh olive leaves (*Olea europaea* L.) sourced from
78 the Agriculture Faculty of Kagawa University in Japan. The eggs (Boris Brown hen strain)
79 were purchased from a retail market in Hyogo Prefecture, Japan. Kureha Corp. (Tokyo, Japan)

80 provided the Krehalon® PVDC casing used as a handmade egg gel stuffer. Analytical-grade
81 chemicals were used for all compounds.

82

83 **The process of preparing the olive leaves extract (OEx4)**

84 Olive leaves were dried for 48 h at a temperature between 25°C and 40°C in a cold-air drying
85 machine (Cool Dry Machinery Co. Ltd., Kagawa, Japan) and subsequently pulverized. The
86 ensuing powder was filtered through a nylon mesh sieve with a pore diameter of 108 µm. Two
87 extracts of olive leaf water were prepared from the sieved olive leaf powder at a temperature
88 of 4°C. An Erlenmeyer flask containing 500 mL of pre-cooled, 4°C distilled water and 50 g of
89 olive leaf powder was swirled for an hour at 4°C using a magnetic stir plate. Centrifugation
90 $8,000 \times g$ and 4°C was performed for 20 min on the homogenates obtained from this procedure.
91 Lyophilization was applied to the supernatant and OEx4 was made from the resulting
92 lyophilized powder. OEx4 was kept in storage at -20°C, until it was needed for additional
93 testing.

94

95 **The process of preparing of Egg White (EW) and Whole Egg (WE) gels**

96 The egg white (EW) gel and whole egg (WE) gels were prepared from fifty hen eggs each.
97 EW was divided from egg yolk (EY) using an egg separator, and the EW was gently collected
98 in a container. WE, after breaking eggs, was gently collected in a separate container. Each EW
99 and WE solution was mixed with a Nissei AM-8 homogenizer (Nihonseiki Kaisha, Tokyo,
100 Japan) was used to stir. Subsequently, either 0.03 or 0.1 g of OEx4 was combined with 100 mL
101 of the EW or WE solution. The egg solution was put into a Krehalon® PVDC casing (Kureha
102 Co., Tokyo, Japan), which had a flat width of 4.0 cm. The casing was heated for 30 min at
103 80°C. Post-heating, the resulting cylindrical gel was approved to rest at room temperature for
104 one hour and was then refrigerated at 4°C for 6 h. Each cylindrical egg gel (with a diameter of

105 17 mm) was then removed from its casing and sectioned at 20 mm intervals using a razor blade
106 for the purpose of the freeze-thaw experiment.

107

108 **Treatment of egg gels with a freeze-thaw cycle**

109 Cylindrical egg gels were utilized to assess how OEx4 affected the freezing and thawing
110 process. Twenty-one sets of cylindrical gels were stored in a lidded polypropylene plastic box,
111 which was kept for five days at -20°C in a still-air freezer (Biomedical Freezer, MDF-436;
112 Sanyo, Japan). The frozen gels were then defrosted in a refrigerator (4°C) for 16 h. After being
113 freeze-thawed, seven cylindrical gel samples were physically inspected. Two or three more
114 freeze-thawing steps were done, and physical analyses were conducted on the remaining 14 gel
115 samples.

116

117 **Assessment of water content**

118 An aluminum foil pan was filled with 2 g of egg gel pieces. Following that, these pieces
119 were dried in a drying cabinet set at $105 \pm 1^\circ\text{C}$ until the sample's weight remained constant.
120 The dehydrated sample was chilled in a desiccator containing silica gel prior to being measured
121 using an precision balance. The water content in the egg gel can be determined using the
122 appropriate formula:

$$123 \quad \text{Water content (\%)} = (W_i - W_f) / W_i \times 100 ,$$

124 where W_f denotes the egg gel's final weight following dehydration and W_i denotes the egg gel's
125 weight before dehydration.

126 **Determining the water-holding capacity of egg gels**

127 The water-holding capacity method was used to quantify the amount of expressible water in
128 the egg gel with slight modifications (Visessanguana et al., 2004). Utilizing a Rheoner II creep
129 meter (model RE 2-3305; built by Yamaden Co., Tokyo, Japan) fitted with a 40 mm diameter
130 cylindrical probe, an egg gel cylinder measuring 17.5 mm in diameter and 10 mm in height
131 was squeezed. The compression was carried out at a crosshead speed of 5 mm/s to 50% strain
132 for a duration of 60 s. The expressible water of the egg gel was determined by applying the
133 subsequent formula:

$$134 \text{ Expressible water (\%)} = (W_i - W_c) / W_i \times 100$$

135 Here, W_i represents the slice of egg gel's weight at initial contact, and W_c represents the weight
136 of the condensed area.
137

138

139 **Assessment of thawing loss**

140 The egg gel's thawing loss was measured using the method of thawing loss with minor
141 modifications (DeFreitas et al., 1997). The following formula was utilized to calculate the
142 thawing loss of the frozen cylindrical gel samples:

$$143 \text{ Thawing loss (\%)} = (W_i - W_f) / W_i \times 100$$

144 In this equation, W_i is the weight of a cylindrical gel before it is frozen, and W_f is the weight
145 of the gel after it has been thawed.

146 **Finding the pH value**

147 A pH meter was used to determine the egg gel's pH level (model Eutech™ PC 700 Multi-
148 parameter Meter, manufactured by Thermo Fisher Scientific, US). This measurement was
149 taken after 3.0 g of the egg gel was homogenized with 27 mL of distilled water using a Digital

150 High Shear D-160 (produced by BIOBASE GmbH, Wolfenbuettel, Germany) at a speed of
151 4,500 rpm for a duration of 30 s at room temperature.

152

153 **Microbiological parameters**

154 Microbiological study of the samples was done in cycles (0, 1, 2, and 3) of frozen storage,
155 with seven replicates per group. That is, at each time point, 21 samples (2 concentrations of
156 olive leaf extract + control) were examined. The Total Plate Count was determined with Plate
157 Count Agar (PCA, Merck, Darmstadt, Germany) incubation at 37.8°C for 24 h and enumerated.
158 All counts of TPC from egg gel sample are expressed as Log CFU/g.

159 **Evaluation of the gel's mechanical and rheological attributes**

160 The viscoelastic property and the egg gel's breaking strength was evaluated using a Rheoner
161 II creep meter. A creep-recovery test was conducted at 25°C to assess the viscoelastic property.
162 The cylindrical gel, which had a diameter of 17.5 mm, was subjected to a continuous tension
163 of 120 N/m² and a height of 10 mm for 1 min at a crosshead speed of 5 mm/s using a plate
164 plunger with a diameter of 40 mm, in order to estimate creep compliance. Following this, the
165 stress was released for 1 min to obtain the stress compliance curve of recovery. The creep-
166 recovery curve was assessed with the use of six-element mechanical model and software
167 provided by the manufacturer (CAS-3305, ver. 1.6). The instantaneous modulus (E_0) of a
168 Hookean body and the Newtonian viscosity (η_N), two key quantities, were identified. Using a
169 cylindrical plunger with a 3 mm diameter. 1.0 mm/s of penetration speed was used to create a
170 stress-strain curve. The stress (N/m²) and strain (%) at the peak of the initial stress-strain curve
171 were used to represent the breaking stress and strain.

172 **Differential scanning calorimetry**

173 Differential scanning calorimetry (DSC) was used to identify the degree of heat
174 denaturation of the proteins in the egg batter. For the DSC analysis, a Setaram Micro-DSC VII
175 Commissioning/Utilizations instrument (produced by SETARAM Instrumentation, Caluire,
176 France) was used. Two hundred milligrams of Al₂O₃ and egg batter were added to a reference
177 pan and a sample pan, respectively. The samples were heated from 20°C to 100°C at a rate of
178 1.2°C per second. The thermal analysis software included with the DSC device was used to
179 examine the calorimetric data.

180

181 **Examination of the gel's microstructure**

182 The microstructure of the egg gel was examined using scanning electron microscopy (SEM).
183 After being sliced into 2-3 mm pieces thick using a knife, the egg gel pieces were fixed for 2
184 hours at 4°C with 2.5% glutaraldehyde in a 100 mM sodium phosphate buffer (pH 7.4). They
185 were then post-fixed with 2% osmium in the same solution. Following fixation, the sample
186 underwent 10 minutes of ethanol dehydration at increasing concentrations (50, 70, 80, 90, 95,
187 and 100% (v/v)), followed by freeze-drying. After being dehydrated, the sample was attached
188 to a bronze stub and covered in gold (DII-29010SCTR; JEOL, Tokyo, Japan). Subsequently, it
189 was examined using a scanning electron microscope (JCM-6000; JEOL) operating at an
190 acceleration voltage of 15 kV.

191 **Assessment of crosslinking in heat-treated protein**

192 OEx4, in quantities of 0.3 and 1.0 mg, was dissolved in 1.0 ml of a 10 mM sodium phosphate
193 buffer (pH 7.0) and subsequently combined with 1.0 ml of a 10 mg/ml egg (WE or EW) protein
194 solution. The egg protein solution, once mixed with OEx4, was subjected to heat treatment at
195 80°C for a duration of 30 min. The dissociation solution (8 M urea and 12.5% SDS) was added
196 in a volume four times larger than the hazy solution that was produced. The protein was
197 dissolved in the dissociation solution by vortexing for 4 min, and the sample was then

198 processed for SDS-PAGE. The specimen was combined with a 4×SDS-PAGE sample buffer,
199 which consisted of a pH 6.7 100 mM Tris-HCl buffer, 2% β-mercaptoethanol, 0.004%
200 bromophenol blue, and 5% SDS) then heated for two minutes at 100°C. An SDS-PAGE gel
201 (12.5% acrylamide separating gels) was loaded with a 10 μl aliquot of the prepared sample
202 solution. Following electrophoresis, the gel underwent staining with a 0.025% solution of
203 Coomassie Brilliant Blue R250.

204 **Statistical analysis**

206 The experiment results were obtained from a completely randomized design of at least three
207 independent replicates for each treatment. Statistical analysis was carried out using SPSS 23.0
208 software by performing a one-way ANOVA and the two-way ANOVA in some experiments.
209 The statistical difference of the mean was ascertained using the Duncan multiple range test at
210 a 95% significant level. The data are reported as mean and standard deviation.

211 **Results And Discussion**

212 **Gel properties of egg gels fortified with OEx4**

214 This study investigated how the addition of OEx4 affects the gel properties of egg gels
215 during the freeze-thaw process. The study compared the gel properties of WE gel with 0.03%
216 and 0.1% OEx4 (Table 1). The results showed that adding OEx4 to the WE gel resulted in a
217 water content similar to the control, with no significant difference ($p > 0.05$). However, the
218 expressible water of the WE gel decreased significantly with the addition of OEx4 ($p < 0.05$),
219 indicating that OEx4 improves the gel's ability to hold water. The breaking stress, elongation,
220 elasticity, and viscosity of WE gels increased with the addition of OEx4 ($p < 0.05$). Thus, OEx4
221 enhances the gel properties of WE gel. Based on the Table 2, the gel properties of EW gel
222 containing OEx4 and found that the water content did not change ($p > 0.05$). However, the
223 expressible water decreased significantly with the addition of OEx4 ($p < 0.05$), indicating an

224 improvement in the gel's water-holding capacity. Importantly, the study found that adding
225 OEx4 to WE or EW solution at 0.03% (w/v) is sufficient, as the impact did not significantly
226 differ from that of 0.1% OEx4.

227 Adding OEx4 improves the elasticity and strength of WE and EW gels by altering the
228 physical properties of the eggs with natural enzymes. The interaction between Polyphenol and
229 protein molecules leads to irreversible effects, typically occurring through C-N or C-S linkage
230 (Li et al., 2021). This process commences with the formation of quinones, which can arise
231 through enzymatic modification in the presence of oxygen or through autoxidation under
232 alkaline conditions (Shahidi and Dissanayaka, 2023). Ozdal et al. (2013) explained that
233 polyphenol oxides help in converting monophenols into ortho-diphenols through hydroxylation,
234 and then rapidly oxidizing them into ortho-quinones. These quinones, which are strong
235 electrophiles, react with nucleophilic residues of proteins or peptides (such as thiol, amino,
236 guanidine, or imidazole) through Schiff base (C=N) and Michael addition mechanisms (C-
237 NH), potentially leading to the formation of protein cross-links. Balange and Benjakul (2009)
238 demonstrated that oxidized phenolic compounds such as ferulic acid, tannic acid, catechin, and
239 caffeic acid improve the water-holding capacity (WHC) of surimi gel, and further research has
240 shown that the oxidation of these polyphenols results in the cross-linking of myofibrillar
241 proteins in surimi. Handa et al. (2001) also suggested a strong relationship between WHC and
242 the physical properties of heat-induced gels of egg whites.

243 **Effect of olive leaf extract on egg protein heat denaturation**

244 Two stages thermally induced gelation were used for the egg proteins. The protein is first
245 heated to a denaturation point. The rate of protein diffusion increases when proteins become
246 denatured (Alleoni, 2006). The second stage involves interactions between denatured protein
247 molecules and other denatured molecules. Proteins that interact for extended periods of time
248 form high molecular weight aggregates. As the aggregates continue to form, they create an

249 elastic gel that can hold water molecules. Thus, understanding the gel characteristics of egg
250 yolk requires a thorough examination of protein denaturation and protein-protein interactions.
251 A single endothermic peak was observed within the temperature range of 82.09°C to 82.34°C,
252 according to Figure 1A for all WE samples. In the meantime, all EW samples showed two
253 sizable endothermic peaks (Figure 1B). The first peak shows in the range of 64.15°C and
254 64.41 °C, and the second peak at 82.15°C and 82.48°C (Renzetti et al., 2020). Two endothermic
255 peaks may be seen in the denaturation of ovalbumin and ovotransferrin in the egg white
256 (Gorinstein et al., 1995). The two highest points are 63.6°C and 76.18°C (Sun et al., 2006).
257 Thus, ovotransferrin was associated with the first peak in the EW thermogram, and ovalbumin
258 was associated with the second and sole peak in the WE thermogram. The peak in WE and EW
259 thermograms changed to higher temperatures at 0.25°C or 0.33°C when olive leaf extract was
260 added. This implies that the heat-induced denaturation of egg proteins is slowed down by olive
261 leaf extract. Egg yolk development depends on ovalbumin, the most common protein in eggs
262 (Razi et al., 2023). When olive leaf extract was present, the denaturation temperature of
263 ovalbumin was relatively close to that of ovalbumin without extract. The characteristics of the
264 gels with and without olive leaf extract changed noticeably as a result of the temperature
265 difference. The appropriate gel binding of egg yolks with olive leaf extract depends on the
266 larger temperature shift of ovalbumin denaturation temperature caused by the extract. To
267 ensure that egg yolks containing olive leaf extract are properly bound, the ovalbumin
268 denaturation temperature must be raised to a higher degree.

269 **The impact of OEx4 on egg gel throughout the freeze-thaw cycle**

270 **Physical and microbiological characteristics**

271 Figure 2 illustrates that both WE and EW gels experienced drip and thawing losses when
272 frozen, leading to a decrease in food viscosity and taste. The thawing loss of the control gel
273 sample is significantly increased by repeating the freeze-thaw cycle ($p < 0.05$), while that of

274 OEx4-gel pieces was stable ($p > 0.05$). The freeze-thaw result of WE and EW gels suggests
275 that OEx4 addition effectively prevents the syneresis from proteinous food gels by frozen
276 storage. The oxidized polyphenols present in OEx4 create robust cross-links with the proteins
277 in egg gel. This interaction plays a crucial role in inhibiting the formation of ice crystals during
278 the freezing process. As a result, the drip loss, which refers to the loss of moisture from the gel,
279 is not reduced after freezing. Polyphenols are natural antioxidants that provide excellent
280 protection against oxidation by neutralizing free radicals (Huang et al., 2019). Elgamouz et al.
281 (2019) discovered that natural antioxidants such as α -Tocopherol, cinnamon, cumin, turmeric,
282 garlic, and ginger can effectively prevent protein denaturation during frozen storage.
283 Additionally, they suggested that incorporating cross-linking into the protein structure of egg
284 gel can effectively reduce drip loss during frozen storage. Furthermore, Rachman et al. (2020)
285 have suggested that OEx4 demonstrates a protective effect against drip loss caused by the
286 thawing of sausages that have been stored in a frozen state. This indicates that OEx4 may offer
287 a potential solution to minimize the negative effects of frozen storage on the quality of chicken
288 meat products.

289 Figure 3 shows changes in the expressible water by repeating the freeze-thaw cycle. In both
290 WE and EW gels, the expressible water of the control gel sample increased ($p < 0.05$) with
291 each cycle. This implies that repeating freeze-thaw induces a decrease in bound water volume
292 concomitant with the increase in the volume of free water (Lv and Xie, 2021). On the other
293 hand, the expressible water of the two gel samples containing 0.03% and 0.1% OEx4 was little
294 changed ($p < 0.05$) by freeze-thaw. This suggests that bound water in OEx4-gel strongly
295 interacted with the frame of gel, even if the freeze-thaw of gel was repeated. As a result, bound
296 water was maintained in its gel matrix. Furthermore, the effect was seen in WE gel which
297 contains lipids as well as EW gel without lipids, suggesting that the high water-holding effect
298 in OEx4 gel is applicable in complicated food gel systems that contain multiple types of

299 ingredients. In a study by Moreno et al. (2010), it was discovered that combining alginate and
300 microbial transglutaminase (MTGase) can prevent a decrease in water-holding capacity (WHC)
301 during freezing storage. Additionally, different saccharides interact with egg protein, reducing
302 water loss during freeze-thaw cycles and delaying the decline in WHC of heat-induced egg gel
303 during freezing (Chen et al., 2022; Zhang et al., 2023). OEx4 also causes crosslinks between
304 proteins, contributing to high freeze-reliance. Furthermore, olive leaf extract, which has a high
305 polyphenol content, possesses potent antioxidant properties, such as scavenging hydroxyl
306 ($\text{OH}\cdot$), superoxide ($\text{O}_2\cdot^-$), and DPPH radicals, as well as chelating ferrous ions (Fe^{2+}). By
307 incorporating polyphenols into eggs before frozen storage, it is possible to prevent the
308 formation of protein carbonyls and dityrosine, reduce the loss of surface hydrophobicity in egg
309 proteins, and enhance the total sulfhydryl content, resulting in less protein degradation and a
310 more intact egg protein structure.

311 Despite the presence of OEx4 in egg gels, repeated freeze-thaw cycles of egg gel induced
312 syneresis to a small degree. Herein, we evaluated how much the syneresis affects pH (Tables
313 3 and 4). In both of WE and EW gels, the pH value of the control gel increased in second cycle
314 of freeze-thaw ($p < 0.05$), while that of OEx4 gels did not change much ($p > 0.05$). When the
315 pH exceeds 7, the protein charge increases, resulting in the formation of a three-dimensional
316 gel by ovalbumin (Ferreira Machado et al., 2007). Gels induced by strong bases primarily rely
317 on ionic and disulfide bonds. Raikos et al. (2007) observed a direct correlation between gel
318 strength and pH for thermally induced whole eggs and egg yolk protein, and a relatively small
319 linear relationship between gel hardness and pH for egg white protein. Additionally, these
320 findings suggest that using OEx4 in protein-based gel food helps maintain the food's pH
321 stability during frozen storage. However, due to the instability of OEx4 in an alkaline
322 environment, quinonoids were formed when OEx4 was added to egg white. For instance, Ai et
323 al. (2019) reported that quinonoids have a strong binding capacity with proteins and promote

324 nonspecific cross-linking between proteins and other molecular interactions, including ionic
325 bonds, hydrophobic interactions, and disulfide bonds.

326 The initial cycle revealed that the microbial contamination of egg gel did not surpass 3 Log
327 CFU/g in any of the OEx4 and control samples ($p > 0.05$). This indicates that such a level of
328 contamination is common, as other studies have also shown (Cwiková and Nedomová, 2014;
329 Necidová et al., 2019). The olive leaf extract had diverse effects on the total plate counts (TPCs)
330 of egg gel. Although the TPCs were initially similar, they varied significantly over the
331 following weeks. In the third cycle of freeze-thawing, TPCs in egg gel with contained olive
332 leaf extract stayed below 3 Log CFU/g, while the control without the olive leaf extract showed
333 bacterial growth up to 3-5 Log CFU/g (Tables 5 and 6). Interestingly, the egg gel control
334 samples without olive leaf extract had the highest TPCs by the end of the observation period
335 (cycle 3). Coliform bacteria are commonly utilized as bacterial indicators to assess the hygienic
336 quality of eggs and egg products (Chousalkar et al., 2021). The absence of coliforms in this
337 case is likely due to the high quality of the eggs and the hygienic processing methods employed
338 in both the egg gel with OEx4 and the control group. The olive leaf extract has been shown to
339 enhance the quality and shelf-life of meat products (Hayes et al., 2010; Alirezalu et al., 2016).
340 Also, Takó et al. (2020) suggested that phenolic compounds derived from plants, including
341 phenolic acids, flavonoids, stilbenes, and tannins, have been shown to inhibit the growth and
342 activity of a variety of microorganisms in frozen food. This includes food-related pathogens
343 and clinically significant bacteria, fungi, and protozoa. These findings highlight the
344 effectiveness of the olive leaf extract in inhibiting bacterial growth and reducing contamination
345 in egg gel.

346 **Mechanical and viscoelastic characteristics**

347 Figures 4 and 5 demonstrate the changes in gel strength of OEx4 egg gels during freeze-
348 thaw cycles for WE and EW gels. The breaking stress of the control gel sample without OEx4

349 decreased significantly ($p < 0.05$) after repeated freezing and thawing. In contrast, the breaking
350 stress of OEx4-containing gels remained stable ($p > 0.05$) in the first and two cycles, with only
351 a slight decrease ($p < 0.05$) of 6 to 9% in the third cycle. The viscosity characteristics of the
352 OEx4-egg gels following freeze-thaw were evaluated using elongation and stability analysis.
353 Figure 6 illustrates changes in the elasticity of WE and EW gels. The elasticity of control
354 samples without OEx4 significantly decreased ($p < 0.05$) with repeated freeze-thaw, with a
355 more significant change in WE gel than EW gel. Conversely, the elasticity of the gel samples
356 containing 0.03 and 0.1% OEx4 remained unchanged ($p > 0.05$) until the third freeze-thaw
357 cycle, where a minimal reduction was observed compared to the control gel. Changes in
358 viscosity parameters are presented in Figure 7, showing a significant decrease ($p < 0.05$) in the
359 viscosity of control gels with repeated freeze-thaw. In contrast, the viscosity of OEx4-gels
360 changed minimally throughout the freeze-thaw cycle. The addition of polyphenols is an
361 efficient method to prevent damage to the physical, chemical, and functional properties of
362 proteins during the freeze thaw cycle (Cao et al., 2020). Additionally, hydrogen bond
363 interactions between the protein amino groups and polyphenol hydroxyl groups favor aggregate
364 formation, improving protein stability. Also, the abundant phenolic hydroxyl groups of
365 polyphenols confer high affinity for metal ions, which prevents the various biochemical
366 reactions triggered by these cofactors (Zhu et al., 2021).

367 Figure 8 illustrates the microstructure of unfrozen and frozen WE gels, revealing a rough
368 texture with large voids in the frozen gels, especially the control gel. However, no such voids
369 were observed in the 0.1% OEx4-gel. Figure 9 depicts the microstructure of freeze-thawed EW
370 gels, showing a rough surface with large voids in the control gel, while the 0.03% OEx4-EW
371 gel was smooth with no large voids but slightly rougher. No such voids were observed in 0.1%
372 OEx4 gels subjected to freeze-thaw cycles. This microstructure of OEx4-egg gels is similar to
373 the microstructure of sodium chloride-egg yolk gels during frozen storage, when sodium

374 chloride was added to the egg yolk gels, more proteins were released from granules and
375 participated in gel formation, this yielding a more compact microstructure (Liu et al., 2018).
376 This result is consistent with the conclusion obtained from Figure 6 and 7 that freezing and
377 thawing treatment contributed to a stronger network of elasticity and viscosity OEx4-egg gels
378 than that of control gels. Likewise, the strong cross-linking of OEx4-egg gels were also shown
379 greater strength than that of the control gels during freeze thaw cycle (Figure 4 and 5). The
380 minimal changes in the morphology of OEx4-egg gels suggest their ability to withstand freeze-
381 thaw cycles, as evidenced by their strength and creep analysis results. The strong freeze
382 resistance of OEx4 gels may be attributed to the stabilization of water behavior in the egg yolk.

383 **Crosslink formation in egg gel**

384 The SDS-PAGE pattern of the protein extraction from the heat-treated OEx4-WE solution
385 is shown in Figure 10A. The remaining bands were thinner compared to the control WE protein.
386 Specifically, α -livetin and ovotransferrin disappeared, while ovalbumin, lysozyme, phosvitin,
387 and β -livetin became thinner. A new high molecular weight band emerged, indicating the
388 formation of a protein complex due to the inclusion of OEx4 (Rachman et al., 2021). Intense
389 bands at over 250 kDa were observed at the top of the separating gel, comprising high-
390 molecular-weight crosslinked proteins (Xue et al., 2022). This indicates non-disulfide-type
391 intermolecular crosslinking among these proteins, resulting in polymer formation. In the SDS-
392 PAGE pattern of the heat-treated OEx4-EW protein solution (Figure 10B), the prominent 75
393 kDa ovotransferrin bands were no longer visible. Instead, intense bands over 90 kDa were
394 observed at the top of the separating gel, containing high-molecular-weight cross-linked
395 proteins, including ovalbumin and lysozyme. Ovalbumin bands in OEx4-egg white gels
396 indicate the formation of a network structure through covalent crosslinking. Wang et al. (2024)
397 discovered that tea polyphenols can shield egg white proteins from damage in alkaline
398 conditions by acting as crosslinking agents, with ovalbumin playing a crucial role. Additionally,

399 tea polyphenols can modify the environment around the crosslinked proteins, making egg white
400 proteins more water-attracting and preventing protein damage, ultimately enhancing the
401 formation of network proteins in egg white gels.

402 To investigate the functional groups of egg proteins involved in protein polymerization,
403 Akazawa et al. (2021) analyzed the proteins' sulfhydryl and primary amino groups. The
404 formation of ortho-quinone through the oxidation of phenolic compounds can lead to the
405 binding of nucleophiles, such as amino or sulfhydryl groups of proteins. This can result in the
406 formation of C–N or C–S bonds, as discussed by Jongberg et al. (2011), Cao & Xiong (2015),
407 and Liu et al. (2017). Akazawa et al. (2021) found that combining olive leaf with egg white
408 protein leads to changes in certain chemical groups, forming larger protein molecules. Adding
409 0.2 grams of olive leaf per gram of egg white protein causes a 24% decrease in primary amino
410 groups and a 77% decrease in sulfhydryl groups. The researchers also observed a significant
411 97% reduction in a compound called oleuropein aglycone or decarboxymethyl oleanolic acid
412 linked to hydroxytyrosol (3,4-DHPEA-EDA) at 49.2 minutes when the compounds were
413 exposed to heat. This compound contributes to the olive leaf extract ability to bind proteins
414 together.

415 **Conclusions**

416 The application of OEx4 was highly effective in preventing egg gels' physical and
417 microbiological deterioration when subjected to freeze-thaw cycles. Thus, OEx4 helps improve
418 the storage stability of frozen egg products. This effect probably is due to the insertion of cross-
419 linkage into the egg protein gel network. The cross-linkage raised the WHC of the gel and the
420 robust gel state formation, as a result, the gel acquired high frozen tolerance.

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612

Tables and Figures

613 Table 1. Gel properties of whole egg gels containing OEx4

Items	Water content (%)	Expressible water (%)	Breaking strength		Viscoelasticity	
			Breaking stress (10^5 N/m ²)	Breaking strain (%)	Modulus of elasticity E_0 (10^4 N/m ²)	Coefficient of viscosity η_N [10^7 Pa·s]
Control	83.15±0.53 ^A	28.85±0.60 ^A	1.13±0.22 ^A	30.21±0.63 ^C	4.85±0.21 ^B	4.78±0.23 ^C
+0.03% OEx4	83.43±0.76 ^A	23.78±0.60 ^B	1.47±0.51 ^A	40.48±0.10 ^B	5.56±0.21 ^A	5.12±0.45 ^B
+0.1% OEx4	83.23±0.09 ^A	22.85±0.23 ^B	1.48±0.31 ^A	42.74±0.75 ^A	5.67±0.74 ^A	5.26±0.63 ^A

614 Data are presented as mean ±SD (n=7).

615 ^(A-C)Means having the same superscripts in the same column are not different ($p > 0.05$).

616 Table 2. Gel properties of egg white gels containing OEx4

Items	Water content (%)	Expressible water (%)	Breaking strength		Viscoelasticity	
			Breaking stress (10^5 N/m ²)	Breaking strain (%)	Modulus of elasticity E_0 (10^4 N/m ²)	Coefficient of viscosity η_N [10^7 Pa·s]
Control	87.04±0.23 ^A	30.85±0.41 ^A	0.99±0.32 ^B	30.98±0.33 ^C	3.85±0.14 ^B	3.78±0.21 ^B
+0.03% OEx4	86.97±0.42 ^A	28.76±0.08 ^B	1.35±0.22 ^A	32.25±0.11 ^B	4.47±0.16 ^A	4.32±0.56 ^A
+0.1% OEx4	87.01±0.25 ^A	27.19±0.03 ^B	1.37±0.44 ^A	34.14±0.15 ^A	4.56±0.32 ^A	4.47±0.67 ^A

617 Data are presented as mean ±SD (n=7).

618 ^(A-C)Means having the same superscripts in the same column are not different ($p > 0.05$).

Table 3. Changes in pH of whole egg gels containing OEx4 during Freeze Thaw Cycle

Items	Cycle 0	Cycle 1	Cycle 2	Cycle 3
Control	7.68±0.42 ^{Ab}	7.76±0.30 ^{Ab}	8.15±0.41 ^{Aa}	8.91±0.41 ^{Aa}
+0.03% OEx4	7.49±0.27 ^{Aa}	7.64±0.24 ^{Aa}	7.66±0.48 ^{Ba}	7.80±0.18 ^{Ba}
+0.1% OEx4	7.41±0.14 ^{Aa}	7.61±0.41 ^{Aa}	7.64±0.10 ^{Ba}	7.78±0.23 ^{Ba}

Mean ±standard deviation, n=7 per cycle and olive leaf extract concentration/ control group.

^(A-C)Means having the same superscripts in the same column are not different ($p > 0.05$).

^(a-c)Means having the same superscripts in the same row are not different ($p > 0.05$).

ACCEPTED

Table 4. Changes in pH of egg white gels containing OEx4 during Freeze Thaw Cycle

Items	Cycle 0	Cycle 1	Cycle 2	Cycle 3
Control	8.77±0.21 ^{Ab}	8.89±0.34 ^{Ab}	9.04±0.31 ^{Aa}	9.19±0.41 ^{Aa}
+0.03% OEx4	8.51±0.04 ^{Aa}	8.69±0.07 ^{Aa}	8.70±0.04 ^{Ba}	8.80±0.41 ^{Ba}
+0.1% OEx4	8.45±0.33 ^{Aa}	8.65±0.51 ^{Aa}	8.68±0.12 ^{Ba}	8.79±0.41 ^{Ba}

Mean ±standard deviation, n=7 per cycle and olive leaf extract concentration/ control group.

^(A-C)Means having the same superscripts in the same column are not different ($p > 0.05$).

^(a-c)Means having the same superscripts in the same row are not different ($p > 0.05$).

ACCEPTED

Table 5. Changes in total plate counts (Log CFU/g) of whole egg gels containing OEx4 during Freeze Thaw Cycle

Items	Total plate counts (Log CFU/g)			
	Cycle 0	Cycle 1	Cycle 2	Cycle 3
Control	2.90±0.42 ^{Ac}	2.96±0.30 ^{Ac}	3.15±0.41 ^{Ab}	4.91±0.41 ^{Aa}
+0.03% OEx4	2.69±0.27 ^{Aa}	2.74±0.24 ^{Aa}	2.77±0.48 ^{Ba}	2.80±0.18 ^{Ba}
+0.1% OEx4	2.41±0.14 ^{Aa}	2.61±0.41 ^{Aa}	2.64±0.10 ^{Ba}	2.78±0.23 ^{Ba}

Mean ± standard deviation, n=7 per cycle and olive leaf extract concentration/ control group.

^(A-C)Means having the same superscripts in the same column are not different ($p > 0.05$).

^(a-c)Means having the same superscripts in the same row are not different ($p > 0.05$).

ACCEPTED

Table 6. Changes in total plate counts (Log CFU/g) of egg white gels containing OEx4 during Freeze Thaw Cycle

Items	Total plate counts (Log CFU/g)			
	Cycle 0	Cycle 1	Cycle 2	Cycle 3
Control	2.67±0.41 ^{Ac}	2.82±0.43 ^{Ac}	3.94±0.56 ^{Ab}	4.19±0.33 ^{Aa}
+0.03% OEx4	2.51±0.05 ^{Aa}	2.67±0.08 ^{Aa}	2.70±0.42 ^{Ba}	2.80±0.21 ^{Ba}
+0.1% OEx4	2.45±0.47 ^{Aa}	2.65±0.40 ^{Aa}	2.68±0.21 ^{Ba}	2.79±0.29 ^{Ba}

Mean ±standard deviation, n=7 per cycle and olive leaf extract concentration/ control group.

^(A-C)Means having the same superscripts in the same column are not different ($p > 0.05$).

^(a-c)Means having the same superscripts in the same row are not different ($p > 0.05$).

ACCEPTED

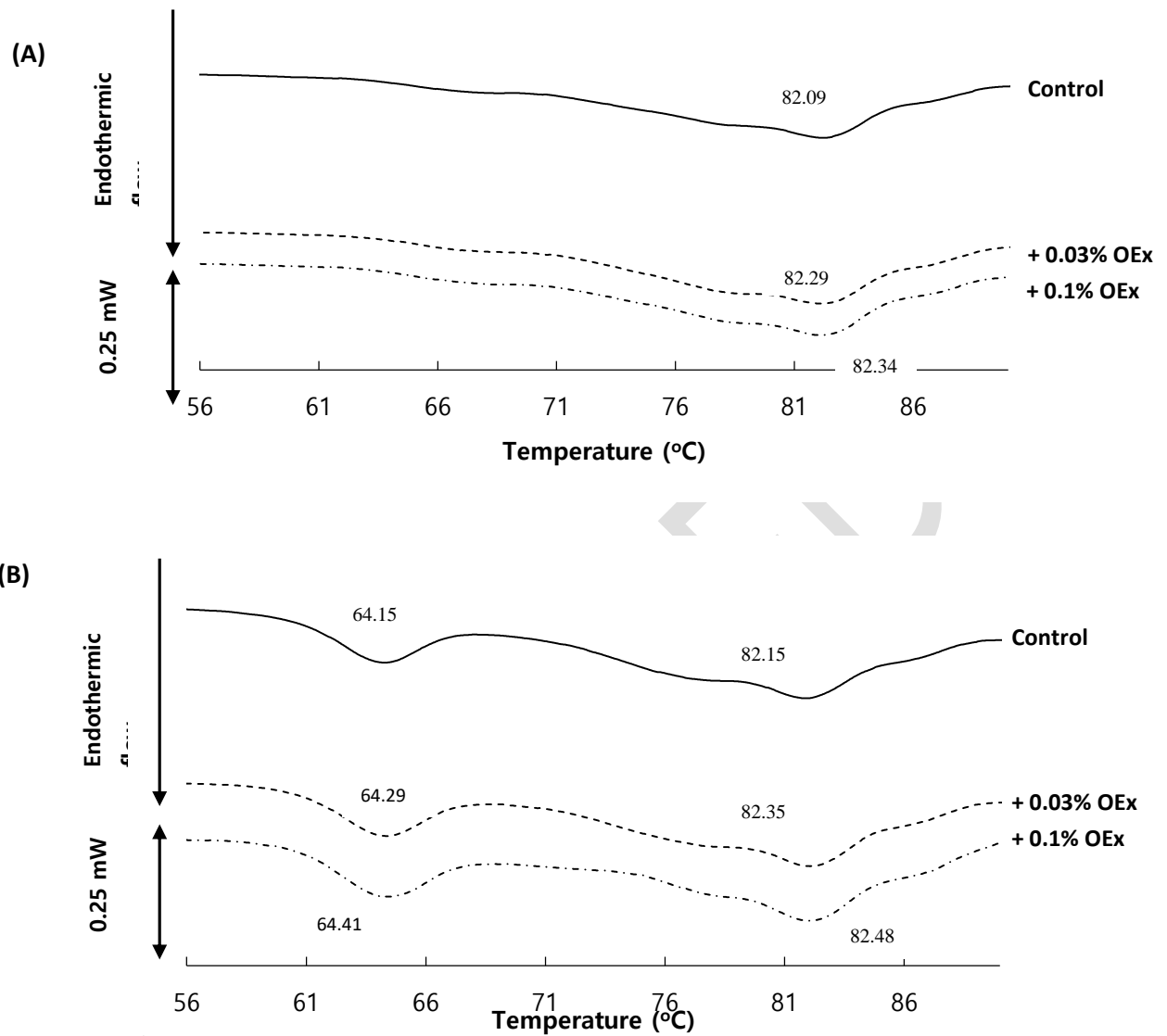


Figure 1. Whole egg (A); and egg white (B) solutions containing OEx4 on DSC Thermogram.

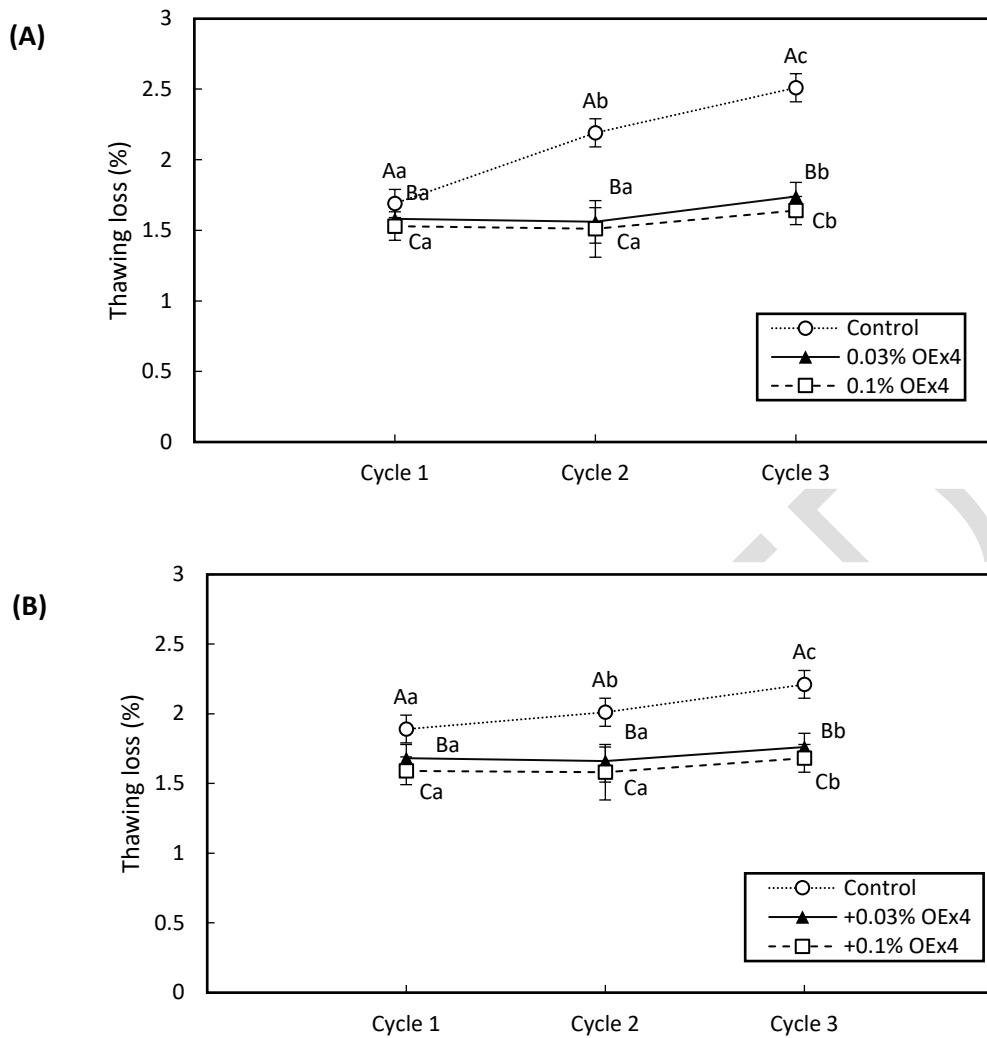


Figure 2. Whole egg gels (A); and egg white gels (B) containing OEx4 on thawing loss during freeze-thaw cycle.

Treatments of olive leaf extract concentration: Control; +0.03% OEx4; and +0.1% OEx4.

^(A-C)Means having the same superscripts for different samples within the same storage periods indicates not different ($p > 0.05$).

^(a-c)Means having the same superscripts for same samples within the different storage periods indicates not different ($p > 0.05$).

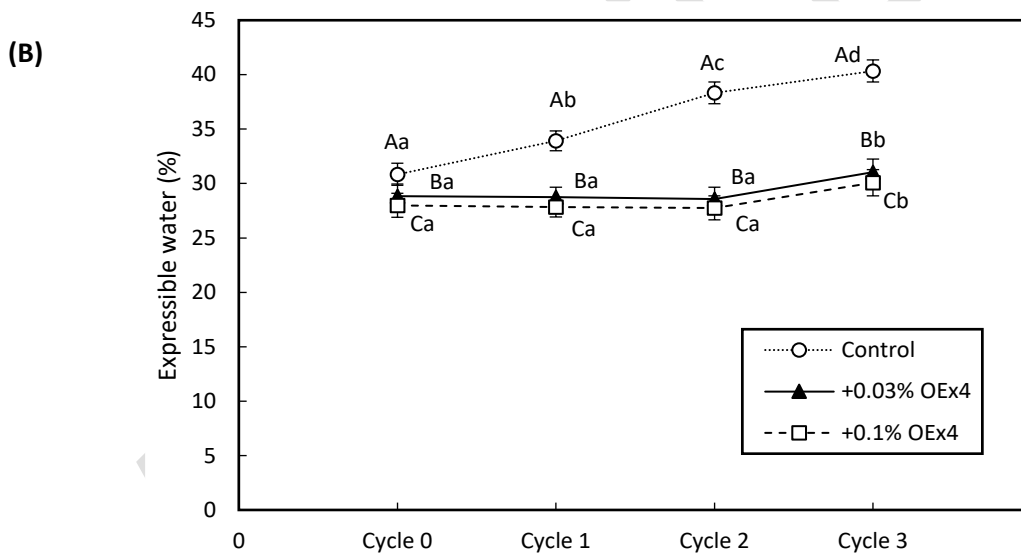
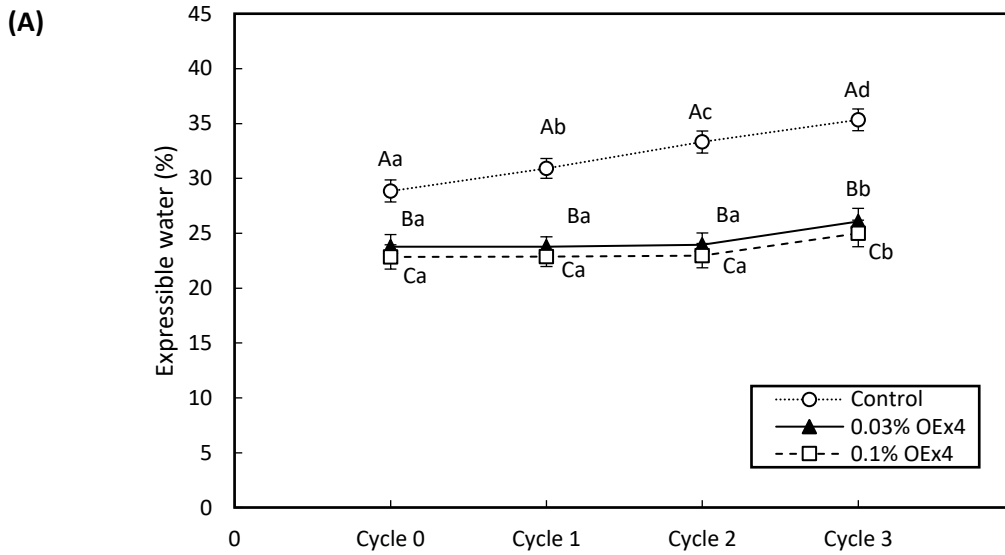


Figure 3. Whole egg gels (A); and egg white gels (B) containing OEx4 on water-holding capacity during freeze-thaw cycle.

Treatments of olive leaf extract concentration: Control; +0.03% OEx4; and +0.1% OEx4.

^(A-C)Means having the same superscripts for different samples within the same storage periods indicates not different ($p > 0.05$).

^(a-c)Means having the same superscripts for same samples within the different storage periods indicates not different ($p > 0.05$).

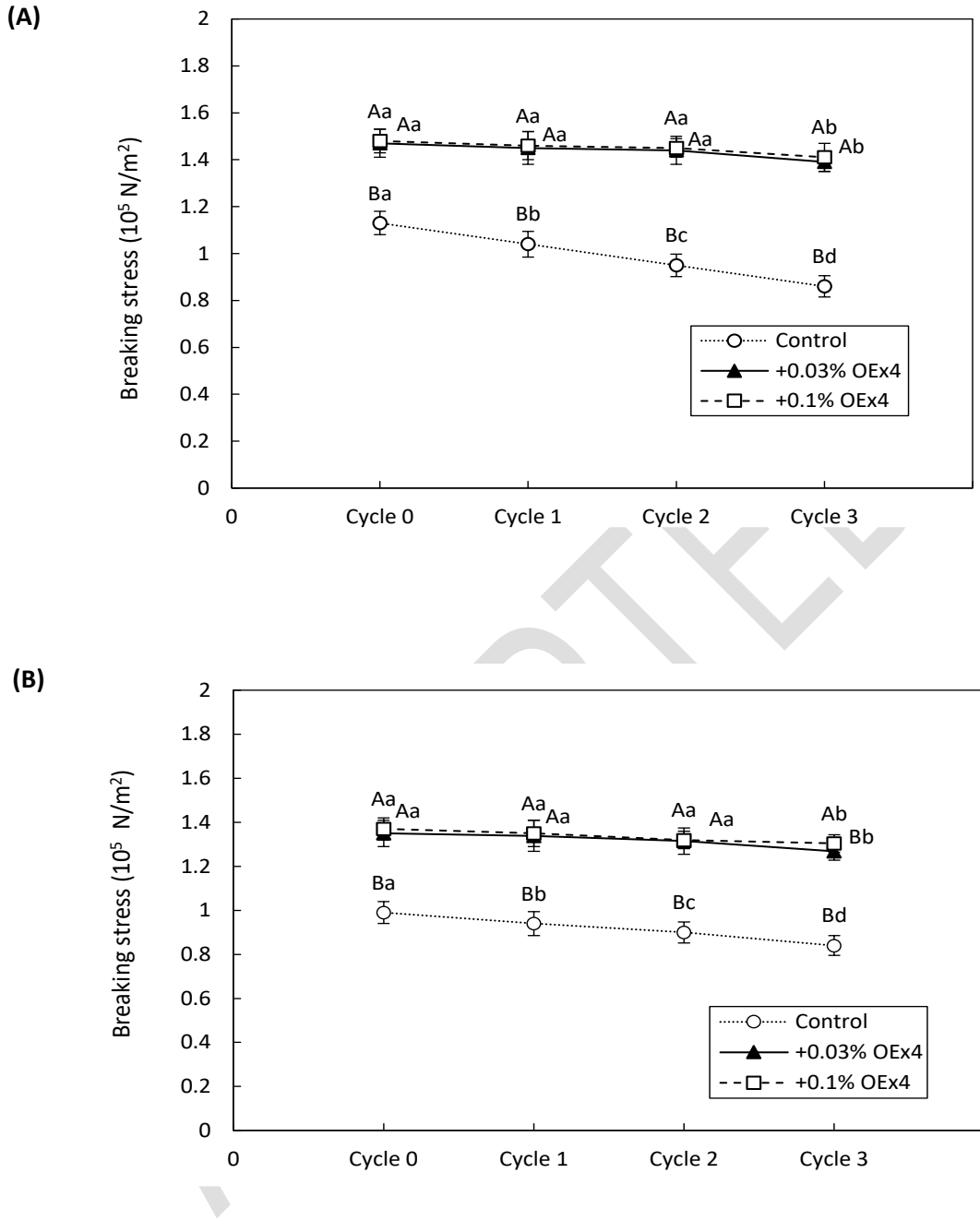


Figure 4. Whole egg gels (A); and egg white gels (B) containing OEx4 on breaking stress during freeze-thaw cycle.

Treatments of olive leaf extract concentration: Control; +0.03% OEx4; and +0.1% OEx4.

^(A-C)Means having the same superscripts for different samples within the same storage periods indicates not different ($p > 0.05$).

^(a-c)Means having the same superscripts for same samples within the different storage periods indicates not different ($p > 0.05$).

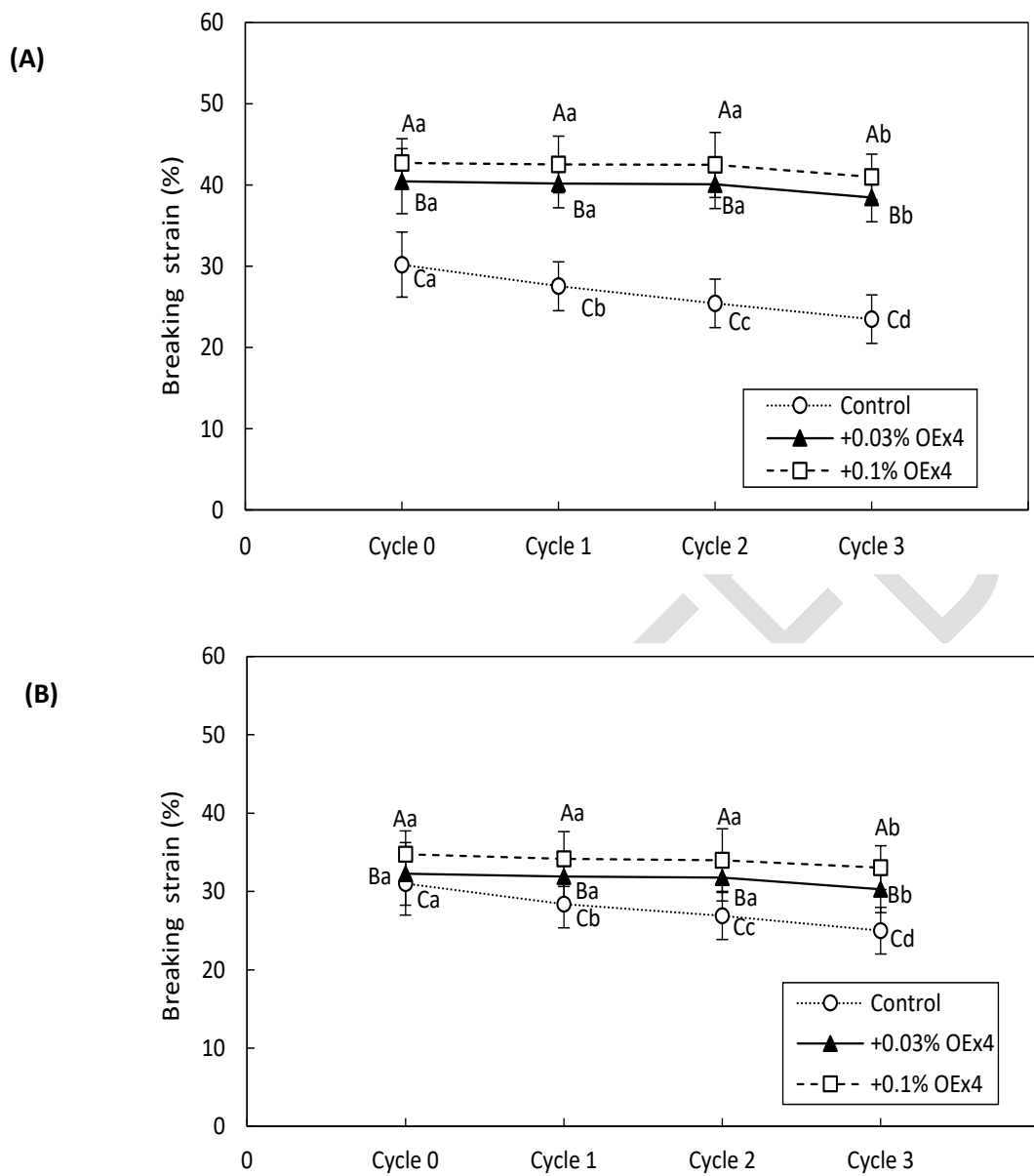


Figure 5. Whole egg gels (A); and egg white gels (B) containing OEx4 on breaking strain during freeze-thaw cycle.

Treatments of olive leaf extract concentration: Control; +0.03% OEx4; and +0.1% OEx4.

^(A-C)Means having the same superscripts for different samples within the same storage periods indicates not different ($p > 0.05$).

^(a-c)Means having the same superscripts for same samples within the different storage periods indicates not different ($p > 0.05$).

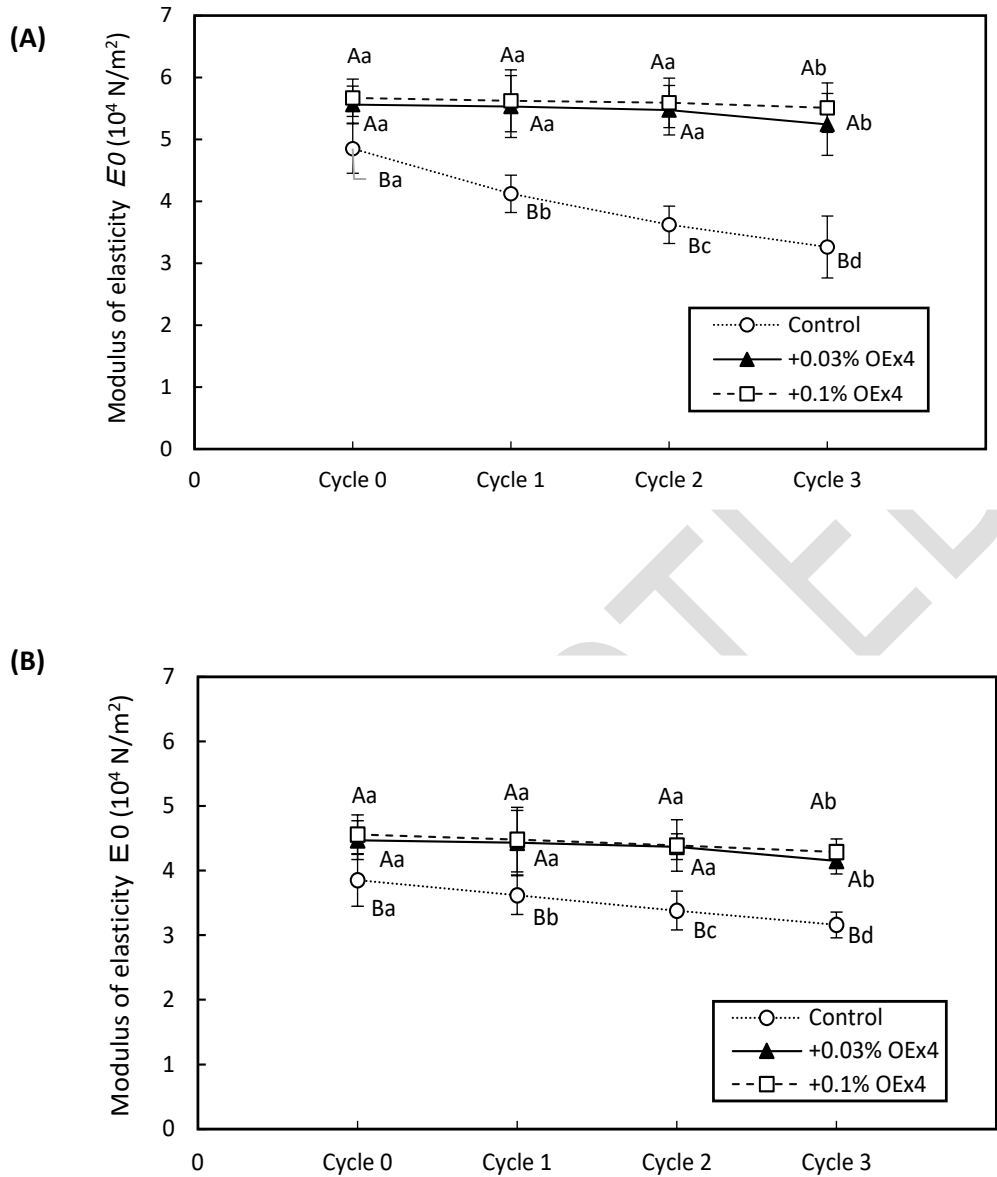


Figure 6. Whole egg gels (A); and egg white gels (B) containing OEx4 on modulus elasticity during freeze-thaw cycle.

Treatments of olive leaf extract concentration: Control; +0.03% OEx4; and +0.1% OEx4.

^(A-C)Means having the same superscripts for different samples within the same storage periods indicates not different ($p > 0.05$).

^(a-c)Means having the same superscripts for same samples within the different storage periods indicates not different ($p > 0.05$).

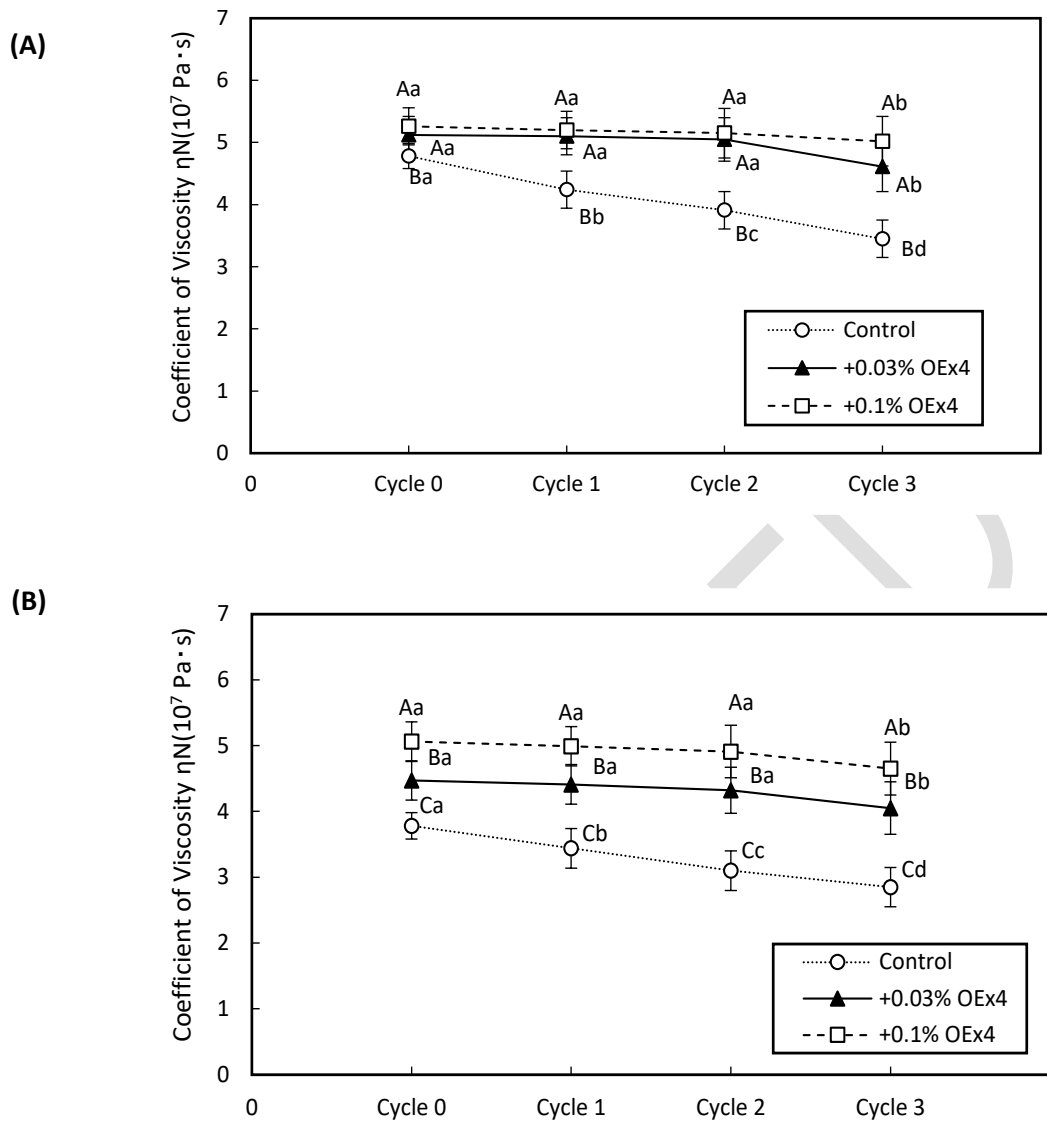


Figure 7. Whole egg gels (A); and egg white gels (B) containing OEx4 on coefficient of viscosity during freeze-thaw cycle.

Treatments of olive leaf extract concentration: Control; +0.03% OEx4; and +0.1% OEx4.

^(A-C)Means having the same superscripts for different samples within the same storage periods indicates not different ($p > 0.05$).

^(a-c)Means having the same superscripts for same samples within the different storage periods indicates not different ($p > 0.05$).

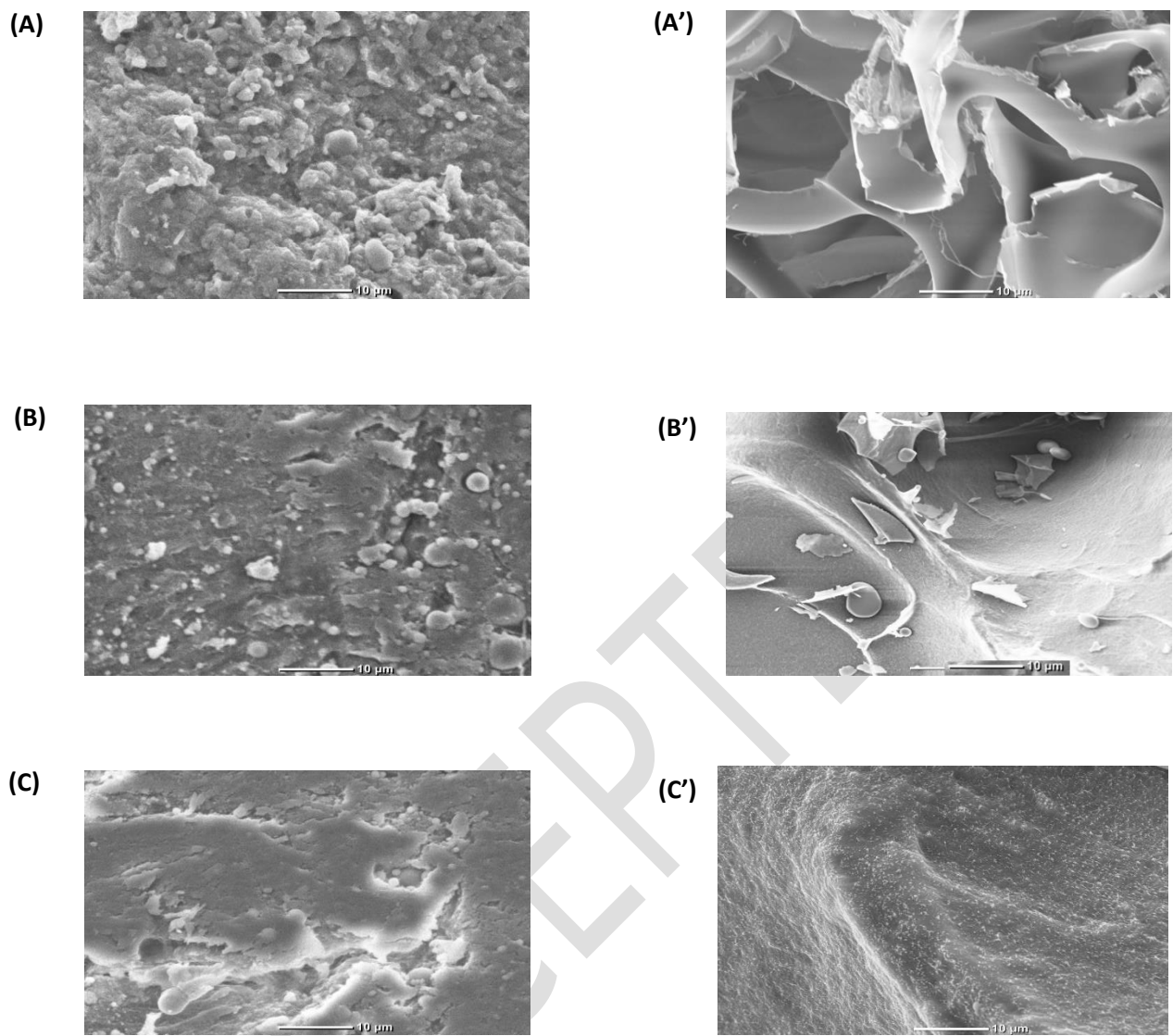


Figure 8. Microstructure of frozen-stored OEx4-WE gel (A) and (A'), control WE gel; (B) and (B'), 0.03% OEx4-WE gel; and (C) and (C'), 0.1% OEx4-WE gel. (A), (B), and (C) show micrographs of unfrozen WE gel, and (A'), (B'), and (C') show micrographs of WE gel thawed after Cycle 3 of freeze thaw. Scale bar, 10 μm .

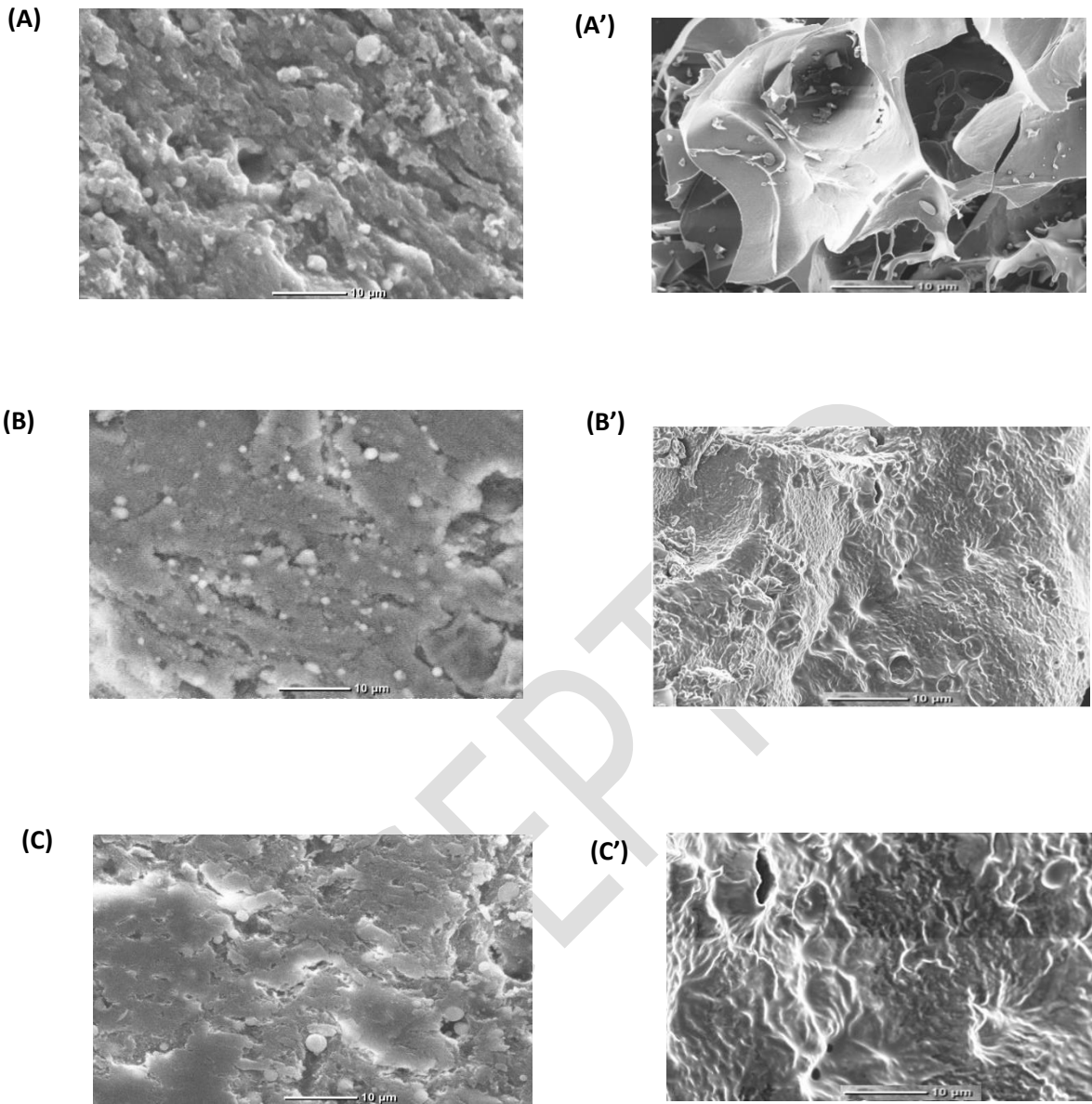


Figure 9. Microstructure of frozen-stored OEx4-EW gel (A) and (A'), control EW gel; (B) and (B'), 0.03% OEx4- EW gel; and (C) and (C'), 0.1% OEx4- EW gel. (A), (B), and (C) show micrographs of unfrozen EW gel, and (A'), (B'), and (C') show micrographs of EW gel thawed after Cycle 3 of freeze thaw. Scale bar, 10 μm .

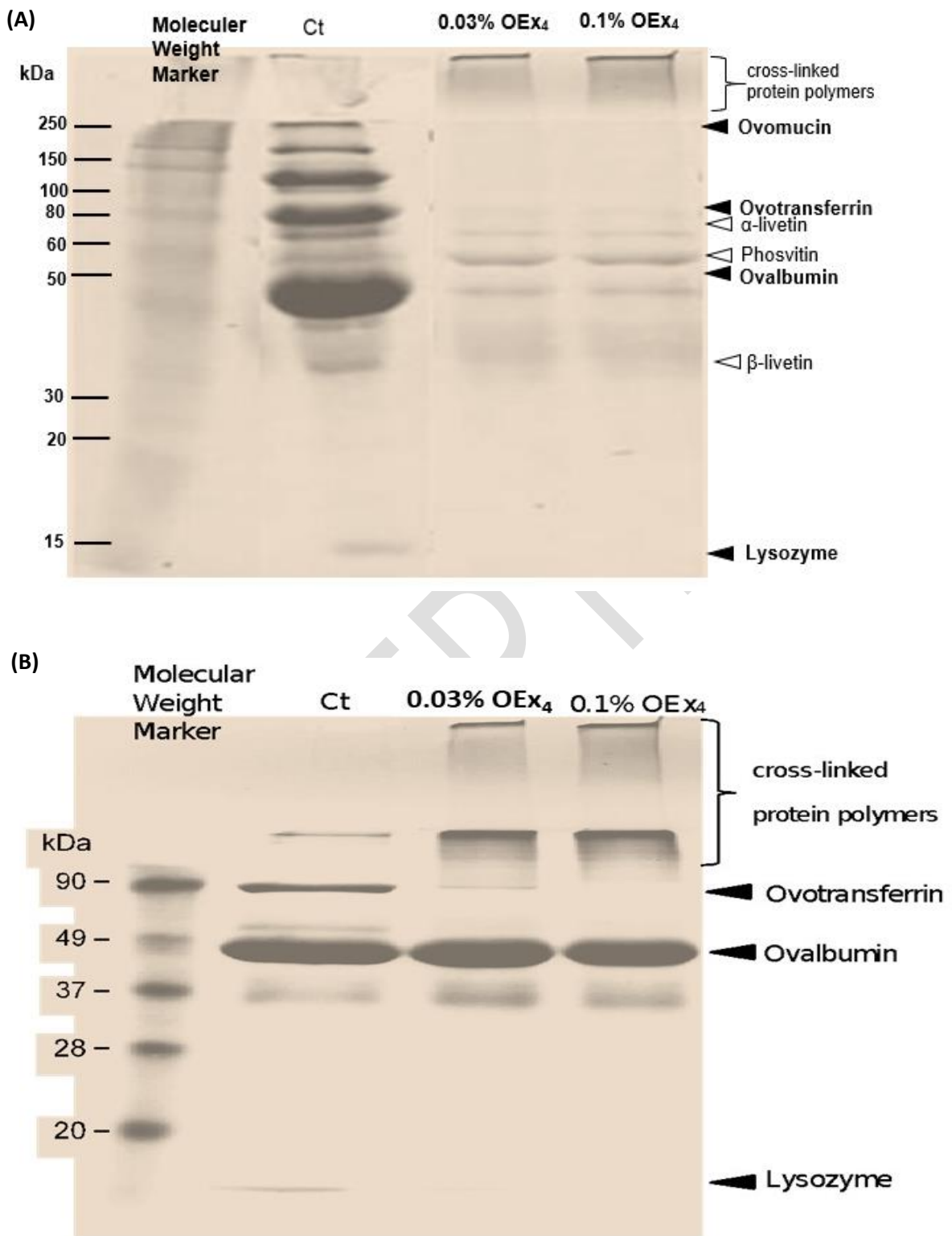


Figure 10. SDS-PAGE pattern of protein extracted from heat-treated OEx4-whole egg solution (A) and OEx4-egg white solution (B).