Electron Microscopic Analysis of the Effects of Tea Extract on Strength Improvement of Egg White Gels

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We examined the effects of several tea extracts containing certain polyphenols on the physical properties of egg white gel. The results of compression tests showed that it was possible to increase the strength of the egg white gel by treatment with various tea extracts. The image analysis of thin sections of egg white gels by transmission electron microscopy (TEM) indicated that the network filaments were larger in the modified gel than in the egg white gel. The thickening of the filaments of the network structure suggests that tea extracts improve the strength of the egg white gel.

Keywords: egg white, electron microscopy, image analysis, polyphenol, protein gel

Introduction

As egg white has favorable functions such as bubble-forming properties, emulsification, and thermocoagulation characteristics, it is being used in the production of various processed foods. The egg white properties such as gel strength or transparency have been improved using various methods such as dry heat treatment (Matsudomi et al., 1991; 1993), addition of various polysaccharides (Matsudomi et al., 1997) and partial digestion by proteolytic enzymes (Kitabatake and Doi, 1985). Recently, egg whites with better bubble-forming properties and improved gel characteristics have been developed and commercialized, and they are used in foods such as fish paste, noodles, and processed meat products. Therefore, technology that improves the function of egg white is important in the food industry.

As phenolic compounds like tannin easily combine with protein, egg white protein has been used to remove surplus tannin in wine by coagulation (Vine, 1997; Sladman and Schmieder, 2002). As a reverse example, tannin in persimmons is used to remove unnecessary proteins in sake (Japanese wine). Complexes of anthocyanin and soybean protein have been prepared and reportedly have high radical-scavenging activity (Huang et al., 2004). Moreover, the protein structure can be easily modified by combination with various phenolic compounds (Haslam, 1998; Hagerman and Butler, 1981; Murray et al., 1994). As the modification of the protein structure can change the physical property of the solution or gel of the protein, optimizing the structure modification using phenolic compounds is thought to be a good technique for improving protein function.

In this study, we attempted to improve the gel characteristics of egg white protein by treatment with phenolic compounds in various tea extracts. We then analyzed the microstructure of egg white gels treated with tea extracts using electron microscopy.

Materials and Methods

Preparation of tea leaf extracts Leaves of black tea (Orange Pekoe, Twining & Co. Ltd, London, England), green tea (Otani Chya-en, Kyoto, Japan) and oolong tea (Kotani Kokufun, Kochi, Japan) were used for the extraction. Tea leaves (10 g) were suspended in 800 mL distilled water. After stirring at 300 rpm for 24 h at room temperature, the suspension was filtered through filter paper and centrifuged at 12,000 × g for 15 min. The supernatant was lyophilized to obtain 2-3 g of dry matter.

Preparation of egg white gels Dried egg white (Taiyo Chemical, Mie, Japan) was dissolved in water (8%, w/v) and centrifuged at 12,000 × g for 15 min. The supernatant (6.9-7.1% dry matter) was used to prepare egg white gels. First, the supernatant (35 mL) containing 7% egg white with or without 40 mg tea leaf extract was incubated in 50-mL disposable centrifuge tubes (Asahi Techno Glass, Tokyo,
Japan) at 45°C for 5 h. Subsequently, the mixture was put into a Teflon vessel (20 mm ø × 10 mm) and incubated at 80°C for 2 h to form a heat-denatured gel; 6 replicate gels were prepared at the same time. To prevent evaporation of the samples, the vessels were heated in sealed petri dishes (Jyusi Shale, Seibu, Higashiosaka, Japan) during incubation. Samples removed from the vessels were kept in the sealed petri dishes until the compression test.

Transparent and brittle gels were prepared from egg white solutions of varying pH, adjusted by adding small amounts of NaOH. The solutions were denatured without pre-incubation at 45°C. Since egg white solutions at alkaline pH were difficult to gel, these solutions were heated in Teflon vessels at 90°C for 2 h to form the gels.

Measurement of the physical properties of egg white gels Stress and strain characteristics of egg white gels were evaluated using a compression tester (Texo Graph, Japan Food R&D Institute, Kyoto, Japan). Each gel (20 mm ø × 10 mm) was compressed using a cylinder probe (0.5 cm², Teflon coated stainless steel) at a speed of 0.05 mm·s⁻¹. Breaking stress and breaking strain were evaluated by Texo Graph software for breaking stress analysis; the software automatically detects sudden declines in strain during gel compression as the point of breaking. The gel strength was evaluated by the value of breaking stress. Each sample was measured six independent times and the results are shown as the mean ± standard deviation.

Dynamic viscoelasticity of the egg white solution with or without tea leaf extracts was measured using a small-amplitude oscillation viscometer (Rheosol G-5000, UBM, Kyoto, Japan). Measurement conditions were as follows: sample volume, 1 mL; probe, corn and plate; corn diameter, 39.96 mm; corn angle, 2.007°; gap, 0.05 mm; sample temperature, 45°C; oscillation, 1 Hz; and deformation, 1°. To prevent evaporation of the sample during measurement, we sealed the sample vessel with low viscosity silicone oil (KF-96-10CS, Shin-Etsu Chemical, Tokyo, Japan). Dynamic viscoelasticity of the 8% egg white solution was <1 Pa (below 96-10CS, Shin-Etsu Chemical, Tokyo, Japan). As dynamic viscoelasticity of the 8% egg white solution was <1 Pa (below the level of accurate detection), we used 20% egg white solution for this experiment.

Electron microscopy For scanning electron microscopy (SEM), small pieces of gel sample (2 × 2 × 2 mm) were fixed with 2% glutaraldehyde solution for 2 h at room temperature. Samples were subsequently washed 3 times with 0.1 M sorbitol. Washed samples were fixed with 1% osmium tetroxide in 0.1 M sorbitol for 30 min at room temperature and washed 3 times with distilled water. After washing with distilled water, samples were dehydrated through a series of ethanol treatments, and dried further by freeze drying in t-butyl alcohol. After coating with gold in an ion sputtering apparatus, samples were observed with a scanning electron microscope (JSM 6460 A, JEOL, Tokyo, Japan).

For transmission electron microscopy (TEM), small pieces of gel sample (2 × 2 × 2 mm) were fixed with 2% glutaraldehyde solution for 2 h at room temperature. Samples were subsequently washed 3 times with 0.1 M sorbitol. Washed samples were fixed with 1% osmium tetroxide in 0.1 M sorbitol for 30 min at room temperature and washed 3 times with distilled water. After washing with distilled water, samples were dehydrated through a series of ethanol treatments, embedded in Quetol 651 resin (Nissin EM, Tokyo, Japan), and polymerized at 60°C for 24 h to form blocks. The blocks were sectioned on an ultramicrotome (Ultracut UCT, Leica, Vienna, Austria) and sections were stained with saturated uranyl acetate for 20 min followed by Sato’s lead citrate solution for 10 min. Sections were observed and photographed with an electron microscope (JEM 1200 EX, JEOL).

Image analysis TEM images were analyzed on a personal computer using image analysis software (Image J, NIH, Bethesda, USA). The threshold of the TEM images was adjusted to convert the original gray scale to black and white (binary image), and images of protein coagulates (black features) were analyzed as particles. To simplify the size distribution analysis of particles, we used the best-fit ellipse analysis of Image J, instead of the segmentation of each particle.

Statistical Analyses Statistical analysis was carried out for each measurement to determine significant differences between means by a t-test using Microsoft Excel.

Results and Discussion

Effects of several tea leaf extracts on stress and strain characteristics of egg white gels Table 1 shows the effect of various tea leaf extracts on breaking stress and breaking strain. The addition of the tea extracts increased the breaking stress and breaking strain of the egg white gel (Table 1); the breaking stress was increased 1.34-, 1.07- and 1.33-fold by adding extracts of green tea, oolong tea and black tea, respectively.

Table 1. Change in physical properties of egg white gels by several tea extracts.

<table>
<thead>
<tr>
<th>Tea extract</th>
<th>Breaking stress (Pa)</th>
<th>Breaking strain (%)</th>
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<tr>
<td>Blank</td>
<td>24000±1100</td>
<td>70±2</td>
</tr>
<tr>
<td>Green tea</td>
<td>32100±4300*</td>
<td>75±5</td>
</tr>
<tr>
<td>Oolong tea</td>
<td>25700±4400</td>
<td>73±5</td>
</tr>
<tr>
<td>Black tea</td>
<td>31800±4800*</td>
<td>80±4**</td>
</tr>
</tbody>
</table>

*,** Significantly different (p<0.05) from blank.
The increase in gel strength accompanied the increase in breaking strain in the presence and absence of black tea extract (Fig. 1), indicating that tea extracts increased flexibility, but not firmness of the egg white gels. As results were similar for each tea, we used only the black tea extract in the following experiments.

**Effects of black tea extract on viscoelasticity of egg white solutions** Strain modulus ($G'$) and loss modulus ($G''$) of egg white solutions containing black tea extract were slightly higher than those of the control (Fig. 2). In addition, viscoelasticity increased with time in the presence of the extract. Thus, changes in the physical properties of the egg white solution due to tea extract seem to affect the egg white gel.

**Analysis of modified egg white gels by SEM** Specimens of egg white gels showed a similar network structure of denatured egg white proteins by SEM (Fig. 3). A relatively thick network structure was sometimes seen in the egg white gel treated with tea extract compared with the gel not treated with tea extract; each SEM image showed no remarkable difference.

**Analysis of modified egg white gels by TEM** TEM images (Fig. 4A and B) showed cross sectional images of the protein coagulate corresponding to the network structure observed in the SEM images. The preliminary experiment of immuno-electron microscopy using anti-ovalbumin antibody revealed that the black dots in TEM images indicate egg white protein (data not shown). We assumed that the modification of the physical properties of the gels was related to the change in the network structure of the protein coagulates.

![Fig. 1. Stress and strain curves of egg white gels. Egg white gels with (----) or without (-----) black tea extract were tested using a compression tester. The gel was compressed using a 0.5-cm$^2$ cylinder probe at a speed of 0.05 mm·s$^{-1}$. Arrows indicate the breaking points of gels.](image)

![Fig. 2. Storage modulus (A) and loss modulus (B) of egg white solutions during incubation at 45$^\circ$C. Dynamic viscoelasticity of egg white solution with (----) or without (-----) black tea extract was measured using a small-amplitude oscillation viscometer. Measurement conditions: sample volume, 1 mL; probe, corn and plate; corn diameter, 39.96 mm; corn angle, 2.007°; gap, 0.05 mm; sample temperature, 45$^\circ$C; oscillation, 1 Hz; and deformation, 1°.](image)
(Clark, 1998) caused by the tea leaf extracts. Therefore, we quantitatively compared the size and number of protein coagulates on 2-dimensional images of ultra thin sections using image analysis software (Image J, NIH). The binary images of the modified gels revealed fewer protein coagulates in the gel (Fig. 4D) compared with the original egg white gel (Fig. 4C). Best-fit ellipse images acquired by particle analysis using Image J revealed that the size distribution of ellipses in the images of the modified gels was larger than that of the egg white gel (Fig. 4E and F). As the protein coagulates in the filaments of the network structure were simulated as ellipses, protein filaments appear to thicken due to tea extract treatment.

**Size distribution of the protein coagulates in the network filaments** From the results of particle analysis, we calculated the number of ellipses by size (pixels). Table 2 shows the size distribution of ellipses classified into four groups. Simulating the protein coagulates as ellipses revealed that the total number of protein coagulates was lower and the ratio of bigger protein coagulates was higher in the modified gels. The average size and number of ellipses (Table 3) calculated from three independent best-fit ellipse image analyses showed the same tendency in the size distribution of protein coagulates.

**Physical properties and microstructure of egg white gels at alkaline pH** To obtain more information about the

![Fig. 3. SEM images of egg white gel with or without black tea extract. Images of egg white gel with (B) or without (A) black tea extract are shown.](image)

![Fig. 4. TEM images of egg white gels and the results of image analysis. A and B, Thin section images of egg white gel with (B) or without (A) black tea extract. C and D, Binary images of (A) and (B), respectively. E and F, Images of best-fit ellipse analysis on (C) and (D), respectively.](image)

<table>
<thead>
<tr>
<th>Size of ellipses (Pixels)</th>
<th>Number of ellipses</th>
<th>Ratio (%)</th>
<th>Number of ellipses</th>
<th>Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 to 50</td>
<td>391</td>
<td>79.0</td>
<td>262</td>
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</tr>
<tr>
<td>51 to 100</td>
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<td>10.5</td>
<td>40</td>
<td>11.4</td>
</tr>
<tr>
<td>101 to 500</td>
<td>49</td>
<td>9.9</td>
<td>40</td>
<td>11.4</td>
</tr>
<tr>
<td>501 to 2500</td>
<td>3</td>
<td>0.6</td>
<td>10</td>
<td>2.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>495</strong></td>
<td><strong>100</strong></td>
<td><strong>352</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

*Significantly different (p<0.05) from blank.

Table 2. Size distribution of ellipses calculated from image analysis of TEM images.
relationship between physical property and microstructure of egg white gel, we prepared egg white gels at alkaline pH. The gelation mechanism of protein is highly dependent on factors such as pH and ionic strength (Hermansson, 1979). It has been reported that ovalbumin, the major protein of egg white, forms transparent and weak gels at alkaline pH (Hatta et al., 1986). Egg white solutions at alkaline pH were also found to form transparent and weak gels by heat-treatment (Fig. 5). As breaking stress and breaking strain were decreased, the egg white gels were thought to become brittle at alkaline pH. Figure 6 shows TEM images of egg white gels at alkaline pH. The protein coagulates decreased markedly in size with increasing pH (Fig. 6B and C). Moreover, the best-fit ellipse analysis of images showed remarkable differences in size and number of protein coagulates (Fig. 6E and F). Figure 7 shows the average size of ellipses in four independent images of best-fit ellipse analysis on TEM images of egg white gels at alkaline pH. The decrease in the size of the ellipses (Fig. 7) and gel strength (Fig. 5) with increasing pH indicates a close relationship between the size of protein coagulates and gel strength. As mentioned before, the size of the protein coagulates influences the thickness of the filaments in the network structure of the egg white gel. These results are more conspicuous than the results of the TEM analysis of Fig. 4. From the results of TEM image analysis, we considered that increasing the thickness of the filaments in the network structure increases the strength of the egg white gel.

Conclusion

The addition of extracts of various teas that contain polyphenols increases the strength of egg white gels. In gels modified by black tea extract, viscoelasticity of the solution is improved before gelation. Moreover, the analysis of thin sections of egg white gels by TEM indicate that the filaments in the network structure are thicker in the modified gel than

Fig. 5. Transparency and strength of egg white gels at alkaline pH. A, Image of egg white gels. B, Means ± SD of six independent compression tests. *Significantly different (p<0.01).

Fig. 6. TEM images of egg white gels at alkaline pH. A-C, Thin section images of egg white gel at pH 7 (A), pH 9 (B), and pH 10 (C). D-F, Images of the best-fit ellipse analysis on images A-C, respectively.

Fig. 7. Size of ellipses in TEM image analysis of egg white gels at alkaline pH. Means ± SD of ellipse size (pixels) calculated after best-fit ellipse analysis on four independent TEM images of egg white gels at alkaline pH are shown. *Significantly different (p<0.01).
in the egg white gel, which is confirmed quantitatively by the size and number of protein coagulates in the filaments of the network structure calculated from the image analysis of ultra thin section images.

References