DOI:10.1021/jf900495n

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Histological Structures of Native and Cooked Yolks from Duck Egg Observed by SEM and Cryo-SEM

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A method was used to fix duck egg yolk while retaining its original sol structure to elucidate the fine structure of native yolk by using fixation with liquid nitrogen and cryo-scanning electron microscopy (cryo-SEM). Native yolk spheres showed a polyhedron shape with a diameter at approximately 50 to 100 μm and packed closely together. Furthermore, the interior microstructure of the native yolk spheres showed that a great amount of round globules ranging from 0.5 to 1.5 μm were embedded in a continuous phase with a lot of voids. After cooking, the sizes of the spheres were almost unchanged, and the continuous phase became a fibrous network structure observed by SEM with chemical fixation probably constituted of low density lipoprotein (LDL). The fine structure of the native yolk can be observed by cryo-SEM; however, the microstructure of yolk granules and plasma from cooked shell eggs can be observed by SEM with chemical fixation.

KEYWORDS: Duck egg; yolk sphere; yolk granules; cryo-SEM

INTRODUCTION

Hen eggs have useful functional properties, such as foaming, emulsifying, whipping, gelling, and coagulability, etc., which are widely used in the food industry (*1*−*3*). Numerous studies have reported on the physical properties of hen eggs and chemical analysis of components (*4*−*6*). Although histological studies have also been reported for a long period of time (*7*−*9*), there are few findings related to fresh native yolks and cooked yolks from shell eggs (*10*, *11*). Such information is needed to understand the structural changes that may occur during storage, cooking and processing and their relationship to functional properties.

The native yolk is a viscous fluid which, is composed of a count of yolk spheres, and these spheres are closely packed in the vitelline membrane (*7*). The hen egg yolk is composed of white yolk spheres and yellow yolk spheres varying in size and content (*12*). Yellow yolk spheres are further classified into light yellow and dark yellow yolk spheres, which are alternately and concentrically layered on each other. However, some studies argue that the yolk mass does not have a concentric structure (*7*, *8*). Yolk spheres are in the pattern of uniform polyhedrons (*10*, *11*). Each of the spheres consists of continuous and discontinuous phases like an emulsion (*13*). The continuous phase is a dispersion consisting of LDL and livetins (*7*, *9*). Spheres with 4−150 μm and granules 0.3−2.0 μm in diameter comprise the discontinuous phase (*7*).

In previous studies, fresh native yolk, which was in the form of a sol and was relatively large, had to be fixed as a whole, and several days were required for fixation (*7*−*9*, *11*). Yolk specimens were fixed by conventional fixation methods with a 10% acrolein solution for 5−7 days and then frozen sections (*8*) or those that were similarly fixed with a 10% formaldehyde solution for 1 week (*14*) were observed. Another study reported that a yolk was fixed by freeze-cutting method with liquid nitrogen, sliced, prefixed with 1% paraformaldehyde and 2.5% glutaraldehyde, then postfixed with 1% osmium tetraoxide, dehydrated, and observed by SEM (*11*). In the latter study, it took about 7 days for sample preparation, and the fixation in the chemical process would induce the morphological changes of yolk spheres. To fix the yolk without deformation by the conventional method, the whole yolk had to be soaked in the fixing solution. When fixed by this method at 5 °C for 7 days, however, only the peripheral part of the yolk (5 mm diameter) was fixed, while the core (about 10 mm) remained unfixed. In the improved freeze-cutting fixation method used by Mineki and Kobayashi (*11*), the peripheral part of the yolk was slightly dissolved, while the inside was inadequately fixed. Furthermore, there have been problems with physical fixation at an extremely low temperature with damage due to freezing inside the specimen. The yolk would be irreversibly converted into a gel when stored at −10 °C for a long time (*15*). To overcome the problem of ice damage, it was necessary to minimize the time in the temperature zone where freezing would occur (−10 to −20 °C). Therefore, a fine structure of the native egg yolk was almost impossible to observe because of its sol structure.

However, the microstructure of cooked egg yolk observed by SEM was much easier than that of native yolk due to its solid structure. The cooked yolk was prepared for SEM by two

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 Published on Web 4/9/2009

methods: First, the sample was cut into small pieces and prepared by glutaraldehyde fixation and osmium−tannic acid−uranyl acetate postfixation (*10*, *16*). After fixation, the sample was dehydrated in ethanol, defatted in chloroform, and critical point dried with CO_2 as the transition fluid. Second, the sample was frozen, freeze-dried, and subsequently defatted in chloroform (*16*). The authors demonstrated that freezing and freeze-drying introduced artifacts due to ice crystal damage and that the removal of fat from egg yolk was essential for the observation of protein matrices. Furthermore, the crumbliness of yolk from cooked shell eggs was attributed to the adjoining polyhedral spheres with no evidence of cross-linking (*10*). However, the microstructure of the interior of the yolk sphere from cooked shell eggs remains unknown.

The functional properties of duck eggs are rarely studied probably because the yields are quite lower than those of hen eggs; therefore, the duck eggs are not practically applied to the food industry. In Taiwan, the duck eggs are usually manufactured as salted egg yolks and pidan (thousand year eggs), which are traditional Chinese products and are very popular in Asia (*17*). Generally, salted eggs can be made by brining whole eggs in saturated saline or by coating the egg with soil paste mixed with salt for about 15 to 30 days and then cooking the eggs at 85 °C for 90 min to obtain the product (*18*). The desirable characteristics of salted egg yolka include orange color, oil exudation, and gritty texture. Because the salted egg yolk becomes solidified and hardened after pickling, the microstructure of the yolk can be easily observed by a microscope or electron microscope using chemical or freeze-drying fixation (*17*, *19*). However, the fresh duck yolk needs to be fixed and observed properly without affecting its original structure. A previous study has reported that the chemical compositions of hen and duck egg yolks were similar (*20*). Although there was no information obtained about the chemistry of the origin of spheres and polyhedrons in duck egg yolks, the knowledge of hen egg yolks could be used to explain that of duck egg yolks.

The greatest advantage of the cryo-SEM over any other technique is that it enables us to closely examine the frozen liquid and very soft specimens (*21*). We suggest that the fresh native egg yolks completely fixed in liquid nitrogen (−196 °C) in a short time would retain their original structure, and then cryo-SEM could be used to observe their fine structure. Therefore, the aim of this study was to examine the method for fixing fresh duck yolk by liquid nitrogen and for observing its microstructure by cryo-SEM while retaining its original structure. A comparative microstructure of the yolk from cooked shell duck eggs (85 °C, 90 min) was observed by SEM with conventional fixation in the chemical process and cryo-SEM with liquid-nitrogen fixation.

MATERIALS AND METHODS

Duck Eggs. One-day old duck eggs produced by a genetically closed flock of *Anas plotyrhyncus* (Tsaiya) were purchased from a local retail market. These eggs weighing 65 to 75 g were washed with water and then drained for 30 min. All experimental eggs were observed within 12 h after purchase. All experiments were conducted in triplicate.

Cryo-SEM Observation of Native Yolk Specimens. A duck egg was thrown and immersed in liquid nitrogen for about 20 min to make sure the whole egg was entirely frozen. The egg was then fractured by striking with a chisel in liquid nitrogen (*22*). The yolk fraction was approximately picked at $3 \times 3 \times 3$ mm^3 in size, loaded on the cryo-specimen holder with colloidal carbon, cryo-fixed in slush nitrogen (−210 °C), then immediately transferred into the vacuum space of the cryo-unit in the frozen state. After coating with gold, the specimen was directly observed by cryo-stage (−176 °C) SEM (ABT-150S, TOPCON, Japan) with a temperature controller (CryoTrans System, Model E7400, BioRad, England) under an accelerating voltage of 5 kV.

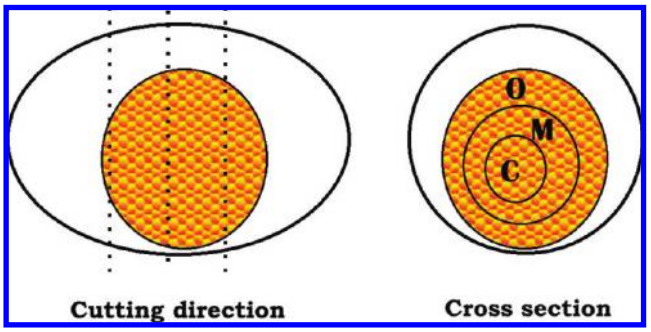

Figure 1. Sampling region of the yolk for SEM. The sample for SEM was prepared from the outer region (O); middle region (M); and center region (C).

Preparation of Cooked Yolk. Shell duck eggs were heated at 85 °C for 90 min in a water bath and then cooled in cold water at 25 °C for 60 min.

Scanning Electron Microscopy. The cooked egg yolks were sliced with a knife into pieces of about to $2 \times 2 \times 5$ mm^3 from outer, middle, and center regions, respectively, according to the method shown in **Figure 1**. In order to prevent sample disintegration during the experimental process, specimens were encased in 3% agar prior to fixation (*16*). Then, specimens were prefixed by 4% glutaraldehyde in 0.075 M phosphate buffer (pH 7.0) at 4 °C for 8 h. After being rinsed with the same buffer for 3 times, each time for 10 min, each specimen was postfixed by 1% osmium tetroxide in the same buffer at 4 °C for 24 h. After being rinsed with the same buffer 2 times, each time for 10 min, postfixed specimens were dehydrated successively in 20, 40, 60, 80, 95, 100, 100, and 100% ethanol, each for 30 min. Samples were critical point-dried with CO_2 as the transition fluid, fractured, mounted, coated with 200 Å gold−palladium, and examined in a SEM (ABT-150S, TOPCON, Japan). The accelerating voltage was 15 kV.

Cryo-SEM. The cooked egg yolks were sliced with a knife into pieces of about $2 \times 2 \times 5$ mm^3 from the middle region according to the method shown in **Figure 1**. The yolk fraction was loaded on the cryo-specimen holder with colloidal carbon, cryo-fixed in slush nitrogen. The following procedure was the same as that described in the previous section.

RESULTS AND DISCUSSION

Native Yolk: Cryo-SEM. For preparing the specimens of fresh native yolk, the whole egg was immersed in liquid nitrogen and rent during freezing. Owing to the rigid and fragile texture of the yolk, a chisel was used to bore the middle region of the yolk (as shown in **Figure 1**) to obtain the specimens. Regarding the structure of the whole yolk, the vitelline membrane was closely packed with yolk spheres similar to those previous reported (*11*, *14*). However, no alternate layered structures of light and dark yolk spheres could be morphologically distinguished. The yolk spheres showed a definite polyhedral structure, and the shape and size of the polyhedrons varied throughout. The yolk spheres in the outer and middle regions of the fresh hen egg yolk were 22 and 60 μm, respectively (*11*).

Native yolk spheres showed the polyhedron shape with a diameter at approximately 50−100 μm and were packed closely together (**Figure 2**). The previous study reported similar results of the polyhedron shape and size of the spheres from native duck yolk by chemical fixation (*19*); however, the surface and edge of the spheres were more uneven and less angular, respectively, compared to those in the present study. Also, sticky components observed in the intervals between the spheres in the previous study were not found in **Figure 2**. We suggested that the yolk spheres were morphologically changed. The result may be due to the different fixation methods used for specimen preparation. The yolks were frozen, cut into a piece of 0.5×0.5 cm, prefixed in 2.5% glutaraldehyde at room temperature, postfixed in 0.1%

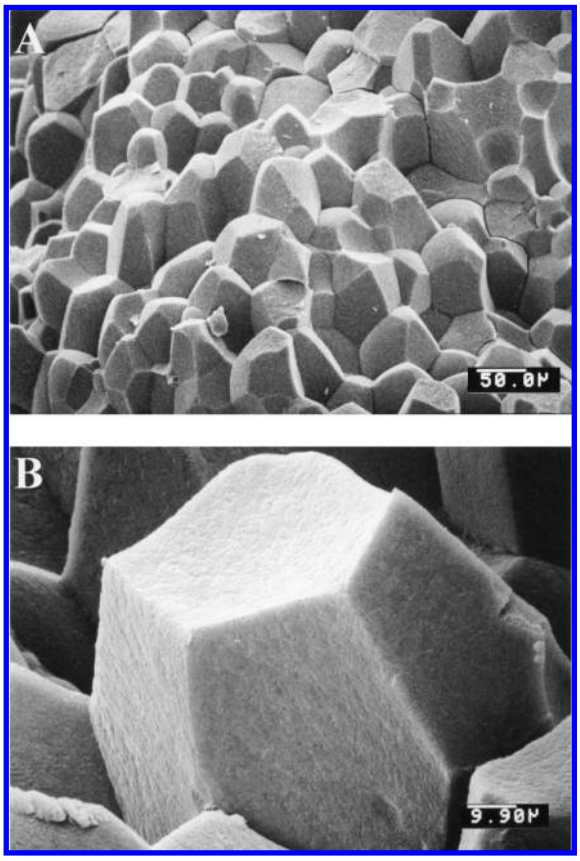

Figure 2. Micrographs of native yolk spheres in the middle region observed by cryo-SEM (**A**) at lower magnification and (**B**) at higher magnification.

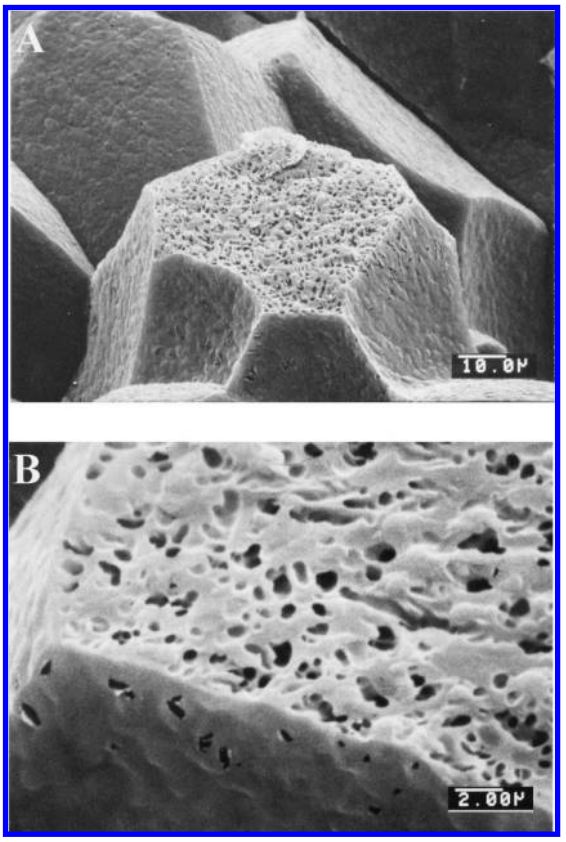

Figure 3. Micrographs of the interior structures of native yolk spheres observed by cryo-SEM (**A**) at lower magnification and (**B**) at higher magnification.

osmium solution, and then dehydrated in gradient concentrations of ethanol solutions (*19*). Therefore, during prefixation, the frozen yolk may melt, and the peripheral part was slightly dissolved (*11*). The latter study reported that the fixation in the chemical process would induce the morphological changes of yolk spheres.

A yolk sphere with its interior exposed is shown in **Figure 3A**. A micrograph of the interior structure of the sphere is shown at higher magnification in **Figure 3B**. The surface of native yolk spheres showed such a membrane structure with irregular veins. Although an interface was apparent, the definite membrane structure may not be concluded (*7*). The interior microstructure of the native yolk spheres showed that a great amount of round globules ranging from 0.5 to 1.5 μm were embedded in a continuous phase with a lot of voids (**Figure 3B**). These round globules were tentatively identified as yolk granules on the basis of their size and shape. Previous studies have reported that the granules ranging in diameter from 0.3 to 1.64 μm present in a continuous phase were observed by SEM (*10*), transmission electron microscopy (TEM) (*11*), light microscopy (*10*), and phase contract microscopy (*7*). The continuous phase (also called plasma), which is an emulsion, mainly consisted of LDL and livetin (*13*). This is the first study to present the microstructures of native duck yolk granules and plasma by SEM. Because of the fixation by liquid nitrogen and observation by cryo-SEM, the morphology of the yolk constitutes may not change under this condition. Furthermore, fixation in the chemical process (including dehydration by ethanol solutions) might induce morphological changes and yolk constitute (especially lipids) loss (*11*).

Cooked Yolk: SEM. Figure 4A−C shows the microstructures of yolk spheres in outer, middle, and center sections, respectively, of the cooked egg yolk observed by SEM. Yolk spheres in all regions were presented in a pattern of uniform polyhedrons ranging from 30 to 75 μm, and they were closely packed with neither voids nor cross-linking (*10*). The yolk spheres in the outer and center sections (about 50 μm in major diameter) were slightly smaller than those in the middle section and were rounded. The surface of the yolk spheres was very slightly uneven but almost smooth. The yolk spheres in the middle section were slender polyhedrons with a relatively uniform size 60 μm in major diameter. The surface of these spheres were slightly uneven but quite smooth (**Figure 4B**). The sizes of the cooked yolk spheres were almost unchanged compared to those of native yolk spheres (**Figure 2**).

The sizes of yolk spheres in the outer and middle regions of the fresh hen egg yolk were 22 and 60 μm, respectively (*11*). Furthermore, the yolk spheres in the center region were larger and more slender than those in the outer region. The yolk spheres of the cooked hen egg yolk showed a size of 60 $\times$ 70 μm (*10*). The fresh duck yolk had polyhedral spheres with a size range of 50 to 100 μm (*19*), which is similar to our result. The results showed that the distribution of yolk sphere sizes in various sections of duck eggs differed from those in hen eggs.

Figure 5A−D shows both surface and interior microstructures of spheres of cooked duck egg yolks observed by SEM. The surface of yolk spheres is constituted with embedded round globules and even holes, and shows a continuously lumpy appearance (**Figure 5A**,**B**). The round globules, ranging from 0.5 to 2.0 μm in diameter, were scattered over the surface. These may have been yolk granules based on their size ranging from 0.2 to 2 μm and shape (*22*). Fujii et al. (*14*) found that yolk spheres were surrounded by walls made of viscous substances. The result showed that yolk spheres of cooked duck yolks were

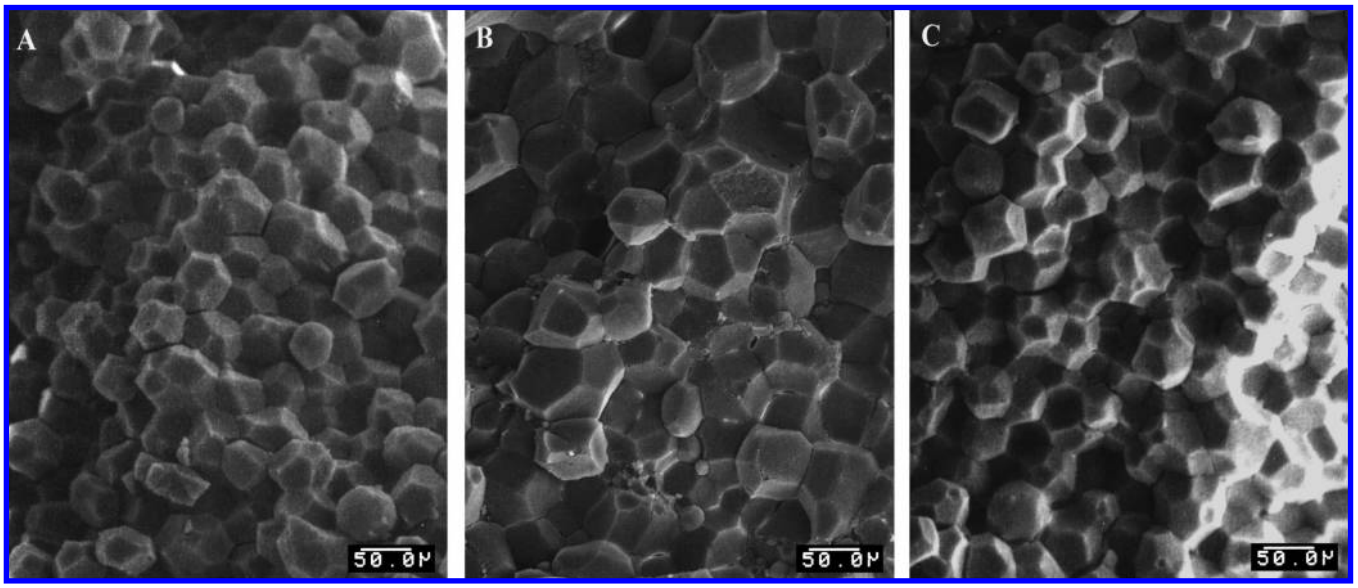

Figure 4. Micrographs of cooked yolk spheres in the (**A**) outer region; (**B**) middle region; and (**C**) center region observed by SEM with chemical fixation.

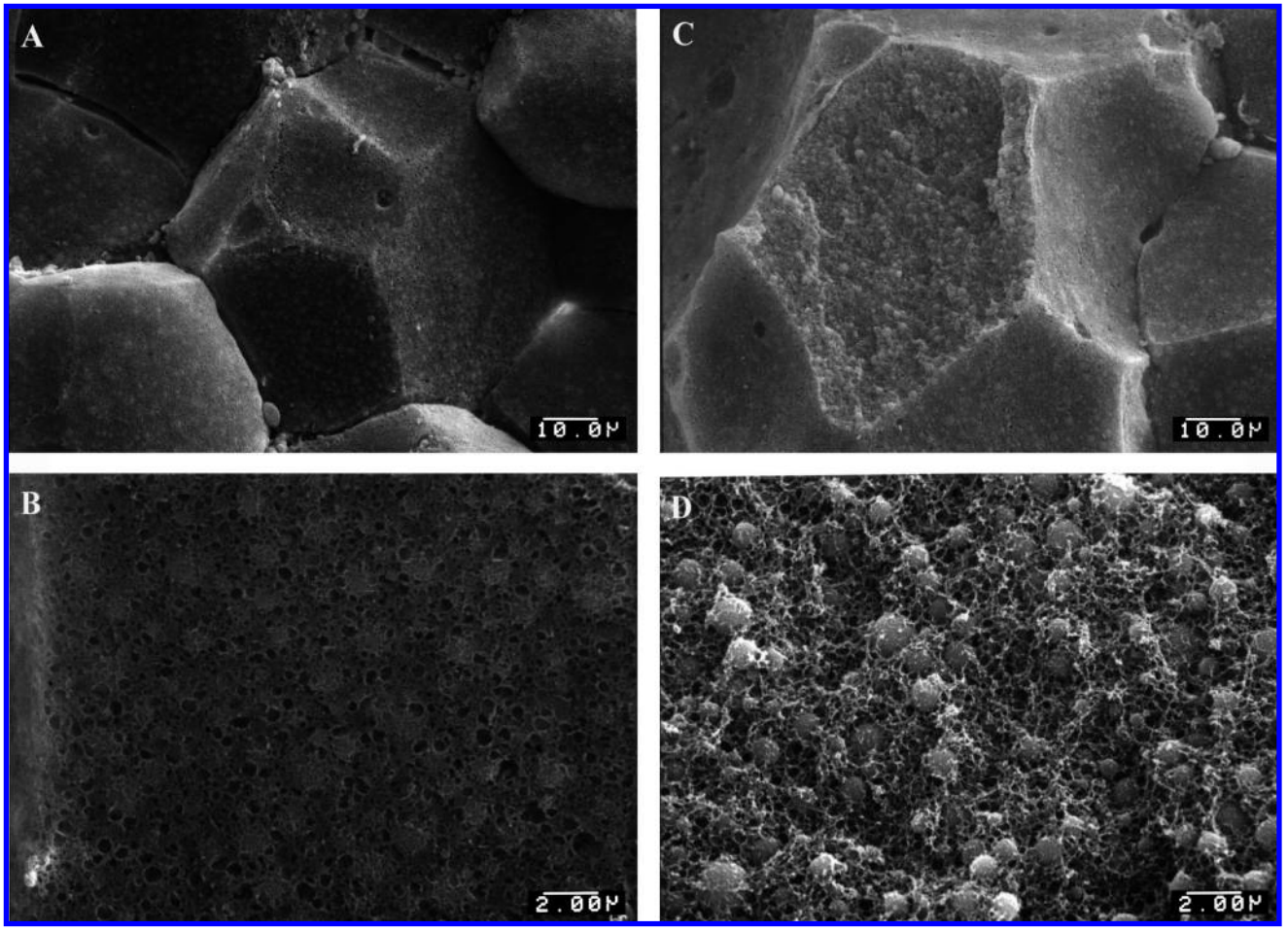

Figure 5. Micrographs of the surface (**A**,**B**) and interior (**C**,**D**) structures of cooked yolk spheres observed by SEM with chemical fixation at (**A**,**C**) lower magnification and at (**B**,**D**) higher magnification.

not surrounded by definite membranes. A similar finding was reported that the yolk spheres had a naked surface and no definite membrane structure by Mineki and Kobayashi (*11*). Also, Bellairs (*7*) found three different surface conditions: the lamellated capsule, the unit membrane-like structure, and the naked surface, and he also stated that it was difficult to decide which of these best represented the original state of the yolk spheres, although reasons were given for believing that yolk spheres were not normally enclosed by membranes identical to cell membranes.

The interior microstructure of the cooked yolk spheres showed that a great amount of round globules ranging from 0.4 to 1.8 μm were trapped in a fibrous network structure (**Figure 5C**,**D**). Also, these round globules were tentatively

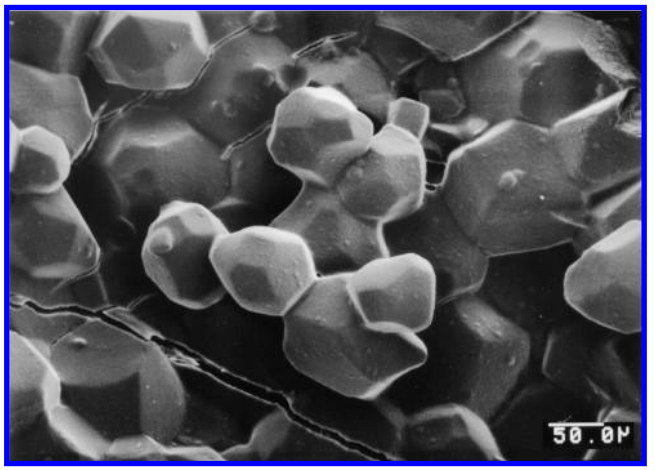

Figure 6. A micrograph of cooked yolk spheres in the middle region observed by cryo-SEM.

identified as yolk granules on the basis of their size and shape. Previous studies reported that the granules were present in a continuous fluid phase (*7*, *10*, *11*), although the results in the present study showed the granules were trapped in a fibrous network structure such as cobwebs. Woodward and Cotterill (*16*) also found a fibrous matrix inside the yolk spheres of egg yolk gels prepared by defatting and freeze-drying; however, the authors stated that the void spaces observed were attributed to ice crystal damage. As compared to the observation in the present study, the structure of the fibrous matrix was different from that of the fibrous network. The fibrous network should be contributed by LDL (*23*). A previous study also reported that the formation of a three-dimensional protein network with a hard, cohesive, rubbery texture occurred in the yolk from the cooked shell hen egg by heating at 85 °C for 30 min (*10*).

Cooked Yolk: Cryo-SEM. Figure 6, and **Figure 7A**,**B** show the yolk granules and surface and interior microstructures of cooked duck egg yolks observed by cryo-SEM, respectively. As compared to the morphology of duck yolk spheres shown in **Figure 4**, the size and shape of yolk spheres were similar to that shown in **Figure 6**. The surface of the yolk spheres showed exhibition of many prominences in various sizes ranging from 0.5 to 5 μm (most of them were 0.5 to 2 μm) (**Figure 7A**). According to the shape and size of the prominences, we suggest that they are yolk granules. In **Figure 7B**, several spherically shaped structures 0.5 to 1.5 μm in diameter cluttered a background, which consisted mostly of 0.2 μm particles. A previous study also reported that in the yolk of cooked shell hen eggs, several granules from 0.5 to 1.5 μm in diameter were held in a background of the small globules (0.1−0.2 μm) (*10*). The spherically shaped structures are suggested to be yolk granules according to their sizes. The interior microstructures of yolk spheres observed by SEM with chemical fixation (**Figure 5**) and cryo-SEM (**Figure 7**) were quite different. We suggested that the small globules, which might be lipid drops and lipoproteins, were partially dissolved and lost during chemical fixation and dehydration (**Figure 5D**). Chemical fixation, whereby the protein is usually denatured with an aldehyde and fixed to retain histological structure, is employed in most cases for electron microscopy. The solutions used for chemical fixation usually bring about problems in dilution and diffusion of a water-soluble specimen in the fixing solution, and denaturation of the specimen. In contrast, the freezing method is a physical fixation process that utilizes the transient change from a liquid phase to solid phase at an extremely low temperature within a few seconds, and the specimen is close to the native structure;

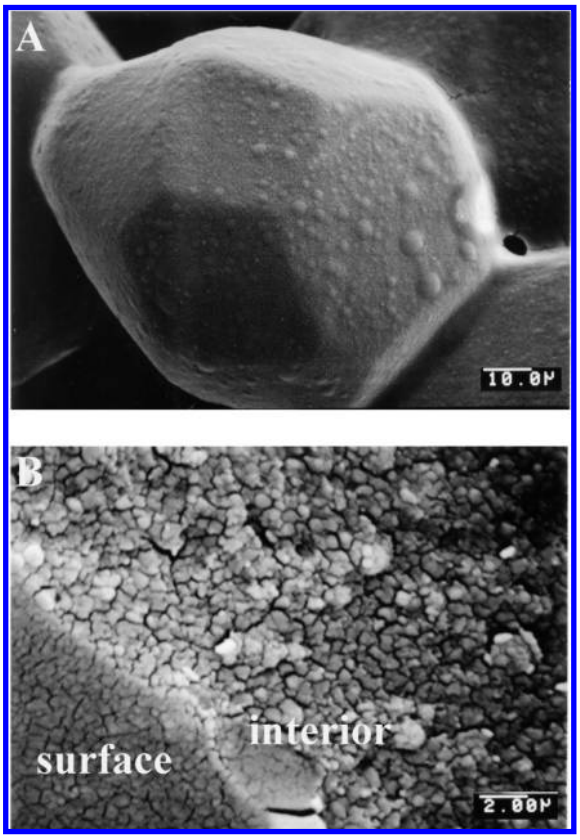

Figure 7. Micrographs of the surface (**A**) and interior (**B**) structures of cooked yolk spheres observed by cryo-SEM.

meanwhile, neither chemical fixation nor dehydrated deformation occurs (*24*). As cryo-SEM was employed, a specimen was physically fixed and observed at an extremely low temperature while retaining its shape and components. However, the specimen would be destroyed under a strong electron beam or long time exposure; therefore, the high magnification image is not easily obtained, and improvement of techniques is required (*25*).

Conclusions. The fine structure of fresh native egg yolk has been impossible to observe because of its sol structure. With the use of an improved method such as the cryo-technique, however, it was possible to understand the histological structure of each part of fresh native yolk. Native yolk spheres showed the polyhedron shape and were packed closely together. Furthermore, the interior microstructure of the native yolk spheres showed that a great amount of round globules were embedded in a continuous phase with a lot of voids. After cooking, the sizes of the spheres were almost unchanged; however, the continuous fluid phase became a fibrous network structure probably constituted by LDL. These findings on the fine structure of duck eggs can help to explain changes that occur in food cookery and other types of food processing.

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Received for Review February 11, 2009. Revised manuscript received March 24, 2009. Accepted March 30, 2009. This study was financially supported by National Science Council, ROC, No. NSC 97-2313-B-039-006.