Preparation for frozen section of steamed brown rice grains

Mariko Onishi^{*1}, Ichiro Shoji^{*2}, Toru Takahashi^{*3}, Michiko Shimomura^{*4},
Yoshimitsu Katoh^{*5}, and Shunji Nagaoka^{*5}

蒸し玄米粒の凍結切片の調製法

大西真理子^{*1}・庄司一郎^{*2}・高橋 徹^{*3}・下村道子^{*4}・加藤好光^{*5}・長岡俊治^{*5}

(^{*1}相模女子大学 (非常勤), ^{*2}郡山女子大学, ^{*3}美作大学, ^{*4}大妻女子大学, ^{*5}藤田保健衛生大学)

^{*1}*Adjunct Professor Sagami Women's University, 2-1-1 Bunkyo, Sagamihara, Kanagawa, 228-0807, Japan*

^{*2}*Faculty of Domestic Science, Koriyama Women's University, 3-25-3 Koriyama, Fukushima 963-8851, Japan*

^{*3}*Faculty of Human Life Science, Mimasaka University, 50 Kitazono, Tsuyama city, Okayama 708-8511*

^{*4}*Faculty of Home Economics, Otsuma Women's University, 12 Sanbancho, Chiyoda-ku, Tokyo 102-8357, Japan*

^{*5}*Fujita Health University School of Health Sciences, 1-89 Toyoake, Aichi 470-1192, Japan*

^{*1}〒228-8533 神奈川県相模原市文京 2-1-1

^{*2}〒963-8851 福島県郡山市開成 3-25-3

^{*3}〒708-8511 岡山県津山市北園町 50

^{*4}〒102-8357 東京都千代田区三番町12

^{*5}〒470-1192 愛知県豊明市沓掛町 1-89

従来、米粒および飯粒の組織切片標本はパラフィン包埋法が利用されてきたが、破断や破面、染色むら、構成成分の剥落等の問題があった。本研究は、蒸し加熱した玄米粒に対して凍結切片法を導入し、玄米粒の形態や組織構造が保持されているか否かを検討した。

蒸し玄米粒の凍結切片法による変形や崩壊の程度および組織構造の保存性を検証するため、蒸し加熱処理した玄米粒をクリオスタットにて-18℃で凍結切片を作製し、トルイジンブルー染色を施した。この標本の超深度形状測定顕微鏡観察により、3次元表面画像から算出された表面粗滑度を、未加熱処理玄米粒の厚切り切断面と比較対照した。その結果、蒸し玄米粒の凍結切片像は未加熱処理玄米粒の組織構造をよく反映しており、顕著な変形や崩壊もなく、胚乳細胞壁の表面粗滑度も定量的に保存されていることが証明された。

次に、蒸し玄米粒の凍結切片法の組織化学的有効性を検証するため、蒸し玄米粒5品種のたんぱく質をニンヒドリン・シッフ反応およびアクロレイン・シッフ反応で染色し、その分布像を比較対照した。両染色法とも、従来報告されている通り、5品種の玄米粒の胚芽、果皮、種皮、糊粉層、胚乳細胞壁、および澱粉複粒周囲を着染した。しかし、その染色性の濃淡に若干の違いが認められた。近縁の国産4品種は表層部背側の胚乳細胞壁が濃染し、背腹軸に沿った中心部は淡染した。中心部の澱粉複粒周囲は染色されなかった。これに対し、ワイルドライスは表層部から中心部にかけて胚乳細胞壁が一様に濃染され、さらに澱粉複粒周囲も濃染されていた。これらのことから、たんぱく質分布の品種差も明瞭に確認でき、蒸し玄米粒の凍結切片法の有効性が高いと考えられる。

更に、ズダンⅣ染色にて、脂質分布についても蒸し玄米粒の凍結切片で検証した。その結果、未加熱玄米粒で報告された結果と同様、胚芽、種皮、および澱粉層の脂質蓄積部位を茶色に着染することを確認した。

以上の結果から、蒸し玄米粒に対する凍結切片作製法は、簡便であり、玄米粒の形態および組織構造、即ち構成成分をよく保持し、染色性にも問題がなく、組織標本作製上有効な方法と判断された。

INTRODUCTION

In response to the current diversification of dietary habits in Japan, many new rice varieties have been developed. Influenced by our acclimated tastes and consumption habits, these new rice varieties are designed to act both as nutritious ingredients and to have a technological functionality. This can only be achieved by paying attention to the local distributions of fats, carbohydrates and proteins in the tissues of the whole rice grains¹⁻³⁾.

Although many reports discussed the distribution of fats, carbohydrates and proteins in brown rice tissue¹⁻⁸⁾, their tissue section of the rice was not clear enough for detailed observations. Furthermore, no clear view of the endosperm cell wall and starch compounds in the rice grains has been observed. This is mainly due to the use of the resin embedding during the preparation of sectioning, which leads to detachment or collapse of the endosperm cell wall and starch compound grain due to the fragility of the rice tissue.

The present study reported advantages of the frozen section method for steamed brown rice grain. The method can maintain the fine morphological structure of the rice. Distributions of the protein, lipid and starch in the tissues of brown rice were observed after staining.

EXPERIMENTAL METHODS

1) Materials

The following five varieties of brown rice were used for the test: *Yamada nishiki* from the Hyogo prefecture harvested in 1992, *Koshihikari* from the Fukushima prefecture in 1998, the newly developed rice variety *Hoshiyutaka*, *Calrose* from Australia, and *Hatsushimo* from Gifu Prefecture in 2001. All were purchased from Toho Food Co., Ltd., Japan. Details of their cultivation conditions were not available.

Canadian wild rice of unknown origin and year of harvesting was purchased from Meijiya, Japan. The cultivation conditions for this rice were also unknown. Wild rice grains were carefully roasted, hulled, and then soaked in water for 24 hours and finally boiled for 15 minutes.

2) Steaming methods of brown rice

Steaming prevented swelling of the tissue and dispersing the protein in the tissue. Furthermore, steaming avoided breakdown of the pericarp and episperm of the brown rice. Each of the four varieties of brown rice, such as *Yamadanishiki*, *Hatsushimo*, *Koshihikari*, and *Hoshiyutaka* was placed into separate small 30 mm diameter laboratory dishes. The rice varieties were heated for 50 min in a steamer with an internal temperature of 99°C af-

ter being washed and soaked for 2 hrs. The temperatures of the core of the grains of the varieties were measured after cutting the rice grains. If the temperature of the rice had not reached 99°C, water had sprayed on the surface of the grains and they were re-steamed.

3) Preparation of frozen sections for observation using a light microscope

The steamed rice grains were embedded in distilled water and frozen at -18°C . The section was cut into $10\mu\text{m}$ thick slices using a cryostat (Sakura Cold Dome CM-501), and was stained with toluidine blue, and then observed using a light microscope. In this study, the $10\mu\text{m}$ thick sections were used for staining and for microscopic observations. Figure 1 shows the location of the microscopic observations of the brown rice grains.

Fig. 1 Locations for microscopic observation of brown rice grain

I: Overall view of the rice grain

II: X1-X1': Cut plane for horizontal cross-section

X2-X2': Cut plane for horizontal cross-section

III: Z-Z': Cut plane for vertical cross-section

Sampling locations for observation are marked as A, B, C, D, E, and F, and correspond to those on Figures 3. Areas marked A, B, C and D are on Figures 4.

IV: Wild rice stained by the Ninhydrin-Schiff methods

"G" indicate locations for Figure 3.

4) Preparation of frozen section for the 3-dimensional observations on the surface of the section

Non-steamed brown rice grains, *Hatsushimo*, were soaked in 10% glycerin¹⁾ for approximately 5 days, and then cut with the cryostat at a 100 μm thickness to observe the 3-dimensional surface images of the cross sections at -18°C . The steamed brown rice was sliced at a 20 μm thickness using the cryostat.

5) Staining and microscopic observations of proteins and lipids

Proteins in the sections were stained using the Acrolein-Schiff method and Ninhydrin-Schiff method. Lipids were stained using Sudan IV after drying at room temperature for one day. The staining procedures are summarized in Table 1. Ninhydrin-Schiff method was modified for use in the present study. The temperature of the ninhydrin solution was set at room temperature to prevent collapse of the section. The staining reagents included acrolein (Katayama Chemical), ninhydrin (Kanto Chemical), Schiff reagent (Muto Pure Chemicals), sulfite solution (sodium sulfite/HCl solution, Muto Pure Chemicals) and Sudan IV (Katayama Chemical).

To prevent the detachment of a section from the slide

glass, the present study employed a method in which the slide is horizontally placed on a dish and then stained by gently adding the solutions onto the slide using a pipette.

The sections were photographed using an Olympus BH-2 optical microscope with Fujicolor ISO 400 film, or with a Nikon COOL PIX 990 digital camera.

6) 3-dimensional observation of the surface of the section of *Hatsushimo* brown rice grains

The frozen sections of the steamed rice grains of the *Hatsushimo* brown rice were stained with toluidine blue (0.05%, pH 7.0; Muto Pure Chemicals, Japan). The 3-dimensional images on the surface of the section was observed using a color laser 3D-profile microscope (Keyence Co. VK-8500) with an x50 objective lens. The horizontal and vertical spatial resolutions were 0.3 μm and 0.2 μm , respectively.

RESULTS AND DISCUSSION

1) Surface properties of the brown rice

Figure 2 shows the surface properties of the non-steamed brown rice (A) and steamed brown rice (C) using the color laser 3D-profile microscope. In the non-steamed rice, the cross-section shows a crystal-like

Table 1 Protein and lipid staining procedures

Protein staining
<ol style="list-style-type: none"> 1. Quickly rinse frozen sections with distilled water. 2. Rinse with 95% alcohol for 3 minutes. 3. React with acrolein* reagent for 3 minutes. 4. Rinse with 95% alcohol for 3 minutes. 5. Wash in running water for 5 minutes then transfer to distilled water. 6. React with Schiff's reagent for 30 minutes. 7. Rinse with sulfite solution, three changes, 3 minutes each to remove Schiff's reagent. 8. Wash in running water for 5 minutes then rinse in distilled water. 9. Mount after dehydration and penetration.
Ninhydrin Schiff's method
<ol style="list-style-type: none"> 1. Quickly rinse frozen sections with distilled water. 2. Rinse with 70% alcohol for a few seconds. 3. React with ninhydrin** reagent for 15 hours. 4. Wash in running water for 5 minutes then transfer to distilled water. 5. React with Schiff's reagent in 15 minutes. 6. Wash in running water for 10 minutes then rinse in distilled water. 7. Mount after dehydration and penetration.
Lipids staining
<ol style="list-style-type: none"> 1. Rinse cut frozen sections with water then rinse lightly in 60% alcohol. 2. React with Sudan IV*** solution at 37°C for 30 minutes. 3. Rinse lightly with 60% alcohol. 4. Wash in running water for 5 minutes then transfer in distilled water. 5. Mount with glycerin jelly.

*Acrolein reagent (10%) is prepared in 95% alcohol.

**Ninhydrin reagent (0.5%) is prepared in 100% alcohol.

***Sudan IV solution (2%) is prepared in 60% alcohol.

Fig. 2 Surface profiles and smoothness measurement of the frozen section of brown rice by color laser 3D-profile microscope. 2A, steamed brown rice and 2C, non-steamed one. Lines marked as X-X' and Y-Y' indicate surface height profiles, including endosperm cell walls. 2B and 2D indicate the surface height profiles, upper; X-X', lower; Y-Y', where symbols (a, b, c, a', b', c') correspond to those in the profiles of 2A and 2C. In X-X' cross-section of 2A, the height differences of a-a', b-b', and c-c' are 9.8mm, 6.5mm and 3.1mm respectively and in Y-Y' 6.1mm, 4.9mm and 12.9mm respectively. In the X-X' cross-section of 2C, the height differences of a-a', b-b' and c-c' are 14.9mm, 7.8mm and 8.6mm respectively and in Y-Y'' 2.4mm, 5.1mm and 4.4mm respectively. Arrows indicate starch granules. Note that starch granules of the steamed rice were swollen.

cleavage plane along the individual endosperm cell wall. Measurements showed the dark portions with light area hollows of 5–8 μm . The surface properties of the non-steamed brown rice (A) and steamed brown rice showed that the pericarp, epispem and aleurone were distinguishable in the cross-sections. The sizes of the endosperm cell walls at the periphery and at 400 μm into the interior were 50–80 μm and 80–110 μm , respectively. Starch granules were observed near the broken endosperm cell walls (A). In contrast, the endosperm cell wall on the surface of the section with toluidine blue-staining of the steamed brown rice was slightly swollen and deformed, but the shape of the cell was well maintained. Collapse of the tissue was not observed. Swelling was slightly observed in the pericarps, episperms and aleurone layers. Starch granules in the endosperm cell walls were slightly swollen (B). These results suggest that the preparation method of the section in the present study can keep the tissue structure intact.

Figures 2B and 2C indicate the corresponding surface height profiles marked in the figures. The tissue slices were stained by toluidine blue. The non-steamed rice

surface (Figure 2A) indicated a clear fracture structure along the cleavage plane, which may be the endosperm cell wall. On the cleavage plane, densely packed starch granules were observed. The surface height profiles (Figure 2B) clearly showed the fracture characteristics. The surface filled with the starch granules showed a flat plane with sharp edges around the endosperm cell walls.

On the other hand, the steamed rice surface (Figure 2C) indicated a much smoother plane with partly deformed starch granules, but with some projection-like barriers. Both the X-X' and Y-Y' cross sections in Figures 2C and 2D revealed the endosperm cell walls. Based on a surface smoothness analysis, the top of the endosperm cell wall was 5–13 μm higher than the starch layer. This result implied that the starch granules might be slowly dissolved from the surface during the staining process. The surface level of the endosperm cell wall was almost constant and therefore no detachment occurred during the staining process. No detachment also occurred in the steamed rice, and the endosperm cell wall was well preserved. This suggested that the macromolecular components, such as the cellulose, hemi-cellu-

Fig. 3 Heterogeneous distribution of the protein in vertical and horizontal sections of steamed brown rice.

Pe: pericarp, sp: spermederm, al: aleurone layer, sr: seed root, eps: epithelium of scutellum, sc: scale, vb: vascular bundle of sutellum

lose and proteins were well stained and could serve for the fine observation of the tissues detail.

2) Protein distribution in steamed brown rice grains

The ninhydrin-Schiff and Acrolein-Schiff methods, as well as the Toluidine blue staining, prevented structural destruction of the endosperm cells in the tissue sections. The ninhydrin-Schiff and Acrolein-Schiff methods were used to observe the protein distribution in the sections of the *Yamadanishiki*, *Hatsushimo*, *Koshihikari*, *Hoshiyutaka*, and wild rice, which contained twice the protein content (about 12-15%) as did the typical Japanese varieties. The Ninhydrin-Schiff method showed slightly clearer images compared to the Acrolein-Schiff method (Figure 3).

In the Japanese varieties, *Yamadanishiki*, *Koshihikari*,

Hoshiyutaka, and *Hatsushimo*, the endoderm cell walls in the dorsal region were weakly stained, while the endosperm cell wall and the area around the starch granules were intensely stained in the wild rice (Figure 3).

Figure 3 shows that the protein in the rice grains varied among the varieties and among the inside and outside of the endosperm in the grains, which agreed with Matsumoto et al.²⁾. The rice proteins are found in the bran, germ and endosperm, and were most abundant in the periphery⁸⁾, which did not contradict the results of the present study. This suggested that the method used in the present study detected the protein distribution.

3) Lipid distribution in steamed brown rice grains

Lipids in the frozen sections of the steamed brown rice were stained using the Sudan IV (Figure 4A-4D: 4A)

Fig. 4 Distribution of lipids in vertical sections of steamed brown rice.

4A: Vertical cross-section of brown rice (*Yamadanishiki*)

4B: Dorsal side of vertical cross-section (*Yamadanishiki*)

4C: Dorsal side of vertical cross-section (*Koshihikari*)

4D: Dorsal side of vertical cross-section (*Hoshiyutaka*)

The arrow represents the cell wall of the endosperm.

Yamadanishiki vertical cross-section of the brown rice. 4B) *Yamadanishiki*, dorsal side of the vertical cross-section, 4C) *Koshihikari*, dorsal side of the vertical cross-section, and 4D) *Hoshiyutaka* dorsal side of the vertical cross-section. The embryo, episperm, and aleurone layers were stained reddish orange, indicating that these tissues contained lipids.

Generally, the lipids in the rice grains are neutral lipids⁹⁾, because the Sudan IV stained the neutral lipids and fatty acids a reddish orange¹⁰⁾. The episperm and aleurone layers including numerous neutral lipids and fatty acids were confirmed. Kurasawa et al. reported that the lipid content of the brown rice was about 2%, and that the lipids were rich in the germ and bran layers¹¹⁾. Furthermore, they reported that the lipid content of the brown rice decreased as the polish rate of the rice increased, but the lipid levels in the endosperm did not decrease¹²⁾. The

glycolipids and phospholipids are found in the endosperm¹³⁾. However, Sudan IV does not stain glycolipids, and thus the distribution of lipids in the endosperm could not be determined.

Yoshizawa et al.¹⁴⁾ and Yanagisawa et al.¹⁵⁾ employed the paraffin sectioning method, documented the presence of lipids in the germ, pericarp, episperm, and aleurone layers, and the cell membrane (wall) of the endosperm cells adjacent to the aleurone layer¹¹⁾¹³⁾. However, we did not find lipids in the endosperm cells (Fig. 4). Such differences in the lipid distributions in the endosperm cells might be due to the dissolution of the lipids in the aleurone layer in the organic solvent used for deparaffinization and due to the migration of lipids to the endosperm in the study of Yoshizawa and Yanagisawa¹⁵⁾. Therefore, lipids might not exist in the cell wall of the endosperm (Figure 4D).

The frozen section method also prevented tissue swelling caused by heating and water immersion. Therefore, the frozen section method had advantages of maintaining the tissue shape, eliminating lipid dispersion, and being easy for preparation.

The present study was conducted to resolve the problems associated with the sample preparation. The steaming of brown rice was easy because tissue fixation was not required, and the degrees of tissue swelling, deformation and destruction caused by heating and freezing were minimal. These results confirmed that the present method was useful for preparing the brown rice tissue sections.

CONCLUSIONS

Tissue deformation and destruction are the major problems associated with the sectioning of cooked or uncooked rice grains by paraffinization. In order to resolve these problems, steamed brown rice grains were used for the preparation and staining of the tissue sections. The present study concluded that:

- 1) The section frozen using a cryostat at -18°C prevented collapse of the tissue as reflected in the fine observation of the rice tissue details.
- 2) The 3-dimensional surface images of the frozen sections revealed that the structure of the endosperm cell wall was well preserved after staining with

toluidine blue.

- 3) Proteins in the frozen sections from five varieties of brown rice were stained by the Ninhydrin-Schiff and Acrolein-Schiff methods. No differences in the protein distributions were observed between these two methods. The germ, pericarp, episperm, aleurone layers, endosperm cell wall and the area around the starch granules were stained reddish purple.
- 4) In all four Japanese varieties, the dorsal region of the superficial layer of the endosperm cell wall was well stained, but it was weak in the central region along the dorsal-ventral axis. However, in the wild rice, the endosperm cell wall and starch granules were intensely stained throughout the grain, from the surface to the central regions.

The frozen section method of the steamed brown rice grain was a simple method to maintain the fine structure of the rice. These things results that this method is useful for preparing tissue sections because the distribution of proteins and lipids does not decrease.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. S. Watanabe at the Daido Hospital, Institute of Occupational Medicine, for his helpful suggestions and comments throughout the course of this study. The authors would also thank Mr. S. Takagi for his expert technical assistance

REFERENCES

- 1) Little R. R., Dawson E. H.: Histology and histochemistry of raw and cooked rice grains, *Food Res.*, **5**, 611-622 (1960)
- 2) Matsumoto K., Yamamoto S., Okazaki F.: Histochemical studies on rice varieties, *Bull. Kochi Gakuen J. College*, **8**, 37-41 (1977)
- 3) Ikeda H., Kimura T., Saeke Y., Ogawa K., Tomoda K., Kuchiba N.: Relation between the physicochemical properties and the structures of cooked rice (part 1), *J. Home Economics Japan*, **47**, 877-887 (1996)
- 4) Matsumoto K., Yamamoto F., Okazaki S.: Histochemical studies on rice varieties, *Bull. Kochi Gakuen J. College*, **8**, 37-41 (1977)
- 5) Okazaki S., Morioka T., Yamamoto E.: Histochemical studies on rice grains, VI, Protein of raw and cooked rice grains on various milling conditions. *Bull. Kochi College*, **24**, 81-87 (1975)
- 6) Ogawa Y., Sugiyama J., Kuensting H., Ohtani T., Hagiwara S., Kohubo M., Kudoh K., and Higuchi T.: Development of Visualization Technique for Three-Distribution of Protein and Starch in a Brown Rice Grain Using Sequentially Stained Sections, *Food Sci. Technol. Res.*, **6**, 176-178 (2000)
- 7) Onishi M., Yamanaka N., Shoji I., Ogawa N.: Physicochemical properties and histochemical studies of rice milled under various conditions, *J. Home Economics Japan*, **48**, 303-313 (1997)
- 8) Matsuda T., Chonan N.: Fine Structure of Cooked Rice, *Jpn. J. Crop Sci.*, **59**, 276-277 (1990)
- 9) Yamada K.: Histochemistry, Nankodo, Tokyo, 1987, 203-205 (in Japanese)
- 10) Committee of Food Histology: Japan Society of Home Economics ed. Histology of foods, In methodology of food histology and their examples, 20-21 (1986)
- 11) Kurasawa F.: Rice and the Improvement, Kenpakusha, 31-35 (in Japanese) (1982)
- 12) Namiki M., Nakamura R., Kawagisi K., Watanabe S.: Contemporary Food Chemistry, Sankyoshuppan, Tokyo, 220-221 (in Japanese) (1985)
- 13) Chikubu S.: Science of Rice, 20-28 (in Japanese) (1995)
- 14) Yosizawa K., Hasuo T., Ishikawa K.: Localization of Lipid in Rice Corn and Dislocation During Teatment Maneuver, *J. Brew. Soc. Japan*, **75**, 432-435 (1980)
- 15) Yanagisawa Y., Takiguchi T., Terada A.: Preparation of thin sliced sections from rice grain, *J. Brew. Soc. Japan*, **77**, 636-638 (1982)