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Ultrastructure of Individual and Compound Starch Granules in Isolation Preparation from a High-Quality, Low-Amylose Rice, Ilpumbyeo, and Its Mutant, G2, a High-Dietary Fiber, High-Amylose Rice

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The ultrastructures of isolated starch granules from Ilpumbyeo (IP), a low-amylose japonica rice, and its mutant, Goami2 (G2), a high-amylose rice, which have extreme contrasts in physicochemical properties, cooking qualities (Kang, H. J.; Hwang, I. K.; Kim, K. S.; Choi, H. C. Comparative structure and physicochemical properties of Ilpumbyeo, a high-quality japonica rice, and its mutant, Suweon 464. *J. Agric. Food Chem.* **2003**, *51*, 6598−6603. Kim, K. S.; Kang, H. J.; Hwang, I. K.; Hwang, H. G.; Kim, T. Y.; Choi, H. C. Comparative ultrastructure of Ilpumbyeo, a high-quality japonica rice, and its mutant, Suweon 464: Scanning and transmission electron microscopy studies. *J. Agric. Food Chem.* **2004**, *52*, 3876−3883), and susceptibility to amylolytic enzymes (Kim, K. S.; Kang, H. J.; Hwang, I. K.; Hwang, H. G.; Kim, T. Y.; Choi, H. C. Fibrillar microfilaments associated with a high-amylose rice, Goami2, a mutant of Ilpumbyeo, a high-quality japonica rice. *J. Agric. Food Chem.* **2005**, 53, 2600−2608), were compared. In isolated preparation, IP consisted entirely of well-separated individual starch granules (ISG), whereas G2 consisted of two populations, the large voluminous bodies and the smaller forms, the ISGs. High-voltage electron microscopy revealed that each of the voluminous bodies consisted of tightly packed smaller subunits, the ISGs, indicating that they represent the compound starch granules (CSGs) of G2. This suggests that the structural as well as functional unit of G2 involved in food processing is, unlike IP and other ordinary rices, not ISG but is primarily CSG. ISGs located at the periphery of CSGs were fused to each other with adjacent ones forming a thick band or wall encircling the entire circumference. The periphery of ISGs separated from CSGs of G2 consisted of thin radially oriented filaments arranged side by side along the entire granule surface, whereas no such filaments occurred in ISG of IP. It appears that the thick band and the peripheral filaments surrounding CSGs and ISGs, respectively, function as a structural barrier that limits the entrance of water into the granules and subsequent absorption, causing the low swelling power, incomplete gelatinization, and finally poor quality of cooked rice in G2.

KEYWORDS: Ultrastructure; starch granules; compound starch granules; high-amylose rice; microfilament; SEM; TEM; high-voltage EM

INTRODUCTION

Recently, a high-amylose rice mutant, Goami 2 (G2), formally called Suweon 464, was developed by mutation breeding via *N*-methyl-*N*-nitrosourea (MNU) treatment of Ilpumbyeo (IP), a low-amylose japonica rice that has an excellent cooking quality (*1*, *2*), at the National Institute of Crop Science, RDA, Suwon, Korea. G2 contains enough amylose to be classified as a high-amylose rice (*3*) and has an unusual B-type X-ray diffraction pattern as a rice starch, a markedly low proportion of short chains in the distribution of glucan-chain fraction of debranched starch, and lower relative crystallinity, all of which would contribute to its unsuitability for ordinary cooked rice (*1*). Indeed, G2 is very low in adhesiveness, cohesiveness, and very high in hardness and chewiness when cooked (I). Although G2 may not be suitable for ordinary cooked rice, however, it has

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been proposed that G2 could be an excellent candidate for other processed "healthy" food products based on its unusually higher contents of dietary fibers, protein, and lipid as compared to those in ordinary japonica rice, including IP (*1*, *2*).

On the basis of our recent studies on physicochemical and ultrastructural comparisons between IP and G2 (*1*, *2*), one of the most significant factors involved in controlling the quality of G2's cooked rice appeared to exist in the morphological and physical properties of the starch granules in the mature kernel. In rice, starch granules are developed in the amyloplasts of endosperm cells where they exist, at the maturity, as compound starch granules (CSGs) in each of which 20−60 individual starch granules (ISGs) are tightly packed (*4*, *5*), and each CSG is derived from a single amyloplast (*5*, *6*).

The development and formation of CSGs in amyloplasts of G2 endosperm cells were similar to those in other ordinary rice varieties except that the average sizes of amyloplasts and individual starch granules (ISG) within them (CSGs) were much smaller than those in IP (*1*, *2*). However, when these two starches in milled rice grains were subjected to the isolation and purification processes, including physical and chemical treatments to remove nonstarch material such as protein, lipid, cell well components, etc., the products of the two showed striking differences. The CSGs in IP grains were completely dissociated (broken) and the ISGs within them were freely released, whereas most of the CSGs in G2 were structurally intact, indicating that ISGs were still retained in CSGs following the isolation processes (*2*). In fact, CSGs of G2 in fractured endosperm cells in the whole grains examined by scanning electron microscopy (SEM) were morphologically indistinguishable from those in the isolation preparations, indicating that the CSGs of G2 are physically tolerant of the stresses involved in physical fracturing and chemical treatments for starch isolation processes (*2*). These indicate that the basic structural as well as functional units of G2, involved in a variety of food processings including cooked rice, are not ISGs but are CSGs in each of which 20−60 individuals are tightly packed. Most commercial starches, including rice, are known to consist of single starch granules (*4*, *7*−*9*).

The ultrastructural studies of processed foods made of G2, such as cooked and malt-treated cooked rices, revealed that the CSGs in milled rice grains survived the processes involved in cooking and malt treatments, thereby retaining their morphological identity (*10*). These indicate that the CSGs of G2 are tolerant of not only physical and chemical treatments involved in mechanical fracturing and starch isolation processes (*2*) but also of cooking and amylolytic enzymes (*10*). These studies (*2*, *10*) support that the functional unit of G2 involved in food processing is not an ISG but is a CSG. The factors involved in determining the capability of retaining the structural integrity of CSGs in G2 after receiving such severe treatments as mentioned above are not known. However, the presence of a novel and unique structural feature, the fibrillar microfilaments embedded in coarsely gelatinized starch matrix of the G2's cooked rice, which are also extremely resistant to amylolytic enzymes, led the authors to believe that these structures could play a major role in the constitutional nature of G2's CSGs (*10*).

The fibrillar microfilaments in CSGs of G2 observed were in the cooked and malt-treat cooked rices, and it raises a question whether they are the products formed as a result of gelatinization and/or retrogradation, the two major events occurring during the cooking, or are a preexisting entity prior to cooking. One of the major objectives of the present study was to ascertain this question by studying the isolated preparation of G2 starch from uncooked law grains and comparing the result with that of its counterpart, IP starch, via conventional transmission electron microscopy (TEM). Another objective was to visualize the internal structure of the large voluminous bodies, which are believed to be the structurally intact CSGs of G2 and, therefore, called as such, present in the isolated preparation. If the bodies are, indeed, CSGs, they should reveal the presence of tightly packed ISGs within each of them. For this study, high voltage electron microscopy (HVEM) was employed because of the technical difficulties faced during the preparation of the specimen (voluminous bodies) for conventional TEM (see Materials and Methods).

MATERIALS AND METHODS

Rices. The sources of milled, raw grains of the mutant, G2, and its original variety, IP, were the same as those used for previous studies (*1*, *2*). To re-ensure the previous results, fresh crops harvested in the 2004 growing season were used. The rice plants were cultivated in a rice experiment field at the National Institute of Crop Science, Suwon, Korea.

Isolation of Starch Granules. Starch granules were isolated following a method described by Hoover and Sosulski (*11*) involving repeated steeping of rice flour in 0.2% NaOH solution. The precipitated starch was further treated with methanol and then ether to remove lipids (*12*). Starch so isolated was dried under atmosphere, ground into powder, and passed through a 100 mesh sieve.

Specimen Preparation. *SEM.* Thoroughly dried, isolated starch granules of IP and G2 were dusted onto the alumium specimen stubs with a fine painting brush. The specimens were sputter coated with gold and viewed with a JSM 5410LV (JEOL) scanning electron microscope at 15 kV.

TEM. Isolated starch granules were fixed overnight at room temperature in a conical effendorf tube (1.5 mL size) containing a modified Karnovsky's fixative (*13*) by placing a small spatula of the granule power in it. Following the fixation, the tube containing the granules was centrifuged in an "Effa" centrifuge tube (Ernest F. Fullam, Inc., Schenectade, NY) at 20 000*g* for 1 h, the supernatant was discarded, and the pellet, consisting of the isolated starch granules, was washed three times with distilled water for 10 min each and postfixed in 2% osmium tetroxide overnight. The pellet was then washed briefly with distilled water and en bloc stained in 0.5% aqueous uranyl acetate overnight before dehydrating in an ethanol series and propylene oxide. The pellet was embedded in Spurr's low viscosity medium (*14*), sectioned with a diamond knife, and double stained with 2% aqueous uranyl acetate and lead citrate before being viewed with Zeiz LEO 906 TEM at 80 kV.

HVEM. Thin sectioning of isolated granules in region blocks, especially those of G2 granules, for TEM (50−80 nm thick) was extremely difficult apparently due to the nature of the block consisting primarily of purified starch granules. Because thicker sectioning of the blocks reduced the tearing of the sections, 1−2 μm-thick sections were made and viewed with a JEOL high voltage electron microscope, JEM-ARM1300S, at 1250 kV that produces high energy electrons, capable of penetrating the tissue sections much thicker than the thin sections for an ordinary TEM operating at 80 kV.

RESULTS AND DISCUSSION

SEM Study of Isolated Starch Granules from IP and G2. *IP.* Isolated starch granules from IP (**Figure 1A**) were, as in the case of the previous study (*2*), similar in granule morphology and sizes, which were polygonal with sharp angles and edges, having an average diameter of 5.2 μm (*1*). The surfaces of the granules were smooth and flat or slightly concaved with no structural debris associated, indicating that the isolation preparation consists primarily of pure individual starch granules (ISG).

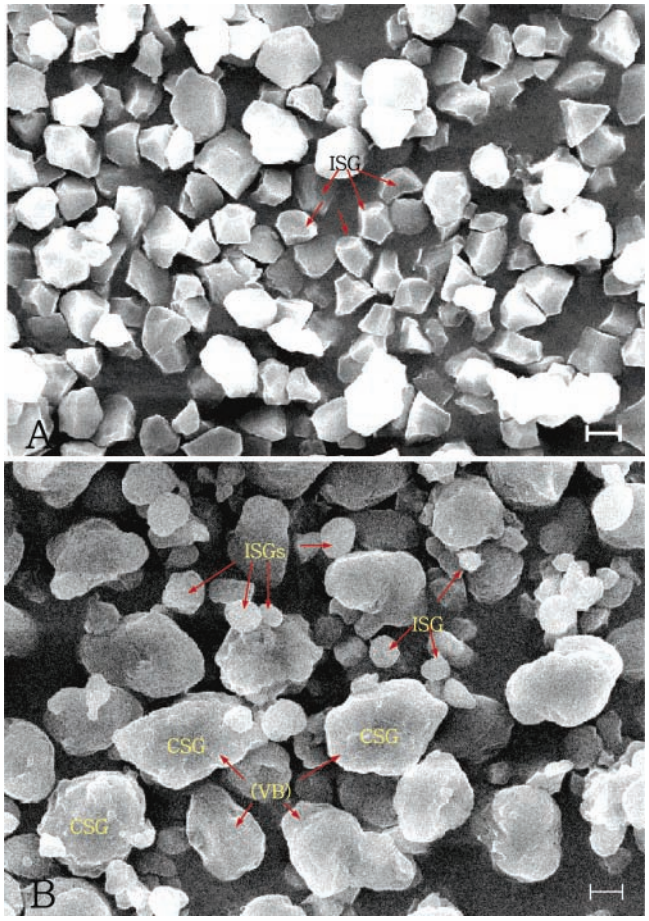

Figure 1. Isolated starch granules from IP (**A**) and G2 (**B**) (scale bar = 3 μm). (**A**) Entire population consists of well-separated individual starch granules (ISG) of similar sizes that are polygonal in shape with sharp angles and edges. (**B**) Two types of population: one consists of smaller, somewhat rounded individual starch granules (ISG), and the other consists of larger voluminous bodies (VB) that are believed to be the compound starch granules (CSG). The surface of the bodies is not smooth but has irregular undulations, protrusions, and linear divisions.

The whole or partially split CSGs present in fractured whole grains (*2*) were not present, indicating that the entire population of the CSGs were totally dissociated during the isolation processes and ISGs tightly packed within them were all released freely.

G2. In contrast with those in IP, the isolated starch granules were heterogeneous in sizes and shapes and could be grouped into two populations (**Figure 1B**): one with those having smaller sizes and rounded or slightly angled circumferences, which represent apparently the ISGs, and the other, the major population of the two, with those large voluminous bodies having roughly spherical or ovoid profiles. These bodies were morphologically indistinguishable to those believed to be the CSGs of G2 endosperm cells shown in fractured whole grains (*2*). This suggests that the voluminous bodies (CSGs) of G2 endosperm cells have survived the severe treatments received during the isolation processes, thereby retaining their structural integrity. In addition, the surfaces of these large bodies were, unlike the smaller granules, not smooth but had irregular undulations and/or protrusions of various shapes and sizes (**Figure 1B**), suggesting that these were caused by the presence of solid structures of some sort in the interior of the voluminous bodies. Although these interior structures are believed to be the ISGs of G2, it is still uncertain ultrastructurally, because the voluminous bodies shown are the images of their surface examined by SEM that show no details of internal organization of the bodies. One of the major objectives of the present study is to ascertain this uncertainty by visualizing the structural details of the interior of the voluminous bodies in the isolated starch preparation of G2 with HVEM.

HVEM Study of Isolated Voluminous Bodies (CSGs) from G2. 1−2 μm-thick sectioning of isolated starch granules for HVEM still produced a considerable extent of tearing and overlapping of the tissue ribbons (slices), causing difficulties in locating the properly sectioned voluminous bodies (CSGs) to examine. In addition, when some CSGs were located, the interior structures of many of them were distorted or disrupted due apparently to poor fixation and/or poor infiltration of the embedding medium into the granules during the preparation of isolated starch granules for electron microscopy (see Materials and Methods). **Figure 2A** shows an area where a number of large CSGs are reasonably well sectioned, exhibiting tightly clustered ISGs within them. This electron micrograph and the others (**Figure 2A** and **B**) demonstrate clearly that the large voluminous bodies in the isolation preparation of G2 starch are indeed the CSGs. Sections of larger CSGs containing more than 20 ISGs were common, but those of smaller ones having only a few, often 2 or 3, ISGs were more common (**Figure 2A** and **B**). Regardless of the sizes of the CSGs, ISGs packed within each of CSGs were separated from the adjacent ones by a thin electron-lucent linear space, apparently an air space (**Figure 2A**−**D**). The central region of each ISG in CSGs had a lightly stained circular halo, representing apparently the hilium area of each single granule (**Figure 2C** and **D**). The spaces separating ISGs that were located at the periphery of CSGs are always oriented radially extending from the central area to the granule surface (**Figure 2C** and **D**). However, these radially oriented spaces did not reach through to the external surface of CSGs, but stopped a short distance underneath the surface, creating externally a thick, dense, and continuous band surrounding the entire circumference of a CSG (**Figure 2C** and **D**).

It is believed that the band corresponds to the coat of the saclike voluminous bodies shown by SEM (*2*), and the electron-dense layer surrounding the entire circumference of the starch granule-packed amyloplasts in developing endosperm cells of G2 demonstrated by TEM (*2*). It is suggested, therefore, that the band functions as a structural barrier that limits the entrance of water and subsequent absorption into CSGs, causing the whole grains of G2 to have an unusually low swelling power and poor gelatinization when cooked.

In a higher magnification, however, the band is not an amorphous structure. The external portion of most bands were serrated in a radial direction with deep and narrow clefts of various depths and widths, exhibiting a sawlike or comblike profile (**Figure 2C**−**F**). Furthermore, some of the teeth of the saw or comb, which appeared as filamentous structures, were further thinned out to exceedingly fine fibrillar elements at the external tips (**Figure 2E** and **F**). When the filaments were sectioned transversely or tangentially, the fibrils at the external tips appeared as minute dots (**Figure 2E**). These structures are similar to those observed in lightly heated potato (*15*) and to those in wet-ground waxy maize starch granules (*16*). It is presumed that the radial serrations and the fibering of the filaments (saw teeth) at the surface of G2's CSGs are a consequence of the orientation of the major components of starch molecules, amylopectin and amylose (*15*, *16*), including amylose−lipid complexes of which G2 should contain unusually high amounts (*1*, *2*, *10*).

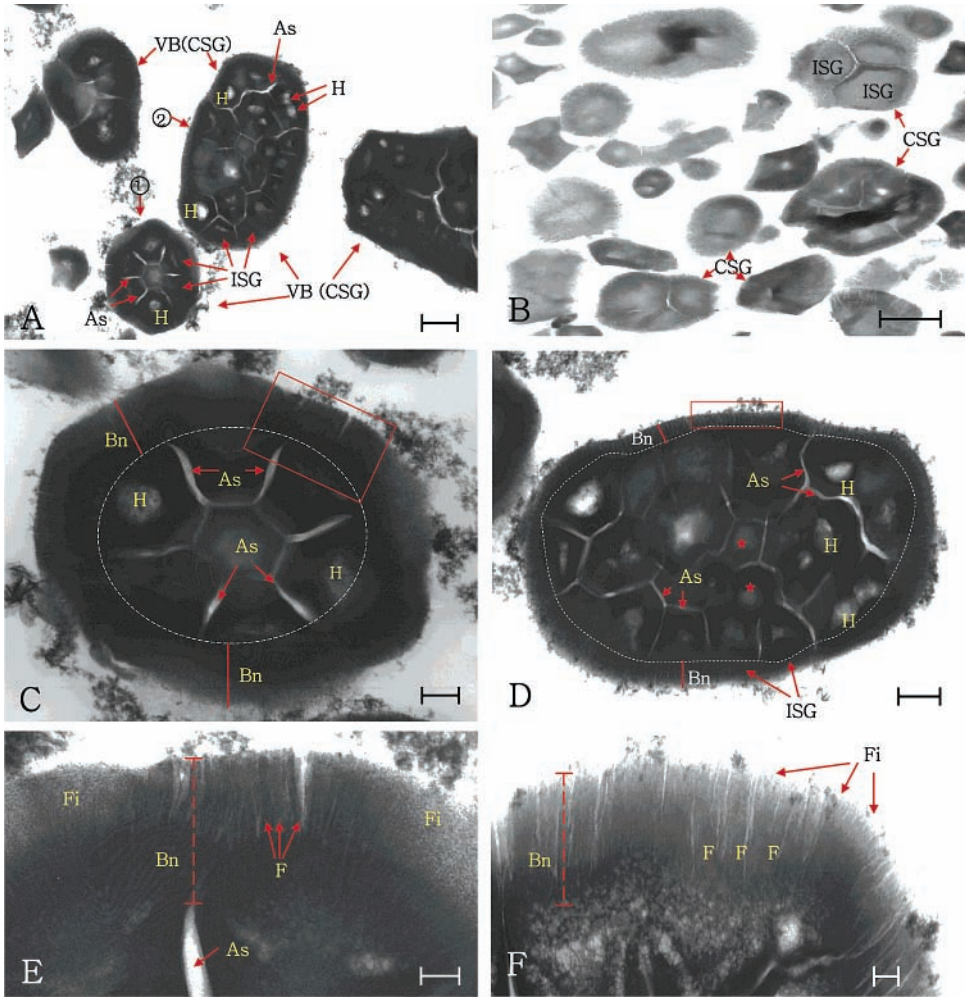

Figure 2. HVEM micrographs of isolated starch granules from G2. (**A**) A low magnification view (scale bar = 2 μm) of the several voluminous bodies (VB), each of which consists of tightly packed individual starch granules (ISG), demonstrating that they are indeed compound starch granules (CSG). ISGs within each CSG are separated from the adjacent ones by an electron-lucent, linear air space (As). (**B**) A low magnification view (bar scale = 2 μm) of an area where CSGs consisting of only a few ISGs, 2 or 3, are grouped. (**C**) A higher magnification view (scale bar = 500 nm) of a CSG-① shown in **Figure 2A**. The spaces separating ISGs, the intergranular spaces (As), are not continuous all the way through the outer surface of the granules, but stopped at a short distance underneath the surface, creating a dense, continuous band (Bn) or wall encircling the entire circumference of the granule. The central area of each ISG has a lightly stained circular halo, hilium region (H). In the outermost region of the band, the presence of radially oriented filaments or rods was discernible (squared area). The structural details of the squared region are shown in **Figure 2E**. (**D**) A higher magnification view (scale bar = 1 μm) of a large CSG-② shown in **Figure 2A** containing more than 20 ISGs, each of which is separated by an intergranular space (As). The structural details of the squared area are shown in **Figure 2F**. * = ISGs located internally below those (ISGs) forming the banded wall of CSGs. (**E**) A higher magnification view of the squared area in **Figure 2C** (scale bar = 200 nm) showing the outermost portion of the band (Bn) encircling the entire circumference of the CSG. Radially oriented filaments (F) are clearly shown. The right and left corners of the figure show the fibrillar structures resulted from the tangential sections of the filaments, indicating that they are made of subfilamentous fibrils (Fi). (**F**) A higher magnification view (scale bar = 200 nm) of the squared area in **Figure 2D** showing radially oriented filaments (F) arranged side by side with various widths and depths. The tip of each filament (F) exhibits the presence of subfilamentous finer fibrils (Fi).

As indicated, G2 is a high-amylose rice with an unusually higher content of lipid as compared to ordinary rice, including IP (*1*, *17*, *18*). The lipid in rice, although present in smaller amounts, has been demonstrated to generate complex molecules as a result of intra- and/or intermolecular interactions with starch components, especially with amylose, during the cooking of whole rice grains (*19*, *20*). It was suggested, therefore, that a large portion of amylose molecules in the cook rice of G2 occurs as lipid complexed forms of amylose (amylose−lipid complexes), and such complexes would contribute to the characteristic ultrastructural feature of G2, particularly the fibrillar filaments embedded in coarsely gelatinized starch matrix, which were absent in the cooked rice of IP, a low-amylose rice with an average amount of lipid as compared to that contained in ordinary rice (*1*, *10*). Later studies, however, presented the evidence that amylose−lipid complexes of cereal starches also occurred in uncooked native starch granules (*21*−*23*). This suggests that the microfilaments in the cooked rice of G2 could be a preexisting structure prior to cooking in raw granules, assuming that the complexes are involved in the formation of them. Although HVEM clearly demonstrated that the saclike, voluminous bodies in the isolation preparation of G2 examined by SEM (**Figure 1B**) were indeed structurally intact CSGs themselves (**Figure 2A**−**D**), structural details of ISGs within them were not discernible due primarily to the thickness of the sections, 1−2 μm, which was too thick to resolve the fine structure like the microfilament of G2. For this reason, thin sections (500−800 Å) from the same tissue blocks used for

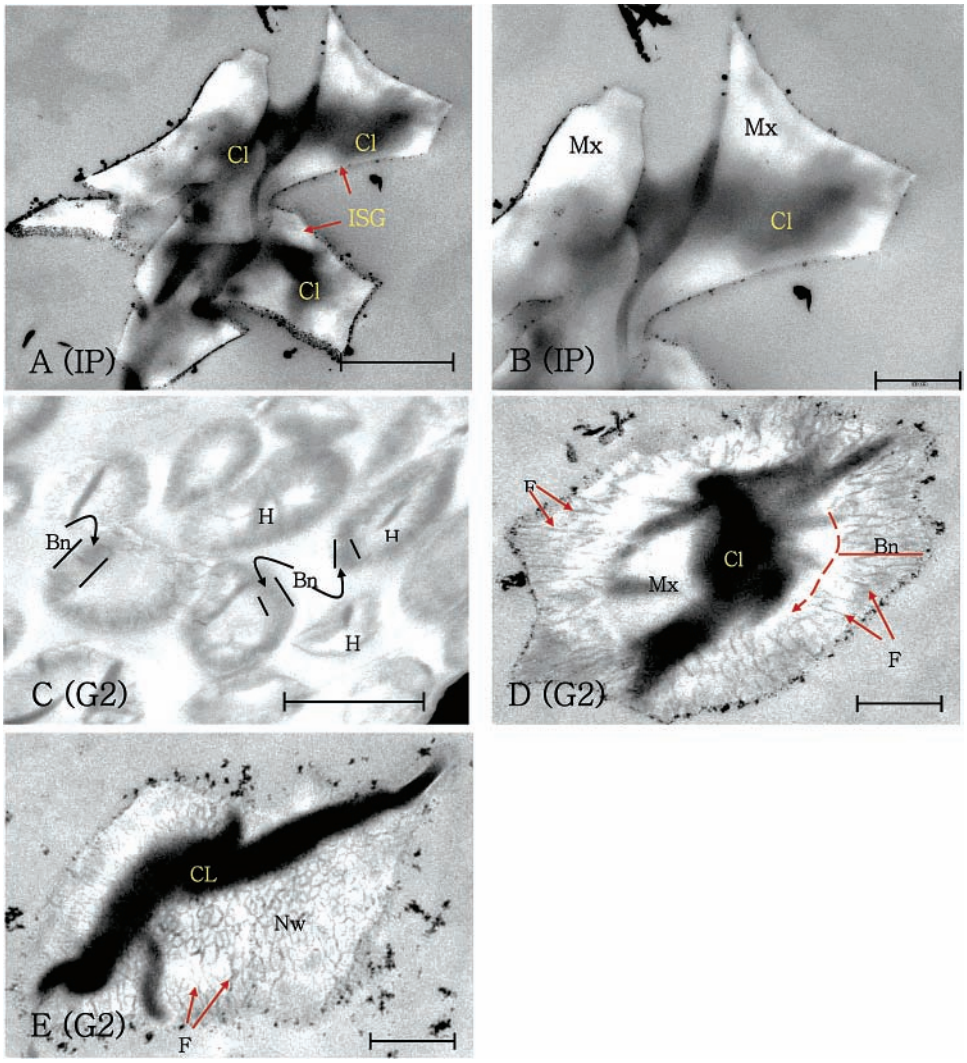

Figure 3. TEM micrographs of isolated starch granules from IP (**A**, **B**) and G2 (**C**–**E**). (**A**) A low magnification view (scale bar = 100 nm) of an area where several individual starch granules (ISG) of IP are grouped. Granules are polygonal in shape having sharp angles and edges. The central area of each granule has slightly dense and irregularly shaped "clouds" that may represent its hilium region where more electron stains are trapped. (**B**) A higher magnification view (scale bar = 50 μm) of the portions of two ISGs. The entire matrix (Mx) of the granules is homogeneously smooth with no structural modification or nonstarch material. (**C**) A low magnification view (scale bar = 200 μm) of an area where a number of individual starch granules (ISG) of G2 are grouped. Most granules are roughly spherical or ovoid having no sharp angles and edges. Many of the granules have either a light or a dense central area, apparently the hilium region (H). The periphery of each granule is somewhat densely stained throughout the entire circumference resulting in the formation a circular band (Bn) in which radially oriented filamentous structures are discernible. (**D**) A higher magnification view of a ISG of G2 (scale bar = 50 μm) demonstrating the presence of the radially oriented filaments (F) in the peripheral band (Bn). The matrix (Mx) between the band and densely stained "cloud" (Cl) is smooth and amorphous. (**E**) A higher magnification view (scale bar = 50 μm) of an isolated starch granule demonstrating the presence of the filaments (F) not only in the periphery but also in the interior of the granule as a weblike network (NW), as a result of a tangential section of a granule periphery. Cl = densely stained hilium region.

HVEM were cut for the conventional TEM to examine the structural details of the isolated ISGs from G2, and the results were compared to those from IP.

TEM Study of Isolated Starch Granules From IP and G2. *IP*. **Figure 3A** illustrates several well-isolated starch granules of IP. The granules have sharp angles and edges with linear boundaries (**Figure 3A** and **B**) similar to those shown in isolated starch granules examined with SEM (**Figure 1A**). The matrix of the granules was homogeneously smooth and flat with no noticeable structural modifications or signs of the presence of nonstarch material (**Figure 3B**). The granules were very low in electron density due apparently to the nature of their chemical composition, the starch, and, therefore, were very lightly contrasted to the background, the embedding medium. Slightly dense and irregularly shaped "clouds" dispersed around the central region of each granule (**Figure 3A**) may represent its hilium area where it consists of different starch molecules from the rest of the granule or of nonpolysaccharide material (*4*, *9*) that may have attracted more electron stains.

G2. **Figure 3C** shows a low magnification view of an area where several ISGs in an isolation preparation of G2 starch are grouped. Most of these granules are, unlike those of IP, roughly spherical or ovoid without sharp angles and edges and are similar to ISGs examined by SEM (**Figure 1B**). Many of these granules had either slightly dense or light central area, apparently a hilium region (**Figure 3C**). One of the most striking differences in ISGs of G2 from those of IP was located at the periphery of each granules where it appeared as a densely stained circular band encircling the entire circumference of each granule (**Figure 3C**). The band often revealed the presence of radially oriented fine

rods or filaments, suggesting that it is the same entity as the banded wall that surrounds the periphery of the CSGs examined with HVEM (compare **Figure 2C** and **D** with **Figure 3C**) in terms of its morphology, location, and electron density. It is further suggested that the ISGs, tightly appressed by the adjacent ones that are located internally below those (ISGs) participating in the formation of the banded wall of CSGs shown in HVEM (**Figure 2D**), would have the same granule morphology as that of separated free ISGs (**Figure 3C**) when they are released from the mother CSGs freely.

In a higher magnification view, the peripheral region of each ISG of G2 exhibited the structural details of the band that surrounds the entire circumference of each granule (**Figure 3D**). The band consisted of radially oriented filaments aligned side by side appearing like "skeletal" elements of a starch granule, which may responsible for determining its shape, roughly spherical. The starch matrix below the banded region near the central hilium area was amorphous (**Figure 3D**), indicating that the molecular architecture of starch in the granule surface and its internal region must be different. As a rule, the filaments were radially oriented toward the granule surface (**Figure 3D**), but, in some granules, they also occurred in the central region (**Figure 3E**). In this case, the filaments are not parallel in alignment but formed some sort of a weblike network (**Figure 3E**). It is suggested that the network represents the filaments that located at the granule surface were sectioned tangentially through the peripheral region of a granule resulting in such configuration.

The filaments present in the periphery of ISGs, in the isolation preparation from the raw grains of G2 (**Figure 3D** and **E**), are believed to be the same entity as the microfilaments shown in cooked rice grain, on the basis of their similarity in morphology, sizes (15−20 nm in diameter), and the consistency of their occurrence only in G2, but not in its counterpart rice, IP, a low-amylose rice (*1*−*3*). The microfilaments in cooked rice seemed to be somewhat more crisp in appearance than those in the ISGs from raw grains, and it may be due to the fact that some starch material associated with them is being leached out of the granules during the cooking, causing an unmasking effect. The presence of the microfilaments in raw ISGs of G2 indicates further that they are not the product formed as a result of gelatinization and during cooking and subsequent cooling of cooked rice as suggested previously (*3*), but are the native in occurrence. This observation strengthens the case that amylose−lipid complexes, present in unusually higher amounts in G2 as compared to in other rice, could be a strong candidate to be involved in the formation of the filaments, because the complexes also occur in native raw starch granules in various forms depending on the type of lipids (*21*−*23*). However, the precise compositional nature of the filaments is unknown, and it is possible that some other components from the starch granules of G2 might have been involved in the formation of them. Whatever the nature of chemical composition, the filaments must be formed by tightly bonded molecules that are firm enough to retain their structural integrity after receiving such harsh treatments involved in cooking and cooling as gelatinization and retrogradation, and also in amylolytic enzymes (*2*, *3*). The occurrence of the filamentous structures in raw starch grains described in this paper has not been previously reported in any other rice.

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