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Heterogeneity of the morphology of growing starch granules at the developing seeds of kidney bean (*Phaseolus vulgaris* L.)

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Abstract

The growth and maturation of starch granules was studied during seed development of *Phaseolus vulgaris* L. cv. Toramame. In very young developing seeds, 4 mm in length, small spherical starch particles of 2-5 μm in diameter were routinely observed. Periodic microscopic observations to seed maturity revealed that the growth and maturation of these small spherical starch particles to mature elliptical-shaped starch grains occurred by at least three distinct stages. In the first stage of growth, the small rounded starch particles elongated in length in either a uni-, bi-, or tri-direction. During this elongating phase of growth, the starch particles attained more than 50% of their maximum length. Microstructural changes such as the formation of protuberances, cavities and terraces were prominent on the surface of the growth fronts. In the second stage, when seeds were larger than 8 mm, elongation growth slowed while the girth of the starch granule growth increased. In developing seeds 12 mm in length, the granular form was completed, and growth rings, positions of the hilum, grooves on the surfaces and ditches on the sides of starch granules were detected. At a final stage of the growth of seeds from 15 to 16 mm, a remarkable enlargement of starch granules occurred.

Starch is the primary reserve of carbon and energy for many plants and has been a subject of a large number of enzymatic studies[1-6]. Starch granule is composed of the linear glucosyl chain, amylose, and the branched form, amylopectin, as well as small amounts of proteins and phosphates[7,8]. Amylose usually comprises about 30% of the starch content with the remaining contributed by amylopectin, although this proportion varies among plant species. In reserve

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid.

organs starch granules exhibit alternating growth rings, suggesting that the synthesis of granule varies.

Several studies[8-10] have been carried out on the overall structure of the starch grain. Based on the presence of thin crystalline lamellae in starch granules, a cluster substructure was suggested to be the unit composition of the granule[11-14]. The crystalline size perpendicular to the molecular axis was found to be 15 nm in potato starch[15]. Studies using electron microscopy[16,17] and X-ray diffraction[18] showed a periodicity of 10 nm in the granule showing the presence of the stack of thin crystalline lamellae (that is, amylopectin clusters as the crystalline component). As a detailed cluster model of amylopectin, Kainuma and French[12] proposed the first left-handed parallel-stranded double helices and this proposal has been supported by Imberty *et al.*[19] and Oostergetel and van Bruggen[20]. Oostergetel and van Bruggen[21] obtained electron micrographs of starch granule fragments, the results of which are interpreted as alterations of crystalline regions with amorphous zones.

Gallant *et al.*[22] proposed "the blocklet concept" on the internal architecture of starch granules based on results from studies using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The blocklet diameter of semi-crystalline shells of wheat starch was small (25 nm), whereas the diameter was larger (80-120 nm) in the crystalline shells. Gallant *et al.*[22] proposed a starch granule structure that was comprised of alternating crystalline hard shells (consisting of large blocklets) and semi-crystalline soft shells (consisting of small blocklets).

Observations on the morphological changes that occur during the growth of the starch granules may lead to insights on how the starch granules are formed and their structure. Such a study, as far as we are aware, has not been conducted, as all morphological studies of the starch grain have been conducted on the mature form. In this study we observed morphological changes of the starch grain in developing kidney beans of varying age. We showed that the growth and maturation of the starch granule are not simple processes. Instead, it entails at least three distinct phases of growth.

MATERIALS AND METHODS

Plant Material

Seeds of kidney bean (*Phaseolus vulgaris* L. cv Toramame) were obtained from a local market in Sapporo. Plants were grown in a field plot at Hokkaido University under natural conditions. Immature seeds of varying age were periodically collected during the growing season. The age of the seeds was assigned according to their length of the major axis and ranged from 4 mm (youngest seeds) to the final mature stage (16 mm).

Microscopy

To observe starch granules, the small developing seeds in the young pods were directly placed on a slide glass, sectioned into thin pieces using a razor blade and then minced using tweezers. In the case of more mature seeds (>8 mm in length), a section of the cotyledon, 1 mm thick, near the embryo was obtained by free hand sectioning. The outer layers of the cotyledon section were removed and the remaining 0.5 mm thick cotyledon sectioned was placed on the slide glass and minced as described above. A small amount of iodine solution (0.005% I_2 -0.05% KI) was then added to the cellular extract on the glass slide. Starch granules, particularly those that were present in seeds of 4 to 6 mm, could easily be distinguished microscopically from other cellular components. In more mature seeds, starch granules were isolated by differential centrifugation. The seeds were homogenized in the presence of extraction buffer (5 mM EGTA, 50 mM Tris-HCl buffer of pH 7.2, 4 mM DTT) and then centrifuged at 1,000g for 10 min. The starch pellet was then washed twice by resuspending in extraction buffer and sedimenting at low centrifugal force. The starch preparation was then stained with iodine solution. The starch preparations were examined with phase contrast optics using an Olympus model BHS microscope (Olympus Optical Co., Tokyo).

In some cases, thin sections of the seeds at several developing stages were prepared by free hand sectioning. The starch granules in cells of the sections were then stained directly on the slide glass as described above.

Sucrose content

About 1 g of seeds was homogenized in small volume of 50 mM Tris-HCl buffer containing of 1 mM EDTA and 1 mM DTT, pH 7.5 with a Polytron (Kinematica), and the homogenate was made up to 10 ml with the same buffer. The supernatant obtained by centrifugation at 8,000g for 30 min was heated at 100 °C for 10 min and centrifuged again. Sucrose content was assayed indirectly from the amount of glucose liberated by hydrolysis of sucrose by invertase (Sigma Chem., baker yeast). Glucose contents were then measured using glucose oxidase-peroxidase method[23,24].

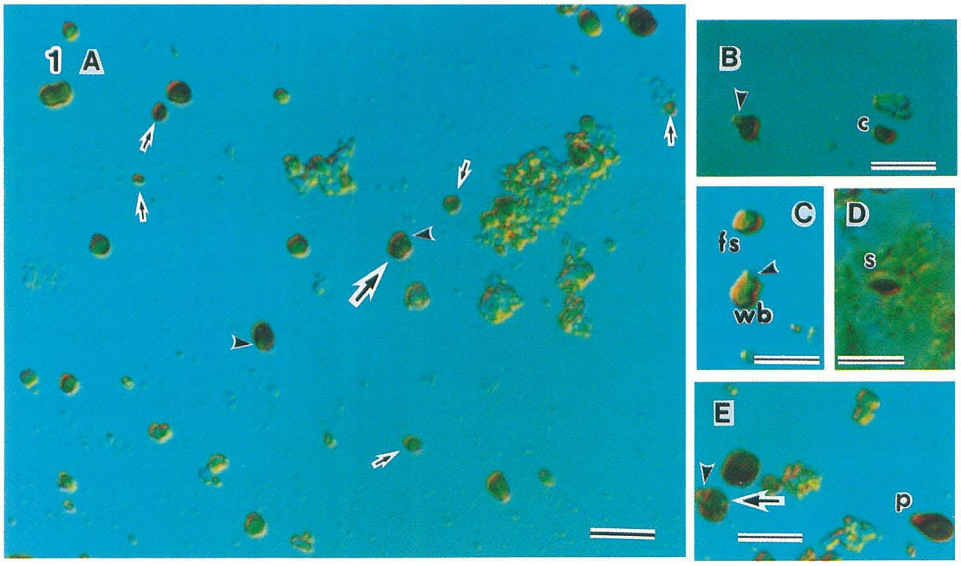
RESULTS

Changes in Morphology at an Early Stage of the Development of Starch Granules from Round to Pointed and/or Elongate Structures

Starch particles first became discernible in very young immature seeds 4 mm in size. Instead of the elliptical-shaped appearance displayed by the mature starch granules, these small starch particles, 2-5 μm in diameter, were more spherical in overall shape (Fig. 1, small arrows). Closer examination revealed that many of these starch particles had small protrubances (Figs. 1A - E, large

Figure 1A. Nomarski differential interference micrographs of juvenile growing starch particles in developing beans in young pods of *P. vulgaris* L. The developing seed of about 4 mm in size was minced with a cutter, and it was then pressed the juice extract from the cut slurry by means of tweezers. To discriminate starch particles from other cellular particles, a minimum amount of the iodine solution was added to the extract on the slide glass and stained slightly. Small arrows, juvenile starch particle detectable by the optics; large arrows, the occurrence of a number of protuberances is seen on the surface; small arrowheads, sites of protuberance formation. At this stage, various profiles of the particles are seen from B through C, D to E such as : c, crescent shapes ; fs, flying saucers ; wb, water bottle shapes ; s, spindle shapes; and p, pear shapes. Scale bar on A = 20 μm and on B to E = 10 μm.

Figure 2A. Nomarski differential interference micrographs of growing starchs in developing seeds of 5 mm in size. The starch particles on the slide glass were prepared, stained and photographed as in Fig. 1. From their growth profiles, elongation reactions are predominanted over thickening ones in width, and formation of filamentous (the bidirectional growth) and triangular (the tridirectional growth) particles are evident. Partially twisted particles are started to form. Particles marked as wb (water bottle shapes) may result from the unidirectional growth. Small arrowheads, sites of protuberance formation; bi, bidirectional growth ; c, crescent shapes; p, pear shapes ; s, spindle shapes ; tr, tridirectional growth. Scale bar = 20 μm. 2B Detailed profiles of growing fronts of filamentous starch particles. Large arrowheads, growing fronts of elongating starch particles; small arrowheads, sites of the protuberance formation; large arrow, the side view of growing starch particles; h, hill-like shapes; asterisks, undulated surfaces. Scale bar = 10 μm.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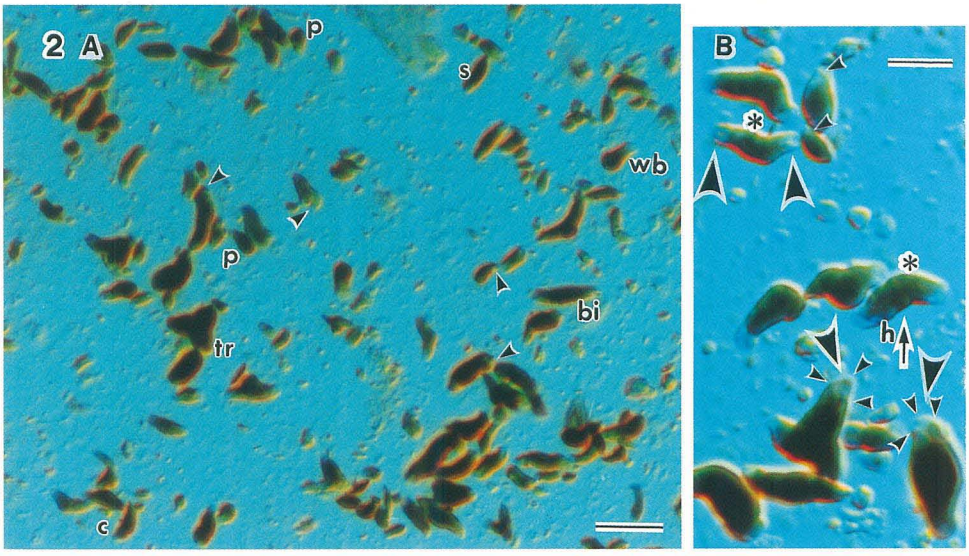
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arrows, small arrowheads) such that the particles took on a crescent-shape (Fig. 1B, c), pear-shaped (Fig. 1E, p), spindle-shape (Fig. 1D, s), flying saucer-shape (fs) and bottle-shape (wb) (Fig. 1C) appearance.

In slightly older developing seeds of 5 mm in size, the small rounded starch particles that were routinely observed in 4 mm seeds were no longer visible. Instead these small rounded particles were transformed into sausage-like structures *via* growth along the main axis (Fig. 2). These starch particles could be divided into two groups based on their size. Most of the starch particles were about 14 to 20 μm in length and 4 to 6 μm in width while a smaller fraction were 6 to 8 μm in length and 2.4 to 4 μm in width (Table 1). Growth of starch particles appear to proceed predominantly towards one or two growing points (Fig. 2, bi). In some instances, triangular-shaped starch particles were observed indicating growth in three directions (Fig. 2, tr). Protuberances were clearly seen on the growing points (Fig. 2B, small arrowheads). Side views of the particles (Fig. 2B, large arrows) exhibited hill-like (h) and undulated surfaces (Fig. 2B, asterisks) in detailed profiles.

In 6 mm seeds, the general pattern of shapes were similar to those seen in 5 mm seeds, except that the number of pointed elongating structures decreased and the starch particles began to thicken (Fig. 3A). Most of the elongating particles were partially twisted and showed characteristic growing fronts (Figs. 3A - E,

Table 1. Growing characteristics of shapes and sizes of starch granules in developing seeds

Seeds sizes (mm)	Sizes of starch granules length (L) and width (W) of major fraction (μm)	L/W	Note
4	2−5		Most of particles are round shapes, but a few variety are also seen
5	14×4	0.29	Crescent, pear and triangle shapes Pointed shapes are frequent at opposite growing points
6	18×6	0.33	The shape are heterogeneous and twisted and sickle shapes are frequent The surface is unclear
8	20×10	0.50	Growth of starch granules in length starts to decrease
12	22×13	0.59	Enlargement of thickening reaction is evident
14	24×16	0.67	Appearance of small starch granules of 4 to 6 mm in diameter takes place
15	30×22	0.69	Starch granules of small sizes decrease
16	33×24	0.73	The presence of ring structures of the surface can be detected microscopically
(mature)	33×24	0.73	

large arrowheads), where the formation of protuberances (small arrowheads) and step structures (white arrows) were generally seen. The surfaces on the sides of the particles were undulated (asterisks). These small protuberance that were always present in the growing front suggest that they are sites of active polyglucan synthesis. These observations indicate that the starch particles grows in both the major and minor axil directions. The formation of node- or tier-like structures on the general part of body surfaces (Figs. 3A - E) appears to be a result of differences in synthetic activities at surface sites of starch microenvironments of the growing particles. The size of the starch particles at this stage varied considerably and ranged from about 10 to 26 μm in length and from 4 to 8 μm in width. The majority of the starch grains were 18 μm in length and 6 μm in width (Table 1). As elongation proceeds, species that had a hook-like appearance and curved or twisted shapes were frequently detected (Fig. 3A). The growing apices were no longer pointed and showed some irregular surfaces.

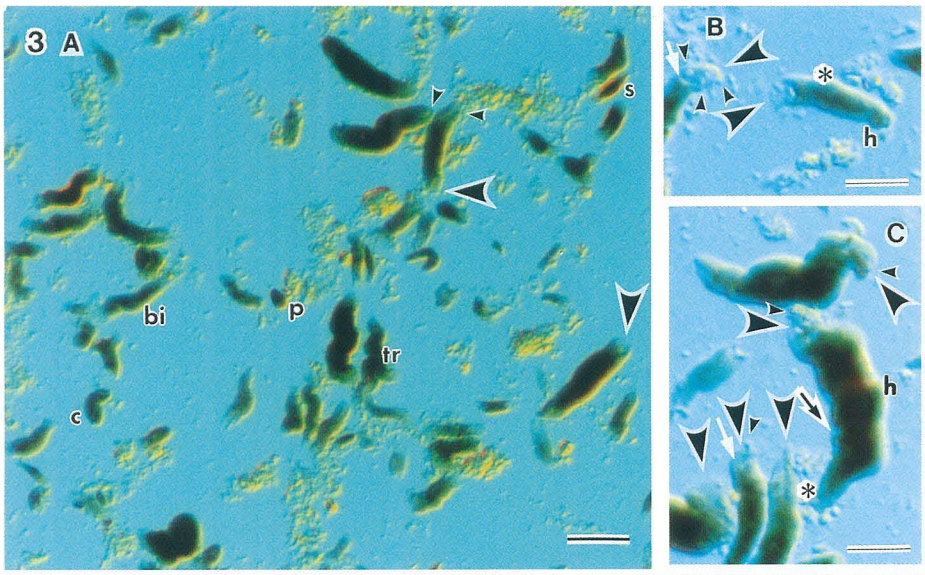
Similar trends in elongation continued in 8 mm seeds but the characteristic growth fronts seen in the 5 and 6 mm seeds were not detectable at this stage (Fig. 4A). The starch particles displayed a variety of shapes as seen in the 5 and 6 mm seeds (Figs. 2A and 3A) including triangular ones (tr). The particles started to form its round and granular shape, and as an indication of the augmentation of thickening reactions, an occurrence of undulated or hill-like surfaces (h) were generally seen, suggesting differences in polyglucan synthesis and accumulation on micro-environments of the starch grain surface. The medium length and width of the starch grains was 20 μm and 10 μm, respectively (Table 1). Hence, the ratio of the length to the thickness (or the width) of the major starch fractions of the starch particles in seeds from 5 through to 8 mm increased from 0.29 to 0.50, indicating that in juvenile stages, starch synthesis proceeded predominantly in the major axis in younger developing seeds and then predominantly in the minor axis as the seeds further developed (Table 1).

Growth towards Formation of Granular Forms at a Middle Stage of the Seed Development

A steady increase in relative reaction rates of the thickening to the enlargement in starch granules was clearly detected microscopically in 12 mm seeds (Figs. 5A - D). The length of the major starch granules was 22 μm and the width was 13 μm (Table 1), resulting in a length/width ratio of 0.59, which is 81% of the value of the mature starch grains. Therefore, the appearance of the growing granules at this stage was quite different from that of the growing particles observed in the seeds of the 5 and 6 mm in size. Starch particles appeared to be premature granular forms not only in shape but also containing distinct features, e.g. grooves or ditches (small arrows), and hilum-like positions (stars) (Figs. 5A - D) characteristic of the mature starch grain. A visible development of growth rings on growing starch surfaces was also detected at this stage (large arrow-

Figure 3. Formation of various elongated and twisted starch particles in 6 mm seeds in size. The preparations of the starch samples on the slide glass and procedures for observations were the same as in Fig. 1. At this stage, in addition to continuous elongation reactions, a random occurrence of hill-like shapes (h) on side position of starch particles is always observed, resulted in the formation of a variety of irregular forms. Large arrowheads, growing fronts of elongating starch particles; small arrowheads, sites of protuberance formation ; large arrows, the side view of growing starch particles ; bi, bidirectional growth ; c, crescent shapes ; p, pear shapes ; s, spindle shapes ; tr, tridirectional growth ; asterisks, undulated surfaces. Note that formation of a number of the protuberances and step structures (white arrows) at the growing fronts (large arrowheads) is frequently seen at this stage. Scale bar on A = 20 μm and on B to E = 10 μm.

Figure 4. Changes in the growth of starch particles from the elongation to rounding and/or thickening in seeds of 8 mm. Preparations of starch samples on the slide glass and staining with the iodine solution were done as in Fig. 1. At this stage, the reaction rate involved in the elongation of starch particles is decreased while that involved in thickening in width is increased. The starch particles start to form its round shape. Note that the surfaces of the starch particles exhibit undulated or hill-like (h) profiles, and the formation of protuberances (small arrowheads) is only infrequently seen. Large arrowheads, grown fronts in the elongation ; large arrows, side views of starch particles ; bi, bidirectional growth ; c, crescent shapes ; p, pear shapes ; tr, tridirectional growth. Scale bar on A = 20 μm and on B = 10 μm.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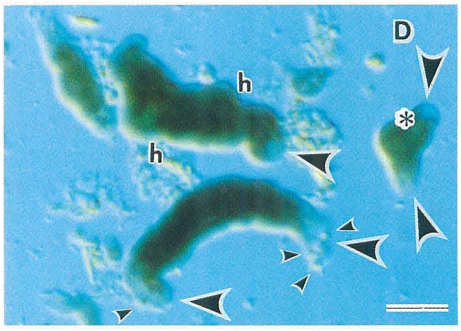
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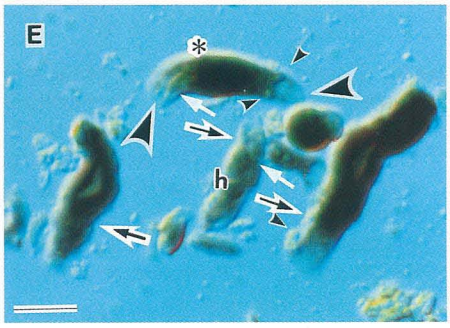
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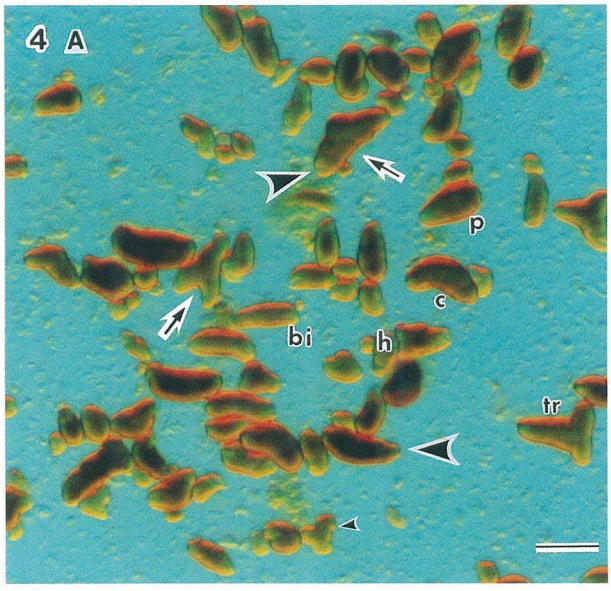
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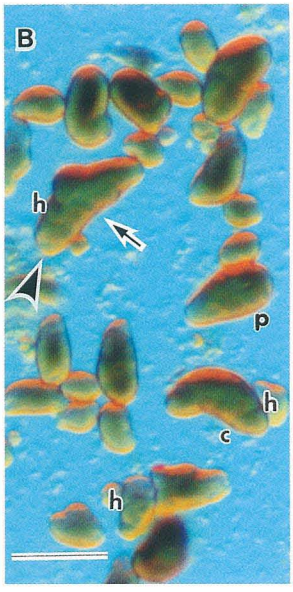
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Figure 5. Progression of rounding of the starch particles and the formation of granular forms in seeds of 12 mm in size. Preparations of starch samples for observation (Figs. 5A and B) were the same as in Fig. 1. Starch granules in Fig. 5C and D were stained with the iodine solution and photographed as in Fig. 1. At this stage, the elongated and twisted forms of the starch particles totally disappeared, and starch shapes have turned to granular forms. Growth rings (arrowheads), hilum positions (white stars), grooves on the surfaces (white asterisks) and ditches on the side (arrows) are detected. Scale bars on 5A and C = 20 μm and on 5B and D = 10 μm.

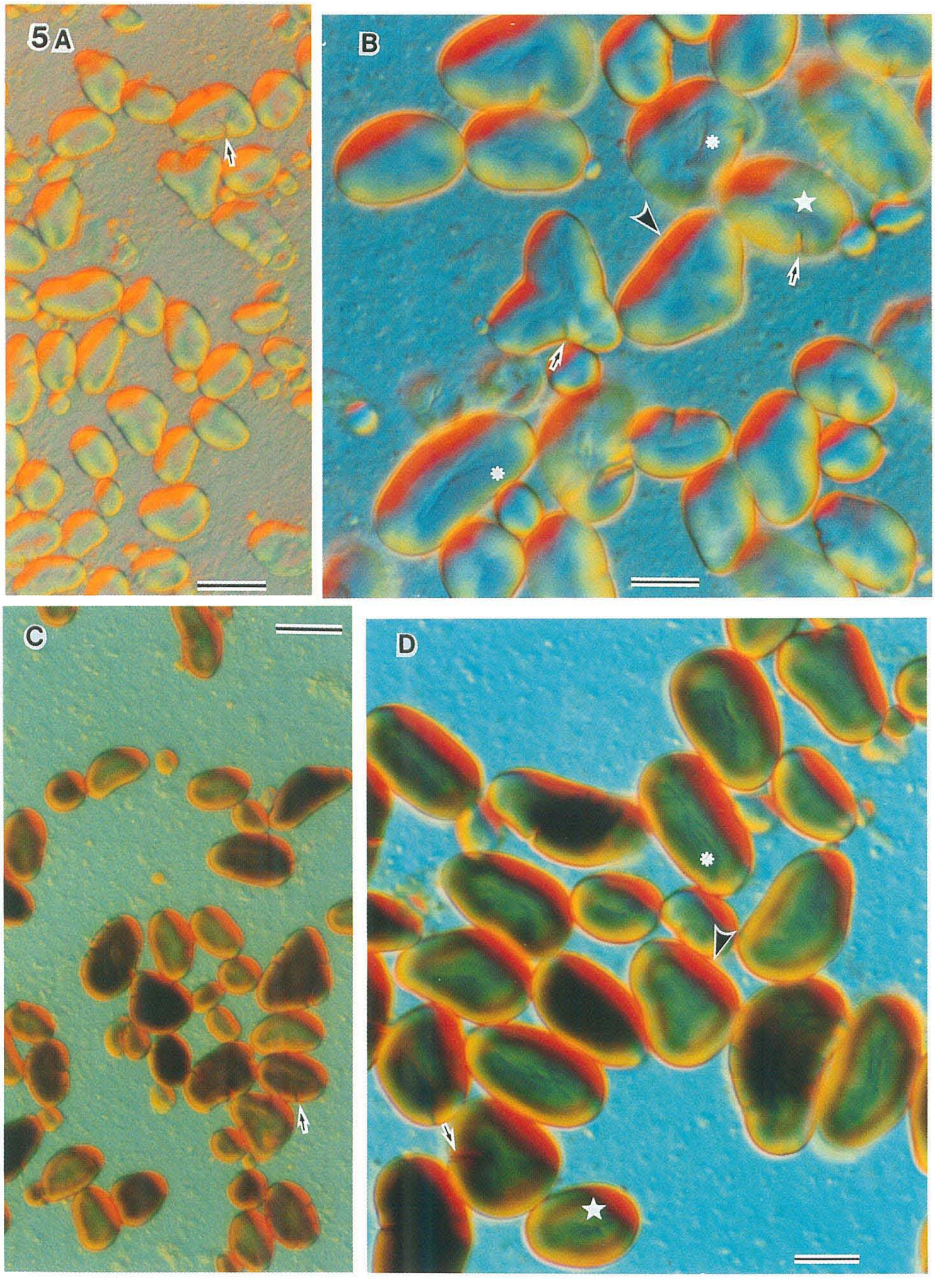
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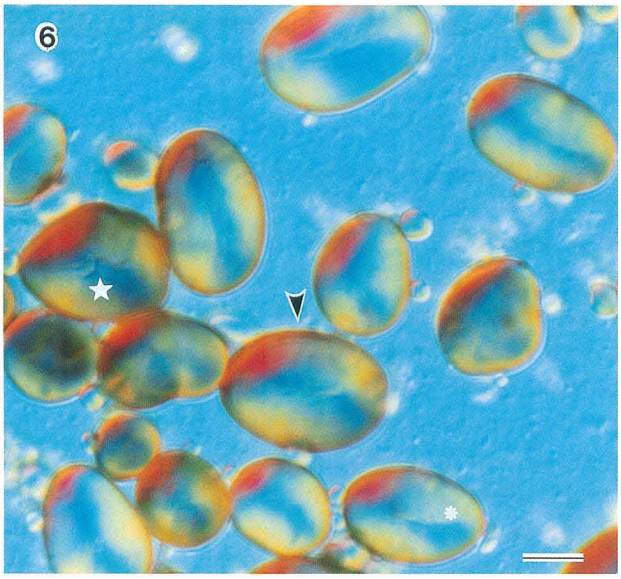
Figure 6. Completion of the transformation of growing starch particles to granular forms in seeds of 14 mm in size. Preparations of starch samples for observation were as in Fig. 1. At this stage, an elliptic form, a typical form of starch granules is finally completed. The granules clearly show grooves (white asterisks), growth rings (large arrows), and hilum positions (white stars). Scale bar = 10 μm.

Figure 7. Populations of starch granules in 15 mm seeds in size. Preparations of starch samples and staining in minimum levels by the iodine solution were as in the Fig. 1. The starch granules are comprised of two groups in size, large (L) and small (S) ones. From differences in stainability in terms of color development, there are two groups, faster (D) and slow (B) rates of reactive ones. Scale bar = 20 μm.

Figure 8. An active enlargement of starch granules at a final stage of seed development in 16 mm seeds in size. Preparations of starch samples and minimum levels of staining by the iodine solution were done as in Fig. 1. The growth of the size in terms of thickness is conspicuous at this final stage of the development of pods. White asterisks, grooves; large arrowheads, growth rings; white stars, hilum position. Scale bar = 10 μm

Figure 9. Local difference in staining responses of the surfaces of developing starch particles to the iodine solution in seeds of 5 mm in size. Preparations of the samples for observation and low levels of staining to allow detectable difference on the sample surface were done as in Fig. 1. The staining of part of the surface is clearly seen (black arrows) while virtually no staining in other part (white arrows) is evident. Scale bar = 10 μm.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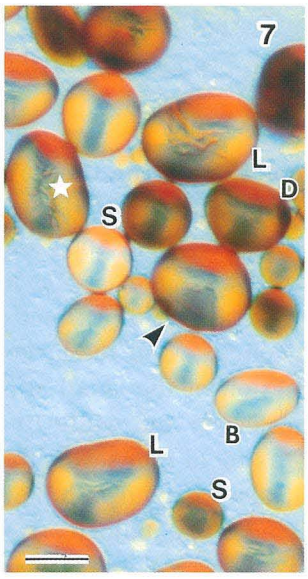
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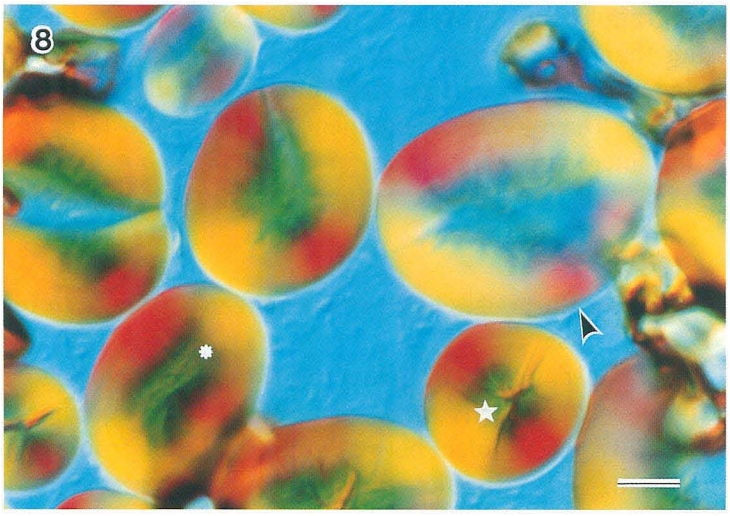
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heads) (Figs. 5B and 5D). These apparent changes in appearance in 12 mm seeds indicate that construction processes of polyglucan/or accumulation modes of the reaction products differ than before that occur in the 5 to 8 mm seeds (Figs. 2, 3 and 4).

Completion of the Construction of Starch Granules in Seeds at Final Stages of Maturation in Pods

In seeds of 14 and 15 mm in size, starch granules reached a typical, elliptic form (Figs. 6 and 7). As a characteristic event of this stage, a number of small starch granules of a round shape of 4 to 6 μm appeared. Therefore, the population comprised of the large (L) and small (S) starch granules. Mechanisms of the formation of the small starch granules in the 14 mm seeds are unknown. The mean length and width of starch granules was 24 μm and 16 μm, respectively. And as to 15 mm seeds, they are 30 μm in length and 22 μm in width.

Starch granules in mature 16 mm seeds had a thickness/length ratio of 0.73 (Table 1). The majority of the starch granules were about 33 μm in length and 24 μm in width. Some large granules show 44 μm (length) and 32 μm (width) of the size. The small starch granules appeared in the 14 mm seeds increased their size, but the size was smaller than the major fraction of the starch granules. At this stage, the scaly or ring structure was detected microscopically all over the surface of starch granules. During the development of seeds from 14 to 16 mm in size, the starch grains increased about 9 μm in the length, but the ratio in length over thickness showed small changes compared with the preceding stage (Figs. 6 and 8).

Local Differences of Staining Responses of Growing Starch Particles to Iodine Solution

In the presence of low concentration of iodine solution, the elongating fronts exhibited extremely low levels of reactivity as seen in Fig. 9. The remarkable differences in the stain-ability were evident between the elongating fronts and the other part of the starch particles. Similar staining patterns were also seen in Figs. 2B and 3E, in that virtually no staining by iodine solution was observed in the front areas including protuberances and close areas. The less reactivity towards low concentration of iodine solution tended to continue until the active elongation apparently ceases as was observed in the 8 mm seeds (Fig. 4A). After this stage, the whole starch surfaces showed similar extents of staining by iodine solution. These observations indicate that major fractions of the building block of the growing site differ from the elongating sites to thickening ones.

Sucrose contents in developing kidney bean seeds

Sucrose contents of kidney bean were determined using various developing seeds (Table 2). It was 8.2 mg/g of seed in 4 mm seeds, in which juvenile starch

particles were seen. In the seeds more than 6 mm in size, sucrose contents ranged from 12.5 to 16.7 mg/g of seed until seeds reached 14 mm in size. The seeds of final stage, 16 mm in size, showed a decrease in sucrose content of 9.5 mg/g of seed, which was almost identical to that observed in mature seeds (9.7 mg/g of seed). These results indicate that during the period of rapid growth rates of the starch grains, the supply of translocated sugar from the source organ to the sink organ does not so change appreciably.

Table 2. Sucrose content in developing seeds

Size of seed (mm)	Sucrose (mg/g of seed)
4	8.2
6	16.7
8	15.2
10	14.1
12	15.8
14	12.5
16	9.5
(mature)	9.7

DISCUSSION

Starch granules in mature seeds of kidney bean, *P. vulgaris* L. cv. Toramame, show almost an elliptic or spherical shape. The population is comprised of large (about 33 μm) and small (about 10 μm) sized granules, and the former occupies the major fraction. In developing pods, however, the elliptic shape is first detectable in seeds of 12 mm in size, and an onset of the formation of the elliptic shape of starch granules starts in seeds of about 8 mm size (Fig. 4). In developing seeds of 4 to 6 mm in size, juvenile starches are long and/or filamentous particles, and they are irregular in terms of sizes and shapes, showing that elongation processes predominantly proceed at the growing front of the particles (Fig. 2). The juvenile starch particles do not show any indications of the presence of growth rings, as could be seen on mature starch granules of the bean (Fig. 8). These observations clearly indicate that processes of the starch granule formation of bean proceed at least in two stages, firstly an elongation and then thickening, and that mechanism involved in processes of the elongation and thickening, in other words, synthetic reactions of unit constituents and following stacking on polysaccharide strata at early stages are different from those of the latter stages. Furthermore, it is clear that the construction of the reported starch granule structure such as alternating crystalline and semi-crystalline zones only occurs in latter stages.

In the present studies, structural changes in the dimension of 1 μm can be detected microscopically, and the length that is equivalent to ca. 10 amylopectin molecules in tandem (2 to 4 μm in length) is easily observed. On the growing fronts, there is the formation of microconstructions, such as protuberances, horse-back structures (or cavities), terraces (or step structures) and so on (Figs. 2, 3 and 4).

Plant starch granules consist of amylopectin and amylose7,8). Starch synthesis starts naturally in the formation of the primer1), and a consecutive forma-

tion of a unit fraction of amylopectin (a cluster) at intervals of 9 nm results concomitantly in the stacking onto starch granules. A unit dimension of amylopectin molecule in average is 200 to 400 nm long and 15 nm wide[25], and part of these amylopectin chains form parallel arrays of double helices that are responsible for the crystalline layers of starch granules, in other words, the growth ring is the reflection of the presence of crystalline layers in the architecture of starch granules as shown earlier[26].

It is necessary to find out general mechanism that account for the above features of the event occurring on juvenile starch particles, shortly as; 1. a transient occurrence of microstructural changes such as the protuberance, 2. the formation of long and irregular forms of starch particle, and 3. an absence of growth rings at early stages and their appearance in latter stages. As the possible explanations, we suggest the event is resulted from the following cellular conditions, that is, 1. an abundant supply of mingled amylopectin molecules of different sizes, particularly longer ones as the major fraction, occurs all the time at the early stages, 2. the condition 1 partly depends on relative levels of related enzyme activities as well, and 3. substrate levels at sink sites are also an important factor that might determine properties of the construct, amorphous or crystalline, on growth fronts of starch granules.

The above suppositions are based on the following considerations. In general, tendencies of the crystal formation are largely determined by the uniformity and concentrations of solute components. In principle, similar conditions can be expected on the formation of starch granules. On slick faces of the generating polyglucan layers on starch granules, set of branched side chains (for example B1 chains) of amylopectin molecules running parallel and in close proximity to each other may allow the prompt formation of double helices, and repetition of the process results in the continued formation of crystalline layers in the slick regions. The chain structure is left-handed parallel-stranded double helices as proposed first by Kainuma and French[12] and confirmed subsequently by Imberty *et al.*[19] and Oostergetel and van Bruggen[20]. Hizukuri showed that in potato amylopectin, appreciable amounts of clusters (10-20%) form inter connections with adjacent clusters[10]. When each unit structure of mixed amylopectin molecules on a given reaction site is largely, resembled physical properties, such as solubilities, of the heterogeneous components facilitates tendencies to form clotted precipitates in the way that is not in stratum, and together with amylose molecules and other minor components, may form an amorphous layer.

Seeds of juvenile pods contain abundant amounts of translocated sugars from the source (Table 2) and low levels of starch branching enzyme activities[27], where due to the low enzyme level, amylopectin molecules of longer ones as the major fraction are synthesized at the same time. Then precipitates or coagulates formed from any combinations of available species of amylopectin molecules, and possibly with amylose molecules, tend to stack onto growing surface layers of

juvenile starch particles. Thereby structural differences among amylopectin molecules or random location of short branched chains of amylopectin clusters disturb the formation of crystalline layers on the growing starch particles, and may result in the formation of the protuberance of ca. 100 nm in diameter on the growing fronts (Fig. 3, G - E). Recently, Gallant *et al.* inferred that a 'blocklet' organization within starch granules detected by the SEM examination and a surface protrusion observed by the atomic force microscopy are the same structures, and that sizes of the 'blocklet' of wheat and potato starch granules are 80 to 120 and 200 to 500 nm, respectively[22]. From the limited occurrence at growing apices of juvenile starch particles, their sizes of ca. 100 nm, and the only possible glucan component of starch particles, we consider that the protuberance is stacks of amorphous forms of polyglucan in nature, and that from the prominent elongation at the apices, a great number of enzymes involved in glucan synthesis are concentrated in the regions around. From these considerations, the structural heterogeneity of amylopectin molecules that contain long size classes as the major fraction seems to cause the formation of irregular and filamentous starch particles.

The following results may support the above inference. Firstly, at early stages of starch granule formation in seeds of the bean, it is expected that supply of photosynthetic materials (the rate limiting substrate, ADPG as well) is enough and efficient as judged from sucrose levels (Table 2). Under the conditions, synthesis of amylose molecules of several lengths may actively proceed, and consequently, results in the formation of amylopectin molecules of different sizes. At mature stages, an average degree of polymerization of amylopectin molecules has found to be 5,270, and the value is, as expected, less than one third of that (17,100) of 8 mm seeds, in that the distribution of the values is asymmetrically inclined to much more larger zones (Takeda *et al.*, unpublished observation). These results indicate that, as maturation proceeds, changes in the size of the amylopectin molecules occur gradually from larger to small ones. Secondly, when the placentae of seeds that is 4, 8 and 12 mm in size, for example, are supplied the same concentrations of assimilates from the source, relative levels of the concentrations on respective sinks (inside the seeds) at an equilibrated state will be (on the assumption that concentrations are reciprocal to their seed volumes and the level in 4 mm seed is assumed to be 1) 0.13 and 0.04, assuming further that all seeds are spherical and velocities of the required reactions for starch synthesis are the same at the respective. Results of analyses of sucrose concentrations at the two different stages indicate that the condition similar to the above inference on changes in substrate levels is taking place at sink sites (Table 2). Thirdly, at latter stages of the seed development, ca. 12 mm in size, an abrupt upsurge of branching enzyme activities is detected[27], the result of which is consistent with an earlier result of developing maize endosperm cells[28]. The results of analysis of low levels of branching enzyme activities appear to substan-

tiate, though it is partly, the above suppositions in terms of the formation of irregular and elongated particles and the absence of crystalline structures at early stage.

Juvenile starch particles elongate in three ways; uni-, bi- and tridirections, and among them uni- and bidirectional growths result in the formation of longer starch particles, while the tridirectional ones form trianguler ones (Fig. 4). The mechanism of the directional growth of juvenile starch particles may also be explained by a locational bias of starch synthase around peripheral zones of growing apices, and local differences in enzyme activities on the particles surfaces result in the cause of the mode of the directional growth. The partial location of starch synthase on the periphery of granule starch particles has been observed in cells of developing potato stolons by immunogold staining[29,30]. The juvenile starch particles of about 0.25 μm in developing potato proplastids show transparent images by transmission electron microscopy that is typical characteristic of starch granules, but they do not sediment as particles (Sagisaka, unpublished observation). These observations suggest that the growing periphery of starch granules and juvenile starch particles contain yet premature states of starch like glucans and a number of required enzymes, where the resin is easily penetrate into the sites of interstice.

Irregular and filamentous starch granules are formed in mutant plant cells defective in a starch branching enzyme[22]. By contrast, at juvenile stages, seeds of the bean that is not defective in any of enzymes also form irregular starch particles similar in shape to that of mutant plant cells. Moreover, no indication for the occurrence of such a directional growth as mentioned above has heretofore been demonstrated in the literature on the field of the starch granule formation, and more importantly, this process determines roughly the length of the starch granules, that is, by 8 mm of the seed size, major fractions of starch particles reach more than 60% of their maximum length by this growth mode. Thereafter, the particles are found to start rounding, and in the seed size by 14 mm, they, together with the trianguler ones, establish an elliptic mature shape. The occurrence of the directional growth and a number of irregular surfaces on the growth fronts before thickening suggests that some mechanisms or undefined proteins that govern the directional growth of starch particles are present on the growing sites of the particle in the early stage.

In algae, a wild type strain of *Chlamydomonas reinhardii per se* forms irregular starch granules in size and shape, and the trend in irregularity in shape is more pronounced in double mutants lacking one of the starch granule-bound starch synthases and a soluble starch synthase[31]. Starch granules formed in the latter mutant contain an appreciable level of short chain amylopectin molecules. In these granules, growth rings are not detected by transmission electron microscopy, but X-ray diffractograms and thermograms of differential scanning calorimeter show an occurrence of crystalline structures. Another mutant missing

the second starch granule-bound starch synthase is shown to form starch granules of low crystallinity that contains amylopectin molecules of a high degree of polymerization[31]. In the present studies using seeds of a cultivar of kidney bean, growth rings in starch granules are first detected in 12 mm seeds in size (Fig. 5), in which an onset of a raise of starch branching enzyme activities concurs, suggesting that a pertinent size of amylopectin molecules for crystallinity (an average degree of the polymerization of 5,270) is started to be synthesized in the cells.

There is no indication of the presence of the blue staining core (the hilum or center) of starch particles in early stages at least by the seed size of 8 mm in size, and the iodine staining is hardly observed near the growing zones (Fig. 4). When seeds grow to about 12 mm in size (Fig. 5), the starch granules in the seeds first show seemingly wrinkled zones, and then the trend in the formation of wrinkly surfaces in the area is strengthened. Even at this stage, it is hard to observe clearly from the iodine staining the core site of starch construct. This result differs from that of Kuipers *et al.*[32]. The low level of reactivities of the elongating fronts towards low concentrations of iodine solution is unexpected findings, and it is hard to describe exact mechanism of the phenomenon that is obviously the reflection of the physical properties, and therefore the glucan contains low levels of amylopectin molecules. Taken these facts together, the facts that we have observed such large differences among studies suggest that we have some unknown variable.

It is of worthy to note that during changes in seed sizes from 14 to 16 mm at final maturation stages, starch granules in the seeds rapidly grow to the extent that the diameter increases roughly 1.4 times (Figs. 6 and 8).

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