Biogenesis of Protein Bodies in Embryonic Axes of Soybean Seeds (Glycine max. cv. Enrei)

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Received November 21, 1991

Protein bodies in embryonic axes of soybean seeds have inclusion structures containing phytic globoids. Biogenesis of the protein bodies during seed development was examined by transmission electron microscopy. Protein bodies in embryonic axes originated from central vacuoles. The central vacuole in embryonic axes subdivided into smaller vacuoles with internal membranous structure. Then the subdivided vacuoles were directly associated with rough endoplasmic reticulum (rER), and were filled with proteinaceous matrix from the peripheral region. The increase of matrix was simultaneous with accumulation of β-cglycinin estimated by SDS-polyacrylamide gel electrophoresis. Glycinin-rich granules that had been found in developing cotyledons were not observed in embryonic axes. After proteinaceous matrix filled the protein bodies, electron-transparent regions presumably surrounded by a single membrane appeared in the matrix. Phytin globoids were constructed in this internal structures of protein bodies as the final step of protein body formation.

Seed storage proteins are reserved in specialized organelles called protein bodies, which are classified into several groups according to their origin and structural complexity.1,2) One of the types of protein bodies is that with inclusion structures composed of apparent internal membrane, electron-transparent globoids and electron-dense globoids of phytic acid and/or protein. In our previous paper3) we showed that protein bodies in soybean embryonic axes belong to this type, while those in cotyledons have a homogeneous matrix with few inclusions. However, the biogenesis of this type of protein bodies was not understood. Here we follow the ultrastructural changes in embryonic axes of soybeans during the seed development to examine how the protein bodies are formed.

Materials and Methods

Plant material. Soybean plants (Glycine max cv. Enrei) were grown in the experimental field of the Agriculture Research Center, Tsukuba, Japan. Seed maturation took about 90 days after flowering (DAF). Embryonic axes and cotyledons were collected from developing seeds which were harvested during 26 to 90 DAF. They were freeze-dried, ground with mortar and pestle to a fine powder, defatted with chloroform/methanol as described by Nagao et al.4) and used for chemical analysis. For electron microscopic study, embryonic axes were fixed and processed as followed immediately after harvest.

Electron microscopy. Isolated embryonic axes were cut into pieces about 8 mm², fixed overnight in 2.5% glutaraldehyde in 0.1 m sodium cacodylate buffer, pH 6.7, and postfixed for 2 hr in 1.0% osmium tetroxide in the same buffer, dehydrated with a 30—100% graded ethanol series (30, 50, 60, 70, 80, 90, 95, and 100% ethanol), and embedded in Spurr’s® epoxy resin. Ultrathin sections were stained with uranyl acetate for 30 min and lead citrate for 1 hr, and examined with a JEM 1200EX microscope.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For protein extraction, 20 mg of defatted sample powder were homogenized in 1 ml of 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS and 5% 2-mercaptoethanol, left for 2 hr at room temperature, and dialyzed against the same buffer. After addition of 0.1 ml of glycerol containing 0.05% bromophenol blue and 3 min of boiling, 10 μl of the protein extracts were put on 10% SDS-polyacrylamide gels and run for 3 hr at 80 V by the method of Laemlli.6) The resulting gels were stained with Coomassie Brilliant Blue R-250 (CBB), or with periodic acid-Schiff’s (PAS) reagent, and scanned on a densitometer (Shimadzu UV 240).

Immunoblot analysis. The antibodies against β-cglycinin and glycginin from soybean cotyledons were generous gifts from Dr. Horisberger of Nestle Co., Ltd. Protein extracts (5 μl) from embryonic axes were put on 5—20% SDS-polyacrylamide gradient gels, and run for 1 hr at 20 mA. Proteins were transferred electrophotothetically onto Immobilon PVDF membrane (Millipore), and after equilibration overnight in TBS-BSA (Tris-buffered saline (TBS), pH 7.5, containing 3% BSA), the membranes were incubated with antibodies against β-cglycinin or glycginin in TBS-BSA for 1 hr at room temperature. The membranes were washed three times for 20 min with TBS-Tween (TBS, pH 7.5, containing 0.1% Tween 20), followed by reaction with peroxidase conjugated goat anti-rabbit IgG antibodies (Organon Teknika) in TBS-BSA for 1 hr at room temperature. The membranes were finally washed three times for 20 min with TBS-Tween. The peroxidase activity on the membranes was detected by a Konica immunostaining HRP kit IS-50B.

Phytic acid estimation. Thirty mg of defatted powder was homogenized in 3 ml of 0.2 M HCl for 2 min by a homocyt (NIT-ON NS50). Phytic acid in the extracts was estimated by the colorimetric method of Haug.8) In this method, phytic acid was precipitated with an acidic iron-III-solution of known iron content, and the decrease of iron in the supernatant was measured with 2,2'-bipyridine solution.

Results

Deposition of protein matrix into subdivided vacuoles

Proteinaceous matrix in the protein bodies in embryonic axes was formed during about 31—50 DAF (Fig. 1). In the early stage of seed development (Fig. 1A), a large part of the cell was occupied by a central vacuole, and subdivision of the vacuoles into smaller vacuoles was observed. Craig et al. documented similar changes in vacuoles during protein body formation in developing pea cotyledons.9) At 40 DAF (Figs. 1B and C), the vacuoles were usually a few μm in diameter. The vacuoles contained membranous inclusions, and were directly associated with rER. Dispersed matrix appeared in the peripheral region of the vacuoles.

Abbreviations: CBB, Coomassie Brilliant Blue R-250; DAF, days after flowering; PAS reagent, periodic acid-Schiff’s reagent; rER, rough endoplasmic reticulum; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.
Fig. 1. Biogenesis of Protein Bodies from Central Vacuoles in Embryonic Axes of Developing Soybean Seeds.
Ultrastructure of embryonic axes of developing soybean seeds at 31 DAF (A), 40 DAF (B and C), and 50 DAF (D) were examined by transmission electron microscopy. AM, amyloplast; LB, lipid body; rER, rough endoplasmic reticulum; V, vacuoles.

Abundance of ribosomes in cytoplasm and rapid increase of proteinaceous matrix in vacuoles indicated that storage proteins in embryonic axes are vigorously synthesized, intracellularly transported, and accumulated at this stage. Electron density of the proteinaceous matrix increased till 50 DAF (Fig. 1D).
We had found large, electron-dense granules in cotyledons at mid-stage of seed development, and discovered that those granules are extremely rich in glycinin, which is one of the major storage proteins in soybean cotyledons, but not in embryonic axes. In this experiment we did not detect similar granules in embryonic axes at mid-stage or any other stage of seed development.

Protein accumulation in embryonic axes

The accumulation of proteinaceous matrix observed

![Fig. 2. Protein Accumulation in Embryonic Axes and Cotyledon during Seed Development.](image)

Extracts from embryonic axes (A and B), and cotyledons (C) of developing soybean seed at 26-91 DAF analyzed by SDS-PAGE. Gel were stained with CBB (A and C), and PAS reagent (B). Peak 1 represents lipoxynase. Peaks 2, 3, and 4 represent \( \alpha', \alpha, \) and \( \beta \) subunits of \( \beta \)-conglycinin, respectively. Peaks 5 and 6 represent acidic subunits of glycinin.

![Fig. 3. Immunoblot Analysis of \( \beta \)-Conglycinin (A) and Glycinin (B) in Embryonic Axes of Developing Soybean Seeds.](image)

Extracts from embryonic axes of soybean seeds at 26 (lane 1), 35 (lane 2), 47 (lane 3), and 68 (lane 4) DAF were analyzed. Bands 1 and 2 represent \( \alpha' \) and \( \alpha \) subunits of \( \beta \)-conglycinin, respectively. Bands 3 and 4 represent acidic subunits of glycinin. Band 5 represents basic subunit of glycinin.

![Fig. 4. Construction of Inclusion Structure of Protein Bodies in Embryonic Axes.](image)

Ultrastructure of embryonic axes in late developing stage (A, 61 and B, 71 DAF) were examined by transmission electron microscopy. PB, protein body; PG, phytin globoid; SG, soft globoid.
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Fig. 5. Changes in Phytic Acid Content in Embryonic Axes during Seed Development. Closed circle and open circle represent phytic acid content in embryonic axes and cotyledons of developing soybean seeds, respectively.

under the electron microscope was simultaneous with the increase of β-conglycinin during seed maturation estimated by SDS-PAGE. Figures 2A and B show the profile of protein accumulation in embryonic axes during seed development. β-Conglycinin, the major storage protein in embryonic axes, increased between 26-47 DAF. It appeared in embryonic axes later than in cotyledon (Fig. 2C), and the accumulation period was shorter than that in cotyledons. Lipoxygenase increased throughout the seed maturation. The peak area of glycinin could not be clearly estimated because of the interference by other numerous peaks.

Neither β-conglycinin nor glycinin was detected in embryonic axes at 26 DAF by the SDS-PAGE analysis (Fig. 2), but by an immunoblot analysis (Fig. 3), β-conglycinin was found at 26 DAF, while glycinin was not detected at this stage. This result indicates that β-conglycinin in embryonic axes accumulated in advance to glycinin, as in cotyledons.

Construction of inclusion structure in protein bodies

Protein bodies in soybean embryonic axes have inclusion structures composed of inner membrane, soft globoids, and phytin globoids. Figure 4 shows that the inclusion structure was formed after deposition of proteinaceous matrix had completed. At 61 DAF, an electron-transparent region surrounded by a single membrane appeared in the proteinaceous matrix (Fig. 4A). Then, phytin globoids were formed in the transparent region as the final step of protein body biogenesis (Fig. 4B), though the phytic acid content in soybean embryonic axes increased constantly from the early stage of seed development (Fig. 5).

Changes in organelles other than protein bodies

Amyloplasts filled with starch granules were abundant at 31–40 DAF (Figs. 1A and B). The starch granules were decreased during the later stage of seed development, and became rare at 61 DAF (Fig. 4A). They changed into plastids in mature seed axes having a spiral membrane structure and crystal inclusions (data not shown). The changes of plastid structure in embryonic axes during seed development were similar to that in cotyledons. Lipid bodies were scattered in the cytoplasm till 40 DAF (Figs. 1B and C). They assembled along cell wall at 50 DAF (Fig. 1D), and around protein bodies at 61 DAF (Fig. 4A).

Discussion

The complex structure of protein bodies in soybean embryonic axes was formed through two steps: first, proteinaceous matrix was transported into subdivided vacuoles from rER, and second, inner structures usually including eminient phytin globoids were constructed in the matrix. It is reported that the stage when the phytin globoids appear in the protein bodies is not common among tissues studied. In some species, phytin globoids in protein bodies are observed from early stages of seed development, and they grow through seed development, but in other species, phytin globoids do not appear until later stages of seed development. Protein body formation of soybean embryonic axes belongs to the latter type, and this might be related to the biological role of embryonic axes and the characteristics of protein body components. But factors that determine the species-to-species, organ-to-organ, and tissue-to-tissue differences in structure and biogenesis process of protein bodies are complicated and still to be discovered.

We could not detect the electron-dense, large granules that are considered to be complexes of glycinin, phytate, and other components in the developing embryonic axes. Since phytic acid content in embryonic axes increased constantly from the early stage of seed development (Fig. 5), a soluble or invisible form of phytate might be deposited, probably in cytoplasm, before the phytin globoids appear in the protein bodies.

In our experiment, direct association of rER with protein bodies was observed while storage protein was rapidly accumulated. Numerous studies showed that Golgi apparatus is involved in the transport of storage protein from rER to protein body or vacuole. Biochemical and cytological evidence support the idea that pea vicilin and other legume proteins related to β-conglycinin are intracellularly transported via modification of the glycosyl side chain in Golgi apparatus. Several researchers indicated that there are transport pathways of storage proteins in mustard and maize seeds bypassing the Golgi apparatus. As for embryonic axes of soybean seed, Ladin et al. observed Golgi apparatus in embryonic axes of developing soybean seed, but they reported that the vesicles secreted from the Golgi apparatus were electron-transparent. Though β-conglycinin in soybean embryonic axes was glycoprotein (Fig. 2B) and immunochemically reactive to antibodies against cotyledonary β-conglycinin (Fig. 3), the glycosylation in embryonic axes had not been examined. The association of rER with vacuoles observed in this experiment shows the possibility that β-conglycinin in soybean embryonic axes was transported directly from rER to vacuoles bypassing the Golgi apparatus. Biochemical research is necessary to clarify whether this pathway is present or not.

Acknowledgments. We thank Dr. A. Nagao for his kind help in sample preparation, and Dr. Horisberger for the gift of antibodies against β-conglycinin and glycinin.

References
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