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Biosynthesis of Storage Proteins in Developing Rice Seeds

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ABSTRACT

Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the starchy endosperm protein of rice (*Oryza sativa* L. Japonica cv Koshihikari) during seed development confirmed that storage protein begins to accumulate about 5 days after flowering. Two polypeptide groups, 22 to 23 and 37 to 39 kilodaltons, the components of glutelin, the major storage protein in rice seed, appeared 5 days after flowering. A 26-kilodalton polypeptide, the globulin component, also appeared 5 days after flowering. Smaller polypeptides (10- to 16-kilodaltons) including prolamin components, appeared about 10 days after flowering. In contrast, the levels of the 76- and 57-kilodalton polypeptides were fairly constant throughout seed development. Transmission electron microscopy and fractionation by sucrose density gradient centrifugation of the starchy endosperms at various stages of development showed that protein body type II, the accumulation site of glutelin and globulin, was formed faster than protein body type I, the accumulation site of prolamin.

The 57-kilodalton polypeptide but not the glutelin subunits was labeled in a 2-hour treatment with [^{14}C]leucine given between 4 and 12 days after flowering to developing ears. *In vivo* pulse-chase labeling studies showed the 57-kilodalton polypeptide to be a precursor of the 22 to 23 and 37 to 39 kilodalton subunits. The 57-kilodalton polypeptide was salt-soluble, but the mature glutelin subunits were almost salt insoluble.

***In vitro* protein synthesis also showed that the mRNAs directly coding the 22 to 23 and 37 to 39 kilodalton components were absent in developing seeds and that the 57-kilodalton polypeptide was the major product. Thus, it was concluded that the two subunits of rice glutelin are formed through post-translational cleavage of the 57-kilodalton polypeptide.**

The major storage proteins of most cereal grains are glutelin and prolamin. In rice grains, however, glutelin is the major protein of the starchy endosperm, constituting at least 80% of the total protein, prolamin accounting for less than 5% (12). Rice glutelin has a mol wt of 6×10^5 according to Tecson *et al.* (27), but Takeda *et al.* (25) demonstrated that it has a heterogeneous mol wt. The increase in glutelin in the developing rice grain coincides with the appearance of PB[1] in the starchy endosperm 7 to 8 DAF (7, 29), and glutelin is found exclusively in PB (7, 26, 30). In a previous paper (26), we reported two types of PB in the starchy endosperm of rice grains and described their isolation. One (type I, PB-I) is spherical with a concentric ring structure, whereas the other (type II, PB-II) is stained homogeneously by osmium tetroxide and does not have this structure. PB-I contains prolamin, and PB-II is rich in glutelin and globulin. The glutelin in PB-II is composed of two principal subunits, the 22- to 23- and 37- to 39-kD complexes, and the prolamin in PB-I is composed mainly of 13-kD polypeptide. The 16- and 10-kD polypeptides also are located in PB-I (26). Other information about PB and about the isolation and characterization of rice storage protein also has been reported (1, 5, 10, 12, 13, 17, 21–23).

Because of the active synthesis and accumulation of relatively few molecular species over a short, well-defined period, the biosynthesis of storage protein in seeds provides an excellent system for studies on the molecular basis of the regulation of specific gene expression. Several storage protein polypeptides of cereals (4, 8, 16, 20) and legumes (9, 11, 24, 28) are synthesized as short-lived precursor polypeptides which may undergo modification during or after translation. Some of these precursors seem to be the equivalents of precursor polypeptides of secretory or membrane proteins in that a 'signal' peptide may be present on the polypeptide (2). Recently, using *in vitro* protein synthesis systems, Croy *et al.* (6) and Tumer *et al.* (28) showed that two major subunits of pea legumin and soybean glycinin are formed from a high mol wt precursor by a posttranslational cleavage that differs from the cotranslational processing of signal peptide. Little, however, is known about the molecular mechanisms which control the synthesis of the storage proteins of rice seeds.

Here, we describe the synthesis of storage proteins in developing rice grains and their accumulation in PB and present evidence for the existence of a precursor of the two major subunits of glutelin. A preliminary account of this work has been published elsewhere (31).

MATERIALS AND METHODS

Plant Materials. Rice (*Oryza sativa* L. Japonica cv Koshihikari) was field-grown at the Research Institute for Food Science, Kyoto University from the middle of May to the beginning of September. Maturation period of rice seeds was about 40 d in the present experiment. Ears were tagged upon flowering and developing seeds were harvested at the desired times after flowering. The immature starchy endosperms were separated from the testae, aleurone tissues, and embryos. These endosperms were used immediately or were stored in liquid nitrogen for protein extraction.

Reagents. Radiochemicals were purchased from the Radiochemical Centre, Amersham, U. K. L-[U-^{14}C]Leucine and L-[^{35}S] methionine had specific activities of 348 mCi/mmol and 800 Ci/mmol. The radioactive concentration of the L-[U-^{14}C]amino acid mixture was 50 μCi/ml. Na-phosphoenolpyruvate and pyruvate kinase were purchased from Sigma Chemical Co. Yeast *t*RNA and mol wt markers for the SDS-PAGE analysis of polypeptides were purchased from Boehringer Mannheim Co., W. Germany. All other reagents were of analytical grade.

Electron Microscopy on Developing PB. Initially, a rice seed was cut into halves with a razor blade. Then one half of the seed was fixed with 3% (w/v) glutaraldehyde and 1% (w/v) osmium tetroxide and embedded in epoxy resin by the method described previously (26). The embedded sample was cut transversely with a glass knife on an ultramicrotome. Thin sections were stained with 7% (w/v) uranylacetate and 2% (w/v) lead acetate and examined in a TEM (Hitachi H-700; Hitachi Co. Ltd., Japan).

[1] Abbreviations: PB, protein body(s); DAF, days after flowering; PAGE, polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; SDG, sucrose density gradient.

Separation of Two Types of PB by SDG Centrifugation. The amount of the two types of PB formed during ripening was determined by SDG centrifugation as described previously (26). The starchy endosperms separated from developing rice seeds (about 50 seeds) at various stages were homogenized in a motar at 0°C with 2 ml of 10 mM Tris-HCl (pH 7.5) containing 0.4 M sucrose. The homogenate was filtered through four layers of gauze, then centrifuged at 7,700*g*, 0°C for 20 min. The precipitate was resuspended in 2 ml of the homogenizing buffer and layered on 26 ml of a 52% to 62% (w/w) linear SDG in 10 mM Tris-HCl (pH 7.5) formed on a 3-ml bed of 65% (w/w) sucrose. Centrifugation was carried out in a Hitachi RPS-25 rotor at 21,000 rpm, 2°C for 5 h. An ISCO density gradient fractionator was used to measure the *A* at 280 and 340 nm. As reported previously (26), PB-I and -II appeared at about 56% and 59% (w/w) sucrose, representing specific gravities of 1.27 and 1.29, respectively.

[^{14}C]Leucine Administration to Ripening Rice Grains. Ears at the appropriate stage of development were detached and their stalks immersed in 50 μl of [^{14}C]leucine (2.5 μCi/7.1 pmol) per ear. When the [^{14}C]leucine had been absorbed, the stalks were transferred to distilled H_2O. The starchy endosperms were dissected from the seeds 2 h after the treatment was begun, and their protein was extracted by homogenizing the endosperms with 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 4 M urea, and 10% (v/v) glycerol at 25°C. In the pulse-chase experiment, after the [^{14}C]leucine had been absorbed, the stalks were transferred to distilled H_2O for a total of 30 min, then to 1 mM L-[^{12}C]leucine for time of 2 h to 8 d.

Alternatively, 10 starchy endosperms (about 100 mg) dissected from immature seeds in the 5 to 10 DAF stages were incubated at 30°C with 40 μl of a cocktail for translation of cell-free protein synthesis that contained 20 mM Hepes-KOH (pH 7.6), 4 mM Mg-acetate, 80 mM K-acetate, 2 mM ATP, 480 μM GTP, 2 mM DTT, 10 mM Na-phosphoenolpyruvate, 4.8 μg of pyruvate kinase (410 units/mg), 0.04 A_{260} unit of yeast *t*RNA, 100 μM each of 19 amino acids (without leucine), and 0.08 μCi of [^{14}C]leucine. After incubation, the reaction was terminated by an addition of 0.2 ml of 4.1 mM L-[leucine], then the reaction mixture immediately was chilled to 0°C, after which protein was extracted as described above.

Incorporation of radioactive amino acids into proteins was determined with a 50 μl sample on a 3 MM filter-paper disc (Whatmann) which then was treated with 10% (w/v) TCA to fix the proteins on the disc (18). The radioassay was made in a liquid scintillation counter.

Fractionation of Storage Protein by Solvents. [^{14}C]Leucine-labeled prolamin, glutelin, and globulin were separated from *in vivo*-labeled immature rice endosperm. Forty starchy endosperms (about 400 mg) dissected from ears at the 10-DAF stage that had been administered the [^{14}C]amino acid mixture for 1 d were ground to fine powder in liquid N_2 in a mortar. The ^{14}C-labeled proteins were extracted sequentially in a 16-ml centrifuge tube by vigorously stirring the sample with 20 ml each of the following extracting solvents for 2 h at 25°C: 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA (solvent A) to extract albumin; 1% (v/v) Triton X-100 in solvent A for membranous protein; 0.5 M NaCl in solvent A for globulin; 60% (v/v) *n*-propanol in solvent A for prolamin; and 62.5 mM Tris-HCl (pH 6.8), 2% (w/w) SDS, and 4 M urea for glutelin and residual proteins. For extraction of rice prolamin, 60% (v/v) *n*-propanol was at least twice as much effective as with 70% (v/v) ethanol. The extracted proteins were precipitated with 2 volumes of acetone at −20°C overnight, after which they were dissolved in the sample buffer (62.5 mM Tris HCl [pH 6.8], 2% [w/v] SDS, 5% [w/v] 2-mercaptoethanol, 4 M urea, and 10% [v/v] glycerol) for SDS-PAGE and treated at 100°C for 3 min.

***In Vitro* Protein Synthesis.** *In vitro* protein synthesis took place when the homogenate of the rice endosperm was added to wheat germ S-23 extract. The homogenate of the rice endosperm was prepared as follows; 5 g of starchy endosperms from seeds at the 10-DAF stage was ground to fine powder in liquid N_2 in a mortar then suspended in 10 ml of the homogenizing buffer (50 mM Tricine-KOH [pH 8.0], 100 mM KCl, 5 mM $MgCl_2$, 0.001% [w/v] polyvinylsulfate, 2 mM 2′,3′-AMP, 1 mM DTT, and 7 mM diethylpyrocarbonate) containing 250 mM sucrose. This suspension was centrifuged at 75*g* for 3 min. The turbid supernatant was layered on a 3-ml cushion of 60% (w/w) sucrose in the homogenizing buffer which then was centrifuged at 500*g* for 5 min. The upper layer on the sucrose cushion was used as the homogenate of the endosperm. Wheat germ S-23 extract was obtained according to the method of Marcus (19) and was stored in liquid N_2. The *in*

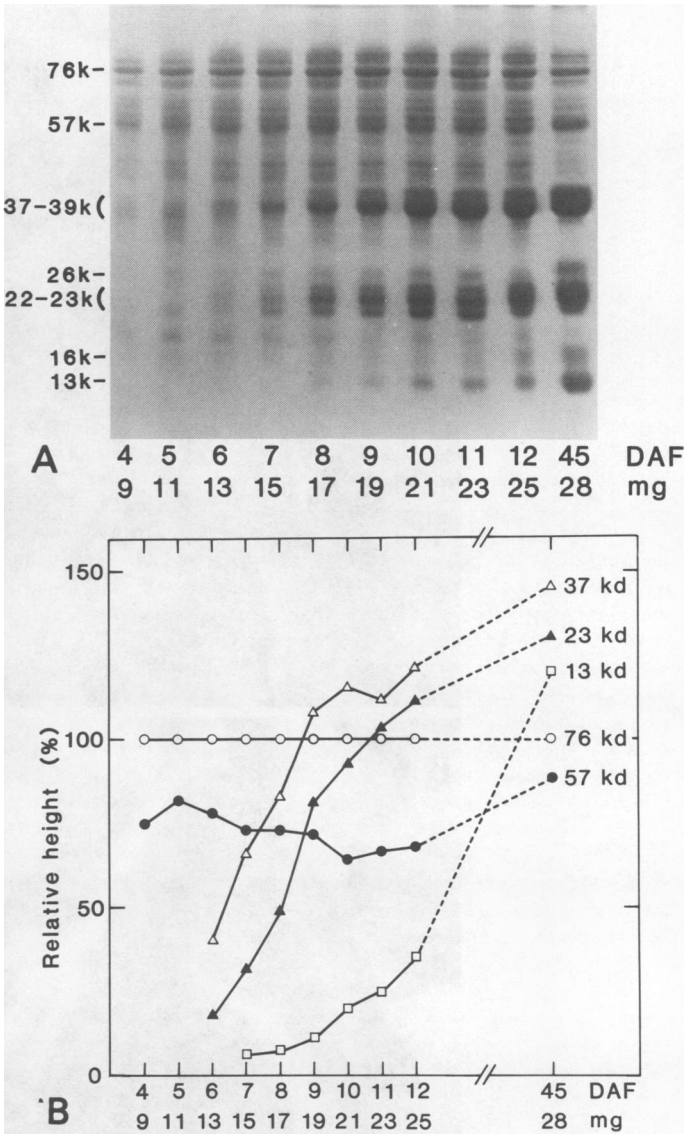

FIG. 1. Polypeptide composition of the starchy endosperm of rice during ripening. Numbers at the bottom of the figures represent the fresh weight of a grain (mg) at the indicated stage (DAF). A, About 20 starchy endosperms at the designated stages of development were extracted with 200 μl of sample buffer and the extracts (approximately 0.3 A_{280} units/20 μl·lane) were fractionated on SDS-PAGE, after which the polypeptides were detected by Coomassie blue staining. The apparent mol wt of the major polypeptides are given in daltons. B, Changes in the relative amounts of polypeptides in developing rice endosperm were obtained as peak heights by densitometric scanning of the electrophoregram. The peak height of the 76-kD polypeptide was designated 100%.

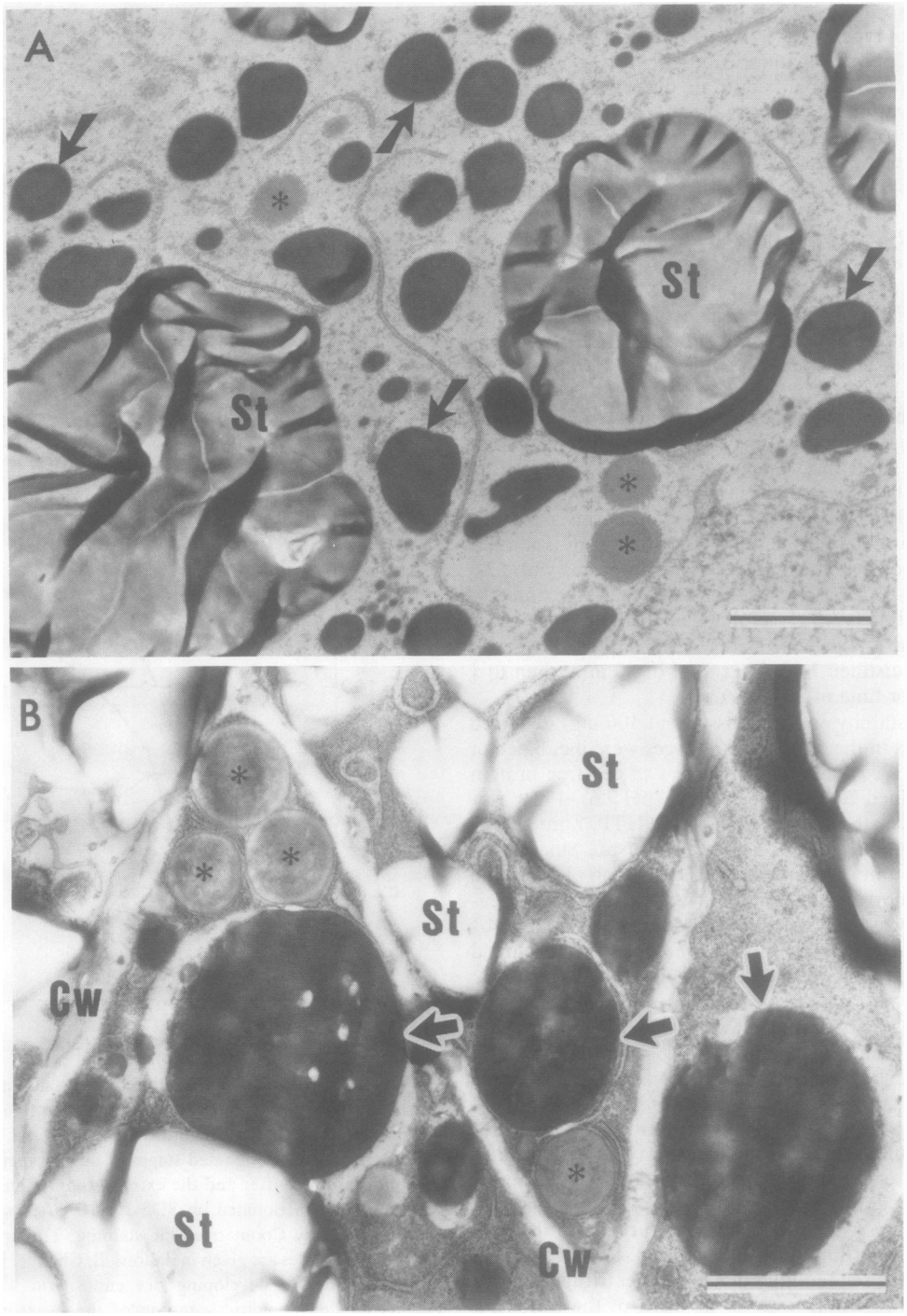

FIG. 2. Transmission electronmicrograms of the starchy endosperm in the ripening stage. Lamellar PB (PB-I, asterisks) and amorphous PB (PB-II, arrows) are present. A, Starchy endosperm at 7 DAF; bar, 3 μm. B, Starchy endosperm at 16 DAF; bar, 2 μm. St, starch granules; Cw, cell wall.

vitro translation mixture (125 μl) had the following composition: 20 mM Hepes-KOH buffer (pH 7.6), 4 mM Mg-acetate, 100 mM K-acetate, 2 mM ATP, 480 μM GTP, 2 mM DTT, 10 mM Na-phosphoenolpyruvate, 15 μg of pyruvate kinase (410 units/mg), 0.12 A_{260} unit of yeast *t*RNA, 100 μM each of 19 amino acids (without leucine), 0.25 μCi of [^{14}C]leucine, 25 μl of the homogenate of rice endosperm, and 60 μl of 8 A_{280} units of wheat germ S-23 extract. The reaction was carried out at 30°C for 30 min, then a 0.025 volume of 4.1 mM L-[^{12}]leucine was added, and incubation was continued for an additional 10 min. Protein was precipitated with 250 μl of 30% (w/v) TCA containing 0.3 M L-[leucine]; this precipitate was washed three times with 5% (w/v) TCA, then twice with acetone. After being dried *in vacuo*, the protein was dissolved in the sample buffer for SDS-PAGE and treated at 100°C for 3 min.

Electrophoresis of Synthesized Polypeptides. Discontinuous SDS-polyacrylamide gels were prepared according to the method of Laemmli (14). Polyacrylamide gels (12 cm long, 14 cm wide, and 2 mm thick) composed of 2.75% (w/v) stacking and 14% or 10% (w/v) running gels were cast in a slab gel apparatus (Atoh Instruments, Tokyo). The electrophoresis buffer contained 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS. Current was applied at 125 v until the bromphenol blue had reached the bottom of the gel. BSA (67 kD), ovalbumin (45 kD), aldolase (40 kD), chymo-

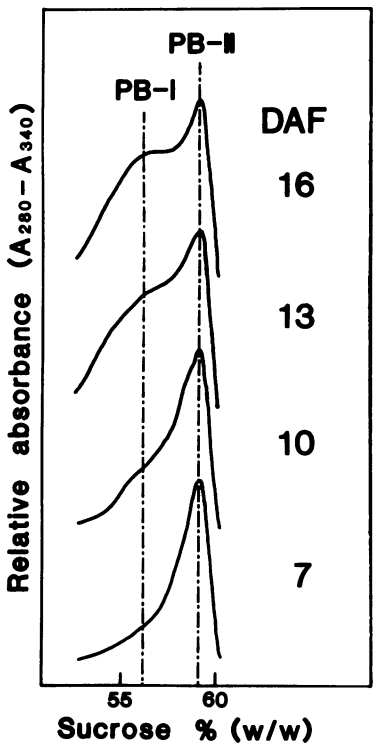

FIG. 3. Relative ratios of PB-I and -II in the starchy endosperm of rice at various stages of ripening. The PB fraction (1 ml) obtained from developing seeds at the indicated stages was layered on 26 ml of 52% to 62% (w/w) linear SDG formed on 3 ml of a 65% (w/w) sucrose bed. After centrifugation at 21,000 rpm for 5 h at 2°C, the absorbances of the fractions at 280 and 340 nm were measured. The relative absorbance equals the height of the peak of PB-II at each stage. PB-I and -II indicate the positions of the specific gravities, 1.27 and 1.29, as in Tanaka *et al.* (26).

trypsinogen *A* (25 kD), and Cyt *c* (12.4 kD) were used as the mol wt markers. After electrophoresis, the proteins were fixed overnight in 50% (w/v) TCA, after which they were stained with 0.1% (w/v) Coomassie brilliant blue G-250, then diffusion-destained in the presence of a destaining pad (Pharmacia) in 10% (w/v) acetic acid. Radioactive proteins were located by autoradiography or fluorography, as described by Bonner and Laskey (3), on Kodak X-Omat R film exposed for 10 to 30 d.

RESULTS

Accumulation of Protein and PB during Endosperm Development. Although the accumulation of protein in developing rice endosperms is greatest during the milky stage, the increase in protein content begins as early as 4 DAF (12). Changes in individual proteins were, therefore, examined from the earliest stage of development at which endosperms could be harvested. The peptide composition of the starchy endosperm protein was analyzed by SDS-PAGE (Fig. 1A). The polypeptide profiles for the 4- to 6-DAF stages were similar, but differed from the profile of mature seeds (45 DAF). The mol wt of the storage protein subunits ranged from 10 to 76 kD for more than 15 bands.

The glutelin of the cultivar Koshihikari consists of two major subunit groups; the 22- and 23-kD group and the 37, 38, and 39-kD group (26). These polypeptide subunits became identifiable at 5 to 6 DAF; thereafter, they predominated (Fig. 1A). The increase in the 22- to 23-kD subunit group paralleled that of the 37- to 39-kD subunit group; the increase in the relative peak height of the densitometric scanning of the 23-kD peptide paralleled that of the 37-kD polypeptide (Fig. 1B). Thus, the synthesis of these subunits was coordinated closely. The 26-kD polypeptide, a component of globulin, also appeared at the same stage as the glutelin subunits. The amounts of the large, 76- and 57-kD polypeptides remained fairly constant throughout ripening. The 76-kD peptide belongs to albumin component and localizes in the starch granules. The 57-kD peptide is not soluble in low-ionic-strength solution. The 13-kD peptide, the major component of prolamin (26), appeared at the 10-DAF stage (Fig. 1, A and B). Thus, the biosynthesis of glutelin preceded that of prolamin.

These results were supported by TEM and SDG-centrifugation analyses of the relative amounts of the two types of PB. TEM images of the starchy endosperm showed that there were already two types of PB in the endosperms at 7 DAF (Fig. 2A). PB in the 7-DAF endosperm were, however, composed mainly of PB-II, and the volume occupied by PB-I was far smaller than that occupied by PB-II. At 16 DAF, although PB-II also was a major type of PB, the relative amount of PB-I had increased compared to values at 7 DAF (Fig. 2B). Furthermore, SDG-centrifugation analysis showed that the PB-rich fraction isolated from the starchy endosperm at the 7-DAF stage was composed mainly of PB-II (Fig. 3). But, from the 10-DAF stage the amount of PB-I gradually increased. This means that PB-II, the accumulation site of glutelin and globulin, is formed earlier than PB-I. PB-I is the accumulation site of prolamin (26); therefore, the early appearance of PB-II is evidence in support of our findings that the components of glutelin and globulin appear earlier than those of prolamin (Fig. 1, A and B).

***In Vivo* Protein Synthesis in Developmental Grains.** Storage proteins were labeled with [^{14}C]leucine for 2 h by an ear culture, then the ^{14}C-labeled proteins were extracted from the starchy endosperm and analyzed by SDS-PAGE and fluorography (Fig. 4). Surprisingly, the labeling pattern on the gel differed from the pattern of protein staining (Fig. 1A). The 57-kD subunits was readily labeled after a 2-h period at all stages, but no ^{14}C was detectable in the 22- to 23- and 37- to 39-kD groups of glutelin at any stage.

The 13-kD polypeptide, a component of the prolamin that accumulates in PB-I (26), was labeled in seeds after the 9-DAF stage. This observation is consistent with the results shown in Figure 1 which show that the biosynthesis of prolamin begins at the 10-DAF stage. Non-PB polypeptides larger than 57 kD also were readily detected 4 to 12 DAF, the extent of their labeling corresponding to the relative intensity of protein staining (Fig. 1).

The slight incorporation of [^{14}C]leucine into the 22- to 23- and 37- to 39-kD polypeptide groups after a 2-h labeling prompted us to perform a pulse-chase labeling experiment (Fig. 5). After a 2-h labeling of ears at the 10-DAF stage which were actively synthesizing glutelin, L-[^{12}C]leucine was administered. As the chase with [^{12}C]leucine continued, the ^{14}C in the 57-kD polypeptide gradually decreased, which suggests a high turnover of this polypeptide. After a 1-d chase, almost all the ^{14}C in the 57-kD polypeptide had disappeared, but the ^{14}C in the 22- to 23- and 37- to 39-kD polypeptide groups increased gradually even after [^{14}C]leucine administration was stopped. The increase in ^{14}C in both polypeptide groups paralleled the decrease of ^{14}C in the 57-kD polypeptide. These observations suggest that the 22- to 23- and 37- to 39-kD polypeptide groups are formed by the splitting of the 57-kD precursor polypeptide.

Incorporation of [^{14}C]leucine into the 13-kD polypeptide took place after a very short period of labeling (Figs. 4 and 5). This means that 57- and 13-kD polypeptides may be synthesized independently and that there is no precursor-product relationship between them.

Essentially similar results were obtained when [^{14}C]leucine or [^{35}S]methionine was administered; however, the incorporation of methionine into the 37- to 39-kD peptide was lower than that of leucine. These observations indicate that the content of methionine

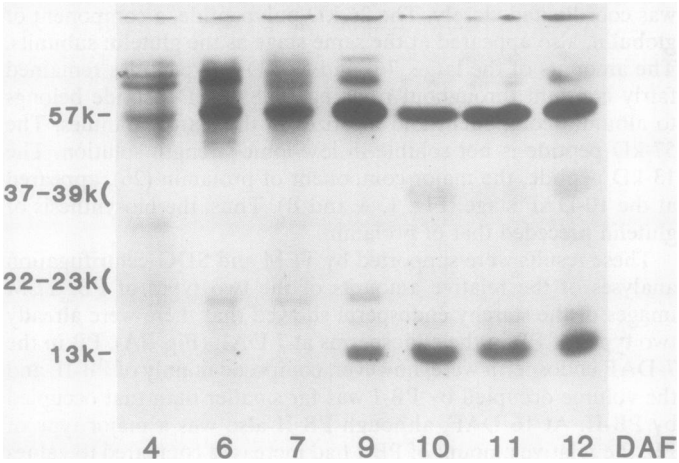

FIG. 4. SDS-PAGE analysis of [^{14}C]leucine incorporation into polypeptides of the starchy endosperm of the rice ear. Rice ears at various stages of development were pulse-labeled with [^{14}C]leucine for 2 h, then the proteins of the starchy endosperm were extracted and fractionated on SDS-PAGE (14% gel) as described in "Materials and Methods." Approximately equal amounts of radioactive protein (1,500 cpm) were loaded in each gel track.

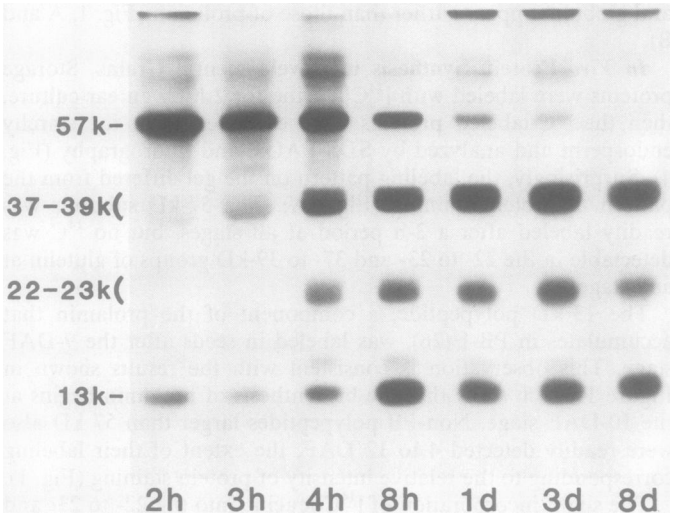

FIG. 5. Pulse-chase labeling of polypeptides with [^{14}C]leucine in developing rice endosperm. [^{14}C]Leucine was administered to rice ears for 30 min at 10 DAF after which it was chased with 1 mM [^{12}]leucine for the designated periods. Polypeptides were fractionated on SDS-PAGE (14% gel) and detected by fluorography. Approximately equal amounts of radioactive protein (about 2,000 cpm) were loaded in each gel track.

in 37- to 39-kD peptides is lower than that in 22- to 23-kD peptides. Incorporation of [^{35}S]methionine in the prolamin component, 13-kD peptide, was not observed, indicating that the peptide doesn't seem to be methionine-rich.

Scarcely any [^{14}C]leucine was incorporated into the protein of incubated isolated endosperms (5–10 DAF). But, when isolated endosperms were incubated in a cocktail for cell-free protein synthesis ("Materials and Methods") a high incorporation of [^{14}C] leucine into protein was observed, about 20% of added [^{14}C]leucine being incorporated into TCA-insoluble material within 30 min. The incorporation patterns for the endosperm system, however, were very similar to those for pulse-labeling experiment of ear cultures (Fig. 4). Mainly 57- and 13-kD polypeptides were synthesized; no synthesis of the 22- to 23- and 37- to 39-kD peptide groups was observed up to 4 h. Thus, the processing of the 57-kD precursor to mature glutelin subunits was not detected in dissected

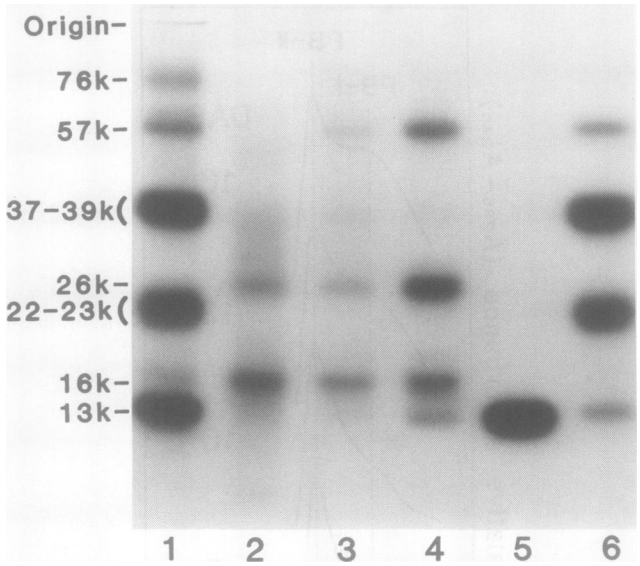

FIG. 6. SDS-PAGE analysis of proteins from the [^{14}C]amino acid-labeled 10-DAF endosperms obtained by fractionation with solvents. Starchy endosperms dissected from ears at the 10-DAF stage that had been administered the [^{14}C]amino acid mixture were ground and the ^{14}C-labeled proteins extracted sequentially with each solvent. Lane 1, total protein; lane 2, 10 mM Tris-HCl (pH 7.5) containing 1 mM (w/v) EDTA soluble protein (albumin); lane 3, 1% (v/v) Triton X-100 soluble protein (membrane protein); lane 4, 0.5 M NaCl soluble protein (globulin); lane 5, 60% (v/v) *n*-propanol soluble protein (prolamin); lane 6, residual protein extracted with the sample buffer for SDS-PAGE (mainly glutelin). Labeled polypeptides were analyzed as in Figure 4.

endosperms in comparison to this processing in the ear.

^{14}C-Labeled albumin, globulin, prolamin, and glutelin fractions were obtained from ears at the 10-DAF stage that had been administered the [^{14}C]amino acid mixture for 1 d. The distribution in the SDS-PAGE of these labeled polypeptides is shown in Figure 6. Labeled, nascent 57-kD polypeptide was present mainly in the 0.5 M NaCl-soluble globulin fraction.

***In Vitro* Protein Synthesis.** We conducted *in vitro* translation experiments to determine whether the mRNAs for the 22- to 23- and 37- to 39-kD polypeptides are absent in developing rice endosperm. Rice endosperm at the 10-DAF stage had a complete set of the polypeptide constituents found in the starchy endosperm of mature rice (Fig. 1). The homogenate of 10-DAF starchy endosperm was prepared as described in "Materials and Methods," then fractionated into RER, membrane-bound polysomes, and free-polysomes. mRNA was isolated from the membrane-bound polysomes according to the methods of Larkins and Davies (15) and Burr and Burr (4). The homogenate, RER, free and membrane-bound polysomes, and mRNA were added to the wheat germ cell-free translation system. No incorporation of [^{14}C] leucine into the two subunits of glutelin was found in any of the fractions. ^{14}C incorporation into proteins by the starchy endosperm homogenate was found mostly in the 57-kD polypeptide with only a little in the 10- to 16-kD polypeptides (Fig. 7) and no labeling of the subunits of glutelin was detectable up to a 90-min incubation of the cell-free system. This pattern was very similar to that obtained after a short period of [^{14}C]leucine administration to the ear and isolated starchy endosperm. The 57-kD peptide also was synthesized by RER and membrane-bound polysomes as templates, but not by free-polysomes. The polypeptides in PB-I (10-, 13-, and 16-kD peptides) were produced by the PB-rich fraction and RER. The translation products produced by the membrane-bound polysomes and mRNAs isolated from the membrane-bound polysomes resembled prolamin subunits in that they had

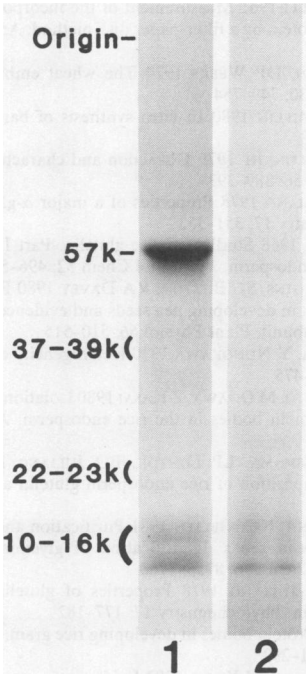

FIG. 7. SDS-PAGE analysis of *in vitro* synthesized polypeptides in a homogenate of rice endosperm at the 5- to 10-DAF stage. The homogenate of developing rice endosperm was prepared as described in "Materials and Methods" and incubated in a wheat germ cell-free translational system for 30 min at 30°C. The total protein was precipitated then fractionated by SDS-PAGE (10% gel). Individual bands were detected by fluorography. Panels show products synthesized in the cell-free system with (lane 1) or without (lane 2) the homogenate of rice endosperm.

the same solubility in 60% (v/v) *n*-propanol and similar, but not identical, electrophoretic properties. Further results of *in vitro* protein synthesis will be reported elsewhere.

Our results indicate that mRNAs coding for the glutelin subunits directly are absent and that the mRNA for the 57-kD polypeptide is present in rice endosperms.

DISCUSSION

SDS-electrophoregrams of the storage proteins of rice during seed development and of *in vivo*-labeled storage proteins showed that the 57-kD polypeptide is a possible precursor of the glutelin subunits. The sum of the mol wt of the 22- to 23- and 37- to 39-kD groups is reasonably close to the weight of 57 kD. And, during development, the 57-kD peptide was synthesized at an earlier stage than the glutelin subunits (Fig. 1). In addition, after short-term labeling of the ears or isolated starchy endosperms while glutelin was rapidly accumulating, the 57-kD polypeptide was labeled, but the subunits of glutelin were not (Fig. 4). Pulse-chase labeling studies also showed that the radioactivity incorporated in the 57-kD polypeptide moved to the 22- to 23- and 37- to 39-kD polypeptides (Fig. 5). Further evidence that the 57-kD polypeptide is cleaved into glutelin subunits is the absence of mRNA coding for the subunits of glutelin and its presence for the 57-kD peptide (Fig. 7).

We concluded that a single polypeptide of 57 kD is synthesized first, then it subsequently is cleaved into the mature, glutelin 22- to 23- and 37- to 39-kD subunit groups by proteolytic enzyme. Immunochemical and peptide-mapping studies of the structural relationship between nascent 57-kD polypeptide and the glutelin subunits are in progress.

The fact that the 57-kD glutelin precursor is a relatively stable polypeptide *in vivo* means that proteolytic cleavage of the precursor takes place post-translationally but not co-translationally. In this respect, the processing of the 57-kD polypeptide differs from the removal of the signal peptide (2). As shown in Figure 7, the post-translational cleavage of the 57-kD precursor requires a highly organized system and the system for proteolysis is very labile.

PB-II seems to correspond to the crystalline PB described by Bechtel and Juliano that is formed from a Golgi body (1). Our electron microscopy also has shown that PB-II is formed through the Golgi body (H. Yamagata *et al.*, unpublished). Therefore, we propose that the processing of the 57-kD polypeptide takes place during transport of the synthesized polypeptide from RER to PB-II *via* the Golgi body. This is backed by our current findings that the mRNA for the 57-kD precursor peptide was present in the membrane-bound polysome, but not in the free-polysome. Since the precursor polypeptide is soluble in a solution of high ionic strength, whereas mature glutelin polypeptides are nearly insoluble (Fig. 6), cleavage may not take place until the precursor polypeptide reaches PB-II.

The synthesis and accumulation of the glutelin molecule in the starchy endosperm of developing rice thus is very different from those processes during zein synthesis in maize seeds (4). During zein synthesis, the precursor polypeptides are only 1,100 and 2,000 D heavier than the native polypeptides. The extra amino terminal sequence acts as a signal when the synthesized polypeptide travels through the membrane. The system for the biosynthesis and accumulation of rice glutelin is very similar to that for legumin and glycinin synthesis in pea and soybean cotyledons in which a precursor, 60-kD polypeptide, is split into 20- and 40-kD polypeptides (6, 28). Ours is the first report to show that post-translational processing also works during storage protein biosynthesis in cereal plants.

The profile of the *in vivo* pulse-labeled products in the 10- to 16-kD polypeptides, which appeared from about 10 DAF, changed with seed development similar to the way in which total protein changed (Figs. 1 and 4). Juliano *et al.* reported that the 16-kD subunits, which seems to correspond to the 13-kD peptide in our experiment (26), is the major component at the 4- to 7-DAF stages in the cultivars IR 26 and IR 480-5-9, and that only traces of the 25- and 38-kD subunits of glutelin are present (29). In contrast, our results showed that the 13-kD peptide appeared at later stages than the 22- to 23- and 37- to 39-kD peptides, the smaller peptides of the storage protein being synthesized later than the larger peptides. These variations may be due to the different cultivars used.

Proteins (10-, 13-, and 16-kD subunits) that accumulated in PB-I were synthesized during the pulse-labeling period as mature polypeptides and were not modified post-translationally (Figs. 4 and 5). We found that PB-I has polysomes attached to the outside of its boundary membrane, whereas PB-II has not (H. Yamagata *et al.*, unpublished). Similar observations have been reported, and PB-I has been thought to be formed directly from RER (1). These reports suggest that the polypeptides in PB-I are synthesized on the membrane-bound polysomes attached to PB-I or RER and that they pass through into the lumen where they aggregate as they do in zein synthesis and deposition in maize PB. The results of the *in vitro* protein synthesis experiments described here support these speculations.

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