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Enzymes of Starch Metabolism in the Developing Rice Grain[1]

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ABSTRACT

The levels of starch, soluble sugars, protein, and enzymes involved in starch metabolism—α-amylase, β-amylase, phosphorylase, Q-enzyme, R-enzyme, and starch synthetase—were assayed in dehulled developing rice grains (*Oryza sativa* L., variety IR8). Phosphorylase, Q-enzyme, and R-enzyme had peak activities 10 days after flowering, whereas α- and β-amylases had maximal activities 14 days after flowering. Starch synthetase bound to the starch granule increased in activity up to 21 days after flowering. These enzymes (except the starch synthetases) were also detected by polyacrylamide gel electrophoresis. Their activity in grains at the midmilky stage (8–10 days after flowering) was determined in five pairs of lines with low and high amylose content from different crosses. The samples had similar levels of amylases, phosphorylase, R-enzyme, and Q-enzyme. The samples consistently differed in their levels of starch synthetase bound to the starch granule, which was proportional to amylose content. Granule-bound starch synthetase may be responsible for the integrity of amylose in the developing starch granule.

Most starch biochemists believe that the starch synthetases (ADP- and UDP-glucose-starch 4-glucosyltransferases) are the enzymes involved in starch synthesis (2, 20). Some investigators believe that phosphorylase is also a synthetic enzyme and that the starch synthetase functions to protect the amylose molecule formed with phosphorylase and starch synthetase from being transformed to amylopectin through the action of Q-enzyme (1, 13). Still others propose a multiple pathway synthesis of starch (4).

Another unsolved problem in starch biosynthesis is the genetic integrity of amylose in the granule of nonwaxy starches. Previous work on developing rice and corn grains showed that starch synthetase bound to the starch granule occurs mainly in nonwaxy granules (3, 20). Akazawa *et al.* (3) also found lower activity of starch synthetase in grain of a japonica rice variety than in grain of an indica variety with a higher amylose content. Badenhuizen and Chandorkar (5,11) reported a positive correlation between starch synthetase activity and amylose content of developing starch granules in several plant species.

To determine the main pathway of starch synthesis, we made a comprehensive survey of changes in the levels of enzymes of starch metabolism—amylases, phosphorylase, starch synthetases, Q-enzyme (α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase or branching enzyme), and R-enzyme (amylopectin 6-glucanohydrolase or debranching enzyme)—during grain development in the rice variety IR8. Lines from the same cross differing in amylose content were used to compare enzyme activities in relation to amylose synthesis. The use of such lines grown in the same crop reduces complicating environmental and genetic factors which accompany studies in which different varieties are compared.

MATERIALS AND METHODS

Samples of developing rice grains (*Oryza sativa* L., variety IR8) were obtained from the experimental field of the Institute at 7, 10, 14, 21, and 28 days after flowering and immediately stored at 0 C. They were dehulled by hand prior to analysis. The grain weight was determined for each sample.

Samples of lines differing in amylose content were grown in a Mylar house in pots containing 6 kg of air-dried soil. One day before transplanting, 10 g of $(NH_4)_2SO_4$ and 8 g Na_3PO_4 were added to the soil, the soil was mixed well, and the pots were flooded. Four 10-day-old seedlings were transplanted per pot and kept under continuous flooding. Panicles were tagged at flowering, and 8 to 10 days later the grains were harvested and then stored at −20 C. They were dehulled by hand prior to enzyme extraction.

Amylases and R-enzyme. Dehulled grains were homogenized at 0 C in a 0.05 M sodium citrate buffer (pH 5.7) with 0.2% $CaCl_2$ (2 ml/g of grain) in a cold mortar and pestle. The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 30,000*g* at 0 C for 10 min. Protein was precipitated by adding saturated $(NH_4)_2SO_4$ solution to the supernatant fluid to 50% saturation. The precipitate was dialyzed at 4 C against the citrate buffer for 5 hr.

Assays for α-amylase and R-enzyme were made with corn amylopectin β-limit dextrin as substrate according to the procedure of Briggs (8) as employed by Greenwood and MacGregor (16) and Shain and Mayer (28). The α-amylase assay was done at pH 5.7. The R-enzyme assay was performed at pH 6.6, after α-amylase was inactivated by incubating the enzyme preparation with 0.007 M EDTA for 30 min. Enzymic activity per grain was based on the decrease in absorbance of the iodine-dextrin complex at 540 nm. Results were expressed in units of iodine-dextrin color.

For the β-amylase assay, the method of Greenwood and MacGregor (16) was employed, but reducing sugars were assayed with a modified reagent of 3,5-dinitrosalicylic acid and phenol (6). The β-amylase activity was expressed as micrograms of maltose liberated per min per grain, corrected for the maltose that had been converted by maltase into glucose. Maltase activity was determined from the glucose content of a portion of the incubation mixture determined by the glucose oxidase method using Glucostat (Worthington Biochemicals Corp.) (32).

Phosphorylase. Two grams of dehulled grains were homogenized at 0 C in 10 ml of 0.05 M tris-maleate buffer (pH 7.0).

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The homogenate was filtered through cheesecloth and centrifuged for 10 min at 27,000g at 0 C. The supernatant fluid was brought to 50% saturation with saturated ammonium sulfate, stirred for 30 min at 0 C, and centrifuged again. The precipitate was dissolved in 0.2 ml of the tris-maleate buffer and dialyzed for 5 hr against the same buffer for the phosphorylase assay.

The phosphorylase assay was essentially that of Lee (19). However, NaF at final concentration of 20 mM was added to inhibit phosphatases (30). The P_i which was formed was determined by the method of Lowry and Lopez (21) with some modifications. Turbidity was removed before color development by centrifugation for 8 min at 550g. The color was allowed to develop for 1 hr. Phosphorylase activity was calculated in terms of millimicrograms of P_i liberated every 5 min per grain.

Phosphorylase activity of the grain decreased progressively during grain storage at −20 C.

Q-enzyme. Q-enzyme was extracted from dehulled rice grain by the same procedure as phosphorylase except that 0.05 M sodium citrate buffer (pH 7.0) was used as the extracting medium instead of tris-maleate buffer. Higher Q-enzyme values were obtained in sodium citrate buffer than in the tris-maleate buffer. Manners *et al.* (23) reported that tris buffer inhibits the activity of corn Q-enzyme.

Q-enzyme activity was assayed at 30 C by the method of Igaue (17) as used by Manners *et al.* (23). The enzyme solution was incubated for 30 min with 7×10^{-3} M EDTA before the assay to inactivate α-amylase. The activity of Q-enzyme per grain was expressed as percentage decrease in absorbance at 660 nm (ΔA_{660}) of the amylose-iodine solution per min.

Starch Synthetases. The procedure for preparing the starch synthetases was adapted from Murata and Akazawa (26). Dehulled grains (1 g) were ground in a chilled mortar at 0 C and allowed to stand for 30 min with 1 ml of 0.07 M phosphate buffer (pH 7.5) containing 0.035 M GSH and 0.07 M EDTA. The mixture was then centrifuged at 0 C at 10,000g for 20 min. The supernatant fluid was siphoned off and used directly for the soluble enzyme assay. The soluble enzyme was extremely labile, and it tended to increase in activity the longer the homogenate was extracted. The starch precipitate was suspended in water, filtered through cheesecloth, and diluted to 50 ml with water. A portion of this starch suspension was analyzed for starch content by the anthrone method (30). The rest was used for the preparation of starch granule-bound enzyme according to Leloir *et al.* (20).

The incubation mixture consisted of 5 μmoles of glycine buffer (pH 8.6), 0.1 μmole of EDTA, 0.05 μmole of KCl, 0.25 μmole of GSH, 3.0 μmoles of NaF, a 0.2-μmole mixture of ADP-glucose-U-^{14}C (International Nuclear Corp.) and ADP-glucose (Sigma Chem. Co.) (1:2, 7,500 cpm), 20 μl of the soluble enzyme, and 3 mg of oyster glycogen (Nutritional Biochemicals Corp.) in a total volume of 40 μl. After 15 min at 37 C, the glycogen was precipitated with ethanol and washed three times according to the method of Mendicino and Pinjani (25).

For the starch granule-bound enzyme, the incubation mixture consisted of 5 μmoles of glycine buffer (pH 8.6), 0.1 μmole of EDTA, 0.05 μmole of KCl, 0.5 μmole of GSH, 0.2 μmole of ADP-glucose-U-^{14}C (7,500 cpm), and 2 or 3 mg of starch granules in a total volume of 20 μl. After 15 min at 37 C, 75% ethanol was added to stop the reaction. The starch granules were washed according to Cardini and Frydman (9).

The washed glycogen or starch granules were hydrolyzed with 0.5 ml of 1 N HCl in a boiling water bath for 1 hr, and the hydrolysate was mixed with 10 ml of Bray's (7) solution. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. Enzyme activity was expressed in micrograms of glucose incorporated per grain during the 15-min incubation period.

Chemical Analyses. Soluble sugars and the starch content of developing IR8 were determined by the anthrone method (24). The blue value of the starch extract in perchloric acid was determined by the method of McCready *et al.* (24). Protein concentration of enzyme preparations was determined by the Lowry method (22).

Zymogram. Zymograms of soluble enzymes involved in starch metabolism were prepared according to Juliano and Varner (18) including the identification of the bands. Electrophoresis was done in 8% polyacrylamide containing 0.06% amylopectin. The identity of the amylases was confirmed with amylopectin β-limit dextrin as the substrate during incubation.

RESULTS

Changes in Starch and Soluble Protein Levels and Enzyme Activities during Grain Development. Starch synthesis was most rapid between the 7th and the 14th day after flowering and was essentially over by the 21st day (Fig. 1). An increase in the amylose content of the starch (indexed by blue value) was also observed between the 7th and 14th days after flowering. Total soluble sugars showed a progressive decrease during this period. The maximal concentration of soluble protein occurred at about the 10th day after flowering. The peak coincided with a rapid rate of starch synthesis in the rice grain. Among the six enzymes with activities measured during grain development, phosphorylase, Q-enzyme, and R-enzyme had their maximal activities during this period of starch synthesis (Fig. 2). The α- and β-amylases had their peak activities after the 14th day, when the soluble protein level was already low. Hence, the specific activity of the amylases are higher than the curves based on an individual grain had indicated, whereas those of the phosphorylase, Q-enzyme, and R-enzyme changed relatively less. By contrast, soluble starch synthetase decreased in activity during grain development. Maltase activity paralleled β-amylase activity during grain ripening. Maltase had previously been reported in the rice grain by Igaue (17).

The activity of starch synthetase bound to the starch granule increased progressively in the grain during grain development as starch was synthesized. Its activity per milligram of starch was also highest in the 21-day-old grain. Based on the combined starch synthetase activity of the grain, the bound enzyme was

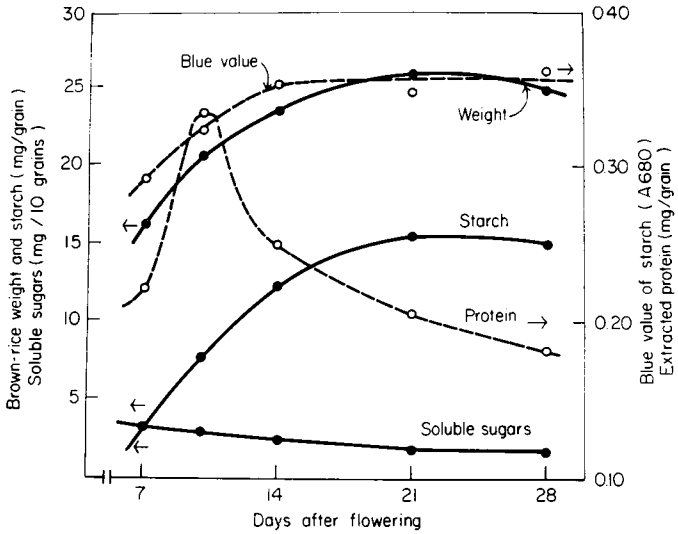

FIG. 1. Changes in the wet weight, starch, soluble sugars, and soluble protein of dehulled (brown) rice and in the blue value of the starch in the developing IR8 rice grain.

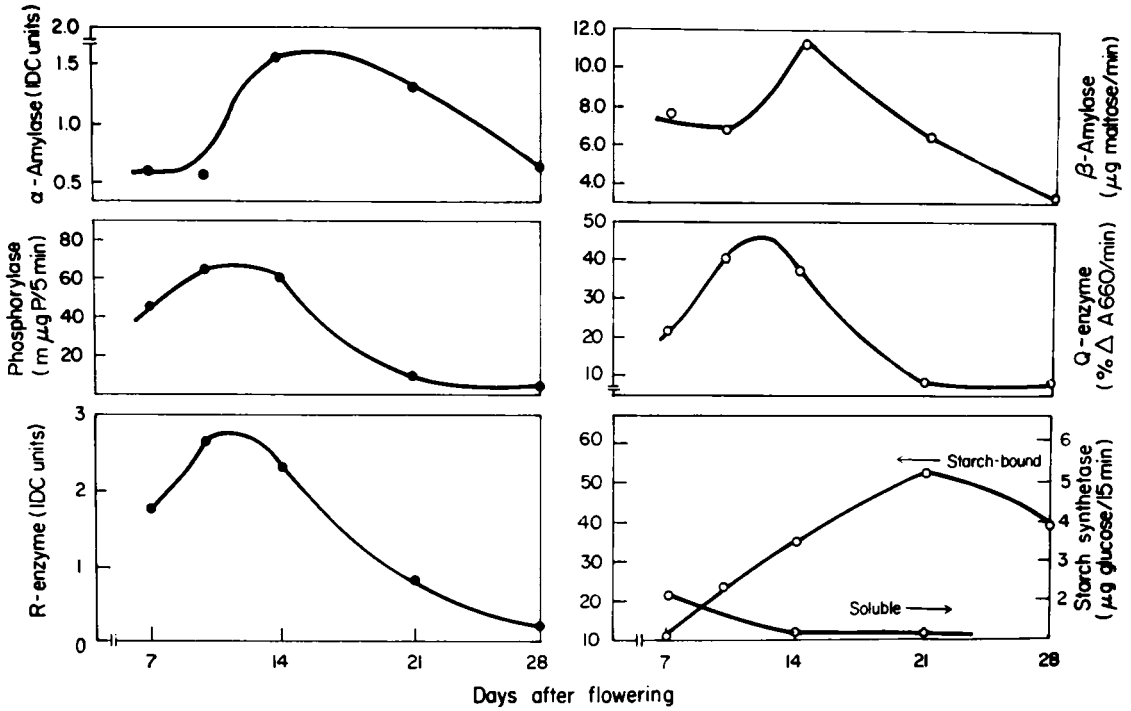

FIG. 2. Changes in the activities (per grain) of α- and β-amylases, phosphorylase, Q-enzyme, R-enzyme, and ADP-glucose starch synthetases n the developing IR8 rice grain.

83% of the total in the 7-day-old sample, 97% in the 14-day-old sample, and 98% in the 21-day-old sample.

These results indicate that the midmilky stage, which corresponds to 8 to 10 days after flowering in tropical rice, is the best stage for studying and comparing starch synthesis in grain of lines differing in amylose content. At this stage, the activities of the synthetic enzymes, phosphorylase, starch synthetase, and Q-enzyme are high. Among the hydrolytic enzymes, the activities of α- and β-amylases are still low at this stage, although R-enzyme activity is high.

The use of $(NH_4)_2SO_4$ to precipitate the enzymes improved the precision of the enzyme assays since the blank values for reducing sugars, P_i, and other metabolites in the enzyme preparations were essentially eliminated. Preliminary experiments showed that nearly all the enzyme activity and most of the soluble protein were in the $(NH_4)_2SO_4$ precipitate. The concentration of the extracted enzymes was also facilitated.

Pattern of Enzymes Involved in Starch Metabolism in the Developing Rice Grain. Eight bands were observed in the zymogram pattern of the extracts (Fig. 3). The slowest moving band colored brown in gels incubated with starch and stained with I_2. It was designated as Q-enzyme. Two of the next three bands were colorless in gels incubated in starch in phosphate buffer and stained with I_2 but were absent with the same substrate in tris-maleate buffer. These three bands were the only ones which synthesized linear side chains (blue color with iodine) from glycogen primer and glucose-1-P. These results are consistent with their identification as phosphorylases. They can degrade starch only in the presence of P_i; this explains their absence in the gel incubated in tris-maleate buffer. The slow band overlapped with the Q-enzyme band. Only the fast phosphorylase band was observed when the electrophoresis was run without amylopectin and the glycogen added with glucose-1-P during incubation. Presumably, phosphorylase complexes with amylopectin to form the two slower phosphorylase bands. This ability of phosphorylase to complex with its substrate may explain why these phosphorylase isozymes can synthesize blue-staining glucans on incubation in glucose-1-P even in the absence of glycogen substrate.

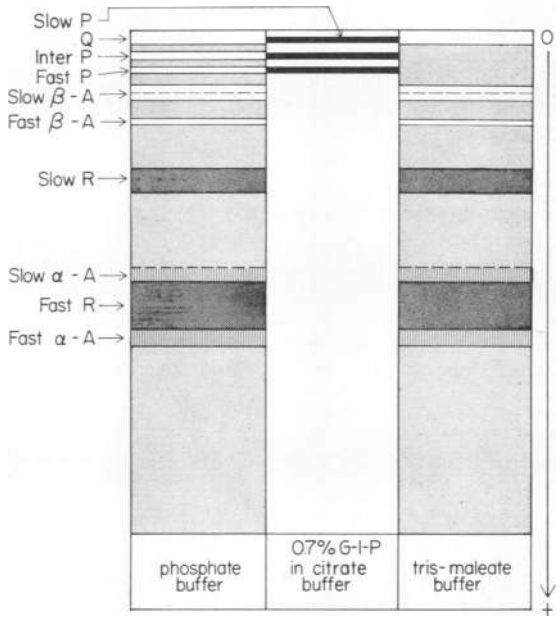

FIG. 3. Schematic zymogram in 8% polyacrylamide containing 0.06% amylopectin of Q-enzyme, phosphorylase (P), α- and β-amylases (A), and R-enzyme in the developing IR8 grain in three incubation media.

The 5th and 6th bands were pink and were present when the gel was incubated in starch in either phosphate or tris-maleate buffer, but they were not detected when the substrate was amylopectin β-limit dextrin. These bands were identified as β-amylase. The 7th and 9th bands gave a deep blue color when the gel was incubated in starch, and a bluish color when amylopectin β-limit dextrin was the substrate. They were considered as R-enzymes. The 8th and 10th bands gave faint, light blue bands with starch as the substrate. They were more readily detected with

amylopectin as the substrate. They were identified as α-amylases. The quantitative assays also showed that α-amylase activity in the grain is low (Fig. 2).

All the 10 bands were present during grain development and in the mature rice grain. In some lines, the slow phosphorylase band was less distinct than the fast band.

Levels of Enzymes in Lines Differing in Amylose Content at the Midmilky Stage of Grain Development. The dehulled grains of the waxy lines were heavier than those of the nonwaxy samples, but they had similar levels of soluble protein per grain (Table I). Synthetase bound to the starch granule in nonwaxy lines was 25 times greater than that in waxy lines, but nonwaxy lines had lower levels of soluble starch synthetase than waxy lines. The samples had similar levels of α-amylase, phosphorylase, and Q-enzyme, but the waxy lines had higher levels of β-amylase (and maltase), and R-enzyme. These differences would be less if the results were expressed per unit weight because of the heavier grain of the waxy lines. However, the specific activities of the enzymes would not be affected since the soluble protein contents of the lines were identical.

In the three pairs of nonwaxy lines differing in amylose content, smaller differences in grain weights were noted (Table II) than in the four IR253 lines (Table I). Soluble protein levels were similar within each pair. The bound starch synthetase was significantly higher in the high amylose sample. The grains had similar levels of soluble starch synthetase and β-amylase (and maltase).

Although some pairs differed in the level of the other enzymes, these differences were not consistent with amylose content among the pairs. One low amylose sample (Chianung 242/2 × Peta) had higher R-enzyme activity than its high amylose counterpart. The second low amylose sample (CP-SLO × Sukanandi) had a lower R-enzyme activity than its paired sample. The third low amylose sample (IR8 × FF 36) had higher activities of R-enzyme and Q-enzyme. Hence, only the bound starch synthetase differed consistently in activity with amylose content.

Considering all the synthetic enzymes assayed, only the level of bound starch synthetase differed consistently with amylose content in the 10 (2 waxy and 8 nonwaxy) lines we studied. Amylose content had a significant positive correlation with the bound synthetase activity of the grain ($r = +0.85$) and a significant negative correlation with the percentage of soluble synthetase ($r = -0.95$). No significant correlation between amylose and the activity of the soluble enzyme was found.

Table I. *Mean Levels (per Grain) of Enzymes of Starch Metabolism at the Midmilky Stage of Two Waxy and Two Nonwaxy IR253 Lines*

Enzyme	Waxy[1]	Nonwaxy[1]	LSD (0.05)
Starch synthetase (μg glucose/15 min)			
Starch granule-bound	0.61	15.3	0.70
Soluble	1.68	0.73	0.65
α-Amylase (iodine-dextrin color units)	0.53	0.50	NS
β-Amylase (corrected, μg maltose/min)	7.98	6.94	0.68
Maltase (μg glucose/min)	9.4	7.0	0.29
Phosphorylase (10^{-2} μg/5 min)	3.91	3.58	NS
Q-Enzyme (% ΔA_{660}/min)	19.3	19.1	NS
R-Enzyme (iodine-dextrin color units)	1.50	1.05	0.22
Hulled grain weight (mg)	22.6	19.2	1.8
Protein (Lowry, μg)	318	316	NS

[1] Mean of two lines. Amylose content (% of starch) of the mature grain was 0% for the waxy and 32% for the nonwaxy samples.

DISCUSSION

The study showed that the activity of starch synthetase increased as the amylose content increased in the developing IR8 grain. A similar trend was observed at the midmilky stage in lines differing in amylose content. These results indicate that this enzyme is responsible for the integrity of amylose in the starch granule, since this was the only enzyme that changed with amylose content.

Although both starch synthetase and phosphorylase have been mentioned as enzymes involved in starch synthesis, the former enzyme is thermodynamically favored for synthesizing starch from sucrose (27). The pathway is almost irreversible, whereas the phosphorylase system is reversible (2). Since phosphorylase activity is high when starch synthesis is rapid, its function may be to produce the primer molecules needed by starch synthetase to synthesize starch. In the ripening grain with a pH of 5 to 6, phosphorylase activity is high (1). We found that the bound starch synthetase had the same activity at pH 6 and pH 8. De Fekete (13) found the same results for the bound starch synthetase of broad-bean cotyledons.

Tsai and Nelson (29) recently reported that two out of four

Table II. *Levels (per Grain) of Enzymes of Starch Metabolism at the Midmilky Stage of Nonwaxy Lines Differing in Amylose Content*

Enzyme	Chianung 242/2 × Peta		CP-SLO × Sukanandi		IR8 × FF 36		LSD[1] (0.05)
	(18.9)[2]	(34.4)[2]	(18.4)[2]	(31.4)[2]	(15.7)[2]	(30.4)[2]	
Starch synthetase (μg glucose/15 min)							
Starch granule-bound	3.62	16.95	3.34	6.23	4.14	17.23	0.22
Soluble	1.50	1.78	0.92	1.19	1.05	1.33	NS
α-Amylase (iodine-dextrin color units)	0.92	1.02	0.57	1.21	0.71	0.89	NS
β-Amylase (corrected, μg maltose/min)	9.8	14.0	10.2	9.8	13.0	8.0	NS
Maltase (μg glucose/min)	3.8	4.1	5.2	4.1	7.6	6.4	NS
Phosphorylase (mμg P/5 min)	25.8	28.9	65.4	34.2	37.4	32.8	NS
Q-Enzyme (% ΔA_{660}/min)	12.5	10.2	9.2	11.0	14.2	8.7	3.9
R-Enzyme (iodine-dextrin color units)	1.40	1.21	0.70	1.48	1.72	0.89	0.15
Hulled grain weight (mg)	18.9	19.3	16.1	17.9	17.4	16.6	0.8
Protein (Lowry, μg)	350	325	291	321	269	271	NS

[1] Low *versus* high amylose sample of each pair.
[2] Amylose content (% of starch) in the mature grain.

corn phosphorylases can synthesize linear chains from glucose and glucose-1-P. However, the phosphorylase isozymes of rice evidently require a primer molecule since they cannot synthesize amylose from glucose-1-P alone. The other degradative enzymes, the amylases and R-enzyme, may have the function of increasing the number of primer molecules by degrading starch molecules.

Based on this pathway, the amylose content of the starch depends on how much starch synthetase and Q-enzyme are on the surface of the developing starch granule. In the waxy granule, linear chains are probably produced by the soluble starch synthetase, but they are readily transformed into amylopectin by Q-enzyme.

In the nonwaxy granule, the starch synthetase level is higher. Amylose molecules are synthesized and are protected from the attack of Q-enzyme by forming an insoluble complex with the starch synthetase. It is interesting that only the activity of starch synthetase changes considerably with amylose content, whereas Q-enzyme activity is relatively constant (Tables I and II). Badenhuizen (4) believes that, inside the granule, the starch synthetase bound to the starch granule finishes the amylose synthesis started by phosphorylase.

Since the weights (and starch levels) of the samples were similar at the midmilky stage, the rates of starch synthesis in these samples must be similar. Hence only the amylose to amylopectin ratio was affected by the observed differences in enzyme levels. For this reason, the ADP-glucose pyrophosphorylase activity, the rate-controlling step in starch biosynthesis (12), was not assayed in our study. Turner (31) reported that the activities of ADP-glucose and UDP-glucose pyrophosphorylases were proportional to rates of starch synthesis in the developing wheat grain.

The distinction between soluble and granule-bound starch synthetases does not seem to be clear cut. Even the waxy starch granule showed some activity of the bound enzyme. Although this may be attributed to the low amylose content (<1%) of waxy rice, it could also be due to soluble enzyme adsorbed on the surface of the waxy starch granule. On the other hand, soluble enzyme is present in the high amylose samples. Since the extraction becomes more efficient the longer the homogenate is mixed with the buffer, the soluble enzyme must have some affinity for the surface of the starch granule. Hence, the observed activity of the bound enzyme must also include the activity of adsorbed soluble enzyme, as suggested also by Chandorkar and Badenhuizen (10). Also, the assay of intact starch granules may not reveal the total activity of enzyme that is complexed with the starch. Mechanical disruption (grinding) of the starch granules increased the activity of the bound enzyme (10, 15). Badenhuizen (4) believes that these two forms may be isozymes. In this connection, Fredrick (14) found isozyme forms of soluble transferases in algae.

Another problem is the nature of the complex between starch synthetase and amylose in the nonwaxy starch granule. Although Murata and Akazawa (26) demonstrated that the soluble synthetase has affinity for the starch granules of nonwaxy rice, this complexing may be just surface adsorption. Most of the bound enzyme in the nonwaxy granule is within the granule. In fact, starch preparations from the rice grain always contain less than 1% residual protein. The starch granules of the lines studied showed similar Kjeldahl protein contents (4–6%) regardless of amylose content. Presumably, the method for purifying starch granules of the rice endosperm does not effectively remove the contaminant storage protein which is essentially insoluble in water.

Our study verified that samples taken at the midmilky stage are appropriate for studying starch biosynthesis in the developing rice grain. We also showed that at the midmilky stage, only the activity of bound starch synthetase was correlated with the amylose content of the starch. Hence this enzyme may be involved in the integrity of amylose and probably in synthesis in the developing nonwaxy starch granule. Although we only measured the activity of ADP-glucose-starch 4-glucosyltransferase in our study, similar results would be expected from the UDP-glucose-starch 4-glucosyltransferase. The ADP-glucose and the UDP-glucose pathways may be both important, because even though the activity of UDP-glucose-starch 4-glucosyltransferase is less than that of ADP-glucose-starch 4-glucosyltransferase, the ripening rice grain has a higher content of UDP-glucose than ADP-glucose (2, 26). We found that the activity of UDP-glucose-starch glucosyltransferase in rice is 30% that of the ADP-glucose-starch synthetase in IR8 starch granules.

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