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Changes in the Activity of Some Hydrolases, Peroxidase, and Catalase in the Rice Seed during Germination[1]

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ABSTRACT

A study was made of the changes in activity of enzymes involved in the breakdown of stored phytin, lipid, and hemicellulose in the aleurone layer of rice seed (*Oryza sativa* L., variety IR8) during the 1st week of germination in the light. Enzyme assays were made on crude extracts from degermed seed, and activities were expressed on a per seed basis. Phytase activity increased within the 1st day of germination. The increase in activity of most other enzymes—phosphomonoesterase, phosphodiesterase, esterase, lipase, peroxidase, catalase, β-glucosidase, and α- and β-galactosidase—closely followed the increase in protein content. Their peak activities occurred by the 5th to the 7th day. Some enzymes, such as β-1,3-glucanase and α-amylase, continued to increase in activity after the 7th day. Phytase, β-1,3-glucanase, and α-amylase followed a similar sequence of production in embryoless seed halves incubated in 0.12 μM gibberellin A_3, but the production of lipase was delayed.

The aleurone layer of cereals is the site of production of hydrolases during seed germination and during incubation of embryoless seed halves or isolated aleurone layers in GA_3 (2, 6–8, 19, 21, 24). Many hydrolases have been studied, including α-amylase (2, 19), protease (6, 19), phytase (10, 15), phosphatase (24), lipase (26), and RNase (2, 19). Phytase has been shown to be located in the protein bodies of the barley aleurone layers (17).

The aleurone layer of rice (*Oryza sativa* L.) is a storage organ for phosphate in the form of phytin (9, 15) and for lipids (12); it is richer in hemicellulose than the endosperm (4). It is devoid of starch granules in the mature grain (9). Jones (8) reported the presence of glyoxysomes, which are responsible for gluconeogenesis of lipids, in the barley aleurone layer. Earlier we have shown that protease is produced in the rice aleurone layer earlier than α-amylase and R-enzyme and probably RNase (19). This paper reports on a study of the sequence in which phosphatases, esterases, and glycosidases are produced in the rice grain germinated in the light and of the sequence in which phytase, lipase, and β-1,3-glucanase are produced in embryoless seed halves in the presence of GA_3. Knowledge of the sequence of biochemical changes in the rice seed during germination is important in our understanding of dormancy, storage quality of wet grain, and seedling vigor.

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MATERIALS AND METHODS

Germination. Rice seeds (variety IR8) were germinated in the light and in the dark and prepared for analysis as previously described by Palmiano and Juliano (19). Freeze-dried, dehulled, degermed seeds were assayed for enzyme activities.

Enzyme Extraction. Ten degermed dehulled grains were homogenized at 0 to 4 C at top speed in a VirTis 45 homogenizer for 3 min with 10 ml of 0.1 M tris-HCl buffer (pH 7.0). The homogenate was centrifuged at 30,000*g* for 10 min at 4 C, and the crude enzyme was used for the assays, except for assays of lipase and β-1,3-glucanase, which were done on the protein fraction of the crude enzyme precipitating between 0 and 80% saturation with $(NH_4)_2SO_4$. Soluble protein was determined by the Folin phenol reagent (11) corrected for the reading of the tris buffer.

Phosphatases and Esterases. The method of Pollard (21) using *p*-nitrophenyl derivatives was used for the assay of phosphomonoesterase, phosphodiesterase, and esterase. The incubation mixture consisted of the crude extract of degermed seed and substrate equivalent to 0.5 μmole of *p*-nitrophenol (monophosphate, bisphosphate, and acetate) in 0.1 M acetate buffer (pH 5.0) at 30 C. Incubation time was 15 min for phosphatases and 1 hr for esterase. To stop the reaction, Na_2CO_3 was added, and the amount of *p*-nitrophenol released was determined colorimetrically at 420 nm. Activities were expressed in μmoles of *p*-nitrophenol formed per hour per seed.

Phytase activity was assayed by the method of Mukherji *et al.* (15) except that incubation time was only 15 min at 30 C at pH 4.0. The amount of phosphate released was measured by the method of Galanos and Kapoulas (3).

Lipase activity was determined by a fluorometric method using 4-methyl umbelliferone butyrate as substrate (20). Incubation time was 3 min at 30 C in 0.1 M phosphate buffer (pH 7.5).

Peroxidase. The incubation mixture for peroxidase assay consisted of pyrogallol, H_2O_2, and crude extract of degermed seed in 0.1 M phosphate buffer (pH 6.0) maintained at 30 C for 3 to 5 min (22). We added 2 N H_2SO_4 to stop the reaction and extracted the purpurogallin that formed with 1-butanol. The concentration of purpurogallin in 1-butanol was measured colorimetrically at 420 nm.

Catalase. The catalase activity of the crude extract of degermed seed was assayed by the titrimetric method of Chance and Maehly (1). The extract and H_2O_2 in 0.1 M phosphate buffer (pH 7.0) was incubated at 30 C for 1 min. The reaction was stopped by adding an excess of 5% H_2SO_4, and the residual H_2O_2 was titrated with 0.05 N $KMnO_4$ solution.

Glycosidases and β-1,3-Glucanase. The crude extract of degermed seed was assayed for α- and β-glucosidases and α- and β-galactosidases by the method of Pollard (21) with *p*-nitrophenyl derivatives of D-glucose and D-galactose as substrates. The incubation mixture consisted of crude extract and 0.5 μmole of *p*-nitrophenyl substrate in 0.1 M acetate buffer (pH 5.0) at 30 C for 1.0 to 2.5 hr.

The extract of degermed seed precipitated by $(NH_4)_2SO_4$ was assayed for β-1,3-glucanase by the method of Jones (7) with 0.1% laminarin as substrate. Incubation time was 1 hr at 37 C in 0.02 M acetate buffer. The reducing sugars released were determined by the 3,5-dinitrosalicylate method (4) with glucose as standard.

Incubation of Seed Halves. Seeds of IR8 rice were cut in half through the short axis, and the embryoless halves were incubated in 0.12 μM (40 μg/liter) GA_3 at 30 C as previously described (19). Activities of phytase, lipase, and β-1,3-glucanase were determined on the incubated seed halves with α-amylase as a marker enzyme (19).

RESULTS

Changes in the levels of enzymes in grains germinated in the light were similar to those in grains germinated in the dark. Although the changes tended to be faster in the dark, the changes in the light have more practical importance, since rice grains are usually germinated in the light and are not used for malting. Hence, only the data for rice germinated in the light are presented in the tables. The dry weight of seeds did not decrease significantly (16–19 mg) during the 1-week period of germination in the light (19). The crude preparations gave essentially a linear relationship between enzyme concentration and assayed activity for the enzymes which were assayed.

Acid Phosphatases. Soluble protein started to increase in the IR8 seed by the 4th day of germination. Both acid phosphomonoesterase and phosphodiesterase increased in activity in the rice endosperm during germination (Table I). The phosphatase levels had increased significantly by the 4th day of germination. Phytase activity increased faster than phosphatase activities (Table I) and was significantly higher in the germinating seed than in the ungerminated seed after steeping for 1 day before any visible sign of germination appeared. It was essentially constant after 4 days of germination. Phytase is probably a different enzyme from phosphomonoesterase since acid phosphatase of embryoless seed halves treated with GA_3 has been reported to have very low specificity for phytin (24). Specific activity of phytase has also been reported to increase during rice germination in the dark (15).

Phosphomonoesterase activity was also significantly higher during germination in the dark than in the ungerminated seed after 4 days of germination, and phosphodiesterase activity was higher after 5 days of germination (18). However, phosphatase activities were slightly higher in seeds germinated in the light than in seeds germinated in the dark.

Esterases. Esterase and lipase showed a similar trend during germination. Both increased in activity during germination, but lipase increased faster during the 1st week of germination than esterase (Table II). In the light, esterase activity was significantly increased after 7 days of germination. Lipase activity of the rice seed leveled off from the 5th to the 7th day of germination. Rice bran lipase has been reported to have no positional specificity for ester bonds of rice bran triglycerides (16). Tavener and Laidman (24) reported an increase in lipase activity in the tissue of wheat grain during germination. In the dark, esterase activity did not increase significantly during the 1st week of rice germination (18).

Table I. *Changes in Activity of Acid Phosphatases in Germinating IR8 Seed*

Germination Time	Soluble Protein	Phosphomonoesterase	Phosphodiesterase	Phytase
days	*μg*	*μmoles p-nitrophenol/hr*		*μmoles P_i/hr*
0	255	2.85	0.35	1.8
1	278	...	...	9.2
2	264	...	...	11.4
3	309	2.96	0.35	12.3
4	324	4.08	0.54	16.9
5	362	5.72	0.83	14.3
6	443	5.92	0.76	18.3
7	360	6.24	1.10	18.8
LSD (5%)	24.1	0.65	0.14	3.3

Peroxidase and Catalase. Endosperm peroxidase level increased during germination, and the increase was already significant after 4 days in the light and remained essentially constant from the 5th to the 7th day of germination (Table II). In the dark, seed peroxidase activity increased faster and was higher than in the light. It was already significantly higher by the 3rd day of germination (18). Strength *et al.* (23) also reported an increase in peroxidase in rice seedlings after 4 days of germination, but the contribution of the seed was not determined. Ito and Hayashi (5) reported no change in peroxidase activity during the 1st day of germination of rice grains.

Catalase activity increased in the endosperm during germination (Table II). Ito and Hayashi (5) reported an increase in catalase activity of rice seeds during the 1st day of germination.

Glycosidases and β-1,3-Glucanase. Activity of α-glucosidase in the seed decreased during germination, but the activities of β-glucosidase and α- and β-galactosidase increased (Table III). The increase in activity was highest for β-galactosidase. Two isozymes of α-glucosidase from ungerminated rice grain showed greater activity on α-1,4- and α-1,3- than on α-1,6-glucosidic linkages (25). In the dark, only β-galactosidase of the four glycosidases increased in activity during the 1st week of germination (18).

The activity of seed β-1,3-glucanase increased during germination and was still increasing after 7 days of germination (Table III). Manners and Marshall (13) also reported an increase in endo-β-1,3-glucanase activity during germination of barley in the dark.

Enzyme Production in Embryoless IR8 Seed Halves in the Presence of GA_3. When an embryoless seed half was incubated

Table II. *Changes in Activity of Esterase, Lipase, Peroxidase, and Catalase in Germinating IR8 Seed*

Germination Time	Esterase	Lipase	Peroxidase	Catalase
days	*μmoles p-nitrophenol/hr*	*μmoles butyrate/hr*	*μmoles purpurogallin/hr*	*μmoles O_2/hr*
0	0.059	0.284	11.5	198
1	...	0.308	...	274
2	...	0.377	...	501
3	0.083	0.422	11.2	1170
4	0.083	0.616	31.9	1190
5	0.109	0.875	45.5	1400
6	0.105	0.914	...	3060
7	0.130	0.777	43.2	2020
LSD (5%)	0.065	0.213	19.2	459

Table III. *Changes in Activity of Glycosidases and β-1,3-Glucanase in Germinating IR8 Seed*

Germination Time	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	β-1,3-Glucanase
days	*μmoles p-nitrophenol × 10²/hr*				*μmoles glucose/hr*
0	5.4	6.3	3.0	4.1	0.56
1	...	...	...	...	0.56
2	4.4	7.0	3.0	4.8	0.44
3	4.4	5.7	4.4	5.0	0.89
4	4.0	6.9	3.2	5.7	1.22
5	2.6	12.8	3.5	11.2	1.22
6	2.8	11.8	4.5	10.4	1.78
7	2.7	10.5	4.6	9.6	3.44
LSD (5%)	0.5	2.2	1.2	1.4	0.94

Table IV. *Changes in Phytase, Lipase, and β-1,3-Glucanase Activities of Embryoless Seed Half of IR8 Incubated in 0.12 μM GA_3*

Incubation Time	Soluble Protein	α-Amylase	Phytase	Lipase	β-1,3-Glucanase
days	*μg*	*ΔA540/hr*	*μmoles P_i/hr*	*μmoles butyrate/hr*	*μmoles glucose/hr*
0	98	1.2	0.088	Trace	0.44
1	99	3.4	0.126	Trace	0.72
2	117	10.8	0.177	0.32	13.2
2.5	129	26.4	0.202	0.99	22.4
3	132	71.4	...	1.51	26.3
4	132	...	0.208	2.50	31.4
5	137	111.5	0.196	4.54	36.6
LSD (5%)	26.5	24.4	0.057	0.69	3.61

in 0.12 μM GA_3 for 5 days, the marker enzyme, α-amylase, increased throughout the period (Table IV). By contrast, phytase activity increased during the first 2.5 days of incubation, then leveled off. Activity of β-1,3-glucanase also increased and followed closely the pattern for α-amylase. Lipase activity was negligible in the unincubated seed half and during incubation in water for 5 days. It started to increase only after 2 days of incubation in GA_3. Addition of 1 mM glutamine or hydroxylamine to GA_3 had no effect on lipase induction. Hence, the sequence data for phytase and β-1,3-glucanase production are the same in seed halves incubated in GA_3 and in germinating grains. However, the production of lipase was delayed in the seed halves incubated in GA_3.

In contrast to the comparable levels of α-amylase and protease production we previously found (19), treatment of seed halves with GA_3 resulted in lower production of phytase but greater production of lipase and β-1,3-glucanase than in the germinating seed in the light. It is interesting that the lipase level in the mature grain is mainly present in the seed half with embryo, indicating that lipase is mainly in the embryo. Activity of α-amylase was higher in this experiment than in the one we previously reported (19).

DISCUSSION

Sequence of Enzyme Production in the Germinating Rice Grain. Phytase showed the earliest increase among the enzymes assayed. Its activity increased within the 1st day of soaking before soluble protein increased in the germinating grain (Table I). A decrease in the level of phytin P and an increase in the level of P_i on the 1st day of rice germination were reported by Kurasawa *et al.* (10) and Mukherji *et al.* (15). Oxygen uptake and ATP level increased during this period, and peak total and specific activities of phytase coincided with peak ATP level in the seed 4 days after germination (19). These results support recent studies which showed that phytin in wheat grain acts mainly as a phosphorus and cation store for the germinating seed (27). Phytin also can complex with rice proteins (22) and inhibit lipase activity (16).

Most of the other enzymes follow closely the increase in soluble protein by the 4th day after germination—phosphatases (Table I); protease (19); esterase, lipase, peroxidase, and catalase (Table II); and β-glucosidase and α- and β-galactosidase (Table III). These enzymes do not increase much in specific activity during the 1st week of germination in the light. Lipase action on lipids is the first step in gluconeogenesis of fat in the aleurone layers. Glyoxysomes are probably present in rice aleurone layer, which has 16 to 20% lipids (9, 12) and is devoid of starch (9), since they have been reported in barley aleurone layer with similar fat content (8). In addition, germination is accompanied by an increase in the activity of catalase, one of the enzymes characteristic of glyoxysomes (8). The increase in lipase activity occurs earlier than the increase in α-amylase activity, indicating that gluconeogenesis of aleurone lipids occurs before the breakdown of endosperm starch in the germinating rice grain (19).

The third class of enzymes are those that are still increasing in activity after 7 days of germination, represented by RNase (19), α-amylase (19), R-enzyme (19), and β-1,3-glucanase.

Sequence of Enzyme Production in Embryoless Seed Halves. Phytase production was verified to occur earlier than lipase and β-1,3-glucanase production in embryoless seed halves incubated in 0.12 μM GA_3 (Table IV). Lipase production was delayed, compared with that in intact germinating seed, but β-1,3-glucanase and α-amylase were still increasing after 5 days of incubation.

The delayed induction of lipase in embryoless seed halves incubated in GA_3 (Table IV) is consistent with the need for a nitrogenous compound in addition to IAA for lipase induction in wheat seed halves (25). GA_3 was unable to replace IAA in the wheat system. Glutamine and hydroxylamine, however, had no effect on lipase induction in rice seed halves incubated in GA_3.

Embryoless seed halves and isolated aleurone layer of barley and wheat treated with GA_3 secrete ATPase, phytase, phosphomonoesterase, phosphodiesterase, glucosidases and galactosidases, peroxidase, and amylase, in that order (21). GA_3 also stimulates the release of β-1,3-glucanase in barley seed halves and follows closely the synthesis of α-amylase in the aleurone layers (7). RNase is produced slightly later than α-amylase in aleurone layers of barley treated with GA_3 (2). In addition, nonhydrolase protein is also released by the aleurone layers of barley on treatment with GA_3 (14). We also found peak levels of soluble protein and protease after about 2.5 days of incubation in GA_3 of embryoless rice seed halves (19). Hence, hydrolases in the rice aleurone layer also are produced in sequence during germination or during incubation in GA_3.

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