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Biochemical Changes in the Rice Grain during Germination[1]

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ABSTRACT

Changes in the content of starch, protein, and RNA and in the activity of their hydrolases in the rice endosperm (*Oryza sativa* L., variety IR8) were determined during the first week of germination without added nutrient both in the dark and in the light. Changes were generally more rapid in the dark than in the light. Oxygen uptake and RNase activity started to increase and the root protruded on the second day, followed by the coleoptile on the third day, and the primary leaf on the fourth day. ATP level was at a maximum on the fourth day. The activity of amylases and R enzyme increased progressively, but that of phosphorylase tended to decrease during starch degradation. A new α amylase isozyme band appeared during germination. Glucose was the major product of starch degradation. Sucrose, maltose, maltotriose, raffinose, and fructose were also detected. Protease activity reached a maximum on the fifth or sixth day and closely paralleled the increase in soluble amino N and soluble protein.

In embryoless seed halves with 0.12 μM gibberellin A_3, peak protease activity occurred in 2.5 days and peak α amylase activity on the fifth day of incubation. The production of α amylase, protease, and R enzyme was inhibited by 40 μM cycloheximide, but only α amylase and R enzyme were inhibited by 20 μg/ml actinomycin D.

Most studies on biochemical changes in the rice grain (*Oryza sativa* L.) during germination have been done on samples germinated in the dark (9, 25, 28). They involved an aspect of either starch or nitrogen metabolism. These studies used different varieties, so they cannot be compared. To obtain a comprehensive picture of the sequence of biochemical events occurring during germination, we studied the changes in activity of degradative enzymes and in their substrates in variety IR8 during the first week of germination both in the light and in the dark. The effect of inhibitors of the synthesis of protein and RNA on levels of α amylase and protease during the germination of embryoless seed halves in the presence of GA_3 was also studied.

MATERIALS AND METHODS

Germination. Rice seeds (variety IR8) were sterilized by soaking them in 1% aqueous NaOCl for 20 min, washed, and steeped in distilled water for 24 hr. After soaking, the seeds were placed on moist filter paper in covered glass dishes and maintained at 30 C in the dark or in the diffuse light of the laboratory at 25 to 27 C. Germination schedules were staggered to permit harvesting of all the samples at the same time. The hull, roots, shoot, and scutellum were removed, and the degermed grains were freeze-dried, stored at −20 C, and used for all the subsequent analyses.

Oxygen Uptake. Oxygen uptake was measured in duplicate from seeds germinated in light for 6 to 86 hr. Ten germinated grains were placed in Warburg flasks with 2 ml of distilled water in the outer well and 0.2 ml 10% KOH in the inner well. Manometric measurements were done at 30 C in the light for 15 min to 12 hr, depending on the age of seedling. Results were expressed as nmoles oxygen evolved per hour per seed.

ATP. Twenty degermed grains were soaked for 4 hr at 0 C in 15 ml 0.4 N $HClO_4$, homogenized 3 min with a VirTis "45" homogenizer, and centrifuged at 500*g* for 10 min. A 5-ml portion of the supernatant fluid was neutralized to pH 6.0 with dilute KOH and passed through Whatman No. 1 filter paper. We assayed an aliquot of the filtrate for ATP by the luciferase system using freeze-dried firefly extract (Worthington Biochemical Corp.) in 0.1 M arsenate buffer, pH 7.4 (2). A Packard TriCarb scintillation counter was used to measure the luminescence emitted for 3 sec with ATP (Calbiochem) as standard.

Carbohydrases. Degermed dehulled grains were homogenized at 0 to 4 C at top speed in a VirTis homogenizer for 3 min with 0.1 M tris-HCl buffer (pH 7.0, with 0.2% $CaCl_2$) (2 to 5 grains/ml buffer). The homogenate was centrifuged at 30,000*g* for 10 min at 4 C, and the crude supernatant fluid was used for the enzymic assays. R or debranching enzyme and α amylase were assayed colorimetrically with amylopectin β limit as substrate (3). The presence of R enzyme was verified using pullulan (Dr. W. J. Whelan, University of Miami) as substrate (19). β Amylase was assayed by the colorimetric determination of maltose released (3). Phosphorylase was assayed by the amount of P_i produced from glucose-1-P and amylopectin, according to Baun *et al.* (3). Activities were expressed in ΔA or μmoles per hour per grain. Zymograms of the extracts in 8 and 10% polyacrylamide gels were determined according to Juliano and Varner (17).

Free Sugars and Starch. Free sugars and starch were extracted and estimated by the anthrone method (23) according to Baun *et al.* (3). Blue values of the starch extract in $HClO_4$ were also assayed at 680 nm (3). Free sugars were separated by descending paper chromatography with butanol:pyridine:water (6:4:3, v/v) as developer (25) for 24 to 30 hr at 25 to 27 C. The location of the sugars was determined from guide strips sprayed with $AgNO_3$-NaOH reagent (34) and identified by the use of authentic sugars. The quantity of individual sugar eluted from the paper chromatogram was determined by the anthrone method.

Protease, Protein, and Free Amino Nitrogen. Degermed dehulled grains were homogenized at 0 to 4 C in a VirTis ho-

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mogenizer at top speed for 3 min in 0.1 M phosphate buffer, pH 7.5, containing mM cysteine·HCl (2 grains/ml buffer). The homogenate was centrifuged at 440*g* for 15 min, and the supernatant fluid was used for the assays. Soluble protein was determined with the Folin reagent (20). Protease activity was assayed by the method of Cruz *et al.* (5) with hemoglobin as substrate. Incubation was done in 0.1 M acetate buffer, pH 3.5, for 90 min at 40 C. Activity was expressed as the increase in absorbance at 280 nm of the supernatant fluid after precipitation with trichloroacetic acid. The presence of cysteine improved the extraction of protease but had no effect on the specific activity of the protease. Total protein was determined with the Folin reagent (20) with crystalline bovine plasma albumin (Armour Laboratories) as standard; free amino nitrogen was determined by reaction with ninhydrin, with L-leucine as standard, according to Cruz *et al.* (5).

RNase and RNA. RNase and RNA were assayed according to Cruz *et al.* (5). Yeast RNA (Nutritional Biochemicals Corp., reagent grade) and bovine plasma albumin were used as the standards.

Incubation of Seed Halves. Grains of IR8 brown rice were cut in half through the short axis. The halves without embryos were sterilized by soaking in anhydrous ethanol for 1 min and in 1% (w/v) NaOCl for 20 min, and washed three to four times with sterile distilled water. Ten halves were incubated in sterilized 25-ml Erlenmeyer flasks containing 0.12 μM (40 μg/l) GA_3 (a gift of Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) at 30 C for varying periods from 0.33 to 7 days in diffuse light. Preliminary experiments showed that this concentration of GA_3 produced higher levels of α amylase and protease in halves that had no embryos than in halves that had embryos when germinated for the same period in distilled water. The halves were then freeze-dried. Ten seed halves were homogenized in 5 ml of appropriate buffer in glass homogenizers at 0 C for α amylase and protease assays as described above. Cycloheximide (Nutritional Biochemicals Corp.), actinomycin D (Nutritional Biochemicals Corp.), and dibutyryl cyclic 3′,5′-AMP (Calbiochem) were added to the incubation medium. Embryoless halves were then incubated for 3 days and were similarly analyzed for α amylase, R enzyme, and protease levels.

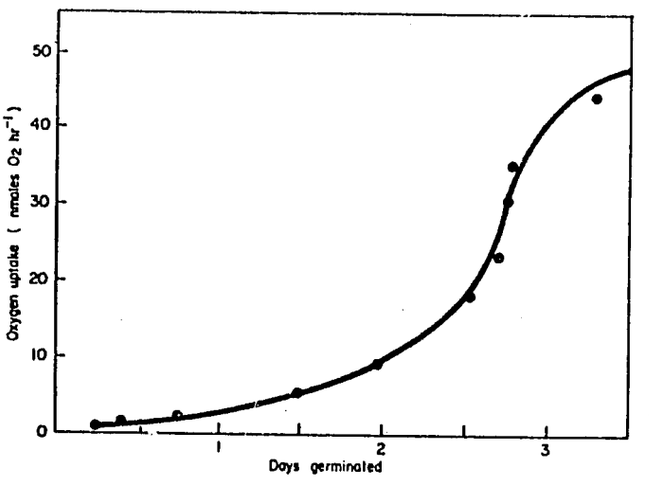

FIG. 1. Changes in oxygen uptake of IR8 grain germinated at 30 C in the light.

RESULTS

Oxygen Uptake, ATP Level, and Dry Matter Loss. Oxygen uptake increased even while the rice seeds were soaking in water (Fig. 1). A soaking period of 18 to 24 hr was optimum for our germination study, which agrees with the value of 18 hr reported by Takahashi (32). The seed swelled during soaking. The radicle was exserted during the second day, the coleoptile appeared on the third day, and the primary leaf appeared on the fourth day. The rapid increase in oxygen uptake coincided with the emergence of the radicle, as also reported by Abdul-Baki (1) for barley. The roots and shoots were longer in samples grown in the dark. ATP level was highest in the grain germinated for 4 days in the light and in the grain germinated for 3 to 4 days in the dark (Table I).

The amount of dry matter in the grain decreased faster in the samples germinated in the dark than in those germinated in the light (Table I). The weight loss was significant after 5 days germination in the dark.

Starch Metabolism. Starch content of the endosperm decreased progressively during germination and was more rapid in the dark than in the light (Table I). The decrease in starch content was significant after 7 days germination in the light and after 5 days in the dark. The blue value of the residual

Table I. *Changes in ATP, Dry Weight, Starch, Free Sugars, and Carbohydrases in Germinating IR8 Grain*

Days Germinated	ATP	Dry Wt	Starch	Blue Value	Free Sugars	α Amylase	β Amylase	Phosphorylase	R Enzyme
	picomoles	*mg*	*mg*	A_{660}	*mg*	$\Delta A_{540}/hr$	*moles maltose/hr*	*μmoles* $P_i \times 10^2/hr$	$\Delta A_{540}/hr$
0	1.00	18.4	16.2	0.348	0.15	0.3	32	4.01	0.46
Light									
2	1.11	18.9							
3	1.57	18.8	15.2	0.368	0.27	1.5	8	4.43	0.90
4	2.52	18.6	14.6	0.353	0.39	3.9	21	4.27	1.3
5	0.72	17.3	13.8	0.346	0.43	11.9	43	4.30	2.3
6							73		4.4
7	0.52	16.0	8.9	0.377	0.71	15.0	99	3.60	7.0
Dark									
2	0.54	19.6		0.364					
3	2.35	17.0	13.9	0.380	0.37	1.0	111	3.65	1.8
4	2.34	17.0	12.4	0.363	0.77	6.3	209	3.19	2.4
5		12.6	10.8	0.345	1.14	14.1	294	2.76	4.5
6				0.393			346		7.6
7	1.02	9.2	5.6	0.373	1.79	55.2	370	1.33	8.7
LSD (5%)	0.73	3.0	4.8	n.s.	0.33	4.5	56	0.88	0.9

starch remained high during germination. An increase in free sugars accompanied the decrease in starch content. The increase in free sugars was significant after 7 days germination in the light and after 4 days in the dark. This increase coincided with the decrease in dry weight and starch of the germinating grain.

Paper chromatography showed that glucose was the major reducing sugar together with lower amounts of fructose, maltose, and maltotriose. The major nonreducing sugar was sucrose together with raffinose. During germination, the levels of all these sugars in the grain increased, but glucose remained the principal reducing sugar and was higher in concentration than the principal nonreducing sugar, sucrose. In the grain germinated 4 days in the dark and in the grain germinated 7 days in the light, the concentration of maltotriose was similar to that of maltose. Our chromatogram was similar to that of Murata *et al.* (25), except that we found that fructose had an $R_{glucose}$ value of 1.1, and sucrose had a value of 0.83. Hence, the spot denoted as fructose by Murata *et al.* was probably sucrose.

The amylases increased in activity in the grain during germination, and the activity was greater in the dark (Table I). The increase in α amylase activity was only significant after 5 days germination in the light and after 4 days in the dark. By 7 days germination, α amylase activity was already at a maximum in grains in the light, but it was still increasing in grains germinated in the dark. Williams (36) reported that α amylase reaches maximum activity in the dark after 11 to 13 days germination, but IR8 shows a lower first peak after 7 days germination. Murata *et al.* (25) reported that activity of α amylase reached a peak after about 10 days germination in the dark.

The activity of β amylase increased during germination and was significantly higher than its activity in the ungerminated grain after 7 days germination in the light and 3 days in the dark (Table I). β Amylase activity was initially higher than α amylase activity, but during 7 days germination it only increased 3-fold in the light and 12-fold in the dark. α amylase level increased 50-fold during 7 days germination in the light and 184-fold in the dark. In contrast to the amylases, phosphorylase activity did not increase during the first week of germination in either light or darkness (Table I). Phosphorylase activity had decreased significantly after 5 days germination in the dark. By contrast, Murata *et al.* (25) reported a 2-fold increase in phosphorylase activity during 8 to 10 days of germination in the dark of another rice variety.

R enzyme activity increased significantly during germination after 4 days in the light and after 3 days in the dark. It increased 15-fold during 7 days germination in the light and 19-fold in the dark. Although α amylase was not completely inhibited by EDTA addition in the R enzyme assay using amylopectin β limit dextrin, the increase in R enzyme was confirmed using pullulan as substrate (19).

Polyacrylamide-gel electrophoresis showed that a third fast α amylase band appeared 4 days after germination in the dark with a mobility of 1.36 relative to the fast α amylase band in mature grain (3). The two other α amylase bands and the two β amylase bands (3) widened during germination and overlapped the two R enzyme bands. In contrast, the slow and intermediate phosphorylase bands, but not the fast phosphorylase band of mature grain, were observed in the grain germinated 2 days in the dark. After 4 days of germination, only the slow phosphorylase band was present. The zymograms supported the results of quantitative assays for these enzymes.

Nitrogen Metabolism. Crude protein started decreasing earlier in the grain than starch, indicating faster nitrogen breakdown and translocation to the embryonic tissues (Table II).

Table II. *Changes in Crude Protein, Soluble Protein, Amino Nitrogen, Protease, RNA, and RNase in Germinating IR8 Grain*

Days Germinated	Crude Protein (N × 5.95)	Soluble Protein	Soluble Amino N	Protease	RNA	RNase
	mg	*μg*	*μg*	$A_{240} \times 10^2/hr$	*μg*	$\Delta A_{240}/hr$
0	1.36	258	2.18	2.8	26	0.54
Light						
2	1.01				16	
3	0.98	264	3.37	6.0	16	2.22
4	0.98	302	3.84	11.2	12	6.84
5	0.92	326	7.75	17.5	18	12.1
6	0.78	340	8.36	14.0		
7	0.77	313	8.03	14.2	7	19.4
Dark						
2	0.83	276			14	
3	0.82	268	9.79	4.4	11	7.68
4	0.70	296	14.25	9.8	11	11.0
5	0.64	304	15.80	16.0	11	7.4
6	0.44	364	16.45	13.2		
7	0.49	310	16.80	13.9	4	11.3
LSD (5%)	0.06	30.8	1.58	3.5	10.4	2.70

Protein level had decreased significantly after 2 days germination both in light and in the dark. The drop in crude protein was faster in the grains germinated in the dark than in those germinated in light. Soluble protein increased during germination up to the sixth day of germination, after which it started to decrease. In fact, soluble protein was the major nitrogen fraction in the grain germinated in the dark for 6 days.

The level of free amino acids increased progressively during the first week of germination. The increase was faster in the dark. The increase in free amino nitrogen was significant after 4 days germination in light and 3 days in the dark. The peak content of free amino nitrogen occurred after 6 days germination in the light, but free amino nitrogen was still increasing after 7 days germination in the dark. Protease activity increased 5- to 6-fold during germination and was highest in the grain germinated for 5 days, after which it levelled off at 14 units. The increasing protease activity may account for the increase in the level of soluble protein and free amino acids. Matsushita (22) also reported a progressive decrease in total endosperm nitrogen during rice germination.

The RNA level in the grain also decreased during germination faster than the starch level, coinciding with the increase in RNase level (Table II). The drop in RNA was significant after 4 days germination in light and after 3 days in the dark. The increase in RNase level was also significant after 4 days germination in light and after 3 days germination in the dark. Its level was still high after 7 days germination. Matsushita (22) similarly found a progressive decrease in rice endosperm RNA during germination.

Production of α Amylase and Protease in Embryoless IR8 Seed Halves in the Presence of GA_3. GA_3 at a concentration of 0.12 μM induced the synthesis of α amylase in embryoless IR8 seed halves. The peak activity occurred after 5 days of incubation (Fig. 2). By comparison, protease activity increased within 2.5 days and decreased afterward and was absent in the sample incubated for 7 days. The concentration of soluble protein reached a peak at about 2 days of incubation and then leveled off to over 90 μg/seed half.

The α amylase level in the dehulled seed halves that contained embryos and were germinated in the light was lower

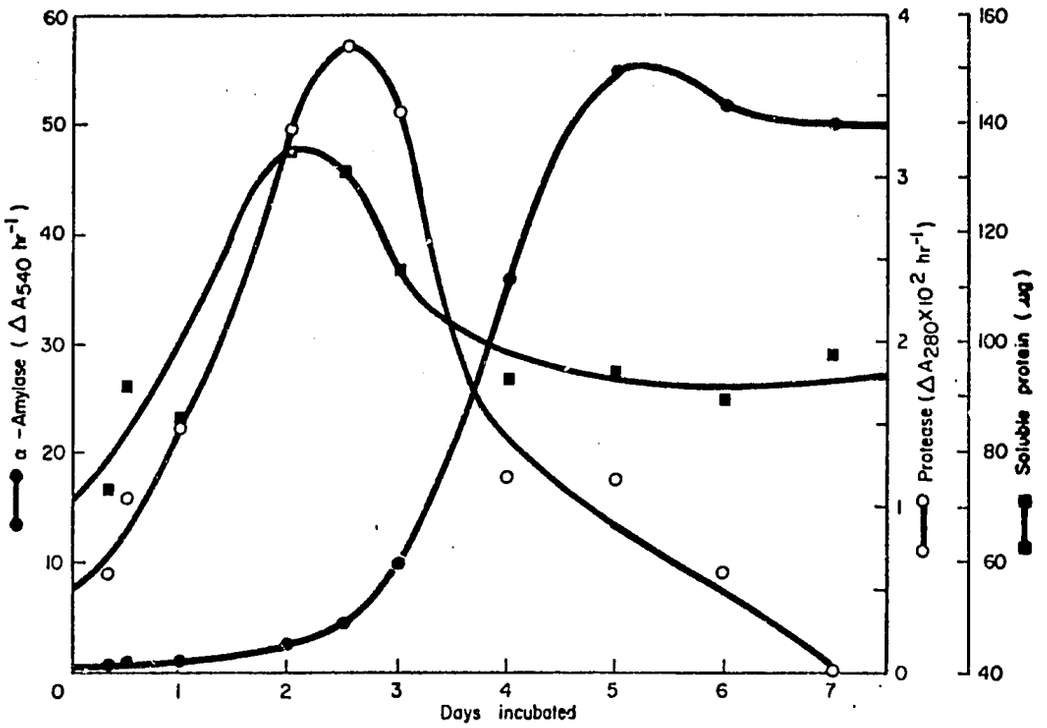

FIG. 2. Changes in α amylase, protease, and soluble protein levels of embryoless seed half of IR8 incubated in 0.12 μM GA_3.

Table III. *Effect of Three Biochemicals on the α Amylase, Protease, and R Enzyme Levels of IR8 Embryoless Seed Half Incubated for 3 Days with GA_3*

Biochemical	α Amylase	Protease	R Enzyme
	$\Delta A_{540}/hr$	$\Delta A_{280} \times 10^2/hr$	$\Delta A_{610}/hr$
GA_3 (0.12 μM) control	8.88	8.6	7.14
Actinomycin D (10 μg/ml)	5.31	8.5	
Actinomycin D (20 μg/ml)	6.42	9.3	3.65
Cyclic AMP (17.6 μM)	5.60	7.2	3.58
Cycloheximide (35.5 μM)	0.85	0.2	0.47
Ungerminated grain	0.90	0.4	0.47

than that of the embryoless seed halves incubated in 0.12 μM GA_3 but higher than that of embryoless halves incubated in 60 nM GA_3. However, in the seed halves containing embryos, peak α amylase activity was reached after 6 days of germination. The protease activity of the embryo-containing seed halves was similar to that of embryoless seed halves incubated in 60 nM GA_3, and it decreased after 3 days of germination.

Effect of Inhibitors on Production of α Amylase, Protease, and R Enzyme. The effect of antibiotics, cycloheximide and actinomycin D, and of cyclic AMP on the production of α amylase and protease was studied on the embryoless seed halves incubated in 0.12 μM GA_3 for 3 days. Preliminary experiments on intact, dehulled grain showed that only cycloheximide inhibited germination. Also, the levels of α amylase and protease after 4 days in the presence of 36 μM cycloheximide were similar to those of the ungerminated grain, although the level of soluble protein in the grains that were treated with cycloheximide was higher. The presence of 10 or 20 μg/ml actinomycin D or 20 μM cyclic AMP reduced the α amylase activity after 3 days of incubation relative to the GA_3-treated control with little effect on protease activity, which was at a maximum at this time (Table III). Cycloheximide at the 36 μM level completely inhibited α amylase and protease production in the embryoless seed halves, and the enzyme levels were similar to those of the ungerminated grain. Production of protease and amylase by isolated barley aleurone layers was also reported to be less inhibited by actinomycin D than by cycloheximide at concentrations similar to those we used (16). Galsky and Lippincott (8) also reported that cyclic AMP inhibited production of α amylase by barley seed halves in GA_3 solution. Duffus and Duffus (6), however, reported that 10 μM cyclic AMP stimulated in 18 hr the α amylase synthesis triggered by GA_3 in slices of barley endosperm.

The production of R enzyme was also induced by incubation in GA_3 (Table III). The R enzyme activity in seed halves was lowered by the presence of actinomycin D or cyclic AMP, while cycloheximide completely inhibited R enzyme production. Our finding of the similar effect of these inhibitors on R enzyme and α amylase production contradicts the reported production of R enzyme in germinating peas under conditions inhibitory to α amylase production (30).

DISCUSSION

Changes in the Rice Grain during Germination. In germinating rice grain, an increase in oxygen uptake preceded the increase in ATP level, which coincided with the period of rapid increase in the activities of hydrolases and degradation of endosperm reserves. Endosperm starch and protein were degraded faster in the dark, reflecting the importance of light or photosynthesis to plant growth even at the seedling stage.

Starch degradation presumably involves the initial action of α amylase on starch granules, since α amylase had a greater increase in activity than β amylase, phosphorylase, or R enzyme, and since a new α amylase isozyme band appeared during germination. The other hydrolases probably help in the complete hydrolysis to glucose of the more soluble products of the initial action of α amylase on the starch granule. Tanaka *et al.* (33) also found new α amylase isozymes during rice

germination by isoelectric-focusing disc electrophoresis in polyacrylamide gel. The major enzyme for starch degradation is considered to be α amylase in germinating rice (25), oats (21), and peas (17). The glucose and other free sugars formed are presumably translocated to the growing plant through the scutellum, where the sugars are converted to sucrose (26).

The increase in β amylase activity during germination with no increase in the number of isozyme bands must be principally the result of a release of latent forms bound to storage proteins in the endosperm protein bodies, as shown in barley (31, 35) and wheat (29). No new isozyme band accompanied the increase in R enzyme activity in germinating rice.

The slight increase in the blue value of starch during germination shows that α amylase does not preferentially attack amylose rather than amylopectin. In contrast, Fukui and Nikuni (7) found that residual starch increased in amylose content in germinating rice grain.

Protein and RNA were hydrolyzed and the activities of protease and RNase increased in the germinating rice grain before the breakdown of starch and the increase in activity of α amylase. A zymogram study of the proteases of the rice grain (18) showed only one protease band in mature grain, in contrast to three in ripening grain (14). The electrophoretic mobility of the single protease band in the mature grain was slightly slower than that of the slowest protease band of ripening grain. Germination results in a broadening of the single protease band without the appearance of new isozyme bands, even at neutral and alkaline pH, in contrast to α amylase. The protease synthesized or liberated during germination probably has properties similar to those of the protease of mature grain.

Ozaki and Horiguchi (28) found that the properties of protease in mature and germinated rice grains were similar. Although our protease assays were done at pH 3.5, Horiguchi and Kitagishi (10) showed that rice protease had two pH optimums, pH 3 and 8, with rice glutelin as substrate. Although protease in the developing grain requires cysteine activation (5), cysteine did not activate protease of germinating grain, as also reported by Ozaki and Horiguchi (28). We found, however, that the addition of cysteine improved the extraction of protease from the grain without affecting its specific activity.

The increase in soluble protein during germination must come from the degradation of glutelin by protease and the synthesis of new soluble proteins (enzymes) from the liberated free amino acids. Our previous study showed that glutelin and globulin of IR8 grain decreased during the first week of germination in the dark as nonprotein N increased, but the levels of prolamin and albumin remained constant (13). Horiguchi and Kitagishi (9) also found a decrease in glutelin and an increase in nonprotein N of the rice grain during germination, but also reported an increase in the albumin level.

GA_3 and Production of α Amylase, Protease, and R enzyme. Hydrolases are presumably synthesized in the aleurone layers of cereal grains in the presence of GA_3. These include α amylase of barley (15) and rice (25, 33), barley protease (16), and barley RNase (4). Although GA_3 addition to germinating rice grain did not accelerate starch degradation and α amylase production (25), certain dwarf rice lines showed a response indicating a low indigenous GA_3 level in these lines (14). The lower α amylase and protease levels in such dwarf line than in its tall counterpart support the reported difference in the contents of GA-like substance in plants of these two lines (15). The short line has almost no GA-like substance.

Embryoless rice seed halves incubated in 0.12 μM GA_3 also showed the faster production of protease than α amylase. The addition of cycloheximide completely inhibited the production of both α amylase and protease, both in intact grain and in seed halves incubated in GA_3. Protease production, however, was not affected by actinomycin D and cyclic AMP addition, in contrast to the reduction in α amylase production in the presence of these inhibitors (Table III). The action of GA_3 on these two enzymes may not be identical. The mature grain has little α amylase activity but much protease activity. In fact, protease is usually present in endosperm and aleurone protein bodies (24, 27). Since *de novo* synthesis of both α amylase and protease has been demonstrated in isolated aleurone layers of barley incubated in GA_3 (4, 16), α amylase must be derived exclusively from this source, while protease is derived both from the aleurone layers and the protein bodies of the endosperm. In addition, mRNA for protease has been reported to be synthesized in developing cottonseed (11), and that may explain why inhibiting effect of actinomycin D, an inhibitor of RNA synthesis, is lower on protease than α amylase. The differences in the period required for the synthesis of these two enzymes in rice may be due to the synthesis of the mRNA for protease during grain development, while the mRNA of α amylase is synthesized during germination. Ihle and Dure (11) similarly found that cycloheximide, but not actinomycin D, inhibited synthesis of protease in germinating cottonseed.

R enzyme production presumably is under the same GA_3 induction as α amylase, as shown by the same action of inhibitors of protein and RNA synthesis on its production in embryoless seed halves in the presence of GA_3 (Table III). Since protease activity was not affected by actinomycin D, it is improbable that R enzyme in rice is produced by the action of protease on preexisting inactive forms of R enzyme, as was reported in germinating peas (30).

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