Enzyme Activities in Preharvest Rice Grains

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The enzyme kinetic parameters (equilibria, specific rate constants, initial velocities, and diffusion resistance constants) of several different enzymes (α-amylase, phospholipase c, acid phosphatase, alkaline phosphatase, peroxidase, and cytochrome c reductase) have been studied in the long-grain rice variety (Lemont) during the preharvest period (14, 30, and 50 days after flowering). During this period before harvest, the enzymic substrate inhibitors increased in all studied enzyme systems. The specific rate constants (activities) of α-amylase, phospholipase c, alkaline phosphatase, and peroxidase increased. On the other hand, the specific rate constant of acid phosphatase decreased and that of cytochrome c reductase remained unchanged during this ripening period. The changes were faster than, but qualitatively similar to, the changes in stored rice grains after harvest. Thus, there was no qualitative discontinuity in these parameters at and after harvest.

The studies of enzymes in developing rice grains are fragmentary. Most of the protein and enzymic rice studies in ripening rice grains concentrated their efforts on isolation and purification. The biological materials usually used for these studies were the embryos during germinations or the rice leaves and, to a lesser extent, the rice grains (Juliano, 1972, 1985).

For example, in the early stages after flowering some work has been done on the isolation and purification of some enzymes, for example, phytin and phytin synthesis (Saio, 1964; Asada et al., 1969; Villareal and Juliano, 1978; Mandac and Juliano, 1978), enzymes related to sucrose and starch metabolism (Baun et al., 1970; Paul et al., 1971; Villareal and Juliano, 1977), lipase and lipoxygenase (Choudhury and Juliano, 1980), cytochrome c oxidase (Tang et al., 1983), and peroxidase (Tang and Pang, 1983). These studies do not describe the enzyme kinetic details and usually compare only the initial velocities of these enzymes mostly in early stages after flowering.

The main objective of this study is to compare the results from the ripening rice to the results from postharvest stored rice (Chrastil, 1990) to see if the preharvest and postharvest changes are continuous or discontinuous processes. The following paragraphs describe in more detail some important rice grain enzymes during the ripening period.

EXPERIMENTAL PROCEDURES

Materials. All chemicals and substrates were analytical reagents of the highest obtainable purity from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI).

Source of Enzymic Activities. A typical U.S. long-grain rice variety (Lemont) was obtained from the Crowley Research Station, Crowley, LA. Rice was planted in the greenhouse in Hoagland–Snyder solution as a nutrient (Yoshida et al., 1976). At selected time intervals after flowering, samples were taken in random order. Grains that differed very much from the average grain size, color, or shape were excluded. Rice grains were cleared of small debris and broken pieces and ground to a flour in a water-cooled micromill (Technilab Instruments, Pequannock, NJ). All samples were ground in the same manner (10 g of grains, 3-min grinding).

The flour was extracted twice by 2 min of sonication and 30 min of shaking with 2 volumes of 0.01 M NaOH (1:2 w/v) at 25 °C. Each suspension was immediately centrifuged at 40000g in a cold (4 °C) rotor for 15 min. Combined cold supernatants were filtered (Whatman No. 2) and used immediately as a source of enzymic activities. The pH of the pooled extracts was almost

neutral, 7.1–7.3. The remaining total enzymic activity in the pellets after extractions was less than 5% (approximated by comparison with the homogenates).

This method extracted over 95% of the total rice grain protein, including albumins, globulins, prolamines, and oryzenins (rice glutelins). The globulin fractions contained over 97% of all enzymic activities, albumin fractions contained the rest, and prolamin and oryzenin fractions were inactive (not shown here).

Protein. For the colorimetric protein determination the rice extract was diluted (1:50 v/v) and the protein determined by the method of Lowry et al. (1951). For the enzyme activity determination the protein in rice extract (E) was added directly to the enzymic reaction mixtures (0.5 mL in 3 mL of reaction mixture) (Table I).

Optimum pH. Optimum pH curves were determined from initial velocities of enzymic reactions at different pH, in 0.2 M buffers (citrate, Tris, or glycine buffers) at 25 °C. These experiments were done prior to kinetic studies. The optimum pH for α-amylase was 7.3; phospholipase c 5.5; acid phosphatase, 5.5; alkaline phosphatase, 8.0; peroxidase, 7.5; and cytochrome c reductase 7.5. Substrates used were amylopectin blue, p-nitrophenyl phosphorylcholine, p-nitrophenyl phosphate, phenylenediamine, and cytochrome c, respectively. Most of these optima were only slightly different from those of pure enzymes (Barman, 1974). Because of the nonspecific substrate (p-nitrophenyl phosphate), the term phosphatases is used in this work for the general wide category of enzymes (which includes not only different phosphatases but also, for example, some esterases).

Stability of Enzymes in Reaction Mixtures. The reaction mixtures were incubated without substrates for different time intervals. After the addition of the substrates, the losses of activity were expressed relative to the initial enzymic activities (at 0 time). The loss of activity in the time range used for the determination of enzymic time curves was less than ±5% and was neglected.

Albumin Extract. Rice flour was extracted twice by 2 min of sonication and 30 min of shaking with 2 volumes of distilled water (1:2 w/v) at 25 °C. Each suspension was immediately centrifuged at 40000g in a cold (4 °C) rotor for 15 min. Combined cold supernatants were filtered (Whatman No. 2) and used immediately as a source of enzymic activities.

Globulin Extract. After albumin extraction, rice flour was extracted twice by 2 min of sonication and 30 min of shaking with 2 volumes of 5% NaCl (1:2 w/v) at 25 °C. Each suspension was immediately centrifuged at 40000g in a cold (4 °C) rotor for 15 min. Combined cold supernatants were filtered (Whatman No. 2) and used immediately as a source of enzymic activities.

Determination of Kinetic Constants. The enzyme kinetic constants were determined by regression analysis of the enzymic

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time curves by means of the time equation (Chrastil, 1988, 1990)

$$P = P_{eq}(1 - \exp(-kEt))^n \tag{1}$$

where P is the product of the enzymic reaction at time t, P_{eq} is the apparent equilibrium (P at $t \rightarrow \infty$), k is the specific rate constant, E is the protein concentration, and n is the diffusion resistance constant. The initial velocities, V_0, were determined by common methods from the experimental values at short reaction times by assuming linearity near the start of the reaction. This assumption is not quite correct, and, especially when $n \ll 1$, the results could be far from reality (Chrastil, 1988). However, when all samples are determined in the same manner, semi-quantitative relative comparisons are still possible.

Determination of α-Amylase. Amylase activity was measured by the modified spectrophotometric method of Rinderknecht et al. (1967) as follows: 10 mg of amylopectin blue, 8 mL 0.2 M Tris buffer (pH 7.5), and 2 mL of enzymic rice flour extract were incubated at 30 °C. After selected time intervals (30, 60, 90, 120, 180, 240, 300, and 480 min), 1 mL of the incubation mixture was pipetted into the small test tube with 3 mL of isopropyl alcohol. The test tube was centrifuged at 5000g for 10 min, and the supernatant was transferred into the spectrophotometric cuvette and read at 595 nm vs H_2O in a reference cuvette.

Determination of Acid Phosphatase. Acid phosphatase was determined according to the method of Bessey et al. (1946) and Torriani (1960), with p-nitrophenyl phosphate as a substrate. The incubation mixture contained 0.1 mL of 3×10^{-2} M p-nitrophenyl phosphate, 2.4 mL of 0.2 M citrate buffer (pH 5.5), and 0.5 mL of the enzymic rice flour extract. After selected time intervals, 0.1 mL of the reaction mixture was pipetted into another small test tube containing 3 mL of 0.5 M NaOH, mixed, and transferred into a cuvette, where it was read at 400 nm vs H_2O.

Determination of Alkaline Phosphatase. Alkaline phosphatase was determined in the same manner as described for acid phosphatase except in Tris buffer (pH 8.0).

Determination of Phospholipase c. Phospholipase c was determined according to the method of Kurioka and Matsuda (1976), using p-nitrophenyl phosphorylcholine as a substrate. The incubation mixture contained 0.1 mL of 6×10^{-2} M p-nitrophenyl phosphorylcholine, 2.4 mL of 0.2 M citrate buffer (pH 5.5), and 0.5 mL of the enzymic rice flour extract. After selected time intervals, the reaction mixture was treated in the same manner as phosphatase.

Determination of Peroxidase. Peroxidase was determined by measuring the oxidation of p-phenylenediamine as a substrate at 452 nm (Kiermeier and Kayser, 1960; Kiermeier and Meinl, 1961). The incubation mixture contained 0.1 mL of 3×10^{-3} M p-phenylenediamine, 0.1 mL of 6×10^{-2} M H_2O_2, 2.3 mL of 0.2 M Tris buffer (pH 7.5), and 0.5 mL of enzymic rice flour extract. The time curve was recorded directly on the spectrophotometer at 452 nm against the reference cuvette without enzymic extract.

Determination of Cytochrome c Reductase. Cytochrome c reductase was determined according to the method of Williams and Kamin (1962). The incubation mixture contained 0.05 mL of 1.5×10^{-2} M NaCN, 0.05 mL of 1.5×10^{-3} M cytochrome c, and 2.3 mL of 0.2 M Tris buffer (pH 7.5). Before the start of the reaction, 0.1 mL of 3×10^{-3} M NADPH$_2$ and 0.5 mL of the enzymic rice flour extract were added. After a stabilization period (15–45 s), which was easily observed on the screen of the spectrophotometer, the enzymic time curve was recorded at 550 nm against the reference cuvette without enzymic extract.

All reaction mixtures were incubated at 30 ± 1 °C. The color intensities were read in the cuvettes on the Shimadzu 260 double-beam spectrophotometer. The blanks were readings made at 0 times.

RESULTS AND DISCUSSION

α-Amylase. From the exponent, n, in eq 1, which was less than 1, it was apparent that the hydrolysis of starch by α-amylase was a diffusion-limited process (Figure 1; Table I). In all cases, the heterogeneous diffusion resistance constants (n) in the time equation (eq 1) were less than 1 (approximately 0.850). Rice development after flowering had little influence on constants (n) and on
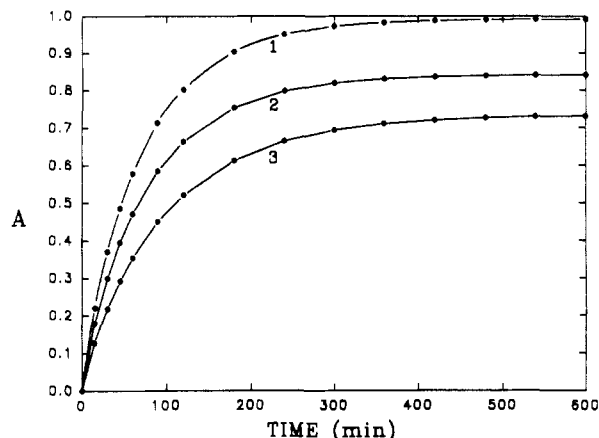

Figure 1. Enzymic time curves of α-amylase: 1, 14 days after flowering; 2, 30 days after flowering; 3, 50 days after flowering.

Table I. Kinetic Constants of α-Amylase[a]

	days after flowering		
	14	30	50
E (g L^{-1})	0.58	0.50	0.39
k (g^{-1} L min^{-1})	0.0217	0.0234	0.0237
P_{eq} (A)	0.992	0.841	0.733
n	0.853	0.848	0.854
V_0 (L g^{-1} min^{-1})	0.021	0.020	0.019

[a] All values are related to absorbance (optical density). The constants k, P_{eq}, and n are averages from triplicates, calculated by regression analysis of time curves. In all cases deviations between triplicates were smaller than ±5% of the mean. All regression coefficients were larger than 0.99.
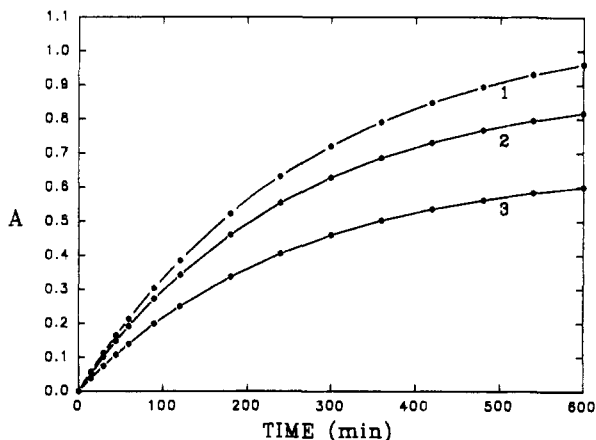

Figure 2. Enzymic time curves of phospholipase c: 1, 14 days after flowering; 2, 30 days after flowering; 3, 50 days after flowering.

apparent initial velocities (V_0), but the specific rate constants (k) slightly increased and the apparent equilibria (P_{eq}) greatly decreased. This means that, during the studied rice grain ripening interval (14–50 days after flowering), the synthesis of this enzyme was not much faster than the general protein synthesis (k increased only slightly). However, from the significant decrease of P_{eq}, we can assume that the relative accumulation of the starch substrate binding proteins increased during this period.

Phospholipase c. This enzymic reaction (Figure 2; Table II) was of the first order ($n = 1$). The initial velocities (V_0) did not change but the specific rate constant (k) increased greatly during 14–50 days after flowering. This means that the synthesis of this enzyme was relatively much faster than the synthesis of other protein. P_{eq} greatly decreased, and thus, as in the case of amylase, we may assume the accumulation of the phospholipid substrate binding proteins increased during this developing period.

Table II. Kinetic Constants of Phospholipase c^a

	days after flowering		
	14	30	50
E (g L^{-1})	0.58	0.50	0.39
k (g^{-1} L min^{-1})	0.0063	0.0080	0.0101
P_{eq} (A)	1.080	0.900	0.661
n	1	1	1
V_0 (L g^{-1} min^{-1})	0.0064	0.0064	0.0063

^{a} All values are related to absorbance (optical density). The constants k, P_{eq}, and n are averages from triplicates, calculated by regression analysis of time curves. In all cases deviations between triplicates were smaller than ±5% of the mean. All regression coefficients were larger than 0.99.
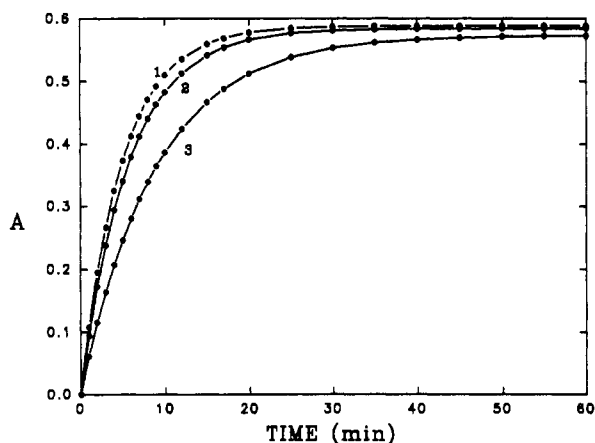
Figure 3. Enzymic time curves of acid phosphatase: 1, 14 days after flowering; 2, 30 days after flowering; 3, 50 days after flowering.

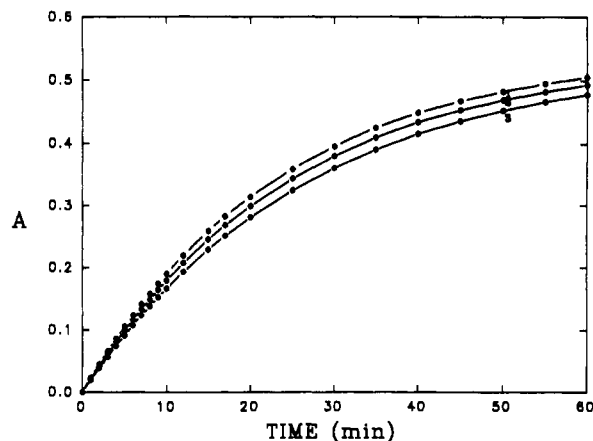**Table III. Kinetic Constants of Acid Phosphatase^a**

	days after flowering		
	14	30	50
E (g L^{-1})	0.58	0.50	0.39
k (g^{-1} L min^{-1})	0.403	0.350	0.288
P_{eq} (A)	0.588	0.584	0.573
n	1	1	1
V_0 (L g^{-1} min^{-1})	0.129	0.128	0.126

^{a} All values are related to absorbance (optical density). The constants k, P_{eq}, and n are averages from triplicates, calculated by regression analysis of time curves. In all cases deviations between triplicates were smaller than ±5% of the mean. All regression coefficients were larger than 0.99.

Acid Phosphatase. This enzymic reaction (Figure 3; Table III) was of the first order ($n = 1$). The initial velocities (V_0) and the equilibrium (P_{eq}) did not change significantly, which meant that there was no significant relative accumulation of the substrate binding inhibitors of this enzyme during 14–50 days after flowering. On the other hand, the specific rate constant (k) decreased greatly, which simply means that the synthesis of this enzyme at this developing stage was relatively slower than that of the other proteins.

Alkaline Phosphatase. This enzymic reaction was of the first order ($n = 1$). The enzyme kinetic characteristics of this enzyme (Figure 4; Table IV) were quite different from those of the acid phosphatase. Although the equilibrium (P_{eq}) did not change (no accumulation of the enzymic substrate inhibitors), the initial velocities (V_0) and the specific rate constants (k) significantly increased. This means that the relative synthesis (to other protein) increased during this period (14–50 days after flowering).

Peroxidase. This enzymic reaction was of the first order ($n = 1$). The initial velocities (V_0) and the specific rate constant (k) increased (Figure 5; Table V). This means that the synthesis of this enzyme in this developing stage

Figure 4. Enzymic time curves of alkaline phosphatase: 1, 14 days after flowering; 2, 30 days after flowering; 3, 50 days after flowering.

Table IV. Kinetic Constants of Alkaline Phosphatase^a

	days after flowering		
	14	30	50
E (g L^{-1})	0.58	0.50	0.39
k (g^{-1} L min^{-1})	0.0734	0.0804	0.0953
P_{eq} (A)	0.548	0.542	0.536
n	1	1	1
V_0 (L g^{-1} min^{-1})	0.039	0.042	0.049

^{a} All values are related to absorbance (optical density). The constants k, P_{eq}, and n are averages from triplicates, calculated by regression analysis of time curves. In all cases deviations between triplicates were smaller than ±5% of the mean. All regression coefficients were larger than 0.99.
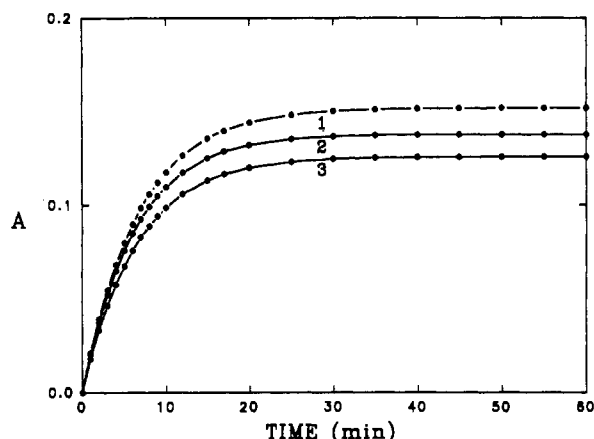
Figure 5. Enzymic time curves of peroxidase: 1, 14 days after flowering; 2, 30 days after flowering; 3, 50 days after flowering.

Table V. Kinetic Constants of Peroxidase^a

	days after flowering		
	14	30	50
E (g L^{-1})	0.58	0.50	0.39
k (g^{-1} L min^{-1})	0.257	0.318	0.392
P_{eq} (A)	0.152	0.138	0.126
n	1	1	1
V_0 (L g^{-1} min^{-1})	0.036	0.040	0.046

^{a} All values are related to absorbance (optical density). The constants k, P_{eq}, and n are averages from triplicates, calculated by regression analysis of time curves. In all cases deviations between triplicates were smaller than ±5% of the mean. All regression coefficients were larger than 0.99.

(14–50 days after flowering) was faster than the synthesis of other protein. The decrease of the equilibrium (P_{eq}) indicated the accumulation of the substrate binding inhibitors of this enzyme during this period.
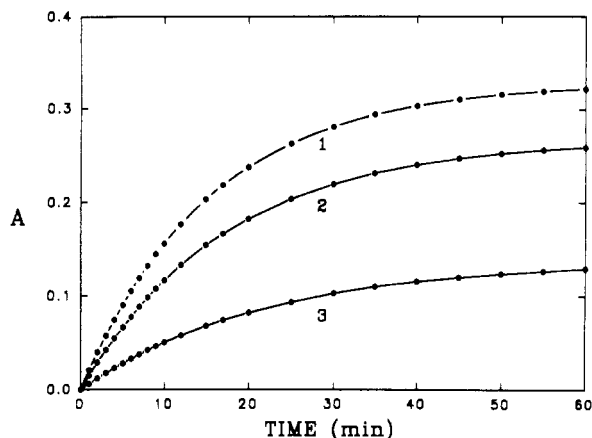
Figure 6. Enzymic time curves of cytochrome *c* reductase: 1, 14 days after flowering; 2, 30 days after flowering; 3, 50 days after flowering.

Table VI. Kinetic Constants of Cytochrome *c* Reductase[a]

	days after flowering		
	14	30	50
E (g L^{-1})	0.58	0.50	0.39
k (g^{-1} L min^{-1})	0.111	0.114	0.116
P_{eq} (A)	0.329	0.268	0.138
n	1	1	1
V_0 (L g^{-1} min^{-1})	0.034	0.030	0.015

[a] All values are related to absorbance (optical density). The constants k, P_{eq}, and n are averages from triplicates, calculated by regression analysis of time curves. In all cases deviations between triplicates were smaller than ±5% of the mean. All regression coefficients were larger than 0.99.

Cytochrome *c* Reductase. This enzymic reaction was of the first order ($n = 1$). The specific rate constants (k) did not change much, which means that the relative synthesis of this enzyme continued hand-in-hand with the synthesis of other protein (Figure 6; Table VI).

Additionally, the great decrease of the equilibrium (P_{eq}) indicated a strong increase of the cytochrome *c* substrate binding inhibitors during the period 14–50 days after flowering. This case is also a typical example of the possible disagreement between the initial velocities (V_0) and the specific rate constants (k) [one of the main reasons for that is the strong dependency of (V_0) on (P_{eq})] [see Chrastil (1988)].

Conclusion. All enzymes studied in this work were present mostly in globulin fractions of rice grains. During the ripening period before harvest (14–50 days after flowering, where the last interval was the harvest time), the enzymic substrate inhibitors increased (decrease of P_{eq}) in all enzymes studied: α-amylase, phospholipase c, alkaline phosphatase, acid phosphatase, peroxidase, and cytochrome *c* reductase. The specific rate constants (activity) of α-amylase, phospholipase c, alkaline phosphatase, and peroxidase increased.

On the other hand, the specific rate constants of acid phosphatase decreased and that of cytochrome *c* reductase remained unchanged. The acid and alkaline phosphatases behaved differently. The specific rate constant (activity) of the acid phosphatase was much higher than that of alkaline phosphatase, but the balance between these two phosphatases changed in favor of the alkaline phosphatase during the ripening period.

Regardless of the rice variety, similar behavior (increase or decrease of P_{eq}, k, or V_0) was found in stored rice grains (Chrastil, 1990). Thus, after harvest during storage, the changes of enzymic reactions are slower but continue in the same trend as before harvest.

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