Influence of Storage on Enzymes in Rice Grains

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Kinetic analysis of protease, amylase, peroxidase, cytochrome *c* reductase, phosphatase, and phospholipase *c* in three typical U.S. rice varieties stored at different temperatures revealed several characteristics of their degradation during storage. Protease and amylase were heterogeneous diffusion-limited systems. On the other hand, peroxidase, cytochrome *c* reductase, phosphatase, and phospholipase *c* were first-order reactions. Cytochrome *c* reductase was the only enzyme that was quickly deactivated by several simultaneous destructive mechanisms (all kinetic constants decreased to 0 after 1 $^1/_2$ years of storage). Specific rate constants and diffusion resistance constants of all other studied enzymes did not change during storage up to 18 months, even at higher storage temperatures (up to 37 °C). On the other hand, the apparent equilibria and the initial velocities decreased. Kinetic analysis has shown that most studied enzymes were not destroyed during storage but were protected by the accumulation of the reversible substrate receptors.

Enzymes are important constitutents of rice and often play roles in the physicochemical and functional properties of rice grains. In rice, they are present not only in embryo or aleurone but also in endosperm, and they retain significant activities after harvest. Some enzymic activities in stored rice have been studied (Kondo and Okamura, 1937; Screenivasan, 1938; Desikachar and Subrahmanyan, 1960; Tani et al., 1964; Matsuda and Hirayama, 1973; Shibuya et al., 1974; Ghosh et al., 1978; Pushpamma and Reddy, 1979; Fox and Mulvihill, 1982; Shewry and Miflin, 1985; Juliano, 1985), but the information is fragmentary and the only outcome we have from these studies is that the initial velocities of the enzymes generally decrease during storage. However, simple initial velocities of enzymic reactions cannot explain all possible chemical and physicochemical mechanisms of the enzymic systems.

More information about the properties of enzymic systems can be obtained from the time curves (Chrastil, 1988a). The product formation by an enzyme can be expressed by the time equation $P = P_{max}[1 - \exp(-kEt)]^n$, where P is the reaction product after time t, E is the enzyme (protein) concentration, k is the specific rate constant, n is a constant characterizing the diffusion and sterical properties of the enzymic system, and P_{max} is the product after $t \rightarrow \infty$.

This time curve method, when compared to the initial velocity kinetics, reveals several additional properties of an enzymic system. First, the Henry–Michaelis–Menten (Segel, 1975) constants V_{max} and K_m can be determined more accurately than just from initial velocities. Second, from the constant n, the diffusion resistance or the consecutive reaction scheme can be determined (Chrastil, 1988a,b). Third, from the apparent equilibrium constant P_{max} the substrate–receptor inhibition (reversible substrate binding by inactive protein) can be determined. This cannot be done from initial velocities. When P_{max} changes, then the apparent initial velocities do not express specific activities because they do not reveal the possible substrate–receptor inhbition. On the other hand, the specific rate constants of the time equation (k) are independent of P_{max}.

EXPERIMENTAL PROCEDURES

Materials. All chemicals and substrates were analytical reagents of the highest obtainable purity from Sigma Chemical Co., St. Louis, MO, or J. T. Baker Chemical Co., Phillipsburg, NJ.

Source of Enzymic Activities. Highly polished (30% removed) rice grains (not older than 1 month after harvest) of three typical U.S. varieties (Lemont, long; Mars, medium; S-201, short) were stored in closed jars at 4, 25, and 37 °C. The relatively large polish was removed to be sure that the polished rice is only endosperm, as machine polishing is not perfect and often irregular. Low (commercial) polish (10% or less) does not always remove all germs and all outer layers. At the beginning and after 5, 10, and 18 months of storage, the samples used for analysis 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 shaking with 2 volumes of 0.01 M NaOH (1:2 w/v) for 30 min at 25 °C. Each suspension was immediately centrifuged at 40000*g* in a cold (4 °C) rotor for 10 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).

Protein. Rice extract was diluted (1:50 v/v) and the protein determined by the method of Lowry et al. (1951). The protein concentration in the enzymic reaction mixture was 6× smaller than in the enzymic extract and was denoted E (Table I).

Optimum pH. Optimum pH curves were determined from 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 protease was 3.5, amylase 4.5, peroxidase and cytochrome *c* reductase 7.5, and phosphatase and phospholipase *c* 5.5 with hemoglobin, amylopectin-blue, *p*-phenylenediamine, cytochrome *c*, *p*-nitrophenyl phosphate, and (*p*-nitrophenyl)phosphorylcholine as substrates, respectively. Most of the optima thus obtained were only slightly different from those of pure enzymes [except amylopectin-blue substrate which had lower optimum pH (4.5) than amylopectin or starch (in the literature it is 5.5)] (Barman, 1974).

Stability of Enzymic Extracts. Enzymic extracts were incu-

Table I. Protein Content in the Enzymic Incubation Mixtures[a]

rice	storage temp, °C	storage time, months 0	5	10
long	4	0.75	0.71	0.63
	25		0.66	0.62
	37		0.57	0.54
medium	4	0.86	0.83	0.75
	25		0.78	0.62
	37		0.71	0.60
short	4	1.01	1.00	0.95
	25		0.99	0.75
	37		0.94	0.66

[a] Protein content E in the incubation mixtures is in grams per liter. Values are averages from triplicates. The variation from the mean was less than ±5%.

bated in 0.2 M buffers at different temperatures for different time intervals. After incubation, the extracts were used immediately for the estimation of reaction velocities. Activity losses were expressed relative to the initial enzymic activities (at 0 time), and the second-order statistical regression stability–time curves were calculated for every pH to fit the experimental results. The stability regression equations were integrated, and the integral middle values were calculated on the time limits, 0–t. All rice enzymic extract stability correction factors obtained in this manner for the maximum reaction times used in the time curve experiments were >0.95 (not shown here) and thus had very little influence on the experimental results.

Albumin Extract. Rice flour was extracted twice by 10 volumes of distilled water. The combined extracts were evaporated under vacuum at room temperature to $^1/_{20}$ of the original volume and deactivated by TCA (final concentration 10% TCA). Precipitated albumins (starch remained in the supernatant) were washed twice with methyl alcohol and acetone and dried under vacuum.

Globulin Extract. Rice flour was extracted three times with 20 volumes of distilled water and then twice with 10 volumes of 5% NaCl. The combined NaCl extracts were dialyzed and evaporated under vacuum at room temperature to $^1/_{20}$ of the original volume, and the globulins were precipitated as described under Albumin Extract.

Determination of Kinetic Constants. The apparent equilibrium constants (P_{max}), specific rate constants (k), and diffusion resistance constants (n) were determined from the regression analysis of the time curves by means of the time equation (Chrastil, 1988a,b). 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 approximation is not quite correct, and when the reaction is diffusion-limited and $n \ll 1$, the results could be far from reality (Chrastil, 1988a). However, when the determination is effected with all samples in the same manner, the relative comparisons are still possible with reasonable accuracy.

Determination of Acid Protease. Proteolytic activity was measured with hemoglobin as a substrate. Commercially pure hemoglobin, which is commonly used as a substrate for proteases, has an advantage of well-known and constant composition and high solubility in buffers. On the other hand, for example, the albumins, globulins, or oryzenins from rice would cause solubility and constant composition problems, to say nothing of the difficulties of preparing them in pure state. The incubation mixture contained 2.5 mL of 30 mg/mL hemoglobin stock solution, 10 mL of 0.2 M citrate buffer (pH 3.5), and 2.5 mL of the enzymic rice flour extract. After selected time intervals, 1 mL of the reaction mixture was transferred into a test tube containing 2 mL of 10% TCA. The mixture was centrifuged, and 2 mL of the supernatant was made alkaline with 2 mL of 1 M NaOH. After 5-min standing, 0.5 mL of 1 M Folin reagent was added and the color read after 30 min at 740 nm vs H_2O.

Determination of α-Amylase. Amylase activity was measured by the modified spectrophotometric method of Rinderknecht et al. (1967). The incubation mixture contained 42 mg of amylopectin-blue in 16.5 mL of 0.2 M citrate buffer (pH 4.5) and 3.5 mL of enzymic rice flour extract. After selected time intervals, 2-mL aliquots were mixed with 3 mL of acetone, centrifuged, and read at 595 nm vs H_2O.

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 at 452 nm against the reference cuvette without enzymic extract.

Determination of Cytochrome c Reductase. Cytochrome c reductase was determined by 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 time curve was recorded at 550 nm against the reference cuvette without enzymic extract.

Determination of Phosphatase. Phosphatase was determined by the methods 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 2 mL of H_2O and 1 mL of 1 M NaOH, mixed, and trasferred into a cuvette where it was read at 400 nm vs H_2O.

Determination of Phospholipase c. Phospholipase c was determined by 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.

All reaction mixtures were incubated at 25 ± 1 °C by shaking on a Nutator mixer. The colors were read in the cuvettes on the Shimadzu double-beam 260 spectrophotometer. The blanks were 0 times.

RESULTS AND DISCUSSION

Protease and α-Amylase. The crude rice extract could be a complicated mixture of proteases, peptidases, and carboxypeptidases, and the kinetic data represent only a net sum of these reactions. On the other hand, the amylase reaction at pH 4.5 is mostly α-amylase reaction.

Kinetic analysis of the time curves of protease and/or amylase has shown that the proteolytic degradation of hemoglobin by proteases and the hydrolysis of amylopectin by α-amylase from rice grains were heterogeneous, diffusion-limited processes. In all cases, the heterogeneous diffusion resistance constants (n) in the time equation were less than 1 (0.71–0.86 for protease and 0.58–0.74 for amylase) (Table II). This resulted in only apparent initial velocities of these reactions (the real initial velocities were close to infinity).

Storage time and/or storage temperature had little influence on constants (n), but the apparent equilibria (P_{max}) and the apparent initial velocities (V_0) decreased in all cases, especially at higher storage temperatures. On the other hand, the specific rate constants (k) did not change significantly during storage even at higher storage temperatures (up to 37 °C) or longer storage times (up to 18 months, not shown here). This indicated that these enzymes (protease and/or amylase) were not deactivated or destroyed. The specific activity [represented by the specific rate constant (k)] of protease was similar in all studied rice varieties, but that of amylase was much lower in short-grain rice (Table II).

Peroxidase. In contrast to protease and amylase, per-

Table II. Kinetic Constants of Protease and Amylase from Stored Rice[a]

	protease				amylase			
	0 month	10 months			0 month	10 months		
	4 °C	4 °C	25 °C	37 °C	4 °C	4 °C	25 °C	37 °C
				Long Grain				
P_{max}, A	0.247	0.217	0.192	0.170	0.240	0.230	0.220	0.185
k, min^{-1} g^{-1} mL	1.3	1.0	1.0	1.1	2.7	2.7	2.4	2.2
V_0, min^{-1} g^{-1} mL	0.78	0.65	0.60	0.50	6.7	3.2	3.2	2.0
n	0.86	0.83	0.81	0.83	0.61	0.71	0.70	0.74
r	0.999	0.999	0.999	0.997	0.998	1.000	0.999	1.000
				Medium Grain				
P_{max}, A	0.150	0.130	0.125	0.115	0.230	0.175	0.160	0.120
k, min^{-1} g^{-1} mL	1.5	1.7	2.0	1.3	3.0	3.2	3.2	3.3
V_0, min^{-1} g^{-1} mL	1.0	0.85	0.80	0.45	7.0	5.3	5.0	3.1
n	0.75	0.78	0.81	0.81	0.59	0.61	0.62	0.63
r	0.999	0.999	0.999	0.999	0.997	0.999	0.999	0.998
				Short Grain				
P_{max}, A	0.346	0.309	0.255	0.245	0.240	0.199	0.165	0.140
k, min^{-1} g^{-1} mL	1.0	1.1	1.4	1.5	1.5	1.2	1.6	1.8
V_0, min^{-1} g^{-1} mL	1.8	1.1	1.0	1.2	3.5	2.2	2.3	2.2
n	0.71	0.78	0.80	0.79	0.58	0.60	0.60	0.61
r	1.000	0.998	0.997	1.000	0.999	1.000	0.999	1.000

[a] V_0 is the apparent experimental initial velocity obtained from short reaction times (0.5–2 h) by assuming the linearity at the start of the reaction (because $n < 1$ these experimental initial velocities were lower than the real initial velocities which were close to infinity); A is the experimental maximum absorbance (see Experimental Procedures). Values are averages from duplicates. The variation from the mean was less than ±6%.

Table III. Kinetic Constants of Peroxidase and Cytochrome *c* Reductase from Stored Rice[a]

	peroxidase				cyt *c* reductase			
	0 month	10 months			0 month	10 months		
	4 °C	4 °C	25 °C	37 °C	4 °C	4 °C	25 °C	37 °C
				Long Grain				
P_{max}, A	0.481	0.210	0.205	0.165	0.136	0.139	0.093	0.037
k, min^{-1} g^{-1} mL	132	152	150	174	157	24	24	28
V_0, min^{-1} g^{-1} mL	63	32	31	29	21	3.3	2.2	1.0
n	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
r	0.998	0.995	0.991	0.995	0.997	0.999	0.999	0.997
				Medium Grain				
P_{max}, A	0.511	0.350	0.320	0.240	0.283	0.278	0.093	0.028
k, min^{-1} g^{-1} mL	93	97	115	112	83	33	29	35
V_0, min^{-1} g^{-1} mL	47	34	30	27	23	3.5	2.7	1.0
n	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
r	0.991	0.996	0.997	0.996	0.998	0.999	1.000	0.999
				Short Grain				
P_{max}, A	0.553	0.590	0.530	0.420	0.450	0.417	0.278	0.139
k, min^{-1} g^{-1} mL	82	75	85	70	84	74	36	29
V_0, min^{-1} g^{-1} mL	45	44	45	29	38	31	10	4.0
n	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
r	0.997	0.998	0.999	1.000	0.998	0.995	0.992	0.997

[a] A experimental maximum absorbance (see Experimental Procedures). Values are averages from duplicates. The variation from the mean was less than ±5%.

oxidase from rice grains reacted as a first-order reaction and $n = 1$ (Table III). The apparent equilibria (P_{max}) and the apparent initial velocities (V_0) decreased very significantly during storage. On the other hand, the specific rate constants (k) did not change significantly during storage even at higher storage temperatures (up to 18 months, not shown here). This indicated that the enzyme was not deactivated or destroyed. The highest specific activity of peroxidase was found in long-grain and the lowest in short-grain rice.

Cytochrome *c* Reductase. Cytochrome *c* reductase was a first-order reaction ($n = 1$) (Table III). The apparent equilibria (P_{max}), the apparent initial velocities (V_0), and the specific rate constants (k) quickly decreased during storage. This indicated that cytochrome *c* reductase, in contrast to other studied enzymes, was almost completely depleted by storage (after 18 months of storage the activity was almost 0, not shown here).

Phosphatase. Phosphatase in enzymic rice grain extracts reacted as a first-order reaction ($n = 1$) (Table IV). The apparent equilibria (P_{max}) (with the same substrate concentration) and the apparent initial velocities (V_0) decreased significantly during storage. On the other hand, the specific rate constants (k) did not change significantly during storage even at higher storage temperatures (up to 37 °C) or longer storage times (up to 18 months, not shown here). This indicated that the enzyme was not deactivated or destroyed. The highest specific activity of phosphatase was found in short-grain and the lowest (up to 20× lower) in medium-grain rice.

Phospholipase *c*. Phospholipase *c* in rice grains reacted also as a first-order reaction ($n = 1$) (Table IV). The apparent reaction equilibria (P_{max}) decreased and were much more influenced by storage than those of phosphatase. On the other hand, the influence of storage on the apparent initial velocities (V_0) was mixed (initial veloc-

Table IV. Kinetic Constants of Phosphatase and Phospholipase c from Stored Rice[a]

	phosphatase				phospholipase c			
	0 month	10 months			0 month	10 months		
	4 °C	4 °C	25 °C	37 °C	4 °C	4 °C	25 °C	37 °C
				Long Grain				
P_{max}, A	0.520	0.490	0.490	0.480	0.780	0.620	0.490	0.350
k, min^{-1} g^{-1} mL	77	87	81	76	1.6	1.9	1.9	2.0
V_0, min^{-1} g^{-1} mL	40	42	40	36	1.3	1.1	0.91	0.70
n	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
r	1.000	0.998	0.999	0.999	1.000	0.985	0.984	0.985
				Medium Grain				
P_{max}, A	0.560	0.450	0.450	0.440	0.570	0.380	0.270	0.190
k, min^{-1} g^{-1} mL	7.1	5.9	6.0	5.6	1.4	1.5	1.8	1.8
V_0, min^{-1} g^{-1} mL	40	26	27	25	0.80	0.56	0.49	0.34
n	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
r	1.000	0.998	0.999	0.999	0.999	0.998	0.990	0.994
				Short Grain				
P_{max}, A	0.546	0.455	0.455	0.445	1.100	0.985	0.930	0.809
k, min^{-1} g^{-1} mL	133	129	144	142	0.8	0.8	1.0	1.2
V_0, min^{-1} g^{-1} mL	72	59	65	63	0.90	0.80	0.93	0.96
n	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
r	1.000	1.000	0.998	1.000	0.999	0.999	0.999	0.998

[a] A is the experimental maximum absorbance (see Experimental Procedures). Values are averages from duplicates. The variation from the mean was less than ±5%.

ities of long and medium grains significantly decreased but that of short grains did not change). The specific rate constants (k) increased during storage in all studied varieties. This indicated that the enzyme was not at all deactivated or destroyed, but may be even partially reactivated or resynthesized during storage. The highest specific activity of phospholipase c was found in long-grain and the lowest in short-grain rice.

Addition of Albumins and Globulins. When the albumin fractions from rice were added to the protease, amylase, peroxidase, cytochrome c reductase, phosphatase, and/or phospholipase c reaction mixtures in the concentration 0.2 g/L albumin protein, all apparent reaction equilibria (P_{max}) decreased (compared to the control without albumin extract) by 10–20%. When the globulin fractions from rice were added to the same enzymic reaction mixtures in the concentration 0.2 g/L globulin protein, all apparent reaction equilibria (P_{max}) decreased by 50–80%. On the other hand, all the other kinetic constants remained unchanged when compared to controls without albumins and/or globulins. Although the albumins or globulins were denatured by TCA during the preparation (to inactivate the enzymes present in these fractions), these results indicated that the substrate–receptor inhibitors might be concentrated more in globulin than in albumin fractions of the rice grains.

Conclusions. The results indicate that the studied enzymes behaved differently during storage. Although the cytochrome c reductase almost completely diminished during storage, the activities of the other studied enzymes did not change and the activity of phospholipase c even slightly increased. The diffusion resistance constants (n) did not change during storage. In agreement with theory and the time equation when n and k are constant but P_{max} decreases, initial velocity must decrease. This was in agreement with our results.

In all cases, after the apparent equilibria (P_{max}) were reached, the activities of the reaction mixtures were renewed by addition of the corresponding substrates (hemoglobin, amylopectin-blue, p-phenylenediamine, cytochrome c, p-nitrophenyl phosphate, or (p-nitrophenyl)phosphorylcholine). The activities were also renewed by the additions of the corresponding pure enzymes (acid protease, α-amylase, peroxidase, cytochrome c reductase, acid phosphatase, or phospholipase c).

All this behavior is characteristic for the reversible substrate–receptor inhibition. The experimental results indicated an accumulation of enzymic substrate inhibitors during storage. The stability of the rate constants indicated not only that the enzymes (except cytochrome c reductase) retained their specific activities but also that the direct enzyme inhibitors (competitive or noncompetitive) were either absent or did not increase during storage.

From these facts it was apparent that storage brought about an accumulation of substrate receptors which were probably concentrated in globulin fractions of rice grains. Pronoucned varietal differences in these characteristics were also found.

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Enzymatic Oxidative Reaction of Catechin and Chlorogenic Acid in a Model System

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Enzymatic oxidation of catechin and chlorogenic acid and their mixture was studied at 20 °C and at pH 3.5 and 6.5. The reaction products were monitored by HPLC with diode array detection. The rate of reaction at pH 6.5 was higher than at pH 3.5. The reaction products of catechin oxidation were dimers and other polymers of catechin with low polarity; chlorogenic acid produced mainly polymers. However, the enzymatic oxidation products of the catechin–chlorogenic acid mixture were mainly copolymers which had higher polarity than catechin or chlorogenic acid and were less brown in color than the oxidation products of catechin or chlorogenic acid alone.

Catechin and chlorogenic acid are the major phenolic compounds found in many fruits such as apples, pears, peaches, plums, cherries, and apricots (Risch and Herrmann, 1988). These two polyphenols are well-known substrates of polyphenol oxidase (PPO). PPO catalyzes the oxidation of diphenols to *o*-quinones, which upon further reaction leads to brown pigments. The enzymatically oxidized phenolic acid *o*-quinones were reported to oxidize other polyphenols, such as flavans, by a coupled oxidation reaction (Cheynier et al., 1988). The *o*-quinone formed by enzymatic or coupled oxidation can also react with a hydroquinone to yield a condensation product (Singleton, 1987). Dimers or oligomers are reported to be generated by condensation of hydroquinone with quinone or by condensation of quinones of phenolic acid and catechin (Cheynier et al., 1988). Oszmianski et al. (1985) reported a rapid depletion of grape seed polyphenols by PPO as compared to that by chemical oxidation. Cilliers and Singleton (1989) found more than a dozen oxidation products from a nonenzymatic oxidative browning reaction of caffeic acid.

The purpose of the present work was to study the PPO reaction products of individual chlorogenic acid and catechin and the mixture of the two at pH 3.5 and 6.5 in relation to browning in model systems.

MATERIALS AND METHODS

Materials. Standard catechin, chlorogenic acid, and tyrosinase (monophenol monooxygenase, polyphenol oxidase; EC 1.14.18.1) were obtained from Sigma Chemical Co. Catechin and chlorogenic acid (2 mM) were dissolved individually or together in 0.02 M acetic acid buffer solutions at pH 3.5 and 6.5 and filtered through 0.45-μm membrane filters. Tyrosinase (0.5 mg/mL) was dissolved in the same buffer solutions.

Sampling. Phenolic solutions (19.5 mL) and the enzyme solution (0.5 mL, 565 units) were thoroughly mixed and incubated at 20 °C with constant agitation on a magnetic stirrer. The oxidation reaction was terminated by acidification of an ali-

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