Phenoloxidase from Shrimp (*Penaeus setiferus*): Purification and Some Properties

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Phenoloxidase (PO) was purified from heads of *Penaeus setiferus* by affinity chromatography. The enzyme catalyzed the oxidation of 3,4-dihydroxyphenylalanine (DOPA) and migrated as a single band in polyacrylamide gels with a molecular weight of 30 000. The procedure achieved a degree of purification of 66-fold, and the overall yield was approximately 9%. The enzyme was stimulated by copper, was most active between pH 6.5 and 7.5, and was most stable at pH 8.0, but very unstable at acid pH. The enzyme was heat stable up to 50 °C but was rapidly inactivated by 30-min incubation at 60 °C and higher temperatures. The optimum temperature for the phenolase–DOPA reaction was 45 °C with a corresponding E_a of 13.7 kcal/mol.

Phenoloxidase (PO; EC 1.10.3.1), also known as phenolase, has been implicated in the browning of fruits, vegetables, and crustaceans (Savagaon and Sreenivasan, 1978). The discoloration on shrimp and other crustaceans commonly called melanosis or blackspot is not appealing to consumers and reduces the market value of these foods. The browning phenomenon has been extensively studied in fruits and vegetables but has not received as much attention in shrimp and other crustaceans. Furthermore, studies carried out on the enzyme derived from crustaceans have employed crude or semipurified extracts that makes it difficult to relate all findings made in such studies exclusively to PO. For instance, it is known that dihydric compounds such as DOPA are very unstable and readily undergo autoxidation to form polymers (Neville, 1975). It is therefore interesting to study the relative contribution of enzymic and nonenzymic oxidation to postmortem melanosis. It is also unclear from studies using crude and/or semipurified PO preparations whether one or more enzymes are involved in the oxidation of phenolic substrates such as DOPA and their derivatives to melanins (Cobb, 1977). A better understanding of the properties and mechanism(s) of action of these enzymes is necessary to enable researchers to evolve ways of controlling the deleterious effects elicited by these enzymes. The need for such an understanding has been rendered more acute by the implication of sulfites added to food (to control melanosis) causing certain health-related problems. Various species of shrimp undergo postmortem discoloration to different extents, which may be related to differences in substrate levels, enzyme levels, or enzyme activities within these various species. White shrimp (*Penaeus setiferus*) is one of the least susceptible to browning. The present work describes the purification and some properties of shrimp phenoloxidase.

MATERIALS AND METHODS

Fresh white shrimps (*P. setiferus*) were obtained from Key West, FL, and promptly decapitated. Heads (carapace) were powdered in liquid nitrogen and stored at −30 °C until needed for use.

DOPA and cyanogen bromide activated Sepharose 4B were purchased from Sigma Chemical Co., St. Louis, MO. Protein standards for molecular weight determination were purchased from Bio-Rad, Richmond, CA, and Brij 35 was purchased from Fisher Scientific Co., Orlando, FL.

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Extraction of Shrimp Phenoloxidase. Shrimp heads were powdered by grinding with liquid nitrogen in a Waring blender. The powder was added to 0.5 M sodium phosphate buffer, pH 7.2, containing 0.05 M NaCl at a ratio of 1:5 (w/v), and the suspension was made up to 0.2% with Brij 35. This was then stirred at 4 °C for 3 h with a magnetic stirrer after which the suspension was centrifuged at 4 °C for 30 min at 8000*g*. The clear supernatant was fractionated with solid ammonium sulfate, and the precipitate between 40% and 75% saturation was collected by centrifugation at 12500*g* for 30 min at 4 °C and redissolved in a minimum volume of extraction buffer (Savagaon and Sreenivasan, 1978). The ammonium sulfate fraction was dialyzed overnight against extraction buffer, then mixed with 3 volumes of cold acetone (−30 °C), and left to stand at −30 °C for 2 h before centrifuging at 8000*g* for 30 min at 0 °C. The acetone precipitate was then lyophilized and stored at −70 °C until needed.

Affinity Chromatography of Acetone Fraction. The acetone powder was dissolved in extraction buffer at a ratio of 1:30 (w/v) with stirring at 4 °C and insoluble material removed by centrifugation at 3000*g* for 30 min at 4 °C. The clear supernatant (acetone fraction) was pumped onto a column packed with approximately 12 mL of DOPA–Sepharose 4B material prepared according to the procedure developed by Pharmacia Fine Chemicals (*Affinity Chromatography*, 1979) at a rate of 12 mL/h. Unbound material was thoroughly washed off the column with extraction buffer, and the bound phenolase was eluted with 5 mM HCl after the procedure of Katoh et al. (1978). The fractions with phenoloxidase activity, as measured by the catalytic conversion of DOPA to dopachrome at 470 nm, were pooled (affinity fraction) and promptly dialyzed against 0.05 M sodium phosphate buffer, pH 6.5.

Electrophoresis and Molecular Weight Determination. The affinity-purified shrimp phenoloxidase was electrophoresed in the absence and presence of SDS in 7.5% polyacrylamide gels according to the methods of Davis (1964) and Laemmli (1970), respectively, and protein zones were detected by staining gels with 0.1% Coomassie brilliant blue (R-250) in 25% propan-2-ol, 10% acetic acid, 0.1% cupric acetate, and deionized water. Protein standards used to estimate the molecular weight of shrimp phenolase were phosphorylase *b* (mol wt 92 500), bovine serum albumin (mol wt 66 000), ovalbumin (mol wt 45 000), carbonic anhydrase (mol wt 31 000), soybean trypsin inhibitor (mol wt 21 000), and lysozyme (mol wt 14 400).

Assay of Phenolase Activity. Shrimp phenolase activity was assayed with DOPA as substrate by a modified form of the spectrophotometric procedure of Horowitz et al. (1960). The assay system comprised 0.2 mL of appro-

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Table I. Purification Scheme for Shrimp Phenoloxidase[a]

step	total vol, mL	total protein, mg	total act., units	sp act., units/mg	yield, %	purificn
crude	215	5968.4	5482.5	0.9	100	1.0
$(NH_4)_2SO_4$ fraction	50	745.1	1650.3	2.2	30.1	2.4
acetone fraction	20	66.5	765.8	11.5	14.0	12.8
affinity fraction	28.8	8.3	489.6	59.0	8.9	65.6

[a] 50 g of shrimp powder was treated as described in the text; data presented are representative of two other trials.

priately diluted enzyme solutions and 2.8 mL of 5 mM DOPA in 0.05 M sodium phosphate buffer, pH 6.5, and the formation of dopachrome was measured at 470 nm and 25 °C on a Gilford spectrophotometer (Model No. 250). The unit of activity was defined as the ΔA_{475nm}/min. Protein concentration in the various fractions was determined by the Biuret method (Gornall et al., 1949) at 540 nm on a Gilford spectrophotometer.

Optimum pH and Stability. The optimum pH of the purified shrimp phenolase was determined by preparing the substrate (DOPA) in various buffer solutions and adding 0.2-mL aliquots of enzyme to 2.8 mL of the buffered substrate and measuring the formation of dopachrome at 470 nm as described above. Compositions of the buffer solutions: 0.1 M sodium citrate–HCl, pH 2.0; 0.1 M sodium citrate–NaOH, pH 4.0; 0.1 M sodium citrate–NaOH, pH 6.0; 0.1 M sodium phosphate, pH 6.5; 0.1 M sodium phosphate, pH 7.0; 0.1 M sodium phosphate, pH 7.5; 0.1 M sodium phosphate–NaOH, pH 8.0; 0.1 M sodium phosphate–NaOH, pH 9.0; 0.1 M sodium phosphate–NaOH, pH 10.0; 0.1 M glycine–NaOH, pH 12.0 as described by Gomori (1955). The concentration of HCl or NaOH used in adjusting pH of buffer solutions was 0.1 M. To determine the influence of pH on the stability of the enzyme, the latter was incubated in various buffer solutions ranging from pH 2.0 to 12.0 for 30 min at approximately 25 °C. The ratio of enzyme stock solution to buffer was 1:2. Residual enzyme activity was measured by applying fixed aliquots of the incubated enzyme to DOPA (pH 6.5) and measuring the rate of dopachrome formation as described above. Nonenzymatic oxidation of DOPA, which proceeds readily above pH 8, was corrected for by using a blank of DOPA at the same pH.

Optimum Temperature and Thermal Stability. The optimum temperature of the shrimp phenolase–DOPA reaction was determined by preequilibrating the substrate at various temperatures in a Gilford spectrophotometer for 5 min prior to the addition of the enzyme, and the rate of dopachrome formation was assayed as described previously. The thermal stability of the enzyme was determined by incubating the enzyme solution at various temperatures for 30 min and then rapidly cooling in an ice bath for 5 min before assaying for residual activity with DOPA at 25 °C.

Influence of Trypsin on Activity of Shrimp Phenolase. The influence of trypsin (bovine pancreas, Type III, Sigma Chemical Co.) on the activity of shrimp phenolase was determined according to the method of Savagaon and Sreenivasan (1978) by comparing the activity of the phenolase (with and without trypsin) on DOPA. Aliquots of the affinity-purified extracts were incubated with or without trypsin in an ice bath for 30 min prior to assay for phenolase activity with DOPA as substrate. The reference sample was treated with equivalent amounts of trypsin in 5 mM HCl without the phenoloxidase.

Influence of Copper on Phenolase Activity. The influence of copper on the activity of PO was determined with a modified form of the procedure by Bailey et al. (1959), and it involved incubating the enzyme solution with or without copper acetate in an ice bath for 30 min prior to assaying for phenolase activity at 470 nm and 25 °C as described previously.

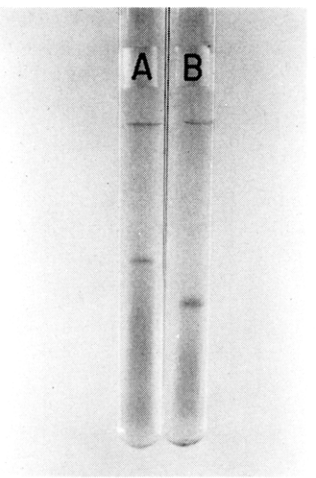

Figure 1. Polyacrylamide gel electrophoresis of affinity-purified PO from heads of *P. setiferus*. Gels A and B were electrophoresed according to the methods of Davis (1964) and Laemmli (1970), respectively. Concentration of protein applied is approximately 8 μg.

Statistical Analysis. Statistical analysis was performed by SAS, GLM procedures (SAS, 1985). A pooled estimate of the standard deviation was performed for all figures, assuming equal population variances ($p = 0.01$) by Hartley's test (Ott, 1984).

RESULTS AND DISCUSSION

Purification of Shrimp Phenolase. A summary of the purification scheme for the shrimp enzyme is presented in Table I. The yield of purified shrimp phenolase was about 8.3 mg of protein/50 g of powdered shrimp heads. The specific activity of the purified enzyme was about 66-fold greater than that of the crude extract. The present procedure is therefore superior to the ion-exchange procedure described by Madero (1982) and Savagaon and Sreenivasan (1978), which achieved 10-fold and a 3-fold purification, respectively.

Electrophoresis and Molecular Weight Determination. The affinity-purified shrimp phenolase migrated as a single band in polyacrylamide gels by both the methods of Davis (1964) and Laemmli (1970) (Figure 1). The R_f of the protein band in the SDS gels corresponded to a molecular weight of 30 000 which falls within the range of 15 000–40 000 reported for phenoloxidases in kiwi fruit (Park and Luh, 1985) and especially close to that of the dominant kiwi fruit phenoloxidase, which was reported by these investigators to be 25 000 Da. With respect to homogeneity, white shrimp phenoloxidase differs from lobster phenoloxidase, which was shown to exist as three isozymes on Sephadex G-100 (Savagaon and Sreenivasan, 1978). The molecular weight of phenoloxidase from *P. setiferus* was different from those derived from *Penaeus aztecus* and *Xyphopenaeus kroyerii*, which were determined by gel filtration as 213 000 and 76 000, respectively (Madero, 1982).

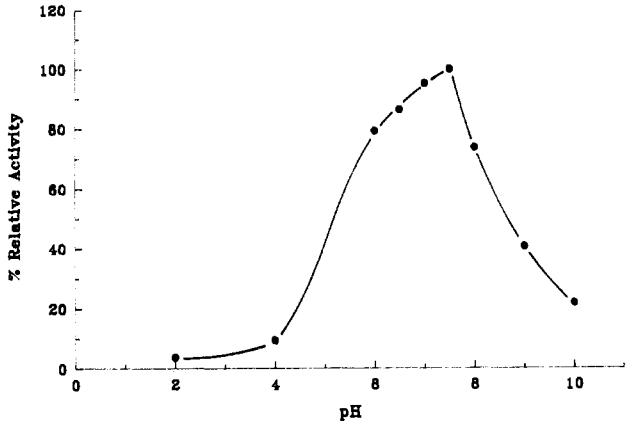

Figure 2. Optimum pH of PO. Data used to plot figure are average values of triplicate results of two experiments. The pooled estimate of the standard deviation was ±3%.

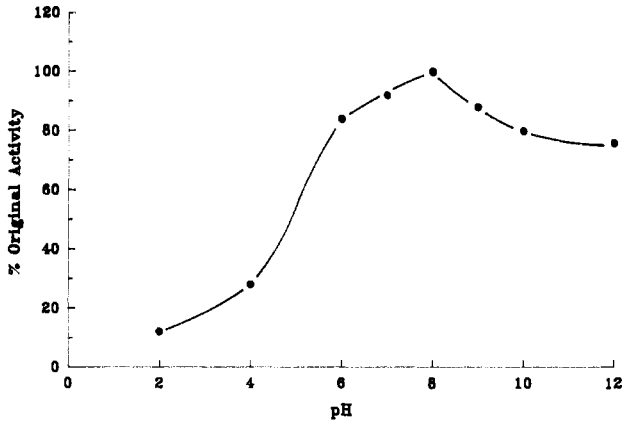

Figure 3. pH stability of PO. Data used to plot figure are average values of duplicate results for two experiments. The pooled estimate of the standard deviation was ±3.8%.

Optimum pH and Stability. The optimum pH for the phenolase–DOPA reaction was 7.5 at 25 °C (Figure 2). Similar pH activity profiles have been reported for other phenoloxidases such as the two kiwi fruit isozymes with optima pH of 6.8 and 7.5 (Park and Luh, 1985) and 8.0 for the enzyme from the crab, *Cancer pagurus* (Pinhey, 1930). However, Aylward and Haisman (1969) reported that the optimum pH of phenoloxidase activity varies considerably with the source of enzyme and with the substrate in a relatively wide range, in most cases between pH 4.0 and 7.0. The shrimp enzyme was most stable at alkaline pH (8.0) and least stable at acid pH (Figure 3). In this respect, shrimp phenolase is similar to phenoloxidases characterized from several other sources (Thomas and Janave, 1973).

Optimum Temperature and Stability. The optimum temperature for the phenolase–DOPA reaction was 45 °C (Figure 4) and corresponds to an E_a of 13.7 kcal/mol (in the range of 20–35 °C). The E_a value was higher than those reported for two kiwi fruit phenoloxidases (4.0 and 7.0 kcal/mol), which might relate to structural differences in these proteins. Bailey et al. (1959) reported E_a of shrimp phenoloxidase as 5.2 kcal/mol. However, these workers used very crude preparations of the enzyme with catechol as substrate and estimated E_a in a lower temperature range (10–30 °C) unlike the present study employing a highly purified enzyme preparation. The enzyme was stable at lower temperatures up to and including 50 °C but unstable at higher temperatures (Figure 5). For instance, it retained only about 10% of its original activity after 30-min incubation at 70 °C. In this respect, shrimp phenoloxidase differed from kiwi fruit phenoloxidases, which retained

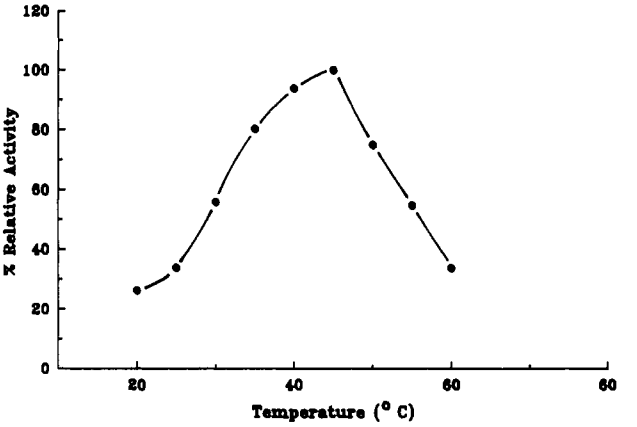

Figure 4. Optimum temperature of PO. Data used to plot figure are average values of triplicate results for two experiments. The pooled estimate of the standard deviation was ±2.3%.

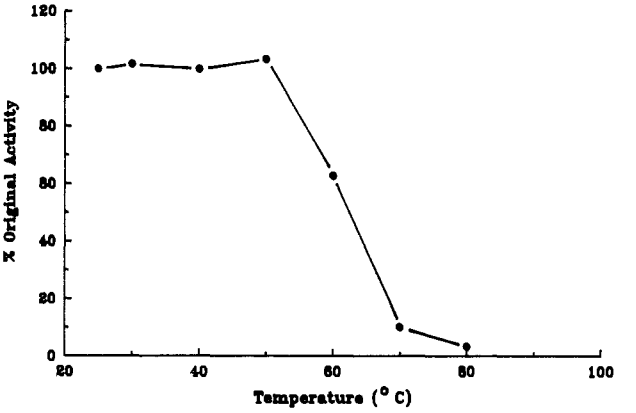

Figure 5. Temperature stability of PO. Data used to plot figure are average values of duplicate results for two experiments. The pooled estimate of the standard deviation was ±4.1%.

Table II. Influence of Trypsin on Phenoloxidase Activity[a]

test	% rel act.
PO (no trypsin added)	100.0[a]
PO (+0.05 mg of trypsin)	99.2[a]
PO (+0.10 mg of trypsin)	101.3[a]
PO (+0.50 mg of trypsin)	100.7[a]

[a] Data presented in Table II are average values of duplicate results for two experiments. Numbers followed by the same letter are not significantly different (α = 0.05) by Duncan's multiple-comparison test.

more than 70% of the original activity after 1-h incubation at 75 °C (Park and Luh, 1985), but was similar to *P. aztecus* phenoloxidase, which was rapidly inactivated after 30 min at temperatures above 35 °C (Madero, 1982). The extreme heat stability of the kiwi fruit enzymes is unusual for phenoloxidases. According to Vamos-Vigyazo (1981), phenoloxidases are not extremely heat stable and are, in most cases, partially or totally destroyed after short exposures to temperatures from 70 °C upward.

Influence of Trypsin on Phenolase Activity. Trypsin does not appear to alter the activity of shrimp phenolase, as shown in Table II. In this respect, the shrimp enzyme differed from other phenoloxidases described by Savagaon and Sreenivasan (1978) and Tolbert (1973). This finding suggests that unlike the lobster and chloroplasts phenoloxidases, described by Savagaon and Sreenivasan (1978) and Tolbert (1973), respectively, the purified shrimp enzyme may not exist in a latent form that requires further activation by a proteolytic enzyme such as trypsin. Trypsin activation was also not observed with crude extracts. It is thus possible that if the enzyme exists

Table III. Influence of Copper on Phenoloxidase Activity[a]

molar ratio (Cu to PO)	% rel act.	molar ratio (Cu to PO)	% rel act.
0:1	100.0[a]	1:2	124.3[c]
1:4	117.4[b]	1:1.25	136.8[d]

[a] Data presented in Table III are average values of duplicate results for two experiments. Numbers followed by the same letter are not significantly different (α = 0.05) by Duncan's multiple-comparison test.

as an inactive precursor, it became activated before the purification process.

Influence of Copper on Phenolase Activity. Addition of copper stimulated the phenolase–DOPA reaction (Table III), and the data presented indicate that the enzyme activity increased with copper concentration. Addition of copper acetate at a molar ratio of 1:1.25 (Cu to PO) increased PO activity by approximately 37% while the same salt at a molar ratio of 1:4 (Cu to PO) only stimulated the reaction by approximately 17%. The values reported in Table III are corrected for catalysis of the reaction by copper. Activation by copper was also observed with the various fractions recovered in the purification process. Various workers such as Bailey et al. (1959) and Mathew and Parpia (1971) have also demonstrated a copper dependency of phenoloxidases.

Conclusions. In conclusion, the 30 000 molecular weight band is phenoloxidase (EC 1.10.3.1) made up of a single polypeptide chain. We also observed that the various fractions from the purification of PO were activated by copper, which eliminates the possibility that activation of the purified enzyme by copper was a simple case of stripping copper from the enzyme with subsequent recovery of activity on adding the ion. Further studies in our laboratory will be designed to determine the kinetic properties of white shrimp PO in relation to properties of homologous enzymes derived from other crustacean species as well as investigate the efficacy of various treatments to prevent melanosis as alternatives to the use of sulfites in crustacean species.

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LITERATURE CITED

Affinity Chromatography. Principles and Methods; Pharmacia Fine Chemicals: Uppsala, Sweden, 1979; p 15.
Aylward, F.; Haisman, D. R. *Adv. Food Res.* **1969**, *17*, 1.
Bailey, M. E.; Fieger, E. A.; Novak, A. F. *Food Res.* **1959**, *25*, 557.
Cobb, B. F., III In Proceedings of the Conference on the Handling, Processing and Marketing of Tropical Fish, Tropical Products Institute, 1977; pp 405–411.
Davis, B. J. *Ann. N.Y. Acad. Sci.* **1964**, *121*, 404.
Gomori, C. *Methods Enzymol.* **1955**, *1*, 138–146.
Gornall, A. G.; Bardawill, C. J.; David, M. M. *J. Biol. Chem.* **1949**, *177*, 751.
Horowitz, N. H.; Fling, M.; Macleod, H. A. Sheoka, N. *J. Mol. Biol.* **1960**, *2*, 96.
Katoh, S.; Kambayashi, T.; Deguchi, R.; Yoshida, F. *Biotechnol. Bioeng.* **1978**, *XX*, 267.
Laemmli, U. K. *Nature (London)* **1970**, *227*, 660.
Madero, C. F. Ph.D. Dissertation, Texas A & M University, 1982.
Mathew, A. G.; Parpia, H. A. B. *Adv. Food Res.* **1971**, *19*, 75.
Neville, A. C. In *Biology of the Arthropod Cuticle*; Springer-Verlag: New York, 1975; pp 116–260.
Ott, L. *An Introduction to Statistical Methods and Data Analysis*, 2nd ed.; Duxbury: Boston, 1984; pp 339–347.
Park, E. Y.; Luh, B. S. *J. Food Sci.* **1985**, *50*, 678.
Pinhey, K. G. *J. Exp. Biol.* **1930**, *7*, 19.
SAS User's Guide: Statistics, 5th ed.; SAS Institute: Cary, NC, 1985.
Savagaon, K. A.; Sreenivasan, A. *Fish. Technol.* **1978**, *15*, 49.
Thomas, P.; Janave, M. T. *J. Food. Sci.* **1973**, *38*, 1149.
Tolbert, N. E. *Plant Physiol.* **1973**, *51*, 234.
Vamos-Vigyazo, L. *CAC Crit. Rev. Food Sci. Nutr.* **1981**, *15*, 49.

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Total Phenolics and High-Performance Liquid Chromatography of Phenolic Acids of Avocado

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Total phenolic concentrations as gallic acid equivalents were determined for leaves, fruits (mesocarp), and seeds (cotyledons) of several cultivated varieties of avocado (*Persea americana* Mill.). Concentrations were generally highest in cotyledons and lowest in the mesocarp of fruits. Phenolic acids were extracted by alkaline hydrolysis and identified with high-performance liquid chromatography, spectrophotometry, and thin-layer chromatography. Sixteen phenolic acids were identified, and in general, every tissue of every variety had the same array. The commonly occurring gentisic acid was not detected.

Plant phenolics include a great diversity of compounds such as simple phenols, phenolic acids, coumarins, flavonoids, tannins, and lignins (Robinson, 1963; Ribereau-Gayon, 1972; Harborne, 1964, 1984). In the strict sense used here, phenolic acids have a benzene ring, a carboxylic acid, and one or more phenyl hydroxyl groups that may become methylated to produce methoxy groups. Phenolic acids commonly occur as esters and/or ethers in combination with various sugars and aliphatic or aromatic acids and hydroxy acids. Most plant phenolic acids are derivatives of either benzoic (benzenecarboxylic; C_6–C_1) or cinnamic (phenylpropanoid or phenylacrylic; C_6–C_3) acids (Roston and Kissinger, 1982). Cinnamic acid is in turn derived by the deamination of the amino acid L-phenyl-

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