Novel Capillary Electrophoresis Technique for the Study of Plant Phenolic Enzymic Oxidation Mechanisms

Kellie M. Shepherd and Tony K. McGhie*

David North Plant Research Centre, Bureau of Sugar Experiment Stations, P.O. Box 86, Indooroopilly, Queensland 4068, Australia

A new enzyme assay for the study of phenolic oxidation in reaction mixtures containing more than one substrate is described. The assay uses capillary electrophoresis to directly measure the disappearance of the substrate(s) over time. The appearance of product can be studied at the same time. The method is rapid, able to be automated, and requires only small amounts of substrate and enzyme. This study found that the rate of phenolic oxidation of a given substrate is influenced by the presence of other substrates in the reaction mixture. When chlorogenic acid was assayed in a binary mixture containing either apigenin, isoorientin, or quercetin, both the rate of disappearance and the overall amount of chlorogenic acid to disappear were reduced, compared to when it was assayed alone. The presence of chlorogenic acid on the other substrates tested caused an increase in their rate of disappearance and in the overall amount consumed compared to when they were assayed alone. This assay system, utilizing rapid separation by capillary electrophoresis, has potential for use with other enzymes, especially when both the substrate and product have similar spectral characteristics, preventing the use of standard spectrophotometric assay systems.

Keywords: *Capillary electrophoresis; chlorogenic acid; phenolics; polyphenol oxidase*

INTRODUCTION

The enzyme polyphenol oxidase (EC 1.10.3.1, PPO) is widely distributed in the plant kingdom and has been detected in most fruits and vegetables. The properties and amount of enzyme present tend to vary depending upon its source and can change with the species, cultivar, and stage of development (Vamos-Vigyazo, 1981). The role of PPO in nature is not clearly understood, but it is thought to be involved in the plant's defense mechanism against mechanical injury and microbial and viral attack (Li *et al.*, 1990; Vaughn *et al.*, 1988). The presence of PPO activity has a large impact on the food processing industry as it is the major class of enzyme involved in the undesirable browning of fruits and vegetables during processing and storage. For example, the formation of color during raw sugar production from sugarcane leads to a reduction in the quality of sugar (Riffer, 1988) and is mostly attributable to the oxidation of phenolic compounds by PPO. PPO is able to catalyze a number of reactions, one of these is the oxidation of *o*-dihydroxyphenols to *o*-quinones in the presence of molecular oxygen (catecholase activity). The quinones produced condense forming highly colored polymers (Cheynier *et al.*, 1988). Quinone formation is reversible in the presence of reducing agents; however, the polymerization step is not. The molecular structure or composition of the polymers has not been defined, but with a better understanding of the enzyme substrate interactions, better methods of limiting or preventing their formation may be devised.

Because of the significance of PPO to the food industry, many assay methods have been developed for PPO. All of these systems have associated limitations. Assay methods include measuring oxygen consumption using an oxygen electrode or Warburg respirometer (Mayer and Harel, 1979), spectrophotometric assays (Gauillard *et al.*, 1993; Waite, 1976), and more recently HPLC techniques (Li *et al.*, 1990). The details of these assay methods are outlined in a review article (Vamos-Vigyazo, 1981). Problems encountered with these existing methods include a lack of specificity, enzyme inactivation by the quinone products formed in the reaction, overlapping substrate/product spectra, interference from endogenous components, the length of time required, limited suitability for a range of substrates, and the inability to assay a mixture containing more than one substrate.

This paper describes first an alternative assay method using capillary electrophoresis to study PPO activity by monitoring changes in substrate and product concentrations over the time course of the reaction. Second, the rate of PPO oxidation of chlorogenic acid was examined alone and in binary mixtures with a range of other phenolic compounds, including isoorientin, luteolin, quercetin, and the monohydroxyphenol apigenin.

METHODS AND MATERIALS

The capillary electrophoresis system used was an Applied Biosystems 270-HT (Foster City, CA). Electrophoretic separations were achieved using a 42 cm × 50 μm i.d. (20 cm from autosampler to detector) capillary, an applied voltage of 18 kV, and detection at 325 nm. The running buffer was 25 mM sodium tetraborate buffer, pH 9.0, and samples were injected for 1.5 s using 12.7 cmHg (1 cmHg = 1333.22 Pa). Each analysis lasted 5 min. Between each analysis the capillary was washed for 0.1 min with 0.1 M NaOH followed by 0.4 min with the running buffer. Enzyme reactions were carried out in 20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid, sodium salt] (Behring Diagnostics, La Jolla, CA), at pH 7.0, in a total volume of 300 μL. The reaction was initiated by the addition of 0.5 μL of enzyme.

Chlorogenic acid was prepared in the assay buffer, while the other substrates were prepared in dimethyl sulfoxide (Mallinckrodt, KY). Chlorogenic acid, quercetin dihydrate, and apigenin were obtained from Sigma Chemical Co. (St. Louis, MO). Isoorientin and luteolin were from Apin Chemicals

* Author to whom correspondence should be addressed.

0021-8561/95/1443-0657$09.00/0 © 1995 American Chemical Society

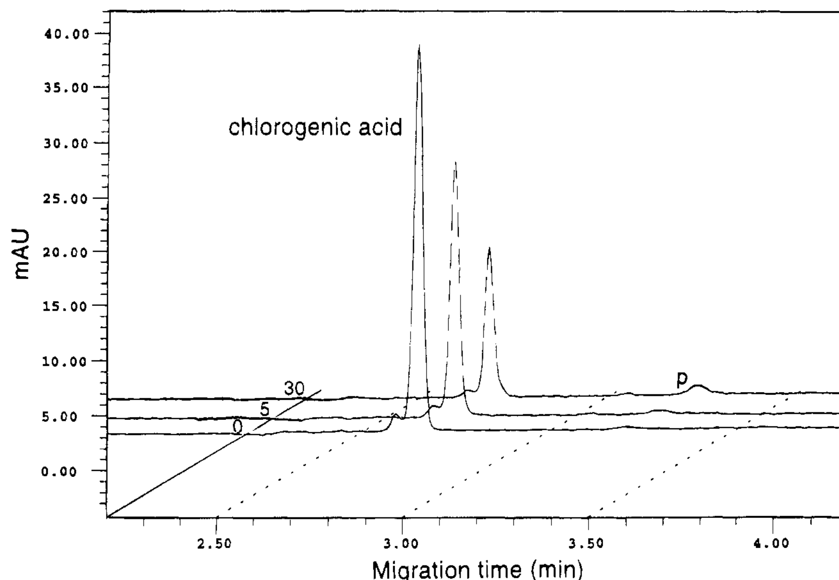

Figure 1. Capillary electrophoresis. Overlaid capillary electrophoresis electropherograms (offset by 60°) show the oxidation of chlorogenic acid (0.25 mM) under standard conditions of pH 7.0 and 30 °C at 0, 5, and 30 min after addition of sugarcane PPO. The products formed are indicated (p).

(Oxon, U.K.). The quinone-trapping compound, L-cysteine (free acid), was from Sigma and prepared in the assay buffer. The enzymes used were mushroom PPO (tyrosinase) from Sigma and sugarcane PPO kindly donated by Carolyn S. Bucheli and Simon P. Robinson (CSIRO Division of Horticulture, Adelaide, Australia). Data points were collected for 90 min. When the concentration of substrate and products had stabilized, the reaction mixture was removed from the capillary electrophoresis system and the reaction products separated from the substrates by gel filtration using Sephadex G-25M prepacked columns (Pharmacia LKB Biotechnology, Uppsala, Sweden). Separation of high and low molecular weight components and analysis of the high molecular weight fraction by capillary electrophoresis were carried out to confirm that polymerization had occurred and to establish the identity of the product peaks in the capillary electrophoresis electropherograms. The molecular size of the products was estimated by differential ultrafiltration through membranes with 3 and 10 kDa molecular weight cutoffs ("Centriprep", Amicon, Beverly, MA).

RESULTS AND DISCUSSION

Activity Assay. The oxidation of chlorogenic acid by PPO was measured by repetitive analysis of enzyme reaction mixtures. Overlaid chromatograms showing a typical time course of chlorogenic acid oxidation are presented in Figure 1. Analysis by capillary electrophoresis provided rapid separation of both the substrate chlorogenic acid and the oxidation products formed enabling PPO enzyme activity to be determined. The peaks with longer migration times were assumed to be the oxidation products because they appeared and then increased in size during the course of the enzyme oxidation reaction. Chlorogenic acid concentration present at each sampling time was calculated by integrating the peak area; a plot showing the reducing concentration with increasing reaction time is shown in Figure 2. The reaction rates, for PPO activity, were calculated from data points collected within 15 min from the start of the reaction. A range of enzyme concentrations was tested, and the reaction rate was found to be directly proportional to the enzyme concentration ($R^2 = 0.95, y = 0.08x + 0.1$), demonstrating that this assay technique is suitable for accurately estimating PPO activity in enzyme extracts.

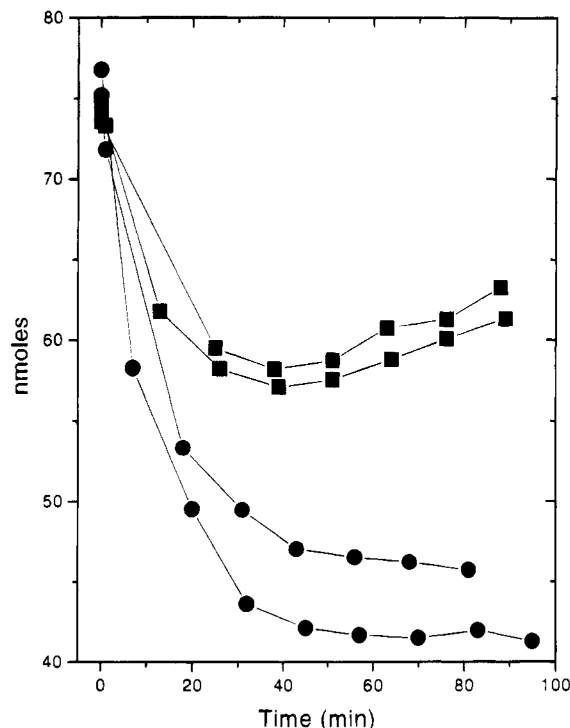

Figure 2. PPO activity assay. PPO activity was calculated from duplicate capillary electrophoresis runs, by plotting peak area of substrate vs time for oxidation of chlorogenic acid (0.25 mM) under standard conditions in the absence (●) and presence (■) of 0.01 mM quercetin.

Measuring substrate disappearance directly has advantages over other methods for determining PPO activity. Determination of PPO activity by capillary electrophoresis is not linked to the formation/consumption of other nonsubstrate compounds, nor does it rely on the formation of secondary products. Problems of overlapping substrate and product spectra encountered in the standard spectrophotometric assays are also avoided. Existing assay methods for determining PPO activity are all based on reactions with single substrates as in the chromogenic assays or do not give information on the rate of phenolic substrate disappearance as in the oxygen consumptionand color formation assays.

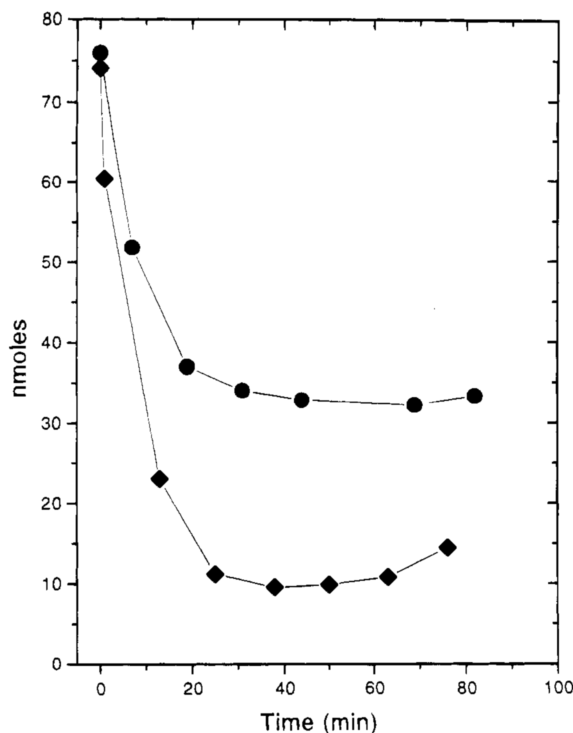

Figure 3. Effect of quinone trap. Oxidation of chlorogenic acid (0.25 mM) is shown in the absence (●) and presence (◆) of 0.75 mM cysteine.

Other advantages of the capillary electrophoresis method include its automation, specificity, and sensitivity, and only small quantities and reaction volumes are required.

A number of spectrophotometric assay systems introduce a quinone-trapping compound into the reaction mixture to block subsequent phenolic quinone polymerization. These complexes are readily detected and used to calculate PPO activity. As shown in Figure 3, including the quinone trap, cysteine increased both the initial rate of the reaction and the overall amount of chlorogenic acid consumed. This finding suggests that spectrophotometric assays which use quinone traps could be significantly overestimating PPO activity in plant and fruit extracts.

Interaction of Chlorogenic Acid with Other Substrates. When PPO oxidation occurs in nature, more than one substrate is usually present. We used the capillary electrophoresis assay technique to investigate the rates of substrate oxidation in binary mixtures of phenolic substrates. An example of the data produced is shown in Figure 4. The overlaid electropherograms are an enzyme activity assay of a reaction mixture which contained chlorogenic acid and the luteolin-based flavone isoorientin. Capillary electrophoresis provided good separation of chlorogenic acid and all four secondary substrates tested. These experiments showed that the rate of chlorogenic acid oxidation was affected by additional substrates in the reaction mixture. For example, when chlorogenic acid was oxidized by PPO in the presence of the flavonoid quercetin (Figure 2), the rate of chlorogenic acid disappearance was reduced compared with the oxidation of chlorogenic acid alone. The rate of chlorogenic acid disappearance was 1285 ± 106 pmol/min (mean ± SD; $n = 2$) when assayed by itself and 595 ± 49 pmol/min (mean ± SD; $n = 2$) when assayed in the presence of quercetin. However, the rate of disappearance of the additional substrate, in this case quercetin, was increased relative to when present alone in the reaction mixture. The rate of quercetin disappearance was 17 ± 1 pmol/min (mean ± SD; $n = 2$) for quercetin alone and 23 ± 4 pmol/min (mean ± SD; $n = 2$) when the reaction mixture contained chlorogenic acid and quercetin. Similar results were also obtained for isoorientin and luteolin (data not presented). The monohydroxyflavone apigenin was tested and found not to be a substrate for sugarcane PPO (data not shown). Furthermore, the overall amount of chlorogenic acid consumed before the reaction reached equilibrium was less when additional substrates were added to the reaction mixture (Figure 2). In comparison, the overall amount of the secondary substrate consumed was increased by mixing with chlorogenic acid (data not shown).

***o*-Quinone Conversion Back to *o*-Phenol.** In the proposed reaction mechanism (Cheynier *et al.*, 1988,

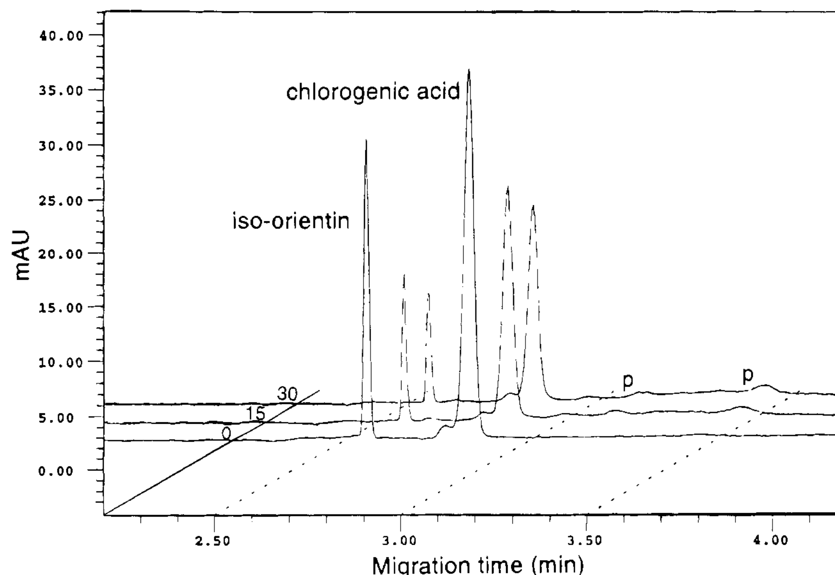

Figure 4. Effect of other substrates on chlorogenic acid oxidation. Overlaid capillary electrophoresis electropherograms (offset by 60°) show the oxidation of chlorogenic acid (0.25 mM) and isoorientin (0.25 mM) under standard conditions of pH 7.0 and 30 °C at 0, 15, and 30 min after addition of sugarcane PPO. Note the separation of the two substrates from each other and the subsequent products formed (p).

1989; Singleton, 1987), the *o*-quinone formed from chlorogenic acid oxidation oxidizes other phenolic substrates and, in the process, chlorogenic acid is regenerated. This cycle would lead to less chlorogenic acid being consumed overall (compared to when it was oxidized separately) when mixed with another phenolic substrate. Also more of the secondary substrate would be consumed (compared to when it was oxidized separately) when mixed with chlorogenic acid. Our findings add support to the proposed mechanism.

The capillary electrophoresis assay method also provided evidence that the reverse reaction of quinone back to the phenol occurs. In Figure 2, the reappearance of chlorogenic acid is indicated by increasing chlorogenic acid concentrations after reaction times of 40 min and is evidence of the reverse reaction occurring. As the enzymic oxidation slows due to inactivation of the enzyme by the *o*-quinones, the reverse reaction predominates with subsequent chlorogenic acid regeneration from the chlorogenic acid *o*-quinone.

Mushroom PPO Activity. PPO is capable of acting on a large number of substrates, its properties varying depending upon its source (Vamos-Vigyazo, 1981). Phenolic oxidation of chlorogenic acid by PPO purified from mushroom was also investigated using the capillary electrophoresis assay method. The results obtained differed from those found for sugarcane PPO. Reaction rates increased for both substrates when they were assayed together relative to when they were assayed separately. The secondary substrates tested with mushroom PPO were luteolin and quercetin. Additionally, the overall amounts consumed before the reaction reached equilibrium apparently did not differ between the single reaction or the mixture (data not shown).

Product Formation. Capillary electrophoresis separation is by electrophoretic mobility differences of components which is due to differences in the molecular charge/size ratios. The oxidation products, as shown in Figures 1 and 4, have a greater migration time (higher electrophoretic mobility) than the starting substrates. To confirm that the products were larger, the products present at the end of the reaction were separated from the starting substrates by gel filtration. The products (as indicated by capillary electrophoresis) eluted in the void volume, confirming that they were of higher molecular size than the substrates. Reanalyses of the gel filtration void volume by capillary electrophoresis confirmed that these high molecular oxidation products were identical to the peaks identified as products in the reaction mixtures. The products with migration times 1.3−1.4 min greater than the monomeric substrates were found to have molecular weights between 3 and 10 kDa, based on their ability to pass through an ultramembrane filter with a 10 kDa molecular weight cutoff but not through a filter with a 3 kDa cutoff. The chemical nature of the polymer products formed was not examined further. According to peak area, apparently more product formed when chlorogenic acid was reacted with another substrate than when it was reacted alone (Figure 5). This could be an indication that *o*-quinones formed from chlorogenic acid polymerize more readily with quinones produced from other phenolic compounds resulting in a faster formation of polymer. The apparent greater amount of product formed from reactions containing substrate mixtures could also be a result of different molar absorption coefficients of the different

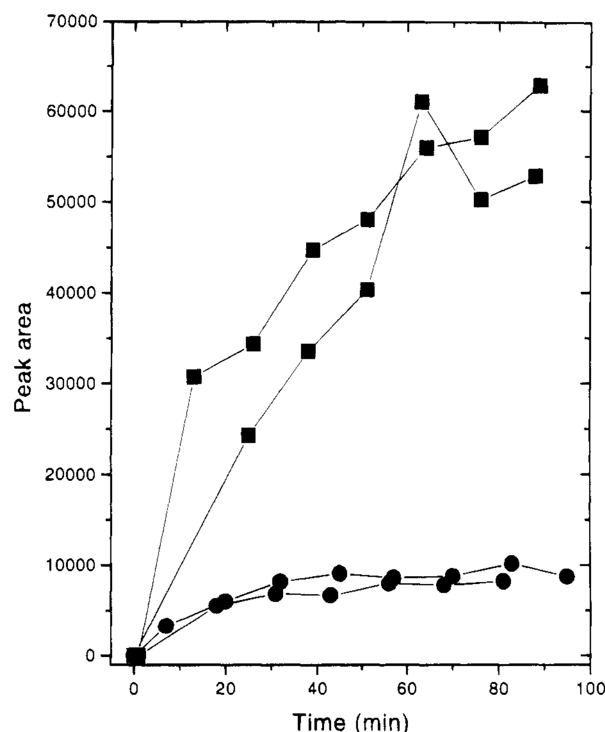

Figure 5. Product formation. In each reaction mixture more than one product peak formed. The combined areas of these peaks formed with time under standard assay conditions are plotted for chlorogenic acid alone (●) and chlorogenic acid and quercetin together (■).

polymer products. The migration times of the products formed were also different indicating the formation of different polymers when the secondary substrate was added. It was also found that the total peak area of the polymer products was considerably less then the loss of peak area for the substrate. This suggests that the molar absorption coefficient of the polymer product is much less than that of the starting substrate. This may be due to loss of phenolic groups or changes in phenolic group pK_a during polymerization.

SUMMARY

Determination of PPO enzyme activity by capillary electrophoresis is potentially a viable alternative to the more commonly used techniques. By measuring the rate of substrate disappearance directly, many of the problems associated with other PPO activity assay methods are avoided. The method proved very useful for investigating the mechanism of enzymic phenolic oxidation with more than one substrate present. Our results show that the composition of substrate mixtures affects the catalysis of individual substrates and supports the previously proposed reaction mechanism.

ABBREVIATIONS USED

PPO, polyphenol oxidase; MOPS, 3-(*N*-morpholino)-propanesulfonic acid, sodium salt.

ACKNOWLEDGMENT

Thanks are due to G. J. Leonard and G. R. Smith for suggestions and to B. D. A. Stickley for reviewing the manuscript. This work was supported by the Sugar Research and Development Corp., Project BS107S.

LITERATURE CITED

Cheynier, V.; Osse, C.; Rigaud, J. Oxidation of grape juice phenolic compounds in model solutions. *J. Food Sci.* **1988**, *53*, 1729–1732.

Cheynier, V.; Basire, N.; Rigaud, J. Mechanism of *trans*-Caffeoyltartaric Acid and Catechin Oxidation in Model Solutions Containing Grape Polyphenoloxidase. *J. Agric. Food Chem.* **1989**, *37*, 1069–1071.

Gauillard, F.; Richard-Forget, F.; Nicolas, J. New spectrophotometric assay for polyphenol oxidase activity. *Anal. Biochem.* **1993**, *215*, 59–65.

Li, J.; Christensen, B. M.; Tracy, J. W. Electrochemical determination of diphenol oxidase activity using high-pressure liquid chromatography. *Anal. Biochem.* **1990**, *190*, 354–359.

Mayer, A. M.; Harel, E. Polyphenol oxidases in plants. *Phytochemistry* **1979**, *18*, 193–215.

Riffer, R. The nature of colorants in sugarcane and cane sugar manufacture. In *Chemistry and processing of sugarbeet and sugarcane*; Clarke, Godshall, Eds.; Elsevier Science Publishers: Amsterdam, The Netherlands, 1988.

Singleton, V. L. Oxygen with phenols and related reactions in musts, wines, and model systems: observations and practical implications. *Am. J. Enol. Vitic.* **1987**, *36*, 69–77.

Vamos-Vigyazo, L. Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Crit. Food Sci. Nutr.* **1981**, *15*, 49–127.

Vaughn, K. C.; Lax, A. R.; Duke, S. O. Polyphenol oxidase: The chloroplast oxidase with no established function. *Physiol. Plantarum.* **1988**, *72*, 659–665.

Waite, J. H. Calculating extinction coefficients for enzymatically produced o-quinones. *Anal. Biochem.* **1976**, *75*, 211–218.

Received for review August 10, 1994. Revised manuscript received December 2, 1994. Accepted December 16, 1994.[⊗]

JF940459A

[⊗] Abstract published in *Advance ACS Abstracts*, February 1, 1995.