Fast Apple (*Malus* × *domestica*) and Tobacco (*Nicotiana tobacum*) Leaf Polyphenol Oxidase Activity Assay for Screening Transgenic Plants

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A spectrophotometric assay method for the analysis of polyphenol oxidase (PPO), in apple and tobacco leaves, has been optimized to increase efficiency in the screening of large numbers of transgenic plants. Crude protein extracts from leaf punches were prepared in a FastPrep homogenizer. The addition of Triton X-100 during extraction resulted in 44 and 74% increases in the PPO activity recovered, from apple and tobacco, respectively. The enzyme kinetics differed markedly between apple and tobacco. Apple leaf PPO was isolated in a latent state and was activated by the addition of SDS. In contrast, tobacco PPO activity was inhibited by SDS, particularly at acidic pH. Apple PPO showed a pronounced pH optimum around pH 6, whereas the pH profile for tobacco PPO was much flatter, with a broad optimum around pH 4. The calculated K_m' value for apple PPO, using 4-methylcatechol as substrate, was 8.1, and for tobacco the K_m was 4.3. The PPO reaction was strongly inhibited by tropolone, a Cu competitor, and restored by the addition of Cu^{2+}. Several factors affecting variability in leaf PPO activity levels in plants are discussed.

Keywords: *Polyphenol oxidase; Malus × domestica; Nicotiana tabacum; extraction; enzyme kinetics*

INTRODUCTION

Many vegetables and fruits become discolored during storage or processing, an action mediated by the enzyme polyphenol oxidase (PPO). PPO is a Cu^{2+}-containing enzyme, widespread in plants, that is synthesized early in tissue development and stored in chloroplasts (van Gelder et al., 1997). When cell membrane integrity is disrupted, phenolic substrates encounter the enzyme and are converted to *o*-quinones in a two-step process of hydroxylation of monophenols to diphenols (monophenolase activity), followed by oxidation of the diphenols to *o*-quinones (diphenolase activity). These highly reactive quinones polymerize with other quinones, amino acids, and proteins to produce colored compounds, and nutrient quality and attractiveness is reduced (Matheis and Whitacker, 1984).

Various approaches for the control, or inhibition, of browning in fresh-cut produce have been investigated (Martinez and Whitaker, 1995), and the search for alternatives to the FDA-banned sulfite treatment is continuing (Richard et al., 1991; Robinson et al., 1993; Sojo et al., 1998). The changing pattern of fresh produce consumption has increased the interest in "fresh-cut", minimally processed fruits (Aylsworth, 1997). Tissue browning is a primary reason that fresh-cut apple slices have not become established as a common produce item and explains, in part, why apple PPO has been studied extensively in recent years (Tan and Harris, 1995; Buta et al., 1999).

A promising approach for the prevention of browning, mediated by PPO, is the production of transgenic plants with lowered PPO activity levels resulting from reduced PPO translation (Bachem et al., 1994). Transgenic apple and tobacco plants, with a PPO transgene fragment, driven by the constitutive CaMV 35S promoter were produced in a previous experiment (B. Li, B. Bhagwat, E. Randall, and W. D. Lane, unpublished results). The shoots were rooted, and then the plants were established in the greenhouse for further characterization. Methods for assay of fruit PPO have been widely described, but leaf PPO is not documented so well (Meyer and Biehl, 1981; Chazarra et al., 1996; Escribano et al., 1997a). Moreover, most papers characterize the kinetic parameters of purified PPOs and are not easily applicable to the analysis of PPO activity when the number of samples is large. Here we describe a simple crude protein extraction method for determining the PPO activity from small samples of leaf tissue. Enzymatic assay parameters were optimized for PPO extracted from apple and tobacco leaf samples. Sources of natural variation in PPO activity levels were identified, and approaches are described to address problems associated with a screening of clones represented by single plants.

MATERIALS AND METHODS

Plant Material. Apple (*Malus* × *domestica* Borkh. cv. Jonagold) and tobacco (*Nicotiana tabacum* L. cv. Samsun) plants were established from tissue culture shoots and grown in soil for several weeks in a greenhouse. In addition to these untransformed control plants, a number of transgenic plants of the same cultivars were assayed. These contained an antisense partial PPO gene sequence of 296 base pairs, under the control of the constitutive CaMV 35S promoter.

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Protein Extraction. Six leaf disks, cut with a 0.5 cm cork borer, for apple, or two leaf disks, cut with a 2 cm cork borer, for tobacco, were taken from each of three sequentially positioned leaves, from midheight on the plant stem. The leaf punch samples were immediately dropped into 2 mL tubes on ice that contained 1 mL of extraction buffer and a FastPrep bead. The extraction buffer consisted of 0.1 M sodium phosphate buffer (pH 6), Triton X-100 (TX-100) at 1 or 2%, and 2% polyvinylpolypyrrolidone (PVPP, Sigma). Following homogenization in a FastPrep apparatus (Savant FP120), set at speed 5.5 for 20 s, the tubes were incubated on ice for 15 min and then centrifuged at 14000 rpm for 10 min. The supernatant was diluted 5 times, for apple, or 20 times, for tobacco, with 50 mM sodium phosphate buffer (pH 6) and either assayed immediately or stored at −80 °C for later use.

PPO Assay. PPO activity was determined by the direct detection of oxidation products at 400 nm, in a Perkin-Elmer spectrophotometer (Tdrive program). Fifty microliters of the extract was mixed with 950 μL of substrate solution at room temperature, and the time drive program was started after 30−60 s, for apple, or immediately, for tobacco. The standard assay consisted of 15 mM 4-methylcatechol in 50 mM sodium phosphate buffer (pH 6) with the addition of 0.1% SDS for apple only. Rates were calculated from the initial linear phase of the curve, which continued for 30−60 or 15−30 s for tobacco and apple, respectively. PPO activity was expressed in units per minute, with 1 unit defined as an increase of 0.001 at OD_{400}. Kinetic constants were determined using nonlinear regression based on least-squares minimization and the Microsoft Excel Solver tools.

RESULTS AND DISCUSSION

Apple and tobacco leaf samples were taken from greenhouse plants about 20 and 40 cm tall, for apple and tobacco, respectively. The *o*-quinone produced by oxidation of the diphenol substrate, 4-methylcatechol, was measured spectrophotometrically in the PPO enzymatic assay. This substrate has been shown to be one of the most reactive for apple PPO (Janovitz-Klapp et al., 1990; Zhou et al., 1993; Tan and Harris, 1995). The steady state rate (V_{ss}) of the reaction was defined as the slope of the linear section of the product accumulation curve (Escribano et al., 1997a). This reaction rate was dependent on enzyme and substrate concentrations, pH, and SDS. The reaction kinetics differed between apple and tobacco PPOs. For apple, the reaction was characterized by the presence of a lag period preceding the linear reaction phase. This lag period decreased as PPO activity increased. Tobacco extracts, on the other hand, produced a linear OD increase immediately following substrate addition. With tobacco, the reaction slowed more rapidly than with apple, so the V_{ss} was calculated from the data points generated during the first seconds of the reaction. To define a protocol for measuring the actual PPO activity in transgenic apple and tobacco plants, the effect of several parameters on leaf PPO activity was investigated.

Effect of TX-100. Because most PPO is sequestered in the chloroplast membrane (van Gelder et al., 1997), the effect of the addition of a detergent to the extraction buffer, TX-100, to enhance recovery of enzyme from extracts, was studied (Figure 1). Without TX-100, the activity measured was 56% of the maximum activity for apple and 26% for tobacco, but addition to extraction buffers of 1% TX-100, for apple, and 2% TX-100, for tobacco, resulted in activities 90% or greater than the maximal for both species. A further increase in TX-100 concentration did not result in greater PPO recovery. The increased PPO activity, recovered when detergent concentration was increased, coincided with increased
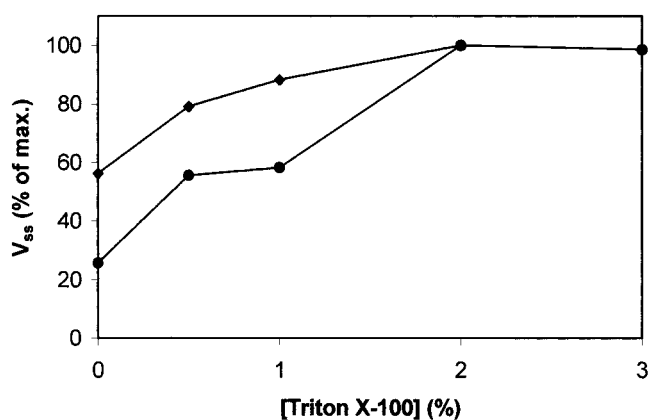

Figure 1. Effect of TX-100 addition during extraction on PPO activity in apple (◆) and tobacco (●). Leaves were homogenized in extraction buffer without detergent and then incubated for 30 min on ice with the appropriate amount of Triton added. Following centrifugation, the extracts were immediately assayed for PPO activity using the standard reaction conditions.
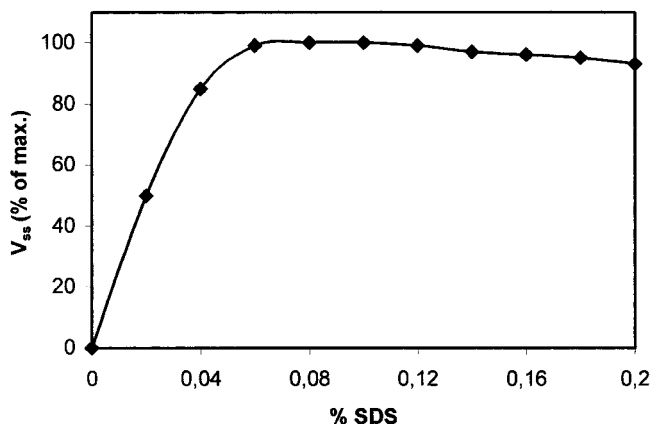

Figure 2. Activation of apple PPO by SDS. The medium contained 50 μL of leaf extract and various SDS concentrations in 50 mM sodium phosphate buffer (pH 6.0), and the reaction was started immediately upon addition of substrate (15 mM final concentration).

chlorophyll extraction, evident from the dark green color of the extracts.

Using purified chloroplasts from Indian tea leaves, Halder et al. (1998) showed that PPO activity was detected only after the addition of 0.7−0.8% TX-100. Although PPO in leaves of apple has not been studied, methods described for extraction of PPO from apple fruit include TX-100, at 0.5−2%, to solubilize the membranes (Janovitz-Klapp et al., 1989; Zhou et al., 1993). Harel et al. (1965) reported that TX-100 preferentially extracted PPO from chloroplasts rather than from mitochondria.

Effect of SDS. An unusual characteristic of PPO is that it exists in an inactive or latent state and is activated by various agents or treatments (Jimenez and Garcia-Carmona, 1996). SDS has been used in many studies to activate PPO, because few other enzymes are known to be activated by it. In apple, PPO activity was negligible in the absence of SDS, at pH 6.0, and increased sharply as SDS concentration was increased from 0 to 0.05% (Figure 2). Maximal activity was observed between 0.06 and 0.12% SDS. The activity was slightly decreased if higher concentrations of SDS were used. At acidic pH, the SDS activation of latent apple PPO was abolished completely (Figure 3). PPO activity, at pH 4.0, was detected only in the absence of SDS.
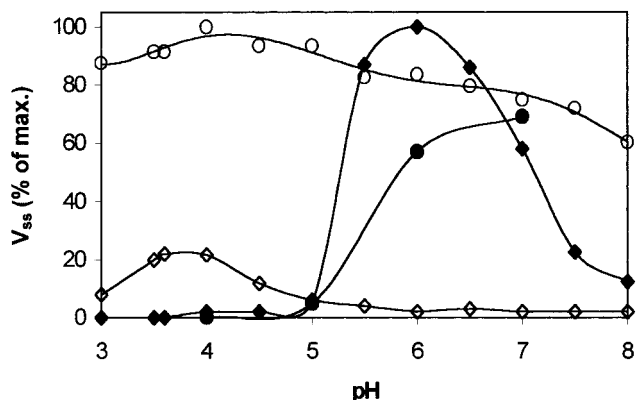
Figure 3. Effect of pH on apple (◆, ◇) and tobacco (●, ○) PPO activity in the presence (solid symbols) or absence (open symbols) of 0.1% SDS. The reaction medium contained 50 μL of extract and 15 mM 4-methylcatechol in 50 mM sodium phosphate/citrate (pH 3.0−7.0), sodium acetate (pH 3.6−5.5), or sodium phosphate (pH 6.0−8.0) buffer.

Figure 4. Effect of different concentrations of 4-methylcatechol on PPO activity in apple (◆) and tobacco (●). The reaction medium contained 50 μL of extract and various substrate concentrations in 50 mM sodium phosphate buffer (pH 6.0) and 0.1% SDS (apple only).

Apple PPO activity at the acidic pH optimum, without SDS, was reduced to slightly >20% of the maximal activity when assayed at pH 6.0 in the presence of SDS. We conclude from this result that most of the apple leaf PPO extracted was in a latent state.

Activation of latent PPO by SDS or acid is a common attribute of PPOs from a number of species (Escribano et al., 1997a). Partially purified latent banana PPO, however, was not activated by acid and retained maximal activity in the presence of SDS throughout the pH range 3.0−7.5 (Sojo et al., 1998). Interestingly, the only apple PPO isozymes studied are ones found in fruit, and SDS was not included in many of the enzymatic assays reported (Janovitz-Klapp et al., 1990; Richard-Forget et al., 1992; Zhou et al., 1993; Oktay et al., 1995; Tan and Harris, 1995). That mature apple fruit PPO is apparently already in an active state may be attributed to the extraction method used (involving ammonium sulfate fractionation) or may be a characteristic of PPOs in this tissue. Nevertheless, Marquès et al. (1995) showed that apple fruit PPO, purified by phase partitioning, was strongly activated by SDS at pH 6.0. They found the activity at pH 4.0 in the absence of SDS to be nearly identical to the activity at pH 6.0 in the presence of SDS. The authors concluded that apple fruit PPO was not in a latent state.

For tobacco leaves, assayed in the presence of 0.1% SDS, PPO activity was abolished completely at pH 4.) and reduced by 40% at pH 6.0. Activity differed only slightly at pH 7.0 in samples prepared with or without SDS. These results indicate that tobacco PPO, in contrast to apple, was extracted in an active state. Mild extraction conditions, based on temperature-induced phase partitioning with TX-114, have been used to purify PPOs, in a latent state, from various sources (Sanchez et al., 1990). We investigated the effect of TX-114, at final concentrations of 8 and 10%, on the latency of extracted tobacco leaf PPO. Although this procedure resulted in the separation of phenols from the supernatant phase that contained the PPO, as evidenced by the rapid browning occurring in the lower phase, we found no difference in PPO activation kinetics with added SDS.

Effect of pH. Apple leaf PPO showed a clear pH optimum with 4-methylcatechol as substrate at pH 6.0 (Figure 3). The PPO activity dropped sharply when the pH was decreased below the optimum of 6.0 to pH 5.5.
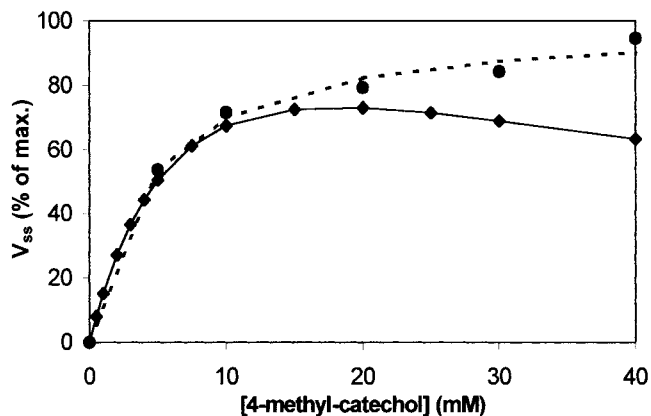
The decrease in activity when the pH was increased above 6.5 was more gradual. A second effect at pH ≥6.5 was that the initial linear reaction rate was not maintained for as long, with a resulting decrease in product formed. In the absence of SDS, a different apple leaf PPO pH optimum of pH 3.5−4.0 was observed. Tobacco leaf PPO revealed a flatter pH profile than apple, with a broad optimum around pH 4.0 (Figure 3). Because tobacco PPO activity at pH 6.0 was close to the maximum activity, this pH was used for convenience in further assays of both apple and tobacco. There was only a slight effect of the type of buffer used on the pH dependency of the activity.

Conflicting results on the pH profile of apple fruit PPOs have been published. Although several studies reported a pronounced acidic pH optimum of 4.5−5.0 with 4-methylcatechol (Janovitz-Klapp et al., 1989; Zhou et al., 1993), a broader optimum of pH 5.0−6.5 (in the presence of SDS) was obtained for Granny Smith pulp (Marquès et al., 1995), and two pronounced pH optima, at pH 6.0 and 9.0, were reported for Amasya apple (Oktay et al., 1995).

Effect of Substrate Concentration. An increase in 4-methylcatechol concentration in the assay resulted in an increase in the steady state rate for both apple and tobacco PPOs (Figure 4). In apple, this was accompanied by a decrease in the lag period. Increasing substrate concentrations also shortened the linear phase of the product accumulation profile for both PPOs, with the reaction rate slowing more rapidly at higher substrate concentration and with a lower maximum OD reading produced. Similar results have commonly been reported for plant PPOs and result from the phenomenon of substrate inhibition and secondary reactions (Janovitz-Klapp et al., 1990; Richard-Forget et al., 1992).

The enzyme kinetics were calculated from the activity plots toward different concentrations of 4-methylcatechol. The tobacco PPO fit well with the Michaelis−Menten equation, producing a K_m of 4.3. Apple leaf PPO activity was best fit with the non-Michaelis−Menten equation for substrate inhibition (Cornish-Bowden et al., 1982). At low substrate concentrations this equation approximates the Michaelis−Menten equation (when the substrate inhibition constant is relatively high; 40 mM in this case). The half-maximal concentration (K_m', a K_m-like value) for apple of 8.1 mM could, therefore, be roughly compared to the tobacco value of 4.3 at low

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concentrations. At higher substrate concentrations it was apparent that tobacco activity continued to increase with increasing substrate concentration, whereas apple PPO showed the typical pattern for substrate inhibition, decreasing in activity after a specific concentration. Variable K_m values for the same substrate have been reported for PPO from different apple fruits (5.2, Janovitz-Klapp et al., 1989; 11.0, Zhou et al., 1993; 7.0, Marquès et al., 1995; 3.1, Oktay et al., 1995; 5.5, Espin et al., 1998). Many species produce multiple PPO isozymes, which may have different enzyme kinetics. On native-PAGE activity gels, it was shown that leaves, flesh, and cultured cells of apple express different isozymes (Murata et al., 1995). Alhtough there exist several PPO genes in apple (Boss et al., 1995), only PPOs expressed in the fruit have been thoroughly characterized. Similarly, kinetic studies of tobacco PPOs have not been carried out before.

Effect of PPO Inhibitor. PPO requires Cu^{2+} as a cofactor in order to be enzymatically active. Tropolone, a competitor of Cu^{2+}, is described as one of the most powerful specific PPO inhibitors (Khan and Andrawis, 1985). When apple leaf extracts were assayed in the presence of 0.5 mM tropolone, >85% of the PPO activity was inhibited, and 2 mM tropolone inhibited apple leaf PPO by 95%. PPO activity was completely restored by including 1 mM $CuSO_4$ in the reaction mixture. Preliminary assays showed that the addition of up to 2 mM $CuSO_4$, alone, also stimulated PPO activity to a maximal increase of 44%, indicating that low Cu^{2+} concentration limited activity in these extracts. This observation appears to be the first report of stimulation of PPO activity by the addition of Cu^{2+} to the assay solution. It would be interesting to determine if Cu^{2+} added to extracts from other sources and species stimulates PPO activity. Clover plants grown in copper-deficient conditions had negligible PPO activity, and Cu^{2+} added at a later stage of growth could not restore activity (Delhaize et al., 1985). Although this result may seem to be contradictory to the Cu^{2+} stimulation observed in the present work, the copper-deficient clover plants may have failed to synthesize PPO at the time and stage of development when this normally occurs, or the clover PPO apoenzyme may have been unstable in the absence of Cu^{2+}.

In parallel studies with tobacco, the addition of 10 mM tropolone resulted in 80% inhibition of PPO activity. Activity was restored to the original levels after the addition of 1 mM $CuSO_4$.

Physiological Factors Affecting PPO Activity. The precise function of PPO during plant development is controversial, and there has been limited progress toward elucidation of the role of PPO (Mayer, 1987). In apple, we investigated the effect of leaf age on PPO activity. Extracts prepared from the youngest, partly unfolded leaf, near the shoot apex, had 3-fold higher PPO activity (expressed in units per minute per milligram of total protein) than the fifth leaf from the shoot tip. When different plants are screened for PPO activity, it is therefore imperative to compare leaves of similar age. As differences in PPO levels between individual leaves were more pronounced in younger leaves, we advise assaying PPO in extracts from fully unfolded leaves taken some distance from the growing tip.

It is also well-known that stress affects PPO activity, and wounding was one source of stress found to have a considerable effect on PPO levels. We observed different PPO activity levels from similar leaves on the same plant when samples were harvested 5 days apart. The leaves harvested later had higher PPO activity, even though they were comparable in age and development. A survey of several crop plants found that only some species responded to wounding by increased PPO activity (Constabel and Ryan, 1998). Severe wounding of apple fruit and leaves caused a strong increase in PPO transcription in, or near, the wounded tissue within several hours (Boss et al., 1995). In tomato, PPO was shown to be systemically induced by wounding, and cis-acting elements, identified in the promoter of one of seven PPO genes, were shown to control the responsiveness to a broad range of signals including wounding (Thipyapong and Steffens, 1997).

The PPO assay described herein was applied to several transgenic genotypes to screen for plants with reduced PPO levels. Individual plants from the same transgenic clone were shown to reveal variable PPO levels depending on the physiological condition of the plant. For example, an apple plant beginning to wilt due to root rot had 6 times more PPO activity than when the same plant was assayed 2 weeks earlier. A significant factor affecting the growth of apple plants derived from tissue culture is variation in the number and size of their roots, a condition that was difficult to control. It is therefore advisable to obtain PPO activity data from 10 or more plants of a single transgenic clone. This requires careful planning of the experiments and the space to grow a large number of plants. Moreover, procedures to ensure that rooting of the tissue culture shoots is maximized should be employed. Rooting of tobacco was generally consistent and efficient, and it was much easier to grow homogeneous tobacco plants than apple plants in the greenhouse. The variation in PPO levels among individual tobacco plants was less than in apple as a result. The herein described leaf PPO assay will be useful for the screening of large numbers of transgenic plants only if precautions are taken to ensure the uniform growth of the plants and the selection of comparable tissue samples.

ABBREVIATIONS USED

K_m, Michaelis−Menten constant of PPO; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; TX-100, Triton X-100; V_{ss}, steady-state rate.

ACKNOWLEDGMENT

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