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Biochemical characterization of two differentially expressed polyphenol oxidases from hybrid poplar

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Two polyphenol oxidase isoforms with distinct expression patterns were identified in hybrid poplar (*Populus trichocarpa*× *P. deltoides*). PPO-1, corresponding to the previously cloned *PtdPPO* (Constabel et al., Plant Physiol. 124: 285–295) was primarily leaf tissue-specific and detected only after wounding. PPO-2 was expressed constitutively in all tissue types tested except mature leaves, with highest expression in very young leaves and conducting tissues such as roots, stems and petioles. These two PPO isoforms were partially purified from hybrid poplar by ammonium sulfate fractionation followed by hydrophobic interaction chromatography. They were found to differ in stability, pH optimum, and activation by SDS. Tests with common phenolic substrates showed that PPO-1 had a broader substrate specificity than PPO-2. The distinct enzymatic properties and expression patterns of these two PPO isoforms suggest that they may have different physiological functions in hybrid poplar.

Keywords: *Populus*; Salicaceae; Catechol oxidase; Phenolase; Catechin

1. Introduction

Polyphenol oxidase (PPO, EC 1.10.3.1) is a common enzyme which uses molecular oxygen to oxidize *o*-diphenols to *o*-quinones, and in some plants also hydroxylate monophenols to *o*-diphenols (monophenolase activity; EC 1.14.18.1). The quinones are reactive compounds which are responsible for the damage-induced browning of many fruit and vegetables (Steffens et al., 1994). Typically, plant PPOs have broad substrate specificities and are able to oxidize a variety of *o*-diphenols. While the physiological function of PPO in many plants is unresolved, in some species there is strong evidence for a role of PPO in defense against insects as well as pathogens (reviewed in Steffens et al., 1994; Constabel et al., 1996). In tomato, PPO induction by the wound signal systemin and the octadecanoid pathway provided strong evidence for a defensive function of PPO against insects (Constabel et al., 1995). A clear role of PPO in pathogen defense was demonstrated by Li and Steffens (2002) using PPO-overexpressing transgenic tomato plants, which show enhanced resistance to *Pseudomonas syringae*. An intriguing feature of PPO is that while this enzyme has been found in many plant species, patterns and levels of expression may differ widely between species (Constabel et al., 1998). This suggests that other physiological roles for PPO still remain to be discovered; for example, a hydroxylation reaction during aurone biosynthesis was recently found to be carried out by a PPO-like enzyme (Nakayama et al., 2000).

Plant PPOs are usually encoded by mid-sized gene families. For example, in tomato there are at least seven PPO genes (Newman et al., 1993), and in potato, five PPO genes were characterized (Thygesen et al., 1995). The genes can have complex temporal and tissue-specific patterns of expression, with high levels of transcript commonly found in flowers, young leaves, trichomes, roots, and tubers (Thipyapong et al., 1997; Thygesen et al., 1995). Only one member of the tomato PPO gene family is wound- and stress-induced (Thipyapong and Steffens, 1997). As in higher plants, multiple forms of polyphenol oxidases have also been described in fungi (Zhang and Flurkey, 1997).

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Previously, a wound- and herbivore-inducible leaf specific PPO from hybrid poplar (*Populus trichocarpa×P. deltoides)* was cloned and proposed to be a component of the inducible defense against leaf-eating herbivores (Constabel et al., 2000). Additional experiments with PPO-specific antibodies showed the presence of two differentially expressed PPO proteins in hybrid poplar. In order to learn more about these PPO isoforms and their possible functions, both hybrid poplar PPOs were partially purified and characterized.

2. Results

2.1. Hybrid poplar contains two major PPO isoforms with distinct patterns of expression

In the course of characterizing a wound-induced PPO cDNA, *PtdPPO* from *Populus trichocarpa×P. deltoides* (Constabel et al., 2000), we observed that in addition to the inducible leaf PPO activity, hybrid poplar contained high levels of PPO activity in petiole and stem tissue. To further characterize this PPO activity, an antibody was raised against recombinant *PtdPPO* (M. Christopher and C.P. Constabel, unpublished data) and then used to perform western blots with different poplar sapling tissues (Fig. 1A). While there was no PPO band in control leaves, the antibody recognized a protein migrating at 59 kDa in wounded leaves. Based on its induced expression, size, and recognition by the PPO antibody, this band likely represents the product of the wound-induced *PtdPPO* gene. Furthermore, in *PtdPPO*-overexpressing transgenic canola plants, the same 59 kDa band was present while being absent in controls, providing direct proof that *PtdPPO* encodes the 59 kDa PPO (C.P. Constabel, unpublished data).

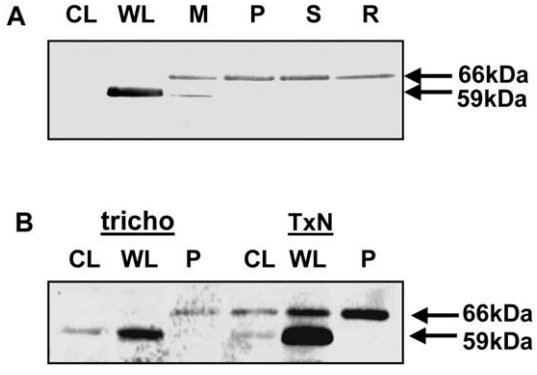

Fig. 1. Western blot analysis of PPO in different tissues of hybrid poplar. Tissues were extracted and blotted as described in Experimental, and analyzed using an antibody raised against recombinant PPO-1. (A). Analysis of *P. trichocarpa×P. deltoides* tissues. (B). Analysis of the *P. trichocarpa* parent and a *P. tremula×P. nigra* hybrid. CL, Control leaf; WL, Wounded leaf; M, Midvein; P, Petiole; S, Stem; R, Root.

The 59 kDa band was not present in petioles, stems, and roots, and was only faintly visible in midvein samples of *P. trichocarpa×P. deltoides* (Fig. 1A). In these tissues, however, the antibody recognized a different protein band, migrating at approximately 66 kDa. The intensity of this band correlated well with PPO activity in crude extracts made from these tissues (data not shown), suggesting that this protein represents a second PPO isoform. Additional western blots with the *P. trichocarpa* parental clone, as well as an unrelated *P. tremula×P. nigra* hybrid, also showed the induced 59 kDa band in wounded leaves and the 66 kDa PPO band in petioles (Fig. 1B). In addition, the 66 kDa band was present in control and wounded leaves of the *P. tremula×P. nigra* hybrid, and small amounts of the 59 kDa band were present in control leaves of both this and the *P. trichocarpa* genotype. We concluded that the 66 kDa PPO band does not represent a second parental allele found only in hybrids, but rather represents a second PPO isoform encoded by a second gene. The wound-induced leaf isoform was named PPO-1, and the constitutive isoform PPO-2. Wounding of the PPO-2 expressing tissues did not increase expression levels discernably on western blots (data not shown).

2.2. Partial purification of PPO isoforms

The distinct patterns of expression of the two PPO isoforms prompted us to undertake partial purification of both proteins, in order to compare their enzymatic properties. Wounded leaf tissue, and stems and petioles were used as starting material for extraction of PPO-1 and PPO-2, respectively. Since *Populus* is known to contain high levels of phenolic compounds, PVP-40, Amberlite XAD-4, and PVPP were included in the extraction buffer as phenolic adsorbants. Protease inhibitor cocktails were also added, as these were found to preserve the integrity of the PPO protein as visualized on western blots.

Preliminary experiments suggested that while the starting material for PPO extraction was highly enriched for either of the PPOs, it contained small amounts of the other isoform. Therefore, the protein extracts were fractionated using ammonium sulfate precipitation, followed by column chromatography for further purification. Anion exchange chromatography on DEAE cellulose was not successful in separating the isoforms (data not shown). However, a hydrophobic interaction column (Phenyl Sepharose CL-4B), was very useful for separating the two PPO isoforms, and allowed us to eliminate the small amount of PPO-2 from the leaf PPO-1 preparation. Western blot analysis of the extracts during this procedure confirmed that the final preparation contained only a single PPO protein band (Fig. 2).

The semi-purified PPO preparations were analyzed using in situ PPO-staining in non-denaturing SDS-poly-

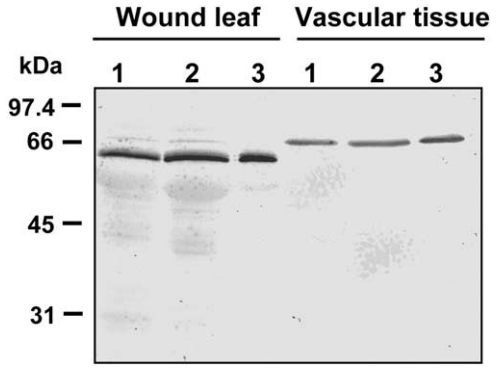

Fig. 2. Western blot analysis showing the major steps of the purification process of both PPO isoforms. Aliquots of active fractions were applied to SDS-PAGE gels, blotted, and analysed using the PPO antibody. 1, crude extract; 2, 40–90% $(NH_4)_2SO_4$ precipitate; 3, semi-purified PPO from Phenyl Sepharose CL-4B column.

acrylamide gels. This technique is based on the observation that plant PPOs retain full activity in the presence of SDS; poplar PPO is extracted in a latent form but is activated by SDS (Constabel et al., 2000). When analyzed on non-denaturing SDS-polyacrylamide gels, only single PPO activity bands were detected for both PPO-1 and PPO-2 preparations, which confirmed successful chromatographic separation of the PPO isoforms (Fig. 3). Interestingly, the difference in migration between the two PPOs as seen on western blots was also retained, with PPO-1 migrating faster than PPO-2. We concluded that each of our semi-purified preparations contained only one of the two PPO isoforms, and that these were suitable for further comparisons.

2.3. PPO stability, heat tolerance and pH optima

To investigate the stability of both PPO isoforms during storage, aliquots of both enzyme preparations were kept at −20 °C, and PPO activity was measured using 25 mM DOPA as substrate. PPO-1 activity decreased to 43% after one week, and continued to decrease until after 4 weeks at −20 °C, only 8% of the original activity remained. By contrast, for PPO-2 no loss of activity occurred in the same 4-week experimental period. Thus, PPO-2 was more stable in storage than PPO-1. Because of this differential stability, fresh PPO preparations were used for all subsequent experiments.

In order to study the tolerance of the PPO isoforms to heat treatment, both PPO preparations were incubated at temperatures between 40–80 °C. Aliquots were removed periodically, cooled on ice, and then assayed for PPO activity. Incubation at temperatures between 40–70 °C stimulated PPO-1 activity. This transient temperature-induced stimulation of PPO was not observed for PPO-2, and PPO-2 activity decreased progressively during incubation at 70 °C. Between 40–60 °C PPO-2 was not inactivated, but maintained 100% of its activity for the full 1 h period. Both isoforms were inactivated rapidly at 80 °C, again with PPO-2 being more sensitive. Therefore, PPO-1 appeared to be more tolerant of high temperature incubations, and was even activated at high temperatures compared to PPO-2. Plant PPOs generally appear to be robust enzymes; recently, a latex PPO from *Hevea brasilensis* was also shown to be very resistant to heat treatment, and at 60 °C this PPO retained 80% of the activity (Witisuwannakul et al., 2002).

When the pH activity profiles of the poplar PPO isoforms were compared, they both showed broad pH preferences (Fig. 4). Using catechol as the substrate, PPO-1 had the highest activity at pH 6, whereas the optimum for PPO-2 was pH 7. PPO-1 had a somewhat narrower pH range than PPO-2, which retained significant activity even at pH 4. The only previous report of poplar PPO, obtained from senescent *P. nigra* leaves, found the highest activity at pH 7.5, but also reported a similarly broad pH preference (Trémolières and Bieth, 1984).

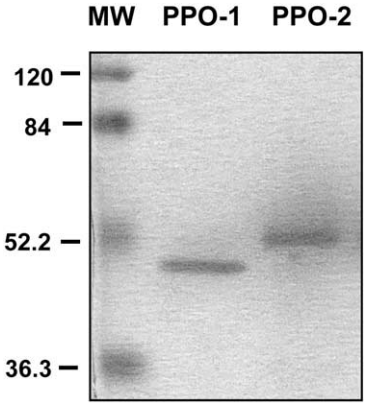

Fig. 3. PPO activity-stained gel of the two partially purified PPO isoforms. Proteins were electrophoresed on 12% SDS-PAGE gels without prior boiling and without ß-mercaptoethanol. Following electrophoresis, PPO activity was visualized using 25 mM DOPA in 100 mM sodium phosphate buffer. MW indicates prestained molecular weight marker.

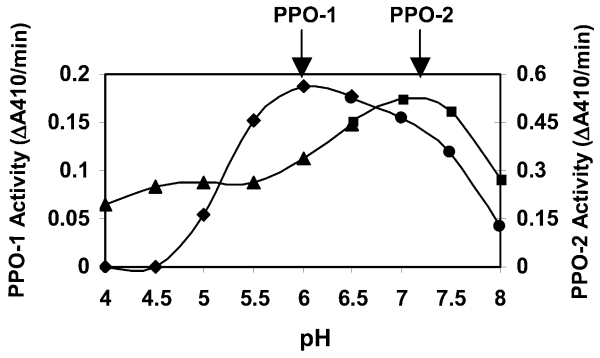

Fig. 4. Effect of assay buffer pH on activity of PPO-1 and PPO-2. 100 mM citrate phosphate buffer was used from pH 4.0 to pH 6.5 and 100 mM sodium phosphate buffer was used from pH 6.5 to pH 8.0. The pH optimum of PPO-1 and PPO-2 activity was measured using 10 mM catechol and the TNB assay.

2.4. Substrate specificities and K_m determinations

A major objective of this work was to identify potential differences in substrate preference between PPO-1 and PPO-2, since these may provide clues as to physiological function. A number of substrates were tested using the 2-nitro-5-thiobenzoic acid (TNB) assay, in which the PPO-generated quinones react with TNB and yield colorless Michael-type adducts (Esterbauer et al., 1977). This spectrophotometric assay monitors the consumption of TNB, and therefore allows for the direct comparison of enzyme rates with different substrates. PPO-1 showed the highest rates of oxidation with methyl catechol, followed by catechin > chlorogenic acid > DOPA > catechol > caffeic acid, in decreasing order (data not shown). This substrate profile is generally consistent with our previous analysis of PPO wound-induced leaves (Constabel et al., 2000). Minor differences were observed, likely because earlier data were obtained using crude leaf extracts. For PPO-2, the substrate preferences were catechol≈methyl catechol > > chlorogenic acid > caffeic acid > > catechin > DOPA (data not shown). Methyl catechol was clearly a good substrate for both enzymes, but surprisingly, catechol was a much poorer substrate for PPO-1 than for PPO-2. Conversely, catechin and DOPA were good substrates for PPO-1 but not for PPO-2. Overall, we concluded that PPO-2 has a greater substrate specificity than PPO-1. As a control, for all experiments and substrates we confirmed that the measured activity was inhibited by the PPO inhibitor tropolone (Kahn and Andrawis, 1985).

The pattern of substrate preference was confirmed by K_m values determined for the best PPO substrates (Table 1). Both PPOs had the lowest K_m with methyl catechol, 0.6 mM and 0.5 mM, respectively. However, PPO-2 had a much larger range of K_m values compared to PPO-1, consistent with earlier observations (see above). Again, the most dramatic differences were found for catechin, DOPA, and catechol, confirming previous findings. Trémolières and Bieth (1984) reported K_m values for *P. nigra* leaf PPO of 5.0 mM for catechol, chlorogenic acid and methyl catechol. We obtained similar values for PPO-1 (leaf PPO) as these authors, with the exception of our calculated K_m for methyl catechol of 0.6 mM (Table 1). This difference could be due to their use of senescent poplar leaves as a source of PPO. In general, the K_m values obtained are in the range commonly observed in plant PPOs with these substrates (Robert et al., 1996; Fujita et al., 1991; Billaud et al., 1996). No appreciable enzyme activity was detected with either PPO isoform using the monophenol 4-hydroxyanisol (Espín et al., 1998), suggesting that there is no monophenolase activity associated with either PPO.

2.5. Effects of inhibitors and activators

Tropolone is a very potent PPO inhibitor (Kahn and Andrawis, 1985) and was the most effective inhibitor for both PPOs, with less than 0.5% of either PPO activity remaining at 200 μM (Table 2). Kojic acid (Chen et al., 1991) and ferulic acid (Walker and McCallion, 1980) were also effective inhibitors of both PPOs. Therefore, both PPOs showed comparable sensitivity to common PPO inhibitors. Cetyltrimethylammonium bromide (CTAB), an inhibitor of laccase (*p*-diphenol oxidase), did not have an inhibitory effect on either poplar PPO isoform (data not shown).

To determine if PPO-1 and PPO-2 differ in their ability to be activated by SDS, both enzymes were assayed at different SDS concentrations. PPO-1 is fully activated with approximately 4 mM SDS, whereas PPO-2 had already achieved maximal activity at 1 mM (Fig. 5). Therefore, PPO-2 appears to be more sensitive to SDS. The identical results were obtained when the experiment was repeated using crude enzyme extracts, indicating that this effect is not dependent on different total protein concentrations (data not shown). There was no inhibitory effect of SDS concentrations greater than 10 mM (data not shown), although such a decrease in activity has been reported for fungal tyrosinases (Pérez-Gilabert et al., 2001). Proteases such as trypsin are also known to activate latent PPOs (Steffens et al., 1994), and both poplar PPO isoforms were activated by trypsin

Table 1
Michaelis–Menten constants (K_m) for PPO-1 and PPO-2[a]

Substrates	K_m (mM)	
	PPO-1	PPO-2
4-Methyl catechol	0.6	0.5
Chlorogenic acid	3.5	6.5
Catechin	3.7	24.3
Catechol	6.6	0.9
DOPA	21.1	79.5

[a] Michaelis–Menten constants (K_m) were determined using the TNB assay. The K_m for each substrate was calculated from a plot of 1/V vs. 1/[S] by the method of Lineweaver and Burk.

Table 2
Effects of inhibitors on PPO-1 and PPO-2 activity[a]

Inhibitors	PPO activity remaining (%)	
	PPO-1	PPO-2
Tropolone (200 μM)	0.43	0.25
Kojic acid (1 mM)	6.1	13.5
Ferulic acid (1 mM)	38.4	68.8
None	100.0	100.0

[a] Enzyme inhibition was determined using the TNB assay with 25 mM DOPA as substrate. Inhibitors were added to final concentrations as indicated.

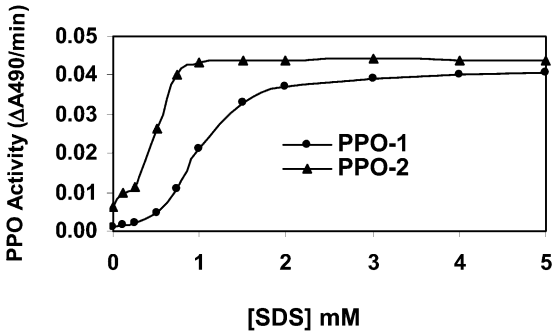

Fig. 5. Effect of SDS on poplar PPO isoforms. SDS was added to the assay buffer at different concentrations and PPO activity was determined immediately using a direct assay with 25 mM DOPA as substrate.

to 70% of maximal activity (data not shown). Analysis by SDS-PAGE and western blotting indicated that trypsin-treatment of PPO resulted in migration on SDS-PAGE at approximately 45 kDa. It thus appears that poplar PPO, like the enzyme from grape, has a protease sensitive site, and that removal of a C-terminal extension exposes the active site or activates it via a conformational shift (Dry and Robinson, 1992).

3. Discussion

Higher plant PPOs are often encoded by multigene families with complex regulation, and different tissues can express very distinct sets of PPO isoforms (Thipyapong et al., 1997; Thygesen et al., 1995). The significance of this complexity is not clear, in particular as the catalytic differences between these PPO isoforms have rarely been investigated. In hybrid poplar there are at least two PPO genes, and western blot analysis confirmed the presence of two major PPO protein isoforms (Fig. 1). PPO-1 was detected primarily in wound-induced leaves, whereas PPO-2 is expressed constitutively in midveins, petioles, stems and roots. We partially purified both PPO isoforms from poplar tissues in order to characterize these enzymes biochemically and determine differences which could provide clues as to differences in function.

Both PPO isoforms differed in stability, heat sensitivity, pH optimum, and activation by SDS; overall, the results are generally typical of plant PPOs, which are stable enzymes and have broad pH optima. Although both poplar PPOs are extracted in a latent form and require activation, PPOs from other plant sources are extracted in fully active form, for example from apricot (Chevalier et al., 1999). The most striking differences between PPO-1 and PPO-2 were observed in substrate preference. Our results indicate that PPO-1 accepts a broader range of substrates compared to PPO-2, as both catechin and DOPA are good PPO-1 sustrates (Table 1). Interestingly, PPOs from a number of plants are not able to oxidize catechins (Ho, 1999; Witisuwannakul et al., 2002). We speculate that this substrate difference between poplar PPO-1 and PPO-2 is indicative of differences in function. PPO-1 is wound-induced and active during tissue disruption due to insect feeding, where cross-linking and amino acid alkylation by the PPO-generated *o*-quinones can lead to a loss of amino acid assimilation by the insect gut. Similarily, PPO-1 may also be involved in pathogen defense. In these defense situations, one might expect selection for an enzyme with a broad range of acceptable substrates, such as PPO-1. Greater specificity, as observed with PPO-2, might indicate a separate physiological function, one that requires oxidation of particular phenolic substrates. The *in vivo* substrates of hybrid poplar PPO are as yet unknown. However, in *P. tremuloides*, there is indirect evidence that the abundant phenolic glycosides tremuloidin and salicortin are broken down in insect guts, releasing catechol as a possible PPO substrate (Clausen et al., 1989; Haruta et al., 2001). By contrast, in tomato leaves the most likely *in vivo* PPO substrate is chlorogenic acid (Li and Steffens, 2002).

Although PPO has been purified from many sources, in only a few studies have different PPO isoforms from the same species been compared directly. Ho (1999) characterized four PPO isoforms from orchid root, and Witisuwannakul et al. (2002) compared two PPOs from *Hevea brasiliensis* latex. In both of these studies, the PPO isoforms compared were very similar. By contrast, our data indicate that poplar PPO-1 and PPO-2 are quite distinct and show clear differences in substrate preference; together with their distinct patterns of expression, this may be indicative of differences in function. While PPO-1 is hypothesized to be an inducible anti-nutritive defense against folivore insects, the role of PPO-2 is unknown. It is possible that PPO-2 has a role in sealing of wounds, in pathogen defense, or other processes, and experiments to study PPO expression in poplar following stress treatments are under way. In addition, other novel roles for PPO have recently been discovered, for example in hydroxylation reactions during the biosynthesis of aurones in snapdragon flower pigments (Nakayama et al., 2000). Future work will focus on the cell-type localization of both PPO isoforms and on the identification of the PPO-2 encoding gene.

4. Experimental

4.1. Plant material

Saplings of poplar hybrid H11-11 (*Populus trichocarpa*×*P. deltoides*) were propagated and grown in environmental chambers with 16 h days at 21 °C as described previously (Constabel et al., 2000). *P. tricho-*

carpa (clone 93-968) was obtained from Dr. Carl Douglas, University of British Columbia, Vancouver, Canada, and *P. tremula*×*P. nigra* (clone 305-40) was obtained from Dr. Michael Carlson, British Columbia Ministry of Forests, Vernon, Canada. Plants were about two months old when used. To obtain wounded leaf samples, leaves were mechanically wounded by crushing leaf blades at the margins with pliers. Each leaf was wounded three times at 2 h intervals. Vascular tissue for purification included the stem and petioles between internode 1 and internode 10. All tissue samples were frozen in liquid N_2 and stored at −80 °C until use.

4.2. PPO assay and protein determination

Unless noted otherwise, all chemicals were purchased from Sigma (Oakville, Ontario). For routine measurements, PPO activity was measured spectrophotometrically by following the conversion of DL-DOPA (dihydroxyphenylalanine) to dopaquinone at 490 nm using a Shimadzu Model UV-1601PC spectrophotometer. DOPA (25 mM) was dissolved in 100 mM citrate phosphate buffer (pH 5.0) with 0.15% SDS and 470 units/ml catalase. Catechol was used as a substrate at a concentration of 10 mM, and the change in absorbance monitored at 410 nm.The assay buffer was aerated for 5 min prior to the assay. For some assays, a coupled TNB (2-nitro-5-thiobenzoic acid) assay was used (Esterbauer et al., 1977). The TNB standard reaction mixture contained 0.1 M citrate–phosphate buffer (pH 6.0), 0.05 mM TNB, and 0.15% SDS. The reaction was initiated by the addition of PPO enzyme, and the decrease in absorbance due to oxidation of TNB was followed at 412 nm. Under the conditions used, reaction rates were linear for at least 2 min. Approximately equal amounts of PPO-1 and PPO-2 activity were used for all comparative experiments. Depending on the experiment, between 20 and 60 units of PPO were used per assay, where one unit is defined as the amount of enzyme converting 1 μmol DOPA to dopaquinone per min, calculated using a molar extinction coefficient of 3600 M^{-1} cm^{-1} (Burton and Kirchmann, 1997). Because the TNB assay is more sensitive, lower amounts of enzyme activity were required for this assay. Protein concentration was determined with the Bradford reagent using bovine serum albumin as a standard (Bradford, 1976). All PPO assays were performed in triplicate and means reported. Variation was less than 5% between assays. All experiments were repeated at least three times.

4.3. Extraction and partial purification of PPO isoforms

Ground frozen plant material (3 g) was suspended in 30 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 1% Triton X-100, 80 mM ascorbic acid, 2% PVP-40, 2% Amberlite XAD-4, 5% PVPP, and protease inhibitor cocktails (Roche Molecular Biochemicals, Montreal), and was stirred on ice for 20 min. The homogenate was centrifuged at 16,000 *g* at 4 °C for 20 min and the supernatant used as the crude extract. Proteins were precipitated sequentially from the crude extract using 40 and 90% $(NH_4)_2SO_4$. Following centrifugation, the protein pellet was suspended in 10 ml of 0.5 M $(NH_4)_2SO_4$ in 10 mM sodium phosphate buffer, pH 7.0. The redissolved protein was loaded on a Phenyl Sepharose CL-4B column (diameter 1.5 cm, length 13 cm) equilibrated with the same buffer and eluted at a flow rate of 45 ml h^{-1}. After washing away unbound proteins with the equilibration buffer, the PPO activity was eluted using a linear gradient (0.5–0 M) of $(NH_4)_2SO_4$ in 10 mM sodium phosphate buffer (pH 7.0), followed by distilled water. The absorbance at 280 nm was monitored with a single path UV monitor and the PPO activity was determined on each 1.5 ml fraction using the DOPA assay. Fractions containing PPO activity were pooled, and the pooled fractions were dialyzed at 4 °C overnight against 100 mM sodium phosphate buffers pH 7.0. The dialyzed samples were aliquoted and kept at −20 °C for use in further experiments.

4.4. Immunoblotting

SDS-PAGE and immunoblotting was carried out using standard procedures (Harlow and Lane, 1988). Polyclonal anti-PPO antiserum was raised against recombinant PtdPPO protein (Christopher and Constabel, unpublished data). The antiserum was diluted 1:15,000 for immunostaining of the blots. Antibody binding was visualized using goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate in a dilution of 1:3000 (BioRad, USA) and alkaline phosphatase color development reagents 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT).

4.5. In-gel staining of PPO activity

Partially purified PPO-1 and PPO-2 (10 μl each), unboiled and without reducing agent, were applied to SDS-polyacrylamide gels and electrophoresed in the presence of SDS. Following electrophoresis, PPO activity was visualized by staining the gels with 25 mM DL-DOPA in 10 mM sodium phosphate buffer (pH 6.0) containing 0.15% (w/v) SDS and 470 units/ml catalase. Gels were stained until no further increase in color was apparent, usually within 30 min. The gels were dried between cellophane sheets and photographed.

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