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SOLANUM BERTHAULTII TRICHOMES CONTAIN UNIQUE POLYPHENOLOXIDASES AND A PEROXIDASE*

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Key Words Index—*Solanum berthaultii*; *Solanaceae*; wild potato; enzymology; polyphenoloxidase; peroxidase.

Abstract—Glandular trichomes of *Solanum berthaultii* leaves contain two unique forms of polyphenoloxidase (α- and β-PPO) and a separate peroxidase. The β-PPO appeared to be a polymer of α-PPO and exhibited a similar K_m but an apparently higher specific activity with chlorogenic acid and caffeic acid. These data are directly related to studies on the insect defensive properties of the trichomes.

INTRODUCTION

Solanum berthaultii, a diploid wild potato, is resistant to several insect species [1–4]. Resistance is mainly dependent on the presence of two types of foliar glandular trichomes [5]. Type A is a short trichome (*ca* 120–210 mu in length) with a tetralobulate membrane-bound gland (50–70 mu in diameter) at its apex. Type B is a longer (*ca* 600–950 mu in length), simple trichome with an ovoid gland at its tip which continuously discharges clear viscous exudate. Following insect contact, the four-lobed heads of Type A trichomes are ruptured releasing a clear exudate onto the insects legs and mouthparts. The exudate rapidly becomes brown and hard, the insects are immobilized and feeding is disrupted [2, 3, 5].

Polyphenoloxidase (PPO) and/or peroxidase (PO) activities are associated with the browning reaction [5–8]. In this paper we demonstrate that (a) the glandular trichomes contain two forms of PPO and a separate PO moiety; (b) the two forms of PPO differ in apparent M_r, v_{max} and specific activity with the probable endogenous substrates and (c) the lower M_r form (α-PPO) is localized exclusively in the trichomes.

RESULTS

Partial purifications of PPO and PO

PO and PPO activities were extracted from leaf trichomes by mechanical disruption of the trichomes with cotton swabs. Those extracts could be stored for at least one month at 4° without any loss of PPO activity; PO activity however disappeared within 6–8 hr after extraction from the trichomes.

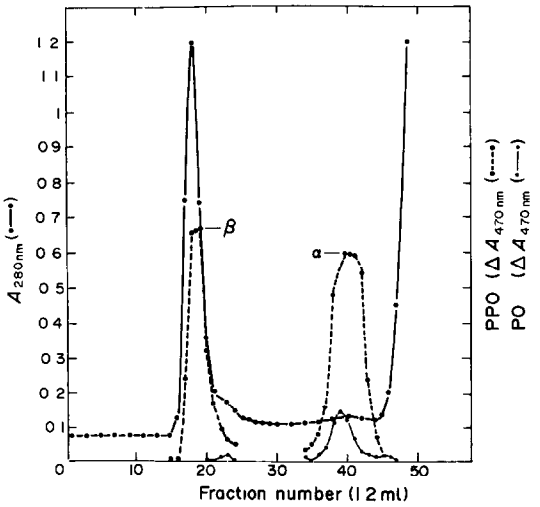

Fig. 1. Elution profile of the leaf trichome PPOs. The leaf trichome extract was applied, in minimal volume (< 0.6 ml), onto an AcA34 column (1.6 × 55 cm) equilibrated with 0.1 M NaPi (pH 7.0) and 50 mM sucrose. Fractions (1.2 ml) were eluted at a flow rate of 10 ml/hr. Assays were performed by the methods described in Experimental.

Two forms of PPO (α- and β-PPO) were separated on ultragel AcA 34 columns (Fig. 1). The β-enzyme eluted with the exclusion volume and appeared to be of high M_r. The pooled fractions were green and visible and UV spectral analysis of the fractions suggested the presence of chlorogenic acid. The α form of PPO eluted near the PO activity but ahead of phenols, and other small molecules which eluted last. The pooled fractions of the α form were clear and did not appear to contain chlorogenic acid by spectral analysis.

Chromatography on Ultragel A6 and CM Cellulose yielded similar elution patterns to those from AcA 34 columns where PO and α-PPO could not be separated. Similarly, electrophoresis on gradients of acrylamide in non-denaturing conditions (Fig. 2) separated the two

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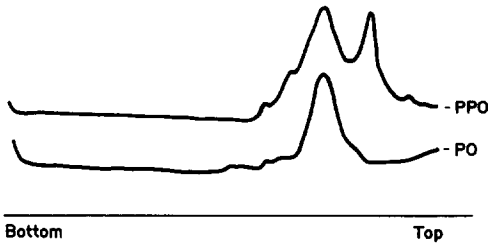

Fig. 2. Acrylamide gel electrophoresis of leaf trichome extracts under non-denaturing conditions. Electrophoresis and enzyme specific substrate-staining were performed by the methods described in Experimental.

forms of PPO but not PO from α-PPO. In this system however the α-PPO peak of activity exhibited several (three–four) shoulders suggesting the presence of isozymes of the α form.

Partial characterization of the two forms of trichome PPO

Ryan et al [5] have reported evidence that the PPO activity in unfractionated trichome extracts has greater affinity for *o*-diphenols than for mono- or tri-phenols thus suggesting the presence of an *o*-diphenolase (*o*-DPO) in trichome exudates. We have partially characterized the enzymic activities of α- and β-PPO by using the procedure of ref. [9]. This procedure is based on substrate specificity and selective inhibition measurements. *ortho*-Diphenolases specifically oxidize diphenols. Their inability to oxidize *p*-diphenols is a typical characteristic. By contrast, *p*-DPO (laccase) oxidizes a wide range of phenolic substrates including mono-, tri- as well as *o*- and *p*-diphenols [10]. Table 1 shows the reactivity of α- and β-PPO toward *o*-dipenols (chlorogenic acid, caffeic acid, catechol, DOPA, protocatechuic acid), *p*-diphenols (gentisic acid, hydroquinone) and an analogue (*p*-phenylenediamine), *m*-diphenol (orcinol), monophenols (*p*-coumaric acid, ferulic acid, guaiacol, *p*-hydroxybenzoic acid) and a triphenol(gallic acid).

Table 1. Reactivity of the two forms of PPO towards various phenolic substrates

Substrate	Specific activity (nmol O_2/min/mg protein) Form α	Form β
o-Diphenols		
1. Chlorogenic acid	0.97	9.75
2. Caffeic acid	0.97	9.79
3. Catechol	0.16	2.69
4. DOPA	0	0.44
5. Protocatechuric acid	0	0
p-Diphenols		
6. Gentisic acid	0	0
7. Hydroquinone	0	0.13
p-Diphenol analogue		
8. *p*-Phenelenediamine	0	0
m-Diphenol		
9. Orcinol	0	0
Monophenols		
10. *p*-Coumaric acid	0	0
11. Ferulic acid	0	0
12. Guaiacol	0	0
13. *p*-Hydroxybenzoic acid	0	0
Triphenols		
14. Gallic acid	0	0

Polyphenol oxidase activity in the presence of various phenolic substrates (10 mM) was measured by the oxygen depletion method described in Experimental.

Both α- and β-PPO oxidized *o*-diphenols (with the exception of DOPA and protocatechuic acid) but could not use any other phenolics as substrates (Table 1) thus suggesting that both forms of PPO are *o*-DPO.

Lerner *et al.* [11] and Walker and McCallion [9] reported that *o*-DPO but not *p*-DPO is strongly inhibited by cinnamic acid, *p*-coumaric acid, polyvinylpyrrolidone (PVP) and phenylhydrazine. The results in Table 2 show that cinnamic acid and *p*-coumaric acid are inhibitors of both forms of PPO; at 8 mM, *p*-coumaric acid was found to be a competitive inhibitor with respect to chlorogenic acid but at 20 mM, *p*-coumaric acid inhibited both forms of PPO in a non-competitive manner; 2% PVP inhibited the two forms only marginally and 1 mM phenylhydrazine had no effect.

Both forms of PPO showed a remarkable independence of the pH of the reaction mix. No peak of activity could be detected with either form of PPO; instead activity increased sharply from pH 4 to 6 and steadily thereafter, from pH 6 to 9. Enzyme kinetic experiments on α- and β-PPO involved oxidation of chlorogenic acid, a probable endogenous substrate. Thin-layer chromatography and HPLC of non-hydrolysed trichome extracts showed that chlorogenic acid was the major phenolic component whereas acid-hydrolysed extracts contained caffeic acid as the major component. Caffeic acid is the breakdown product resulting from hydrolysis of chlorogenic acid. Lineweaver–Burk [12] plots showed that the two forms of PPO were not inhibited by high concentrations of substrate, chlorogenic acid (up to 20 mM), the predominant phenol found in non-hydrolysed trichome extracts. In contrast, black poplar PPO was inhibited by high concentrations of chlorogenic acid [13]. The K_m calculated for α-PPO (0.85 mM) was only slightly lower than that calculated for β-PPO (K_m = 1.37 mM) suggesting similarities in affinity of the two forms of PPO for

Table 2. Effect of various inhibitors of *o*-DPO function on the α- and β-forms of PPO

Substrate	Specific activity (% of control) Form α	Form β
Chlorogenic acid (10 mM)	100	100
Chlorogenic acid + 1 mM phenylhydrazine	100	100
Chlorogenic acid + 2% (w/v) PVP	92	93
Chlorogenic acid + 20 mM cinnamic acid	71	72
Chlorogenic acid + 8 mM *p*-coumaric acid	84	81
Chlorogenic acid + 20 mM *p*-coumaric acid	47	57

Phenol oxidase activity was measured by the oxygen depletion method described in Experimental.

chlorogenic acid. Both forms of PPO exhibited the same lower affinity for caffeic acid (K_m = 3.4 mM). However, the V_{max} calculated for β-PPO (V_{max} = 119 μmol oxygen consumed/min/mg protein) was far greater than that of α-PPO (V_{max} = 5 μmol oxygen consumed/min/mg protein). Accordingly, the specific activity of the β-PPO was also greater that of the α-PPO (Table 1). However, the two forms of the enzyme are not pure (Fig. 3) and the differences in v_{max} and specific activity appear to be due in part to greater contamination in the α-PPO preparations.

M_r *estimation of α-and β-PPO*

M_rs were estimated on different types of columns since their large differences in M_r prevented the use of a single column. The α-PPO migrated on AcA 34 column (M_r range: 25 000–325 000) with an R_f corresponding to a M_r of 58 000 ± 7000 (average of five experiments) (Fig. 1). The standard curve was constructed with a field of standards ranging from 53 000 (M_r triosephosphate isomerase) to 550 000 M_r (ribulose bisphosphate carboxylase). The PO peak of activity migrated with an R_f corresponding to a M_r of 72 000 ± 7000. The β-PPO eluted with the exclusion volume on AcA 34 (M_r > 325 000) (Fig. 1) but on an Ultragel A6 column (accurate up to M_r: 2.4×10^6), it migrated with an R_f corresponding to a M_r of 1.2×10^6 in a field of M_r standards ranging from 106 000 (hexokinase) to 1.33×10^6 (phosphorylase kinase).

Electrophoresis of the α- and β-PPO fractions from AcA34 columns on SDS slab gels revealed that the major band in the α-PPO fraction migrated with an R_f corresponding to a M_r of 51 000 (Fig. 3), consistent with the previous M_r estimation of 59 000 obtained on AcA 34 column. However, even though the β-PPO fraction exhibited only one major band and a few minor bands on SDS gels (Fig. 3), its major band appeared to co-migrate with the major band found in the α-PPO fractions, giving the β-PPO an estimated M_r of 51 000 or 4.25% of the M_r estimated by column chromatography. The two enzyme forms also yielded a similar proteolytic degradation product at 32 000 M_r after mild digestion in the presence of 0.1 μg trypsin/ml for 10 min at 8–10° [Fig. 3, lane 3 (β-PPO) and lane 5 (α-PPO)].

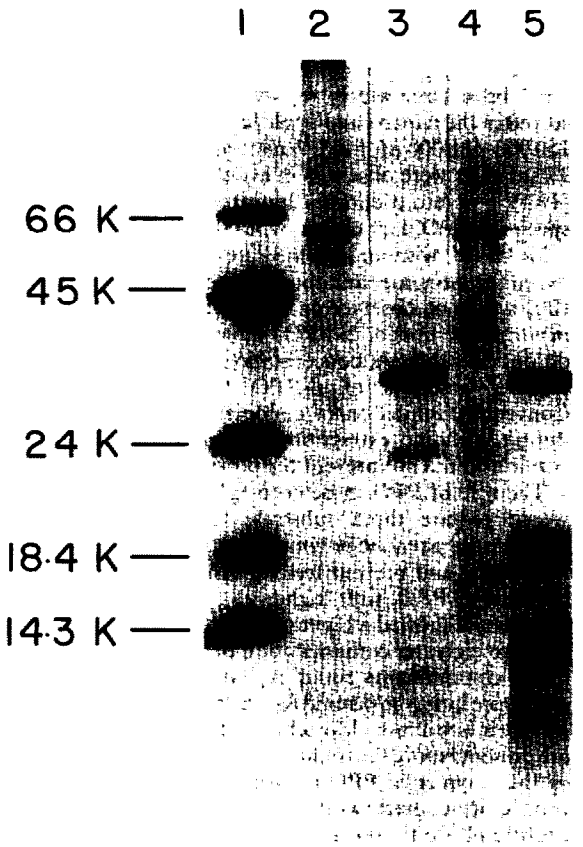

Fig. 3. SDS-PAGE of α- and β-PPO. The two activity peaks eluted from the AcA 34 column were concentrated and electrophoresed on SDS gels by the method of ref. [24]. The calibration curve was determined using (lane 1) bovine serum albumin (M_r: 66 000), egg albumin (45 000), trypsinogen (24 000), β-lactoglobulin (18 4000) and lysozyme (14 300) as protein markers. The β-PPO (20 μg) was loaded in lane 2 and, after trypsin treatment, in lane 3. The α-PPO (20 μg) was loaded in lane 4 and, after trypsin treatment, in lane 5.

Isoelectric focusing (IEF) of PPO and PO from trichomes, the cytosol and chloroplasts

Catechol-stained IEF gels showed one major band and several minor bands of PPO activity (5.0 < pH < 5.6) (Fig. 4). A slight variation (± 0.2 pH unit) in the estimated

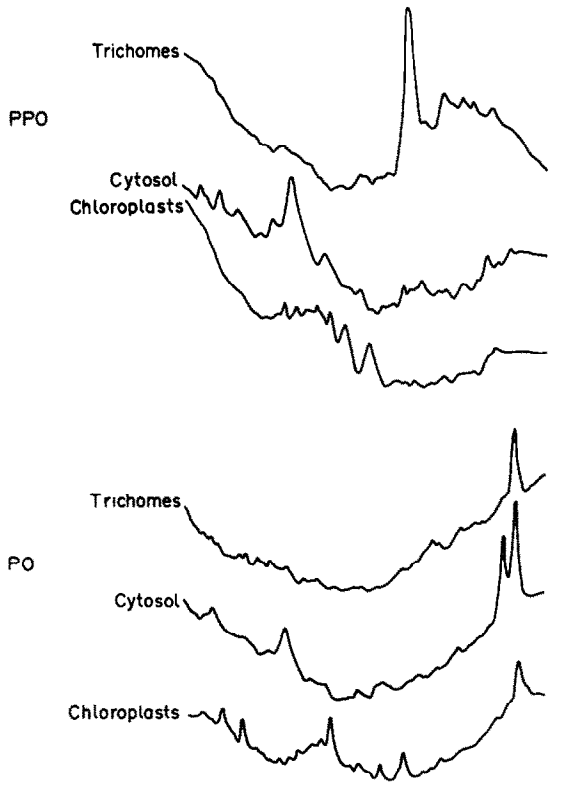

Fig. 4. Isoelectric focusing of PPOs and POs extracted from trichomes, cytosol and chloroplasts of potato leaves. The various leaf fractions were electrophoresed by isoelectric focusing on 5% acrylamide gels in 3.5–9.5 pH range. The gels were stained for PPO or PO activity in enzyme specific substrate solution as described in Experimental. The stained gels were scanned by densitometry.

isoelectric point (pI) of each band was observed from experiment to experiment; the relative intensity of the minor bands also varied. These variations were attributed to the variability in general health of the greenhouse-grown plants from which the samples were taken from day to day. A strong band of PPO activity was also found at the point of application of the trichome extract. This band was probably β-PPO because focusing of β-PPO fractions from the AcA34 column yielded a single band of PPO activity at multiple points of application.

Even though the PO activity in trichome extracts was found to comigrate with the α-PPO in molecular sieves, its low pI ($3.0 < pH < 3.3$) facilitated separation from the α-PPO on IEF (Fig. 4).

On IEF gels the trichomes yielded several bands of PPO activity located between pH 5.0 and pH 5.6 (Fig. 4). The cytosol and the chloroplast also yielded several peaks of PPO activities (Fig. 4). These PPOs were located, for the most part, in the basic zone of the pH gradient, above pH 6.0; the area below pH 6.0 containing only minor bands (cytosol) or no band at all (chloroplast). Thus the leaf trichome PPOs appeared to be unique, with no counterparts in the leaf chloroplast or cytosol.

Comparison of trichome PO with peroxidases from leaf chloroplasts and cytosol showed that chloroplasts and cytoplasm contain more forms of PO than the trichomes (Fig. 4). The two largest peaks however, located in the acidic zone of the IEF gel ($3.0 < pH < 3.3$) were present in the trichome, chloroplast and cytosol extracts.

DISCUSSION

The co-purification of PO and PPO reported here has also been observed during the isolation of PPO from black poplar [13] and tomato [14]. The tomato enzymes eluted at identical positions on column chromatography and electrophoresis and had identical isoelectric points. The potato trichome PO and α-PPO activities also comigrated on AcA34 columns and electrophoresis but their pIs were sufficiently different to afford a good separation by isoelectric focusing (Fig. 4). The data thus show that the PO and PPO activities found in potato trichomes are not associated with the same enzyme but rather are attributable to the presence of two distinct enzymatic species.

Both forms of potato trichome PPO oxidized several *o*-diphenols but, with the exception of weak hydroquinone oxidation by β-PPO, did not oxidize other classes of phenols (Table 1). That specificity for *o*-diphenols is a characteristic of *o*-DPO. The K_m values of α and β-PPO for chlorogenic acid ($K_{m_\alpha} = 1.37$ mM; $K_{m_\beta} = 0.84$ mM) were similar and characteristic of the affinity of plant catechol oxidases for phenolic substrates ($K_m \approx 1$ mM) [10]; *p*-coumaric acid inhibited α- and β-PPO to the same extent (Table 2). At 8 mM, *p*-coumaric acid was found to be a competitive inhibitor with respect to chlorogenic acid but at 20 mM, *p*-coumaric acid inhibited both forms of PPO in a non-competitive manner, suggesting that at low concentration, *p*-coumaric acid acts on the binding site for phenolic substrates but that at higher concentrations, it acts at a different site, possibly an 'inhibitor site'. Cinnamic acid, even at high concentrations, inhibited only partially the two forms of trichome PPO. Phenylhydrazine was shown to act on PPOs at a specific 'inhibitor site' [15]. However, phenylhydrazine showed no effect on trichome PPO (Table 2), suggesting the absence of such an inhibitor site in this system. PVP was shown to inhibit plant PPOs irreversibly, by combining with the enzyme–substrate complex even in the absence of substrate [16, 17]. However the potato leaf trichome PPOs were unusual in being virtually uninhibited by PVP (Table 2). Thus the data suggests that the trichome enzymes are PPOs even though some characteristics make them unique.

Even though the α- and the β-PPO have widely different M_rs on AcA34 columns, SDS-PAGE data showed that both enzymatic species migrated with an estimated M_r of 51 000. The presence of phenolics adsorbed to the β-PPO could have produced this artefact. Phenolics associated with β-PPO, however, rather than its size appear to prevent the migration of the enzyme on isoelectric focusing gels. It is unlikely that the pI of the enzyme corresponded to its point of application on the gel since purified β-PPO did not migrate from multiple points of application on IEF gels.

The results are consistent with the presence, in trichome extracts, of (a) a PPO with characteristics of a σ-DPO. Its estimated M_r was 58 000; (b) two to four isozymes of α-PPO with slightly different pI and M_r and (c) a polymer with *ca* 24 subunits of α-PPO and a M_r of 1.2×10^6.

The presence of a large PPO polymer appears to be unique to the potato trichomes. Even though polymers of PPO have been widely reported, the largest PPO observed in the banana and apple leaves was estimated to be 150 000–180 000 M_r [10]. While aggregates of 2, 4, 8, and 17 subunits were observed in avocado and potato tubers [18,19], potato trichomes appeared to contain only two species of PPO, a monomer of 58 000 M_r and a polymer of 1.2×10^6 M_r without intermediates. Interconversions between the monomer and the oligomer as in the apple fruit [20] were not observed in potato trichomes. In several *in vitro* experiments, α-PPO, submitted to various conditions of substrate concentrations and the presence of priming amounts of β-PPO failed to polymerize. Conversely, inhibition of endogenous phenol oxidation during trichome extraction with cysteine, DTT or ascorbic acid did not prevent the polymerization of β-PPO.

The role of PPO in defence against insect infestation would require rapid substrate turnover in order to produce the phenolic polymers that would glue the insect mouthpart and prevent feeding. Thus the presence of a polymer of PPO, with higher v_{max} would be consistent. Our results showed a higher v_{max} for the polymer of PPO but also a greater contamination of the α-PPO fractions. These contaminants could explain, at least in part, the difference in v_{max} measured for the monomer and polymer PPOs. It is still not clear whether the two forms of PPO are present before the trichomes are ruptured by the insect or the polymer of PPO is formed after the monomer is primed its contact with the phenolic substrates upon rupture of the trichomes.

It is possible that the PO found in trichome extracts are only contaminants from the leaf cytosol or chloroplasts. But whether PO is endogenous to the trichome or not, its role in the defence of the plant against insects has not been established. However the trichome PPOs are likely involved in the plant defence mechanism against insects since they are unique to the trichomes and appear different from the PPOs found in the leaf cytosol and chloroplasts. Their restriction to the trichome and their biochemical complexity strongly suggest a role in the defence of the plant against insects.

EXPERIMENTAL

Trichomes extracts were collected at 4° from *Solanum berthaultii* (Hawkes) clone PI 310927-11 with cotton swabs dampened with 50 mM sucrose, 100 mM NaPi (pH 7.0) and 10 mM cysteine [6]. Trichomes were discharged by 3–5 light passes of the damp swab over the abaxial leaf surfaces as described in ref. [21]. This operation was performed on a glass plate kept on ice. Trichome exudate was collected by application of gentle pressure on the swab.

Enzyme purification. The trichome extract was squeezed from the cotton swabs and chromatographed at room temp. on an ultragel AcA 34 column (1.6 × 55 cm) equilibrated with the extraction buffer without cysteine. The flow rate was maintained at 10 ml/hr. Fractions showing substantial PPO activity were pooled and stored frozen.

Trichome phenol characterization. Leaflets (150–200) were wiped with cheesecloth that had been soaked in 60% EtOH. The cheesecloth was then boiled in 60% EtOH for 30 min, frozen overnight, thawed at room temp. and sonicated for 5 min. The cheesecloth was removed from the solution and the remaining extract squeezed out. The total extract was filtered and evaporated to dryness. After resuspension in 30% EtOH and three extractions with Et_2O, the alcohol fraction was divided evenly into two aliquots. Both aliquots were evaporated to dryness. One aliquot (for analysis of non-hydrolysed phenols) was resuspended in MeCN–H_2O (4:1), passed through a 0.5 μm Millipore fluoropore filter and retained for TLC and HPLC. The second aliquot (for analysis of hydrolysed phenols) was dissolved in 2 M HCl, boiled for 30 min, cooled and extracted three times with Et_2O. The Et_2O extracts were combined, evaporated to dryness, redissolved in MeCN–H_2O (4:1), passed through a 0.5 μm Millipore fluoropore filter and retained for HPLC. Part of this preparation was evaporated to dryness and redissolved in 80% EtOH for TLC.

One dimensional TLC was performed on silica gel G or GF with toluene–ethyl formate–formic acid (5:4:1) [22]. Phenols were visualized with Folin's reagent and fumed with NH_4OH.

HPLC was performed with a Bondapak C-18/Corasil guard column (Waters Associates). The mobile phase was 5% HOAc adjusted to pH 3.0 with KOH or NaOH and delivered at a flow rate of 1.0 ml/min with a pressure of 1000–1500 psi. Column effluent was monitored at 280 mμ.

PAGE. Non-denaturing 8–12% acrylamide tube gels were run in Tris–glycine discontinuous system according to the method of ref. [23] for 4 hr at 4 V per tube. The gels were then stained for PPO or PO activity and scanned at 470 nm on a Gilford spectrophotometer using a special tube gel scanner. Denaturing 12% acrylamide slab gels were run in SDS–Tris-glycine discontinuous system according to the method of Laemmli [24] for 14 hr at 40 V. The gels were then stained in Coomassie Brilliant Blue R 250 [0.025% in H_2O–MeOH–HOAc (63:30:7)], destained in H_2O–MeOH–HOAc (63:30:7), dried and scanned with a LKB model 2202 laser densitometer.

Subcellular fractionation. Potato leaves were wiped twice by the cotton swab technique described above before grinding with a mortar and pestle in 0.3 M sucrose, 35 mM NaPi (pH: 7.4) and 15 mM cysteine. The brei was filtered through one layer of Miracloth and centrifuged for 10 min at 12 000 g. The supernatant was used as our cytosolic fraction. The pellet was resuspended in grinding buffer supplemented with 0.3% Triton X-100 and centrifuged at 12 000 g for 10 min. The supernatant contained the solubilized chloroplast components and was used for the chloroplast fraction.

Isoelectric focusing. Crude subcellular or trichome extracts and partially purified PPO fractions were layered (without swabs) on a LKB PAG plate (pH 3.5–9.5 in a 5% acrylamide gel) in drops of 5–50 μl and electrophoresized for 1 hr at 30 W at 10°. The gel was stained in a PO stain (0.1 M NaOAc, pH 5.0, 2.7 mM 3-amino-9-ethylcarbazole, 5.6% dimethylformamide and 0.37% H_2O_2) or a PPO stain (0.1 M NaPi, pH 7.0, 0.3 M catechol and 90 mM *p*-phenylenediamine) for 5–10 min and dried on a B10-RAD gel dryer or photographed on Polaroid type 55 film. The dried gel or its photograph was then scanned by laser densitometry.

Enzymatic activities. Polyphenol oxidase and peroxidase activities were measured spectrophotometrically at 470 nm with 45 mM catechol and 4.5 mM *p*-phenylenediamine. Alternately, kinetic measurements were determined by monitoring O_2-consumption with a Yellow Springs Instruments Co. oxygen probe and Model 53 oxygen monitor, in 50 mM sucrose, 100 mM NaPi pH 7.0 at room temp. Initial rates were calcd by drawing tangents to the kinetic curves.

Protein estimation. Protein was estimated by the alkaline phenol method as total N_2. The presence of endogenous phenolics in the trichome extracts as well as in the β-PPO fractions did not interfere with the assay of N_2. Protein samples (50 μl) were boiled in 2 ml of 1 M H_2SO_4 for 4–6 hr. The samples were cooled and mixed with 3.0 ml of 0.6 M NaOH, 12.5 mM NaEDTA and 200 mM $KNaC_4H_4O_6 \cdot 4H_2O$. One ml of 26% (w/v) phenol in 4.7 M NAOH was added to the assay. Finally, 0.8 ml of 4.2% Na hypochlorite was added and each tube was vortexed. After 5 min, absorbance was measured at 625 nm. Colour was stable for up to 24 hr. A standard curve was prepared with BSA.

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