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Biochemical Characterization of the Suberization-Associated Anionic Peroxidase of Potato[1]

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The anionic peroxidase associated with the suberization response in potato (*Solanum tuberosum* L.) tubers during wound healing has been purified and partially characterized at the biochemical level. It is a 45-kD, class III (plant secretory) peroxidase that is localized to suberizing tissues and shows a preference for feruloyl (*o*-methoxyphenol)-substituted substrates (order of substrate preference: feruloyl > caffeoyl > *p*-coumaryl ≈ syringyl) such as those that accumulate in tubers during wound healing. There was little influence on oxidation by side chain derivatization, although hydroxycinnamates were preferred over the corresponding hydroxycinnamyl alcohols. The substrate specificity pattern is consistent with the natural substrate incorporation into potato wound suberin. In contrast, the cationic peroxidase(s) induced in response to wound healing in potato tubers is present in both suberizing and nonsuberizing tissues and does not discriminate between hydroxycinnamates and hydroxycinnamyl alcohols. A synthetic polymer prepared using *E*-[8-^{13}C]ferulic acid, H_2O_2, and the purified anionic enzyme contained a significant amount of cross-linking through C-8, albeit with retention of unsaturation.

Suberization is a tissue-specific process whereby cell walls become impregnated with a poly(phenolic) matrix coincident with the deposition of a poly(aliphatic) matrix between the plasmalemma and carbohydrate cell wall (for review, see Bernards and Lewis, 1998). While the nature of the phenolic matrix remains incompletely defined, it has recently been shown that in potato (*Solanum tuberosum* L.) tubers it comprises primarily hydroxycinnamic acids (especially no. **3**; see Scheme 1 for numbering), and their derivatives (especially no. **3b**) (Bernards et al., 1995; Negrel et al., 1996). It has also been shown indirectly that in the suberized tissues of *Quercus suber* (Gil et al., 1997) and *Clivia miniata* (Schreiber, 1996; Zeier and Schreiber, 1997), there is a significant amount of hydroxycinnamic acid (especially nos. **1** and **3**) present in the cell walls. These data suggest that the poly(phenolic) component of suberized cell walls is unique and distinct from cells that are lignified, where the poly(phenolic) matrix comprises oxidatively cross-linked hydroxycinnamyl alcohols (i.e. the monolignols **5a**, **6a**, and **7a**) (Lewis and Yamamoto, 1990).

The macromolecular assembly process whereby monomeric hydroxycinnamic acids (and/or their derivatives) are transported to and subsequently incorporated (i.e. polymerized) into the carbohydrate cell wall matrix remains undefined. By analogy to the oxidative cross-linking model accepted for lignification, it has been hypothesized that the phenolic component of suberized cell walls is polymerized via a peroxidase/H_2O_2-mediated process (Kolattukudy, 1980). In this regard, an anionic peroxidase has been shown to be both temporally and spatially associated with the wound-induced suberization process in potato tubers (Borchert, 1978; Borchert and Decedue, 1978; Espelie and Kolattukudy, 1985; Espelie et al., 1986).

While the suberin-associated anionic peroxidase has not been fully characterized biochemically, it has been cloned and its molecular biology studied. Thus, a cDNA clone of the wound-induced anionic peroxidase of potato (Roberts et al., 1988; Roberts and Kolattukudy, 1989) was used to isolate a genomic clone (containing two tandemly oriented anionic peroxidase genes of 96% and 87% homology) from tomato (*Lycopersicon esculentum* Mill.) (Roberts et al., 1988). Expression studies of one of these genes, *TAP 1* (tomato anionic peroxidase 1), demonstrated both a stress-induced and a developmentally regulated role for this peroxidase (Mohan and Kolattukudy, 1990; Mohan et al., 1993a, 1993b; Sherf and Kolattukudy, 1993). However, with its expression silenced in antisense tomato transformants, the incorporation of phenolics into the cell walls of wounded fruits (judged cytochemically by autofluorescence) continued unabated (Sherf et al., 1993). Thus, the suberin-specific role for the anionic peroxidase of potato and tomato remains tenuous, and more definitive evidence is required to unambiguously assign this specific function to it.

The specificity with which purified peroxidases oxidize different phenolic substrates (e.g. Converso and Fernandez, 1995; Marquez and Dunford, 1995; Pomar et al., 1997; Loukili et al., 1999), and characterization of the in vitro products formed when they are reacted with specific phenolic substrates (e.g. Lewis et al., 1987; Zimmerlin et al.,

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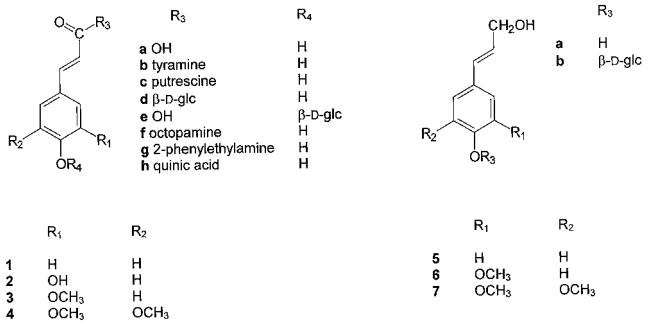


Scheme 1. Hydroxycinnamic acid and hydroxycinnamyl alcohol derivatives used as substrates in this study. The aromatic ring substitution pattern is denoted by a number (i.e. hydroxycinnamates 1–4 and hydroxycinnamyl alcohols 5–7), while derivatives are denoted by lowercase letters. Not every possible derivative was used; refer to Table II for a complete listing of the 25 phenolics used in the substrate specificity study.

1994; Wallace and Fry, 1995) may provide clues to their in vivo role(s). However, for most peroxidase isoforms, these properties remain untested. We describe our progress in characterizing the wound-induced anionic peroxidase from potato at the biochemical level, particularly with respect to some of its basic biochemical and enzymological properties, including a major substrate specificity assessment.

MATERIALS AND METHODS

General

Solvents were of analytical or HPLC grade. Hydroxycinnamic acids (referred to by numbers as given in Scheme 1) **1a**, **2a**, **3a**, and **4a**, chlorogenic acid **2h**, and coniferyl alcohol **6a** were purchased from Aldrich. *p*-Coumaryl **5a** and sinapyl **7a** alcohols were a kind gift of Dr. Norman G. Lewis (Washington State University, Pullman). *N*-Feruloyloctopamine **3f** was a kind gift of Dr. Jonathan Negrel (Institut National de la Recherche Agronomique, Dijon, France). All other substrates were either synthesized according to published protocols or isolated from natural sources (see below). Horseradish peroxidase type VIII (anionic) was purchased from Sigma.

Plant Material

Potato (*Solanum tuberosum* cv Russet Burbank) tubers were obtained from Monashee Mountain Seed Potatoes (Lumby, British Columbia, Canada), a member of the British Columbia Seed Potato Growers Association, and propagated in the Prince George, British Columbia, Canada, area. Tubers were harvested each fall and stored at 5°C in the dark until used. Suberization was induced by slicing surface-sterilized tubers into 0.5- to 1-cm-thick cross-sectional pieces, and incubating them in sterile Magenta boxes as described previously (Bernards and Lewis, 1992). For purification of the anionic peroxidase, acetone powders (Espelie and Kolattukudy, 1985) prepared from the mechanically removed suberized layers of 7-d wound-healed tubers were used.

Isoform Analysis

Total soluble protein extracts were prepared separately from 1 g each of suberized and nonsuberized (i.e. the tissue immediately underlying the suberized layer) tissues collected 7 d post wounding, for 30 min on ice in 10 mL of cold extraction buffer (50 mM potassium phosphate, pH 7.5, containing 300 mM Suc, 20 mM KCl, 10 mM DTT [added fresh at the time of extraction], 3 mM EDTA, and 0.1 mM $MgCl_2$). After centrifugation at 13,500*g*, the supernatant was desalted (model P6-DG, Bio-Rad) into 25 mM Bis-Tris-iminodiacetate, pH 7.1, and chromatofocused on a Mono-P HR 5/5 column (Pharmacia) over a 7.1 to 3.5 pH range. The pH gradient was generated with buffer (Polybuffer 74, Pharmacia, pH adjusted to 3.5 with saturated iminodiacetate) at a flow rate of 0.5 mL min^{-1}. Fractions (0.5 mL) were assayed spectrophotometrically using both guaiacol/H_2O_2 (20 mM/10 mM; 470 nm) and ferulic acid/H_2O_2 (0.15 mM/2 mM; 310 nm).

Purification of Anionic Peroxidase

All purification steps were performed at 4°C or on ice. Column fractions were assayed for peroxidase activity spectrophotometrically using 20 mM guaiacol and 20 mM H_2O_2 in acetate buffer (20 mM, pH 5.0) by following the oxidation of guaiacol at 470 nm. Column eluants were monitored at 280 nm.

A total of 100 g of acetone powder (obtained from approximately 530 g of suberized layers) was extracted in 20 separate 5-g batches. For each batch, proteins were extracted with 40 mL of cold extraction buffer (50 mM potassium-phosphate, pH 7.5, containing 300 mM Suc, 20 mM KCl, 10 mM DTT [added fresh at the time of extraction], 3 mM EDTA, and 0.1 mM $MgCl_2$) on ice for 30 min with occasional stirring. After squeezing through eight layers of cheesecloth, the extract was centrifuged (10,000*g*, 20 min, 4°C) and the supernatant was immediately loaded onto a 2.5- × 28-cm Sephadex G25-M column (Pharmacia) pre-equilibrated with 25 mM Tris-HCl, pH 7.5, and gravity eluted with 25 mM Tris-HCl, pH 7.5, to separate the proteins from low-molecular-mass phenolics present in the extract. The protein fraction was collected and brought to 50% saturation with solid $(NH_4)_2SO_4$. After centrifugation at 20,000*g* for 10 min at 4°C, the supernatant was brought to 90% saturation with solid $(NH_4)_2SO_4$ and recentrifuged at 20,000*g* for 10 min at 4°C). The 50% to 90% $(NH_4)_2SO_4$ pellets were reconstituted in a minimum volume of 25 mM Tris-HCl, pH 7.5, stored at −40°C. The 50% to 90% $(NH_4)_2SO_4$ pellets were pooled, desalted into 25 mM Bis-Tris, pH 7.1 (Sephadex G25-M, 2.5- × 28-cm), concentrated by ultrafiltration (YM 10 membrane, Amicon, Beverly, MA), loaded onto a 2.5- × 100-cm Sephadex G100 column (Pharmacia) pre-equilibrated with 25 mM Bis-Tris, pH 7.1, in five 8- to 10-mL batches, and eluted with 25 mM Bis-Tris, pH 7.1, at 0.2 mL min^{-1}.

Fractions from Sephadex G100 containing peroxidase activity were pooled, concentrated to 10 mL by ultrafiltration (YM 10 membrane, Amicon), and loaded onto a 1.5- × 18-cm polybuffer exchanger (PBE) column (Pharmacia)

pre-equilibrated with 25 mM Bis-Tris, pH 7.1). Proteins were eluted first with equilibration buffer followed by a pH gradient (7.1−3.5) generated with buffer (Polybuffer 74 diluted 1:8 with water) at pH 3.5 at 0.5 mL min^{-1}. Fractions containing peroxidase activity were pooled, desalted, and concentrated by ultrafiltration (YM 10 membrane, Amicon) into 25 mM Bis-Tris, pH 7.1.

The anionic peroxidase from PBE was loaded onto a 1.0- × 6.5-cm DEAE Sepharose Fast Flow column (Pharmacia) pre-equilibrated with 25 mM Bis-Tris, pH 7.1, and eluted with a salt gradient (0–300 mM NaCl in 25 mM Bis-Tris, pH 7.1, over 60 min) at 1 mL min^{-1}. Fractions containing peroxidase activity were pooled, desalted, and concentrated by ultrafiltration (YM 10 membrane, Amicon) into 25 mM Bis-Tris, pH 7.1. The cationic peroxidase from PBE was retained without further purification.

Protein concentrations were estimated by the micromethod modification of the Bradford assay (Bradford, 1976) using commercially available dye reagent (Bio-Rad) and bovine-γ-globulins as standards, according to the manufacturer's instructions. The concentration of pure enzyme was estimated using a molar extinction coefficient (ϵ_{405}) value of 105 mM^{-1} cm^{-1}.

SDS-PAGE

SDS-PAGE was carried out using 14% acrylamide gels essentially as originally described (Laemmli, 1970) and modified for the Bio-Rad Mini Protean system according to the manufacturer's instructions but without boiling. Gels were silver-stained using a modified protocol of de Moreno et al. (1985). After successive fixing with 20% TCA (minimum 2 h) and MeOH:HOAc:H_2O (4:1:5) (3 × 15 min), gels were rinsed with water (2 × 15 min) and treated with 0.1% $AgNO_3$ (1 h). After rinsing (2 × 10 s), protein bands were visualized using successive washes (3 × 100 mL) with a developer solution (3% [w/v] Na_2CO_3 containing 0.0185% [w/v] formaldehyde). Color development was stopped using 2.3 M citric acid (7.5 mL/100 mL developer solution).

Gels were internally calibrated using a low-molecular-mass marker kit (Pharmacia) containing phosphorylase B (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.1 kD). Peroxidase activity was visualized in gels without prior silver-staining using guaiacol/H_2O_2 (50 mM each in 50 mM acetate buffer, pH 5), after first rinsing the gels with water (2 × 15 min) to remove SDS. The reaction was stopped by removing the substrates and rinsing the gels with water.

Calibrated Molecular Sieving Chromatography

A Bio-Prep SE 100/17 column (Bio-Rad, molecular mass range 5–100 kD) was calibrated using thyroglobulin A (670 kD void volume estimate), IgG (150 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), myoglobin (17 kD), RNase A (13.7 kD), and vitamin B_{12} (1.3 kD total volume estimate). Standard solutions (5 mg mL^{-1}) of BSA, carbonic anhydrase, and RNase A were prepared in elution buffer (20 mM Tris-HCl, pH 7.5, containing 150 mM KCl). The remaining standards were part of a calibration kit (Bio-Rad) and were prepared according to the manufacturer's instructions. Samples were loaded individually (100 μL) and eluted with elution buffer at 0.25 mL min^{-1}. For purified anionic peroxidase, 100 μL of a 5 μM solution in elution buffer was used. Fractions (0.25 mL) were collected and assayed for activity using guaiacol/H_2O_2.

Chemical Deglycosylation

Purified anionic peroxidase (35 μg) was deglycosylated according to the method of O'Donnell et al. (1992) and analyzed by SDS-PAGE followed by silver staining.

Enzyme Assays

Potato anionic peroxidase, potato cationic peroxidase, and horseradish type VIII (anionic) peroxidase were assayed at a final concentration of 0.5 nM. A molar extinction coefficient of 105 mM^{-1} cm^{-1} was used to adjust their concentrations. For ascorbate and guaiacol substrates, the method of Amako et al. (1994) was used essentially as described, except guaiacol was substituted for pyrogallol. For phenolic substrate specificity assays, solutions were preincubated at 40°C. The following quasi-rapid mixing method, based on that described by Rasmussen et al. (1995), was employed: anionic peroxidase (1 nM) and phenolic substrate (0–0.4 mM), in assay buffer (50 mM citrate buffer, pH 4.5 or 6.5, containing 1 mM $CaCl_2$) was placed in one syringe (3 mL), while H_2O_2 (4 mM) in assay buffer (total 3 mL) was placed in another. Equal volumes (1 mL) of each solution were mixed by simultaneous injection into a flow cell (75 μL internal volume) and the initial rate of substrate disappearance was monitored for 30 s. Triplicate reactions were measured for each syringe filling and each substrate concentration, with each repeated at least three times. Slopes (in absorbance units min^{-1}) were measured for the initial, linear phase of the reaction (usually over 5–10 s). The data were fitted to straight lines using Wolfe-Hanes transformations, and apparent maximum rates (V_{max}^{app}) values were extrapolated from intercepts.

Synthesis of Phenolic Substrates

N-(Hydroxycinnamoyl)tyramine derivatives **1b**, **2b**, **3b**, and **4b** and the 2-(phenyl)-ethylamine analog **3g** were synthesized according to the method of Villegas and Brodelius (1990). *N*-(Hydroxycinnamoyl)putrescine derivatives **1c** and **3c** were synthesized according to the method of Malmberg (1984) and purified on a 1.5- × 30-cm polyamide column (model SC6, Machery-Nagel, Duren, Germany, pre-equilibrated with water) eluted with water. Hydroxycinnamyl alcohol-4-*O*-β-D-glucosides **5b**, **6b**, and **7b** were synthesized via reduction of the corresponding hydroxycinnamoyl-ethyl esters using diisobutylaluminum hydride (Terashima et al., 1995). Hydroxycinnamate-4-*O*-β-D-glucosides **1e**, **3e**, and **4e** were synthesized using the same basic procedure as for **5b**, **6b**, and **7b**, but incorporating ester hydrolysis (10% [w/v] KOH in MeOH for 1 h followed by acidification [HCl] and extraction into ethylac-

etate) in place of the reduction with diisobutylaluminum hydride. The identity of each product was verified by NMR spectroscopy (^{1}H and ^{13}C) and comparison with published spectral data.

E-[8-^{13}C]Ferulic Acid

Piperidine (50 μL) was added to a suspension of vanillin (97.3 mg, 0.64 mmol) and [2-^{13}C]malonic acid (120.2 mg, 1.14 mmol, 1.8 equivalents) in freshly distilled pyridine (1 mL). The resulting yellow solution was stirred at 55°C for 17 h. The yellow pyridine solution was cooled to room temperature, poured into a 6 M solution of HCl (6 mL), and stirred vigorously for 15 min. The aqueous mixture was extracted with EtOAc (4 × 10 mL) and the organic solubles were combined, dried ($MgSO_4$), concentrated in vacuo, and chromatographed on silica gel (EtOAc:CH_2Cl_2:MeOH, 5:5:1) to produce a yellow solid (94.6 mg, 76%). ^{1}H-NMR (300 MHz, acetone-d_6): δ 3.92 (3H, *s*, Ar-OMe), 6.38 (1H, *dd*, $J_{H7\text{-}H8}$ = 15.9 Hz, $J_{C7\text{-}H8}$ = 160.8 Hz, H-8), 6.87 (1H, *d*, $J_{H5\text{-}H6}$ = 8.2 Hz, H-5), 7.14 (1H, *dd*, $J_{H2\text{-}H6}$ = 1.8 Hz, $J_{H5\text{-}H6}$ = 8.2 Hz, H-6), 7.34 (1H, *d*, $J_{H2\text{-}H6}$ = 1.8 Hz, H-2), 7.59 (1H, *dd*, $J_{H7\text{-}H8}$ = 15.9 Hz, $J_{C7\text{-}H7}$ = 2.7 Hz, H-7), 8.2 (1H, br *s*, exchangeable with D_2O, Ar-OH). ^{13}C-NMR (75 MHz, acetone-d_6): δ 116.3 (C-8).

Isolation of Phenolic Substrates

The 9-*O*-β-D-Glc esters **1d** and **3d** were isolated from young tomato leaves after first feeding the appropriate hydroxycinnamate precursor (10 mM in water) for 2 to 3 d (Harborne and Corner, 1961). For sinapoyl Glc **4d**, 4-d-old radish seedlings were used. In either case, a total phenolic extract was prepared (80% aqueous MeOH) from 35 to 100 g fresh weight of plant material, concentrated in vacuo (<40°C) until aqueous, filtered, and applied to a 2.5- × 25-cm polyamide SC6 column (Machery-Nagel) pre-equilibrated with water. The hydroxycinnamoyl glucosides were eluted from the polyamide column with water and concentrated in vacuo (<40°C). Free sugars were precipitated at 4°C by repeatedly adding (four times) cold MeOH (to 80% [v/v]) to the aqueous fraction, followed by filtration and concentration in vacuo to remove the MeOH. Crude, sugar-free samples were loaded onto a water-equilibrated 1.5- × 20-cm Sephadex LH20 column (Pharmacia), and the phenolic glucosides eluted with a stepwise gradient of MeOH (100 mL each of 0%, 25%, 50%, 75%, and 100% [v/v] MeOH). Fractions containing hydroxycinnamoyl conjugates were selected using their distinctive UV spectra as a marker. Final purification was achieved using a semipreparative 25- × 100-mm HPLC C_{18} column (NovaPak, Waters). Glucosides were eluted with an isocratic gradient (3% [v/v] acetonitrile in water) at 9 mL min^{-1} and identified on the basis of their ^{1}H-NMR spectra.

p-Coumaroylglucose 1d

Isolated as an amorphous powder (5 mg). UV (MeOH, λ_{max}) 330 nm. ^{1}H-NMR (300 MHz, MeOH-d_4): δ 3.37–3.46 (4H, *m*, Glc protons 2′, 3prime], 4′, 5′), 3.69 (1H, *dd*, J = 12.0 Hz, 4.8 Hz, H-6′B), 3.85 (1H, *dd*, J = 1.0 Hz, 12.0 Hz, H-6′A), 5.57 (1H, *d*, $J_{H1'\text{-}H2'}$ = 7.6 Hz, H-1′), 6.38 (1H, *d*, $J_{H7\text{-}H8}$ = 15.9 Hz, H-8), 6.38 (2H, *d*, $J_{H5\text{-}H6}$ = 8.7 Hz, H-3, H-5) 7.49 (2H, *d*, $J_{H2\text{-}H3}$ = 8.7 Hz, H-2, H-6), 7.73 (1H, *d*, $J_{H7\text{-}H8}$ = 16.0 Hz, H-7).

Feruloylglucose 3d

Isolated as an amorphous powder (38 mg). UV (MeOH, λ_{max}) 330 nm. ^{1}H-NMR (300 MHz, MeOH-d_4): δ 3.31–3.43 (4H, *m*, Glc protons 2′, 3′, 4′, 5′), 3.66 (1H, *dd*, J = 12.1 Hz, 4.5 Hz, H-6′B), 3.82 (1H, *dd*, J = 1.6 Hz, 12.0 Hz, H-6′A), 3.86 (3H, *s*, Ar-OMe), 5.54 (1H, *d*, $J_{H1'\text{-}H2'}$ = 7.6 Hz, H-1′), 6.38 (1H, *d*, $J_{H7\text{-}H8}$ = 15.9 Hz, H-8), 6.79 (1H, *d*, $J_{H5\text{-}H6}$ = 8.2 Hz, H-5), 7.07 (1H, *dd*, $J_{H2\text{-}H6}$ = 1.6 Hz, $J_{H5\text{-}H6}$ = 8.2 Hz, H-6), 7.18 (1H, *d*, $J_{H2\text{-}H6}$ = 1.8 Hz, H-2), 7.70 (1H, *d*, $J_{H7\text{-}H8}$ = 16.1 Hz, H-7).

Sinapoylglucose 4d

Isolated as pale yellow needles from water (28 mg). UV (MeOH, λ_{max}) 330 nm. ^{1}H-NMR (300 MHz, MeOH-d_4): δ 3.34–3.50 (4H, *m*, Glc protons 2′, 3′, 4′, 5′), 3.70 (1H, *dd*, J = 8.0 Hz, 4.5 Hz, H-6′B), 3.84 (1H, *d*, J = 1.8 Hz, H-6′A), 3.88 (6H, *s*, Ar-OMe), 5.59 (1H, *d*, $J_{H1'\text{-}H2'}$ = 7.9 Hz, H-1′), 6.44 (1H, *d*, $J_{H7\text{-}H8}$ = 15.9 Hz, H-8), 6.93 (2H, *s*, H-2, H-6), 7.72 (1H, *d*, $J_{H7\text{-}H8}$ = 15.9 Hz, H-7).

Product Formation

Polymeric products were prepared by the slow addition (0.8 mL h^{-1}) of H_2O_2 (50 mM, 10 mL, in 10 mM phosphate buffer, pH 7) to a stirring solution (10 mL, 10 mM phosphate buffer, pH 7) of pure potato anionic peroxidase (0.14 mg) and either ferulic acid **3a** (19.4 mg, 0.1 mmol) or *E*-[8-^{13}C]ferulic acid (19.5 mg, 0.1 mmol) in a 40°C water bath. All solutions were bubbled with N_2 gas prior to use. After 24 h, the reaction mixture was deep red, and the product was precipitated with the addition of a few drops of concentrated HCl, collected by centrifugation (1250*g*, 10 min, room temperature), and washed with water (two times), collecting the precipitate by centrifugation as above. The final pellet was freeze-dried to yield a dark orange powder, reconstituted in 1 mL of 0.1 M NaOH, loaded onto a 1.5- × 25-cm Sephadex G25-M column (Pharmacia) pre-equilibrated with 0.1 M NaOH, and eluted with 0.1 M NaOH at 1.8 mL min^{-1}. The UV-absorbing eluant (A_{280}) was collected, acid precipitated with HCl, washed with water, and freeze-dried as above to yield 10 mg (52%). BSA and ferulic acid were used to estimate the void and total volumes, respectively, of the column used. For NMR, equal amounts of either natural abundance or ^{13}C-enriched reaction product were dissolved separately in 1 mL of 0.1 M KOH in D_2O. A drop of DMSO-d_6 was added as an internal standard.

RESULTS

Isoform Analysis

Wounding of potato tubers induced at least three groups of peroxidase isoforms, cationic, neutral, and anionic, in

the suberizing tissue isolated from a 7-d-old wound site (Fig. 1). By contrast, the nonsuberized tissue underlying the suberized layer contained predominantly cationic and neutral forms, with only trace amounts of the anionic forms. All three groups of isoforms oxidized both ferulic acid **3a** and guaiacol, albeit with different specific activity. For example, the cationic isoforms oxidized ferulic acid **3a** approximately 1.5 times faster than guaiacol, while the anionic form oxidized ferulic acid **3a** approximately 2.5 times faster than guaiacol. The neutral peroxidase oxidized both substrates equally well.

Purification of a Wound-Induced Anionic Peroxidase

Potato anionic peroxidase was readily purified to apparent electrophoretic homogeneity (Fig. 2, lane b) from wound-induced tubers through a combination of size-exclusion and anion-exchange chromatography (Table I). Enzyme activity (using guaiacol as the substrate) was used as the basis for selection at each step. The final product, representing approximately 20% of the original (i.e. total) activity (measured using ferulic acid as substrate) was recovered in a total yield of 3.5 mg and had a Reinheitszahl value (ratio of heme A_{405} to protein A_{280}) of 2.7. Typical values reported for purified peroxidases range from 1.6 (Kwak et al., 1995) to 4.1 (Converso and Fernandez, 1995), with most in the 2.6 to 3.3 range (Zimmerlin et al., 1994; Padiglia et al., 1995; Rasmussen et al., 1995). The relatively low pI of the protein (approximately 3.5) and its small size (approximately 45 kD) facilitated purification. Remarkably, the enzyme retained its H_2O_2-dependent activity in the SDS-PAGE gel (Fig. 2, lane d), confirming that the isolated protein was a peroxidase.

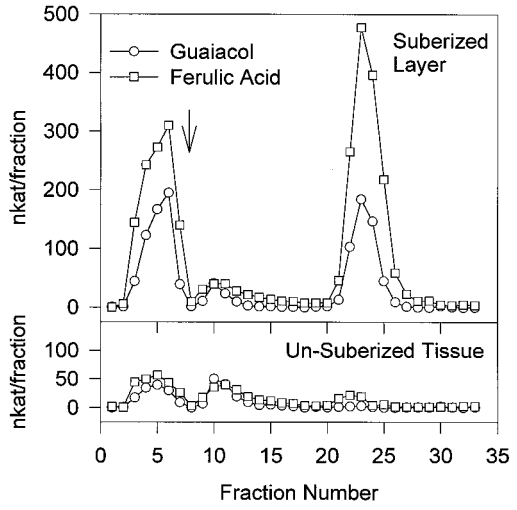


Figure 1. Isoform analysis of wound-induced peroxidases of potato tubers. Total soluble proteins were extracted from either the (mechanically removed) suberized layer or the unsuberized tissue immediately below the suberized layer of 7-d wound-healed potato tubers, and chromatofocused on a Mono-P HR 5/5 column (Pharmacia). Proteins were loaded at pH 7.1, and the pH gradient (7.1–3.5 over approximately 15 min) started after all of the unbound protein had been washed through (indicated by an arrow). Fractions were assayed separately for activity using both ferulic acid and guaiacol.

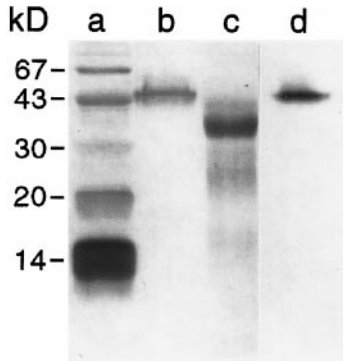


Figure 2. SDS-PAGE analysis of purified anionic peroxidase from potato electrophoresed in a 14% acrylamide gel under denaturing conditions, before (lane b) and after (lane c) treatment with TFMS. Lane a, Molecular mass markers, including BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.1 kD). Lane d, Activity stain using guaiacol/H_2O_2. Proteins in lanes a to c were visualized by silver staining. After staining, gels were dried onto cellulose acetate sheets (Bio-Rad) and scanned to generate digital images.

Potato Anionic Peroxidase Characterization

The purified potato anionic peroxidase has a molecular mass of 45.8 kD, based on SDS-PAGE (Fig. 2, lane b). Deglycosylation with trifluoromethane sulfonic acid (TFMS) yielded a 35.3-kD protein (Fig. 2, lane c). Calibrated molecular-sieving chromatography predicted a molecular mass of 44.9 kD for the purified protein (data not shown). The enzyme displayed a broad temperature optimum between 40°C and 60°C (data not shown) and a pH optimum of 4.5 for phenolic acids and 6.5 for monolignols (Fig. 3). In both cases, the pH optima were broad, and neutral conjugates (e.g. **3b**) were equally good substrates at either pH (data not shown). For convenience, all substrates except the hydroxycinnamyl alcohols (**5a**, **5b**, **6a**, **6b**, **7a**, and **7b**) were assayed at pH 4.5.

Substrate Specificity of Potato Peroxidases

Anionic Peroxidase

Twenty-five different phenolic compounds were tested as substrates (Table II; Scheme 1). The potato anionic peroxidase showed a strong preference for substrates with *o*-methoxyphenol-substituted aromatic ring systems. Thus the hydroxycinnamates **3a**, **3b**, **3c**, **3d**, **3f**, and **3g** were all excellent substrates, while (in decreasing order) the caffeoyl (**2a**, **2b**, and **2h**), *p*-coumaroyl (**1a–1d**), and sinapoyl (**4a–4d**) compounds were less effective. The hydroxycinnamyl alcohols (**5a**, **6a**, and **7a**) were poorer substrates than the corresponding hydroxycinnamates, but still showed the same pattern of maximal activity with the *o*-methoxyphenol-substituted coniferyl alcohol **6a**. As expected, protection of the phenolic hydroxyl groups (i.e. the initial site of oxidation by peroxidase) with Glc moieties

Table I. *Purification summary for the anionic peroxidase isolated from potato tubers during wound healing*

Anionic peroxidase was purified from potato tubers 7 d after wounding.

Purification Step	Total Protein	Total Activity[a]		Purification	Recovery	RZ[b] Value
	mg	*μkat*	*μkat/mg protein*	*fold*	*%*	
Crude	2,552	12,731	5	1	100.0	ND[c]
AS pellet[d]	790	8,578	11	2.2	67.4	ND
SG 100	108	4,775	44	8.9	37.5	0.4
PBE	11.2	2,803	250	50.2	22.0	1.6
DEAE	3.5	2,517	719	144.2	19.8	2.7

[a] Using ferulic acid as a substrate. [b] RZ, Reinheitszahl value (A_{405}/A_{280}) in deionized water. [c] ND, Not determined. [d] 50% to 90% ammonium sulfate pellet.

(e.g. **1e**, **3e**, **4e**, **5b**, **6b**, and **7b**) prevented their oxidation by the enzyme. The potato anionic peroxidase readily oxidized guaiacol, both in the presence and absence of *p*-chloromercuribenzoic acid (*p*CMB) (up to 200 μM), while ascorbate was a very poor substrate (data not shown).

Cationic Peroxidase

A subset of the phenolics tested as substrates for the anionic peroxidase, including the hydroxycinnamates **1a**, **2a**, **3a**, and **4a** and coniferyl alcohol **6a**, were also tested with the partially purified cationic peroxidase(s) of potato (Table III; Scheme 1). In contrast to the specificity apparent for the anionic peroxidase, the cationic peroxidase(s) oxidized ferulic acid **3a** and coniferyl alcohol **6a** equally well. A similar trend in preference for aromatic substitution patterns was observed. The (descending) order of substrate preference for the cationic isoform(s) was feruloyl > caffeoyl > syringyl > *p*-coumaryl.

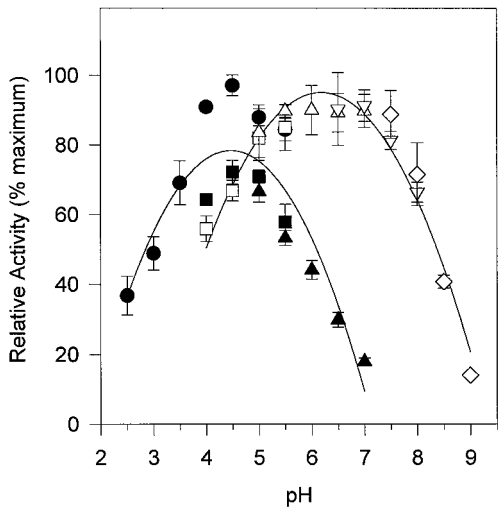


Figure 3. Dependence of phenolic oxidation rate by potato anionic peroxidase on pH. The rate of oxidation of ferulic acid **3a** (black symbols) and coniferyl alcohol **6a** (white symbols) was measured spectrophotometrically in buffers of differing pH. The buffers used (all at 50 mM) were citrate (●), acetate (■, □), His (▲, △), phosphate (▽), and Tris (◇). Oxidation rates were measured in triplicate. Error bars represent ±1 SD.

Potato Anionic Peroxidase Reaction Products

The acid-insoluble product(s) obtained from the slow addition of H_2O_2 to an enzyme/ferulic acid solution appeared to be polymeric (M_r > 5,000), on the basis of its elution in the void volume of a Sephadex G25-M column (Fig. 4). The natural abundance polymer had only a single weak resonance in its ^{13}C-NMR spectrum, corresponding to the methoxyl carbon (δ 56.8 ppm), owing to the heterogeneous nature of the polymer as well as the low abundance of sample (data not shown). In the ^{13}C-NMR spectrum obtained for the polymer prepared from *E*-[8-^{13}C]ferulic acid (Fig. 5), however, major resonances wereapparent at δ 171.8, 124.2, 122.9, 118.0, 105.4, and 59.6 ppm, with minor resonances observed at δ 136.3, 135.3, 130.4, and 55.3 ppm.

DISCUSSION

The Anionic Potato Peroxidase and Suberization

Wounding of potato tubers results in a gradual increase in total soluble peroxidase activity over a period of 5 to 7 d (Borchert, 1978; Roberts et al., 1988). This involves the synthesis of new protein and is preceded by the accumulation of mRNA (Roberts et al., 1988). However, plants typically contain multiple peroxidase isoforms and it is not surprising that several (i.e. cationic, neutral, and anionic) are induced in potato under wound healing conditions (Borchert, 1978; Borchert and Decedue, 1978; Fig. 1), with the cationic and anionic forms predominating. Since suberization is restricted to the two to three cell layers immediately below the wound site (Borchert, 1978; Borchert and Decedue, 1978; Kolattukudy, 1980), and the anionic isoform(s) is immunocytochemically (Espelie et al., 1986) and biochemically (Fig. 1) localized to this region, it is strongly implicated in the suberization process.

Potato Anionic Peroxidase Characterization

Heme peroxidases are classified as either class I (intracellular), class II (fungal secretory), or class III (plant secretory), largely based on their structure (i.e. carbohydrate content, number of bound Ca^{2+} atoms, number of disulfide bridges, etc.) (Welinder, 1985; O'Donnell et al., 1992), sub-

Table II. *Substrate specificity of potato anionic peroxidase*

Maximum catalytic rates were determined for each substrate by measuring the initial rate of their consumption over a range of concentrations (0–0.2 mM) with a fixed concentration of H_2O_2 (2 mM). The rate values were predicted from the intercepts of Wolfe-Hanes plots. For all assays, the purified potato anionic peroxidase was used at a 0.5 nM final concentration.

Substrate (refer to Scheme 1)	V_{max}^{app}	Relative Activity[a]
	pkat	
1a *p*-Coumaric acid	9.5	3.1
1b *N*-(*p*-Coumaroyl)tyramine	70.6	22.9
1c *N*-(*p*-Coumaroyl)putrescine	51.1	16.6
1d *p*-Coumaroylglucose	50.0	16.2
1e *p*-Coumarate-4-*O*-β-D-glucoside	<1[b]	0.3
2a Caffeic acid	163.4	53.1
2b *N*-Caffeoyltyramine	129.6	42.1
2h Chlorogenic acid	137.7	44.7
3a Ferulic acid	307.8	100
3b *N*-Feruloyltyramine	318.6	103.5
3c *N*-Feruloylputrescine	366.0	118.9
3d Feruloylglucose	379.9	123.5
3e Ferulate-4-*O*-β-D-glucoside	<1[b]	0.3
3f *N*-Feruloyloctopamine	258.1	83.8
3g *N*-Feruloyl-(2-phenyl)-ethylamine	376.0	122.2
4a Sinapic acid	34.6	11.2
4b *N*-Sinapoyltyramine	32.2	10.5
4d Sinapoylglucose	15.9	5.2
4e Sinapate-4-*O*-β-D-glucoside	<1[b]	0.3
5a *p*-Coumaryl alcohol	3.6[b]	1.2
5b *p*-Coumaryl alcohol-4-*O*-β-D-glucoside	2.2[b]	0.7
6a Coniferyl alcohol	174.6	56.7
6b Coniferin	<1[b]	0.3
7a Sinapyl alcohol	12.5[b]	4.1
7b Syringin	2.2[b]	0.7

[a] Ferulic acid set to 100%. [b] Maximum rate measured; substrate did not show typical saturation kinetics, precluding the use of the Wolfe-Hanes transformation.

Table III. *Substrate specificity for selected peroxidase isoforms*

Values represent relative rates of oxidation of the substrates listed, with ferulic acids arbitrarily set to 100%. Isoforms are grouped as either cationic or anionic.

Plant Source	Isoform	Substrate					Reference
		p-Coumarate **1a**	Caffeate **2a**	Ferulate **3a**	Sinapate **4a**	Coniferyl Alcohol **6a**	
Cationic peroxidases							
Potato		4.9	33	100	12	105	Present work
Horseradish	IX	121	NA[a]	100	1.7	NA	Takahama (1995)
V. myrtillus	C1	14	64	100	NA	150	Melo et al. (1997)
	C2	61	69	100	NA	128	Melo et al. (1997)
Soybean	Leaf	NA	174	100	NA	NA	Schmitz et al. (1997)
	Stem	NA	466	100	NA	NA	Schmitz et al. (1997)
Anionic peroxidases							
Potato		3.1	53	100	11	57	Present work
Horseradish	VII	44	NA	100	3.6	NA	Takahama (1995)
	VIII	18	NA	100	6.6	NA	Takahama (1995)
	VIII	6.3	64	100	5.1	64	Present work
Soybean	Leaf-1	NA	160	100	NA	NA	Schmitz et al. (1997)
	Leaf-2	NA	0[b]	100	NA	NA	Schmitz et al. (1997)
	Stem	NA	0	100	NA	NA	Schmitz et al. (1997)

[a] NA, Data not available. [b] 0, No measurable activity.

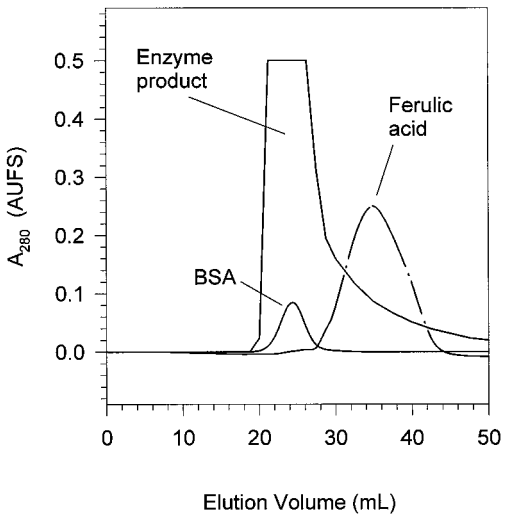


Figure 4. M_r characterization of the polymeric product prepared by the incubation of ferulic acid/H_2O_2 with purified anionic peroxidase. The acid-insoluble precipitate collected after incubation of anionic peroxidase with ferulic acid/H_2O_2 was dissolved in 0.1 M NaOH and eluted from a Sephadex G25-M column. BSA was used to mark the void volume of the column. The ferulic acid monomer was used to mark the total volume of the column.

strate preference (i.e. ascorbate versus guaiacol), and sensitivity to *p*CMB (Amako et al., 1994). For example, class I intracellular peroxidases, typified by ascorbate peroxidase, prefer ascorbate as substrate and are inhibited by *p*CMB, while class III peroxidases (the so-called guaiacol or secretory peroxidases) prefer guaiacol as substrate and show no sensitivity to *p*CMB (Amako et al., 1994). For potato anionic peroxidase, the large (10.7 kD) shift in molecular mass after treatment with TFMS, its preference for guaiacol over ascorbate as substrate, and its insensitivity to *p*CMB clearly distinguish it as a class III peroxidase.

In the present study, we found that the highly anionic peroxidase from potato had a molecular mass of approximately 45 kD, as determined by both SDS-PAGE and calibrated molecular-sieving chromatography. While this molecular mass is consistent with that reported earlier (Espelie and Kolattukudy, 1985), and with that for other anionicperoxidases of solanaceous species (e.g. Pomar et al., 1997), it is not consistent with that predicted by the published amino acid sequence (Roberts et al., 1988). Since the latter was deduced from a cDNA showing three possible start sites, it could not be used with confidence to predict the molecular mass of the native protein. Instead, as a tightly folded, globular protein, its elution from a molecular-sieving column can be taken as a good first approximation. In this case, both the SDS-PAGE (45.8 kD) and molecular-sieving chromatography (44.9 kD) predictions were in close agreement. While the influence of the carbohydrate side chains on sieving behavior cannot be discounted, the deglycosylated protein still had a molecular mass approximately 6 kD greater than that predicted (Roberts et al., 1988) for the unglycosylated protein.

Substrate Specificity

The reaction catalyzed by peroxidase is both complex (Scheme 2) and fast, and does not follow simple Michaelis-Menten kinetics (e.g. Nakajima et al., 1991). The initial step involves the binding of H_2O_2 by the Fe(III) heme, followed by its oxidation, cleavage of the O-O bond, and the subsequent formation of a ferryl (Fe[IV]=O)-porphorin π-cation radical (referred to as compound I), accompanied by the release of water. Next, the first of two reducing substrates (e.g. R-OH in Scheme 2) binds and donates one electron to compound I, reducing the porphorin cation and resulting in a ferryl (Fe[IV]=O) enzyme (referred to as compound II). The reducing substrate is released as a radical (e.g. R-O$^{\cdot}$ in Scheme 2). In the last step of the cycle, a second reducing substrate binds and donates an electron to Fe(IV)=O, resulting in the reduction of the heme to Fe(III) and, with the addition of two protons, the release of water. A second radical is generated in the process. Thus, the stoichiometry of the reaction involves 2 mol phenolic substrate oxidized for each mole of H_2O_2 reduced, with different binding affinities for the phenolics for compounds I and II.

Coupled with the apparent inhibition observed when the concentration of H_2O_2 is disproportionately large, simple Michaelis-Menten constant determinations become increasingly difficult. While it is possible to measure rate constants for the individual reactions shown in Scheme 2 (Rasmussen et al., 1995; Converso and Fernandes, 1996; Rodriguez-Lopez et al., 1996), it requires special rapid kinetics equipment. Therefore, in determining the substrate preference of the potato anionic peroxidase, we have employed a relatively simple, quasi-rapid mixing spectrophotometric assay in which the initial rate of phenolic substrate depletion was used as an indicator of relative activity

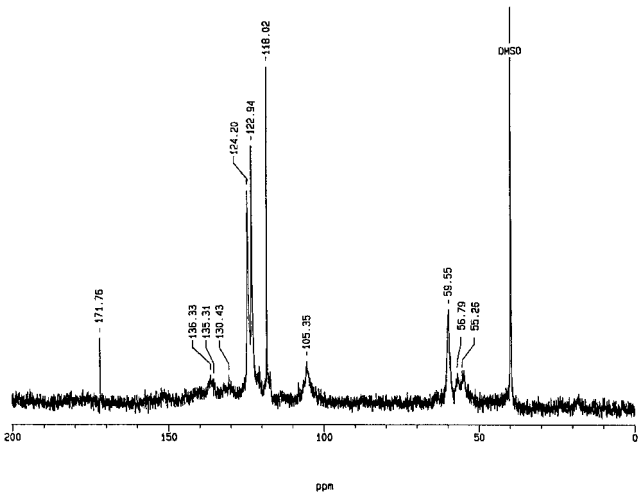
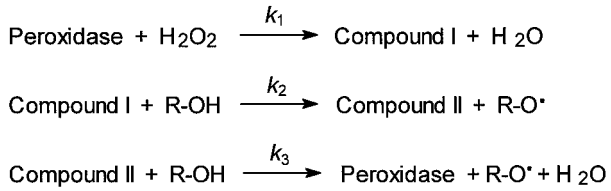


Figure 5. Solution-state ^{13}C-NMR spectroscopic analysis of the polymeric product prepared by the incubation of *E*-[8-^{13}C]ferulic acid/H_2O_2 with purified anionic peroxidase. Ten milligrams of the polymeric product collected from a Sephadex G25-M column was dissolved in 0.1 M KOH prepared in deuterated water. A drop of DMSO was added as an internal standard. With the exception of the resonance at 56.79 ppm, all resonances are due to the enhanced C-8 of the initial substrate.



Scheme 2. General reaction mechanism for peroxidase. See text for detailed description of each step. R-OH, Phenolic substrate; R-O·, phenolic radical.

under conditions of saturating H_2O_2 (i.e. saturation or steady-state kinetics). Rapid mixing is essential to monitor the reaction, since the initial rate is only linear for about 5 to 10 s (depending on substrate concentration). Our procedure allowed monitoring within 1 to 2 s of mixing. Under the conditions used, the rate of ferulic acid oxidation was linear with respect to enzyme concentration (data not shown), indicating that the reaction was being monitored at saturating concentrations. For most substrates, saturation kinetics were observed, but for others (especially **1e**, **3e**, **4e**, **5a**, **5b**, **6b**, **7a**, and **7b**) saturation was not achieved. For these compounds, oxidation rates were very low relative to ferulic acid **3a** (Table II), and the maximum rate observed is reported.

The reduction of compound II (step 3 in Scheme 2) is rate limiting when there is sufficient H_2O_2 present (Rodriguez-Lopez et al., 1996); however, there are substantial differences in the rate of compound II formation (step 2 in Scheme 2) depending on the reducing substrate (Takahama, 1995; Takahama et al., 1996). For example, the rate of sinapic acid **4a** oxidation by horseradish peroxidase isoforms can be greatly enhanced by the addition of small amounts of either *p*-coumaric **1a** or ferulic **3a** acids to the reaction mixture (Takahama, 1995), presumably because the latter (especially **3a**) react to form compound II more readily than sinapic **4a** acid alone. Consequently, the "rates" shown in Table II reflect the relative efficiency with which the enzyme can use each substrate for its complete catalytic cycle.

The substrates used were selected because many (i.e. **2c**, **2h**, **3b**, **3c**, and **3f**) are known to accumulate in potato tubers during would healing (e.g. Malmberg, 1984; Bernards and Lewis, 1992; Borg-Olivier and Monties, 1993; Negrel et al., 1996), and are potential "natural" substrates for the enzyme. Other compounds (e.g. the hydroxycinnamoyl-9-*O*-β-D-glucosides **1d**, **3d**, and **4d**) are common in solanaceous plants (Harborne and Corner, 1961). The monolignols 5 to 7 were also included since they are known to be incorporated into suberizing cell walls, albeit in minute amounts (Borg-Olivier and Monties, 1989, 1993). The remaining substrates represent the free acids, analogs, and/or modifications of the "natural" substrates. For example, *N*-feruloyl-(2-phenyl)-ethylamine **3g** represents a dehydro analog of *N*-feruloyltyramine **3b**.

The highest rate of enzyme activity was measured with feruloyl derivatives as substrate (Table II). Indeed, the enzyme seems particularly sensitive to the aromatic substitution pattern of its substrates, and showed a marked preference for those that are *o*-methoxyphenol substituted. The (descending) order of substrate preference is feruloyl > caffeoyl > *p*-coumaryl ≈ syringyl, with little influence by side chain derivatization. Coincidentally, the major compounds accumulating in potato tubers induced to suberize via wounding (with the exception of chlorogenic acid **2h**) are ferulic acid derivatives, and their ready oxidation by the potato anionic peroxidase implicates them (and the enzyme) in the suberization process. Interestingly, the oxidation of *N*-feruloyl-(2-phenyl)-ethylamine **3g** was more rapid than that of *N*-feruloyltyramine **3b**, indicating that the enzyme discriminates between the hydroxycinnamate and tyramine ends of the substrate.

In order to place the substrate specificity of the potato anionic peroxidase in context, we measured the relative oxidation of the hydroxycinnamic acids **1a**, **2a**, **3a**, and **4a**, as well as coniferyl alcohol **6a** by a partially purified cationic peroxidase preparation from wounded potato tubers, as well as a commercially available anionic horseradish peroxidase (Table III). In addition, literature data for nine other peroxidase isoforms for which comparative data are available are included. (Note that the literature contains many reports in which only one hydroxycinnamate substrate was used, usually ferulic acid **3a**, but these do not provide comparative values and are not considered here.) Whereas most isoforms show a similar preference for *o*-methoxyphenol substituted substrates (i.e. ferulic acid **3a**), some differences are apparent. For example, the descending order of substrate preference for the anionic horseradish peroxidase isoforms VII and VIII is feruloyl > caffeoyl > *p*-coumaroyl > syringyl, while that for the cationic isoform IX is *p*-coumaryl > feruloyl > syringyl (Takahama, 1995).

The two cationic peroxidases from *Vaccinium myrtillus* also preferentially oxidize ferulic acid **3a** over other hydroxycinnamic acids, although they differ in their ability to oxidize *p*-coumaric acid **1a** (Melo et al., 1997). In contrast, three soybean peroxidase isoforms (two cationic and one anionic) more readily oxidized caffeic acid **2a** than ferulic acid **3a**, while two others (both anionic) were unable to oxidize caffeic acid **2a** at all (Schmitz et al., 1997). In general, the cationic peroxidases listed in Table III oxidized coniferyl alcohol **6a** equally well or better than ferulic acid **3a**, while anionic ones more readily oxidized ferulic acid **3a**. For the wound-induced anionic peroxidase of potato, all hydroxycinnamates appeared to be more readily oxidized than their corresponding hydroxycinnamyl alcohols (Table II). Thus, of the peroxidase isoforms induced upon wounding of potato tubers, the anionic one appears predisposed to favor oxidation of the major phenolic compounds that accumulate coincidentally. Since some of these (especially **3c**) become oxidatively cross-linked in the suberized cell walls of tubers, the anionic peroxidase is implicated in the process.

Product Analysis

Horseradish peroxidase has often been used to generate dehydrogenation polymers of coniferyl alcohol (e.g. Lewis et al., 1987; Lewis and Yamamoto, 1990). The type of product obtained depends on the rate at which the substrates

(i.e. coniferyl alcohol and H_2O_2) are added to the enzyme (see Saake et al., 1996; Guan et al., 1997). In the present study, the slow addition of H_2O_2 to a stirring solution of purified anionic peroxidase and ferulic acid **3a** yielded a polymer of $M_r > 5{,}000$, as judged by chromatography on a Sephadex G25-M column (Fig. 4). More than half (i.e. 52%) of the original monomer was recovered in this polymeric fraction. Based on a monomer molecular mass of approximately 194 g mol^{-1}, the recovered material represents a polymer with a minimal degree of polymerization of 26. By contrast, either the rapid addition of H_2O_2 or the addition of both substrates at once to a stirring solution of purified anionic peroxidase yielded mainly low-M_r, acid-soluble products, not unlike those reported by Zimmerlin et al. (1994) (data not shown).

NMR spectroscopic analysis (Fig. 5) revealed two important features of the in vitro polymeric product(s) formed by the slow addition of H_2O_2 to a stirring solution of *E*-[8-^{13}C]ferulic acid and the purified enzyme. First, only a small proportion of the total enhanced resonances (approximately 30%) correspond to that of the original *E*-[8-^{13}C]ferulic acid (i.e. δ 118.0 ppm), indicating that the majority of carbons originating from C-8 of the monomer were found in a modified electronic environment, including their involvement in cross-linking. Second, the majority of resonances were found in the olefinic region of the spectrum (i.e. δ 110–150 ppm), indicating that C-8 of the original monomer retains its unsaturation, despite being cross-linked with other monomeric units. This feature of in vitro peroxidase/H_2O_2-generated polymers represents a deviation from polymers generated using horseradish peroxidase and monolignols (e.g. Lewis et al., 1987; Guan et al., 1997), where the side chain carbons were reduced during coupling. It is consistent, however, with the retention of side chain unsaturation noted when [2-^{13}C]Phe was administered to suberizing potato tubers (Bernards et al., 1995). Recently, Ralph et al. (1994) described a number of ferulic acid dehydrodimers present in grass cell walls, some of which show carbon resonances consistent with those observed in the polymeric product generated by the anionic peroxidase. Curiously, the enhanced signal at 171.8 ppm (Fig. 5) suggests that a minor proportion of the ferulic acid monomers underwent oxidation at C-8 to a carbonyl during the reaction with anionic peroxidase. Notwithstanding this interpretation, the analysis of the polymers generated in vitro by the anionic peroxidase of potato requires further study.

CONCLUSIONS

The macromolecular assembly of the aromatic domain in suberized tissues is hypothesized to involve a peroxidase/H_2O_2-mediated free radical coupling process. One candidate peroxidase in potato tubers is the highly anionic isoform that is induced by wounding. The biochemical evidence presented here supports this contention on two counts. First, the anionic peroxidase is restricted to the suberizing tissues in the immediate vicinity of the wound site. Second, the anionic peroxidase of potato prefers *o*-methoxyphenol-substituted hydroxycinnamates (typical of those that accumulate in tubers during wound healing and incorporated into the suberized cell wall) to other phenolic substrates (order of substrate preference: guaiacyl > caffeoyl > *p*-coumaryl ≈ syringyl) including hydroxycinnamyl alcohols. This contrasts with the cationic peroxidase(s) of potato, which is found in the tissues underlying the wound site (in addition to the suberizing tissues), and does not discriminate between ferulic acid **3a** and coniferyl alcohol **6a**. The purified anionic enzyme readily formed dehydrogenative polymers from ferulic acid **3a** in the presence of H_2O_2 that are characterized by a high level of cross-linking (potentially through side chain C-8) and a high degree of retention of side chain unsaturation. In general, the data presented in this paper are consistent with the involvement of the anionic peroxidase isoform of potato in the polymerization of the poly(aromatic) domain during suberization, although definitive proof awaits further investigation.

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