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CELL WALL-BOUND CONIFERYL ALCOHOL OXIDASE ASSOCIATED WITH LIGNIFICATION IN CONIFERS

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Key Word Index—*Pinus*; *Abies*; *Larix*; *Picea*; Gymnospermae; cambial growth; enzymes; glycoprotein; leaves; lignin; secondary wall development; tracheid differentiation; wood formation.

Abstract—An enzyme utilizing O_2 to oxidize coniferyl alcohol into a lignin-like product is described. This enzyme has been found firmly bound to cell walls in developing xylem of *Pinus strobus*, *Abies balsamea*, *Larix laricina*, *Picea rubens* and *Pinus banksiana*. In each of these conifers, oxidase activity arises in synchrony with initiation of lignification and disappears with termination of lignification. By combined gas chromatography-mass spectrometry, the enzyme was found to oxidize coniferyl alcohol into dehydrodiconiferyl alcohol and pinoresinol, and by UV microscopy it was determined that under aerobic *in vitro* conditions the enzyme converts coniferyl alcohol into hydrophobic globules similar to guaiacyl lignin. By isoelectric focusing, several oxidase isoenzymes were resolved; all appear to be glycoproteins.

INTRODUCTION

Although Freudenberg [1] was of the opinion that an oxidase such as laccase was involved in lignification, it was later concluded that dehydrogenative polymerization of monolignols such as *trans*-coniferyl alcohol occurs exclusively through peroxidase/H_2O_2 [2, 3]. The observation [4] that *E*-coniferin (i.e. the 4-*O*-*β*-D-glucopyranoside of *trans*-coniferyl alcohol) accumulates in primary-walled radially enlarged cambial derivatives in the absence of associated lignification suggested that, prior to initiation of tracheid differentiation, these cells lack competence to convert coniferyl alcohol into lignin. During the course of investigations to explain this, we found evidence for an enzyme in lignifying cell walls that can oxidize coniferyl alcohol using O_2.

RESULTS AND DISCUSSION

The species investigated were *Abies balsamea* (L.) Mill., *Larix laricina* (Du Roi) K. Koch, *Picea rubens* Sarg., *Pinus banksiana* Lamb. and *Pinus strobus* L. In agreement with a previous report [5], resumption of cell division activity in the main stems of these species occurred first near the crown base, leading to the formation of a zone of primary-walled radially enlarged ('RE') cells on the xylem side of the cambial zone ('CZ') [6] in early May, before bud break. Following formation of the RE zone, bordered-pit development, secondary wall polysaccharide deposition and lignification were initiated sequentially beginning in foliated stem regions, progressing to more basal regions, and giving rise to a zone of cells undergoing secondary wall formation and lignification ('SL') adjacent to the previous year's annual ring.

Evidence for cell wall-bound oxidase activity first emerged during histochemical investigations into cambial peroxidase in relation to progressive development of the RE and SL zones. In the absence of exogenous H_2O_2, oxidation of *p*-anisidine HCl was not detected in CZ or RE of any species; however, *p*-anisidine oxidation was associated with initiation of the SL, red oxidation product being detected first at cell corners and at sites of bordered-pit development, the locations where lignification commences in the still primary-walled cells during early stages of tracheid differentiation [7–9]. With continuing SL development, the ability for *p*-anisidine oxidation appeared in the compound middle lamella and then in developing secondary walls, in synchrony with occurrence of lignification at these sites. In the absence of exogenous H_2O_2, *p*-anisidine oxidation was restricted to the SL zone. In the presence of H_2O_2, both primary and secondary cell walls in cambium, RE, SL and mature wood zones developed red to purple colouration within a few minutes. In other words, all stem samples, regardless of species, stage of cambial growth, or position within the tree, displayed the continual ubiquitous presence of active peroxidase.

The spatiotemporal correlation between appearance of an oxidizing factor in cell walls and occurrence of lignification was evident over the stems of whole trees. The patterns of oxidizing activity found in the main stem of red spruce (*P. rubens*) having RE only (May 9th) and with SL (May 25th) are presented in Fig. 1, as determined by microscopy of live sections incubated on H_2O_2-free *p*-anisidine solution. Before initiation of SL, no *p*-anisidine oxidation occurred despite development of CZ and RE (Fig. 1, May 9th). Oxidation on May 25th (Fig. 1) occurred only in SL cells, and both SL cell number and associated oxidizing actvity decreased basally, disappearing in lower stem regions. It deserves emphasis that the cambium in the older (> seven years) stem regions had generated RE several weeks before either RE or SL development were initiated in younger foliated stem regions; nevertheless, the *p*-anisidine oxidizing activity

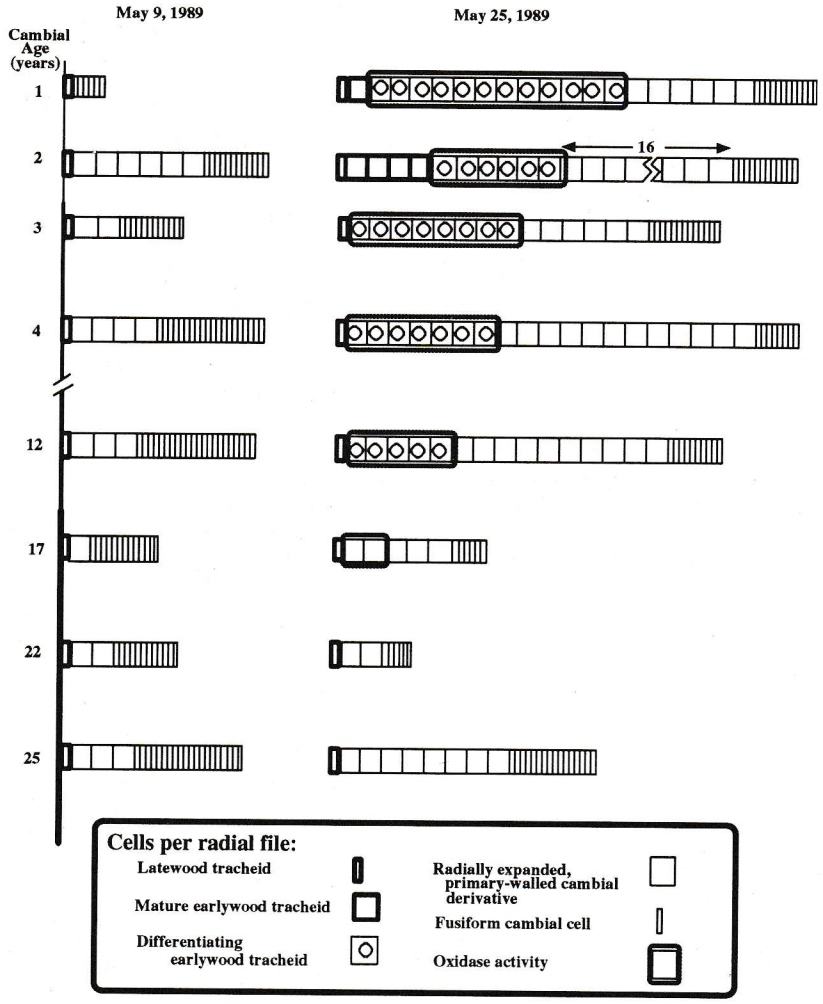

Fig. 1. Pattern of springtime resumption of cambial growth and development of *p*-anisidine oxidizing activity in *Picea rubens*. Hand-cut transverse sections of freshly harvested stems were floated on 20 mM *p*-anisidine HCl (without added H_2O_2) at room temperature for 4 hr, then examined by brightfield microscopy.

first appeared in the upper crown of all species. By mid-June the basal stem regions contained the SL zone, at which time *p*-anisidine oxidizing activity was detected there. As the first early-wood tracheids underwent autolysis and maturated, the *p*-anisidine oxidizing activity in their cell walls disappeared. The developmental pattern of *p*-anisidine oxidation described here for red spruce was confirmed with all five species during 1989 and again in spring 1990.

We attempted to isolate the non-H_2O_2-requiring factor oxidizing *p*-anisidine by scraping SL tissue from mature wood following bark peeling [10]. For comparison, RE tissue was also collected from stems earlier in the season, before development of the SL zone. Both before and after PO_4-buffer/NaCl extraction, RE cell wall residues were devoid of histochemically detectable *p*-anisidine oxidizing activity (Fig. 2). In contrast, SL cell wall residues contained a wall-bound factor capable of oxidizing *p*-anisidine (Fig. 3). No oxidizing activity was found, however, in $(NH_4)_2SO_4$ (90% saturation) precipitates from extracts [0.05 M PO_4-buffer (pH 7.0)/0.5 M NaCl] of >1 kg quantities of SL tissue. The oxidizing factor was not solubilized from SL walls by either detergent (0.5% Triton-X-100) or salt solutions reported to extract proteins from higher plant cell walls, viz. 0.5 M NaCl, 2 M $CaCl_2$, 3 M LiCl [11].

Following thorough extraction, digestion of the oxidizing SL cell wall residue for 16 hr at room temperature with a mixture of pectinase and cellulase resulted in solubilization of >95% by weight of the material. After centrifuging the digest, the supernatant was concentrated by centrifugal ultrafiltration to yield a fraction (>10 000 M_r) containing abundant protein; however, this soluble protein-enriched fraction was devoid of *p*-anisidine oxidizing activity. Repeated re-suspension (distilled water) and centrifuging of the insoluble pellet in water yielded a SL particulate preparation ('SL PP') enriched in bound protein that oxidized *p*-anisidine, *p*- and *o*-phenylenediamine, dopamine, gallic acid and *o*-dianisidine, but not guaiacol, in the absence of added H_2O_2.

A soluble high M_r (>10 000) *p*-anisidine oxidizing factor from SL PP ('SL PP SOF') was obtained by salt

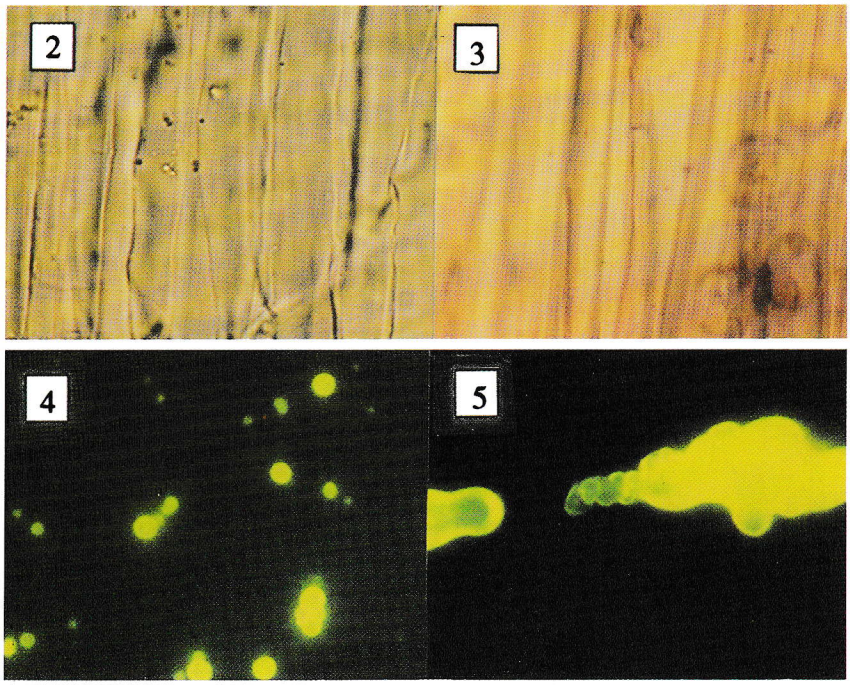

Fig. 2. Brightfield photograph of thoroughly washed (phosphate buffer, NaCl) CZ and RE cell wall residues in radial longitudinal view after incubation in *p*-anisidine HCl (20 mM) for 24 hr (20°). Little, if any, *p*-anisidine oxidation occurred. Magnification × 1000.

Fig. 3. Brightfield photograph of thoroughly washed (phosphate buffer, NaCl) SL cell wall residues in radial longitudinal view after incubation in 20 mM *p*-anisidine HCl for 24 hr (20°). Oxidizing activity (pink to brown) is evident throughout the walls, particularly at peripheries of developing bordered pits and at cell–cell contacts. Magnification × 1000.

Fig. 4. UV microscopy of an aqueous solution showing autofluorescent globules which developed *in vitro* during incubation of coniferyl alcohol with SL PP SOF. Magnification × 670.

Fig. 5. Higher magnification UV fluorescence micrograph of hydrophobic globules developing *in vitro* from coniferyl alcohol incubated with SL PP SOF. Magnification × 2280.

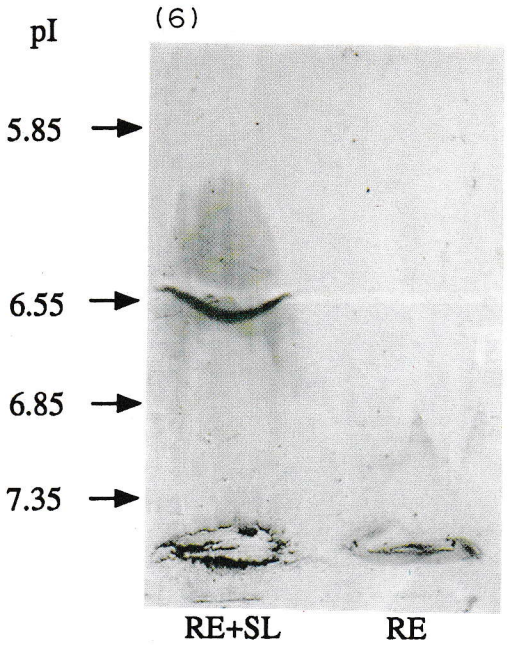

Fig. 6. IEF (pH 3–9, cathodal loading) showing oxidase activity [0.25% (w/v) *p*-phenylenediamine] obtained from *Pinus strobus* SL cambial derivatives by pectinase-cellulase digestion of cell wall material followed by centrifugation and repeated washing of the undigested residue. RE+SL: SL PP from zone of cells undergoing secondary wall polysaccharide deposition and lignification, showing a singular band (*pI* 6.55) of oxidase activity. RE: RE PP from zone of radially expanded primary walled cambial derivatives exhibiting no microscopically detectable evidence for bordered pit development and no histochemically detectable oxidase activity. Standard *pI*s: 5.85 = bovine carbonic anhydrase B, 6.55 = human carbonic anhydrase B, 6.85 = horse myoglobin (acidic band), 7.35 = horse myoglobin (basic band).

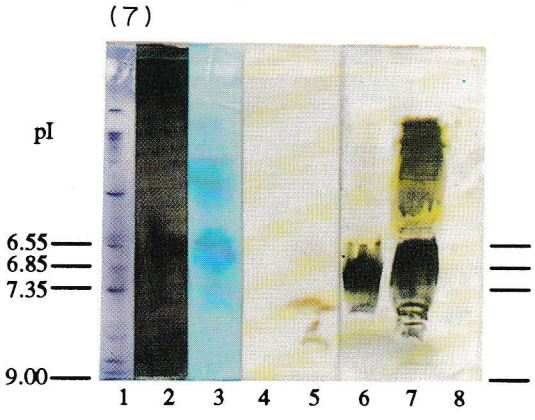

Fig. 7. IEF (pH 3–9, cathodal loading) of solubilized oxidizing activity from developing xylem of *Pinus strobus*. Lane 1: markers *pI* 6.55 = human carbonic anhydrase B, 6.85 = horse myoglobin (acidic band), 7.35 = horse myoglobin (basic band). Lane 2: SL PP SOF stained with 0.25% (w/v) *p*-phenylenediamine. Lane 3: SL PP SOF stained with 0.5% (w/v) Alcian blue. Lane 4: solubilized RE PP stained with 20 mM *p*-anisidine HCl. Lane 5: SL PP SOF stained with 20 mM *p*-anisidine HCl. Lane 6: solubilized high M_r fraction from RE PP stained with silver. Lane 7: SL PP SOF stained with silver. Lane 8: blank (pectinase-cellulase-gentamycin carried through the SL PP SOF workup procedure) stained with silver.

extraction of SL PP (see Experimental). Incubation of coniferyl alcohol with SL PP SOF resulted in the initially clear filter-sterilized solution becoming cloudy; the control solution (coniferyl alcohol—no SL PP SOF) remained clear. Using incident UV (autofluorescence) microscopy [9, 12], the cloudiness was determined to be due to water-insoluble highly fluorescent yellow-green globules (Figs 4, 5). These globules, although more intensely autofluorescent, were identical in emission colour to guaiacyl lignin as it occurs in walls of conifer wood.

Isoelectric focusing (IEF, pH 3–9) of *P. strobus* SL PP suspensions repeatably yielded a singular dominant band (*pI* 6.5) of oxidizing activity when gels were stained with *p*-phenylenediamine (Fig. 6); however, a non-focused residue invariably remained at the loading point (~*pI* 7.5). With other species (not shown), SL PP yielded a singular band at *pI* 7.0 and a non-focused residue. Negative reaction of these oxidizing bands with 3,3′,5,5′-tetramethylbenzidine-H_2O_2 suggests that these proteins lack a heme [13]. No IEF band of oxidizing activity was associated with RE particulate preparations (Fig. 6), nor with pectinase–cellulase 'blanks'.

Following solubilization, the *P. strobus* band of oxidase activity was resolved by IEF into at least three components near *pI* 6.5 (a preliminary report [14] of *pI* ~9 was in error) as detected by *p*-phenylenediamine oxidation products (Fig. 7, Lane 2); however, *p*-anisidine was oxidized only weakly in this region and was more strongly oxidized near the loading point (~*pI* 7.5) and *pI* 8 (Fig. 7, Lane 5). Unfortunately, except for the loading point, protein corresponding to the bands of oxidizing activity could not be detected by Coomassie staining. When silver stained, the bands near *pI* 6.5 could not be resolved from a heavy protein concentration overlaying their isoelectric point (Fig. 7, lane 7); however, dense silver staining at the loading point (Fig. 7, lane 7) corresponded to *p*-anisidine oxidation products at this *pI* (Fig. 7, lane 5). Following IEF, solubilized RE PP gave no *p*-anisidine oxidation products (Fig. 7, lane 4) although it did silver stain (Fig. 7, lane 6) whereas blanks (cellulase–pectinase processed through the procedure in the absence of cambial tissue) did not (Fig. 7, lane 8). A positive Alcian blue response (Fig. 7, lane 3) corresponded to the loading point and the three bands near *pI* 6.5, suggesting that they are glycoproteins [15]. The optimum oxidation of *o*-dianisidine by SL PP occurred near pH 7 (Fig. 8); hence, this pH was used in subsequent investigations.

Both SL PP and SL PP SOF (*P. strobus*) oxidized *p*-anisidine (Fig. 9) and coniferyl alcohol (Fig. 10). Concentrated solutions of SL PP SOF had a high protein content (e.g. 2.3 mg ml^{-1}); however, most of this was oxidatively inactive. This low activity agrees with the bulk of the protein in the preparations being non-oxidizing (Fig. 7). For SL PP SOF, the oxidizing rate when exposed to air at room temperature was (for *o*-dianisidine) 0.3 nmol $(\mu g_{protein}\ hr)^{-1}$. During oxidation of *o*-dianisidine by SL PP, O_2 was consumed, and within a sealed test tube under flowing N_2, SL PP was oxidatively inactive.

Following incubation of coniferyl alcohol with either SL PP or SL PP SOF for two hours, combined GC-mass spectrometry revealed that two major metabolites had accumulated: dehydrodiconiferyl alcohol and pinoresinol (Fig. 11). Coniferin, when incubated with SL PP, was hydrolysed to coniferyl alcohol in substantial amounts (apparently due to the presence of SL PP-bound β-glucosidase activity), and dehydrodiconiferyl alcohol and

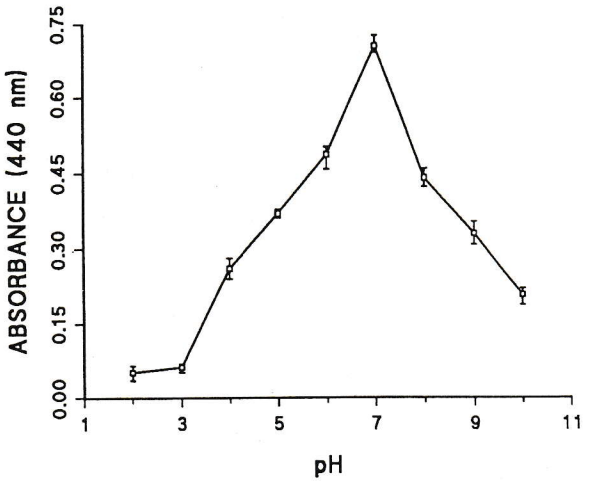

Fig. 8. Oxidation of *o*-dianisidine 2HCl (0.016 mmol) by SL PP (4 μg bound protein) over pH 2–10. Buffers (2.0 ml, 0.05 M) were HCl/KCl (pH 2.0), citrate-phosphate (pH 3.0–5.0), phosphate (pH 6.0–8.0), Tris (pH 9.0) and carbonate/bicarbonate (pH 10.0). Standard error of means are shown for three replicates.

pinoresinol were again confirmed by GC-mass spectrometry as reaction products.

Syringaldazine, negative histochemical reaction of which prompted Harkin and Obst [3] to conclude that lignification is catalysed exclusively by peroxidase, was not oxidized by *P. strobus* SL PP. When incubated for an extended period with a highly concentrated aliquot of SL PP SOF, syringaldazine was oxidized, but slowly in comparison with coniferyl alcohol or *p*-anisidine. The evident low affinity of this coniferyl alcohol oxidizing glycoprotein for syringaldazine probably explains why Harkin and Obst [3], investigating *P. strobus* and other conifer species used by us, detected "not even the faintest trace" of syringaldazine oxidation.

SL PP did not oxidize either putrescine or spermidine, suggesting that it is distinct from the diamine oxidase discovered in mature xylem apoplast of angiosperms [16, 17]. Repeated attempts to demonstrate that SL PP or SL

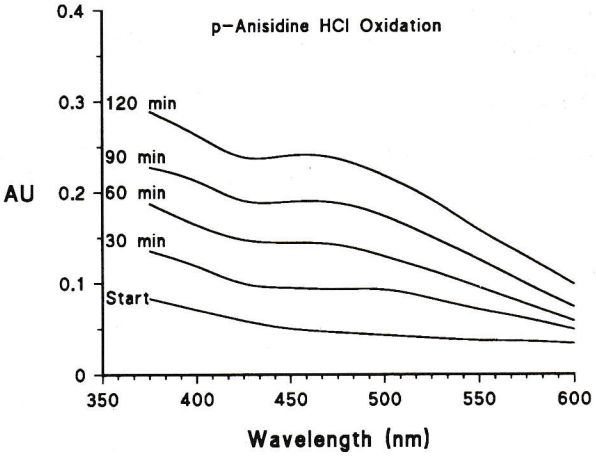

Fig. 9. Oxidation of *p*-anisidine HCl (10 mM) by SL PP SOF (116 ± 2 μg protein) in 0.05 M phosphate buffer, pH 7.0.

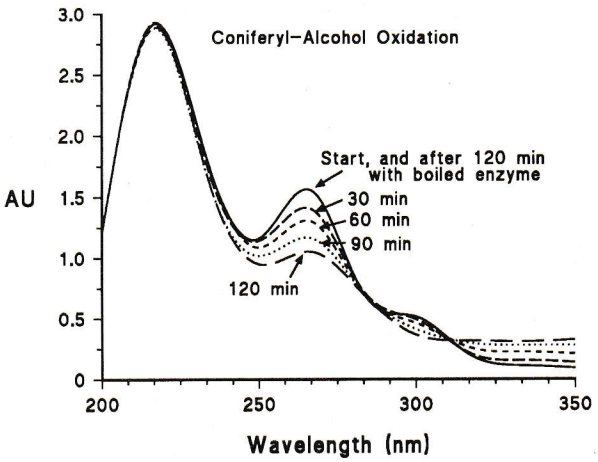

Fig. 10. Oxidation of coniferyl alcohol (70 μM) by SL PP SOF (116 $\pm$ 2 μg protein) in 0.05 M phosphate buffer, pH 7.0.

PP SOF generates H_2O_2 during oxidation have all been unsuccessful.

The essentially perfect spatiotemporal correlation between appearance of cell wall-bound *p*-anisidine oxidizing activity and occurrence of lignification, together with the finding that both *p*-anisidine and coniferyl alcohol are oxidized by SL PP SOF, suggests that these glycoproteins, presumably isoenzymes, be termed 'coniferyl alcohol oxidase'. It remains to be determined whether coniferyl alcohol oxidase has copper as active redox site. Coniferyl alcohol oxidase is seemingly stable when stored in solution at $-18°$; hence, assuming we did not discard or inactivate a significant proportion of the enzyme during workup, it appears that the enzyme occurs only in minute amounts, and only during active lignification, quite in contrast to peroxidase.

During tracheid differentiation in conifers, lignification occurs slowly, the process not uncommonly extending over a period of several weeks within walls of individual tracheids. Our observations suggest lignification during tracheid differentiation will not occur at all in the absence of active cell wall-bound coniferyl alcohol oxidase; hence, it would appear that it is O_2 rather than H_2O_2 that is reduced during lignification, There is evidence that O_2 becomes in short supply during *in vivo* wood formation [18], and it follows that O_2 availability could be a factor regulating the rate of lignification. On the other hand, initiation of lignification in springtime occurs when O_2 levels are relatively high, suggesting that competence for initiating lignification resides in either gene expression for, or activation of cell wall-bound coniferyl alcohol oxidase.

EXPERIMENTAL

Reagents. *E*-Coniferin was crystallized in-house from *P. strobus* cambial extracts [4]. Coniferyl alcohol (Fluka) and other compounds (Sigma, except where indicated) were purchased.

Trees. Healthy trees between 10 and 30 years old growing in the University of New Brunswick Forest, Fredericton, NB, Canada, were selected for all aspects of this research.

Microscopy. The progression of early-wood development was determined by bright-field microscopy using live samples taken from successive points over the length of the main stems of freshly felled trees from late spring to early summer. Peroxidase activity was investigated by floating hand-cut transverse sections of live stem material on the surface of freshly prepared solutions [either 1.0 mM *p*-anisidine HCl (Eastman Organic) with 10 mM H_2O_2 (Fisher Sci.) or 1.0 mM *p*-anisidine HCl] for 4 hr at room temp. The sections were rinsed and mounted in H_2O and examined by bright-field microscopy. The *p*-anisidine reagent rapidly yields a distinctively yellow-orange chromophore in walls of mature tracheids, and upon addition of H_2O_2 the reagent gives a pink (weak, e.g. Fig. 3), red-brown (strong) or dark purple (intense) colour. As measured by UV/Vis spectroscopy, aq. *p*-anisidine HCl has an absorption maximum at 286 nm. With peroxidation (horseradish peroxidase/H_2O_2), the products initially had an absorption maximum at 546 nm which shifted to 508 nm after 16 hr.

Incident UV autofluorescence microscopy was done with a Reichert-Jung Polyvar (B-1 filter: bandpass 450–495 nm, longpass > 520 nm) using established methods [9, 12].

Preparation of secondary wall particulate preparation (SL PP). Following bark peeling during the period of cambial growth [10], scrapings from the xylem surface were frozen in liquid N_2, pulverized to a fine powder with mortar and pestle, and maintained in liquid N_2. Frozen powder (100 g_{fw}) was extracted for 4 hr with 0.05 M phosphate buffer (pH 7.0, 1 l, 4°) containing 1% Triton-X 100 (Sigma), then filtered through 4 layers cheese cloth and squeezed to damp dryness. The insoluble tissue residue was extracted with continuous stirring in 0.5 M NaCl (1 l, 4°) for 16 hr

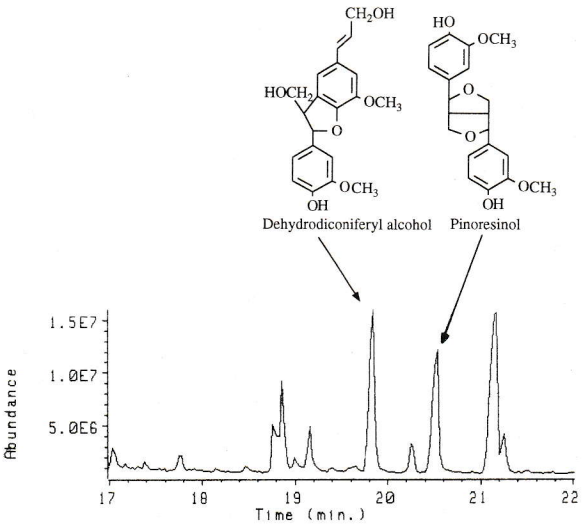

Fig. 11. GC-MS total-ion current fragmentogram (*m/z* 70–700) of trimethylsilyl (TMSi) derivatives of ethyl acetate-soluble fraction after incubating SL PP with coniferyl alcohol in pH 7.0 phosphate buffer for 2 hr (25°). Mass spectrum of R_t 19.845 min: *m/z* 574 $[M]^+$, 559 (M − 15), 484, 454, 382, 323, 310, 293, 280, 233, 223, 209, 204, 193, 179, 161, 147, 133, 129, 115, 103, 89, 77, 75, 73: dehydrodiconiferyl alcohol, 3X TMSi. Mass spectrum of R_t 20.535 min: *m/z* 502 $[M]^+$, 487 (M − 15), 393, 293, 277, 263, 252, 247, 235, 223, 217, 209, 205, 194, 179, 166, 149, 131, 117, 115, 105, 103, 89, 81, 77, 75, 73: pinoresinol, TMSi × 2. The several small peaks near 19 min were present at similar abundance in the control (coniferyl alcohol without SL PP). The small peak at 20.25 min and the one large and one small peak after 21 min were present at similar abundance in SL PP without coniferyl alcohol.

and filtered through 4 layers of cheese cloth. The insoluble tissue residue was transferred to 250-ml centrifuge bottles, suspended in double all-glass distilled H_2O (4°), and centrifuged (10 000 *g*, 4°, 20 min), repeating this centrifugation step 4 times and discarding each supernatant. The resulting pellet was suspended in double all-glass distilled water (1 l) to which was added pectinase (*Aspergillus niger*, Sigma P-5146, 50 mg, 6.9 U mg^{-1}), cellulase (*Trichoderma reesi*, Serva 16421, 125 mg, 0.5 U mg^{-1}) and gentamycin (62.5 mg, Flow). The mixture was stirred for 16 hr at room temp. (~25°), then centrifuged (10 000 *g*, 20 min, 4°) and the supernatant discarded. The pellet was re-suspended in double all-glass distilled H_2O (4°) and centrifuged again as described, repeating this washing step 6 times. The final pellet comprises 'SL PP' (or 'RE PP' when only radially expanded primary-walled cambial derivates were investigated). All 5 species were investigated to the SL PP stage.

Preparation of solubilized oxidizing factor from SL PP (SL PP SOF). Re-suspended SL PP from *P. strobus* was incubated in 0.5 M phosphate buffer (pH 7.0, 20 ml, 4°, 20 hr) containing NaCl (0.5 M) and gentamycin (50 mg), then centrifuged (20 000 *g*, 4°, 30 min) and the supernatant transferred to a Centriprep 10 (Amicon) tube. Following centrifuging (2455 *g*, 4°, 15 min), the ≤10 000 M_r dialysate was decanted to waste, double all-glass distilled water (2.0 ml, 4°) was pipetted to the retentate, and repeat centrifuging of the Centriprep 10 was done 4 times as described. Gentamycin (5.0 mg) was added with 2.0 ml H_2O before the final centrifugation was done (2455 *g*, 4°, 60 min). The final retentate (≥10 000 M_r) containing solubilized oxidizing factor from SL PP ('SL PP SOF') was transferred to a vial, capped, and stored at −18°.

Protein estimation. Soluble protein was estimated by the Bradford method [19]. The same method with modifications [20] was used for spectrophotometric semi-quantitative estimation of bound protein content in suspensions (SL PP) of total volume 2.1 ml.

Isoelectric focusing. Isoelectric focusing was performed on PhastGel (Pharmacia) IEF 3-9 minigels (43 × 50 × 0.35 mm) using a PhastSystem (Pharmacia) according to the manufacturer's specifications. Suspensions (RE PP, SL PP) or solutions (SL PP SOF) were loaded on to gels and IEF was performed at 15° under conditions described in 'Separation Technique file no. 100' (Pharmacia PhastSystem User's Manual, Pharmacia LKB Biotechnology, Sweden). Markers used were: bovine carbonic anhydrase B, human carbonic anhydrase B, horse myoglobin (acidic band), horse myoglobin (basic band).

Coomassie staining (~20 ng protein detection limit) and silver staining (~1 ng protein detection limit) of PhastGel IEF media were carried out in a PhastSystem Development Unit (Pharmacia) according to the protocols in Table 1 of PhastSystem Development Technique File no. 200 and File no. 210, respectively (Pharmacia PhastSystem User's Manual, Pharmacia LKB Biotechnology, Sweden). For heme and glycoprotein determinations, gels were fixed in 20% trichloroacetic acid, washed and destained (30% MeOH + 10% HOAc in distilled H_2O), and stained for 10 min in the stated reagents prepared in the same destaining solution. Gels were destained for 10 min until the background had cleared. To determine oxidase activity, gels were incubated for 16 hr at 37° in 0.25% (w/v) *p*-phenylenediamine or 20 mM *p*-anisidine.

Enzyme assays. Assays were performed using 0.05 M phosphate buffer (2.0 ml, pH 7.0) at 37° with 50 µl aliquots (≡116 ±2 µg protein) of *P. strobus* SL PP SOF. Controls were boiled *P. strobus* enzyme; checks (substrate only, no added protein) were also done. Investigated compounds included *p*-anisidine (10 mM), *p*- and *o*-phenylenediamine (10 mM), dopamine (10 mM), gallic acid (10 mM), *o*-dianisidine (10 mM), guaiacol (20 mM), coniferyl alcohol (70 µM), and syringaldazine. Absorbance changes were monitored using a UV/Vis spectrophotometer (HP8452, Hewlett-Packard, 2 nm photodiodes).

Syringaldazine oxidation was assayed as follows: 2.2 ml sodium phosphate buffer (0.1 M, pH 6.5) and 0.5 ml SL PP suspension (≡40 µg bound protein) or 50 µl SL PP SOF (≡116 µg protein) were pipetted into a cuvet and equilibrated at 30°. Syringaldazine (300 µl of 0.078 mg ml^{-1}, in MeOH) was added to the reaction mixture and the increase in absorbance at 530 nm was recorded for 120 min.

To investigate the possibility of diamine oxidase activity, putrescine (5.36 mmol) or spermidine (5.35 mmol) were added to 0.1 M guaiacol in phosphate buffer (0.1 M, pH 7.2). To this was added horseradish peroxidase (Sigma P-8250, 5 U) and SL PP (8 µg protein). The reaction mixture, with appropriate controls, was incubated for 16 hr at room temp. and absorbance read at 416 nm. Following these spectrophotometric readings, addition of a trace amount of H_2O_2 confirmed that absence of guaiacol oxidation could not be explained in terms of inactive peroxidase.

The possibility of H_2O_2 generation by *P. strobus* SL PP (8 µg protein) was investigated using 20 mM guaiacol with horseradish peroxidase. The reaction mixture with appropriate control was incubated for 16 hr at room temp. and absorbance read at 416 nm.

Gas chromatography-mass spectrometry. Coniferyl alcohol (1.0 mg) was added to 0.1 M phosphate buffer (2.0 ml, pH 7.0) and incubated for 2 hr (25°) with or without *P. strobus* SL PP (80 µg protein). The activity of *P. strobus* RE PP and the contribution of an equivalent weight of SL PP without added substrate were also investigated. The experiments were done in triplicate and stopped by extracting with EtOAc (2.0 ml × 4), combining the 4 EtOAc fractions and drying under N_2. The dry residue was derivatized with *N*, *O-bis*-(trimethylsilyl)trifluoroacetamide (Pierce), heating for 1 hr at 70° before GC-MS. Splitless injections (2 µl) were made on to a 0.18 mm (i.d.) × 16 m DB-1 (J&W Scientific, Folsom, CA) open tubular column (0.4 µm cross-linked methyl silicone) at 40° (deactivated splitless injection liner, injection port 200°), purging the injection port after 1 min at 40°. Temperature programming at 16° min^{-1} to 300° followed. Carrier gas (He) flowed at 0.55 ml min^{-1} (35.7 cm sec^{-1}). Electron impact (70 eV) data were collected using a mass spectrometer (HP5970B hyperbolic quadrupole mass filter with *m/z* 0.1 steps, Hewlett Packard) at 0.68 scans sec^{-1} over the range *m/z* 70–700. Data were stored, analysed and used to generate hard-copy reports using a HP59970C ChemStation (Hewlett Packard). Following development of methods to solubize the oxidizing factor, the experiment was repeated using SL PP SOF and the results with SL PP were confirmed.

Role of oxygen. Following N_2 purging, a solution of *o*-dianisidine (0.016 mmol) in 0.05 M phosphate buffer (2.2 ml, pH 7.0) with *P. strobus* SL PP (8 µg protein) in a stoppered (red rubber Suba seal) glass test tube was continuously flushed free of oxygen by maintaining the headspace under gently flowing N_2. N_2-flushed, control (*o*-dianisidine solution containing SL PP under air in a sealed test tube) and check (*o*-dianisidine solution under air in a sealed test tube) solutions were left standing for 16 hr at ambient temp. After 16 hr, *o*-dianisidine + SL PP incubated under N_2 yielded 440 nm absorbances <0.05. The *o*-dianisidine check solution (no SL PP) incubated under air gave <0.1 AU. Controls exhibited absorbance >3.2.

After 20 hr of incubation under air in stoppered test tubes at ambient temp. aliquots (2 µl) of the headspace above *o*-dianisidine (0.016 mmol) solutions with and without SL PP (8 µg protein) were analysed in triplicate by GC-MS using a 0.18 mm i.d. cross-linked methylsilicone column (J&W Sci., DB-1) at 30° isothermal with He flowing at 0.6 ml min^{-1} in splitless mode,

purging the injection port 0.1 min after each injection. Selected ion monitoring at m/z 32 was done using a 5890A gas chromatograph (Hewlett Packard) with direct inlet to 5970B (Hewlett Packard) source. Under these conditions, O_2 was consumed at the rate of 1 ml hr^{-1}.

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