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Electrophoretic analysis of coniferyl alcohol oxidase and related laccases

Gradient gel electrophoretic methods enabled a distinction to be made between coniferyl alcohol oxidase (CAO) of lignifying cell walls and a p*I* ~ 9 pine "laccase" recently implicated in lignification (*Science* 1993 *260*, 672). Following treatment of a partially purified protein mixture from developing xylem of *Pinus strobus* with 2-[*N*-morpholine]ethanesulfonic acid (MES) buffer, isoelectric focusing and sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicated that CAO had been selectively precipitated by MES and thereby purified to electrophoretic homogeneity. Purified CAO was determined to be a cell-wall-bound glycoprotein (38% glycan), M_r 107 500, p*I* 7.6, pH and temperature optima 6.3 and 30°C, respectively. By graphite-furnace atomic-absorption analysis, CAO contained one copper atom per protein molecule. Proteins obtained from lignifying cambial derivatives of conifers (family *Pinaceae*) and from *Rhus typhina* bark were compared with CAO and the p*I* ~ 9 pine "laccase" following electrophoresis and Western blotting. For *Abies balsamea, Larix laricina, Picea rubens, Pinus banksiana, Pinus taeda,* and *R. typhina,* the isoelectric points of oxidatively active bands were identical to those of purified CAO. In addition, for all species only the p*I* 7.6 band was immunoreactive with antibodies against periodate-deglycosylated CAO.

1 Introduction

The identity of the enzyme(s) catalyzing the oxidative polymerization of monolignols during lignin synthesis is currently a matter of controversy. Recent reviews by Dean and Eriksson [1] and O'Malley *et al.* [2] trace the historical developments chronologically. Initially, both peroxidase (EC 1.11.1.7) and laccase (*p*-diphenol: oxygen oxidoreductase, EC No. 1.10.3.2) were perceived to catalyze lignification, but subsequent research indicated that laccase either was not present or was inactive during wood formation. By default, therefore, peroxidase emerged as the sole catalyst promoting lignification. This conclusion was recently challenged, however, with our report of an O_2-requiring, non-H_2O_2 utilizing "coniferyl alcohol oxidase" (CAO) that was spatio-temporally correlated with the seasonal progression of *in situ* lignification in conifers [3, 4]. More or less concomitantly, occurrences of "laccase" in angiosperms [5–7] and another conifer species [8] were reported. CAO from *Pinus strobus* was reported to be a "laccase-like" glycoprotein of p*I* ~ 7 obtainable as a soluble enzyme only after cellulase-pectinase digestion of actively lignifying cell walls [4]. A "laccase" having p*I* ~ 9 and extractable from lignifying xylem of *Pinus taeda* in molar $CaCl_2$ solution also has been reported [2, 8]. The differing isoelectric points and extractabilities of these two enzymes has raised questions pertaining to their possible roles in lignification and relation to one another. As detailed below, definitive protein purification in combination with biochemical and immunological procedures have been used to investigate this problem.

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Abbreviations: CAO, coniferyl alcohol oxidase; **DAF,** 2,7-diaminofluorene; **PPD,** *p*-phenylenediamine; **SL PP SOF,** solubilized oxidizing factor from particulate fraction of lignifying secondary walls [4]

2 Materials and methods

2.1 Materials

Precast PhastGel separation media, PhastGel chemicals and calibration kits (molecular weight and p*I*) were obtained from Pharmacia. All other chemicals used were of analytical grade and purchased from Sigma unless indicated otherwise. Immobilon-P (polyvinylidene difluoride, PVDF) protein-blotting membrane was supplied by Millipore. The main stems of healthy trees (*Abies balsamea* (L.) Mill., *Larix laricina* (Du Roi) K. Koch, *Picea rubens* Sarg., *Pinus banksiana* Lamb., and *Pinus strobus* L.) aged between 10–30 years and growing in the University of New Brunswick (UNB) forest were sources of developing, actively lignifying xylem. The green inner bark (cortex) from one-year-old branches of *Rhus typhina* L. growing on the UNB campus was also investigated. This cortical tissue does not lignify; however, it is well known that the latex of *Rhus* spp. is a rich source of laccase. Frozen tissue from *Pinus taeda* L. was kindly provided by Dr. R. Sederoff and colleagues and confirmed by microscopy to consist of lignifying cambial derivatives.

2.2 Methods

2.2.1 Enzyme extraction and purification

2.2.1.1 Conifers

A semi-purified solubilized enzyme extract ("SL PP SOF", [4]) containing CAO activity was prepared from salt-extracted, actively lignifying cell-wall residues of each of the six conifer species by cellulase-pectinase digestion as fully described elsewhere [4]. For comparison, a "cell-wall protein fraction" was also obtained from the same six conifer species by extracting freshly harvested, actively lignifying cambial derivatives with a molar $CaCl_2$ solution using the method of Bao *et al.* [8].

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To purify CAO to electrophoretic homogeneity, SL PP SOF was filtered through glass microfiber (Whatman GF/F); next CAO in the protein solution was precipitated as follows. One mL of filtered SL PP SOF, containing 5–6 mg protein, was diluted tenfold in cold (4°C) MES buffer (10 mM, pH 5.0, 4°C), incubated for 30 min at 4°C, vortexed and centrifuged at 20×10^3 *g* (15 min, 4°C). The supernatant was discarded and the pellet was washed twice with 10 mL cold MES buffer. The washed pellet was dissolved in 500 µL of Tris-HCl buffer (50 mM, pH 8.0) and dialyzed against the same buffer. Aliquots of the purified enzyme were stored at –20°C.

2.2.1.2 *Rhus typhina*

Laccase activity was purified from *R. typhina* bark (500 g_{fw}) using the procedure of Nakamura [9] with slight modifications. The latex-containing cortex was extracted with sodium acetate buffer (50 mM, pH 5.5, 4°C) for 1 h. After passing the extract through four layers of cheese cloth, 1000 mL of ice-cold acetone was mixed with 2000 mL of the crude extract using vigorous stirring, and the mixture was allowed to stand for 30 min. Following centrifugation (22×10^3 *g*, 4°C, 15 min), the pelleted precipitate was cleared of pigment by repeatedly washing with ice-cold acetone until a colorless solution was obtained. After centrifugation and discarding of the final supernatant, the washed precipitate was extracted overnight in double-distilled water (200 mL, 4°C). Solid ammonium sulfate to 65% saturation was added with gentle stirring to the crude, imperfectly dissolved precipitate. The mixture was allowed to stand for 3 h, centrifuged (22×10^3 *g*, 4°C, 30 min), and the supernatant decanted (pellet discarded) and brought to saturation with solid ammonium sulfate. The mixture was allowed to stand overnight at 4°C, then centrifuged (22×10^3 *g*, 4°C, 30 min). The pelleted precipitate was dissolved in a minimum volume of sodium acetate buffer (50 mM, pH 5.5) and extensively dialyzed at 4°C against the same buffer for 48 h. The dialyzed enzyme preparation was filtered through glass fiber (Whatman GF/F) concentrated by centrifugal dialysis (Centriprep-10, Amicon), and stored at –20°C.

2.2.2 Protein estimation

Soluble protein was quantified by a semimicro assay based on the Bradford method using bovine serum albumin as standard [10].

2.2.3 Spectrophotometric enzyme assay

Purified CAO (30 µg) was incubated at 30°C in 1.5 mL coniferyl alcohol (70 µM, Fluka) in phosphate buffer (50 mM, pH 6.3). The spectrophotometric 'blank' consisted of the above solution at the moment of adding protein. Coniferyl alcohol without enzyme was used as control. Decrease in absorbance at 264 nm was measured (HP 8452 UV/Vis spectrophotometer, 2 nm photodiodes, Hewlett Packard). One unit of the enzyme is defined as the amount catalyzing the oxidation of 1 nMol of coniferyl alcohol per hour at pH 6.3 at 30°C.

2.2.4 Deglycosylation of protein

To estimate the M_r of completely deglycosylated CAO, purified enzyme was treated with trifluoromethanesulfonic acid [11]. Mild periodate oxidation, a procedure that destroys all glycan epitopes of glycoproteins [12], was used to prepare deglycosylated CAO as antigen and for subsequent immunological procedures.

2.2.5 Polyclonal antibody production

Four- to six-week-old female BALB/c mice were immunized intra-peritoneally (i.p.) with 250 µg of either purified native or purified deglycosylated CAO emulsified in Freund's complete adjuvant. The mice were subsequently boosted i.p. with the same immunogen in Freund's incomplete adjuvant at monthly intervals on two occasions. The final immunization consisted of 250 µg of enzyme in sterile phosphate-buffered saline (pH 7.4) injected intra-venously. Four days later, mice were bled by heart puncture. The serum from each was separated and decomplemented [13], filter sterilized and stored at –20°C. The antisera were used without further purification.

2.2.6 Isoelectric focusing and polyacrylamide gel electrophoresis

The enzyme preparations were electrofocused on minigels (PhastGel IEF 3–9, Pharmacia) according to PhastSystem separation technique file number 100 (Pharmacia). SDS-PAGE was done on PhastGel Gradient 8–25 minigels (Pharmacia) using a PhastSystem (Pharmacia) according to the manufacturer's specifications.

2.2.7 Staining of electropherograms

Staining of protein in minigels (Pharmacia) was performed with a PhastSystem Development unit (Pharmacia) according to the manufacturer's protocols using either Coomassie Brilliant Blue R-250 or silver. IEF gels were stained for oxidase activity by incubating in *p*-anisidine hydrochloride (20 mM, Kodak Chemicals), *p*-phenylenediamine dihydrochloride (PPD, 10 mM), or 2,7-diaminofluorene (DAF, 0.68 mM) for 16 h at 37°C. IEF gels were stained for glycoprotein using either Alcian Blue [14] or a periodic acid-Schiff staining procedure established for the Pharmacia PhastSystem [15].

2.2.8 Electroblotting and immunodetection

Proteins separated on IEF PhastGels were electroblotted onto a PVDF membrane using the PhastTransfer semidry transfer kit (Pharmacia) according to the manufacturer's instructions. After transfer, membranes were blocked (1 h, 37°C) in milk buffer, composed of Tris-buffered saline (TBS: 0.9% NaCl in 20 mM Tris/HCl), containing 5% w/v powdered nonfat milk (Carnation). The membranes were incubated (4 h, 20°C) in a 1:1000 dilution of polyclonal antibody in milk buffer, then washed for 3×5 min in milk buffer followed by incubation (2 h, 20°C) in a 1:1000 dilution of alkaline phospha-

tase-conjugated goat anti-mouse immunoglobulins (Bio-Rad 170–6520) in milk buffer. Membranes were again washed in milk buffer (20°C), followed by a final wash in TBS (20°C). The washed membranes were incubated (15–30 min, 20°C) in solution with alkaline phosphatase substrates (0.56 mM 5-bromo-4-chloro-3-indolylphosphate, 0.48 mM nitroblue tetrazolium in Tris/HCl buffer (10 mM, pH 9.5)) to visualize sites of antigen-antibody reaction. The reaction was stopped by rinsing in double-distilled H_2O.

2.2.9 Graphite-furnace atomic absorption analysis of copper

Known weights of purified CAO from samples of lignifying *P. strobus* xylem were analyzed in triplicate using a graphite tube analyzer (Varian GTA96) in an atomic absorption unit (Varian AA400), interpolating copper content from a copper nitrate calibration curve. Silver-staining of IEF gels gave no evidence for p*I* 9 protein in any of the CAO samples analyzed for copper. Blanks, comprising solvents and buffers in the absence of protein from lignifying tissue, served as controls.

3 Results and discussion

3.1 Purification of CAO

Using standard procedures such as preparative IEF, electroelution from polyacrylamide gels, ion-exchange high performance liquid chromatography, and liquid-column chromatography (ion-exchange and Concanavalin-A matrices), purification of CAO beyond that described by Savidge and Udagama-Randeniya [4] could not be satisfactorily achieved. In the course of evaluating these standard procedures, it was observed that when SL PP SOF was treated with prechilled MES buffer, CAO precipitated, leaving oxidatively inactive protein in solution. Temperature, pH, and buffer molarity as described above have been optimized for the CAO precipitation procedure. As indicated in Table 1, MES precipitation of CAO from buffered extracts of 0.3 kg_{fw} lignifying cambial derivatives reliably yields > 2000 units of CAO activity.

Table 1. Purification of coniferyl alcohol oxidase from *Pinus strobus*

Enzyme fractionation step	Total protein mg	CAO units	Specific activity U mg^{-1}	% Yield[a)]
"SL PP SOF"	31.1	4752.08	152.8	100
MES precipitate	6.7	2052.96	306.4	43
MES supernatant	21.3	432.06	20.3	9

a) Based on CAO activity estimates.

MES precipitation of SL PP SOF from *P. strobus* yields a homogeneous preparation of CAO, the purity of which has been repeatedly confirmed by obtaining a singular polypeptide band on SDS PAGE gels (Fig. 1). The glycoprotein nature of purified CAO was confirmed by both Alcian Blue [14] and Schiff's staining [15]. CAO had a molecular mass as estimated by SDS-PAGE of 107.5 ± 3.0 kDa, and that of the deglycosylated protein was 67 ± 1 kDa (Fig. 1). Hence, the carbohydrate content of CAO appears to be 38% by weight. The pH and temperature

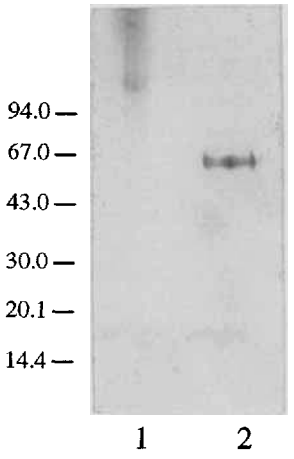

Figure 1. SDS-PAGE of purified CAO. Silver-stained electropherogram (PhastGel Gradient 8–25) of purified CAO (lane 1) and of the deglycosylated [11] form of the enzyme (lane 2). Standard molecular-mass (subunit) markers (kDa): phosphorylase b 94.0; albumin 67.0; ovalbumin 43.0; carbonic anhydrase 30.0; trypsin inhibitor 20.1; alpha-lactalbumin 14.4.

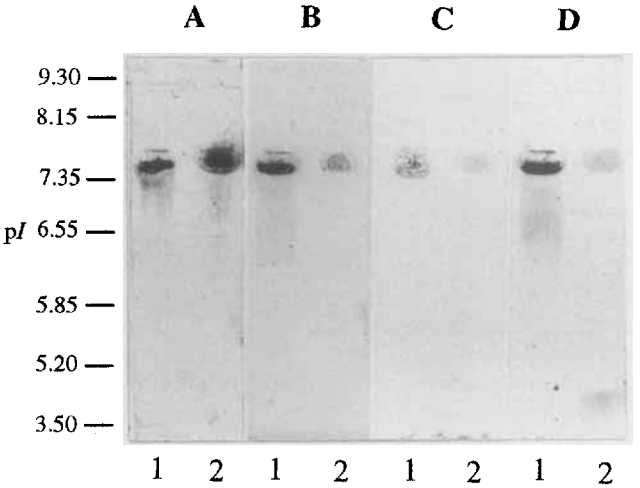

Figure 2. IEF of purified enzymes. CAO (lane 1) and *Rhus* laccase (lane 2) were applied to an IEF (3–9) PhastGel (1 µg protein per lane); following electrophoresis they were stained with (A) silver, (B) 10 mM PPD, (C) 20 mM *p*-anisidine hydrochloride, and (D) 0.68 mM DAF. Standard *p*I markers: trypsinogen 9.30; lentil lectin (acidic band) 8.15; horse myoglobin (basic band) 7.35; human carbonic anhydrase B 6.55; bovine carbonic anhydrase 5.85; *beta* lactoglobulin A 5.20; amyloglucosidase 3.50.

optima of the enzyme were 6.3 and 30°C, respectively, for coniferyl alcohol (70 µM, in 50 mM phosphate buffer) as substrate. The isoelectric point of CAO, as defined by IEF using both Coomassie Blue (not shown) and activity staining (*p*-anisidine and DAF) was p*I* 7.6 (Fig. 2). Silver and PPD staining of isoelectrofocused CAO also revealed intense staining of the band at pH 7.6, but with an accompanying smeared (diffused) region extending from p*I* 7.6 to p*I* ~ 6.55 (Fig. 2).

3.2 Enzyme visualization

The original histochemical evidence for CAO was based upon an enzyme capable of oxidizing both *p*-anisidine

and coniferyl alcohol [4]. IEF of SL PP, a preparation containing particulate CAO activity, on IEF 3–9 PhastGels resulted in a band at p*I* 6.55 with strong PPD-oxidizing and poor *p*-anisidine-oxidizing activity, whereas the loading zone (p*I* ~ 7.5) oxidized both PPD and *p*-anisidine [4]. By incubation of SL PP in 0.5 M NaCl, soluble CAO ('SL PP SOF') activity was obtained [4]. After IEF 3–9, SL PP SOF displayed at least three protein bands near p*I* 6.5 [4]. Again, these p*I* ~ 6.5 bands oxidized PPD strongly but *p*-anisidine only weakly, *p*-anisidine oxidation being restricted to the loading zone (p*I* ~ 7.5) [4]. Following MES precipitation of protein from SL PP SOF, purified *p*-anisidine- and coniferyl-alcohol-oxidizing CAO was found to focus at p*I* 7.6. The MES precipitation step eliminated the p*I* 6.55 band, but when stained with silver or PPD there remained a smeared, diffused region extending from p*I* 7.6 to p*I* 6.55. This diffuse zone did not oxidize *p*-anisidine. Hence, although there clearly are PPD-oxidizing proteins of lower p*I* in SL PP, SL PP SOF, and the MES precipitate of SL PP SOF, only the p*I* 7.6 band just below the loading zone (p*I* 7.7–8.5) oxidizes both coniferyl alcohol and *p*-anisidine. It is possible that the oxidatively active bands of lower p*I* are related to CAO but this remains to be investigated.

3.3 Copper content

Copper was confirmed by graphite-furnace atomic-absorption analysis to be present in CAO preparations both before and after MES precipitation. However, in agreement with our observation that CAO does not yield a blue solution diagnostic of laccase, the absence of absorbance at 600–615 nm indicated type I copper was not present. The copper content in purified CAO was calculated to be 1.2–1.3 copper atoms per protein molecule. The MES-precipitation method inactivated CAO to some extent (Table 1). MES also was observed to form insoluble complexes with solutions of copper salts (data not shown); hence, reduced CAO activity following MES precipitation may be due to removal of some copper from the protein. MES treatment of laccases from *Rhus typhina* and *Pyricularia oryzae* also reduced the coniferyl alcohol oxidizing activities of these enzymes.

3.4 SL PP SOF and $CaCl_2$ extracts

The presence of CAO activity as determined by spectrophotometric analysis of coniferyl alcohol oxidation was detected in both SL PP SOF and $CaCl_2$ extracts of lignifying tissue from *Abies balsamea, Larix laricina, Picea rubens, Pinus banksiana, Pinus strobus, and Pinus taeda.* Following IEF (pH 3–9) of both types of extract, incubation of the gels with *p*-anisidine or DAF yielded an identical staining pattern for all conifer species: only one protein band at p*I* 7.6 was oxidatively active (Fig. 3). However, with PPD as substrate, a smeared staining pattern characteristic of purified *P. strobus* CAO was obtained (Fig. 3).

In agreement with the report by Bao *et al.* [8], $CaCl_2$ extracts of lignifying tissue from all six conifer species contained p*I* 9 protein in bands that stained with both silver and Coomassie Blue (Fig. 3). Based on the Fig. 3 evidence, the band at p*I* 9 could consist of several proteins of p*I* ≥ 9, as the pH gradient in Fig. 3 did not extend beyond p*I* 9; however, separations using pH 3.5–9.5 gels revealed the presence of only a singular protein band focusing at p*I* ~ 9 (data not shown). The p*I* 9 band was readily extracted from protoplasm of lignifying tissue using molar $CaCl_2$. However, unlike CAO, the band was not obtained following cellulase-pectinase digestion of residual lignifying wall material. For every species, the p*I* 9 band as extracted from the protoplasm of lignifying tissue in our lab was oxidatively inactive with *p*-anisidine, DAF or PPD (Fig. 3). In contrast, upon investigating partially purified p*I* 9 "laccase" from *P. taeda* (as provided to us by Bao *et al.*), we observed DAF oxidation to be associated with a minor band at p*I* 9, and this band also oxidized PPD but not *p*-anisidine. The major band of oxidase activity in this partially purified "laccase" preparation focused at p*I* 7.6, and that band oxidized PPD and *p*-anisidine as well as DAF. Cell-wall-bound enzymes obtained from conifers by cellulase-pectinase digestion of lignifying tissues yielded identical banding patterns on IEF gels to those of $CaCl_2$ extracts, the p*I* 7.6 band being the major band of oxidase activity with the same three substrates.

To rule out the possibility that the p*I* 9 protein as extracted by us had been inactivated during workup, sev-

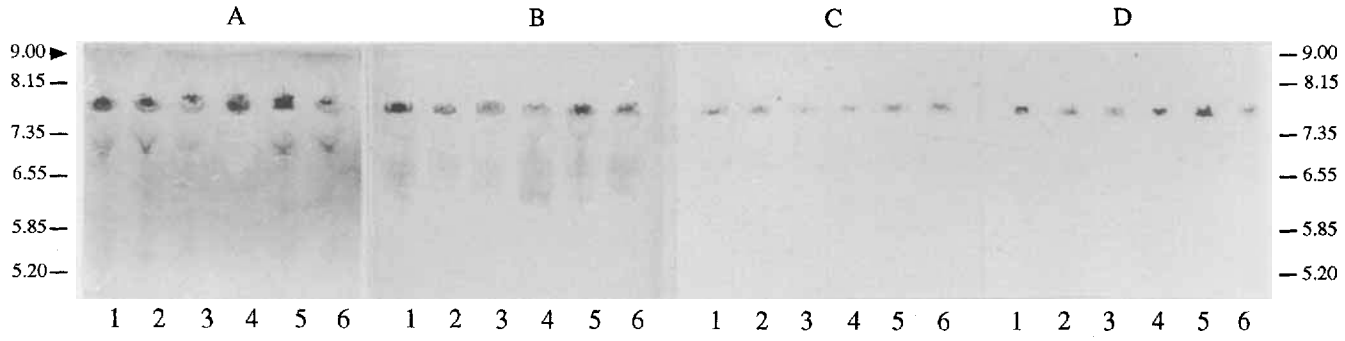

Figure 3. Activity staining of $CaCl_2$ extracts from lignifying tissues of conifers. Cell-wall protein fractions of *Larix laricina* (lane 1), *Picea rubens* (2), *Abies balsamea* (3), *Pinus taeda* (4), *Pinus strobus* (5), and *Pinus banksiana* (6) were electrofocused on pH 3–9 gels and stained with (A) 0.1% Coomassie Brilliant Blue R-250, (B) 10 mM PPD, (C) 20 mM *p*-anisidine hydrochloride, and (D) 0.68 mM DAF. The arrow on (A) indicates a p*I* ~ 9.0 Coomassie Blue stained band present in developing xylem of all six conifer species but devoid of oxidative activity. p*I* markers as in Fig. 2 caption.

eral different batches of tissue from each conifer species were $CaCl_2$ extracted and processed by carefully following the procedure of Bao *et al.* [8]. Nevertheless, the p*I* 9 band invariably was oxidatively inactive. In contrast, the p*I* 7.6 band from each of the six species invariably was active and oxidized *p*-anisidine, DAF and PPD. Our inability to confirm the work of Bao *et al.* [8] cannot be explained in terms of species differences because we examined lignifying tissue from loblolly pine (*P. taeda*), the species previously reported to contain a p*I* 9 "laccase" [2, 8].

3.5 Blotting

Following IEF, $CaCl_2$ extracts were blotted onto a PVDF membrane and probed with mouse sera raised against either native or deglycosylated forms of CAO. No immunostaining was observed with the preimmune control mouse serum. Antiserum against deglycosylated CAO reacted only with the protein band localized at p*I* 7.6, agreeing with DAF and *p*-anisidine oxidation bands (Fig. 4). Antibody against native CAO recognized the p*I* 7.6 proteins but also showed blotchy staining patterns over a range of lower p*I* values, similar to that seen on PPD-incubated gels, plausibly due to differences in the carbohydrate moeity of the CAO molecule. With both types of sera, deglycosylated CAO was recognized as a concise band at p*I* 7.6 (Fig. 5). Antiserum against deglycosylated CAO also recognized native CAO and p*I* 7.6 proteins in $CaCl_2$ extracts of *P. taeda* and the five other conifer species. The similar yet nonidentical immunostaining patterns shown by the two types of antisera indicate that the antigenic determinants in native CAO were not exclusively proteic. On Western blots, neither of the two sera recognized the protein band at p*I* 9.0, corroborating the distinction between this protein and CAO (Fig. 4).

3.6 Comparison of CAO with laccase

CAO and the laccase from *Rhus typhina* latex were identical in isoelectric points (Fig. 2). The *R. typhina* laccase p*I* is distinct from the p*I* 7.9 and p*I* 8.9 values reported for *Rhus vernicifera* laccases [16]. Oxidative staining of IEF 3–9 gels with PPD and *p*-anisidine resulted in identical staining patterns for CAO and *R. typhina* laccase. DAF incubation revealed an additional acidic band that was silver stained with difficulty (Fig. 2). Antisera against deglycosylaed CAO recognized only the p*I* 7.6 band of *R. typhina* laccase (Fig. 5). Following IEF (3–9) of *R. typhina* laccase and fractionation of the gel by pH, only the one protein band at p*I* 7.6 showed oxidation of coniferyl alcohol as detected by spectrophotometric analysis. The inability of a purified laccase from *R. vernicifera* (Japanese lacquer) to oxidize coniferyl alcohol was a contributing factor in rejecting an oxidizing role of plant laccase in lignification [17]. However, coniferyl alcohol was rapidly oxidized by in-house purified *R. typhina* laccase *in vitro*.

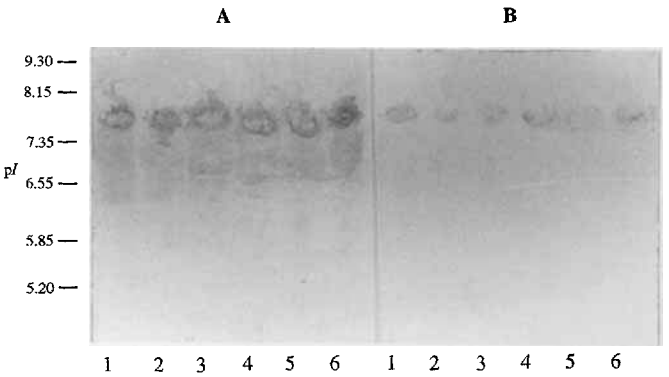

Figure 4. Immunostaining of Western blots of $CaCl_2$ extracts from lignifying tissues of conifers. Cell-wall protein fractions of *Larix laricina* (lane 1), *Picea rubens* (2), *Abies balsamea* (3), *Pinus taeda* (4), *Pinus strobus* (5), and *Pinus banksiana* (6) were electrofocused on pH 3–9 gels, transferred to a PVDF membrane by semidry transfer technique, and probed with polyclonal antibodies raised against (A) native CAO and (B) deglycosylated CAO. p*I* markers as in Fig. 2.

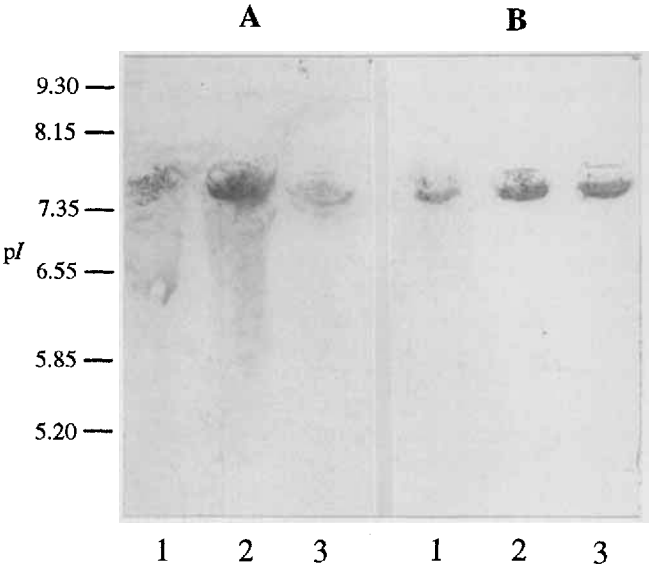

Figure 5. Immunodetection of electroblots of purified enzymes. Three µg of protein of *Rhus typhina* laccase (lane 1), native CAO (2), and deglycosylated CAO (3) were electrofocused on pH 3–9 IEF PhastGel, blotted onto PVDF membrane by semidry transfer technique and immunostained with polyclonal sera raised against the (A) native and (B) deglycosylated form(s) of CAO. p*I* markers as in Fig. 2.

4 Concluding remarks

In conclusion, this study has revealed that CAO is generally present in cell walls of actively lignifying xylem of conifers, that it shares properties with *R. typhina* laccase, and that it is distinct from the p*I* 9 "laccase" of Bao *et al.* [8]. Gradient gel electrophoresis offers a versatile tool for the comparison of enzymes not only with respect to molecular properties such as M_r and p*I* but also with regard to substrate (activity staining) and immunological specificities.

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5 References

[1] Dean, J. F. D., Eriksson, K.-E. L., *Holzforschung* 1994, *489*, 21–33.
[2] O'Malley, D. M., Whetten, R., Bao, W., Chen, C.-L., Sederoff, R., *Plant J.* 1993, *4*, 751–757.
[3] Savidge, R. A., Randeniya, P. V., *Biochem. Soc. Trans.* 1992, *20*, 229 S.
[4] Savidge, R., Udagama-Randeniya, P., *Phytochemistry* 1992, *31*, 2959–2966.
[5] Driouich, A., Laine, A.-C., Vian, B., Faye, L., *Plant J.* 1992, *2*, 13–24.
[6] Sterjiades, R., Dean, J. F. D., Eriksson, K.-E. L., *Plant Physiol.* 1992, *99*, 1162–1168.
[7] Sterjiades, R., Dean, J. F. D., Gamble, G., Himmelsbach, D. S., Eriksson, K.-E. L., *Planta* 1993, *190*, 75–87.
[8] Bao, W., O'Malley, D. M., Whetten, R., Sederoff, R. R., *Science* 1993, *260*, 672–674.
[9] Nakamura, T., *Biochim. Biophys. Acta* 1958, *30*, 44–52.
[10] Appenroth, K. J., Augsten, H., *Z. Med. Lab. Diagn.* 1986, *27*, 327–331.
[11] Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, Jr., L. E., Weber, P., *Anal. Biochem.* 1981, *118*, 131–137.
[12] Laine, A.-C., *Electrophoresis* 1988, *9*, 841–844.
[13] Hudson, L., Hay, F. C., *Practical Immunology*, Blackwell Scientific Publications, London 1989, p. 19.
[14] Wardi, A. H., Michos, G. A., *Anal. Biochem.* 1972, *49*, 607–609.
[15] Van-Seuningen, I., Davril, M., *Electrophoresis* 1992, *13*, 97–99.
[16] Graziani, M. T., Antonilli, L., Sganga, P., Citro, G., Mondovi, B., Rosei, M. A., *Biochem. International* 1990, *21*, 1113–1124.
[17] Nakamura, W., *J. Biochem.* 1967, *62*, 54–61.