Trees (1995) 10: 102–107

ORIGINAL ARTICLE

Preethi V. Udagama-Randeniya · Rodney A. Savidge

Coniferyl alcohol oxidase – a catechol oxidase?

Received: 7 November 1994 / Accepted: 20 February 1995

Abstract The physico-chemical properties of coniferyl alcohol oxidase (CAO), a copper containing glycoprotein spatiotemporally associated with lignification in conifers, is reported here. By electron paramagnetic resonance spectroscopy, only type 3 copper was indicated in CAO. CAO oxidizes several laccase substrates; however, it is not a blue-copper protein and monoclonal antibodies against both native and deglycosylated CAO did not recognize any of several laccases. The N-terminal sequence of CAO, H_2N-X E L A Y S P P Y X P S, was non-homologous with known enzymes. Transparent copper, tetrameric structure, amino-acid composition, phenylhydrazine and tropolone inhibition, and SDS enhancement of CAO activity indicate that CAO is an *o*-diphenol oxidase.

Key words *Pinus strobus* L. · Polyphenol oxidase · Tracheid differentiation · Wood formation · Cell wall · Lignin

Introduction

Peroxidases in cell walls of vascular plants have for many years been considered to be solely responsible for the dehydrogenative polymerization of monolignols during lignification; however, increasing evidence points to one or more oxygen-dependent enzymes participating in lignin biosynthesis (Driouich et al. 1992; Savidge and Udagama-Randeniya 1992; Sterjiades et al. 1992, 1993; Bao et al. 1993; McDougall et al. 1994; Liu et al. 1994). None of these recently discovered phenol oxidases is yet well characterized. Here, the primary objective was to arrive at a proper enzymological classification of coniferyl alcohol oxidase (CAO), a laccase-like wall-bound enzyme spatiotemporally correlated with lignification in conifer species (Savidge and Udagama-Randeniya 1992; Udagama-Randeniya and Savidge 1994).

CAO was previously characterized as a glycoprotein having a pI of 7.6, a single copper atom per polypeptide, and pH and temperature optima of 6.3 and 30 °C, respectively (Savidge and Udagama-Randeniya 1992; Udagama-Randeniya and Savidge 1994). By SDS PAGE, the molecular weight of deglycosylated CAO was determined to be 67 000, and the native molecule migrated to a point where a non-glycosylated protein would yield M_r 107 500. Hence, we deduced the glycan content to be 38% (Udagama-Randeniya and Savidge 1994). However, a reviewer pointed out that the glycan moiety does not bind SDS. The previous estimates (Udagama-Randeniya and Savidge 1994) of native CAO M_r and glycan content are, therefore, probably inaccurate.

In this study, CAO was purified to electrophoretic homogeneity using described methods (Savidge and Udagama-Randeniya 1992; Udagama-Randeniya and Savidge 1994) and further characterized by UV/Visible and EPR spectroscopy, by amino-acid analysis, and by N-terminal sequencing. The ability of CAO to utilize various substrates, and its kinetic properties as affected by various inhibitors, were determined. The specificity of anti-CAO monoclonal antibodies for CAO and other proteins was also investigated.

Materials and methods

Chemicals

All chemicals used were of analytical grade and were obtained from Sigma Chemical Company unless indicated otherwise. Electrophoretic purity reagents were purchased from BioRad. Precast PhastGel IEF minigels and pI calibration kits were obtained from Pharmacia. Polyvinylidene difluoride (PVDF-ProBlott) membrane was supplied by Applied Biosystems.

P. V. Udagama-Randeniya[1] · R. A. Savidge (✉)
Faculty of Forestry and Environmental Management, University of New Brunswick, Fredericton, NB, Canada E3B 6C2

Present address:
[1] Zoology Department, University of Colombo, Colombo, Sri Lanka

Purification and molecular characterization of CAO

Extraction of CAO from actively lignifying xylem of *Pinus strobus* L. (eastern white pine), precipitation and purification using 2-[*N*-morpholino]ethanesulfonic acid (MES), and estimation of pI were done as previously reported (Savidge and Udagama-Randeniya 1992; Udagama-Randeniya and Savidge 1994).

SDS PAGE

SDS PAGE was carried out on 7.5% gels using samples of purified enzyme extracted under reducing conditions. Minigels (84 × 52 × 0.75 mm) were cast and electrophoresed using a Mini Protean II electrophoresis system (BioRad) according to the manufacturer's specifications.

EPR analysis of copper

A sample of purified active CAO was analyzed by EPR on a custom built spectrometer, taking standard precautions including a positive EPR-control for types 1 and 2 Cu^{++} (Mattar and Sutherland 1991).

Substrate specificity and effectors

The ability of CAO to oxidize various substrates was determined using reaction mixtures consisting of 30 µg of purified enzyme in 1.5 ml phosphate buffer (50 mM, pH 6.3, 30 °C) containing known substrate concentrations using a UV/Vis spectrophotometer with 2 nm photodiodes (Hewlett Packard, HP 8452). Reaction rates of CAO with different substrates were determined on the basis of decrease or increase in absorbance at the absorbance maximum of the oxidized product, analyzing only the initial rate of reaction. Substrates in the absence of CAO were used as controls.

Several known effectors of phenol oxidases were tested for their effects on activity in a reaction mixture containing 30 µg of CAO in 1.5 ml 0.68 mM 2,7-diaminofluorene (DAF). Each compound was tested at a final concentration of 1 mM. The increase in absorbance at 600 nm (oxidation product of DAF) was monitored spectrophotometrically and compared with that occurring without the effector.

Production of anti-CAO monoclonal antibodies

Female BALB/c mice (4–6 weeks old) were immunized intra-peritonially (i.p.) with 250 µg antigen immulsified in Freund's complete adjuvent. Purified CAO in its native form, and the deglycosylated protein obtained by mild periodate oxidation (Laine and Faye 1988), were both used as antigens. The mice were subsequently boosted at monthly intervals on two occasions with CAO in Freund's incomplete adjuvant administered i.p. The final immunization, injected intravenously, consisted of 250 µg antigen suspended in sterile phosphate buffered saline. On the 4th day following the final immunizing dose, mouse immune spleen cells were fused with the P3-X63-Ag8.653 (P_3X) myeloma cell line at a ratio of 10:1 using 50% (w/v) polyethylene glycol 1450 according to the method of Galfre et al. (1977) with slight modifications. Fused cells were washed and resuspended at a concentration of 10^6 total cells per milliliter in RPMI 1640 (Flow) containing 10% fetal bovine serum (Hyclone) and seeded into 24-well plates (Flow). The following day, HAT selective medium was dispensed into each well to a final concentration of 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine. Ten to fourteen days after the fusion, supernatants of wells with growing hybrid clones were screened by an indirect enzyme linked immuno-sorbent assay (ELISA) using the appropriate form of purified CAO as antigen. Selected hybrids were cloned twice by the limiting dilution method. Immunoglobulin was precipitated with ammonium sulphate from spent culture supernatant and stored at −20 °C for further use.

Indirect microplate ELISA

Indirect ELISA was performed as described by Cheung et al. (1986) with modifications. Optimal dilution of antigen and conjugate to be used in the assay were pre-determined by checkerboard titration using reference positive (immune mouse) and negative (normal mouse) control sera. Antigen was immobilized on solid support by coating individual wells of micro-ELISA plates (Flow) with 0.5 µg protein in 100 µl TRIS-buffered saline (TBS), pH 7.4, overnight at 4 °C. The following day, protein solutions were flicked off plates, wells were filled with 100 µl blocking buffer (1% BSA in TBS), and the plates were incubated for 1 h at 37 °C to block non-specific protein adsorption sites. After four washes (0.1% Tween-20 in TBS), 100 µl undiluted hybridoma culture supernatant was dispensed to each well and incubated at 37 °C for 2 h. The plates were washed eight times as before. Culture supernatant of the P_3X myeloma cell line was used as control antibody. EIA grade affinity purified goat anti-mouse IgG alkaline phosphatase conjugate (BioRad) was used at a dilution of 1:1000 in dilution buffer (1% BSA in washing buffer). Freshly diluted conjugate (100 µl) was dispensed into each well and incubated for 1 h at 37 °C. After washing eight times, 200 µl of *p*-nitrophenylphosphate (1 mg/ml) in diethanolamine buffer (pH 9.8) was added to each well as substrate. The plates were incubated at 37 °C and the reaction stopped after 1 h by adding 50 µl 3*N* NaOH. The absorbance of each well was read at 405 nm using a Titertek Multiscan MCC/340 ELISA reader (Flow Laboratories, UK).

Isoelectric focusing and electroblotting

Purified enzyme preparations were electrofocused on PhastGel IEF 3–9 minigels (Pharmacia) according to PhastSystem separation technique file number 100 (Pharmacia). The proteins thus separated were electroblotted onto PVDF membrane, probed with spent hybridoma culture supernatant and immunodetected as previously described (Udagama-Randeniya and Savidge 1994).

Analysis of amino-acid composition

CAO (86 µg) was dissolved in 300 µl 0.1% trifluoroacetic acid in water. Manual hydrolysis of the sample was carried out under argon at 160 °C for 40 min prior to phenylisothiocyanate (PTC) derivatization. Analysis of a 5 µl aliquot was done on an Applied Biosystems 420A amino acid analyzer using an on-line reversed phase high performance liquid chromatograph (Applied Biosystems 130A with C-18 narrow bore cartridge) to analyze amino acids (254 nm detection). The system was calibrated (Pierce Standard H), using norleucine as internal standard for derivatization and apomyoglobin (sperm whale, 25 pmols) as the standard for hydrolysis. Using this method, tryptophan, cysteine and hydroxyproline were not analyzed, and glutamine and asparagine were included in the glutamate and aspartate pools, respectively.

N-terminal sequencing

Following SDS PAGE of MES-purified CAO, minigels were soaked in transfer buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid – 10% methanol, pH 11.0, 5 min). Proteins (250 µg CAO) were electrotransferred (250 mA, 15 min) onto PVDF membrane (Problot, Applied Biosystems) using a mini trans-blot electrophoretic transfer cell (Bio-Rad) (Matsudaira 1987). PVDF membranes were washed in double-distilled H_2O (5 changes, 5 min, ambient temperature), stained with 0.1% Coomassie blue R-250 in 50% methanol (5 min, ambient temperature), and then destained in 50% methanol – 10% acetic acid (5 min, ambient temperature). The membranes were finally rinsed in double-distilled H_2O (5 min, ambient temparature), air dried and stored at −18 °C between sheets of blotting paper. N-Terminal sequencing of protein on PVDF Problot membranes was done according to general protocol (Hewick et al. 1981) by Ms. France Dumas (NRC-Biotechnology Research Institute, Montreal, Canada) using an auto-

Table 1 Substrate utilization by *Pinus strobus* CAO. Specific activity is the number of micromoles of substrate oxidised per hour per milligram of CAO

Substrate	Molarity (mM)	Specific activity (μMol h^{-1} mg^{-1})
Coniferyl alcohol	0.07	0.306
p-Coumaryl alcohol	0.08	0.151
L-Ascorbic acid	0.5	0
p-Cresol	20	0
L-Tyrosine	1	0
Guaiacol	20	0
Syringaldazine	0.1	0.005
Hydroquinone	5	4.52
Catechol	20	2.2
Pyrogallol	20	6.12
Gallic acid	20	0.108
DL-Dopa	20	1.38
p-Anisidine (HCl)	20	0.48
o-Dianisidine-2HCl	1	1.06
o-Phenylenediamine	10	2.0
p-Phenylenediamine	10	8.34
2,7-Diaminofluorene	0.68	8.06

mated Edman-degradation system (Model 470A gas-phase sequencer equipped with on-line model 120A phenylthiohydantoin analyzer (Applied Biosystems)). Blotted protein (25–50 pmol) was loaded onto the sequencer with a glass-fiber filter disk (trifluoroacetic acid pretreated). A standard sequencing program (03RPTH, Applied Biosystems) was employed. Based on Tyr giving 26.1 pmoles at cycle 5 and 24.2 pmoles at cycle 9, the average yield was 98%. For the whole sequence, the major signal corresponded to 70% of the total protein.

Results

Activity studies

CAO oxidized both mono- and di-phenols (Table 1). The endogenous monolignols coniferyl alcohol and *p*-coumaryl alcohol were oxidized more slowly than several chromogenic (synthetic) substrates. Very slow but definite oxidation of syringaldazine was observed. No oxidation of tyrosine, *p*-cresol, guaiacol or ascorbic acid was detected. CAO readily oxidized *p*-phenylenediamine and DAF, and also oxidized catechol, a common substrate of catechol oxidase.

Table 2 Effect of diphenoloxidase effectors on the activity of CAO from *Pinus strobus*. Inhibition (%) is based on CAO oxidation of DAF at pH 6.3, 30 °C, under conditions described in the text

Effector (1 mM)	Inhibition (%)
EDTA	25
Potassium cyanide	90
Sodium azide	84
Diethyl-dithiocarbamate	86
Ascorbic acid	100
Phenylhydrazine	100
p-Coumaric acid	30
trans-Cinnamic acid	0
Ferulic acid	93
CTAB	43
Tropolone	38
PVP	0

Using DAF as substrate, CAO activity was inhibited by effectors specific for catechol oxidase (cinnamic acid derivatives, tropolone, phenylhydrazine) and by CTAB, an inhibitor of laccases (Table 2). In contrast, CAO activity was increased 185% in the presence of SDS.

UV/Visible and EPR spectra

The UV/Vis profile of CAO showed absorption maxima at 210 and 258 nm and was devoid of any absorbance in the visible region. Lack of an EPR signal for purified CAO indicated that there was no conventional type 1 or type 2 Cu^{2+} present. The presence of type 3 copper can, therefore, be assumed (Mayer and Harel 1979; Mayer 1987).

Anti-CAO monoclonal antibodies

Seven monoclonal antibodies (MAbs), two against native CAO (CAO-3, CAO-4) and the remainder raised against deglycosylated CAO, were selected from initial screenings

Table 3 Cross-reactivity of anti-CAO monoclonal antibodies as determined by ELISA; each value is the average of triplicate absorbance (405 nm) readings. RE cell walls were digested with cellulase-pectinase as described by Savidge and Udagama-Randeniya (1992); supernatant was from a cellulase-pectinase digest of cell walls as described by Savidge and Udagama-Randeniya (1992); and RE: MES pellet was obtained from 2 M $CaCl_2$-soluble MES-precipitatable protein from cell walls as described by Udagama-Randeniya and Savidge (1994)

Antibody: Antigen	P_3X	CAO-1	CAO-2	CAO-3	CAO-4	CAO-5	CAO-6	CAO-7
CAO (purified)	0.043	0.027	0.008	1.657	1.938	0.037	0.019	0.022
RE[a]: cell sap	0.089	0.037	0.128	0.053	0.070	0.195	0.092	0.127
RE: NaCl soluble after cell sap removal	0.098	0.010	0.207	0.219	0.067	0.258	0.208	0.198
RE: digested primary walls	0.087	0.017	0.029	0.235	0.046	0.158	0.083	0.072
Supernatant of RE digest	0.037	0.026	0.039	0.138	0.030	0.126	0.069	0.032
RE: MES pellet	0.061	0.032	0.048	0.054	0.019	0.169	0.060	0.076
SL[a]: 0–35%$(NH_4)_2SO_4$ precipitate from cell sap	0.042	0.020	0.018	0.023	0.013	0.029	0.032	0.031
SL: 35–75%$(NH_4)_2SO_4$ precipitate from cell sap	0.032	0.010	0.026	0.003	0.021	1.034	0.021	0.018
Cellulase	0.095	0.023	0.063	0.045	0.052	0.075	0.047	0.012
Pectinase	0.045	0.019	0.022	0.015	0.012	0.038	0.032	0.041

[a] *RE* = primary-walled radially expanded cambial derivatives; *SL* = expanded lignifying cell. RE and SL were scraped from mature wood into liquid nitrogen, pulverized and extracted as described (Savidge and Udagama-Randeniya 1992)

Table 4 Cross-reactivity of anti-CAO monoclonal antibodies with known oxidases as determined by ELISA, each value is the average of triplicate absorbance (405 nm) readings.

Antibody: Antigen	P_3X	CAO-1	CAO-2	CAO-3	CAO-4	CAO-5	CAO-6	CAO-7
CAO (deglycosylated)	0.020	1.995	0.505	1.155	1.462	1.272	0.679	0.727
CAO (native)	0.043	0.027	0.008	1.657	1.938	0.037	0.019	0.022
Anionic HRP[a]	0.041	0.034	0.033	0.143	0.034	0.160	0.020	0.004
Cationic HRP	0.054	0.023	0.017	0.164	0.050	0.323	0.011	0.037
Tyrosinase[b]	0.023	0.022	0.015	1.201	0.158	1.355	0.019	0.353
Rhus laccase[c]	0.046	0.031	0.021	0.034	0.027	0.117	0.011	0.020
Fungal laccase[d]	0.039	0.012	0.010	0.016	0.003	0.125	0.015	0.038
Diamine oxidase	0.227	0.119	0.203	0.198	0.298	0.305	0.230	0.221
Xanthine oxidase	0.055	0.016	0.036	0.030	0.005	0.211	0.028	0.312
Cytochrome oxidase	0.060	0.004	0.018	0.011	0.021	0.040	0.019	0.045

[a] Horseradish peroxidase
[b] Tyrosinase as supplied by Sigma is contaminated with catechol oxidase
[c] *Rhus* cortical enzyme prepared in house (Udagama-Randeniya and Savidge 1994)
[d] Derived from *Pyricularia oryzae*

by ELISA. Cross-reactivity studies, using proteins from different growth stages of cambial tissue and also with commercially available oxidases, were performed by ELISA with these MAbs (Tables 3, 4). MAbs A/CAO-1 and A/CAO-4 were selected as being CAO-specific for future immunolocalization and molecular biological studies. When probed by ELISA, only MAbs A/CAO-3 and A/CAO-4 recognized both native and deglycosylated forms of CAO, the others reacting positively with only the deglycosylated protein. On the other hand, all seven MAbs recognized both native and deglycosylated proteins by immunodetection on Western blots (Fig. 1).

Amino-acid composition of CAO

The amino acid composition of CAO is shown in Table 5. CAO has glutamine-glutamate, glycine, alanine, leucine and valine as major constituents. Histidine, tyrosine and methionine were minor constituents.

Table 5 Amino-acid composition of CAO from *Pinus strobus*. Cysteine, tryptophan and hydroxyproline were not analyzed

Amino acid	Composition (%)
ASX[a]	8.5
GLX[b]	10.3
SER	6.3
GLY	9.8
HIS	2.2
ARG	4.7
THR	5.2
ALA	9.7
PRO	5.6
TYR	2.7
VAL	7.6
MET	2.8
ILE	4.7
LEU	9.4
PHE	4.9
LYS	5.5

[a] Aspartic acid and asparagine
[b] Glutamic acid and glutamine

N-Terminal sequence of CAO

Sequencing was attempted on four distinct protein bands electrotransferred from an SDS-PAGE gel onto PVDF membrane. The two higher molecular weight bands contained the identical N-terminal sequence, viz. H_2N-?-Glu-Leu-Ala-Tyr-Ser-Pro-(Pro)-Tyr-?-Pro-(Ser), where ( ) indicates a weak signal and ? no signal. Multiple signals were obtained from the two lower molecular weight bands, indicating that each consisted of more than one protein.

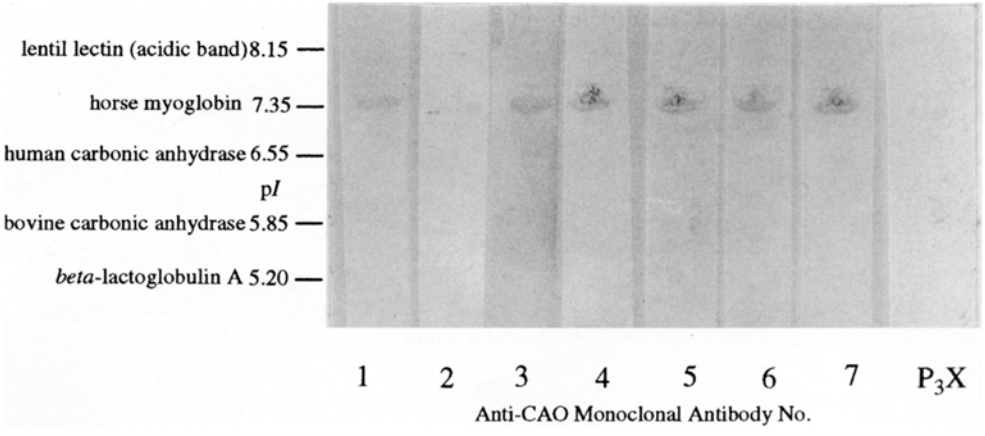

Fig. 1 Immunostaining of Western blots of purified CAO; 3 µg native enzyme was electrophoresed on pH 3–9 IEF PhastGel, blotted onto PVDF membrane by semi-dry transfer technique and probed with spent culture supernatant of monoclonal antibodies to CAO-1 to CAO-7 and the P_3X myeloma cell line. *Standard pI markers* are shown on the left

Discussion

The classification of polyphenoloxidases (PPOs) remains highly subjective (Mayer and Harel 1979; Mayer 1987). The current tripartite nomenclature is as follows: monophenol monooxygenase (tyrosinase) E.C. 1.14.18.1, hydroxylation of monophenols to *o*-diphenols; catechol oxidase (*o*-diphenol: oxygen oxidoreductase) E.C.1.10.3.1, oxidation of *o*-diphenols to *o*-quinones; laccase (benzenediol: oxygen oxidoreductase) E.C. 1.10.3.2, oxidation of *o* and *p*-quinols, aminophenols and phenylenediamine) (Webb 1984). CAO does not oxygenate tyrosine or *p*-cresol (both monophenols), hence is not a monophenol monooxygenase.

Substrates and inhibitors are commonly used as criteria for distinguishing between catechol oxidase and laccase (Mayer and Harel 1979; Mayer 1987; Dean and Eriksson 1994), but with CAO this approach did not unambiguously differentiate between the two. Metal-ion chelators not specific to copper, such as azide and EDTA, are inhibitors of laccase, but may also act on catechol oxidase (Mayer and Harel 1979). Inhibitors such as cyanide and diethyldithiocarbamate which act on copper do not satisfactorily differentiate between the PPOs (Mayer and Harel 1979).

CAO exhibits catechol oxidase activity in oxidizing *o*-dihydroxy phenols, in having its activity inhibited by *p*-coumaric and ferulic acids, and in being activated by SDS (Walker and McCallion 1980). CAO is also similar to reported catechol oxidases in being inhibited by tropolone (Kahn and Andrawis 1985), an inhibitor having no effect on laccases (Sterjiades et al. 1993). Moreover, phenylhydrazine, a specific inhibitor of catechol oxidase, completely inhibits CAO activity (Mayer and Harel 1979).

CAO is laccase-like in oxidizing both *ortho-* and *para*-dihydroxy phenols, syringaldazine and *p*-phenylenediamine (Walker and McCallion 1980). CAO is also laccase-like in not oxidizing *p*-cresol and in being inhibited by CTAB but not by PVP (Walker and McCallion 1980). Apart from oxidizing both *o-* and *p*-quinols, the ability of CAO to act on aminophenols, with special reference to *p*-phenylenediamine, is further evidence for CAO being a laccase (Webb 1984). Moreover, failure of CAO to oxidize tyrosine and *p*-cresol, and the ability of CAO to oxidize DOPA, are laccase as opposed to catechol oxidase properties (Simon et al. 1979).

Structural features can also be used to distinguish PPOs. Laccases are monomeric enzymes having several copper atoms, a characteristic blue colour (600 nm absorbance), and readily detectable EPR spectra for types 1 and 2 copper (Mayer 1987; Dean and Eriksson 1994). Catechol oxidases, in contrast, are polypeptide tetramers having copper but without characteristic colour or EPR spectra (Mayer and Harel 1979; Mayer 1987; Gregory and Bendall 1966). In previous work (Savidge and Udagama-Randeniya 1992; Udagama-Randeniya and Savidge 1994), CAO was characterized as a copper containing oxidase showing "laccase-like" activity but immunologically (polyclonal antibodies) distinct from the pI 9 "laccase" reported by Bao et al. (1993). In the present study, CAO lacked 600 nm absorbance and yielded no EPR evidence for type 1 or 2 copper. None of our protein-specific monoclonal antibodies against deglycosylated CAO recognized either *Rhus typhina* or fungal laccases, indicating that CAO presents amino-acid antigenic sites distinct from those of laccases.

In terms of amino-acid composition, CAO and catechol oxidase are related in their contents of basic amino acids and amino acids with aliphatic side chains (alanine, isoleucine, leucine, valine; Mayer and Harel 1979). Both CAO and catechol oxidase contain similar, elevated quantities of aspartic and glutamic acids, whereas the glutamate content of laccase is approximately half that of aspartate (Mayer and Harel 1979; Sterjiades et al. 1992). In contrast to laccase and in agreement with catechol oxidase, CAO has a low methionine content (Mayer and Harel 1979). The amino-acid composition of CAO is very similar to that reported for an "*o*-diphenol oxidase" from potato (Balasingam and Ferdinand 1970). The N-terminal sequence of CAO was not homologous to known proteins in the Swiss-Prot data bank.

Considered altogether, the low copper content, the absence of an EPR signal for copper, the lack of any detectable absorbance in the visible spectrum, the amino-acid composition, inhibition by tropolone and phenylhydrazine, and activation by SDS all point to CAO being a catechol oxidase (Mayer and Harel 1979; Walker and McCallion 1980). Most enzymes are inactivated by SDS; however, polyphenol oxidases appear to be an exception (Moore and Flurkey 1990). The mechanism of activation as well as physical changes accompanying presumed SDS binding remain to be elucidated.

Polyphenol oxidases which appear to be in an intermediary stage between catechol oxidase and laccase have been previously reported. Laccases characteristically contain three types of copper distinguishable by EPR (Polacheck et al. 1982). Types 1 and 2 Cu^{2+} are the primary sites engendering the high level of catalytic activity in laccases. Type 1 Cu^{2+} is responsible for the strong blue colour of laccases. Type 2 Cu^{2+} is indispensable for catalytic activity of laccases, but evidently is not necessary for catechol oxidase activity (Mayer and Harel 1979). Type 3 copper is EPR nondetectable, in contrast to types 1 and 2, and appears to be the sole type of copper present in catechol oxidases as well as being present in the blue copper oxidases. A phenol oxidase extracted from tea leaves showed a typical laccase absorption spectrum, and the purified enzyme was blue in colour, but the best substrates were *o*-dihydric phenols with quinol and *p*-phenylenediamine being oxidized slowly (Gregory and Bendall 1966). A membrane-bound phenol oxidase from the pathogenic fungus *Cryptococcus neoformans* shows affinity to *o*-dihydric phenols, but diethylthiocarbamic acid, a copper chelator, had no effect on that enzyme (Polacheck et al. 1982). The presence of only type 3 copper in CAO is in agreement with its relatively low in vitro activity and further agrees with the fact that lignification occurs very slowly in vivo.

In conclusion coniferyl alcohol oxidase, a glycoprotein situated in compound middle lamella and secondary walls of conifers in association with lignification and previously described as a copper-containing 'laccase-like' phenol oxidase, was characterized in terms of amino-acid composition, N-terminal sequence, electron paramagnetic resonance signal, substrate utilization, response to effectors, and specificity of recognition by monoclonal antibodies. In terms of substrate utilization, CAO exhibits properties of both laccase and catechol oxidase; however, the supplementary evidence points clearly to CAO being a catechol oxidase.

Acknowledgements We thank S. Mattar for EPR analysis of copper, W. H. Morrison III for providing monolignols, B. Dunbar for amino-acid analyses, and DNAX Institute, Palo Alto, Calif. for the P_3X myeloma cell line. This research was supported by Forestry Canada and the Natural Sciences and Engineering Research Council of Canada.

References

Balasingam K, Ferdinand W (1970) The purification and properties of a ribonucleoenzyme, o-diphenol oxidase, from potatoes. Biochem J 118:15–23

Bao W, O'Malley DM, Whetten R, Sederoff RR (1993) A laccase associated with lignification in Loblolly pine xylem. Science 260:672–674

Cheung A, Leban J, Shaw AR, Merkli B, Stocker J, Chizzolini C, Sander C, Perrin LH (1986) Immunization with synthetic peptides of a *Plasmodium falciparum* surface antigen induces antimerozoite antibodies. Proc Natl Acad Sci USA 83:8328–8332

Dean JFD, Eriksson K-EL (1994) Laccase and the deposition of lignin in vascular plants. Holzforschung 48S:21–33

Driouich A, Laine A-C, Vian B, Faye L (1992) Characterization and localization of laccase forms in stem and cell cultures of sycamore. Plant J 2:13–24

Galfre G, Howe SC, Milstein C, Butcher GW, Howard JC (1977) Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 266:550–552

Gregory RPF, Bendall DS (1966) The purification and some properties of the polyphenol oxidase from tea (*Camellia sinensis* L.). Biochem J 101:569–581

Hewick RM, Hunkapiller MW, Hood LE, Dreyer WS (1981) A gas-liquid solid phase peptide and protein sequenator. J Biol Chem 256:7990–7997

Kahn V, Andrawis AW (1985) Inhibition of mushroom tyrosinase by tropolone. Phytochemistry 24:905–908

Laine A-C, Faye L (1988) Significant immunological cross-reactivity of plant glycoproteins. Electrophoresis 9:841–844

Liu L, Dean JFD, Friedman WE, Eriksson K-EL (1994) A laccase-like phenoloxidase is correlated with lignin biosynthesis in *Zinnia elegans* stem tissues. Plant J 6:213–224

Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membrane. J Biol Chem 262:10035–10041

Mattar SM, Sutherland D (1991) Oxidation-reduction behavior of bianthrone and bianthronyl in basic dimethyl sulfoxide solutions. J Phys Chem 95:5129–5133

Mayer AM (1987) Polyphenol oxidases in plants – recent progress. Phytochemistry 26:11–20

Mayer AM, Harel E (1979) Polyphenol oxidases in plants. Phytochemistry 18:193–215

McDougall GJ, Stewart D, Morrison IM (1994) Cell-wall-bound oxidases from tobacco (*Nicotiana tabacum*) xylem participate in lignin formation. Planta 194:9–14

Moore BM, Flurkey WH (1990) Sodium dodecyl sulfate activation of a plant polyphenoloxidase. J Biol Chem 265:4982–4988

Polacheck I, Hearing VJ, Kwon-Chung KJ (1982) Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. J Bacteriol 150:1212–1220

Savidge RA, Udagama-Randeniya PV (1992) Cell wall-bound coniferyl alcohol oxidase associated with lignification in conifers. Phytochemistry 31:2959–2966

Simon LT, Bishop DS, Hooper GR (1979) Ultrastructure and cytochemical localization of laccase in two strains of *Leptosphaerulina briosiana* (Pollaci) Graham and Luttrell. J Bacteriol 137:537–544

Sterjiades R, Dean JFD, Eriksson K-EL (1992) Laccase from sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. Plant Physiol 99:1162–1168

Sterjiades R, Dean JFD, Gamble G, Himmelsbach DS, Eriksson K-EL (1993) Extracellular laccases and peroxidases from sycamore maple (*Acer pseudoplatanus*) cell suspension cultures. Reactions with monolignols and lignin model compounds. Planta 190:75–87

Udagama-Randeniya P, Savidge R (1994) Electrophoretic analysis of coniferyl alcohol oxidase and related laccases. Electrophoresis 15:1072–1077

Walker JRL, McCallion RF (1980) The selective inhibition of *ortho*- and *para*-diphenol oxidases. Phytochemistry 19:373–377

Webb EC (1984) Enzyme nomenclature 1984: recommendations of the nomenclature committee of the International Union of Biochemistry on the nomenclature and classification of enzyme-catalysed reactions. Academic Press, Orlando San Diego New York London Toronto Montreal Sydney Tokyo