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Purification and Characterization of Cinnamyl Alcohol Dehydrogenase Isoforms from the Periderm of *Eucalyptus gunnii* Hook[1]

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Cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) isoforms were purified from the periderm (containing both suberized and lignified cell layers) of *Eucalyptus gunnii* Hook stems. Two isoforms (CAD 1P and CAD 2P) were initially characterized, and the major form, CAD 2P, was resolved into three further isoforms by ion-exchange chromatography. Crude extracts contained two aliphatic alcohol dehydrogenases (ADH) and one aromatic ADH, which was later resolved into two further isoforms. Aliphatic ADHs did not use hydroxycinnamyl alcohols as substrates, whereas both aromatic ADH isoforms used coniferyl and sinapyl alcohol as substrates but with a much lower specific activity when compared with benzyl alcohol. The minor form, CAD 1P, was a monomer with a molecular weight of 34,000 that did not co-elute with either aromatic or aliphatic ADH activity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis demonstrated that this protein was very similar to another CAD isoform purified from *Eucalyptus* xylem tissue. CAD 2P had a native molecular weight of approximately 84,000 and was a dimer consisting of two heterogenous subunits (with molecular weights of 42,000 and 44,000). These subunits were differentially combined to give the heterodimer and two homodimers. SDS-PAGE, western blots, and nondenaturing PAGE indicated that the CAD 2P heterodimer was very similar to the main CAD isoform previously purified in our laboratory from differentiating xylem tissue of *E. gunnii* (D. Goffner, I. Joffroy, J. Grima-Pettenati, C. Halpin, M.E. Knight, W. Schuch, A.M. Boudet [1992] Planta 188: 48–53). Kinetic data indicated that the different CAD 2P isoforms may be implicated in the preferential production of different monolignols used in the synthesis of lignin and/or suberin.

CAD is one of the two branch enzymes of general phenylpropanoid metabolism specific to hydroxycinnamyl alcohol (monolignol) synthesis catalyzing the conversion of hydroxycinnamaldehydes to the corresponding alcohols (Grisebach, 1981). Monolignols are the monomeric precursors of the complex phenolic polymer lignin that, after cellulose, constitutes the most abundant biopolymer on Earth and accounts for up to 30% dry weight of secondary xylem in woody species.

In the plant, the rigidity and hydrophobicity of the lignin polymer is important for mechanical support (Monties, 1989), water conduction (Northcote, 1989), and defense (Vance et al., 1980). However, although they are of vital importance to the survival of all higher land plants, high lignin levels are problematical in the agro-industrial exploitation of various plant species. Therefore, numerous biotechnological programs are focused on the biosynthesis of this polymer with the intention of reducing total lignin content in commercially important tree and forage crop species.

As well as being the precursors of lignin, recent work has shown that monolignols also constitute the monomeric precursors of other phenolic compounds such as lignans (Lewis and Yamamoto, 1990) and suberin (Kolattukudy, 1987). Suberin is a complex polymer with a structure that has still not been fully elucidated. It consists of an aliphatic and an aromatic domain, and analysis has shown that monolignols are the monomeric precursors of the latter domain. Unlike lignin, suberin is not implicated in mechanical support and water conduction but it is intimately concerned with the defense of the plant (Bostock and Stermer, 1989), where, like lignin, it plays an important role in the response to mechanical wounding or pathogen attack. In higher plants showing secondary growth, it is the suberized and often lignified cell layers (phellem) of the periderm that, after the loss of the cuticle, constitute the physical barrier between the environment and the underlying plant tissues and that are responsible for preventing moisture loss and opportunistic pathogen attack.

Although the down-regulation of monolignol synthesis in xylem tissue would be economically advantageous, it is clear that such down-regulation could also have potentially undesirable effects on, for example, the synthesis of surface, "defense lignin," or suberin. However, it is also possible that tissue-specific isoforms of such enzymes may exist that, in conjunction with the use of tissue-specific promoters, could lead to the independent manipulation of different product (e.g. lignin/suberin) levels in different tissues. Although CAD has previously been purified and characterized from whole organs (stems [Mansell et al., 1974; Halpin et al., 1992], roots [Rhodes and Wooltorton, 1974], pine megagametophytes [O'Malley et al., 1992]), cell-suspension cultures (Wryambik and Grisebach, 1975; Galliano et al., 1993), and differentiat-

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Abbreviations: ADH, alcohol dehydrogenase; CAD, cinnamyl alcohol dehydrogenase; HMW, high molecular weight; LMW, low molecular weight.

ing xylem tissue (Savidge, 1989; Goffner et al., 1992; O'Malley et al., 1992), it has not been purified from other lignified plant tissues and so little is known concerning the nature of tissue-specific forms of this enzyme.

Following the observation that CAD activity could be detected in the surface layers of *Eucalyptus gunnii* Hook branches, it was hypothesized that (a) this enzyme might be implicated in the synthesis of surface lignin monolignols or the monomeric precursors of the aromatic domain of suberin, and (b) it might represent a tissue-specific isoform of this enzyme. To test these hypotheses we have purified and characterized CAD isoforms from the periderm of *E. gunnii* Hook and compared them with isoforms previously characterized in differentiating xylem tissue from the same clone.

E. gunnii Hook was chosen as the experimental system first because of the molecular tools and data already available concerning *Eucalyptus* xylem CAD (Goffner et al., 1992), second because it is a tree species of economic importance and is already the target of transformation experiments, and third because a knowledge of the enzymes and genes present in different tissues could, potentially, lead to a strategy for the independent manipulation of lignin/suberin in different tissues.

MATERIALS AND METHODS

Plant Material

Periderm tissue was obtained from the trunks of young (4-year-old) *Eucalyptus gunnii* Hook trees (clone No. 800859) growing on the AFOCEL (Association Fôret Cellulose) Plantation at Longages, southern France, toward the end of the growing season (September 15–20, 1992). Material was scraped into a beaker with a scalpel, dropped directly into liquid nitrogen, and stored at −80°C until needed. Previous detailed microscopic examination had resulted in a thorough knowledge of surface layer anatomy, which enabled the removal of periderm tissue without contamination from other lignifying tissue (i.e. secondary phloem).

Microscopic and Histochemical Analysis

For general anatomical investigations, semithin (30- to 60-μM) transverse sections of *E. gunnii* branches (2 to 3 cm in diameter) were made on a Reichart Wood Microtome. Sections were stained as follows: for general anatomical study, safranin O/fast green, carmine/iodine green (Johansen, 1940); for lignin detection, Weisner reaction, Mäule reaction, Cross and Bevan reaction (Monties, 1987); for suberin detection, sudan black, sudan III/IV, oil red O (Gahan, 1984). For more detailed investigation of the bark (secondary phloem, cortex, and periderm), samples were fixed in formalin:70% ethanol (1:9, v/v), dehydrated in a graded ethanol series, and embedded in paraffin. Semithin sections (20 μM) were then cut on a rotary microtome and stained as above.

Lignin Monomer Analysis

Thioacidolysis and subsequent monomeric product analysis of *Eucalyptus* periderm samples were performed in the laboratory of Dr. B. Monties (Institut National de la Recherche Agronomique, Grignon, France) essentially as described by Lapierre et al. (1986).

Purification

CAD isoforms were purified using a combination of ion-exchange and affinity chromatography. All steps were carried out at 4°C, and CAD activity was monitored by the oxidation of coniferyl alcohol. Samples showing activity were pooled, desalted, and buffer exchanged by centrifugation through Sephadex G-25 columns (Penefsky, 1977) before proceeding to the next chromatographic step.

Preparation of Crude Extract

Frozen periderm tissue (250 g) was ground up in a coffee mill prechilled with liquid nitrogen and extracted (buffer:tissue 4:1 [v/w]) by stirring with a magnetic bar for 45 min in cold extraction buffer (250 mM Tris-HCl, 100 mM sodium ascorbate, 10% polyvinylpolypyrrolidone, 5% ethylene glycol, 2% PEG, 0.1% β-mercaptoethanol, and the protease inhibitors PMSF [1 mM], leupeptin [1 μM], and pepstatin [1 μM], pH 7.5). Crude extract was filtered through two layers of Miracloth, and a sample was removed, desalted, and assayed for activity. Crude extract was then clarified by centrifugation (18,000*g*, 45 min) and the supernatant was taken on to the next purification step.

Amberlite XAD-7

Amberlite XAD-7 polyphenol-absorbing resin (Sigma) equilibrated in 250 mM Tris-HCl, 5% ethylene glycol, and 5 mM DTT was added to the crude, filtered extract (resin:extract [1:10, w/v]) and stirred for 5 min before filtering through two layers of Miracloth.

Ammonium Sulfate Precipitation and Concentration

The crude extract was brought to 20% saturation with solid $(NH_4)_2SO_4$ and then centrifuged (18,000*g*, 45 min). The resulting supernatant was then brought to 80% saturation with solid $(NH_4)_2SO_4$ and centrifuged (18,000*g*, 45 min), and the resulting pellet was resuspended in 100 mM Tris-HCl, 5% ethylene glycol, 5 mM DTT, pH 7.5.

DEAE-Sephacel

The resuspended pellet was desalted into DEAE-Sephacel start buffer (20 mM Tris-HCl, 5% ethylene glycol, 5 mM DTT, pH 7.5) and then loaded onto a 60-mL DEAE-Sephacel (Pharmacia) column (IBF-Sepracor, 1.6 cm diameter) connected to a Bio-Rad Econo system low-pressure liquid chromatography system at a flow rate of 0.25 mL min^{-1}. Elution was performed with a 180-mL linear gradient of 20 to 400 mM Tris-HCl, pH 7.5, containing 5% ethylene glycol, 5 mM DTT at a flow rate of 0.25 mL min^{-1}.

CAD 1P

The minor form of CAD (CAD 1P) eluted on DEAE-Sephacel was desalted into 2′,5′ ADP-Sepharose start buffer (20 mM Tris-HCl, 5% ethylene glycol, 5 mM DTT, pH 7.5)

and loaded onto a 10-mL 2′,5′ ADP-Sepharose (Pharmacia) column (IBF-Sepracor, 1.6 cm diameter) at a flow rate of 0.2 mL min^{-1}. Although CAD activity did not bind to the column, active fractions were collected, combined, concentrated, and used in further characterization of this form.

CAD 2P

The major form of CAD (CAD 2P) eluted on DEAE-Sephacel showed partial resolution into two unequal peaks (CAD 2Pa, CAD 2Pb). The main peak (CAD 2Pb) was then further purified by affinity chromatography on 2′,5′ ADP-Sepharose.

2′,5′ ADP-Sepharose

Active CAD fractions (CAD 2Pb) were desalted into 2′,5′ ADP-Sepharose start buffer (100 mM Tris-HCl, 5% ethylene glycol, 5 mM DTT) and loaded onto a 10-ml 2′,5′ ADP-Sepharose (Pharmacia) column (IBF-Sepracor, 1.6 cm diameter) at a flow rate of 0.2 mL min^{-1}. The column was then washed with 10 mL of 4 mM NAD (to eliminate NAD-dependent enzymes), rinsed with 20 mL of start buffer (0.3 mL min^{-1}), and then eluted with a 40-mL linear gradient of 0 to 8 mM NADP in start buffer at a flow rate of 0.25 mL min^{-1}.

Mono-Q

CAD activity eluted from 2′,5′ ADP-Sepharose was desalted into Mono-Q start buffer (20 mM Tris-HCl, 5% ethylene glycol, 5 mM DTT) and loaded on a Mono-Q HR 5/5 column (Pharmacia) connected to a fast protein liquid chromatography system (Pharmacia) at a flow rate of 0.2 mL min^{-1}. CAD activity was eluted with a 120-mL linear gradient of 0 to 300 mM NaCl in start buffer at a flow rate of 1 mL min^{-1}.

Enzyme Assays

CAD was assayed spectrophotometrically by both the oxidation of hydroxycinnamyl alcohols and the reduction of the corresponding aldehydes. The reverse reaction (alcohols to aldehydes) was monitored spectrophotometrically by the increase in A_{400} due to the production of the aldehyde. The forward reaction was determined by the change in A_{340} due to the disappearance of NADPH and the aldehyde as described by Wryambik and Grisebach (1975). The assay was carried out in 0.5 mL of reaction mixture containing, for the reverse reaction, 100 mM Tris-HCl, pH 8.8, 2 mM alcohol, 2 mM NADP, and 5 to 50 μL of sample; and, for the forward reaction, 100 mM KH_2PO_4/Na_2HPO_4, pH 6.25, 200 μM aldehyde, 2 mM NADPH, and 5 to 50 μL of sample. Aldehydes were initially dissolved in ethylene glycol monomethyl ether and then diluted with either Tris-HCl (pH 8.8) or KH_2PO_4/Na_2HPO_4 (pH 6.25) for the determination of molar extinction coefficients (given below) at 400 and 340 nm, respectively.

Tris-HCl buffer (pH 8.8):
p-Coumaraldehyde, 18.9×10^3 M^{-1} cm^{-1}
Coniferaldehyde, 17.6×10^3 M^{-1} cm^{-1}
Sinapaldehyde, 14.7×10^3 M^{-1} cm^{-1}
KH_2/Na_2HPO_4 buffer (pH 6.25):
p-Coumaraldehyde, 15.95×10^3 M^{-1} cm^{-1}
Coniferaldehyde, 19.45×10^3 M^{-1} cm^{-1}
Sinapaldehyde, 15.9×10^3 M^{-1} cm^{-1}

Coniferyl alcohol was obtained commercially (Fluka), as were sinapaldehyde and coniferaldehyde (Sigma); *p*-coumaraldehyde was synthesized by the chemistry department at Université Paul Sabatier; and sinapyl alcohol and *p*-coumaryl alcohol were the kind gifts of Drs. N. Lewis and L. Davin (Washington State University, Pullman).

NADP-dependent aromatic ADH activity was assayed spectrophotometrically as described by Mansell et al. (1974) by the increase in A_{340} of reduced NADP. The assay mixture (0.5 mL) contained 100 mM Tris-HCl, pH 8.8, 20 mM benzyl alcohol (as substrate), 2 mM NADP, and 5 to 50 μL of sample. NAD-dependent aliphatic ADH activity was assayed as described by Mansell et al. (1974) by monitoring the increase in A_{340} due to the reduction of NAD. The reaction mixture (0.5 mL) contained 100 mM Tris-HCl, pH 8.8, 25 mM ethanol (as substrate), 2 mM NAD, and 5 to 50 μL of sample.

TLC Analysis of Reaction Products

The standard assay was scaled up 10 times and run at 30°C for 1 h, reaction products and substrates were extracted with ethyl acetate, spotted onto TLC plates ($Merck_{254}$), and separated using $CHCl_3$:methanol (19:1, v/v). Products and unconverted substrates, together with authentic standards, were detected under UV light (254 nm) and by staining with phloroglucinol-HCl as described by Galliano et al. (1993).

Protein Estimation

Protein content of samples was determined using the Bio-Rad microassay (Bradford, 1976).

SDS-PAGE

Denaturing PAGE was performed as described by Laemmli (1970) with either 10 or 12% acrylamide gels. Polypeptides were stained with either Coomassie brilliant blue or silver nitrate (Damerval et al., 1987).

Native Gel Electrophoresis

Activity staining (CAD, aliphatic/aromatic ADH activity) was as described by Mansell et al. (1974). Nondenaturing gels (7.5% acrylamide) were incubated for 45 min at 37°C in the dark in an assay solution containing 10 mL of 100 mM Tris-Gly, pH 8.8, 1.5 mg of nitroblue tetrazolium, 0.1 mg of phenazinemethosulfate, 2.5 mg of NADP/NAD, and 2.5 mg of coniferyl alcohol/25 μL of ethanol (aliphatic ADH activity)/25 μL of benzyl alcohol (aromatic ADH activity). For subsequent SDS-PAGE analysis of activity bands, native gel slices were frozen in liquid nitrogen and ground up, and protein was extracted with 100 mM Tris-HCl, pH 7.5, 5 mM DTT, and 5% ethylene glycol.

Western Blots

Protein samples were transferred to a nitrocellulose membrane using a semidry electroblot apparatus (Bio-Rad). Membranes were incubated with polyclonal or monoclonal antibodies raised against *Eucalyptus* xylem CAD 2 (Goffner et al., 1992) and CAD 1. (*Eucalyptus* xylem CAD 1 antibodies were prepared from approximately 100 μg of pure protein prepared in our laboratory by Eurogenetec [Seraing, Belgium] according to standard procedures, and they were the kind gift of Dr. D. Goffner). Immunocross-reactivity was detected following incubation with secondary antibodies linked to either alkaline phosphatase or horseradish peroxidase by development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, or enhanced chemiluminescence (ECL, Amersham), respectively.

Native Mol Wt Estimation

The native mol wt of Mono-Q-purified CAD 2Pb samples was determined by nondenaturing gradient PAGE essentially as described by Andersson et al. (1974). Acrylamide gels (1.5-mm thick, 4 to 30%, Tris-borate-EDTA) were prepared using a gradient mixer in a mini-protean cell (Bio-Rad). High mol wt standards (Sigma) and samples were loaded and the gel was run at 150 V for 15 h.

RESULTS

Anatomical and Histochemical Investigation

Examination of transverse sections of *E. gunnii* branches revealed that the morphology is complex; internal phloem with associated sclerenchyma (lignified phloem fibers) is present, and the cortex is divided into chlorenchyma and parenchyma containing sclerids with thick, lignified secondary cell walls. Initial staining of wood-microtome-cut sections with sudan black or phloroglucinol-HCl suggested that the periderm contained only suberized cells; however, sequential staining of paraffin-embedded sections with the two dyes revealed the presence of both suberized and lignified cell layers (Fig. 1) as reported previously (Chattaway, 1952). Histochemical analysis using the Weisner, Mäule, and Cross and Bevan reactions (data not shown) suggested that *Eucalyptus* periderm lignin was richer in guaiacyl units than the corresponding xylem tissue.

Lignin Monomer Analysis

Analysis of the monomeric products of thioacidolysis revealed that the syringyl:guaiacyl molar ratio for *Eucalyptus* periderm samples was 1.86 (38 μmol and 20.4 μmol syringyl and guaiacyl monomers, respectively, per g oven-dried, preextracted [Lapierre et al., 1986] tissue) as compared with 2.29 (296 μmol and 129 μmol syringyl and guaiacyl monomers, respectively, per g oven-dried, pre-extracted [toluene/ethanol, ethanol, water] tissue) for *Eucalyptus* mature xylem, indicating that the former is relatively richer in guaiacyl units. Although such an observation is supported by our histochemical analysis, thioacidolysis will also liberate monomeric degradation products from the aromatic portion of suberin present in the periderm, and further work is necessary to confirm the observed monomeric composition of *Eucalyptus* surface lignins.

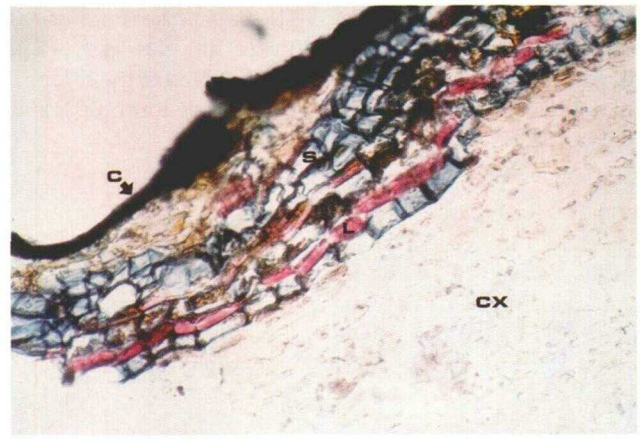

Figure 1. Semithin cross-section of *E. gunnii* periderm from a young stem stained with phloroglucinol-HCl and sudan black. C. Remains of cuticle; S, layers of suberized cells; L, layers of lignified cells; CX, cortical tissue; ×400.

Purification of CAD

Purification of periderm CAD was difficult, primarily because of the extremely high levels of phenolic material, but also as a result of the comparatively low level of total protein (10 to 20 times less than in crude xylem extracts). The initial yield of both total protein and CAD activity was enhanced by the inclusion of 100 mM sodium ascorbate in the extraction buffer and an increase in the polyvinylpolypyrrolidone level to 10%. Although neither CAD 1P nor CAD 2P was purified to homogeneity, enzyme activity was correlated with discrete bands on SDS gels. Purification and recovery values are summarized in Table I.

Two forms of CAD were separated on DEAE-Sephacel, and their elution profiles with respect to aromatic ADH activity and aliphatic ADH activity are shown in Figure 2, a and b, respectively. The first form (CAD 1P) eluted at a concentration of approximately 200 mM Tris and represented only 4% of total CAD activity. The second form (CAD 2P) eluted as an asymmetric peak at approximately 300 mM Tris, together with aliphatic and aromatic ADH activity, and represented the great majority of CAD activity.

CAD 1P

Attempts to further purify CAD 1P by affinity chromatography on 2′,5′ ADP-Sepharose were only partially successful, since activity did not bind to the column. However, SDS-PAGE (data not shown) of pooled 2′,5′ ADP-Sepharose samples revealed that the protein pattern was considerably simplified in comparison with that observed previously on DEAE-Sephacel. Since CAD 1P represented only 4% of the total observed CAD activity, it was not possible to purify this minor form further, so the pooled 2′,5′ ADP-Sepharose samples were used in further characterization of CAD 1P.

Table I. *Purification of CAD isoforms from Eucalyptus periderm*

Purification Step	Protein	Activity	Specific Activity	Recovery[a]	Purification[b]
	mg	*nkat*	*nkat mg^{-1}*	%	
Filtered	208	158.5	0.76		
Postcentrifugation	217.5	150	0.69	94.6	0.9
XAD-7	220.5	142.5	0.65	89.9	0.9
20% supernatant	195	128.5	0.66	81.1	0.9
20-80% pellet	120	108	0.9	68	1.2
CAD1P					
DEAE-Sephacel	1.8	1.8	1	1.1	1.3
2′,5′ ADP-Sepharose	0.06	1.5	25	0.9	33
CAD2P					
DEAE-Sephacel	7.4	48.4	6.57	30.5	8.6
2′,5′ ADP-Sepharose	0.26	38.6	150.9	24.3	198.5
Mono-Q					
CAD peak 2Pbi	0.0135	9.4	693.3	6	916.2
CAD peak 2Pbii	0.0034	7	2074.1	4.4	2729.1

[a] Percentage recovery for individual isoforms is given in terms of total isoform activity/total initial activity. [b] Purification factor for individual isoforms is given in terms of isoform specific activity/initial specific activity.

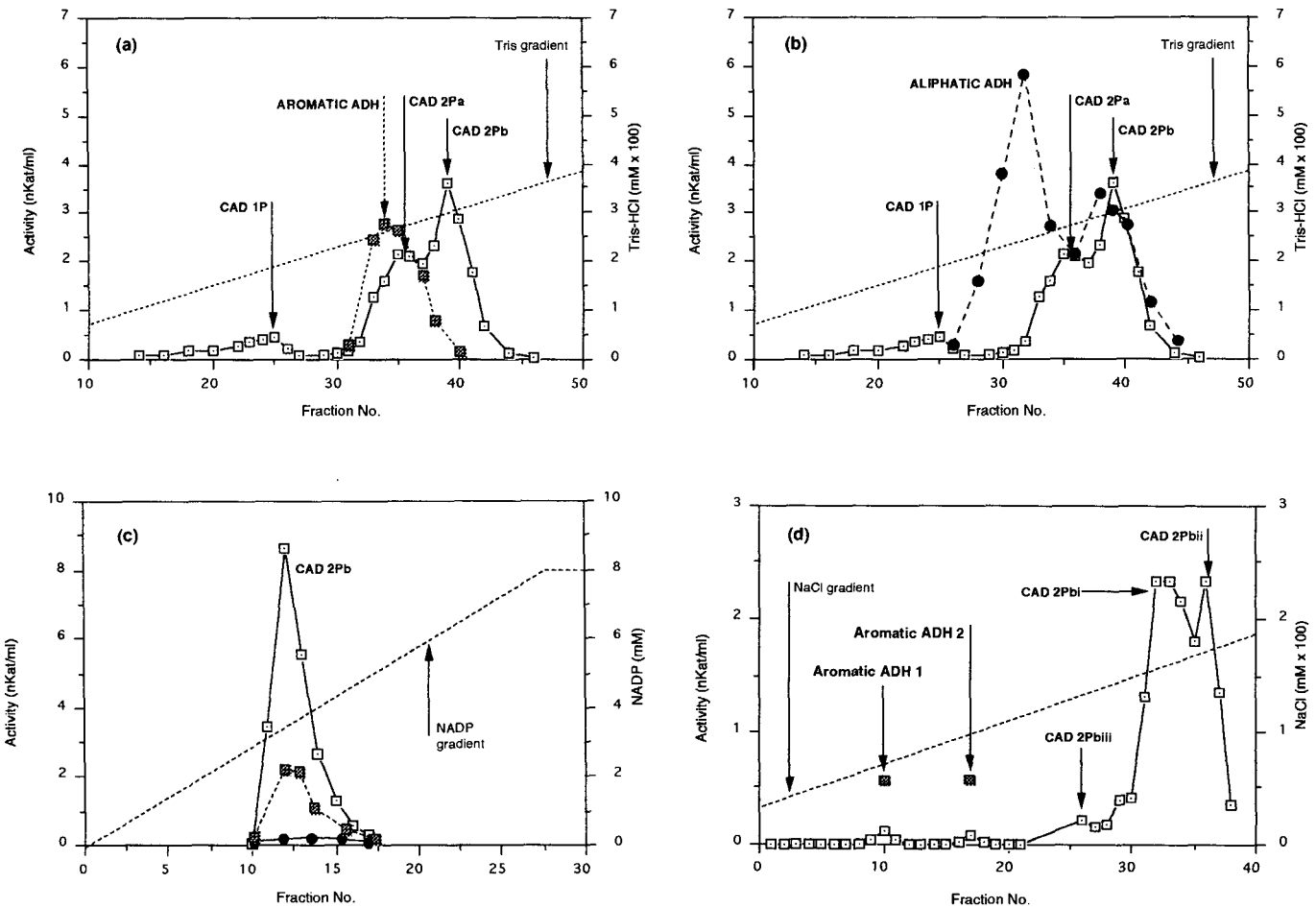

Figure 2. Purification of periderm CAD. a, Chromatogram of periderm CAD isoforms and aromatic ADH activity on DEAE-Sephacel. b, Chromatogram of periderm CAD isoforms and aliphatic ADH activity on DEAE-Sephacel. c, Chromatogram of CAD 2Pb and aliphatic/aromatic ADH on 2′,5′ ADP-Sepharose. d, Chromatogram of CAD 2Pb and aromatic ADH on Mono-Q. ⊡, CAD activity; ▩, aromatic ADH activity; ●, aliphatic ADH activity.

Table II. *Specific activities of Mono-Q-purified periderm ADH peak 2*

Substrate	Specific Activity
	nkat mg^{-1}
Coniferyl alcohol	7.7
Sinapyl alcohol	3.8
p-Coumaryl alcohol	0
Benzyl alcohol	84.8

CAD 2P

CAD 2P eluted as two incompletely resolved peaks (CAD 2Pa and CAD 2Pb) on DEAE-Sephacel; only the main peak (CAD 2Pb) was taken further in the purification. Affinity chromatography of CAD 2Pb fractions on 2′,5′ ADP-Sepharose (Fig. 2c) resulted in the almost complete elimination of NAD-dependent aliphatic ADH activity.

Mono-Q chromatography (Fig. 2d) revealed that CAD activity eluted as two major peaks (CAD 2Pbi and CAD 2Pbii), which showed no aromatic/aliphatic ADH activity, and a third, minor peak (CAD 2Pbiii). Two other smaller peaks of CAD activity were eluted early on in the gradient; however, these fractions also showed aromatic ADH activity with a higher specific activity (Table II) for benzyl alcohol than for hydroxycinnamyl alcohols and, therefore, it was presumed that these fractions contained an aromatic ADH capable of utilizing coniferyl alcohol as a substrate.

Confirmation of the Identity of Periderm CAD Isoforms

CAD 1P

Although further purification of CAD 1P did not prove possible, SDS-PAGE and western analysis of CAD 1P samples and semipurified *Eucalyptus* xylem CAD 1 (Goffner et al., 1992) revealed that they showed identical migration patterns (Fig. 3), suggesting that these two proteins are indeed very similar and that CAD 1P is a monomer with a mol wt of approximately 34,000. The high molecular mass band (>66 kD) observed in both the CAD 1P sample and the semipurified xylem CAD 1 sample may represent either a contaminating but immunologically similar protein or a precursor polypeptide of the 34-kD CAD isoform, and work is currently underway in our laboratory to define more closely the relationship between these two polypeptides.

CAD 2P

SDS-PAGE of CAD Mono-Q fractions (CAD 2Pbi and CAD 2Pbii) (Fig. 4a) revealed that CAD 2Pbi samples contained two very closely spaced bands (44 and 42 kD), whereas CAD 2Pbii samples contained only the 44-kD band; in addition, all samples contained one band at 30 kD (the apparent band at 66 kD was observed in all lanes, including blank ones, and was presumed artifactual). When CAD 2Pbi samples were run on the same gel with semipurified *Eucalyptus* xylem CAD 2, and western blotted (Fig. 3), it was observed that both samples showed identical migration of the 44- and 42-kD bands (xylem CAD 2 is reported to be a heterodimer consisting of a 44- and 42-kD subunit [Goffner et al., 1992]).

Western blot analysis (Fig. 4b) revealed that antibodies (both polyclonal and monoclonal) raised against xylem CAD 2 showed cross-reactivity with the 44- and 42-kD bands but not with the 30-kD band—no cross-reactivity was observed with polyclonal antibodies raised against xylem CAD 1. Since *Eucalyptus* xylem CAD 2 is a heterodimer, these results suggested that the periderm doublet (44- and 42-kD bands) was the equivalent of this enzyme and that activity was associated with the dimer and not with the 30-kD band, and, by association, that activity in the CAD 2Pbii fractions was associated with the 44-kD band.

Further proof that activity was associated with the 44- and 42-kD bands was obtained when CAD 2Pbi and CAD 2Pbii samples were run on nondenaturing PAGE gels. Three parallel lanes were run for each sample (Fig. 5); the first lane was stained for CAD activity, the second was silver stained, and the third lane was cut into 5-mm sections. Protein was extracted from each 5-mm slice, run on SDS-PAGE, and silver stained. SDS-PAGE of protein extracted from the 5-mm slices corresponding to activity gave rise to either a doublet (44 and 42 kD: CAD 2Pbi samples) or a single band at 44 kD (CAD 2Pbii samples), proving that activity was, indeed, associated with the 44- and 42-kD bands. Confirmation of enzyme identity in purified fractions was accomplished by TLC analysis of reaction products (data not shown).

Native Mol Wt Estimation

Estimation of the native mol wt of the Mono-Q CAD 2Pb samples by nondenaturing gradient gel electrophoresis revealed that CAD 2Pbi had a native mol wt of approximately 84,000, and CAD 2Pbii had a native mol wt of approximately 82,000 (data not shown).

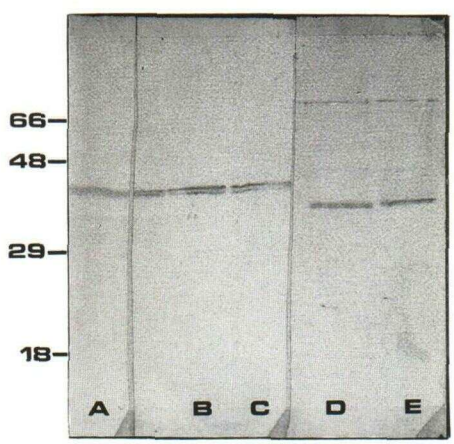

Figure 3. Western blot of *Eucalyptus* xylem and periderm samples separated by SDS-PAGE (12% gel). Lane A, Crude xylem; lane B, pure xylem CAD 2; lane C, Mono-Q-purified periderm CAD 2Pbi; lane D, semipurified xylem CAD 1; lane E, semipurified periderm CAD 1P. Lanes A through C incubated with xylem CAD 2 polyclonal antibodies; lanes D and E incubated with xylem CAD 1 polyclonal antibodies.

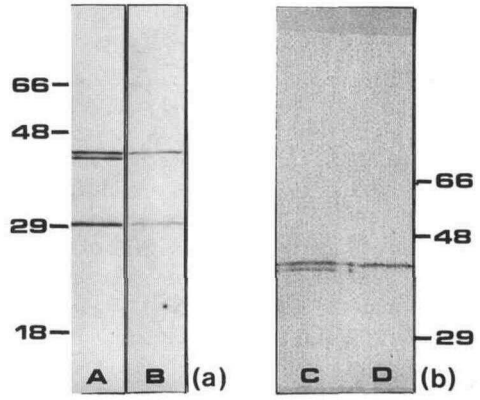

Figure 4. CAD 2Pb polymorphism. a, Silver-stained SDS gel (12%) of Mono-Q-purified periderm CAD 2Pb; lane A, CAD 2Pbi; lane B, CAD 2Pbii. b, Western blot of Mono-Q-purified periderm CAD 2Pb samples separated by SDS-PAGE (10% gel); lane C, CAD 2Pbi; lane D, CAD 2Pbii. Membrane incubated with xylem CAD 2 polyclonal antibodies.

Native Activity Gels

When post-Mono-Q samples (CAD 2Pbi and CAD 2Pbii) were run on native gels and stained for CAD activity (data not shown), they each gave rise to a single band with an R_F value of 0.34 and 0.37, respectively. A post-DEAE-Sephacel sample (corresponding to the combined CAD 2Pb fractions taken on to the next purification step) gave rise to a broad "smear" of activity with an R_F value of 0.31 to 0.37 (data not shown). Native gel R_F values were also determined for aliphatic and aromatic ADH activity (in post-DEAE-Sephacel samples). All R_F values are given in Table III.

CAD 2 Polymorphism

The differences in R_F values on CAD activity gels for CAD 2Pbi and CAD 2Pbii samples together with the observations that (a) these samples show slight differences in migration on nondenaturing gradient gel electrophoresis, (b) SDS-PAGE analyses of CAD activity bands show either a doublet (CAD 2Pbi) or a single band (CAD 2Pbii), and (c) CAD is reported (Savidge, 1989; Goffner et al., 1992; Halpin et al., 1992; O'Malley et al., 1992) to exist as either a homodimer or heterodimer, strongly suggest that active *Eucalyptus* periderm CAD 2P may exist in at least two different configurations. The first configuration (as observed in *Eucalyptus* xylem CAD) is a heterodimer composed of a 44- and 42-kD subunit (HeteroCAD), whereas the second is a homodimer composed of two 44-kD subunits (HMW HomoCAD).

Table III. *Aliphatic/aromatic ADH and CAD activity band R_F values obtained on nondenaturing PAGE*

Enzyme/Isoform	R_F Value
CAD 2Pbi (post-Mono-Q)	0.34
CAD 2Pbii (post-Mono-Q)	0.37
Mixed CAD 2Pb (post-DEAE)	0.31–0.38
Aliphatic ADH (post-DEAE)	0.27
Aromatic ADH (post-DEAE)	0.23

In light of this apparent CAD 2P polymorphism, reevaluation of the Mono-Q chromatogram (Fig. 2d) led us to hypothesize that the minor peak (CAD 2Pbiii) of CAD activity eluting just prior to CAD 2Pbi may represent the other possible configuration of CAD 2P subunits—the homodimer of the smaller subunit. SDS-PAGE and western analysis of these samples (Fig. 6) demonstrated the presence of only the lower (42 kD) band, indicating that CAD 2P also exists as the LMW homodimer.

Aromatic ADHs

SDS-PAGE (data not shown) of the two minor peaks of aromatic ADH activity observed on Mono-Q (Fig. 2d) revealed that one sample (ADH 1) contained a 41-kD polypeptide, whereas the other sample (ADH 2) contained three polypeptides (39.5, 41, and 43 kD). None of the bands corresponded to either of the periderm CAD 2P subunits. Western analysis (data not shown) showed no immunogenic cross-reactivity between any of the ADH polypeptides and either CAD 1 or 2 antibodies, suggesting that although these ADHs are capable of utilizing hydroxycinnamyl alcohols as substrates, they do not correspond to either of the CAD isoforms currently characterized in *Eucalyptus*.

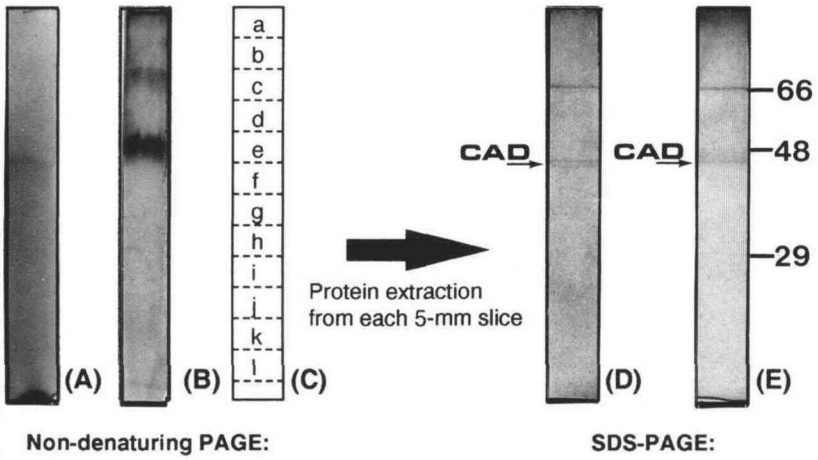

Figure 5. Confirmation of CAD 2Pb identity by nondenaturing PAGE and SDS-PAGE. Lane A, Activity-stained CAD 2Pbi sample; lane B, silver-stained CAD 2Pbi sample (CAD 2Pbii samples gave similar results); lane C, cut into 5-mm slices (a–l) and protein extracted from each slice; lane D, SDS-PAGE of protein extracted from 5-mm slice corresponding to CAD 2Pbi activity; lane E, SDS-PAGE of protein extracted from 5-mm slice corresponding to CAD 2Pbii activity.

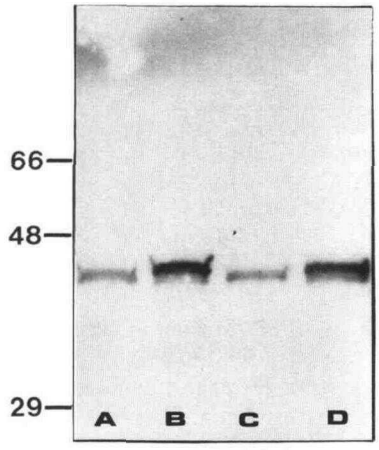

Figure 6. Enhanced chemiluminescence autoradiogram of western blot with Mono-Q-purified CAD 2Pb. Lanes B and D, CAD 2Pbi; lanes A and C, CAD 2Pbiii. Membrane incubated with *Eucalyptus* xylem CAD 2 polyclonal antibodies.

Kinetic Data

Kinetic parameters (K_m, V_{max}) determined for HeteroCAD and HMW HomoCAD by Lineweaver-Burk and Eadie-Hofstee plots are given in Table IV.

Although the HeteroCAD K_m values show that the heterodimer exhibits similar substrate affinities for both aldehydes and alcohols, when both V_{max} and V_{max}/K_m are taken into account, the heterodimer works more efficiently with the aldehydes than the corresponding alcohols, as would be expected given the forward sense of the reaction. The HMW HomoCAD K_m values reveal that this form shows a higher affinity for the aldehydes than for the corresponding alcohols. The HMW HomoCAD V_{max}/K_m value with sinapaldehyde is approximately 3 times that observed with coniferaldehyde. These data indicate that HMW HomoCAD works most efficiently with sinapaldehyde, whereas HeteroCAD works equally efficiently with both sinapaldehyde and coniferaldehyde. Both forms use only NADP as a cofactor; no activity was observed with NAD as cofactor.

DISCUSSION

Histochemical studies of the surface layers of the bark from *E. gunnii* Hook branches and stems revealed that, as previously observed by Chatterway (1952), the periderm of *E. gunnii* consists of clearly defined layers of suberized and lignified cells, suggesting a strict developmental control of phenolic wall material deposition. The observation that *Eucalyptus* periderm lignin is more rich in guaiacyl units than corresponding xylem lignin is interesting in light of results (Biggs, 1984) showing that lignin laid down in response to mechanical wounding and/or pathogen attack is also guaiacyl-rich, suggesting that the physicochemical properties of this lignin type may be more suited to a defensive/barrier role.

The isolation of CAD from *Eucalyptus* periderm tissue strongly suggests that this enzyme is implicated in the synthesis of surface, "defense lignins" in the dermal layers of plants showing secondary growth. The enzyme could also be involved in the synthesis of suberin in these surface layers.

The observation that two CAD isoforms could be separated on DEAE-Sephacel is similar to the situation previously observed in *Eucalyptus* xylem tissue (Goffner et al., 1992) and soybean cell-suspension cultures (Wryambik and Grisebach, 1975). The elution profile on DEAE-Sephacel clearly demonstrates the complex mix of ADHs present in a crude extract—two to three CAD isoforms, two aliphatic ADH isoforms, and a single aromatic ADH can all be detected. Although affinity chromatography eliminated aliphatic ADH activity, aromatic ADH activity co-eluted with CAD 2Pb and gave rise to two further isoforms on Mono-Q, both of which were also able to utilize coniferyl alcohol as a substrate. A full understanding of the enzymic complexity of crude extracts (especially when enzymes exhibiting multiple substrate specificity are involved) is of considerable importance, since measurement of enzyme activity (in crude extracts) is often

Table IV. *Kinetic parameters for Mono-Q-purified periderm CAD 2Pb*

K_m and V_{max} values determined by Lineweaver-Burk and Eadie-Hofstee plots.

Substrate	K_m	V_{max}	V_{max}/K_m	V_{max}/K_m
	μM	nkat mg^{-1}		%
HeteroCAD (Mono-Q peak 2Pbi)				
Coniferaldehyde	4.5	2103.5	467.4	100
Sinapaldehyde	6.8	2758.3	409.4	87.6
p-Coumaraldehyde	5.1	1244.2	244	52.2
Coniferyl alcohol	2.3	539.2	234.6	50.2
Sinapyl alcohol	6.6	717	108.7	23
p-Coumaryl alcohol	35	812	23.2	0.05
HMW HomoCAD (Mono-Q peak 2Pbii)				
Coniferaldehyde	5.2	1579.8	303.4	36
Sinapaldehyde	2.5	2107.3	843.9	100
p-Coumaraldehyde	15.5	2107.3	136.3	16.2
Coniferyl alcohol	23.1	1816.9	78.2	9.3
Sinapyl alcohol	4.5	561.8	124.4	14.7
p-Coumaryl alcohol	63.8	1149.6	17.8	2.1

used in physiological studies or for monitoring the effects of introduced genes in transgenic plants.

SDS-PAGE and western analysis results clearly show that the two periderm CAD isoforms initially separated (CAD 1P, CAD 2P) are, if not identical, very similar to those previously characterized in *Eucalyptus* xylem tissue. The report (Galliano et al., 1993) that, in spruce, the same CAD isoform is apparently involved in both "natural" lignin synthesis and "stress/wounding" lignin synthesis suggests that this isoform is utilized whenever and wherever lignin biosynthesis takes place. Such data strongly suggest that *Eucalyptus* xylem and periderm lignin synthesis is also mediated by the same CAD isoform.

The sizes of the periderm CAD 2P subunits are in good agreement with values observed previously. CAD purified from poplar (Savidge, 1989) is reported to be a homodimer (40-kD subunit), as is the CAD from spruce (Luderitz and Grisebach, 1981) (41.7-kD subunit) and loblolly pine (O'Malley et al., 1992) (44-kD subunit), whereas the CAD purified from tobacco (Halpin et al., 1992) appears to be a heterodimer (44- and 42.5-kD subunits). The observation that *Eucalyptus* periderm CAD 2P exists as a homodimer as well as a heterodimer was unexpected, since *Eucalyptus* xylem CAD has been reported to exist as a heterodimer (Goffner et al., 1992). The Mono-Q gradient used in periderm CAD 2P purification was twice as shallow as that used in the xylem purification, and it is possible that the latter gradient was insufficient to resolve the different CAD isoforms. Interestingly, when the cloned *Eucalyptus* xylem CAD 2 gene (Grima-Pettenati et al., 1993) is expressed in *Escherichia coli*, it gives rise to a functional homodimeric protein, and work is currently underway to determine which of the two CAD subunits is involved.

Comparison of the kinetic properties of the HMW homodimer and the heterodimer reveals clear differences in the affinity of the different forms for their in vivo substrates (hydroxycinnamaldehydes), which, as suggested by Luderitz and Grisebach (1981) in the case of spruce, may be of physiological significance with regard to the synthesis of different lignin types.

Lignin may be classified into three main types, H, G, and S (Monties, 1987), depending upon its monolignol composition. Typical gymnosperm lignin (G lignin) is made up primarily of coniferyl alcohol units, whereas the lignin of woody angiosperms (G-S lignin) is generally made up of a mixture of coniferyl and sinapyl alcohol moieties. The type of lignin varies not only from species to species, but also within a plant and within the same tissue/cell type, depending on its developmental stage and physiological state (Lewis and Yamamoto, 1990). Although clear differences in substrate affinities have been demonstrated between comparable enzymes (e.g. ferulate 5-hydroxylase [Chapple et al., 1992], *O*-methyltransferase [Kuroda et al., 1975], CAD [Goffner et al., 1992; O'Malley et al., 1992; Galliano et al., 1993]) from gymnosperms and dicotyledons, which offer a convincing explanation for the observed differences in monomeric lignin composition between these groups, little is known concerning the underlying enzymic mechanism controlling the synthesis of the various lignin types in the different tissues of woody angiosperms.

The existence of three native CAD isoforms (resulting from different subunit combinations) each exhibiting differences in their affinity for the three hydroxycinnamaldehydes may represent such a mechanism for the synthesis of the various lignins observed in dicotyledons. For example, when V_{max}/K_m values are compared it is clear that, in *Eucalyptus*, the "synthesis/assembly" of the CAD heterodimer would be expected to result in the production of a mixed guaiacyl-syringyl lignin, whereas the synthesis/assembly of the HMW homodimer could be expected to result in the production of a predominantly syringyl rich lignin—it would obviously be of interest to fully determine and compare the kinetic parameters of the LMW homodimer.

The genetic significance of periderm CAD 2P polymorphism is unclear. The results of Southern analysis in *Eucalyptus* (Grima-Pettenati et al., 1993) suggest a single CAD gene, in which case the two CAD subunits may represent allelic differences as previously reported in loblolly pine (O'Malley et al., 1992). However, the observed differences in V_{max}/K_m values between the CAD 2P heterodimer and homodimer strongly suggest that the different CAD 2P isoforms do play different physiological roles and that the two CAD subunits may well represent the products of two different genes (rather than allelic variants), as has been previously observed with maize (Freeling, 1974), rice (Xie and Wu, 1989), and soybean (Newman and VanToai, 1991) ADH. Maize ADH is the product of two unlinked genes, *Adh-1* and *Adh-2*, which dimerize to give three electrophoretically distinct isoforms (two homodimers and the heterodimer); similar *Adh* gene systems have also been reported in rice and soybean. In these examples, the various ADH isoforms exhibit different tissue/organ specificity and induction patterns, demonstrating that they have different physiological roles.

Although further study is obviously required to confirm that the observed differences in *Eucalyptus* CAD 2 isoform kinetics are of physiological significance, it is possible that a fuller understanding of their genetic basis could offer to the numerous biotechnological programs currently working on modifying the total lignin content of trees and animal food crops a valuable "fine-tuning" mechanism by which lignin quality as well as quantity could be modified.

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