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PURIFICATION AND PROPERTIES OF CINNAMYL ALCOHOL DEHYDROGENASE FROM HIGHER PLANTS INVOLVED IN LIGNIN BIOSYNTHESIS

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Key Word Index—*Forsythia suspensa*; Oleaceae; cinnamyl alcohol oxidoreductase; cinnamyl aldehydes, cinnamyl alcohols; stereospecificity; lignification.

Abstract—The purification and characterization of an NADP(H) specific cinnamyl alcohol oxidoreductase is reported. The enzyme, which has been purified 600×, shows an absolute specificity for the cinnamyl moiety. The reaction is readily reversible but is strongly inhibited by aldehyde substrates. The enzyme belongs to class A of NAD(P) specific oxidoreductases. The distribution of this activity throughout a wide variety of taxonomically different plant groups as well as plant parts has revealed a possible correlation with lignin biosynthesis.

INTRODUCTION

ALCOHOL dehydrogenases oxidize a wide range of alcohols and have a broad specificity.[1] In contrast to numerous enzymes known to act on aliphatic alcohols, published work on aromatic alcohol dehydrogenases has been minimal. Aliphatic alcohol dehydrogenases oxidizing aromatic compounds have been reported.[2–4] Enzymes showing an expressed specificity towards aromatic alcohols have been isolated from bacteria,[5–8] fungi[9–11] and animal tissues.[12,13] Cofactor studies reveal some of these enzymes to be NAD specific,[7,8] while others have an NADP specificity[6,10–13] or react with both dinucleotides.[4,5]

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Only recently an enzyme of this type has been reported from a higher plant.[14] In that study an NADP specific aromatic alcohol dehydrogenase from potato tubers was isolated and partially purified. The preparation was active with a variety of substituted benzaldehydes, including at least one aldehyde of the cinnamyl class. It was also postulated that this may be the terminal enzyme on the metabolic pathway from cinnamic acids to the cinnamyl alcohols, which are the precursors for lignin biosynthesis.

Recently we have reported the isolation of three distinct enzymes from stem tissue of *Forsythia* sp. and *Salix alba* which catalyze the production of coniferyl alcohol from ferulic acid[15,16] and coumaryl alcohol from *p*-coumaric acid.[17] From these tissues we have also isolated a very active, NADP dependent aromatic alcohol dehydrogenase which is capable of catalyzing the reversible conversion of coniferyl aldehyde to coniferyl alcohol. We now report on the purification and properties of this enzyme as well as its distribution in a wide variety of plants and plant tissues.

RESULTS

Purification of cinnamyl alcohol dehydrogenase

Cinnamyl alcohol dehydrogenase was extracted from young stem sections of *Forsythia suspensa*. The purification procedure outlined in Table 1 resulted in a 600× purification of this activity. The final fraction was obtained with a 23% recovery of total activity and had a specific activity of 3500 mU/mg protein. During purification the activity was followed by analytical polyacrylamide disc electrophoresis. The crude extract revealed the presence of at least three bands which showed activity with coniferyl alcohol and NADP (Fig. 1). The two upper bands also showed activity with ethanol and benzyl alcohol indicating that they had broader substrate specificities. During the final purification step, these activities were lost, leaving a single band with specificity for cinnamyl alcohol substrates and NADP.

TABLE 1. PURIFICATION OF CINNAMYL ALCOHOL DEHYDROGENASE

Fraction	Volume (ml)	Protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Recovery (%)	Purification (×-fold)
Crude extract	192	506	2935	5·8	100	—
$(NH_4)_2SO_4$(40–65%)	9·9	134	2449	15·9	83	2·7
Sephadex G-200	80·5	41·7	1656	39·7	56	6·9
DEAE cellulose	18·9	3·5	984	281	34	48·4
Hydroxyapatite	19·2	0·2	701	3504	23	604

Stability

Crude enzyme preparations or those stored as eluted from the columns showed complete loss of activity within 1 week at 0°. When concentrated fractions from the columns were stored in 20 or 50% glycerol and held at −18°, the enzyme was stable for several

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months. However, if these glycerol containing fractions were frozen, 50% of the activity was lost within 24 hr.

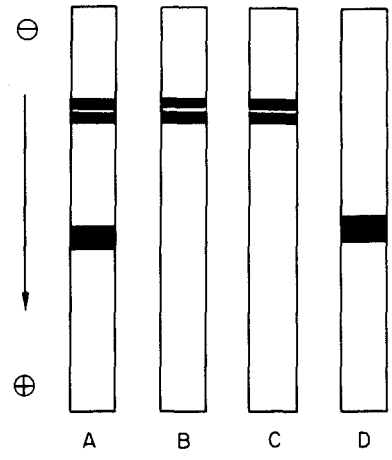

FIG. 1. DISTRIBUTION OF ALCOHOL DEHYDROGENASE FROM *Forsythia* AFTER POLYACRYLAMIDE GEL ELECTROPHORESIS.
In gels (A)–(C), a crude extract concentrated with $(NH_4)_2SO_4$ was used; in (D), the purified enzyme was analyzed. The substrates employed were: (A) and (D) coniferyl alcohol; (B) ethanol; (C) benzyl alcohol.

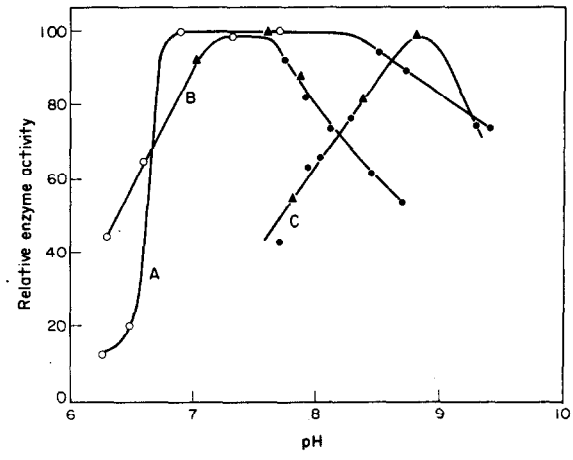

FIG. 2. EFFECTS OF pH ON ACTIVITY AND STABILITY OF CINNAMYL ALCOHOL DEHYDROGENASE.
The enzyme reaction mixtures were assayed at the indicated pH values as described in the experimental section. To test the effect of pH on stability, the enzyme was exposed for 10 min at 30° to the pH values given with subsequent determination of the activity at pH 8·8. (A) stability; (B) reduction of coniferyl aldehyde; (C) oxidation of coniferyl alcohol. (○) Potassium phosphate buffer; (▲) Tris–HCl buffer; (●) potassium borate buffer.

Characteristics and properties

The cofactor requirements for the dehydrogenase activity from purified preparations are shown in Table 2. No activity was observed in the absence of NADP(H). No other cofactor requirements could be demonstrated and no loss of activity was observed after dialysis. Under the conditions of the standard assay (see Experimental) for both the aldehyde reduction and alcohol oxidation, the reaction was linear for approximately 4 min. Aldehyde reduction was linear with protein concentrations up to 0·4 μg/assay which is equivalent to 0·7 mU; alcohol oxidation was linear to a protein concentration of 0·14 μg/assay, or 0·16 mU of activity.

TABLE 2. COFACTOR REQUIREMENTS FOR CINNAMYL ALCOHOL DEHYDROGENASE

Reduction of coniferyl aldehyde		Oxidation of coniferyl alcohol	
System	Reaction rate (nmol/min)	System	Reaction rate (nmol/min)
Complete	0·87	Complete	0·78
Minus NADPH	0	Minus NADP	0
Minus NADPH, plus NADH	0	Minus NADP, plus NAD	0
Minus enzyme	0	Minus enzyme	0

NAD(H) was added in the same concentration as NADP(H). For experimental details, see text.

Molecular weight. The molecular weight was estimated to be 80000.

pH. The effect of pH on enzyme stability and both the oxidation and reduction reactions is shown in Fig. 2. In borate or Tris buffer, the optimal pH was 7·6 for aldehyde reduction and 8·8 for alcohol oxidation. The enzyme was very unstable below pH 6·8 for periods as short as 10 min. Maximum stability was observed between pH 6·8 and 8·4.

Temperature. The maximum initial reaction rate was obtained at 40°. However, instability accelerated at about 30° with rapid inactivation occurring above 40°. Activation energy was calculated to be 5105 cal/mol.

Substrate concentration. A normal substrate saturation curve was obtained with both NADP and NADPH with saturation at approximately 0·2 mM. At a 35 μM coniferyl aldehyde and 200 μM alcohol concentration, the K_m for NADPH was $5{\cdot}2 \times 10^{-5}$ M and for NADP $1{\cdot}6 \times 10^{-5}$ M.

Strong substrate inhibition was observed with all aldehydes tested. At a fixed concentration of 0·2 mM NADPH, the optimal concentrations for maximum velocity were: 200 μM for cinnamaldehyde, 70 μM for *p*-coumaryl aldehyde and 35 μM for coniferyl aldehyde. Only slight inhibition was observed with the alcohols tested and maximum velocity was obtained at 0·2–0·6 mM.

Table 3 shows the effect of a variety of inhibitors upon enzyme activity. The data indicate that a sulfhydryl group is essential for activity. Inhibition by *p*-nitrophenylacetate suggests that an imidazole group may also be essential.[18]

Stereospecificity. Stereospecificity in the reduction of coniferyl aldehyde was determined by testing A and B forms of NADP-[4-^{3}H(n)]. The coniferyl alcohol was isolated and its specific activity determined. With the A form the alcohol showed a specific activity of 2614 dpm/nMol and with the B form, 15 dpm/nMol. Thus, this dehydrogenase is of the class A type.

TABLE 3. INACTIVATION OF CINNAMYL ALCOHOL DEHYDROGENASE BY ELECTROPHILIC AGENTS

Reagent	Inhibition relative to control (%)		
	Concn of reagent		
	0·01 mM	0·1 mM	1·0 mM
p-Chloromercuribenzoate	0	75	100
p-Diazobenzenesulfonate	0	0	0
5,5'-Dithio-*bis*-(2-nitrobenzoate)	33	100	100
N-Ethylmaleimide	0	0	0
Iodoacetamide	0	0	0
Iodoacetate	0	0	0
o-Iodosobenzoate	0	50	67
p-Nitrophenylacetate	0	86	100

Enzymatic activities were measured in the standard assay (see Experimental). Samples were preincubated for 5 min at 30° prior to the addition of NADP.

Substrate specificity. The enzyme gave no reaction with the following aliphatic alcohols: methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol and geraniol. No reaction was observed with the following aromatic alcohols: benzyl, 2-hydroxybenzyl (salicyl),

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4-hydroxybenzyl, 2,3-dihydroxybenzyl, 4-hydroxy-3-methoxybenzyl (vanillyl), 3,4-methylenedioxybenzyl (piperonyl), phenylethyl, 4-hydroxy-3-methoxyphenylethyl (homovanillyl), 4-β-D-glucoconiferyl (coniferin), 4-hydroxy-3-methoxyphenylpropyl (hydroconiferyl). No reaction was observed with the following aldehydes: benzaldehyde, 2-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 3-methoxybenzaldehyde, 3,4-methylenedioxybenzaldehyde (piperonal), 2,5-dimethoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, 4-hydroxy-3,5-dimethoxybenzaldehyde (syringa), 2-nitrobenzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde and 2,6-dichlorobenzaldehyde.

Reactions were observed only when cinnamyl alcohols (Table 4) and cinnamaldehydes were used as substrates. The results obtained with the wide variety of alcohols tested indicate that, besides being specific for the cinnamyl moiety, the activity of this enzyme is maximized when the substrate is substituted in the 3 or 4 position of the ring. The following K_m values were determined for five cinnamaldehydes: cinnamaldehyde, 114 μM; 4-hydroxycinnamaldehyde (*p*-coumaryl), 17 μM; 4-hydroxy-3-methoxycinnamaldehyde (coniferyl), 17 μM; 2-nitrocinnamaldehyde, 98 μM; and 4-nitrocinnamaldehyde, 97 μM.

TABLE 4. SPECIFICITY OF CINNAMYL ALCOHOL DEHYDROGENASE AGAINST VARIOUS CINNAMYL ALCOHOLS

Substrate	Relative enzyme activity* (%)	K_m (μM)
Cinnamyl alcohol	39	156
4-Hydroxycinnamyl alcohol (*p*-coumaryl alcohol)	112	132
4-Methoxycinnamyl alcohol	290	34
4-Hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol)	100	32
3,4-Dimethoxycinnamyl alcohol	384	—
3,4-Methylenedioxycinnamyl alcohol	383	36
4-Hydroxy-3,5-dimethoxycinnamyl alcohol (sinapyl alcohol)	(+)†	—
4-Methylcinnamyl alcohol	128	—
2,4-Dimethylcinnamyl alcohol	90	—
4-Chlorocinnamyl alcohol	55	—
2,4-Dichlorocinnamyl alcohol	13	—
2,6-Dichlorocinnamyl alcohol	6	—
4-Bromocinnamyl alcohol	51	—

Conditions are described in Experimental, except for the varied substrates. Reactions with *p*-coumaryl and coniferyl alcohol were assayed at 400 nm. With all other substrates, the increase of NADPH was followed at 340 nm.

* Activities compared to the reaction rate determined with coniferyl alcohol = 100%.

† Sinapyl alcohol is converted; color development of the aldehyde formed prevents quantitative determination.

Distribution of alcohol dehydrogenase within different plant tissues and taxonomic groups

Table 5 shows the enzyme activities obtained from a large number of plant taxa. Only low levels of enzyme were found in the bryophytes and pteridophytes tested. In the Monocotyledonae, the highest activity was found in leaves of *Zea mays*. In the Dicotyledonae tested the highest levels of dehydrogenase were found in cambial region samples, whereas the lowest levels occurred in leaf samples and tissue cultures. *Acer* and *Sorbus* had the highest enzyme levels of 250 mU/g fr. wt and 220 mU/g fr. wt, respectively. *Sorbus* had the highest specific activity of 170 mU/mg. The gymnosperms gave the highest enzyme levels of all the plants tested. Several species had activity levels of above 500 mU/g fr. wt with *Picea* being the highest at 1300 mU/g fr. wt and a specific activity of 352 mU/mg.

TABLE 5. OCCURRENCE OF CINNAMYL ALCOHOL DEHYDROGENASE IN VARIOUS PLANTS

Taxonomic group	Species	Alcohol dehydrogenase Total activity (mU/g fr. wt)	Alcohol dehydrogenase Specific activity (mU/mg protein)
Bryophyta	*Catharinea undulata*	1·7	1·3
	Sphagnum sp.	1·0	3·3
Pteridophyta	*Athyrium filix-femina*	2·2	4·5
	Equisetum arvense	3·0	0·4
	E. palustre	5·5	1·1
	Nephrolepis exaltata	0·5	0·3
	Pteris cretica	10·6	7·1
	P. wimsetti	3·1	0·8
	Salvinia natans	1·2	1·7
	Selaginella kraussiana	17·8	7·3
	S. martensii	3·9	1·1
	Woodwardia radicans	0·05	0·5
Spermatophyta			
Gymnospermae	*Abies nobilis*	289	161
	Gingko biloba	52	63
	Larix leptolepis	257	143
	Metasequoia glyptostroboides	55	15
	Picea abies	1300	352
	Pinus peuce	576	172
	P. strobus	170	162
	Pseudotsuga menziesii	637	253
	Taxus baccata	154	41
	Thuja plicata	41	33
Angiospermae			
Monocotyledonae	*Avena sativa*		
	Coleoptiles (12-day-old)	7·1	4·9
	Leaves (7-day-old)	1·0	0·2
	Leaves (12-day-old)	18	3·7
	Hordeum sp.		
	Leaves (7-day-old)	12	2·5
	Secale cereale		
	Leaves (3-day-old)	7·4	2·3
	Zea mays		
	Leaves (7-day-old)	56	19
Dicotyledonae	*Acer campestre*	247	40
	A. saccharinum	215	54
	Betula verrucosa	33	38
	Cichorium endive		
	Tissue culture	1·3	5·3
	Corylus sp.	119	23
	Daucus carota		
	Tissue culture	5·6	11
	Forsythia suspensa		
	Stem	57	77
	Young leaves	38	13
	Mature leaves	7·1	1·2
	Meristem	39	16
	Tissue culture	3·5	4·3
	Helianthus annus		
	Hypocotyls (12-day-old)	2·3	4·4
	Heracleum mantegazzianum		
	Phloem	2·2	3·2
	Xylem	17·5	32·0
	Parenchyma tissue	3·4	4·1

TABLE 5. *Continued*

Taxonomic group	Species	Alcohol dehydrogenase Total activity (mU/g fr. wt)	Specific activity (mU/mg protein)
Dicotyledonae—*cont.*	*Ilex aquifolium*	43	9·0
	Ligustrum vulgare	1·7	2·5
	Linum usitatissimum		
	Tissue culture	10	42
	Liriodendron tulipifera	93	23
	Morinda citrifolia		
	Tissue culture	29	42
	Pisum sativum		
	Seedlings (12-day-old)	32	6·5
	Platanus acerifolia	68	35
	Populus italia	160	21
	P. tremula	198	58
	Prunus serotina	41	122
	Quercus robur	54	12
	Q. rubra	148	60
	Salix alba	145	40
	Sambucus racemosa		
	Cambium	58	15
	Bark	10	2·7
	Sorbus aucuparia	54	168
	S. domestica	221	47
	Syringa vulgaris	16	8·1

In *Forsythia* we examined a variety of plant parts and found that the dehydrogenase activity was highest in young stems. Young leaves and meristem regions showed enzyme levels slightly more than half those of stem sections, however, the specific activity in these was only about 1/6 as high. Only low levels of enzyme could be detected in mature leaves or tissue cultures. In *Heracleum* it was possible to separate the xylem, phloem and parenchyma tissues;[19] the highest enzyme levels were found to be associated with the xylem. Only low levels were found in both the phloem and parenchyma.

DISCUSSION

The properties of the enzyme described herein reveal it to be a Class A, NADP(H) specific, cinnamyl alcohol dehydrogenase. The results demonstrate that it has an absolute specificity for the cinnamyl moiety, can catalyze the reaction readily in either direction, and is strongly inhibited by aldehyde substrates and several sulfhydryl-group inhibitors.

Crude extracts of *Forsythia* stem tissue possessed at least three different alcohol dehydrogenases. During purification, which was achieved at 600×, the two non-specific activities were lost and only a single enzyme band remained.

Only one other aromatic alcohol dehydrogenase has been reported from higher plants.[14] This enzyme possessed broad aromatic substrate specificities. The preparation from *Forsythia* is the first reported of an alcohol dehydrogenase with an absolute specificity towards cinnamyl substrates. This result, along with those obtained of the distribution of this activity in the different plant tissues and taxonomic groups, provides a broad

[19] ZIEGLER, H. (1958) *Planta* **51**, 186.

basis for the discussion of lignification mechanisms. It would appear that the role of this enzyme in normal plant development is to provide the cinnamyl alcohol moieties necessary for lignin biosynthesis. In general, the highest levels of enzyme activity and specific activity were found in those plants and tissues which produce the most copious amounts of lignin (e.g. cambial regions of woody Dicotyledonae and gymnosperms). In older tissues, or in taxonomic groups which are thought to lack or produce only minimal amounts of lignin (e.g. bryophytes), only very low levels of enzyme activity could be detected. Thus, rather than an aromatic alcohol dehydrogenase such as that isolated from potato, participating in the synthesis of lignin precursors, perhaps this process involves a very specific dehydrogenase which functions only in this pathway. This is also suggested by the finding that the activity of a cinnamate-CoA ligase and a cinnamoyl-CoA reductase can be isolated from this same tissue[16,20] and more recent results have also shown that the activity of the ligase is, in general, highest in those tissues which are capable of producing lignin. Thus, lignin producing tissues appear to contain the entire enzyme system which is necessary for the conversion of cinnamic acids to the corresponding alcohols.

The significance of the inhibition of the dehydrogenase by aldehydes is not readily apparent. However, it may play a regulatory role. Although we have demonstrated aromatic alcohol dehydrogenase activity in many different plants, only the specificity of the *Forsythia* enzyme has been established. It will be necessary to examine the specificities of the aromatic alcohol dehydrogenases in various other plants before a more generalized understanding of its role in lignin biosynthesis can be reached.

EXPERIMENTAL

Plant material. Forsythia suspensa (Thunberg) Vahl. var. fortunei (Lindl.) Rehd. was phytotron grown under 16 hr photoperiod and 25–20° day–night temp cycle. Relative humidity was maintained at 70 ± 5%. All other plant material was collected from the University Botanical Garden or greenhouses.

Chemicals and isotopes. Coniferyl alcohol was obtained from Fluka AG (Buchs, Switzerland). Dihydroconiferyl alcohol was prepared by catalytic hydrogenation of coniferyl alcohol. Coniferin was a generous gift from Prof. K. Freudenberg, Heidelberg. For the preparation of sinapyl alcohol, the corresponding glucoside (syringin) was isolated from bark of lilac stems[21] and the free alcohol was obtained after hydrolysis with β-glucosidase (E.C. 3.2.1.21). Other cinnamyl alcohols (4-methoxy-, 3,4-methylenedioxy-, 4-methyl-,2,4-dimethyl-, 4-chloro-, 2,4-dichloro-, 2,6-dichloro-, 4-bromo) were prepared as follows: to 250 mg of the corresponding cinnamic acid, an excess of $SOCl_2$ and catalytic amounts of *N,N*-dimethylformamide were added at room temperature. After crystal dissolution, residual $SOCl_2$ was removed *in vacuo*. The remaining acyl chloride was dried over KOH, dissolved in absolute THF and reduced with an excess of $NaBH_4$ for 2–3 hr. The solvent was evaporated and 10 ml of cold water was added to the residue. After acidification with 2 N HCl, the alcohol was extracted with Et_2O and purified on silica gel GF_{254} in $CHCl_3$–C_6H_6–EtOAc–MeCOEt (7:2:1:3). Recoveries were 20–50%. Homovanillyl alcohol was prepared according to Arlt *et al.*[22] For the preparation of *p*-coumaryl aldehyde, *p*-coumaric acid was converted to acetyl-*p*-coumaric acid which in turn was converted to the corresponding acyl chloride with $SOCl_2$. Reduction was performed with an excess of $LiAlH[(Me)_3CO]_3$ in absolute THF. After the addition of water and KOH, the reaction mixture was acidified with 2 N H_2SO_4 and extracted with Et_2O. *p*-Coumaryl alcohol was the main product, but small amounts of the corresponding aldehyde could be obtained. The product was recrystallized from C_6H_6 yielding pale yellow needles (m.p. 139–141°).[23] Coniferyl aldehyde was prepared by reduction of 4-(tetra-*O*-acetyl-β-D-glycosyloxy)-3-methoxycinnamoyl chloride with $NaBH(OMe)_3$ and subsequent removal of the protecting group by hydrolysis with NaOMe and β-glucosidase. 2-Nitro- and 4-nitrocinnamyl aldehyde were prepared according to Baeyer and Drewsen.[24] NADP-[4-^{3}H(n)] was prepared from NAD-[4-^{3}H(n)] by phosphorylation with NAD-kinase (E.C. 2.7.1.23). Labelled NADP was purified on Dowex 1X8 (formate) according to Wang *et al.*[25] Final purification was by PC in isobutyric acid–NH_3–H_2O (66:1:33). A

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and B forms of NADPH-[4-^{3}H(n)] were prepared by stereospecific reduction according to Cornforth *et al.*[26]

Enzyme assay. The activity of the enzyme preparations was determined at pH 7·6 for the conversion of aldehyde to alcohol and at pH 8·8 for the reverse reaction. Coniferyl aldehyde shows a strong absorption peak at 400 nm at both of these pH values whereas the absorption of equimolar coniferyl alcohol is less than 1% at this wavelength. The absorption is linear up to a concn of 20 μM. An extinction coefficient of $18{\cdot}5 \times 10^6$ cm^2 × mol^{-1} was determined for the aldehyde at pH 8·8 and $8{\cdot}8 \times 10^6$ cm^2 × mol^{-1} at pH 7·6 at 400 nm. For the determinations of the conversion of alcohol to aldehyde, the reaction mixture contained in a total volume of 0·5 ml: 50 μmol Tris–HCl, pH 8·8; 0·1 μmol NADP; 0·1 μmol coniferyl alcohol and 0·5 mU of enzyme. In the conversion of aldehyde to alcohol, the reaction mixture contained in a total vol. of 0·5 ml: 50 μmol Tris–HCl, pH 7·6, 0·16 μmol NADPH; 0·016 μmol coniferyl aldehyde, and 0·5 mU of enzyme. One mU of enzyme activity is defined as the amount of enzyme oxidizing 1 nmol of alcohol or reducing 1 nmol of aldehyde per minute at 30°. This corresponds to a change in A_{400} of 0·035/min for alcohol oxidation and 0·016/min for aldehyde reduction.

Enzyme preparation. For the studies related to the distribution of the alcohol dehydrogenase in various plants, the enzyme was isolated as follows. For the woody plants the bark was removed and approximately 1–2 mm of tissue was scraped from the surface of the wood. For herbaceous plant material, stems and leaves were used. Three g of tissue were frozen in liquid N_2 and powdered. An eq. wt of pre-wet polyclar AT and 15 ml 0·1 M borate buffer, pH 7·8 containing 20 mM mercaptoethanol, were added. The extract was stirred at 4° for 1 hr, squeezed through cheesecloth and centrifuged at 48 000 *g* for 25 min. The supernatant was fractionated with 35–70% solid $(NH_4)_2SO_4$ and the pellet was resuspended in 1·5 ml buffer. Prewashed BioRad AG 1 × 4, 100–200 mesh (0·6 g) was added and after stirring for 15 min the mixture was filtered through cotton. Assays were done immediately. Protein was determined by the Lowry method[27] as modified by Potty.[28]

For enzyme purification studies young lignifying stems of *Forsythia* were freed of extracambial tissue, cut into small segments and homogenized and extracted as above. The exchange-resin treated enzyme solution obtained from 40 g of powder was fractionated with 40–65% $(NH_4)_2SO_4$ and the pellet was resuspended in a max vol. of 5 ml buffer. This solution was further fractionated on a Sephadex G-200 column which had been equilibrated with the 0·1 M borate-mercaptoethanol buffer. The combined enzyme was then dialysed overnight in 0·05 M Tris–HCl, pH 7·6, before being applied to a column of DEAE-cellulose (Whatman DE-52, 1·5 × 2 cm). The column was washed with 50 ml of the Tris–HCl buffer and then eluted with 50 ml portions of 0·10, 0·16 and 0·20 M KCl in buffer. The enzyme activity, which was eluted in the 0·16 M fraction was combined and applied to a hydroxylapatite column (0·5 × 2 cm). The column was washed with 20 ml of 0·05 M Tris–HCl, pH 7·6 and then eluted stepwise with 20 ml portions of 0·01, 0·05 and 0·1 M phosphate in buffer. The enzyme activity, which was eluted in the 0·05 M phosphate fraction, was pooled, brought to 50% glycerol concentration, and stored at −20°. Protein determinations were made by the Lowry method.[27]

Polyacrylamide gel electrophoresis. Standard techniques for gel electrophoresis were employed. The gels (pH 8·9, 7·5% acrylamide; upper gels omitted) were developed in pH 8·3 Tris–glycine buffer at 4 mA/gel. After development, the gels were removed and assayed. The stock assay solution contained the following: NADP, 2·5 mg; nitroblue tetrazolium, 1·5 mg; phenazinemethosulfate, 0·1 mg; and coniferyl alcohol, 2·5 mg. Tris buffer, 0·1 M, pH 8·8 was used to bring the final stock volume to 10 ml. 1·5 ml of this solution was added to each gel and the gels were incubated at 30° in reduced light. After 20–30 min, or when maximum color development had occurred, the gels were removed, washed in water and photographed. For assays using benzyl alcohol or ethanol, it was necessary to use an alcohol concentration 10× higher than for coniferyl alcohol.

Molecular weight determination. The molecular weight was estimated on a Sephadex G-200 column in 0·05 M Tris–HCl, pH 7·6, containing 0·1 M KCl as described by Andrews.[29]

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