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MULTIPLE FORMS AND SPECIFICITY OF CONIFERYL ALCOHOL DEHYDROGENASE FROM CAMBIAL REGIONS OF HIGHER PLANTS

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Abstract—Alcohol dehydrogenases of 89 species of plants from the Bryophyta, Pteridophyta, Gymnosperms and Angiosperms were examined by starch gel electrophoresis for their substrate and coenzyme specificities. High activities and multiple bands were observed with EtOH and NAD in most species. The same, but weaker banding patterns were also observed with benzyl alcohol and salicin. When coniferyl alcohol was used as substrate, activity was found only with NADP as coenzyme and the resulting bands were distinct from those obtained with the other substrates. Most plants tested had only one or occasionally a second coniferyl alcohol dehydrogenase band. *Salix* species were an exception, with multiple bands found in each of the species tested.

INTRODUCTION

Recent work has demonstrated the presence in plants of aromatic cinnamyl alcohol dehydrogenases (NADP(H) specific cinnamyl alcohol oxidoreductase) which are evidently involved in the reversible conversion of a cinnamyl aldehyde to the corresponding alcohol [1,2]. The enzymes isolated from *Forsythia* stems and soybean cell-suspension cultures have an absolute specificity for the cinnamyl moiety and may occur in multiple forms. This enzyme, along with hydroxycinnamate:CoA ligase and hydroxycinnamyl-CoA reductase, has been extracted primarily from lignifying tissues and catalyzes the final reaction in the reduction of hydroxycinnamic acids to the corresponding alcohols, the monomeric lignin precursors [3,4]. Furthermore, the presence of similar enzyme activity has been detected in a great variety of other plants although the substrate specificity of these forms has not been determined [1].

The purpose of this report is to examine the substrate and cofactor specificity of the enzyme in a wide variety of plant groups so that a generalized understanding of its role in lignin synthesis may be gained. In addition to its specificity, we are interested in the variability of the enzyme, i.e. is it similar in different plants and does it occur in multiple forms? In soybean cultures, for example, two forms of the enzyme have been demonstrated which have different preferences for various cinnamyl alcohols [2].

Multiple forms or isoenzymes have been detected in a wide variety of enzymes. These may represent products of different loci which have similar functions, products of different alleles of the same locus (allozymes) or different conformational states of the same protein. Isoenzymes are believed to have functional significance, in at least some situations, by increasing an organism's biochemical versatility [5]. For example, it has been demonstrated that maize plants, heterozygous for particular alcohol dehydrogenase alleles, may have increased biochemical versatility due to the favorable features of each allelic product [6,7].

Utilizing the technique of starch gel electrophoresis, we show that the enzyme is indeed specific for the cinnamyl alcohol moiety and NADPH in all plants examined, that it is less variable than other alcohol dehydrogenases examined (e.g. ethanol dehydrogenase) and that with the striking exception of *Salix* and a few other plants, it does not occur in electrophoretically detectable multiple forms. In addition to the coniferyl and EtOH substrates, these enzyme preparations were also tested with benzyl alcohol and salicin (an aromatic glucoside), since these latter compounds are not involved in lignin biosynthesis.

RESULTS

High levels of coniferyl alcohol dehydrogenase activity (expressed as nkat/g fr. wt in Table 1) were found among dicots (mean activity, 2.7) and gymnosperms (mean, 5.7) with low levels occurring in the bryophytes (mean, 0.25), pteridophytes (mean, 0.50) and monocots (mean, 1.25). This is in accord with results previously reported [1]. Visible bands were nearly always detected on the electrophoretic starch gels when the activity of the extracts was greater than 1 nkat/g fr. wt. Starch gels were utilized because they lend themselves readily to side by side comparisons and mixing experiments. The overall banding patterns which were obtained were highly resolved.

Alcohol dehydrogenases, active with EtOH as substrate and NAD as cofactor, were found in 76 of 89 plants tested. Nearly all EtOH–NAD patterns observed consisted of multiple bands, and the position and number of these bands was highly variable from plant to plant (Fig. 1). Similar banding patterns were also obtained when coniferyl alcohol, benzyl alcohol, and

Table 1. Occurrence of cinnamyl alcohol dehydrogenase in various plants

Taxonomic group	Species	R_m value of bands	Alcohol dehydrogenase Total activity (nkat/g fr. wt)	Alcohol dehydrogenase Specific activity (nkat/mg protein)
Bryophyta	*Sphagnum* sp.	NB	0.25	0.80
Pteridophyta	*Equisetum hyemale*	NB	0.16	0.18
	Lycopodium hupaziaselago	NB	0.36	0.30
	Polypodium aureum			
	rachis	NB	0.83	0.42
	rhizome	NB	0.42	0.55
	Psilotum triquetrum	NB	0.80	0.50
	Salvinia sp.	NB	0.20	0.58
	Selaginella haematodes	NB	1.03	0.65
Spermatophyta	*Ginkgo biloba*	NB	1.13	15.00
Gymnospermae	*Larix decidua*	68	14.00	8.75
	Metasequoia glyptostroboides	69	4.40*	3.88*
	Picea abies	68	14.08*	13.86*
	Pinus banksiana	68	3.13	7.82
	P. ellottii	68	8.86	7.18
	P. palustris	67	0.83*	1.20*
	P. pinea	67	1.50*	5.12*
	Pseudotsuga menziesii	68	4.88	8.26
	Taxodium distichum	72	0.18	0.28
	Taxus baccata	68	7.13*	4.42*
Angiospermae Monocotyledoneae				
	Cocos nucifera	NB	0.23	0.83
	Cyperus papyrus	NB	0.06	0.60
	Musa acuminata	NB	0.23	0.83
	Oryza sativa	NB	8.25	2.20
	Saccharum officinarum	NB	0.32	1.73
	Tillandsia usneoides	NB	0.15	2.05
	Zea mays	68	0.65	0.47
	Zingiber officinale	NB	0.12	0.27
Dicotyledoneae	*Acer campestris*	71	5.20*	3.55*
	A. dickii	69	4.10*	2.00*
	A. ginalla	71	1.23	6.16
	A. monspessulanum	71	4.13	6.33
	A. negundo	70	0.82	0.95
	A. platanoides	71	7.53*	4.85*
	A. platanus	71	2.30	2.40
	A. pseudoplatanus	71	5.00*	2.32*
	A. rubrum	71	2.35*	1.01*
	A. saccharinum	67, 69, 71	3.80*	1.92*
	A. tataricum	71	6.63*	3.70*
	Baccharis halimifolia	NB	0.23	0.16
	Betula pendula	NB	1.23	1.58
	Carpinus betulus	65, 68	2.96	1.00
	Casuarina equisetifolia	67	2.80	3.28
	Cephalanthus occidentalis	NB	0.86	0.48
	Cinnamomum camphora	68	3.16*	1.42*
	Dalbergia sissoo	NB	0.10*	0.01*
	Dais cotinifolia	73	8.33	2.82
	Datura suaveolens	NB	1.38	0.82
	Eriobotrya japonica	65, 68	0.26	1.05
	Eucalyptus camaldulensis	71	1.02*	2.03*
	Eugenia uniflora	NB	0.05	0.20
	Euonymus kiautschovicus	66	0.10	0.22
	Fagus sylvatica	69	1.26	2.27
	Forsythia suspensa	trace	0.90	1.38
	Grevillea robusta	68	0.26	0.27
	Ilex aquifolium	67	1.63	1.63
	I. opaca	69	0.25	0.06
	Liriodendron tulipifera	67	3.90*	2.08*
	Melia azedarach	69	1.16*	0.50*
	Parkinsonia aculeata	64	2.30	2.18
	Persea americana	NB	0.12	0.60
	Platanus acerifolia	69	2.82	1.92
	Populus deltoides	67	0.48	0.38
	Prunus caroliniana	NB	1.72	5.13

Table 1 (*Cont.*)

Taxonomic group	Species	R_m value of bands	Alcohol dehydrogenase Total activity (nkat/g fr. wt)	Alcohol dehydrogenase Specific activity (nkat/mg protein)
Dicotyledoneae	*P. padus*	67, 69	0.63	1.05
	Quercus laevis	69	—	0.55*
	Q. palustris	67	0.13	4.00
	Salix agrophylla	60, 62, 64, 66, 71, 75	4.18*	2.13*
	S. alba	66, 71, 75	3.03*	1.18*
	S. aurita	65, 67, 71, 75	5.73	3.16
	S. calodendron	60, 62, 65, 66, 69, 71	3.70*	1.02*
	S. caprea	65, 67, 71, 75	4.83	3.96
	S. chrysotela	65, 66, 69, 71	3.73*	1.46*
	S. daphnoides var. pulchra	68, 72, 75	4.05	1.82
	S. elegantissima	65, 66, 69, 71	3.13	1.45
	S. karolewiana	65, 66, 69, 71	3.13	1.45
	S. laureana	65, 66, 69, 71	4.96	2.50
	S. matsudana	66, 71, 75	4.33	2.22
	S. muscina	65, 67, 71, 75	4.46*	2.43*
	S. nigricans	65, 67, 71, 75	6.50	3.08
	S. purpurea	53, 56, 59, 62, 64, 67, 71, 75	4.22*	1.28*
	S. rehderiana	54, 56, 58, 60, 63, 66, 68, 71	3.50*	1.52*
	S. sitchensis	65, 67, 71, 75	6.33	2.73
	S. spaethii	65, 66, 69, 71	3.13	1.35
	S. viminalis	65, 67, 71, 75	7.90	4.70
	Sambucus kamtschatica	67	2.48	1.05
	S. simpsonii	71	0.38	1.13
	Schinus terebinthifolius	68	1.13*	1.33*
	Sorbus aria	72	1.92	2.73
	Syringa vulgaris	63, 69	1.70	1.22
	Viburnum suspensum	66	0.26	0.20

NB—no bands. *Average of more than one determination.

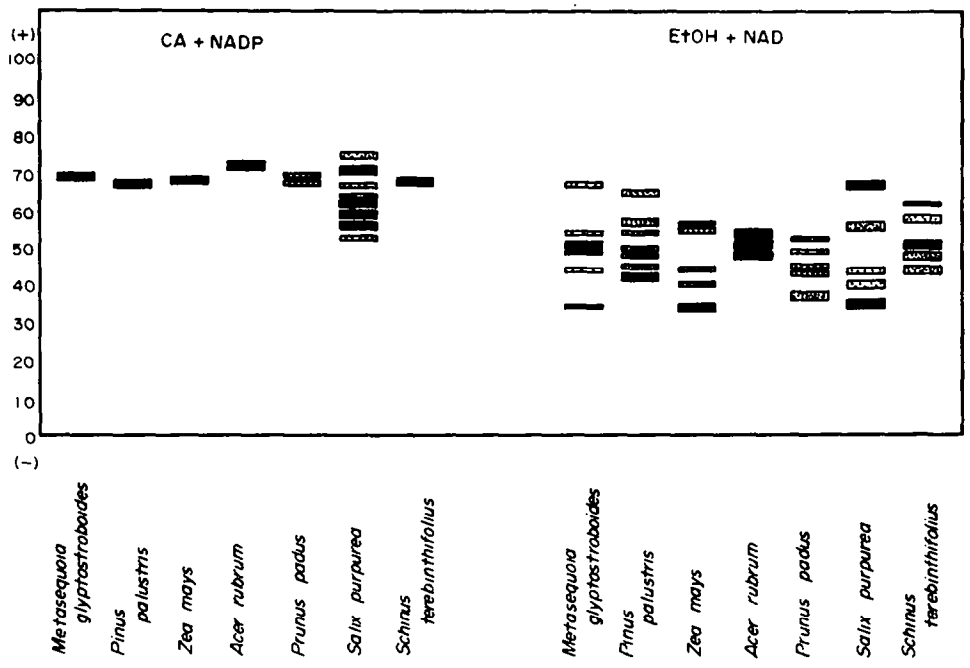

Fig. 1. Representative variation in coniferyl alcohol (CA) and ethanol dehydrogenase found among those plants studied.

salicin were tested with NAD. With these substrates the patterns were usually less intense, and, frequently, some bands were absent.

The EtOH–NAD banding pattern was not observed when NADP was used as a coenzyme with either the EtOH, benzyl alcohol or salicin substrates. In fact, very little activity was observed when NADP was the coenzyme, except when coniferyl alcohol was the substrate. The EtOH–NAD banding pattern appears to be the result of a highly variable multiform enzyme which is coenzyme (NAD) specific but not substrate specific.

With coniferyl alcohol and NADP, strong banding was found in most plants and the R_m values of these bands differed from those obtained with the other substrates. Rarely (in 3 species tested) faint bands analogous to the coniferyl alcohol–NADP bands were detected with coniferyl and NAD. Thus in 95% of the plants tested this dehydrogenase activity was specific for both coenzyme and substrate.

In addition to demonstrating high substrate and coenzyme specificity, this enzyme showed little variability (Fig. 1, Table 1). Excluding the bryophytes, pteridophytes and monocots which had very low activity levels, 65 of 74 species tested produced visible bands with coniferyl alcohol and NADP. The R_m values of these bands ranged from 53 to 75 with most occurring between 66 and 71. With so little variation, the differences between species might appear to be the result of experimental error. That at least some of the observed differences between species are real, however, was confirmed by mixing experiments where extracts from two different sources were mixed and electrophoresed. The bands from both sources were visible at their normal positions in the mixed situation, or, if the bands normally migrate to the same position, there was apparent reinforcement at those positions. We have also done mixing studies with different genera, and, in each case, the individual bands of each were detected and reinforcement of common bands was also observed.

Extracts of some plants showed multiple bands, e.g. *Picea abies*—2, *Acer saccharinum*—3, *Carpinus betulus*—2, *Eriobotrya japonica*—2, *Prunus padus*—2, *Syringa vulgaris*—2, and all of the species of Salix examined. Not only did all of the species of *Salix* exhibit multiple bands (average 4.5), but the greatest number of multiple bands (eight) was found in two of the Salix species, *S. purpurea* and *S. rehderiana*.

CONCLUSIONS

The results of this study substantiate earlier work which demonstrated that an alcohol dehydrogenase enzyme having high activity with the cinnamyl moiety exists in those plant tissues which produce large amounts of lignin (e.g. cambial regions of dicots and gymnosperms) [1]. The earlier studies also showed that in *Forsythia* stems and soybean cell-suspension cultures the enzyme has an absolute specificity for cinnamyl substrates [1,2]. This report extends those studies and demonstrates that there is a similar enzyme with absolute specificity for a cinnamyl substrate and NADP in a variety of other plants. On the starch gels, bands located at the R_m values given in Table 1 were produced, with the exception of rare traces, only with coniferyl alcohol and NADP. In addition to its specificity, the enzyme appears to be very conservative. The R_m values of the enzyme were nearly always between 66 and 71. With the exception of *Salix*, most plants exhibited only one band. It should be noted, however, that the absence of multiple bands is not proof of absence of multiple forms of the enzyme. Multiple forms may exist but migrate to the same position on the gel and, hence, are not distinguishable [8]. Compared with ethanol dehydrogenase, however, the coniferyl alcohol dehydrogenase appears to be much less variable, perhaps indicating that its role in plants is important and tolerant of only small variations. These observations support the proposition that cinnamyl alcohol dehydrogenase is a widespread and important enzyme which occurs in significant concentrations in lignin producing tissues and has the specific role of providing the cinnamyl alcohol moieties necessary for lignin synthesis.

The occurrence of multiple forms of the cinnamyl alcohol dehydrogenases is not unexpected. Multiple forms of many different enzymes have been observed, including other alcohol dehydrogenases in plants [9–11]. What is surprising are the differences observed between *Salix* and the other genera examined. It is possible that the multiple bands in *Salix* are artifacts generated by the electrophoretic conditions, but if this is so, multiple bands would be expected in the other species which were tested under identical conditions. Alternatively, the multiple bands could be artifacts resulting from substances or internal cellular conditions unique to *Salix*. The fact that the number of other alcohol dehydrogenase bands was not noticeably higher in *Salix*, than in other genera, and the fact that mixing experiments with *Salix* and other species resulted in no increase in band number argues against this possibility.

Duplicated genes may also produce multiple bands. For example, if the ancestral diploid plants, from which an allopolyploid was derived, contained different allelic forms of a gene, it is likely that multiple bands would be produced [12]. If this is true with coniferyl alcohol dehydrogenase, we might expect to see more bands in those plants with high chromosome numbers and hence probably polyploid. In *Salix* such a correlation does not exist, in fact, *S. purpurea* with 8 bands has only 38 chromosomes while several other *Salix* species (e.g. *S. laureana*, 76; *S. nigricans*, 114; *S. daphnoides* var. *pulchra*, 152) with fewer bands have more chromosomes. Duplicated genes fixed for alternative alleles, hence capable of producing multiple bands, may be produced without polyploidy. The multiple alcohol dehydrogenase bands observed in *Clarkia franciscana* have evidently been produced in this manner [13]. Although the origin of the multiple bands in *Salix* cannot yet be ascertained, it seems likely that they are genetically determined.

At this point the functional significance of multiple bands in *Salix* can only be theorized. *Salix* is believed to be a highly specialized genus [14]. It is very successful, occurring widely in temperate and arctic zones. One adaptive strategy, believed to be common among wild plants, is the development of all purpose genotypes able to adapt to many situations [15]. The presence of multiple enzyme forms, such as occur in coniferyl alcohol dehydrogenase, may indicate a form of specialization leading to biochemical and phenotypic flexibility and hence all purpose genotypes in *Salix*.

EXPERIMENTAL

Plant material. All material was collected from the University of South Florida or Ruhr University Botanical Gardens

or greenhouses. *Salix* species were propagated from cuttings derived from systematically determined trees growing in the University of Munich Botanical Garden.

Enzyme preparation. Procedures followed as described in ref. [1].

Starch gel electrophoresis. A 12.8% concentration of Connaught Starch (Connaught Labs, Canada) was used in a 15.2 mM Tris–3.7 mM citrate buffer, pH 7.9. The electrode buffer was 0.3 M borate, pH 8.6. Each gel was developed at a constant 300 V and the gels were cooled with ice during the 2 hr required for a 10 cm development. Protein preparations from 4–8 plants were absorbed individually onto paper wicks (capacity 20 μl) and placed into the origin slice on the left half of a gel. A duplicate set of wicks was placed in the right half. After the electrophoretic run was completed the gel was cut to separate the halves. The whole gel was then sliced horizontally into 3 equal slices. Thus from a single gel we were able to obtain 6 separate slices, each containing the same protein samples separated under identical conditions. Each gel slice was placed in a shallow dish and tested with the following combination of substrates and cofactors: to each gel 0.3 ml of a 1% soln of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), 0.1 ml of a 1% soln of phenazine methosulfate and 0.1 M Tris–HCl buffer (pH 8,8) to a total vol of 20 ml was added. To the odd numbered gels was added 2 mg NAD and to the even numbered ones NADP. Gels 1 and 2 were tested with 1.5 mg coniferyl alcohol as substrate. Gels 3 and 4 were tested with 0.7 ml 95% EtOH. Gels 5 and 6 were tested with either 1 ml of H_2O saturated benzyl alcohol or 1.5 mg of salicin. The gels were incubated in the dark at 37° for 1 hr before being washed in H_2O and photographed.

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