Purification and Characterization of Cinnamyl Alcohol Dehydrogenase from Tobacco Stems

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ABSTRACT

Cinnamyl alcohol dehydrogenase (CAD) is an enzyme involved in lignin biosynthesis. In this paper, we report the purification of CAD to homogeneity from tobacco (Nicotiana tabacum) stems. The enzyme is low in abundance, comprising approximately 0.05% of total soluble cell protein. A simple and efficient purification procedure for CAD was developed. It employs three chromatography steps, including two affinity matrices, Blue Sepharose and 2'5' ADP-Sepharose. The purified enzyme has a specific cofactor requirement for NADP and has high affinity for coniferyl alcohol (K_m = 12 micromolar) and coniferaldehyde (K_m = 0.3 micromolar). Two different sized polypeptide subunits of 42.5 and 44 kilodaltons were identified and separated by reverse-phase HPLC. Peptide mapping and amino acid composition analysis of the polypeptides showed that they are closely related, although not identical.

Lignin is one of the major organic materials in the biosphere, being second only to cellulose in abundance. It is synthesized from phenylpropanoid units by a complex sequence of reactions, some catalyzed by enzymes involved in general phenylpropanoid metabolism and others by branch path enzymes specific for lignan and lignin biosynthesis. One of these branch path enzymes is CAD,2 which catalyzes the reduction of the three cinnamyl aldehydes (sinapaldehyde, para-coumaraldehyde, and coniferaldehyde) to the corresponding cinnamyl alcohols that are the direct monomeric precursors of the lignin polymer. The relative proportions of these monomers varies in lignin from different species and it has been suggested that this may be brought about by the particular substrate specificity of CAD from different sources (4, 8). Due to its critical role in lignin biosynthesis, CAD is also a potential target enzyme for biotechnology directed toward modulating the quality and quantity of lignin in plants. Indeed, chemicals that cause inhibition of CAD activity in vivo have been shown to reduce the lignin content of plants (2).

Despite growing interest in CAD and the other lignin branch pathway enzymes, they have not been extensively studied. Angiosperm CAD has previously been purified to homogeneity only from poplar (13). A putative CAD clone was isolated from a bean (Phaseolus vulgaris) cDNA library (14) using an antiserum raised against the poplar enzyme. However, subsequent sequence comparison of this cDNA with another recently cloned cDNA encoding maize malic enzyme (12) revealed extensive homology—over 73% in the coding region (15). Thus, it is likely that this clone does not encode CAD. These results probably reflect the limitations of cross-species immunological screening of cDNA libraries and suggest that homologous probes may be necessary for CAD cDNA identification. To produce such specific probes and to reevaluate and expand the information available on angiosperm CAD, we have investigated the properties of the enzyme from tobacco (Nicotiana tabacum cv Samsun). We report here the purification of a tobacco protein that we have confirmed to be authentic CAD by a number of criteria.

MATERIALS AND METHODS

Plant Material

Tobacco (Nicotiana tabacum cv Samsun) plants were grown in John Innes compost No. 3 in a greenhouse under a 16 h light/8 h dark cycle. Six-week-old plants were used for enzyme extractions.

Buffers

The following buffers were used for enzyme purification:

A = 100 mm TRIS-HCl, pH 7.5, 10 mm mercaptoethanol.
B = 10 mm TRIS-HCl, pH 6.8, 10 mm mercaptoethanol.
C = 20 mm TRIS-HCl, pH 7.5, 10 mm mercaptoethanol, 5% (v/v) ethylene glycol (Pierce). D = 100 mm TRIS-HCl, pH 6.8, 10 mm mercaptoethanol, 5% (v/v) ethylene glycol.

Enzyme Purification

Procedures prior to Blue Sepharose chromatography were carried out at 4°C. Subsequent procedures were performed at room temperature. Six-week-old tobacco stems (4.8 kg processed in eight 600 g batches) were frozen in liquid N_2, crushed with a hammer, and homogenized in buffer A in a Waring blender. The homogenate was centrifuged at 13,000 g for 30 min and the pellet was discarded. Solid ammonium sulfate was added to the supernatant to bring it to 70% saturation and proteins were precipitated at 4°C for 30 min. The precip-
itate was collected by centrifugation at 13,000 g for 45 min. The pellet was resuspended in 0.9 volumes of buffer A supplemented with 5% (v/v) ethylene glycol. To remove material that did not resuspend, the extract was centrifuged at 34,000 g for 15 min and the supernatant was decanted.

The supernatant from each homogenate preparation was desalted by passage of 2.5 mL aliquots through PD-10 columns (Pharmacia), eluting in 3.5 mL buffer B. Pooled PD-10 eluates (50−100 mL) were then applied at a flow rate of 150 mL/h to a 5 × 10 cm Blue Sepharose (Pharmacia) column equilibrated in buffer B. The column was washed in at least six column volumes of buffer B including one column volume supplemented with 4 mM NAD (400 mL/h). Elution of CAD was performed with a gradient of 0 to 4 mM NADP in buffer B. CAD-containing fractions were identified by enzyme assay, pooled, and 5% ethylene glycol added. Eluates from two Blue Sepharose separations were combined prior to Mono Q chromatography.

The pooled eluates were then applied (2 mL/min) to a fast protein liquid chromatography Mono Q column (HR 5/5, Pharmacia) equilibrated in buffer C. The column was washed in buffer C until the absorbance at 260 nm dropped to baseline levels. Proteins were eluted in a linear gradient of buffer C with 20 to 400 mM TRIS-HCl, pH 7.5. Selected CAD-containing fractions from two Mono Q separations were pooled and applied to PD-10 columns, eluting with buffer D. The PD-10 eluate was applied to a 1 × 10 cm column of 2′,5′-ADP-Sepharose (Pharmacia) equilibrated in buffer D (20 mL/h). The column was washed with six column volumes of buffer D including one column volume supplemented with 4 mM NAD. Specific elution of CAD was performed in a linear gradient of buffer D with 0 to 4 mM NADP.

**SDS-PAGE**

Denaturing electrophoresis was performed in 12.5% polyacrylamide gels in the presence of SDS according to the method of Laemmli (5). Proteins were stained with Coomassie brilliant blue or silver reagent. Molecular mass markers used were lactalbumin (14.2 kD), trypsin inhibitor (20.1 kD), trypsinogen (24 kD), carboxic anhydrase (29 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), egg albumin (45 kD), and BSA (66 kD) (Sigma).

**Native Gel Electrophoresis**

For activity staining, samples were run on 12.5% homogeneous Phast-gels (Pharmacia). For subsequent analysis of native bands by SDS-PAGE, larger native gels were used following the procedure of Hedrick and Smith (3). After electrophoresis, the gels were rapidly stained in Coomassie brilliant blue and native protein bands were excised and equilibrated for 1 h in 0.625 mM TRIS-HCl, pH 6.8, 3% (w/v) SDS, 15% (v/v) mercaptoethanol, 10% (v/v) glycerol. The slices were then transferred to SDS-PAGE sample buffer, boiled for 3 min, and transferred to the bottom of sample wells in a 10% SDS gel.

**Protein and Enzyme Determinations**

Total protein estimates were determined by the Bio-Rad assay system (1) using γ-globulin as standard. CAD enzyme activity was determined by measuring the increase in absorbance at 400 nm when coniferyl alcohol was oxidized to coniferaldehyde (16). The assay was performed for 10 min at 30°C in a total volume of 1 mL in 100 mM TRIS-HCl, pH 8.8, containing 0.1 mM coniferyl alcohol (Aldrich), 0.2 mM NADP, and 5 to 100 μL protein extract. Assays measuring the reduction of coniferaldehyde were performed according to Sarni et al. (13).

**Activity Staining of Native Gels**

After electrophoresis, 12.5% homogeneous Phast-gels were incubated in reduced light for 30 min at 30°C in 10 mL of 100 mM TRIS-HCl, pH 8.8, containing 1.5 mg nitroblue tetrazolium, 0.1 mg phenazinemethosulfate, 2.5 mg NADP, and 2.5 mg coniferyl alcohol or 25 μL ethanol according to the method of Mansell et al. (9).

**Reverse-Phase HPLC**

An aliquot of the pure CAD sample (33 μg) was applied to a C8 reverse-phase HPLC column (2.1 mm × 150 mm, SGE) equilibrated in 0.08% (v/v) TFA. Proteins were eluted with a gradient of 0 to 90% acetonitrile in 0.08% TFA and the elution was monitored by absorbance at 214 nm.

**Determination of Native Mol Wt**

A CAD-containing sample eluted from Blue Sepharose was run through a PD-10 column and eluted in 100 mM TRIS-HCl, 150 mM NaCl, 10 mM mercaptoethanol. The sample was applied to a precalibrated Superdex 200 gel filtration column (Pharmacia), equilibrated, and eluted with the same buffer. Eluting fractions were assayed for CAD activity. The calibration markers were β-amylase, alcohol dehydrogenase, BSA, carbonic anhydrase, and Cyt c (Sigma).

**RESULTS**

**Purification of Cinnamyl Alcohol Dehydrogenase**

The tobacco CAD enzyme was purified by successive chromatography on Blue Sepharose, Mono Q, and 2′,5′-ADP-Sepharose columns. This procedure, considerably improved from previously published methods (13), relied on the inclusion of two affinity columns for its efficiency. We found that the specificity of affinity elution from these columns could be maximized by washing the matrix with NAD before elution of CAD with NADP. In particular, this improvement enabled the early elimination from the sample of all contaminating alcohol dehydrogenases. Figure 1 shows the result of this elution procedure from the first column, Blue Sepharose. Activity staining of eluted proteins in native gels showed that the NAD wash (lane 2) removed a number of alcohol dehydrogenases that apparently had dual cofactor affinity because they could also be eluted with NADP if the NAD wash was omitted (lane 1). One dehydrogenase, however, was not eluted with NAD and could be specifically eluted with NADP (lane
3) after the NAD wash. This polypeptide alone exhibited CAD activity because it was the only alcohol dehydrogenase that activity stained when coniferyl alcohol rather than ethanol was supplied as substrate (lane 4).

The SDS-PAGE profile of samples taken at various stages during this purification procedure is shown in Figure 2. The complexity of the protein pattern in the crude homogenate (lane 2) and after ammonium sulfate fractionation (lane 3) was greatly reduced by Blue Sepharose chromatography (lane 4), less than 1% of the protein originally loaded onto the column being present in eluted CAD-containing fractions. Subsequent Mono Q chromatography removed two prominent bands of approximately 45 and 60 kD (lane 5) and also eliminated NADP from the sample. This allowed the cofactor to be used again for affinity elution of CAD from the final column, 2′5′-ADP-Sepharose, which removed all remaining contaminating proteins (lane 6). The NADP-eluted fractions with CAD activity contained only a closely spaced doublet of polypeptides of 42.5 and 44 kD and a faint band at around 70 kD. These three polypeptides had similar peptide maps (see results below) and therefore all represented CAD protein. The 70 kD band was not always seen in gels (e.g. see lane 7) and was therefore probably a native form of the 42.5 and 44 kD polypeptides that was not completely reduced during the preparation of the sample. Silver staining of the 2′5′-ADP-Sepharose NADP-eluate also confirmed its purity and no trace of any bands other than the two CAD polypeptides was observed (lane 7).

Table 1 summarizes the purification data. Overall a 1811-fold purification of the CAD enzyme was achieved, suggesting that the enzyme originally represented 0.05% of the total protein in the homogenate. The purified enzyme had a specific activity of 173 nkat/mg using coniferyl alcohol as substrate.

The recoveries shown represent only the proportion of the CAD-containing fractions that were carried on to the next purification step.

**Determination of Native Mol Wt**

The mol wt of the native CAD enzyme was determined by chromatography on a Superdex 200 column calibrated with β-amylase, alcohol dehydrogenase, BSA, carbonic anhydrase, and Cyt c. CAD activity eluted with an apparent molecular size of 79 kD, suggesting that the native protein is a dimer.

**Separation and Characterization of the Two CAD Polypeptides**

The pure CAD sample was fractionated by RPLC (Fig. 3A) and eluted as two sharp and distinct peaks. No other protein peaks were evident. SDS-PAGE of the two RPLC peaks showed the earlier eluting peak (peak 1) to be enriched in the

**Table 1. Purification of Cinnamyl Alcohol Dehydrogenase from Tobacco Stems**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Protein mg</th>
<th>Specific Activity nkat/mg</th>
<th>Purification -fold</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>18,412</td>
<td>0.1</td>
<td>100</td>
<td></td>
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<tr>
<td>Ammonium sulfate</td>
<td>4,146</td>
<td>0.3</td>
<td>2.7</td>
<td>61</td>
</tr>
<tr>
<td>Blue Sepharose</td>
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<td>20</td>
<td>208</td>
<td>25</td>
</tr>
<tr>
<td>Mono Q</td>
<td>4.4</td>
<td>66</td>
<td>690</td>
<td>16</td>
</tr>
<tr>
<td>2′5′-ADP-Sepharose</td>
<td>0.6</td>
<td>173</td>
<td>1,811</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 1. Nondenaturing gel electrophoresis of alcohol dehydrogenases eluted from Blue Sepharose by NAD and NADP. Gels were stained for alcohol dehydrogenase activity using ethanol (A) or coniferyl alcohol (B) as substrate. Lanes 1 and 4, NADP eluate; lane 2, NAD eluate; lane 3, NADP eluate after NAD wash.

Figure 2. SDS-PAGE of fractions containing CAD activity taken at different stages of purification. Lane 1, molecular mass standards BSA (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), and trypsin inhibitor (20.1 kD); lane 2, crude homogenate; lane 3, ammonium sulfate precipitate; lane 4, Blue Sepharose eluate; lane 5, Mono Q eluate; lane 6, 2′5′-ADP-Sepharose eluate. Proteins were stained with Coomassie blue. Lane 7, 2′5′-ADP-Sepharose eluate stained with silver reagent.
44 kD polypeptide, whereas the later eluting peak (peak 2) contained the 42.5 kD polypeptide (results not shown).

The total amino acid composition of the polypeptide in each of these RPLC peaks was determined. This revealed very similar but not identical overall compositions for the two proteins (data not shown).

The relationship between the 42.5 and 44 kD CAD polypeptides was examined by chemical and enzymic peptide mapping. The RPLC-separated peaks 1 and 2 were digested with trypsin and the peptide products fractionated. Overall, great similarity in the peptide pattern of the two samples was revealed (Fig. 3B). Several peptide peaks eluted with identical retention times from both samples. However, other peptide peaks displayed slightly different retention times in each sample, suggesting small differences in hydrophobicity or size. This suggested that although the 42.5 and 44 kD CAD polypeptides are closely related, they are not identical. Chemical cleavage of the two CAD polypeptides with N-chloro-succinimide/urea (7) (results not shown) confirmed their overall similarity.

**Subunit Composition of Native CAD**

The pure CAD sample was run on a 4 to 30% native gel. Activity staining of this gel was unsuccessful, but staining with Coomassie blue revealed two protein bands (Fig. 4, lane 1). These bands were excised, equilibrated, and boiled in SDS sample buffer and transferred to adjacent wells of an SDS polyacrylamide gel. After SDS-PAGE and silver staining, each native band displayed a polypeptide profile containing both 42.5 and 44 kD bands (Fig. 4, lane 4 for upper native band; lane 5 for lower native band), which was identical to the pattern in the original pure CAD sample (lane 3). This confirms that both polypeptides are components of the native enzyme and may suggest that it is a heterodimer.

**Enzyme Specificity and Kinetics**

Apparent $K_m$ values and maximum velocities of the reversible reaction catalyzed by CAD were determined by measuring the reaction rate at various concentrations of coniferyl alcohol or coniferaldehyde. The $K_m$ for each substrate was calculated from a double reciprocal Lineweaver-Burk plot. Tobacco CAD exhibits a $K_m$ of 0.3 $\mu$M/L for aldehyde reduction, whereas for alcohol oxidation the $K_m$ is 12 $\mu$M/L.

**DISCUSSION**

In this report, we describe the purification and preliminary characterization of the tobacco CAD enzyme. The pure enzyme consists of two similar but apparently different sized polypeptides of 42.5 and 44 kD. Several criteria confirm the identity of the isolated polypeptides as CAD. (a) At an early stage of purification, activity staining revealed only one protein in the sample capable of using coniferyl alcohol as substrate. When this activity stained band was subjected to SDS-PAGE, only the 42.5 and 44 kD polypeptides were apparent. (b) Silver staining and RPLC confirmed that the purified CAD sample contains only these same two polypeptides, whereas enzyme and protein assays showed that it had a high CAD specific activity, over six times that of the isolated poplar enzyme (13). (c) The pure CAD enzyme has a specific cofactor requirement for NADP and has a high affinity for coniferaldehyde, one of its natural substrates in vivo, exhibiting a $K_m$ of 0.3 $\mu$M/L. This is nearly 40 times lower than the concentration of coniferyl alcohol at the $K_m$ for the reverse reaction and is therefore consistent with the proposed role for CAD in the synthesis of cinnamyl alcohol lignin precursors.

The native and subunit mol wts of the tobacco CAD enzyme are in good agreement with those previously reported. The angiosperm enzyme from poplar is composed of 40 kD subunits (13), whereas the gymnosperm enzyme has subunits of 42 kD in spruce (8) and 44 kD in loblolly pine (11). The identification of two different sized polypeptide subunits of the tobacco enzyme is unusual, however, because the poplar,
spruce, and loblolly pine enzymes are reported to be homodimers of identical subunits. Although the two tobacco subunits appear to be very similar polypeptides, small differences between them in terms of size, amino acid composition, and chemical and enzymic digestion patterns have been demonstrated. Both polypeptides are present in tobacco extracts in approximately equal amounts, even when protease inhibitors are included during the early purification steps, and tests for secondary modification such as glycosylation have proved negative (results not shown). Moreover, the difference in amino acid composition between the two polypeptides suggests that they are indeed separate gene products. These may arise as a consequence of the genetic lineage of *N. tabacum*, which is an allotetraploid hybrid, containing the complete chromosome complements of two parental species. A number of tobacco proteins, for example the enzyme acetolactate synthase (6), have been shown to be encoded by two distinct genes, each contributed by one progenitor species. In the case of acetolactate synthase, this gives rise to two very similar proteins that differ to only 0.7% at the amino acid level (6). Alternatively, the two CAD polypeptides could suggest the existence of different CAD isozymes in tobacco. In a survey of 89 different plant species examined by starch electrophoresis and activity staining, Mansell et al. (10) found that multiple forms of CAD were observed in a few species, although most plants apparently contain only one CAD isoform.

Attempts to differentiate between these two possibilities and to elucidate the relationship between the two CAD subunits and the native protein proved inconclusive. Although only one form of native CAD protein was detected by gel filtration and on Phast-gels, two forms were seen on longer 4 to 30% gradient gels (Fig. 4). Because these two forms were visualized by Coomassie blue staining and could not be activity stained, they may represent different inactive conformations of the enzyme generated during electrophoresis and do not necessarily suggest that different native forms of CAD exist in vivo. However, both these native bands and the native band from Phast-gels that retained activity were shown to contain both 42.5 and 44 kD polypeptides when transferred to SDS gels. Thus, it seems likely that at least some native CAD exists as a heterodimer of 42.5 and 44 kD polypeptides. This may suggest that the two CAD polypeptides are more likely allelic variants rather than components of different homodimeric isozymes. Full elucidation of this problem will be greatly simplified by the generation of CAD-specific antibodies and the isolation of CAD clones, allowing investigation of the tissue-specific and temporal expression of the two CAD polypeptides.

The chromatography procedures described here provide an improved method for the purification of CAD to homogeneity from different tissues. Although CAD is not an abundant protein, representing less than 0.05% of total soluble protein in tobacco stems, the simplicity of the purification method, comprising only three chromatography steps, allows reasonable amounts of enzyme to be purified with facility. This will allow the determination of CAD amino acid sequences and the production of CAD-specific antisera, rapidly enabling the isolation of a genuine CAD clone.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**