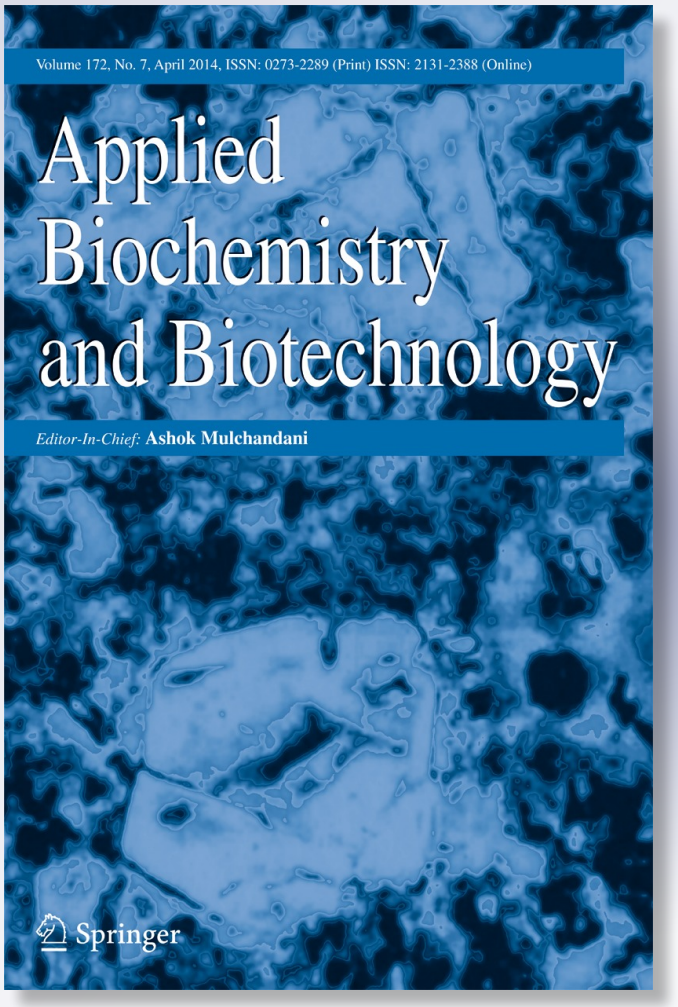
Purification and Characterization of a Zinc-Dependent Cinnamyl Alcohol Dehydrogenase from Leucaena leucocephala, *a Tree Legume*

Brijesh Pandey, Veda P. Pandey, A. K. Shasany & U. N. Dwivedi

Applied Biochemistry and Biotechnology
Part A: Enzyme Engineering and Biotechnology

ISSN 0273-2289
Volume 172
Number 7

Appl Biochem Biotechnol (2014)
172:3414-3423
DOI 10.1007/s12010-014-0776-7



Springer

Appl Biochem Biotechnol (2014) 172:3414–3423
DOI 10.1007/s12010-014-0776-7

Purification and Characterization of a Zinc-Dependent Cinnamyl Alcohol Dehydrogenase from *Leucaena leucocephala*, a Tree Legume

Brijesh Pandey · Veda P. Pandey · A. K. Shasany · U. N. Dwivedi

Received: 2 October 2013 / Accepted: 3 February 2014 /
Published online: 16 February 2014

Abstract A cinnamyl alcohol dehydrogenase (CAD) from the secondary xylem of *Leucaena leucocephala* has been purified to homogeneity through successive steps of ammonium sulfate fractionation, DEAE cellulose, Sephadex G-75, and Blue Sepharose CL-6B affinity column chromatographies. CAD was purified to 514.2 folds with overall recovery of 13 % and specific activity of 812. 5 nkat/mg. Native and subunit molecular masses of the purified enzyme were found to be ~76 and ~38 kDa, respectively, suggesting it to be a homodimer. The enzyme exhibited highest catalytic efficiency (*K*cat/*K*m 3.75 μM^{-1} s^{-1}) with cinnamyl aldehyde among all the substrates investigated. The pH and temperature optima of the purified CAD were pH 8.8 and 40 °C, respectively. The enzyme activity was enhanced in the presence of 2.0 mM Mg^{2+}, while Zn^{2+} at the same concentration exerted an inhibitory effect. The inclusion of 2.0 mM EDTA in the assay system activated the enzyme. The enzyme was inhibited with caffeic acid and ferulic acid in a concentration-dependent manner, while no inhibition was observed with salicylic acid. Peptide mass analysis of the purified CAD by MALDI-TOF showed a significant homology to alcohol dehydrogenases of MDR superfamily.

Keywords Cinnamyl alcohol dehydrogenase · *Leucaena leucocephala* · Lignin · MALDI-TOF · Zinc-dependent alcohol dehydrogenase

B. Pandey · V. P. Pandey · U. N. Dwivedi (✉)
Department of Biochemistry, University of Lucknow, Lucknow 226007, India
e-mail: upendradwivedi@hotmail.com

A. K. Shasany
Genetic Resources and Biotechnology Division, Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India

Present Address:
B. Pandey
Amity Institute of Biotechnology, Amity University, Lucknow, 226010 Uttar Pradesh, India

Introduction

Cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) catalyzes the reversible conversion of hydroxycinnamyl aldehydes to their corresponding alcohols, before their oxidative polymerization to lignin, a major constituent of the plant cell wall [1–3]. Lignin imparts rigidity and strength as well as resistance to physical, chemical, and biological attacks to plants [4, 5]. CAD has been reported to be implicated in the regulation of biosynthesis of lignin which has great relevance in the optimal utilization of the plant biomass for various purposes such as paper or biofuel/bioenergy production [1]. Thus, in the past 20 years or so, genes of lignin biosynthetic pathway have been extensively used to manipulate lignin content as well as composition in a wide variety of plants using metabolic engineering approaches.

CAD has been purified and/or characterized from various plants such as soybean, tobacco, *Eucalyptus gunnii*, wheat, loblolly pine, and a bacterium *Helicobacter pylori* [6–11]. CAD in gymnosperms and angiosperms displays distinct features. Gymnosperm CADs are believed to be highly specific for the reduction of coniferaldehyde [12], whereas angiosperm CADs have been shown to have significant affinity for both coniferaldehyde and sinapaldehyde [2, 4]. However, in aspen (*Populus tremuloides*), a novel sinapyl alcohol dehydrogenase (SAD), which was shown to be specifically involved in the reduction of sinapaldehyde in vitro, has been reported [13].

Native molecular weights of CAD from *Forsythia suspensa*, *Glycine max*, *Pinus taeda*, *Picea abies*, etc. are reported to be 80, 69, 82, and 72 kDa, respectively [2, 6, 10, 14]. Most of the CADs from various sources are reported to have temperature optima of 37ºC. CAD from a number of sources has been reported to have pH optima of 8.8 and 9.2 [6, 11]. A wide variation in *K*m has been reported for various alcoholic and aldehydic substrates [8, 11, 15, 16]. The CADs from a number of angiospermic plants show higher affinities for coniferyl and sinapyl alcohols and lower affinity with cinnamyl alcohol [2], while the *H. pylori* CAD has been reported to have higher affinity for cinnamyl alcohol [11].

The CADs from various sources have been reported as zinc-dependent metalloenzyme. Two Zn^{2+} atoms per subunit have been reported in *Saccharomyces cerevisiae* CAD [17]. Similarly, in the *H. pylori* CAD, Zn^{2+} has been reported to exhibit structural and catalytic functions [11]. The *Bemisia argentifolii* CAD exhibited Zn^{2+}-mediated oligomerization [18].

In the present study, we report purification and characterization of a zinc-dependent CAD, from secondary xylem of stem of a multipurpose tree legume *Leucaena leucocephala* which is a perennial tree and grows well in diversified edaphic and climatic conditions. This plant has multifarious significance as forage, timber, firewood, sand binder, N_2 fixer, and as raw material in paper–pulp manufacturing [19, 20].

Material and Methods

Plant Material

The stem pieces of >3 cm diameter were collected from 4- to 5-year-old field-grown plants of *L. leucocephala* L. var. K-29 growing in the garden of Department of Biochemistry, Lucknow University. The bark was peeled off and secondary xylem was scrapped and used as source of enzyme.

Enzyme Extraction

Two hundred grams secondary xylem scraping of *L. leucocephala* was homogenized in five volume (*w*/*v*) extraction buffers containing Tris-HCl pH 7.5 (100 mM), PVPP insoluble (0.1 %), and β-mercaptoethanol (20 mM). The homogenate was filtered through two folds of muslin cloth and supernatant, obtained after centrifuging at 12,000×*g* in Sorvall RC5C at 4 °C for 25 min, was used as crude enzyme extract for purification.

Enzyme Assay

Enzyme assay was done according to the protocol of Campbell and Ellis [21] with some modifications. Assay system for the reaction consisted of coniferyl alcohol (200 μM), $NADP^+$ (200 μM), Tris-HCl buffer (100 mM, pH 8.8), and a suitable aliquot of the enzyme preparation in a final volume of 3 mL. The increase in absorbance was measured at 340 nm using a UV-visible spectrophotometer (Elico model 169). For the forward reaction (physiological reaction), enzyme activity was assayed by monitoring decrease in absorbance at 340 nm, using coniferyl aldehyde (Aldrich) (200 μM), NADPH (200 μM), and Tris-HCl buffer pH 6.5 (100 mM). CAD activity was expressed in terms of *nkat* (kat=moles of substrates consumed per second) taking molar extinction coefficient of NADPH as 6.22×10^3 M^{-1} cm^{-2}.

Protein was estimated by the method of Bradford [22] using bovine serum albumin fraction V as standard.

In-Gel Activity Staining

In-gel activity staining of CAD was done using the protocol of Loulakakis and Roubelakis-Angelakis [23] with slight modifications. After native polyacrylamide gel electrophoresis (PAGE), the gel was washed with cold water and immersed in 20 mL staining solution containing coniferyl alcohol (200 μM), $NADP^+$ (200 μM), Tris-HCl buffer pH 8.8 (100 mM), nitroblue tetrazolium (1.2 mg), and phenazonium methosulfate (0.2 mg). The gel was incubated at 37 °C for 15–30 min in dark with shaking at 40 rpm. The color was developed in around 15 min, then, the reaction was stopped by washing with distilled water, and the gel was stored in 5 % acetic acid solution till analysis.

CAD Enzyme Purification and Physicochemical Characterization

The crude supernatant was subjected to 20–80 % ammonium sulfate fractionation and the precipitated protein was pelleted by centrifuging at 12,000×*g* for 25 min at 4 °C in Sorvall RC5C centrifuge. The pellet was further suspended in a column equilibration buffer (CEB) containing Tris-HCl pH 7.5 (10 mM), β-mercaptoethanol (7 mM), and ethylene glycol (1 %) and dialyzed overnight against the same with changes of the buffer thrice. The dialyzed enzyme was loaded onto a glass column, packed with equilibrated diethyl amino ethyl (DEAE)–cellulose matrix with CEB. The protein was eluted with NaCl gradient (0–500 mM) in CEB. The fractions containing high specific activity were pooled and concentrated by Maxy Dry Lyophilizer (Heto). The concentrated sample was subjected to gel filtration chromatography using Sephadex G-75 matrix preequilibrated with CEB. The protein was eluted using CEB. The high specific activity-containing fractions were pooled and loaded onto the Blue Sepharose CL-6B column preequilibrated with CEB. The bound protein was eluted with gradient (0–500 μM) of $NADP^+$ in CEB. Throughout the process, cold condition (4 °C) was maintained except for the Blue Sepharose affinity

chromatography step where the enzyme was processed at 25 °C as it could not bind to the resin at 4 °C.

Native molecular mass of the enzyme was determined by Sephadex G-200 gel exclusion chromatography using standard molecular mass markers as described by Wyrambik and Griesebach [6]. Subunit molecular mass of the purified enzyme was determined by discontinuous sodium dodecyl sulfate PAGE (SDS-PAGE) (10 % resolving and 3 % stacking gel).

*K*m for different cinnamyl alcohol (coniferyl alcohol, sinapyl alcohol, and cinnamyl alcohol) and cinnamyl aldehyde (coniferyl aldehyde and cinnamyl aldehyde) substrates was determined for this purified CAD using Lineweaver–Burk plot. Effects of temperature and pH on the CAD activity were investigated using coniferyl alcohol as substrate and $NADP^+$ as cofactor. The effect of EDTA, divalent cations, and phenolics (caffeic acid, ferulic acid, and salicylic acid (SA)) on the activity of CAD was investigated by varying concentrations of EDTA, salts ($CaCl_2$, $MgCl_2$, and $ZnCl_2$), and phenolics with coniferyl alcohol as substrate.

Matrix-Assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS) of CAD

Sample Preparation

Coomassie-stained band of active protein of interest was manually excised from the gel with a scalpel and placed for sample preparation as per protocol provided by the manufacturer.

MALDI-TOF Mass Spectrometry

MS spectrum was acquired in the reflector positive ion mode on MALDI Time-of-Flight (MALDI-TOF)/TOF mass spectrometer (Applied Biosystems 4700 Proteomics Analyzer, Framingham, MA, USA), which uses a 200-Hz diode-pumped neodymium:yttrium–aluminum–garnet (Nd:YAG) laser operating at 355 nm. The instrument was operated in the delayed extraction mode. Spectra were obtained by accumulation of 1,000 consecutive laser shots, and laser intensity used was in the range of 5,000 to 6,000. Close external calibration for MS was performed with 4700 Cal Mix (Applied Biosystems, USA), a standard mixture of six peptides des-Arg1-bradykinin (904.4681), angiotensin I (1296.6853), Glu1-fibrinopeptide B (1570.6774), ACTH [clip 1-17] (2093.0867), ACTH [clip 18-39] (2465.1989), and ACTH [clip 7-38] (3657.9294). Only baseline corrections were applied to the raw data.

Database search was performed with mass spectrometry data using Global Proteome Server (GPS) v3.5 software (Applied Biosystems, USA) equipped with MASCOT (Matrix Science) search engine. Only monoisotopic masses were used for searching against the National Center for Biotechnology Information (NCBI) nr database. The maximum peptide precursor tolerance was set between 40 and 50 ppm. At most one missed cleavage for tryptic peptides was allowed, and the modifications accepted were carbamidomethyl cysteines as fixed modification.

Results and Discussion

Purification of CAD Enzyme

CAD from the stem secondary xylem of *L. leucocephala* was isolated and purified through successive steps of ammonium sulfate fractionation, DEAE cellulose, Sephadex G-75, and Blue Sepharose CL-6B affinity column chromatographies. The purification chart for

Table 1 Purification chart of CAD from old stem secondary xylem of *Leucaena leucocephala*

Steps	Protein (mg)	Activity (nkat)	Specific activity (nkat/mg)	Recovery (%)	Fold purification
Crude	415	657	1.58	100	–
Ammonium sulfate fractionation (20–80 %)	342	579	1.69	88	1.07
DEAE cellulose column chromatography	12	359	29.6	55	18.9
Sephadex G-75 gel filtration chromatography	1.5	123	82	19	51.8
Blue Sepharose CL-6B affinity column chromatography	0.104	84.5	812.5	13	514.2

L. leucocephala CAD is given in Table 1. CAD was purified to 514.2 folds with overall recovery of 13 % and specific activity of 812.5 nkat/mg.

Molecular Weight Determination

The native molecular weight of the purified CAD was determined by Sephadex G-200 column chromatography. The result is presented in Fig. 1a and the native molecular weight was found to be ~76 kDa. Analysis of the purified CAD on SDS-PAGE revealed a single band corresponding to ~38 kDa (Fig. 1b), suggesting that the purified CAD is a homodimer. Size of *L. leucocephala* CAD was found to be close to that reported from other sources such as *F. suspensa* (80 kDa), *P. taeda* (82 kDa), and *P. abies* (72 kDa) [2, 10, 14].

Physicochemical Characterization of CAD

Effect of Substrates

Effect of various substrates on the CAD activity was investigated. Results are presented in Table 2. The *K*m for coniferyl, sinapyl, and cinnamyl alcohols were found to be 5.0, 6.2, and

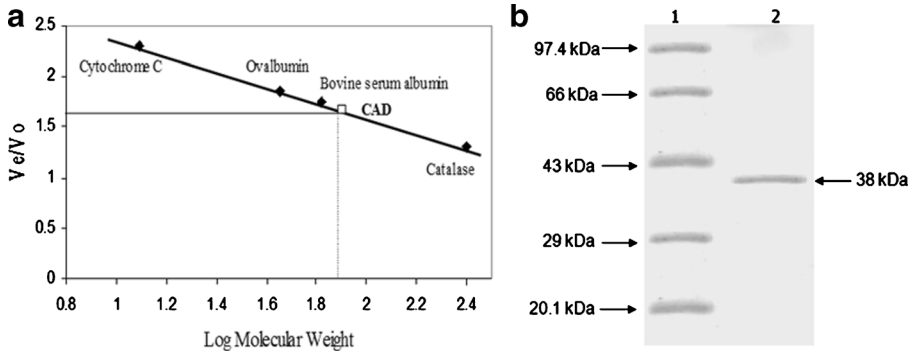


Fig. 1 **a** Native molecular weight determination of purified CAD by Sephadex G-200 gel filtration chromatography. The standard proteins used were catalase (240 kDa), bovine serum albumin fraction V (66 kDa), ovalbumin (43 kDa), and cytochrome C (12 kDa). **b** SDS-PAGE analysis of purified CAD, stained with Coomassie Brilliant Blue. *Lane 1* indicates molecular weight marker and *lane 2* purified CAD

Table 2 Kinetic parameters and substrate specificities of purified CAD from *L. leucocephala*

Substrates	*K*m (μM)	*V*max (nkat/mg)	*K*cat (s^{-1})	*K*cat/*K*m (μM^{-1} s^{-1})
Coniferyl alcohol	5.0±0.45	910.00	8.75	1.75
Cinnamyl alcohol	3.8±0.4	666.25	6.41	1.69
Sinapyl alcohol	6.2±0.55	1,495.00	14.38	2.32
Coniferyl aldehyde	4.5±0.5	1,722.50	16.56	3.68
Cinnamyl aldehyde	1.5±0.23	585.00	5.63	3.75
NADP	8.47±0.85	958.75	9.22	1.09
NADPH	1.5±0.3	1,592.50	15.31	10.21

3.8 μM, respectively. No activity was observed with methanol and ethanol as alcoholic substrates. On the other hand, *K*m for aldehydic substrates, namely, cinnamyl and coniferyl aldehydes, were found to be 1.5 and 4.5 μM, respectively. *K*m for $NADP^+$ with coniferyl alcohol as substrate was found to be 8.47 μM, while *K*m for NADPH with coniferyl aldehyde as substrate was found to be 1.5 μM. The purified enzyme exhibited higher *K*cat/*K*m (enzyme efficiency) with aldehydic substrates than those of alcoholic substrates with maximum enzyme efficiency for cinnamyl aldehyde followed by coniferyl aldehyde (3.75 and 3.68 μM^{-1} s^{-1}, respectively) (Table 2). Enzyme efficiency of *Leucaena* CAD with coniferyl aldehyde is very close to that of tobacco CAD, whose suppression led to the change in S:G ratio with increase in sinapyl aldehyde in lignin [24]. The finding of highest enzyme efficiency with cinnamyl aldehyde is similar to CAD from bacterium *H. pylori* where the highest *K*cat/*K*m (5,480± 2,853.68 μM^{-1} s^{-1}) has been reported with cinnamyl aldehyde as substrate [11]. *Leucaena* CAD exhibited higher efficiency with sinapyl alcohol (*K*cat/*K*m 2.32 μM^{-1} s^{-1}) among the three aromatic alcoholic substrates analyzed (Table 2). This finding was in agreement with other reported CADs from angiosperms which are reported to favor sinapyl and coniferyl alcohols as substrates more than other alcoholic substrates [2]. For sinapyl alcohol and coniferyl aldehyde, *K*m values close to what has been obtained for *Leucaena* CAD have been reported for *Eucalyptus* (an angiospermic tree) CAD (6.6 and 4.5 μM, respectively) [8]. The wide variation in enzyme efficiencies for various substrates may be attributable to variation in the lignin composition in a species-specific manner [5]. The kinetic data obtained for *Leucaena* CAD clearly indicated that it had higher affinities for corresponding aldehydes, and hence, in physiological condition, it seems to favor the conversion of cinnamyl aldehydes to corresponding alcohols as suggested by Wyrambik and Grisebach [6] in case of soybean CAD. Inability of this CAD to use aliphatic alcohols (ethanol and methanol) as substrates further supports its involvement in the monolignol biosynthesis.

Effect of pH and Temperature on Purified CAD

The effect of pH and temperature on purified CAD was investigated. The purified enzyme exhibited considerably good activity over a broad pH range (6.5 to 9.0) with maximum activity at pH 8.8 (Fig. 2). CAD exhibited considerably good activity between 30 to 42 °C with maximum activity at 40 °C, beyond which activity declined sharply. CAD from a number of sources has been reported to have pH optima of 8.8 [11]. However, for other CADs, pH optima of 9.2 have also been reported [6, 11]. Most of the CADs in literature are reported to have temperature optima of 37 °C. *Leucaena* CAD, however, was having little higher temperature optima.

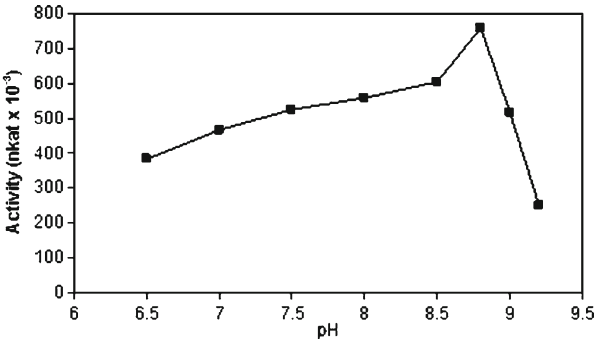


Fig. 2 Effect of pH on activity of purified *L. leucocephala* CAD

Effect of Metal Ions and EDTA

Effects of Mg^{2+}, Ca^{2+}, and Zn^{2+} on the activity of purified CAD were investigated. Results are presented in Fig. 3a. It was found that Mg^{2+} (up to 2 mM) showed activating effect (30 % increase in activity), Ca^{2+} showed no significant effect, while Zn^{2+} showed inhibitory effect (24 % decrease in activity) at similar concentrations. Effect of EDTA was also investigated on the activity of CAD. EDTA up to 2 mM was found to activate the enzyme and beyond that, it exhibited inhibition. At 2 mM EDTA concentration, about 35 % increase in the enzyme activity was observed. The EDTA in combination with Mg^{2+} at equimolar concentration (2 mM each) showed no effect on CAD activity (Fig. 3a). The inhibition of CAD by Zn^{2+} and activation by EDTA was quite unusual. Therefore, we have further investigated the effect of Zn^{2+}/EDTA on the activity of CAD by performing in-gel activity staining as well as native PAGE analysis of the protein. Data are shown in Fig. 3b. It is noteworthy that Zn^{2+} treatment led to the formation of a bigger oligomer of CAD which was catalytically inactive. EDTA treatment prevented the formation of the bigger CAD oligomer and hence increased the CAD activity (Fig. 3b). In case of CAD of *H. pylori*, Zn^{2+} has been shown to have dual roles, namely, structural and catalytic [11]. Similarly, two Zn^{2+} atoms per subunit have been reported by Valencia et al. [17] in a dimeric alcohol dehydrogenase from *S. cerevisiae* (ScAdh6P), which belong to medium-chain dehydrogenase/reductase (MDR) superfamily, of which Zn^{2+}-containing cinnamyl alcohol dehydrogenases are members.

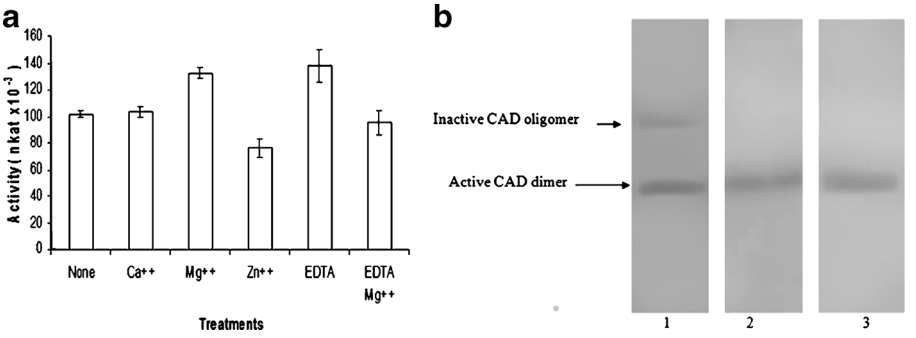


Fig. 3 **a** Effect of divalent cations and EDTA on the activity of CAD. *Error bars* indicate standard deviation from the mean value. **b** *Gels 1 and 3* indicate Coomassie Brilliant Blue staining of purified CAD in the presence of 2 mM Zn^{2+} and EDTA, respectively. *Gel 2* indicates in-gel activity staining of CAD (with 2 mM Zn^{2+})

A recombinant CAD from *L. leucocephala* has been shown to possess catalytic Zn^{2+}-binding domain closer to substrate-binding pocket and structural Zn^{2+}-binding domain bulging out from major lobe [25]. Moreover, Zn^{2+}-mediated oligomerization has been reported by Banfield et al. [18] in NADP(H)-dependent reductase of *B. argentifolii*, where the structural Zn^{2+}-binding loops have been shown to be involved in oligomer formation. The formation of higher molecular weight oligomer of CAD has also been reported from *H. pylori* in the absence of DTT [11].

Effect of Phenolics

Effect of caffeic acid, ferulic acid, and salicylic acid (SA) on the activity of this CAD was investigated. Caffeic acid and ferulic acid both directly inhibited the enzyme activity in a concentration-dependent manner (at 2 mM concentration 54 and 42 % inhibition, respectively). However, salicylic acid did not exhibit any inhibition at all. Insignificant changes in CAD activity, when subjected to SA treatment (in vitro), indicated that altered lignification in response to SA treatment via SAR [26] in general and any possible change in CAD expression in particular may not be due to antagonistic or synergistic effect of SA on existing CAD.

MALDI-TOF Analysis

Purified CAD was subjected to MALDI-TOF analysis. Data are presented in Table 3. MALDI-MS analysis of the purified protein, using the GPS software v3.5 (Applied Biosystems, USA), on NCBI indicated no significant homology with the reported CADs, though it showed some similarity with peptide mass fingerprints of alcohol dehydrogenase (*Populus alba*, *Populus nigra*, and *Populus tremula*) and monodehydroascorbate reductase (*Cucumis sativus*). It also showed some similarity with a short-chain dehydrogenase/reductase of *Solanum tuberosum* (Table. 3). The purified CAD cross-reacted with a polyclonal antibody against a CAD from *L. leucocephala* (data not shown). This observation supported that the purified protein is a CAD. Furthermore, in *Arabidopsis*, at least 17 genes have been annotated as CAD [16]. Out of these, some are still lacking the physicochemical characterization at protein level. In view of a large gene family for CAD as reported in *Arabidopsis*, it may be possible that the present CAD of *Leucaena* may be a new one, not yet characterized. Its similarity to prokaryotic (*H. pylori*) and lower eukaryotic alcohol dehydrogenase (*S. cerevisiae*) along with its ability to catalyze conversion of cinnamyl aldehydes to corresponding alcohol further adds to its importance. Since this enzyme works well with all the hydroxycinnamyl alcohol and aldehydes tested, this

Table 3 Result of MALDI-TOF analysis of purified CAD from *L. leucocephala* showing similarity as searched on NCBI

Serial no.	Enzyme	Accession no.	Molecular weight	Peptide	Scores
1	Alcohol dehydrogenase 1 (*Populus tremula*)	gi|33945797	22,987.7207	5	44.1
2	Alcohol dehydrogenase 1 (*Populus tremula*)	gi|33945809	22,959.7207	5	44.1
3	Alcohol dehydrogenase 1 (*Populus alba*)	gi|33945837	22,945.71094	5	44.1
4	Alcohol dehydrogenase 1 (*Populus nigra*)	gi|33945861	22,959.75977	5	44.1
5	Short-chain dehydrogenase/reductase (*Solanum tuberosum*)	gi|50346893	29,630.96094	4	38.1

can be a better target for silencing. It is worth mentioning here that many enzymes specific to substrates (SAD) have not proved to be good targets for lignin alteration [24]. Further exploration of this CAD, at gene level, along with silencing is required to support the assumed role of this enzyme in lignin biosynthesis and enzyme diversity in the monolignol biosynthesis in woody plants.

Conclusion

A homodimeric CAD was purified from *Leucaena*, a tree legume of importance to paper industry. EDTA activated this CAD by preventing zinc-mediated formation of inactive oligomer. The *Leucaena* CAD catalyzed the physiological reaction efficiently; its peptide mass analysis by MALDI-TOF shows homology to alcohol dehydrogenases. Based on the present data, *Leucaena* CAD seems to be unique and interesting. Further characterization of this CAD at gene and knockout levels is needed.

Acknowledgments The financial support from Council of Scientific and Industrial Research, New Delhi, is gratefully acknowledged. We are also thankful, for the assistance, to Department of Higher Education, Government of Uttar Pradesh, under the Center of Excellence in Bioinformatics program; Department of Biotechnology (DBT), Government of India, New Delhi, under the BIF program; and Department of Science and Technology under Promotion of University Research and Scientific Excellence (DST-PURSE) program.

References

1. Kaur, H., Shaker, K., Heinzel, N., Ralph, J., Galis, I., & Baldwin, I. T. (2012). *Plant Physiology, 159*, 1545–1570.
2. Mansell, R. L., Gross, G. G., Stoeckigt, J., Franke, H., & Zenk, M. H. (1974). *Phytochemistry, 13*, 2427–2436.
3. Saballos, A., Ejeta, G., Sanchez, E., Kang, C., & Vermerris, W. (2009). *Genetics, 181*, 783–795.
4. Boudet, A. (2000). *Plant Physiology and Biochemistry, 38*, 81–96.
5. Weng, J. K., & Chapple, C. (2010). *New Phytologist, 187*, 273–285.
6. Wyrambik, D., & Grisebach, H. (1975). *European Journal of Biochemistry, 59*, 9–15.
7. Halpin, C., Knight, M. E., Grima-Pettenati, J., Goffner, D., Boudet, A., & Schuch, W. (1992). *Plant Physiology, 98*, 12–16.
8. Hawkins, S. W., & Boudet, A. M. (1994). *Plant Physiology, 104*, 75–84.
9. Pillonel, C., Hunziker, P., & Binder, A. (1992). *Journal of Experimental Botany, 43*, 299–305.
10. O'Malley, D. M., Porter, S., & Sederoff, R. R. (1992). *Plant Physiology, 98*, 1364–1371.
11. Mee, B., Kelleher, D., Frias, J., Malone, R., Tipton, K. F., Henehan, G. T. M., & Windle, H. J. (2005). *FEBS Journal, 272*, 1255–1264.
12. Kutsuki, H., Shimada, M., & Higuchi, T. (1982). *Phytochemistry, 21*, 19–23.
13. Li, L., Cheng, X. F., Leshkevich, J., Umezawa, T., Harding, S. A., & Chiang, V. L. (2001). *Plant Cell, 13*, 1567–1585.
14. Luderitz, T., & Grisebach, H. (1981). *European Journal of Biochemistry, 119*, 115–124.
15. Boerjan, W., Ralph, J., & Baucher, M. (2003). *Annual Review of Plant Biology, 54*, 519–546.
16. Kim, S. J., Kim, M. R., Bedgar, D. L., Moinuddin, S. G. A., Cardenas, C. L., Davin, L. B., Kang, C., & Lewis, N. G. (2004). *Proceedings of the National Academy of Science, 101*, 1455–1460.
17. Valencia, E., Larroy, C., Ochoa, W. F., Parés, X., Fita, I., & Biosca, J. A. (2004). *Journal of Molecular Biology, 341*, 1049–1062.
18. Banfield, M. J., Salvucci, M. E., Baker, E. N., & Smith, C. A. (2001). *Journal of Molecular Biology, 306*, 239–250.
19. Rastogi, S. & Dwivedi, U.N. (2003), In: Plant genetic engineering: applications and limitations. (Singh R. P. & Jaiwal, P. K., Eds.) Sci. Tech. Pubs., Houston, U.S.A., pp. 317-360.
20. Rastogi, S., & Dwivedi, U. N. (2006). *Biotechnology Progress, 22*, 609–616.

21. Campbell, M. M., & Ellis, B. E. (1992). *Planta, 186*, 409–417.
22. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
23. Loulakakis, & Roubelakis-Angelakis (1996). *Physiologia Plantarum, 96*, 29–35.
24. Barakate, A., Stephens, J., Goldie, A., Hunter, W. N., Marshall, D., Hancock, R. D., Lapierre, C., Morreel, K., Boerjan, W., & Halpin, C. (2011). *Plant Cell, 23*, 4492–4506.
25. Pandey, B., Pandey, V. P., & Dwivedi, U. N. (2011). *Protein Expression and Purification, 79*, 197–203.
26. Lee, D., Ellard, M., Wanner, L. A., Davis, K. R., & Douglas, C. J. (1995). *Plant Molecular Biology, 28*, 871–884.