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Purification and characterization of cinnamyl alcohol-NADPH-dehydrogenase from the leaf tissues of a basin mangrove *Lumnitzera racemosa* Willd.

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Cinnamyl alcohol-NADPH-dehydrogenase (CAD), the marker enzyme of lignin biosynthesis was purified from the leaf tissues of a basin mangrove *Lumnitzera racemosa* by ammonium sulphate precipitation, followed by anion-exchange, gel filtration and affinity chromatography. The molecular mass of the CAD enzyme was determined as 89 kDa, by size elution chromatography. SDS-PAGE of CAD revealed two closely associated bands of 45 kDa and 42 kDa as heterogenous subunits. The optimum *p*H of CAD was found to be 4.0. K_m for the substrates cinnamaldehyde, coniferaldehyde and sinapaldehyde was determined. Cinnamaldehyde showed higher K_m value than sinapaldehyde and coniferaldehyde. The correlation of activity of CAD with the amount of lignin was found less significant in *L. racemosa,* compared to plant species of other habitats viz., mesophytes, xerophytes and hydrophytes, suggesting that CAD possibly exhibits physiological suppression due to the saline habitat of the plant.

Keywords: *Lumnitzera racemosa*, cinnamyl alcohol-NADPH-dehydrogenase**,** basin mangrove.

Cinnamyl alcohol-NADPH-dehydrogenase (CAD, EC1.1.1.195) has attracted considerable interest, because of its specific role in lignin synthesis[1]. It is a major enzyme of phenylpropanoid metabolism, which catalyzes the last step of monolignol synthesis by reduction of hydroxycinnamyl aldehydes to the corresponding hydroxycinnamyl alcohols[2,3]. Its catalytic property in reducing the cinnamaldehydes viz., sinapaldehyde, coumaraldehyde and coniferal-dehyde to the corresponding cinnamyl alcohols, the direct monomeric precursors of lignin polymer in tobacco, maize, poplar, eucalyptus and oil palm is well established[4-8]. However, CAD is expressed in cells that do not make lignin. Also, it expresses higher activity in response to stress, pathogenic elicitors and wounding. Thus, it is regulated by both development and environmental pathways, like phenylalanine ammonia lyase (PAL), peroxidase (POD), the enzymes of phenylpropanoid metabolism[9,10]. CAD is found to be the potential target enzyme for modulating the quality and quantity of commercially important forest trees through genetic engineering[11,12].

Lumnitzera racemosa, a mangrove has shown a luxuriant growth in certain pockets of coastal belts of Kerala. As a basin or interior mangrove, it is characterized by infrequent tidal action, varied salinity and low water turbulence[13]. Hence, the salinity varies in the habitat of the plant. Previous reports on *L. racemosa* indicate its terrestrial adaptability by correlating the activities of PAL, CAD and POD with other taxa of basin mangroves during lignification[14,15]. CAD exhibits higher rate of activity in all the mangrove taxa. The remarkable variation of CAD activity in mangroves compared to land plants, such as mesophytes and xerophytes was interpreted as the effect of the saline habitat[15]. The present study has been an attempt to purify and characterize the enzyme from leaf tissues of *L. racemosa* Willd.

Materials and Methods

DEAE-cellulose, Sephacryl S 200, Blue Sepharose, cinnamaldehyde, coniferaldehyde, sinapaldehyde and dehydroconiferyl alcohol were obtained from Sigma Chemical Co., St. Louis, MO, USA. Acrylamide, bis acrylamide, Coomassie brilliant blue R-250, sodium dodecylsulphate (SDS), polyethylene glycol (PEG) and TEMED were from SD Fine Chemicals, India. Other chemicals used were of highest purity.

Tender and mature leaves of *Lumnitzera racemosa* were used. As control, selected plants from mesophytes: *Murraya exotica, Tecoma stans, Hamelia patens, Quassia amara, Ixora coccinea*;

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hydrophytes: Eichhornia crassipes, *Nymphaea stellata*, *Limnophila heterophylla*, *Limnanthemum cristatum*, *Hydrilla verticillata* and xerophytes: *Muehlenbeckia platyclados*, *Euphorbia tirucalli*, *Opuntia dillenii*, *Ruscus aculeatus* and *Casuarina equisetifolia* were used.

The frozen tissue was homogenized, using the extraction medium containing 0.1 *M* Tris-HCl buffer, 20 m*M* 2-mercaptoethanol and 0.5% PEG at 4°C[5,16]. The homogenate was centrifuged at 20,000 *g* for 20 min under refrigerated condition and the supernatant was used as the enzyme source for further purification.

Activity of CAD was determined by the method described earlier[16]. The oxidation of NADPH caused a linear decrease in absorbance, which was recorded spectrophotometrically at 340 nm initially and after 15 min of incubation at 30°C. One unit of CAD is equivalent to μmoles of NADPH oxidized, causing the linear decrease in absorbance per 15 min at 30°C. All the purification steps were carried out at 4°C, except for Blue Sepharose affinity chromatography[11] and the crude enzyme was then fractionated by $(NH_4)_2SO_4$ precipitation at 85% saturation. The enzyme protein precipitated was centrifuged at 20,000 *g* for 20 min. The resuspended $(NH_4)_2SO_4$ precipitate was dialyzed against 20 m*M* Tris-HCl (*p*H 7.6), 10 m*M* mercaptoethanol and 0.25% PEG for 24 hr. The dialysate was loaded on a DEAE-cellulose column and the column was eluted (3 ml fractions) with linear gradient of 20 to 400 m*M* Tris-HCl (*p*H 7.6), 10 m*M* mercaptoethanol and 0.25% PEG at a flow rate 1 ml/min.

The concentrated fractions from DEAE-cellulose column were then loaded on a 50 ml column of Sephacryl S 200 and the column was eluted with 10 m*M* Tris-HCl buffer (*p*H 7.6), 10 m*M* mercaptoethanol and 0.25% PEG. Fractions from Sephacryl S 200 were loaded on to a Blue Sepharose column for affinity chromatography. The column was eluted with a linear gradient of 5 to 80 μ*M* NADP in 10 m*M* Tris.HCl (*p*H 7.6), 10 m*M* mercaptoethanol and 0.25% PEG and fractions (5 ml) were collected, using a flow rate 0.5 ml/min. The change in absorbance at 280nm was used to monitor the yield.

The purity was checked by HPLC reverse phase C-4 column, following the procedure described earlier[11]. The polypeptide pattern of purified enzyme after affinity chromatography was studied by SDS-PAGE using 10% polyacrylamide gel[17]. The purified enzyme fraction was subjected to native PAGE and the activity was localized in the gel by nitro blue tetrazolium salt method[17,18]. The molecular mass of purified fraction was determined by gel filtration column, using bovine serum albumin (68 kDa), ova albumin (43 kDa), ATPase (100 kDa) and lysozyme (14.3 kDa) as standards. The void volume was determined by the elution profile of blue dextran.

The optimum *p*H of CAD activity was determined by assaying the enzyme at a range of *p*H 2.0 to 10.0. The enzyme CAD was assayed for substrate specificity using cinnamaldehyde, coniferaldehyde and sinapaldehyde at varying concentrations. The K_m value was determined by the Line weaver-Burk plot.

Protein content of the sample at each stage of purification was determined by Bradford method[19]. BSA was used as the standard. Lignin was isolated by acetyl bromide method and quantified spectrophotometrically at 280 nm[20]. Dehydroconiferyl alcohol polymerizate was used as standard.

Results and Discussion

Purification of CAD

The crude extract of CAD showed a specific activity of 4.2 units/mg protein, as determined by the assay method. After $(NH_4)_2SO_4$ precipitation and desalting, the enzyme exhibited a specific activity of 19 units/mg proteins. The enzyme was further purified to apparent homogeneity by DEAE-cellulose, Sephacryl S 200 and blue Sepharose chromatography (Table 1). The enzyme precipitate was passed through DEAE-cellulose column and eluted with Tris-HCl buffer, *p*H 7.6 (Fig. 1a and b). The concentrated enzyme fraction was further purified by Sephacryl S 200 and the elution profile showed a single major peak in the 9th fraction with specific activity 598 units (Fig. 1 c and d). The degree of purification was further increased to near homogeneity by dye affinity chromatography. Fig. 1e and f show the elution profile of CAD from blue Sepharose affinity chromatography and the enzyme was eluted with NADP gradient of 5 to 80 μ*M*. Thus, the protocol yielded a purified CAD with specific activity 2775 units, with a low protein content of 80 μg. Overall, the specific activity increased about 661-fold with 11.4% yield of activity. The purity of protein was confirmed by reverse phase-HPLC chromatogram, showing a single peak (Fig. 1g).

Table 1—Purification profile of CAD from leaves of *Lumnitzera racemosa*

[The per cent yield, specific activity and fold of purification represent the different stages of purification from crude extract, $(NH_4)_2 SO_4$ (85%), DEAE-cellulose (linear gradient of 20-40 m*M*, Tris.HCl (*p*H 7.6) gel filtration (10 m*M* Tris.HCl, *p*H 7.6) and affinity chromatography 5-80 μ*M* NADP in 10 m*M* Tris-HCl, *p*H 7.6)]

Purification steps	Enzyme activity (Units)	Yield (%)	Total protein (mg)	Specific activity (Units/mg)	Fold purification
Crude extract	1946	100	468	4.2	1
$(NH_4)_2 SO_4$ (85%)	1410	72.5	74	19	4.5
DEAE-cellulose	987	51	5.2	190	45
Sephacryl S-200	323	16.6	0.54	598	142
Blue Sepharose	222	11.4	0.08	2775	661

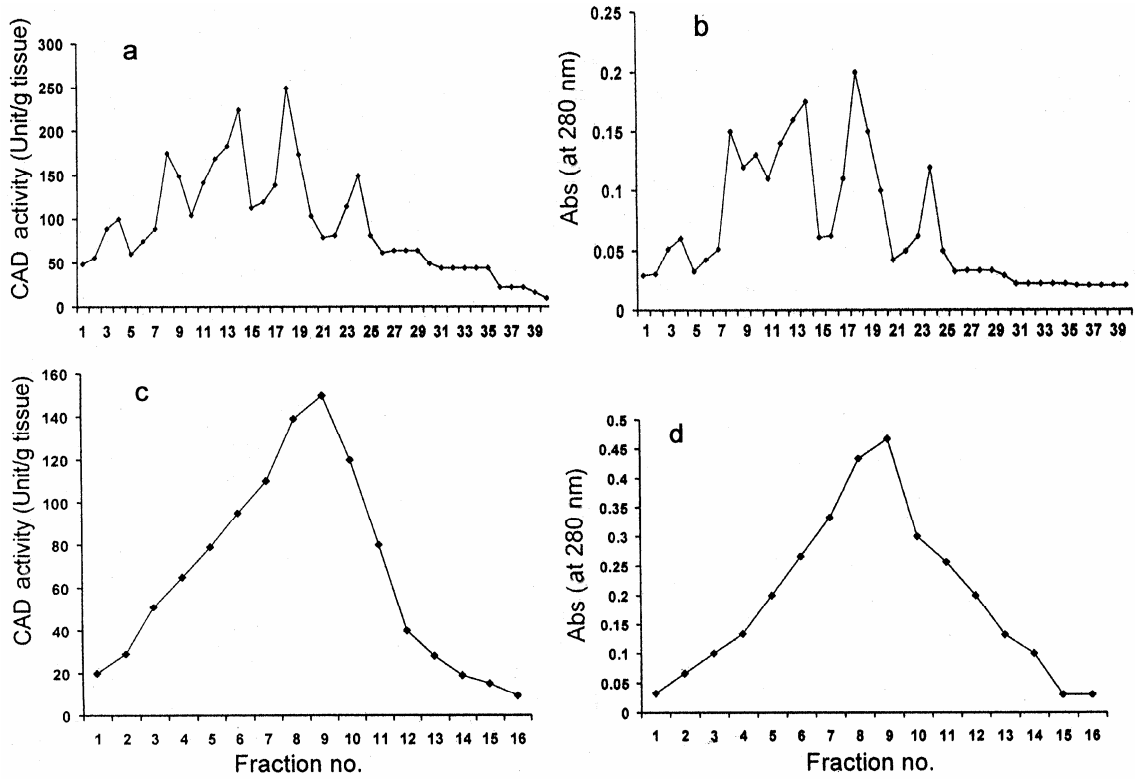

Fig. 1—(**a** & **b**): Purification of CAD by DEAE-cellulose chromatography and elution by Tris-HCl buffer gradient 20 to 400 m*M* (*p*H 7.6) at a flow rate of 1 ml/min slurry of DEAE-cellulose (1 g/100 ml) was prepared in 0.05 *M* Tris HCl buffer *p*H 6.8, 5 m*M* mercaptoethanol and 0.25% PEG, at 4°C. Each fraction was assayed for CAD and protein content was monitored at 280 nm; (**c** & **d**): Elution profile of CAD from gel filtration column (Sephacryl S-200) eluted by Tris buffer (10 m*M*, *p*H 7.6) at a flow rate 1.2 ml/min Sephacryl S-200 was equilibrated with 10 m*M* Tris-HCl buffer *p*H 7.6, 10 m*M* mercaptoethanol and 0.25% PEG, at 4°C, change in absorbancy at 280 nm was used to monitor the elution of bound protein

Physical properties

The molecular mass of CAD was determined by size-elution chromatography. The apparent molecular mass of the purified CAD is estimated as 89 kDa from the markers. The molecular mass of native CAD is roughly the sum of 45 kDa and 43 kDa, estimated for the denatured CAD by SDS-PAGE (Fig. 2). The band pattern of SDS-PAGE indicates that CAD enzyme of *L. racemosa* is composed of two heterogenous subunits of very similar molecular mass. The heterodimer nature of CAD of *L. racemosa* was similar to *Eucalyptus* CAD[7,21] and differs from CADs isolated from soyabean, spruce and poplar, which are homodimers[15,22,23].The activity of CAD was checked by native gel assay. The thick band indicates the localization site of CAD activity (Fig. 3).

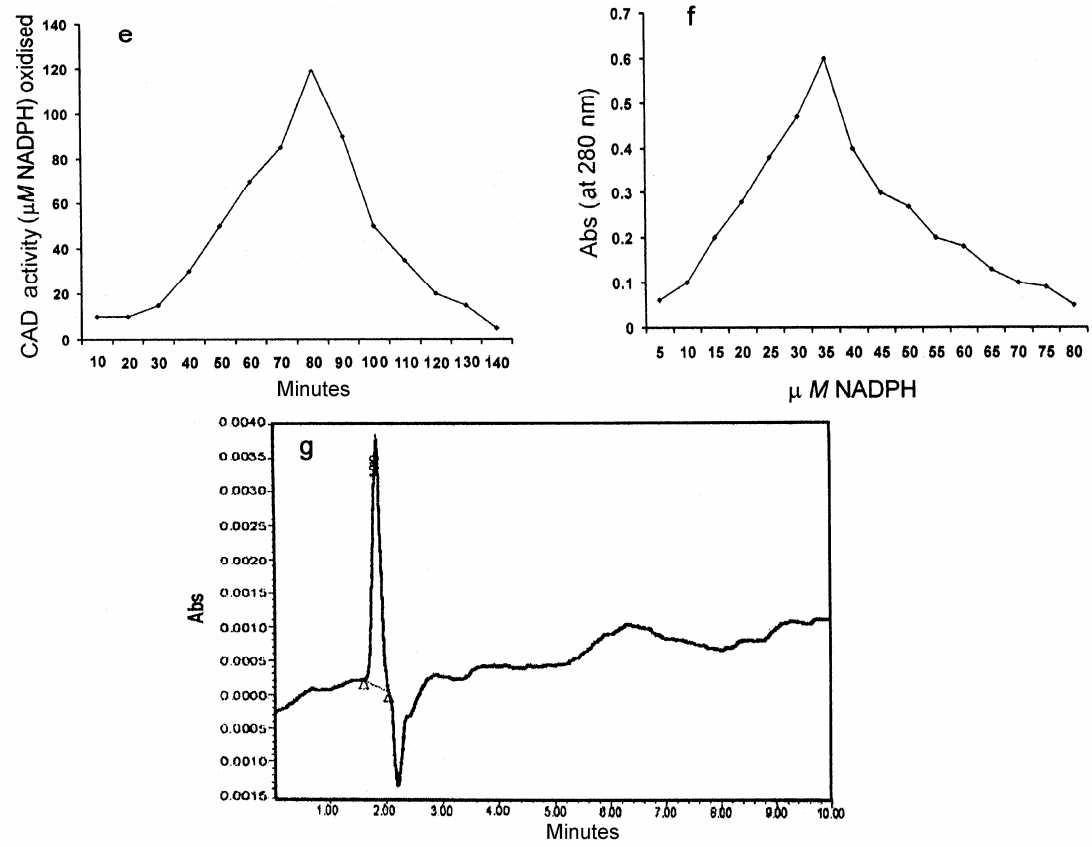

Fig. 1—(**e** & **f**): Elution profile of CAD from blue Sepharose affinity chromatography [The enzyme was eluted with NADP gradient of 5 to 80 μ*M* NADP Tris-buffer (*p*H 7.6) at a flow rate of 0.5 ml/min at room temperature, change in absorbency at 280 nm was used to monitor the elution of bound protein]; (**g**): Chromatogram showing the peak of CAD. [Rp-HPLC with C- 4 column eluted using a linear gradient of acetonitrile in 0.5% elution time was 50 min at a rate of 1 ml/ min.The peak indicates the homogeneity of the enzyme protein]

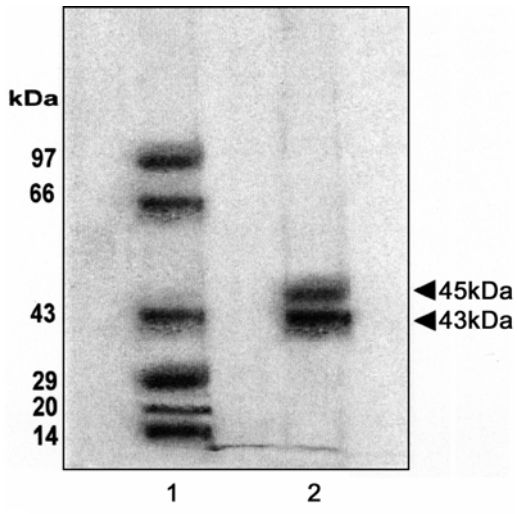

Fig. 2—SDS-PAGE of the purified protein stained with Coomassie brilliant blue [The concentration of acrylamide was 10% for separating gel and 4% for stacking gel. Lane 1, SDS marker; and lane 2, CAD protein after affinity chromatography composed of 2 heterogenous subunits of similar molecular mass 45 kDa and 43 kDa)

Effect of *p*H

The effect of *p*H on CAD activity is shown in Fig. 4. The optimum *p*H of CAD is found to be 4, which is different from that reported for the enzyme from loblolly pine and soyabean[11,16]; CAD is also reasonably stable over a broad range of *p*H 3-7. In earlier lignification studies on land plants, the enzyme CAD was routinely assayed, using the reduction reaction by converting cinnamaldehyde to cinnamyl alcohol at an optimum *p*H 7.6[5,11,16] . By oxidation reaction using coniferyl alcohol as substrate, the *p*H optimum was reported as approx. 8.8[2]. Thus, the optimal activity of CAD at *p*H 4 in *L. racemosa* indicates functional diversion of the enzyme from alkaline to an acidic range (Fig. 4). The affinity of the enzyme towards acidic range can be correlated with *in vivo p*H 4.5 of leaf tissue. Previous studies on the role of CAD in lignin synthesis indicate the *p*H optimum as 7.6 to 8.8[24-26]. Since the *p*H optimum of CAD in the alkaline range indicates the active phase of the lignin synthesis, the change in *p*H optimum of CAD in

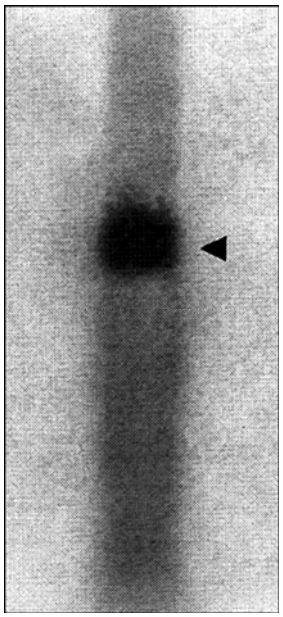

Fig. 3—NATIVE-PAGE of CAD showing the activity in the gel by tetrazolium salt method [Incubation medium contained nitro BT 40 mg, dimethyl foramide 200 µl, 0.5 *M* NADPH 8.4 mg, menadione 3.5 mg, 0.1 *M* phosphate buffer *p*H 7.45. The arrow indicates the localization site of CAD activity]

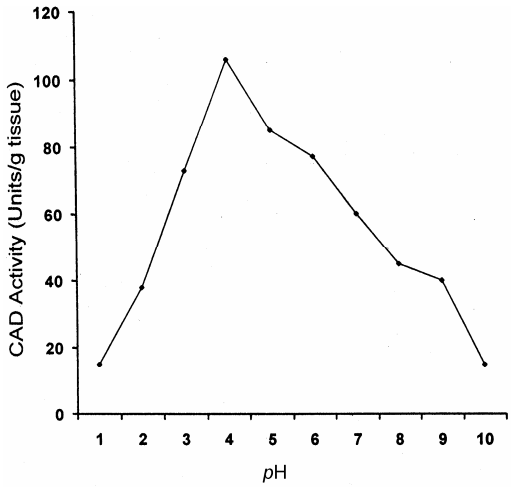

Fig. 4—Effect of *p*H on CAD of *L. racemosa* optimum activity of CAD is at *p*H 4 [Activity was measured at *p*H 2-10 assayed using the reduction reaction of cinnamaldehyde to cinnamyl alcohol optimum activity is at *p*H 4]

L. racemosa suggests the physiological suppression of the enzyme[11].

Correlation of CAD and lignin

The *in vitro* activity of the CAD was checked by quantifying the end product lignin. Lignin content of *L. racemosa* was found to be negligible (69 µg/g tissue), in contrast with the high level of enzyme activity (35.81 units/g tissue). This physiological correlation of CAD with lignin content was further studied in plant species of varied habitat such as *Murraya exotica, Tecoma stans, Hamelia patens, Quassia amara, Ixora coccinea* (mesophytes); *Eichhornia crassipes, Nymphaea stellata, Limnophila heterophylla, Limnanthemum cristatum, Hydrilla verticillata* (hydrophytes); *Muehlenbeckia platyclados, Euphorbia tirucalli, Opuntia dillenii, Ruscus aculeatus, Casuarina equisetifolia* (xerophytes); *Lumnitzera racemosa, Avicennia alba, Rhizophora apiculata, Bruguiera cylindrica,* and *Acanthus ilicifolius* (mangroves). Five species from each group were selected and each value was a mean of 20 replicates. Significant CAD activity is found in the plant species of mangroves, mesophytes and xerophytes except hydrophytes, indicating adaptability of the plants towards terrestrial habitat. Lignin content shows significant variation among various groups and high lignin content observed in the case of xerophytes can be reasonably interpreted as the xeric adaptation. Lignin content in hydrophytes very well matches with low CAD activity. In mangroves, the lignin content did not express a positive correlation with the CAD activity, suggesting the eco-physiological impact of the habitat (Table 2).

Table 2—Mean activity of CAD and lignin content of different plant groups

[Five species from mangroves, mesophytes, xerophytes and hydrophytes were selected for quantifying CAD activity and lignin content. For assay of CAD 50 µl 20 m*M* Zn SO_4, 1.15 ml 0.1 *M* KH_2PO_4 buffer *p*H 7.6, 600 µl 1 m*M* NADPH, 600 µl enzyme extract and 60 µl 1 m*M* cinnamaldehyde record changes in absorbancy with time at 15 min of incubation at 30°C and for lignin, known dry tissue with 3 ml 25% acetyl bromide in acetic acid at 70°C in water bath for 30 min, cool add 0.9 ml 2*N* NaOH, 0.1 ml 7.5 *M* hydroxylamine hydrochloride diluted with 1% acetic acid read absorbency at 280 nm)

Habitat	CAD activity (Units)	Lignin (mg/g tissue)
Mangroves	48.84 ± 0.1822	1.14± 0.2
Mesophytes	72.42 ± 0.1822	4.53 ± 0.2
Hydrophytes	8.77 ± 0.1822	0.072 ± 0.2
Xerophytes	53.16 ± 0.1822	38.76 ± 0.2

Substrate specificity and kinetics

The substrate specificity of the purified CAD was studied, using the primary substrates coniferaldehyde, cinnamaldehyde and sinapaldehyde. CAD showed significant activity towards all the three substrates.

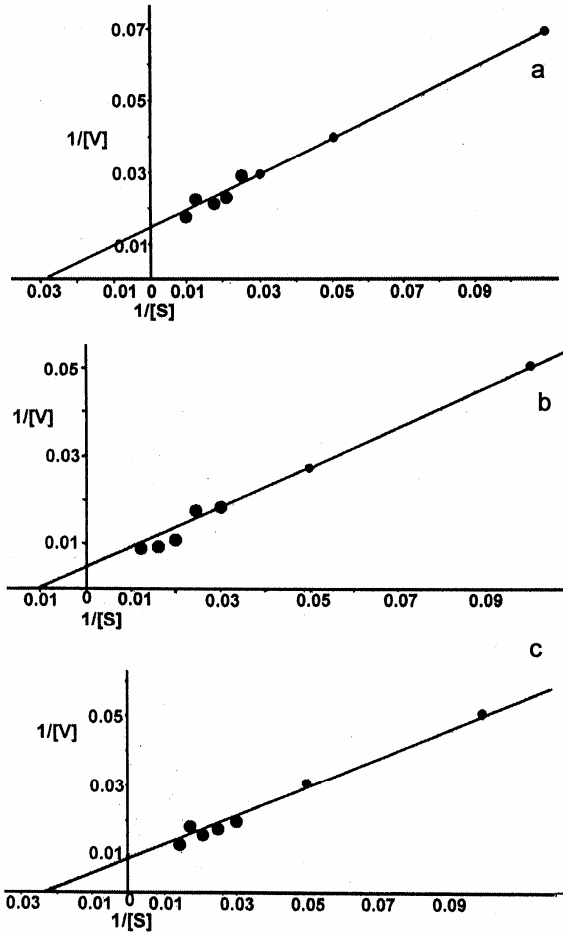

Fig. 5—Determination of K_m value by Line weaver-Burk plot of CAD from *L. racemosa* using different substrate concentrations [(a): coniferaldehyde; (b) cinnamaldehyde; and (c) sinapaldehyde with varying concentrations from 10 μ*M* to 100 μ*M)*

The K_m value was calculated from Line-weaver Burk plot. The K_m value is higher for cinnamaldehyde (59 μ*M*) than sinapaldehyde (40 μ*M*) and coniferaldehyde (33.3 μ*M*) (Fig. 5). The affinity of the enzyme towards NADPH was also determined from K_m value. The K_m for NADPH showed higher affinity towards cinnamaldehyde and coniferaldehyde (100 μ*M*), than sinapaldehyde (58.8 μ*M*). The kinetic data clearly indicates the affinity of *L. racemosa* CAD towards cinnamaldehyde, even though the three substrates are specific for lignin formation in plant system[4,11,27,28].

Conclusion

The results presented herein indicate the physiological suppression of CAD, the key enzyme involved in lignin synthesis in plants. The increased level of CAD activity and the low content of end product lignin suggest the influence of salinity in the growth of basin mangroves.

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