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PURIFICATION AND CHARACTERIZATION OF CINNAMYL ALCOHOL-NADPH-DEHYDROGENASE FROM YOUNG FRUIT TISSUES OF OIL PALM *(ELAEIS GUINEENSIS)* TENERA VARIETY

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Cinnamyl alcohol-NADPH- dehydrogenase (CAD) the key lignifying enzyme in shell synthesis and fibre ring formation in oil palm – *tenera* fruits was extracted and purified by ammonium sulphate precipitation, followed by anion –exchange and gel filtration chromatography. The approximate molecular mass of the enzyme protein was estimated as 75KDa from the polypeptide pattern of denatured enzyme by SDS-PAGE. Two subunits of 40KDa and 35KDa were found as two prominent bands in the SDS - PAGE. The zymogram by native PAGE revealed two isozyme bands. The optimum pH was found to be 7.6. The purified enzyme expressed high specific activity (948.89 units/mg protein) and 10.68% recovery with a purification fold of 94.8. The *K*m values for the substrates cinnamaldehyde and NADPH were estimated as 430 µM and 425 µM respectively. The high *K*m value for the substrate cinnamaldehyde clearly indicates the active role of CAD in shell synthesis and fibre ring formation in oil palm *(Elaeis guineensis)* fruits *tenera* variety. The role of cinnamic acid (CA) as a precursor of lignin synthesis during fibre ring and shell formation was analyzed under RP- HPLC. It could be noticed that the concentration of CA and lignin formation during the development of the pericarp exhibits reciprocal relationship.

Keywords: Cinnamic acid; Cinnamyl alcohol-NADPH-dehydrogenase; *Elaeis guineensis*; Isozyme; Lignin; Purification; Shell synthesis.

Introduction

Cinnamyl alcohol-NADPH-dehydrogenase has attracted considerable interest for decades because of its specific role in lignin synthesis by reducing hydroxy cinnamaldehyde to hydroxy cinnamyl alcohols (CAD, EC1.1.1.195). This enzyme has been considered as an indicator of lignin biosynthesis because of its specific role at the end of monolignol biosynthetic pathway[1]. The catalytic property of this enzyme in reducing the three cinnamaldehydes viz sinapaldehyde, p – coumaraldehyde and coniferaldehyde to the corresponding cinnamyl alcohols, the direct monomeric precursors of lignin polymer has been well established in Tobacco, Maize, Poplar, Eucalyptus and Oil palm-*dura* and *tenera* by biochemical studies[2-7]. Hence it is a potential target enzyme for biotechnology directed towards modulating the quality and quantity of lignin in plants[8]. Polymerization of cinnamyl alcohol to lignin, the last step of lignin synthesis has been attributed to two different classes of enzymes such as peroxidase (POD) and laccases.

Oil palm (*Elaeis guineensis* jacq.) is the most productive tropical oleaginous crop in the world. Under this species there are three varieties – *dura, pisifera* and *tenera*. Dura is thick shelled, tenera is thin shelled and pisifera is shell less. However, confusion may arise in some cases of dura and tenera where fruits possess thin and thick shells respectively. The alternative method to identify tenera from such thin shelled dura is the presence of a fibre ring in the mesocarp around the shell. The role of cinnamyl alcohol – NADPH – dehydrogenase (CAD) and other enzymes such as phenylalanine ammonia lyase (PAL) and peroxidase (POD) during shell synthesis and fibre ring formation in tenera variety has already been reported[9,10]. So the objective of the present study is to purify and characterize the enzyme CAD from the fruits of tenera variety.

Materials and Methods

Plant material: Healthy *tenera* palms were identified from the experimental plantations of National Research Centre for oil palm, Regional station, Palode, Thiruvananthapuram. Fruit samples from different palms were collected separately at 4 weeks after pollination (WAP) and pooled for the study.

Chemicals used: DEAE- Cellulose, Sephacryl S-200 and Cinnamic acid were obtained from Sigma Chemicals Co., St. Louis, MO, USA. Acrylamide, bis acrylamide, coommassie brilliant blue R-250, Sodium dodecyl sulphate (SDS), poly

ethylene glycol (PEG) and TEMED were from SD Fine chemicals, India.

Quantification of lignin: The lignin in the fruit tissue was isolated by acetyl bromide method and estimated spectrophotometrically at 280 nm using the internal standard dehydroxy coniferyl alcohol[11].

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) analysis of soluble phenols: The different phenolic acids in the fruit were separated by RP – HPLC and quantified using internal standards[12].

Isolation and assay of CAD: Frozen fruit tissue was homogenized in 0.1 M Tris HCl buffer containing 20mM 2-mercapto ethanol and 0.5% polyethylene glycol (PEG) at pH 7.6. The homogenate was filtered and centrifuged at 16000 rpm for 20 min. The supernatant was used as a source enzyme for purification. The activity of CAD was determined by reacting the enzyme with cinnamaldehyde to produce cinnamyl alcohol via oxidation of NADPH[13]. One unit of CAD is equivalent to one micromole of NADPH oxidized causing a linear decrease in absorbance per 15 min at 30°C.

Purification and characterization of CAD: The crude enzyme was subjected to $(NH_4)_2 SO_4$ precipitations and fractionated at 85% saturation. The precipitated enzyme protein was resuspended and dialyzed against 20mM Tris HCl (pH 7.6), 10mM mercapto ethanol and 0.25% PEG for 24hr. The dialysate was loaded on a 50ml DEAE cellulose column (anionic exchanger) and eluted (3ml fractions) with linear gradient of 20-100 mM Tris- HCl (pH 7.6); 10mM mercaptoethanol and 0.25% PEG at a flow rate of 1ml / min[8]. After screening for protein and enzyme activities, fractions rich in enzyme activity were pooled together and loaded on a 50ml column of Sephacryl S-200 and were eluted with the elution buffer Tris-HCl (pH 7.6). Fractions of 3ml each were collected using a flow rate of 1.2ml / min. The change in absorbance at 280 nm was used to monitor the yield.

The purity and homogeneity of the purified fraction of the enzyme was checked by electrophoresis in native - PAGE and the activity was localized in the gel by nitro blue tetrazolium salt method. A zymogram was prepared by incubating the gel in assay medium for 30 minutes in darkness[14]. The molecular mass of the enzyme was determined using Sephacryl S 200 column. Bovine serum albumin (68kDa), ova albumin (43kDa), ATPase (100kDa) and lysozyme (14.3kDa) were used as standards. The molecular mass and polypeptide pattern were determined by SDS-PAGE using 10% polyacrylamide gel[13].

The effect of pH on the enzyme activity was studied by using 0.1M KH_2PO_4 / Na_2HPO_4 buffer and cinnamaldehyde as substrate in the pH range of 4.6 to 12.6. Thermal stability of the enzyme protein was determined by assaying CAD using varying range of temperatures (0 - 50°C) in KH_2PO_4/ Na_2HPO_4 buffer (0.1M at pH 7.6).

The effect of substrate concentration on the reaction rate was studied using the substrate cinnamaldehyde and the cofactor NADPH at varying concentrations. The K_m values and V_{max} were determined from the saturation curve at pH 7.6.

Results and Discussion

Purification data: The specific activity of the CAD enzyme in the crude extract was found to be10 units/mg protein (Table 1). After $(NH_4)_2 SO_4$ precipitation and dialysis the enzyme was purified to apparent homogeneity by DEAE-cellulose followed by Sephacryl S-200 chromatography. Figure 1 demonstrates the activity profile of CAD at a range of molar concentrations using the elution buffer Tris-HCl (20 mM to 140 mM) at pH 7.6.The concentrated enzyme was further purified by Sephacryl S-200 and the elution profile showed a single major peak in the 4th fraction with specific activity 948 units (Fig.2) Thus the protocol yielded a purified enzyme with 94.8 fold of purification and 10.6% recovery. A gradual increase in specific activity was noticed at each step of purification procedure and reached the maximum value i.e., 948 units/mg protein at gel filtration (Table 1).

Physical properties

Isoforms of CAD: The purified fraction of CAD was subjected to native -PAGE and a zymogram was prepared by incubating the gel in assay medium for 30 minutes in darkness. Figure 3a represents the isozyme pattern of CAD. The two isozyme bands strongly suggest a dimeric form of CAD.

SDS-PAGE: The apparent molecular mass of the purified enzyme was estimated as 75kDa by gel filtration using known standard markers. In SDS-PAGE two prominent bands were obtained, having individual molecular masses of 40kDa and 35kDa respectively. The band pattern of SDS

Table 1. Purification profile for CAD from Oil palm young fruits.

Purification step	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Recovery (%)	Fold purification
Crude extract	4000	400	10	100	1.0
Ammonium sulphate (35-85%)	2519	61.4	41	62.98	4.0
DEAE Cellulose	1743	7.0	249	43.58	24.9
Sephacryl S-200	427	0.45	948	10.68	94.8

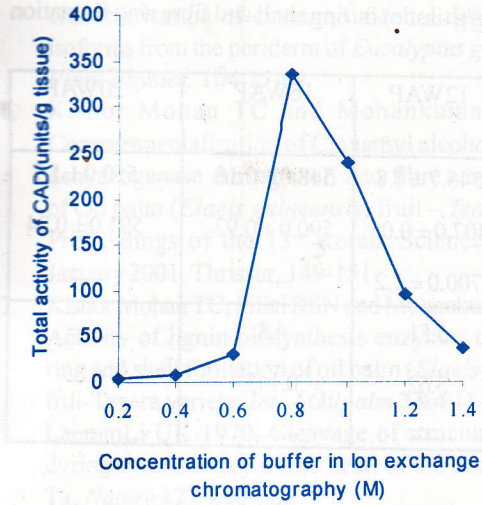


Fig.1. Purification of CAD by DEAE- cellulose chromatography and eluted with Tris HCl buffer gradient 20 to 140 mM at pH 7.6.

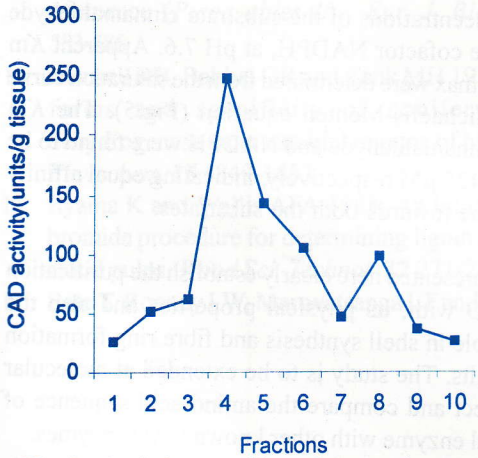


Fig.2. Activity profile of CAD from Gel filtration chromatography showing single peak at 4th fraction.

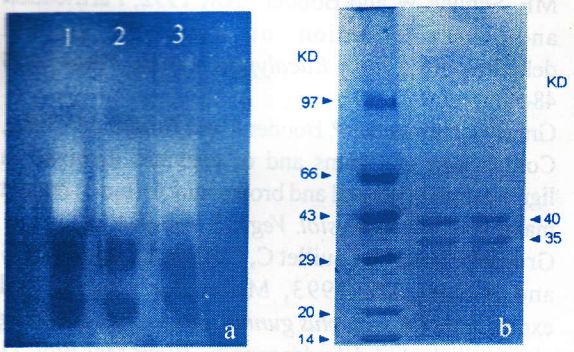


Fig. 3.(a) Zymogram of purified CAD of fruit tissue indicating the isozyme bands by Native PAGE. The bands were identified by Tetrazolium salt method.

(b) The SDS-PAGE showing the polypeptide pattern of purified fraction of CAD separated by Ion exchange and Gel filtration chromatography.

– PAGE indicates that the enzyme CAD is a heterodimer with two subunits α and β (Fig.3b). Recently, the heterodimeric nature of this enzyme has been reported in a basin mangrove *Lumnnitzera racemosa*[15]. The molecular mass of CAD estimated in oil palm fruit was slightly lesser than those reported in loblolly pine, spruce and poplar[8,16, 17].

Effect of pH and temperature: The effect of pH on CAD is shown in figure 4a. The enzyme showed maximum activity at the pH 7.6 with cinnamaldehyde as substrate in 0.1M KH_2PO_4/Na_2HPO_4 buffer. Previous studies on the role of CAD in lignin synthesis indicate the pH optimum as 7.6 to 8.8[18]. Figure 4b represents the residual activity of CAD after thermal incubation. The CAD enzyme showed a maximum activity at 20°C indicating the stability of the enzyme at low temperature.

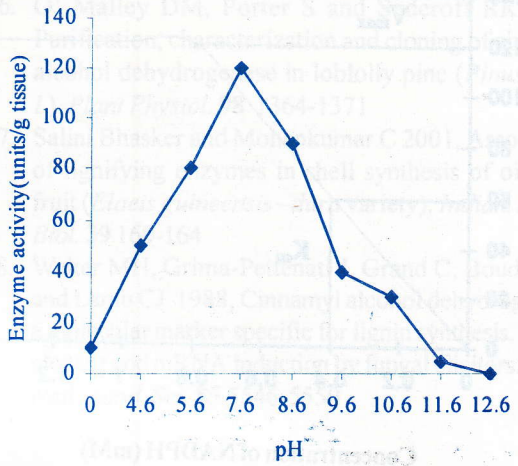
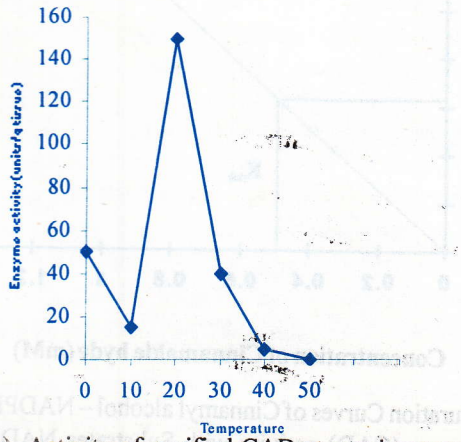


Fig.4.(a) Activity of Cinnamyl alcohol –NADPH-dehydrogenase (CAD) under different pH using cinnamaldehyde as substrate.



(b) Activity of purified CAD at varying temperature (0-50°C) with a single peak at 20°C.

Correlation of CAD with lignin and phenolic acid: The in vitro activity of the enzyme was checked by quantifying the lignin. The lignin content expresses a positive

Table 2. Distribution of Lignin (µg/g tissue) and Cinnamic acid content (µg/g tissue) during shell and fibre ring formation in *Tenera* variety.

Concentration (µg/g tissue)	Fruit tissue	4WAP	8WAP	12WAP	16WAP	20WAP
Lignin	Mes	338.6 ± 1.28	468.2 ± 0.6	546.7 ± 1.8	548.0 ± 0.16	550.0 ± 1.7
	Fr	–	404.0 ± 0.5	407.0 ± 0.05	590.0 ± 0.92	591.0 ± 0.24
	End	342.0 ± 0.37	689.0 ± 0.61	700.0 ± 1.2	—	—
Cinnamic acid	Mes	9.74	8.43	5.51	4.8	—
	Fr	—	0.68	3.62	0.143	—
	End	7.35	0.63	—	—	—

Mes = Mesocarp Fr = Fibre ring End = Endocarp

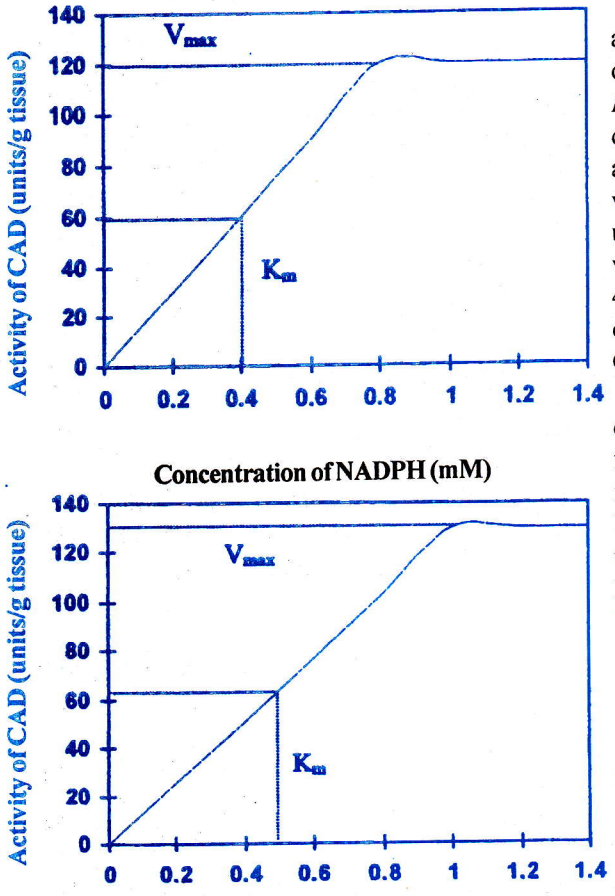


Fig. 5. Saturation Curves of Cinnamyl alcohol – NADPH – dehydrogenase (CAD) activity with Substrates NADPH and Cinnamaldehyde indicating their Vmax and Km values.

correlation with CAD activity confirming its role in shell formation. Distribution of cinnamic acid (CA) and the lignin content during fibre ring and shell formation is presented in table2. The lignin content and the amount of CA showed a negative correlation from 4 to 20WAP confirming the role of CA in lignin biosynthesis[2].

Kinetics of CAD: Kinetic studies were performed with different concentrations of the substrate cinnamaldehyde as well as the cofactor NADPH, at pH 7.6. Apparent *K*m values and V max were determined from the saturation curve using the Michaelis-Menten equation (Fig.5). The *K*m values for cinnamaldehyde and NADPH were found to be 430µM and 425 µM respectively, indicating equal affinity of the enzyme towards both the substrates.

Conclusion

The results presented here clearly establish the purification data of CAD with, its physical properties and also the functional role in shell synthesis and fibre ring formation in tenera fruits. The study is to be extended at molecular level to detect and compare the amino acid sequence of this potential enzyme with other known CAD enzymes.

References

1. Goffner D, Joffroy I, Grima-Pettenati J, Halpin C, Knight ME, Schuch W and Boudet AM 1992, Purification and characterization of Cinnamyl alcohol dehydrogenase from *Eucalyptus* xylum. *Planta* **188** 48-53
2. Grand C, Parmentier P, Boudet A and Boudet AM 1985, Comparison of lignins and of enzymes involved in lignification in normal and brown midrib (bm 3) mutant maize seedlings. *Physiol. Veg.* **23** 905-911
3. Grima-Pettenati J, Feuillet C, Goffner D, Borderies G and Boudet AM 1993, Molecular cloning and expression of *Eucalyptus gunnii* cDNA clone encoding cinnamyl alcohol dehydrogenase. *Plant Mol.Biol.* **21** 1085-1095
4. Halpin C, Knight ME, Grima-Pettenatti J, Goffner D, Boudet A and Schuch W 1992, Purification and characterisation of cinnamyl alcohol dehydrogenase from tobacco stems. *Plant Physiol.* **98** 12-16
5. Hawkins SW and Boudet AM 1994, Purification and

characterisation of cinnamyl alcohol dehydrogenase isoforms from the periderm of *Eucalyptus gunnii* Hook. *Plant Physiol.* **104** 75-84

6. Kishor Mohan TC and Mohankumar C 2001, Compartmentalization of Cinnamyl alcohol-NADPH-dehydrogenase during shell and fibre ring formation of Oil palm (*Elaeis guineensis*) fruit – *Tenera* variety. Proceedings of the 13th Kerala Science Congress, January 2001, Thrissur, 149-151
7. Kishor Mohan TC, Pillai RSN and Mohankumar C 2004, Activity of lignin biosynthesis enzymes during fibre ring and shell formation of oil palm (*Elaeis guineensis*) frui-Tenera variety. *Int. J. Oilpalm* **3&4** 11-15
8. LaemmLi UK 1970, Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. *Nature* **227** 680-685
9. Luderitz T, Schatz G and Grisebach H 1982, Enzymic synthesis of lignin precursors-purification and properties of 4-coumarate CoA ligase from cambial sap of spruce (*Picea abies L*). *Eur. J. Biochem.* **123** 583-586
10. Mansell RB, Babbel GR and Zenk MH 1976, Multiple forms and specificity of coniferyl alcohol dehydrogenase from cambial regions of higher plants. *Phytochem.* **15** 1849-1853
11. Iiyama K and Wallis AFA 1988, An improved acetyl bromide procedure for determining lignin in wood and wood pulps. *Wood Sci. Technol.* **22** 271-280
12. Beta T, Rooney LW, Marovatsanga LT and Taylor JRN 1999, Phenolic compounds and kernel characteristics of Zimbabwean sorghums. *J.Sci. Food Agri.* **79** 1003-1010
13. Morrison TA and Buxton DR 1993, Activity of Phenylalanine ammonia lyase, Tyrosine ammonia lyase and Cinnamyl alcohol dehydrogenase in maize stalk. *Crop Sci.* **33** 1264-1268
14. Morrison TA, Kessler JR, Hatfield RD and Buxton DR 1994, Activity of two lignin biosynthesis enzymes during development of a maize internode. *J.Food Sci.Agric.Tech.* **65** 133-139
15. Murugan K, Arunkumar NS and Mohankumar C 2004, Purification and characterization of cinnamyl alcohol-NADPH-dehydrogenase from the leaf tissues of a basin mangrove *Lumnitzera racemosa* Willd.*Indian J. Biochem.Biophy.* **41** 96-101
16. O' Malley DM, Porter S and Sederoff RR 1992, Purification, characterization and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (*Pinus taeda L*). *Plant Physiol.* **98** 1364-1371
17. Salini Bhasker and Mohankumar C 2001, Association of lignifying enzymes in shell synthesis of oil palm fruit (*Elaeis guineensis –dura* variety). *Indian. J. Exp. Biol.* **39** 160-164
18. Walter MH, Grima-Pettenati J, Grand C, Boudet AM and Lamb CJ 1988, Cinnamyl alcohol dehydrogenase, a molecular marker specific for lignin synthesis. cDNA cloning and mRNA induction by fungal elicitors. *Proc. Natl. Acad. Sci.* **85** 5546-5550