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Short communication

# Probing the active site of cinnamoyl CoA reductase 1 (Ll-CCRH1) from *Leucaena leucocephala*

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ABSTRACT

Lack of three dimensional crystal structure of cinnamoyl CoA reductase (CCR) limits its detailed active site characterization studies. Putative active site residues involved in the substrate/NADPH binding and catalysis for *Leucaena leucocephala* CCR (Ll-CCRH1; GenBank: DQ986907) were identified by amino acid sequence alignment and homology modeling. Putative active site residues and proximal H215 were subjected for site directed mutagenesis, and mutated enzymes were expressed, purified and assayed to confirm their functional roles. Mutagenesis of S136, Y170 and K174 showed complete loss of activity, indicating their pivotal roles in catalysis. Mutant S212G exhibited the catalytic efficiencies less than 10% of wild type, showing its indirect involvement in substrate binding or catalysis. R51G, D77G, F30V and I31N double mutants showed significant changes in $K_m$ values, specifying their roles in substrate binding. Finally, chemical modification and substrate protection studies corroborated the presence Ser, Tyr, Lys, Arg and carboxylate group at the active site of Ll-CCRH1.



## 1. Introduction

Lignin plays a major role in normal growth and development of plants; however, it is considered as an obstacle in number of agro-industrial processes, such as pulping and paper manufacture, forage digestibility and biofuel production. In plants, biosynthesis of monolignols is a specialized branch of phenylpropanoid metabolism (Supplementary Fig. S1), a complex series of branching biochemical reactions responsible for synthesis of variety of products like lignin, flavonoids and hydroxycinnamic acid conjugates [1]. Cinnamoyl CoA reductase (CCR, EC 1.2.1.44) catalyze the first committed step in monolignol biosynthesis and considered as a first regulatory point in lignin formation [2]. CCR carries out the NADPH dependent reduction of various hydroxycinnamoyl CoA esters to corresponding hydroxycinnamaldehydes and vice versa [3,4]. Previous studies on CCR from various plants were basically focused on isolation, cloning, molecular characterization and downregulation aspects [5–12].

Functional attributes of any protein or enzyme are always correlated and dependent on 3D structures. Three dimensional structures of enzymes often provide significant information to understand catalysis mechanisms, substrate specificity and to modify the enzymes with altered specificity and/or advance catalytic function. Although CCR is one of the most investigated enzyme of lignin biosynthesis pathway, its three dimensional structure remain to be determined. The lack of the three dimensional crystal structure of CCR has precluded a clarification of functional active site residues involved in substrate binding and catalysis.

*Leucaena leucocephala*, a multipurpose nitrogen fixing tropical legume, has a tremendous prospective as a raw material (30–40%) for paper and pulp industry in India. As a step toward the active site characterization of Ll-CCRH1 (GenBank: DQ986907), homology modeling was carried out to predict 3D structure and catalytic active site residues. To confirm the roles of putative active site residues, fourteen different Ll-CCRH1 mutants were prepared by site directed mutagenesis and assayed for catalytic activity. To corroborate our mutational studies, chemical modification of Ll-CCRH1 using various amino acid group specific chemical reagents has been carried out. Finally, substrate protection studies of Ll-CCRH1 confirmed presence of modified amino acid residues at the active site.

*Abbreviations:* Ll-CCRH1, *Leucaena leucocephala* cinnamoyl CoA reductase 1; Pg, phenylglyoxal; NAI, N-acetyl imidazole; CA, citraconic acid; PMSF, phenylmethylsulfonyl fluoride; DEPC, diethylpyrocarbonate; WRK, Woodward's reagent K; NEM, N-ethylmaleimide; pHMB, p-hydroxymercurybenzoate; NBS, N-bromosuccinimide.

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## 2. Methods

### 2.1. Heterologous expression and purification of wild type recombinant CCR from L. leucocephala (Ll-CCRH1)

Heterologous expression, purification and assay of the enzyme with cinnamoyl CoA esters were carried out as described earlier [13].

### 2.2. Site directed mutagenesis of Ll-CCRH1 active site residues

Site directed mutagenesis was performed using the Quikchange Lightening Site Directed Mutagenesis Kit (Stratagene, USA) according to manufacturer's instructions. The wild type recombinant Ll-CCRH1 cDNA in pET30b (+) vector was used as template for PCR. Primers used for mutagenesis studies are shown in Supplementary Table S1. Putative positive clones were picked, and plasmids were isolated and sequenced. For double substitution mutant construction, F30V and R51G mutant plasmids were used as template.

### 2.3. Expression, purification and biochemical characterization of Ll-CCRH1 mutants

After sequencing, mutant plasmids were transformed into *Escherichia coli* strain BL21 (DE3) cells for protein expression. The generated Ll-CCRH1 mutants were purified and, the standard enzyme assay and substrate kinetics studies were also performed similarly as that for wild type recombinant Ll-CCRH1.

### 2.4. Chemical modifications and substrate protection studies of Ll-CCRH1

Modification of arginine, tyrosine, lysine, serine, histidine, tryptophan, cysteine residues and carboxylate groups by phenylglyoxal (Pg), N-acetyl imidazole (NAI), citraconic acid (CA), phenylmethylsulfonyl fluoride (PMSF), diethylpyrocarbonate (DEPC), N-bromosuccinimide (NBS), N-ethylmaleimide (NEM)/p-hydroxymercurybenzoate (pHMB) and Woodward's reagent K (WRK) respectively, were carried out using methods described previously [14–16].

Substrate protection studies were carried out by incubating enzyme with various concentrations of feruloyl CoA (0.01–0.2 mM) for 5–10 min prior to treatment with modifying agents and then assayed modified enzyme with proper reaction controls.

### 2.5. Homology modeling and substrate docking

Homology modeling and docking simulation studies of Ll-CCRH1 were carried out by methods as described in Supplementary Method S1.

## 3. Results and discussion

### 3.1. Characterization of Ll-CCRH1 active site mutants

Based on the active site identification studies and interactions observed in modeling/docking simulations (discussed in Sections 3.4–3.6), site directed mutant proteins were generated to confirm the functional role of putative residues involved in substrate binding or catalysis. Substitutions type mutations were designed to have minimal effect on secondary structure and 3D conformation of Ll-CCRH1 protein. Fourteen different Ll-CCRH1 mutants were constructed, cloned, over-expressed and purified to homogeneity before assaying enzyme activity.

#### 3.1.1. Catalytic residues identification for Ll-CCRH1

Complete loss of activity was observed in mutants Y170H, K174M and S136A with feruloyl CoA as substrate (Table 1), indicating its critical role in catalysis. Surprisingly, in modeling and docking, no interactions were observed for putative K174 residue with substrate; but mutational analysis and chemical modification studies of wild type recombinant Ll-CCRH1 proved essentiality of lysine in CCR reactions (Table 1). Also an apparent 2.5-fold increase in $K_m$, 7-fold reduction in $k_{cat}$ and 15-fold decrease in $k_{cat}/K_m$ for S212G suggesting its indirect role in binding (holding reactive groups in proper orientation) or in catalysis (holding the catalytic conformation) (Table 1).

#### 3.1.2. Substrate binding residues in Ll-CCRH1

Mutants, R51G, V200E, D77G, F30V and I31N displayed higher $K_m$ values (up to 4-fold), indicating lower affinity toward substrate, while catalytic efficiencies for theses mutants were notably decreased to 2–3.5-folds (Table 1); suggesting their role in substrate binding and its stabilization in CCR reactions. To strengthen our findings, double substitution mutants F30V–I31N and R51G–D77G were generated from F30V, I31N, R51G and D77G mutants. F30V–I31N mutant showed approximately 7-fold increase in $K_m$ and 8-fold decrease in $k_{cat}/K_m$ values; while R51G–D77G displayed 5-fold increase in $K_m$ and around 9-fold reduction in specificity constant suggesting essential role of F30, I31, R51 and D77, amino acids in substrate binding at or near the active site. Lastly, histidine residue (present in proximity of active site pocket) mutant also showed a reduction about 4–4.5-folds in $k_{cat}/K_m$ and $K_m$ (Table 1). H215 residue has not shown any interactions in docking simulations like K174, and also no significant changes in kinetic parameters were observed. But the probable involvement of histidine was confirmed by chemical modification studies (Section 3.2). Thus, H215 may be involved in substrate binding and stabilization, but not in catalysis.

#### 3.1.3. Possible role of K174 in hydride transfer

The S99 residue, which was supposed to be most important in the transfer of hydride ion from NADPH to substrate showed no significant loss of Ll-CCRH1 activity with any of the substrates after mutation (Table 1). Kinetic parameters for the S99G mutants were in the same order of magnitude as compared to wild type. Thus, role of S99 remains doubtful in CCR mediated reactions. The critical residue involved in hydride transfer still remains a matter of question. Here, K174 in Ll-CCRH1 might be performing the same function of hydride transfer as in template 2C29 [17]. K174 interactions might be reducing the pKa of Y170; thus facilitating proton transfer from Y170.

The observed $K_m$ values vary greatly (1.2–8-folds) among different mutants indicating affinity of mutant enzyme with the substrate. For mutants L64W, H215L and S99G, no significant change in $K_m$ values were observed compared to wild type. The velocity of enzyme reaction, $V_{max}$ is expressed as U mg$^{-1}$ min$^{-1}$, specific activity of individual mutant enzyme. The significant variation of $V_{max}$ values (upto 7-fold decrease) itself suggests the differential kinetic behavior of mutant enzymes. The observed $V_{max}$ values also seem to be reflecting in $k_{cat}$, turn over number of enzyme indicating catalytic efficiency of individual mutant. Finally $k_{cat}/K_m$, specificity constant which takes all parameters $K_m$, $V_{max}$ and $k_{cat}$ into consideration, gives brief idea about catalytic properties of that enzyme. An apparent 2–5-fold decrease in $k_{cat}/K_m$ values were observed for mutants R51G, V200E, D77G, F30V, I31N, H215L mutants. No significant change in specificity constant values was noted in mutants L64W and S99G, where slight increase in affinity for substrate was observed. On the other hand, significant reduction (up to 15-folds) was observed for the same in S212G and double mutants, F30V–I31N and R51G–D77G. Complete loss of