CHAPTER 4(A)

HETEROLOGOUS EXPRESSION OF

LEUCAENA LEUCOCEPHALAL
CAD GENE, ITS PURIFICATION & CHARACTERIZATION
A. Heterologous expression of *Leucaena leucocephala* CAD gene, its purification and characterization

4.1 Introduction

Analysis of gene function is of central importance for the understanding of physiological processes. Expression of genes in heterologous organisms has allowed the isolation of many important genes (e.g. for nutrient uptake and transport) and has contributed a lot to the functional analysis of the gene products. Heterologous expression systems are powerful tools for isolating new genes and for characterizing proteins from all organisms.

An efficient way to clone and simultaneously prove the function of a gene is functional expression in heterologous host cells. Heterologous expression of plant genes provides a new technique for determining gene-product function.

Cinnamyl alcohol dehydrogenase catalyses the conversion of p-hydroxy-cinnamaldehydes to the corresponding alcohols and is considered a key enzyme in lignin biosynthesis (Poratles *et al.*, 2005). The enzyme kinetics of few tree species (Lacombe *et al.*, 1997; Leple *et al.*, 1998) has been studied. Hence functional expression of CAD gene in heterologous system will be of immense help in understanding the characteristics and kinetics of the CAD enzyme.

The pET 30b(+) vector from Novagen was used as the heterologous expression system. The pET system is the powerful system for cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell’s resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein within few hours after induction.

Purification of the recombinant protein is done under fully denaturing conditions using His-Tag fusion proteins and His-Bind immobilized metal affinity chromatography.

In the previous chapter isolation, cloning and characterization of full length CAD gene was done. Clone LlCADc2 was used for heterologous expression analysis.
4.2 Materials and methods

4.2.1 Cloning of CADc2 in pET 30b (+) vector

CADc2 fragment from clone LICADc2 was cloned in pGEM-T Easy vector by incorporating the restriction sites Nde I and Sal I in the primers CAD AF and CAD AR respectively. High fidelity Taq pol (Pfx Invitrogen) was used to amplify CADc2 using AF pET (CAD AF with Nde I) and AR pET (CAD AR with Sal I) primers using LICADc2 plasmid as template. Plasmid LICADc2 was diluted 100 times and 1 μl was used as a template. PCR was performed as described in section 2.17. A 1 kb band was amplified. The band was cut, purified, ligated in pGEM-T Easy vector and transformed in E.coli XL1 MRF cells. Clones with CADc2 fragment with Nde I and Sal I restriction sites were screened by inoculating few colonies in 5 ml LB (Ampicillin 100 μg/ml) tubes. Isolated individual plasmids were restriction digested with Nde I and Sal I enzymes to confirm the integration of CADc2 insert.

PCR cycling condition:

<table>
<thead>
<tr>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
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<tbody>
<tr>
<td>1</td>
<td>95 ºC</td>
<td>5 min</td>
</tr>
<tr>
<td>35</td>
<td>95 ºC</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>60 ºC</td>
<td>30 s</td>
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<tr>
<td></td>
<td>72 ºC</td>
<td>1 min</td>
</tr>
<tr>
<td>1</td>
<td>72 ºC</td>
<td>5 min</td>
</tr>
<tr>
<td>1</td>
<td>4 ºC</td>
<td>hold</td>
</tr>
</tbody>
</table>

The CADc2 fragment with the Nde I and Sal I restriction sites was directionally cloned in pET 30b(+) vector (Figure 4.1 & 4.2). Colony PCR was done to screen the recombinant pET 30b(+) clones. Integration of CADc2 fragment in pET30b (+) was confirmed by digestion with Nde I and Sal I.
Figure 4.1 Vector map of pET 30. pET 30b (+) is shown in box.
Figure 4.2: Strategy for directional cloning of CADc2 in pET30b(+) vector.

4.2.2 Recombinant CAD protein expression and purification from inclusion bodies

4.2.2.1 Recombinant CAD protein expression

A single isolated bacterial colony from freshly streaked plates (grown on LB agar medium containing 50 μg/ml kanamycin) was used to inoculate 5 ml liquid LB medium containing the same concentration of the antibiotic. Culture was grown overnight with shaking at 225 rpm at 37 °C. One ml aliquot of each culture was used to inoculate 100 ml liquid cultures containing 50 μg/ml kanamycin. Once the cultures reached OD_{600} 0.4 - 0.5, recombinant protein expression was induced by addition of isopropyl -β-D-thiogalactopyranoside (IPTG), and the culture was grown for 4–6 h at 37 °C with
shaking at 150 rpm. Recombinant protein extraction was done according to the protocol described in Section 2.21.1.

Four positive recombinant pET 30b(+) clones were screened for CAD protein over-expression on 10% SDS PAGE (Section 2.21.3). The clone showing maximum over-expression was chosen for further studies.

4.2.2.2 Purification of recombinant CAD protein

His-tagged recombinant CAD protein was purified by metal chelate affinity chromatography. The initial stage of His-tagged protein purification is based on the remarkable selectivity and high affinity of patented Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive histidine residues, the 6xHis-tag NTA, which has four chelating sites for nickel ions, binds nickel more tightly than metal-chelating purification systems that have only three sites available for interaction with metal ions. The extra chelation site prevents nickel ion leaching, providing a greater binding capacity and high-purity protein preparations. Purification of recombinant CAD protein carrying a 6xHis-tag was conducted using Ni NTA Agarose beads (Qiagen) (Section 2.21.2). Purity of protein was checked on 10% SDS PAGE (Section 2.21.3).

4.2.3 Raising polyclonal antibody against purified CAD protein in Rabbit

The purified CAD protein was used for raising polyclonal antibodies in New Zealand White rabbit.

4.2.3.1 Pre-treatment of serum:

Rabbit immune serum (stored at -70 °C), containing polyclonal antibodies against the CAD antigen, was thawed overnight at 4 °C. The Serum was kept at 55 °C for one hour and the immune serum was centrifuged at 12,000 g for 15 min at 4 °C. Aliquots according to the requirements were made and stored at -70 °C. The antiserum aliquots were thawed overnight again at 4 °C prior to use.
4.2.3.2 Determination of titre of antibodies

ELISA was performed to determine the titre of first, second, third and fourth bleed of rabbit serum (Section 2.23.1). Once the antibody titre was determined then a fixed dilution of antibodies was used for rest of the experiments.

4.2.4 Extraction and purification of recombinant CAD protein in soluble form

The recombinant CAD protein was extracted in soluble form for the CAD enzyme activity assay. The extraction and purification was done according to the protocol mentioned in section 2.24.1.

4.2.4.1 Standardization of time for protein expression in soluble form

The information obtained from above experiment was utilized for this experiment. Four flasks with 50 ml LB broth (kanamycin 30 μg/ml) were inoculated with O/N grown culture of *E. coli* BL21 harboring PK4pET recombinant plasmid. Induction with 1.0 mM IPTG was done and the cells were grown till *A*₆₀₀ reached 0.5. The cultures were grown at different temperatures (20-37 °C) and different durations (4 to 6 h) for optimization of maximum expression of recombinant CAD protein in soluble form. One flask as an uninduced control sample was used before inducing with IPTG. The soluble fraction of cell lysate was analysed on 10% SDS-PAGE.

4.2.4.2 Purification of recombinant CAD protein in soluble form

The extracted recombinant CAD protein was purified from the crude cell lysate using the Ni NTA Agarose beads (Qiagen) under non-denaturing conditions (Section 2.24.1.2).

4.2.5 CAD enzyme assay

The activity of the purified recombinant CAD enzyme was assayed according to the method given by Wyrambik (1975). The protocol for enzyme assay is given in section 2.24.3. The activity was calculated according to the formula given below

$$\text{Activity (U/ml)} = \frac{\Delta A_{340}/\text{min} \times 1000}{6.22 \times 150}$$
Where, 1000 is the reaction volume in µl, 150 is the volume of enzyme in µl and 6.22 is the extinction coefficient of NADPH. The activity is expressed as units/ml. The protein concentration is measured using the Bradford method. The specific activity is expressed as nKat/mg (1 Unit = 16.67 nkatals)
4.3 Results and discussion

4.3.1 Cloning of CADc2 in pET 30b(+)

4.3.1.1 Incorporation of restriction sites and PCR

The CADc2 gene specific primers CAD AF and CAD AR were modified to incorporate the \textit{Nde} I site at the 5' end and \textit{Sal} I site at 3' end of CADc2.

AF pET – 5' ATA TAT T\textbf{CAT AT} GGG AAG CAT TGA A 3'
AR pET - 5' ACG C\textbf{CGT CGA CCT} GAT GAT CAT CAA GTT TGC TG 3'

A 1 kb band was amplified. The band was cut, purified, ligated in pGEM-T Easy vector and transformed in \textit{E. coli} XL1 Blue cells. Clones with CADc2 fragment with \textit{Nde} I and \textit{Sal} I restriction sites were screened by inoculating few colonies in 5 ml LB (Ampicillin 100 μg/ml) tubes. Isolated individual plasmids were restriction digested with \textit{Nde} I and \textit{Sal} I enzymes to confirm the integration of CADc2 insert.

4.3.1.2 Directional cloning of CADc2 fragment in pET 30b(+) vector

The above clone in pGEM-T Easy vector was designated as pGEMPK4. This clone was restriction digested with \textit{Nde} I and \textit{Sal} I restriction enzymes and 1 kb fragment was purified. pET 30b(+) vector DNA was also digested with same restriction enzymes and purified. A 1 kb fragment from pGEMPK4 was directionally cloned in purified restriction digested pET 30b (+) vector. Ligation mixture was transformed in \textit{E. coli} XL1 competent cells and plated on LB-agar plate (kanamycin 50μg/ml). Colonies for recombinant plasmids were screened by colony PCR method (Figure 4.3). Plasmid was isolated from clones showing 1 kb band in colony PCR reactions. The plasmid was digested with \textit{Nde} I and \textit{Sal} I to confirm the integration of pGEMPK4 fragment in pET 30b (+) vector.
The recombinant pET 30b(+) plasmids harboring CADc2 gene was confirmed after sequencing. The sequence was translated using proteomic tools available on www.expasy.ch and was checked for in frame translation up to HIS tag. Above recombinant plasmids were mobilized in *E. coli* BL21 strain for over-expression.

**4.3.2 Recombinant CAD protein expression and purification from inclusion bodies**

**4.3.2.1 Recombinant CAD protein expression**

*E. coli* BL21(DE3) cells transformed with recombinant pET 30b(+) plasmids were screened for over-expression. Four positive recombinant clones were screened for recombinant CAD protein over-expression. The clones were designated as PK1pET, PK2pET, PK3pET and PK4pET. A ~40 kD protein was expressed in all clones as analysed on 10% SDS PAGE. Maximum expression was found in clone PK4pET, which was then used for all experiments (Figure 4.4).
Figure 4.4 10% SDS PAGE: Lane 1, 3, 4- Inclusion bodies from clones PK1pET, PK4pET and PK2pET respectively. Lane 2, 5- Cell lysate from clones PK4pET and PK2pET respectively. Lane 8- Protein molecular weight marker.
4.3.2.2 Purification of recombinant protein

Large-scale production and purification was done using the protocol described in section 2.21.1 and 2.21.2. The purified protein was approximately 40 kD in size and was eluted at 200 mM imidazole concentration. It was found that four hours of post-induction at 37 °C was enough to achieve the purification of CAD protein from inclusion bodies (Figure 4.5).

![Image of SDS PAGE](image_url)

**Figure 4.5** 10% SDS PAGE: Lane 1-Inclusion bodies. Lane 2- Flow through. Lane 3- Wash 1. Lane 4-8- Eluent 1, 2, 3, 4 and 5 respectively.

Purified CAD protein was dialysed against 1X PBS buffer supplemented with 1 mM DTT and 0.1 mM PMSF overnight with two changes of fresh buffer. Dialysed CAD protein was concentrated, quantified by Bradford assay (Bradford reagent, Promega, USA) and given for raising antibodies in New Zealand rabbit.
4.3.3 Raising antibodies in rabbit:

300 μg of purified protein was used for first injection in New Zealand rabbit to raise antibodies. Same amount of protein was used for booster doses. Antibody titer of first, second, third and fourth bleed was determined by ELISA. Titer of third bleed is ~1:153600 (Figure 4.6). The titre of third and fourth bleed is almost the same. 3rd bleed serum dilution of 1:10000 was used for further experiments.

![Figure 4.6 Graph showing antibody titre of third and fourth bleed.](image)

4.3.4 Extraction and purification of recombinant CAD protein in soluble form

PK4pET and PK1pET clones were used for extraction of recombinant protein in soluble form to test the enzyme activity. Temperature and time required after induction was standardized for maximum CAD protein expression in soluble form that is in cell lysate. PK4pET showed the maximum CAD expression in soluble form. The optimum parameters were as follows, after initial growth at 37 °C, the cells were induced with 1mM IPTG. Before induction cells were cooled to 15 °C and were grown for 16 h at 15 °C after induction (Figure 4.7).
Figure 4.7 Analysis of recombinant CAD protein expressed under temperature and duration on 10% SDS PAGE: Lane1, 3, 6 and 8- PK4pET cell lysate at 15 °C for 16 h, 20 °C for 16 h, 20 °C for 6 h and 37 °C for 4 h respectively. Lane 2, 4, 7 and 9-PK1pET cell lysate at 15 °C for 16 h, 20 °C for 16 h, 20 °C for 6 h and 37 °C for 4 h respectively. Lane 10- purified CAD protein for comparison.

The recombinant CAD protein from cell lysate was purified using the Ni-NTA agarose beads under non-denaturing conditions. The protein was eluted in the second fraction at 250 mM imidazole concentration.

4.3.5 CAD Enzyme assay

The activity of the recombinant CAD enzyme was assayed using the three substrates namely coniferaldehyde, cinnamaldehyde and sinapaldehyde. 1.156 µM of substrate was taken for the assay. The second eluent fraction was used as the CAD enzyme. The quantity of substrate, enzyme and NADPH was same for all the assay reactions. For each reaction decrease in absorbance at 340 nm was monitored for 3 min (Figure 4.8). The specific activity was expressed as nKat/mg of protein (Table 4.1). The results show that CAD specific activity is maximum for cinnamaldehyde. The results can be further validated by enzyme kinetic studies. Even so this data gives preliminary idea about substrate preference of *Leucaena* CAD enzyme.
Figure 4.8 Graphs showing activity of CAD enzyme with coniferaldehyde (A), cinnamaldehyde (B) and sinapaldehyde (C).

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<tr>
<th>Substrate</th>
<th>Specific Activity (nKat/mg)</th>
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<tbody>
<tr>
<td>1 Coniferaldehyde</td>
<td>4.052330913</td>
</tr>
<tr>
<td>2 Sinapaldehyde</td>
<td>12.89378018</td>
</tr>
<tr>
<td>3 Cinnamaldehyde</td>
<td>25.41916664</td>
</tr>
</tbody>
</table>

Table 4.1 Specific activities of recombinant CAD enzyme for coniferaldehyde, sinapaldehyde and cinnamaldehyde substrates.
4.4 Conclusions

- The CADc2 fragment isolated from *L. leucocephala* was directionally cloned in pET 30b(+) expression system.
- Recombinant CAD protein was standardized for over-expression and purified from inclusion bodies. A 40 kD purified protein, from inclusion bodies, was used to raise polyclonal antibodies in New Zealand rabbit.
- CAD enzyme activity for substrates like cinnamaldehyde, coniferaldehyde and sinapaldehyde, was determined using purified CAD protein extracted in soluble form. Preliminary data show that recombinant CAD enzyme has maximum specific activity with cinnamaldehyde.
- The functional expression of recombinant CAD protein confirms that the cDNA isolated from *L. leucocephala* encodes for cinnamyl alcohol dehydrogenase protein.