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Cloning, Expression and Purification of *Chalcone Synthase* from *Solanum Tuberosum*

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Abstract: *Chalcone synthase (**CHS**) gene was amplified by PCR from cDNA of Solanum tuberosum leaf tissue. The gene was cloned into pGEMT easy vector and sequenced. It was then ligated into pET 30 vector to construct recombinant plasmid pET30-**CHS**. The recombinant was transformed into E.coli BL21 strain and the target gene was over expressed using IPTG. Optimum condition of IPTG concentration and temperature for over expression was found to be 1 mmol L^{-1} and 37ºC respectively. The results showed that the His-tag- **CHS** was highly heterogonous expressed in the inclusion bodies into the host cell. However, the His-tag- **CHS** fusion protein was purified using affinity chromatography with Ni^{2+}-NTA resin column. SDS-PAGE analysis demonstrated that the host with pET30-CHS generated a 42 kD His-tag-CHS fusion protein.*

Keywords: *Chalcone synthase; fusion protein; plasmid; cloning; purification; expression*

Abbreviations: *CHS-Chalcone synthase,* ***RT****-Reverse transcription,* ***CHI****-Chalcone isomerase,* ***IPTG****-Isopropyl β-D-1-thiogalactopyranoside,* ***PKS****-Polyketide synthase enzymes,* ***cDNA*** *-Complementary DNA,* **mRNA**-*Messenger RNA,* ***PAGE****-Polyacrylamide gel electrophoresis*

I. Introduction

Chalcone synthase (CHS) is an enzyme ubiquitous to higher plants and belongs to a family of polyketide synthase enzymes (PKS) known as type III PKS. Type III PKSs are associated with the production of chalcones, a class of organic compounds found mainly in plants as natural defense mechanisms and as synthetic intermediates. *CHS* was the first type III PKS to be discovered[1]. It is the first committed enzyme in flavonoid biosynthesis[2]. The enzyme catalyzes the conversion of 4-coumaroyl-CoA and malonyl-CoA to naringenin chalcone. *CHS* exists as a homodimeric protein with each monomer approximately 42-45 kDa in size [3]. *CHS* contains a five layer *αβαβα* core, a location of the active site and dimerization interface that is highly similar to thiolase-fold containing enzymes. The dimerization interface contains both hydrophobic and hydrophilic residues and is generally flat except for a pair of N-terminal helices that lay entwined across the top. The enzyme is localized in the cytosol, associating with the endoplasmic reticulum membrane [4]. In another study, it was shown that *CHS* and CHI co-localize at the nucleus as well [5]. *CHS* is constitutively expressed in plants but can also be induced to express by light, UV light, pathogens, elicitors and wounding. The *CHS* promoter contains a G-box motif with a sequence of CACGTG. This has been shown to play a role in response to light [6]. Other light sensitive domains include Box I, Box II, Box III, Box IV or three copies of H-box (CCTACC) [7]. CHS, as the first committed step in the flavonoid pathway, facilitate production of flavanoids, isoflavonoid-type phytoalexins and other metabolites to protect the plant from stress [8]. *CHS* expression is also involved in the salicyclic acid defense pathway. Being aromatic compounds, flavonoids strongly absorb UV light through a photoreceptor-mediated mechanism which effectively protects the plants from DNA damage. *CHS* is involved in a broader, more general phenylpropanoid pathway which serve as precursors to a range of plant metabolites important to human health such as antioxidants, anti-inflammatory agents, anti-allergens, and even anti-oncogenic products [9].

In this paper we present details of cloning, sequencing, over expression and purification of *chalcone synthase* gene from commercially cultivated potato plant, *Solanum tuberosum* variety-- *Pukhraj*, from Aurangabad, Maharashtra, India.

II. Materials and methods

2.1 Isolation of total RNA and cDNA synthesis.

Total RNA was isolated from leaf material of *Solanum tuberosum* plant by using plant RNA extraction kit (Sigma Chemical Co. USA). cDNA was synthesized from isolated total RNA using oligo(dt) primer(Promega).

2.2 Amplification of full chalcone synthase gene by PCR

The gene for ***CHS*** was amplified from cDNA using ***CHS*** specific primers(Forward:5' *ATA TAT CAT ATG GTC ACC GTG GAG CAG TAT* 3' with NdeI restriction site underlined, Reverse: 5' *ATA TAT CTC GAG AGT AGC AAC ACT GTG* 3' with XhoI restriction site underlined).PCR reaction was performed using 40ng of cDNA along with forward and reverse primers(10pmol each),200µM of dNTP's, 1.5mM Mgcl2 and 3U of high fidelity DNA polymerase(Promega, USA).The amplification cycle was initial 5 minutes denaturation, after that 35 cycles of denaturation at 94ºc for 20sec, annealing at 58ºc for 30sec and extension 72ºc for 1.30min.Final extension 72ºc for 7min.The PCR amplified product was analysed on 1% agarose gel along with DNA molecular weight marker.

2.3 Cloning and characterization of chalcone synthase gene

The amplified ***CHS*** gene fragment was gel purified and ligated into pGEMT easy vector (Promega, USA).The recombinant plasmid *(pGEMT-**CHS**)* was characterized using E.coRI restriction digestion and analysed on 1% agarose gel for the insert. The ***CHS*** gene in recombinant plasmid *pGEMT-**CHS*** was sequenced in Eurofins Genomics, Bangalore and sequence was blast in NCBI.

2.4 Cloning of the chalcone synthase gene into prokaryotic expression vector.

The recombinant pGEMT-CHS plasmid set for restriction digestion with the restriction enzyme NdeI and XhoI.The restriction digestion product was gel purified and cloned in prokaryotic expression vector pET30(Invitrogen) and transformed into BL-21cell line **[10].** The recombinant plasmid was validated by the use of above restriction enzyme pairs and agarose gel electrophoresis. The recombinant plasmid was named as *pET30-**CHS***

2.5 Expression, Purification of chalcone synthase protein in E.coli

Cloning of ***CHS*** into the *pET30b+* vector and expression in wild type *E.coli* strain *BL21* (DE3) enhanced the ***CHS*** production. Level of transcription of ***CHS*** gene in this system depends on the–IPTG induction. We conducted an experiment for the expression of ***CHS*** protein under conditions of 180 rpm shaking culture at 37ºc and induced by IPTG 1.0 mmol L^{-1} for 2 h, 4hrs, 6 hrs, 8hrs, to observe the difference in protein expression among the transformants. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. Pellets were re-suspended in 6 ml lysis buffer. Cells were lysed by sonication for 5 minutes at amplitude 70 on a sonifier cell disruptor. MgSO4 of final concentration of 10 mM and lysozyme of final concentration 100µg/ml was added to the disrupted cell and kept at 37ºC for 30 minutes. It was centrifuged at 10000 rpm for 10 minute and supernatant was saved as lysate and pellet was re-suspended in 2 mL sonication buffer. Suspension was again sonicate for 1 min at70 amplitude to disrupt the inclusion bodies and the disrupted inclusion bodies were dissolved in 3 mL of dispersion buffer and an aliquot of 20 µL checked on SDS PAGE to check the expression**[11].** Affinity chromatography with Ni^{2+}-NTA Resin Column was used to purify the ***CHS*** fusion protein. The recombinant protein, among several other bacterial proteins is loaded on affinity matrix column. The column was kept at 4ºC for 1 hr for binding of recombinant protein to Ni+ beads. After 1 h, flow through was collected in different tubes. Column was washed with two bed volume of washing buffer containing 20 mM imidazole. The 6x His-tagged bind protein was eluted in 4 aliquots of elution buffer 0.5 mL each containing150 mM imidazole. Protein elution was monitored by measuring the absorbance at 280 nm of collected fractions. The eluted protein was analyzed by SDS-PAGE.

III. Results and Discussion

3.1 RNA isolation and cDNA first strand synthesis by Reverse Transcription

The total RNA isolated from leaf material of *solanum tuberosum* plant is presented in Figure 1A. Complementary DNA (cDNA) which was synthesized from a mature mRNA template in a reaction catalyzed by the enzyme reverse transcriptase is presented in Figure 1B. The resulting molecule is a DNA-RNA hybrid and the process is called as cDNA 1st strand synthesis.

3.2 The construction and identification of pET30-*CHS* prokaryotic expression vector

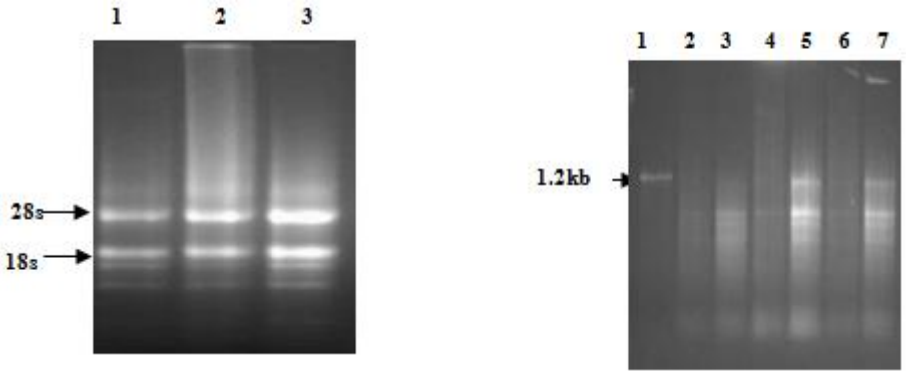
The ***CHS*** gene of the length of about 1200 bp was amplified from cDNA. The gene was then cloned in *pGEMT easy* vector (Promega, USA) which was confirmed by the restriction digestion experiment using *E.coRI* (Figure 2A). The sequencing analysis confirmed the amplified gene to be nearly identical to the target gene. Sequence was deposited in the NCBI Gen Bank (ID **KF285826** and UNIPROT ID **T1YXX9**). The recombinant plasmid carrying the ***CHS*** gene was digested with restriction enzyme pairs (*NdeI, XhoI*) *and* sub-cloned into the expression vector pET30b. Further, the recombinant plasmid was validated by the use of same restriction enzyme pairs i.e. *NdeI and XhoI* and the same was named as *pET30-CHS*(Figure2B).The targeted gene ***CHS*** in the recombinant plasmid has a length of about 1.2 kb, while its vector *pET30* has a length of 5.4 kb

3.3 Protein expression and purification

The *pET30-**CHS*** recombinant plasmid carrying *E.coli* BL21, colony was grown at 37ºc in LB broth containing 50µg per ml kanamycin,when OD600 of the broth reached to 0.6,it was induced with IPTG.The protein profile of the unpurified recombinant CHS protein on SDS-PAGE are shown in fig 3A & 3B.The results of SDS-PAGE showed that a distinct band of about 42 kD in molecular weight existed in the induced bacteria lysate transformed with *pET30-**CHS*** (Figure 4B). The results indicated that recombinant plasmid *pET30-**CHS*** produce protein ***CHS*** upon induction with IPTG. The expression of the fusion protein was increased gradually with increase of induction time up to 6 hours (Figure 3A).The inclusion proteins expressed in *E.coli* was purified using the urea-denaturation procedure. After purification under denaturing conditions using Ni-agarose affinity chromatography,(Huang et al.2008)[12]**.**The purified protein was observed as single protein with a molecular weight of 42kDa on SDS-PAGE (Fig4).The recombinant protein with polyhistidine fusion tag facilitated the easy purification of fusion protein. The expressed protein in our study was found in insoluble inclusion bodies. The formation of IB of over produced protein and its renaturation are common with prokaryotic expression system[13]**.**The expressed recombinant protein, in our study, was eluted with elution buffer oh pH4.5.The observed molecular weight of recombinant pET30CHS in present study was 42kDa. The purity of the protein fractionated with Ni^{2+}-NTA resin is about 90% **(Figure 4).** The concentration of the protein was detected to be 500µg/mL.

IV. Figures

Fig 1

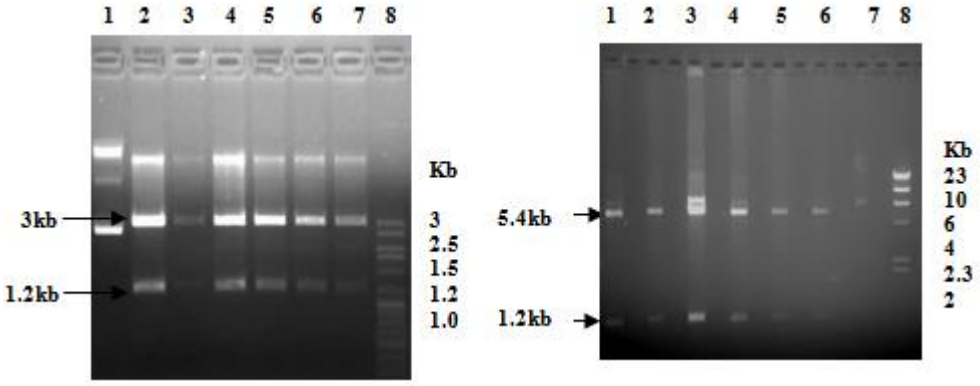


(A) **(B)**

Fig 1 Isolation of RNA (A) and synthesis of cDNA first strand (B).

Upon completion of RNA isolation and cDNA first strand synthesis, agarose gel (1%) was run at constant volt (80amp) for 1hr. **(A)**Lane 1-3-Totall RNA isolated from *solanum tuberosum* leaf sample.**(B)**Lane 1-7- cDNA 1st strand synthesis from mRNA.

Fig 2

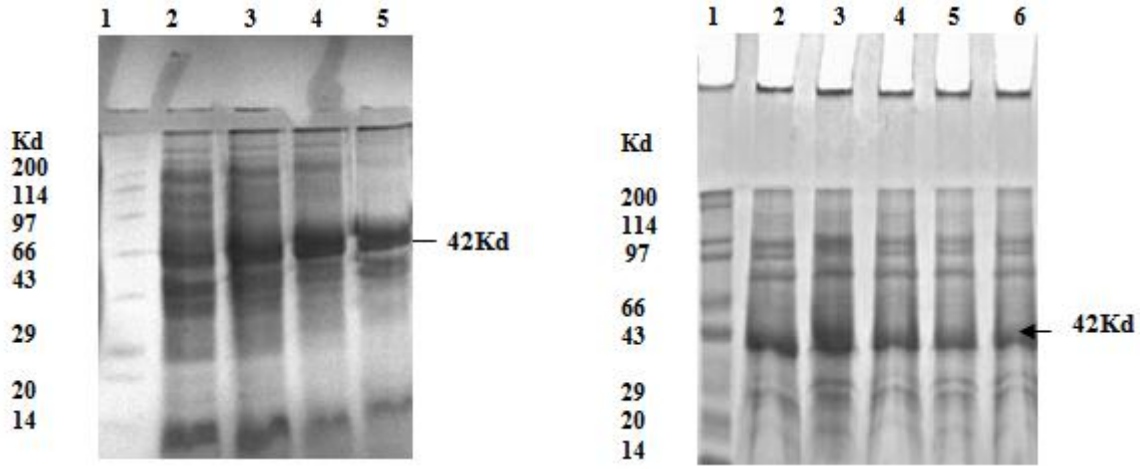


(A) **(B)**

Fig 2 Characterization of recombinant plasmids.

Upon completion of restriction digestion, agarose gel (1%) was run at constant volt (80amp) for 1hr. (**A)** Restriction digestion of *pGEMT-CHS* by *E.coRI,* Lane 1-uncut plasmid, Lane 2-7-clone which contain *chalcone synthase* gene, Lane 8- 3kb Maker (100bp- 3kb**). (B)** Restriction digestion of *pET30-CHS* by *NdeI and XhoI,* Lane 1-6.*Chalcone synthase* gene clone in *pET30* vector cut by *Nde1&Xho1* restriction enzyme, Lane 7-Uncut plasmid, Lane 8-Lamda *HindIII* digest marker

Fig 3



(A) **(B)**

Fig 3 over expression of recombinant *CHS* protein in *E. coli*

[A] Effect of different IPTG induction time on the expression of ***CHS*** protein, Lane 1: low molecular weight protein marker (14-200), Lane 2-6: 42Kd induced ***CHS*** protein from lysate induced by IPTG 1mmol/ml for 2,4,6,8 hrs. Highest expression observes at 6hrs, this show in lane no 4. [B] SDS page (12%) of *E.coli BL21 (pET30+CHS)* induced protein from lysate. Lane 1: low molecular weight protein marker (14-200), Lane 2-6:42Kd induced CHS protein from lysate.

Fig 4

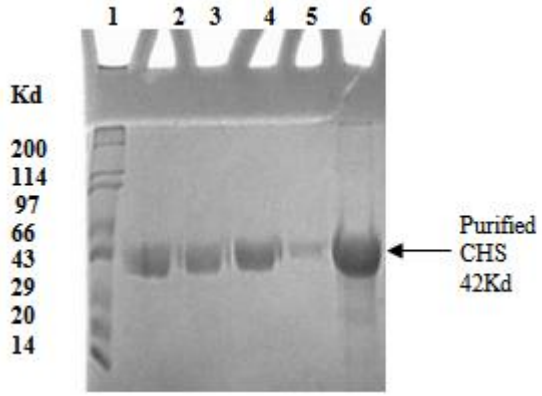


Fig 4 - Purification of the *chalcone synthase* protein

SDS PAGE (12%) of Ni^{+} beads purified ***CHS*** protein from lysate. Lane 1: Low molecular weight protein marker (14-200)

Lane 2-6 :Ni^{+} beads purified ~42Kda CHS protein.

V. Conclusion

Chalcone synthase is an important enzyme for production of chalcone.The organic compounds found mainly in plants as natural defense mechanisms. Purification of CHS from leaf of *Solanum tuberosum* has two major problems- first, the source material is season dependent and secondly, purification from green tissues is cumbersome and expensive. Therefore expression of CHS in *E.coli* is an important alternative to purify the protein for its characterization and other studies.

The result indicate that, over expression of CHS in *E.coli* was found at 37ºC for 6hrs when induced by1mmol/ml IPTG .CHS was purified using Ni agarose affinity chromatography with about 90% purity. A(His)6 tag at the N-terminus helps in purification of fusion protein on Ni-column. The concentration of the protein was detected to be 500µg/mL.

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