Cloning and Expression of *Taxus* Acyltransferase cDNA

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A new full-length acyltransferase cDNA was obtained from *Taxus chinensis* by homology-based cloning strategy. The cDNA has an open-reading frame of 1,275 nucleotides, which encodes 425 amino acids with a calculated molecular weight of 47,241 Da and an estimated pI value of 5.93. The deduced amino acid sequence resembles the sequences of other cloned acyltransferases (56–61% identity; 71–75% similarity) involved directly in taxol biosynthetic pathways. This cDNA was expressed in *Escherichia coli* using the expression vector pET-32a(+). The expression band corresponds to the calculated mass plus the *N*-terminal fusion protein derived from the vector.

Key words: Taxus chinensis, Acyltransferase, Expression in *E. coli*
Data Accession No: AY326950

Introduction

The diterpene natural product taxol, first discovered in the 1960's, has been an effective anticancer drug for the treatment of a variety of human cancers. The limited supply of taxol from natural sources and the lack of a commercially viable total synthesis require alternative means of taxol biosynthesis production. Increasing applications of taxol in chemotherapy have spurred the isolation of many additional taxoids, with the hope of finding the additional compound with better activity in pharmaceutical use (Baloglu and Kingston, 1999).

The biosynthesis of taxol from plant primary metabolism is a very complex process, including at least 20 steps to construct its tetracyclic skeleton and to add of the various hydroxyl and acyl functional groups (Jennewein and Croteau, 2001). The tricyclic structural core and the aminophenylpropanoyl side chain of the taxoids undergo both *O*- and *N*-acyl group transfer reactions, including acetylation, benzoylation, long-chain alkenoyl- and alkanoylation, and cinnamoylation (Walker *et al.*, 2002b). These diverse enzymatic acylations are, in part, responsible for generating an excess of 350 naturally occurring taxane diterpenoids in *Taxus* species (Baloglu and Kingston, 1999). Over the past several years, major advances have been made in cloning acyltransferase genes from *Taxus* by reverse genetics (Walker and Croteau, 2000a), homology-based PCR cloning (Walker and Croteau, 2000b) and DD-RT-PCR-based cloning methods (Hu *et al.*, 2002). There are five acyltransferase genes that have been identified to participate in the taxol biosynthetic pathway, including taxadien-5α-ol-*O*-acetyltransferase (Walker *et al.*, 2000), 10-deacetylbaccatinIIIβ-10-*O*-acetyltransferase (Walker and Croteau, 2000a), taxane 2α-*O*-benzoyltransferase (Walker and Croteau, 2000b), 3′-*N*-debenzoyl-2′-deoxytaxol *N*-benzoyltransferase (Walker *et al.*, 2002a) and taxoid C-13 *O*-phenylpropanoyltransferase (Walker *et al.*, 2002b). Many results from taxol biogenetic schemes show that these regioselective CoA thioester-dependent acylations contribute to the function of taxol in promoting tubulin polymerization and stabilization and are important for the bioactivity of taxol (Kingston, 2001). The utilization of these acyltransferase genes may facilitate increased production of taxol and may allow the biosynthetic generation of second-generation taxol analogs possessing greater bioactivity and improved water solubility with modified acyl groups and substitution patterns (Walker and Croteau, 2001).

This paper reports a new acyltransferase gene cloning from *Taxus chinensis* callus cultures, which produced C-14 oxygenated taxanes at over 2% of dry weight (Cheng *et al.*, 1996), by a homology-based PCR cloning strategy and presents a procedure to establish the expression of a recombinant *trx*A-acyltransferase fusion protein in *E. coli* as a soluble enzyme.

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Materials and Methods

Bacterial strains and plasmids

E. coli (Takara, Dalian, China) and *E. coli* BL21*trxB*(DE3) (Novagen, Darmstadt, Germany) were used as the hosts for plasmid amplification and gene expression, respectively. Plasmid pMD18T (Takara) and pET-32a(+) (Novagen) were used as vectors for cloning and expression, respectively.

Taxus chinensis cDNA library construction

To facilitate the isolation of clones encoding hydroxylase and acyltransferase involved in taxol/taxoid biosynthesis, a cDNA library was constructed. Total RNA was extracted from *Taxus chinensis* callus cells using the single-step method with acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). The poly (A) + mRNA was purified by Magnesphere (Promega, Madison, USA) and a cDNA library of *T. chinensis* was constructed by the λ gt10 cDNA library Kit (Promega) using conditions recommended by the manufacturer.

Preparation of the probe DBAT10

The probe was PCR-amplified from genomic DNA of *Taxus cuspidata* using 5′-ACTCGTTTTACATGTGGAGG-3′ as forward primer and 5′-CCCCCACCCAAAGTCTAC-3′ as reverse primes. The two PCR primers were designed on the basis of a published *Taxus* 10-deacetylbaccatinIII-10-*O*-acetyltransferase gene sequence. Genomic DNA of *Taxus cuspidata* was isolated using the Rogers and Bendich (1985) procedure as template. DNA amplification was performed using Taq and Pfu DNA polymerase (Invitrogen, Carlsbad, USA) under following conditions: 94 °C for 4 min, 35 cycles at 94 °C for 45 s, 52 °C for 1.5 min and 72 °C for 2.5 min and, finally, 72 °C for 7 min. The resulting 849-bp amplicon was separated by agarose gel electrophoresis and the product was extracted from the gel, ligated into the pMD18T vector, and transformed into *E. coli* DH5 α cells. Plasmid DNA was prepared from several individual transformants and the inserts were fully sequenced. Sequence result revealed that this gene fragment (designated probe DABT10) is a part of the *Taxus* 10-deacetylbaccatinIII-10-*O*-acetyltransferase gene.

Screening Taxus chinensis cDNA library

For library screening, DABT10 was amplified by PCR, and the resulting amplicon was gel-purified. 100 ng of DABT10 was labeled as probe. The hybridization probe was used to screen membrane lifts of 5×10^5 plagues grown in *E. coli* C_{600} *Hf1* according to the manual of ECL direct nucleic acid labeling and detection systems RPN 3001 (Amersham Pharmacia Biotech, Buckinghamshire, England). The plaques affording positive signals were purified through an additional round of hybridization. The size of the cDNA insert was determined by PCR using two arm primers of the λ gt10 vector under the following conditions: 94 °C for 4 min, 30 cycles at 94 °C for 45 s, 56 °C for 1.5 min and 72 °C for 2.5 min and, finally, 72 °C for 7 min. Size-selected inserts (> 1.2 kb) were partially sequenced from both ends for the purpose of sorting and a full-length acyltransferase gene was acquired.

Nucleotide and protein sequence analysis

The full-length acyltransferase insert fragment (acquired as above and designated TCA1) gene and deduced amino acid sequences were compared to those available at the GenBank and were aligned by the BLASTP and BLASTX program. The nucleotide sequence reported in this work has been deposited in the GenBank database under the accession number AY326950.

Construction of the expression vector pET32TCA1

The full-length TCA1 cDNA sequence was amplified by PCR and placed in the *Eco*RI (Takara) site at its 5′-terminal and in the *Xho*I (Takara) site at its 3′-terminal by using the forward primer 5′-GCGATATCGAATTCATGGTGAAGCCCTCTATTCCTCT-3′ and the reverse primer 5′-CCGGATCCCTCGAGTCATTCTGCAGTCACATATTTGT-3′, respectively. The PCR product and the pET-32a(+) vector were digested with *Xho*I and *Eco*RI, purified, and ligated, resulting in the plasmid pET32TCA1. The ampicillin-resistant transformants were screened by colony PCR. The resulting expression vector for the acyltransferase gene, designated pET32TCA1, was extracted and digested with both *Eco*RI and *Xho*I endonucleases for confirmation. The sequence of the insert and frame were further confirmed by DNA sequencing.

Bacterial expression

The expression plasmid pET32TCA1 was used to transform *E. coli* strain BL21*trxB*(DE3) competent cells. The recombinant acyltransferase was expressed in transformed cells according to the manufacturer's instructions (Novagen). The selected acyltransferase clone was toothpicked into 20 ml liquid LB medium supplemented with 100 μg ampicillin ml^{-1} and 20 μg kanamycin ml^{-1} and grown overnight for further colony activation. A 1:100 dilution in 100 ml LB medium was supplemented with 100 μg ampicillin ml^{-1} and 20 μg kanamycin ml^{-1} and was grown at 37 °C to $OD_{600\,nm}$ = 0.7–0.8, and then induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The cultures were incubated at 15 °C with shaking (150 rpm) for 16 h, harvested by centrifugation (5,000 × *g*, 10 min). The induced cells were disrupted in buffer A (25 mM sodium phosphate, pH 7.4) by sonication and the supernatant was recovered by centrifugation at 20,000 × *g* for 30 min at 4 °C. The level of expression of acyltransferase as a soluble protein in recombinant *E. coli* was monitored by SDS-PAGE.

Polyacylamide gel electrophoresis

The expression of the interesting enzyme was determined by SDS-PAGE. SDS-PAGE was conducted on a 10% (v/w) polyacrylamide gel by the method of Laemmli (1970). The low molecular weight standard from MBI Fermentas (Fermentas, Hanover, USA) was used. Protein bands were stained with silver nitrate.

Results and Discussion

Cloning and nucleotide sequence analysis of the TCA gene from Taxus chinensis

To clone acyltransferase genes from *Taxus chinensis*, the homologue-based PCR cloning strategy was used as mentioned in Materials and Methods. The probe DABT10 harboring a 849 bp fragment was obtained by PCR using genomic DNA from *Taxus cuspidata* as template. Sequence analysis revealed that this fragment did not include any intron. A full cDNA insert fragment TCA1 was obtained when *Taxus chinensis* cDNA library was screened by the probe DABT10. The cDNA contains an open reading frame of 1,275 nucleotides and encodes a deduced protein of 425 amino acids residues with a calculated molecular weight of 47,241 Da, consistent with that of other monomeric plant acyltransferases (≈ 50 kDa), and an estimated pI value of 5.93 (Pierre and De Luca, 2000). The complete nucleotide sequence and the putative amino acid sequence are shown in Fig. 1. The deduced amino acid sequence does not appear to encode any *N*-terminal targeting information, consistent with the purified acyltransferase enzymes from *Taxus* species with operationally soluble nature and probable cytosolic location of the monomeric enzyme (Walker *et al.*, 2000).

Database searches revealed TCA1 has no homology to any known gene, suggesting that it represents a new gene found in *Taxus* species. The deduced amino acid sequence resembles the sequences of other cloned acyltransferases (56–61% identity; 71–75% similarity) involved directly in taxol biosynthetic pathways. Additionally, TCA1 possesses the HXXXDG motif (residues H147, D151, and G152, respectively) that was found in a family of acyltransferases as the putative acyl group transfer motif that may function in acyl group transfer from acyl-CoA to substrate alcohol (Pierre and De Luca, 2000). Although five acyltransferases present in the final production of taxol have been identified, the complete course of the taxol biosynthetic pathway may involve metabolites that are transiently acylated and deacylated for the purpose of organellar targeting of flux regulation. We deduced that TCA1 maybe a new acyltransferase gene involved in taxol/taxoids biosynthetic pathways according to multiple acyltransferase sequence alignment and phylogenetic tree results (Fig. 2 and Fig. 3).

However, alignment of the TCA1 sequence with these defined acyltransferases of plant origin and with a range of the most closely related deduced protein sequence (of undefined function) from plants showed rather little overall homology (< 32% identity). Since substrates of these encoding acyltransferases were almost certainly varied widely, the corresponding active site binding determinants would also be expected to vary substantially (Pierre and De Luca, 2000). In the taxol/taxoids biosynthesis pathway, taxol/taxoids undergo both *O*- and *N*-acyl group transfer reactions, including acetylation, benzoylation, long-chain alkenoyl- and alkanoylation, and cinnamoylation. According to the sequence alignment result and

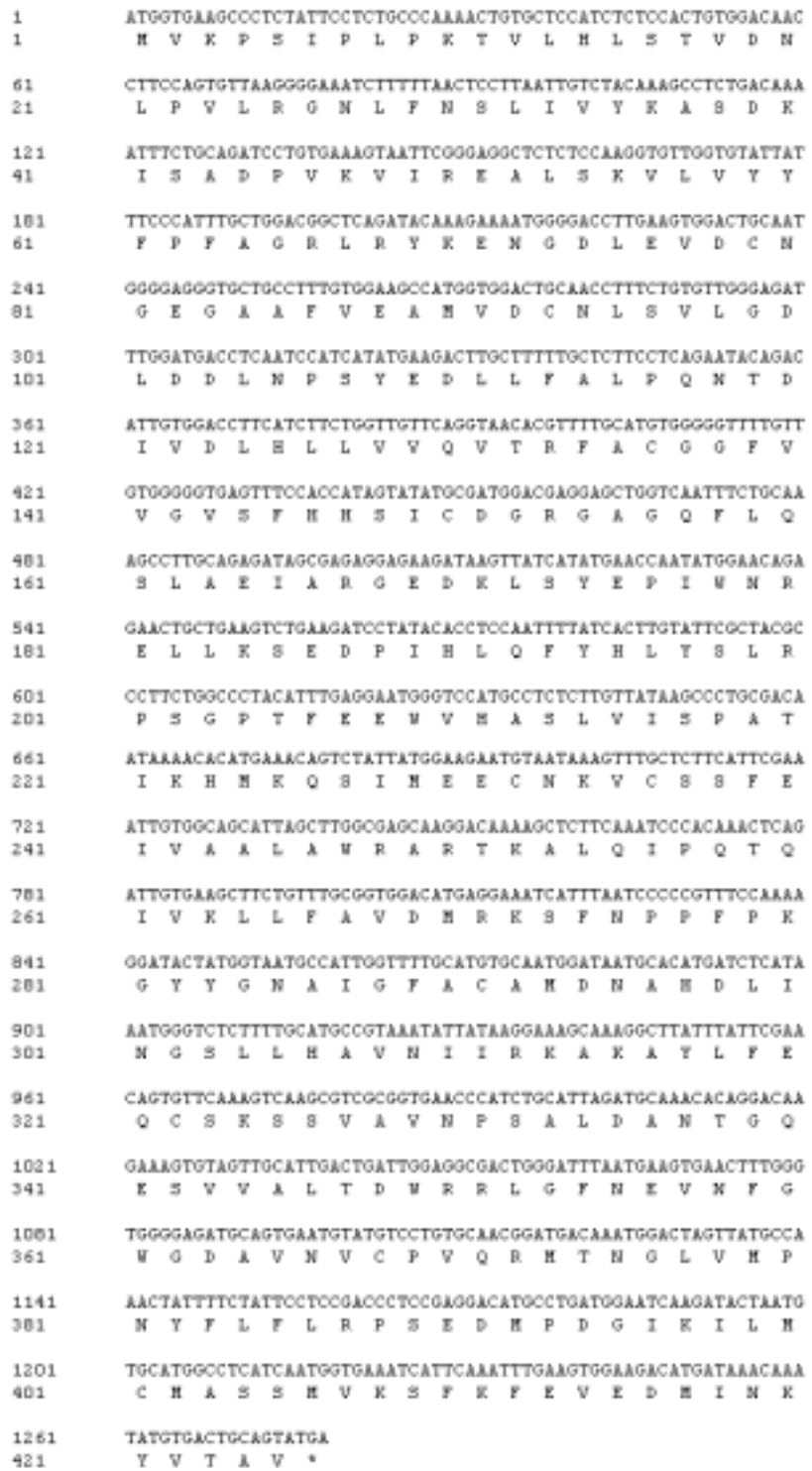

Fig. 1. Nucleotide and predicted protein sequence of TCA1 from *Taxus chinensis* (AY326950). Nucleotide and amino acid number are shown on the left. Both strands were sequenced completely.

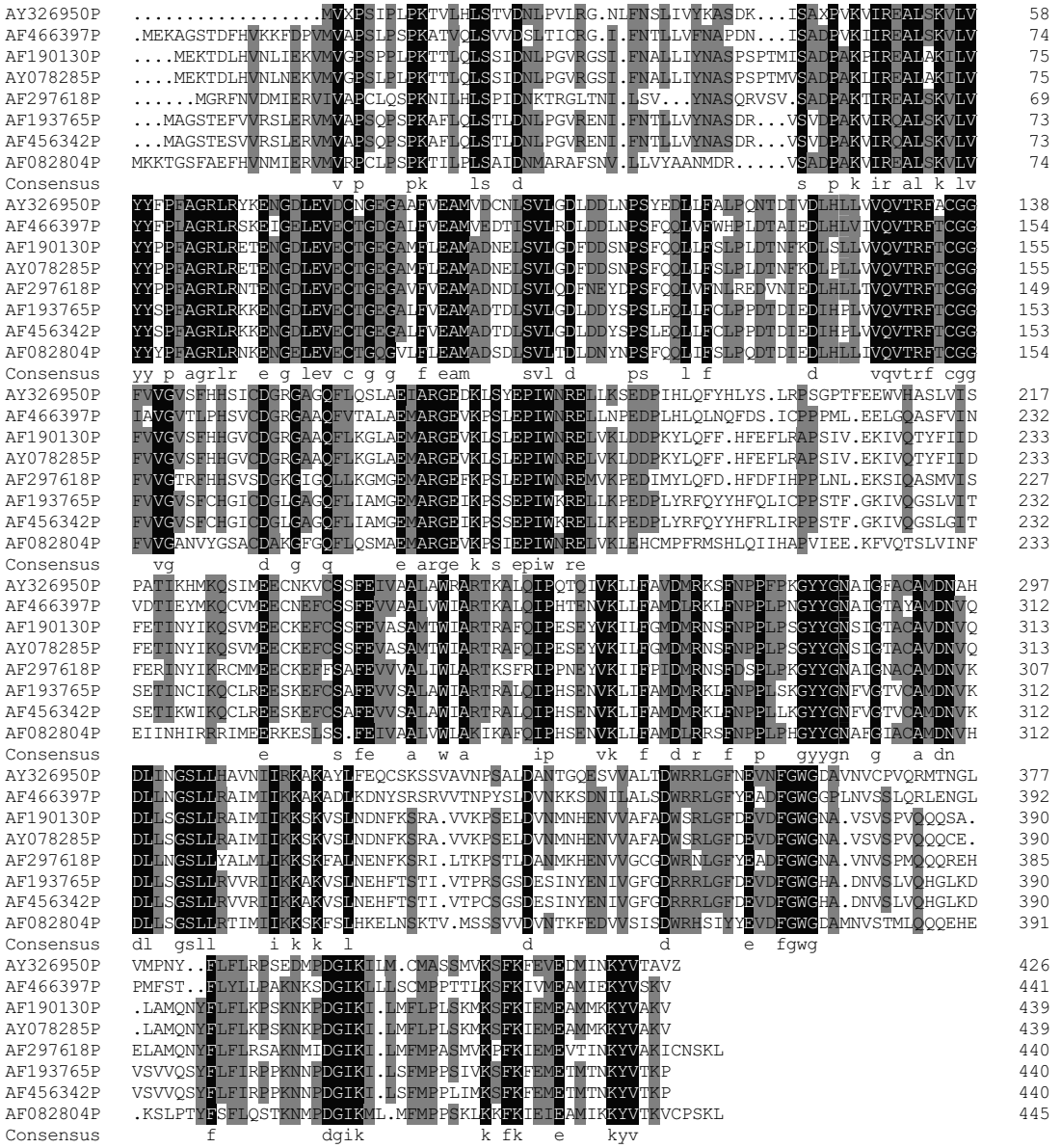

Fig. 2. Multiple alignment of the putative amino acid sequence of TCA1 with those of other cloned acyltransferases involved in taxol biosynthesis. The accession numbers of these acyltransferase sequences used were: taxane 2α-*O*-benzoyltransferase from *Taxus cuspidata* (AF297618), taxadien-5α-ol-*O*-acetyltransferase from *Taxus cuspidata* (AF190130), 10-deacetylbaccatinIIIβ-10-*O*-acetyltransferase from *Taxus cuspidata* and *Taxus baccata* (AF193765 and AF456342), 3′-*N*-debenzoyl-2′-deoxytaxol-*N*-benzoyltransferase from *Taxus canadensis* (AF466397), taxoid C-13 *O*-phenylpropanoyltransferase from *Taxus cuspidata* (AY082804), new cloned acyltransferase TCA1 from *Taxus chinensis* (AY326950).

relative amount of long-chain alkenoyl- and alkanoylation taxanes isolated from *Taxus chinensis* callus cells (Cheng *et al.*, 1996), we further deduced that TCA1 might be a new functional acyltransferase related to C-14 oxygenated taxane of long-chain alkenoyl- and alkanoylation.

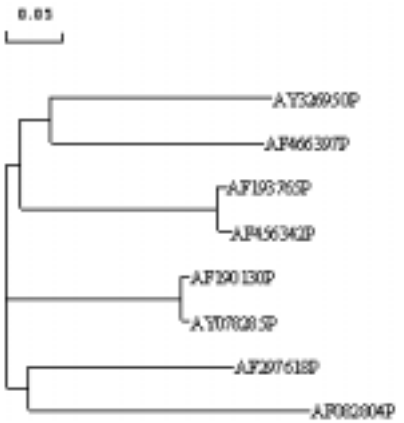

Fig. 3. Phylogenetic tree representation of sequence alignment of acyltransferase from *Taxus.* The tree is based on the alignment of all sequenced *Taxus* acyltransferases and is built by the neighbor-joining method according to Saitou and Nei's procedure (1987). The accession numbers of these acyltransferase sequences used were the same as in Fig. 2.

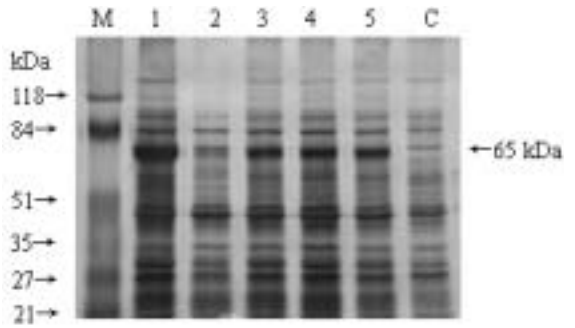

Fig. 4. 10% SDS-PAGE assay for the acyltransferase expression induced by variation of temperature and concentration of IPTG. The protein was analyzed on 10% SDS-PAGE stained with silver. A 15 μl protein sample was applied. Lane M, low molecular weight standards; lanes 1, 2, 3, 4 represent expression induction at 15 °C, 37 °C, 25 °C, 20 °C respectively by addition of 0.5 mM IPTG; lane 5 represents expression induction at 15 °C by addition of 1 mM IPTG; lane C represents expression induction at 15 °C by addition of 0.5 mM IPTG using the void vector pET-32a(+).

Expression of TCA1

The amplified TCA1-coding fragment (1,278 bp) from *Taxus chinensis* cDNA library was inserted into the pET-32a(+) vector, resulting in an expression vector for the new acyltransferase, designated pET32TCA1. The thioredoxin gene *trx*A within the cDNA the pET-32a(+) of vector was conserved to increase the solubility of foreign proteins in *E. coli* (Yasukawa *et al.*, 1995). Although native monomeric acyltransferases are generally soluble, we obtained a high-level expression of proteins by gene-fusion approach because it applies optimal translation initiation to prevent inclusion body formation and protein degradation (Forrer and Jaussi, 1998). In this work, a thioredoxin gene *trx*A was fused to the 5′-terminal of the acyltransferase gene of interest in order to increase the solubilization (Prinz *et al.*, 1997). First, we applied the usual culture conditions for expression of pET32TCA1 in our system. *E. coli* BL21*trxB*(DE3) cells transformed with the plasmid pET32TCA1 were grown in 100 ml LB medium containing 100 μg ampicillin ml^{-1} and 20 μg kanamycin ml^{-1} at 37 °C with agitation at 250 rpm until an $OD_{600\,nm}$ of 1 was reached. IPTG (1 mM) was then added to the medium, and incubation continued at 37 °C for 10 h. Under these conditions, the recombinant protein was expressed mostly as an insoluble inclusion body in *E. coli* determined by SDS-PAGE analysis (data not shown). Through further trial-and-error experiments, a set of culture conditions that allowed production of a reasonable amount of soluble enzyme was established as follows: decreased IPTG (0.5 mM) at mid growth phase ($OD_{600\,nm}$ of 0.7–0.8) on induction; reduced culture temperature (15 °C) and agitation speed (150 rpm) with an extended incubation time (16 h) after induction. The SDS-PAGE result shows that a protein with an apparent molecular mass of 65 kDa appeared, supporting that the new acyltransferase gene clone could be normally translated into a complete *trx*A-fusion protein as we predicted (Fig. 4).

The enzyme was expressed with a fused $(His)_6$-tag and purified via Nickel-affinity chromatography using a HiTrap Chelating HP column. The protein was eluted at approx. 80 mM imidazole and purified in a single step (data not shown). The purified His-tagged fusion-protein would be used for further enzymatic characterization. It will facilitate the detailed understanding of this putative acyltransferase gene. The utilization of these acyltransferase genes may facilitate increased production of taxol and may allow the biosynthetic generation of second-generation taxol analogs possessing greater bioactivity and improved water solubility with modified acyl groups and substitution patterns.

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