Video Article

Overexpression and Purification of Human *Cis*-prenyltransferase in *Escherichia coli*

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Abstract

Prenyltransferases (PT) are a group of enzymes that catalyze chain elongation of allylic diphosphate using isopentenyl diphosphate (IPP) *via* multiple condensation reactions. DHDDS (dehydrodolichyl diphosphate synthase) is a eukaryotic long-chain *cis*-PT (forming *cis* double bonds from the condensation reaction) that catalyzes chain elongation of farnesyl diphosphate (FPP, an allylic diphosphate) *via* multiple condensations with isopentenyl diphosphate (IPP). DHDDS is of biomedical importance, as a non-conservative mutation (K42E) in the enzyme results in *retinitis pigmentosa*, ultimately leading to blindness. Therefore, the present protocol was developed in order to acquire large quantities of purified DHDDS, suitable for mechanistic studies. Here, the usage of protein fusion, optimized culture conditions and codon-optimization were used to allow the overexpression and purification of functionally active human DHDDS in *E. coli*. The described protocol is simple, cost-effective and time sparing. The homology of *cis*-PT among different species suggests that this protocol may be applied for other eukaryotic *cis*-PT as well, such as those involved in natural rubber synthesis.

Video Link

The video component of this article can be found at https://www.jove.com/video/56430/

Introduction

Prenyltransferases are a group of enzymes that catalyze chain elongation of allylic diphosphate using isopentenyl diphosphate (IPP) *via* multiple condensation reactions [1,2]. Z-type enzymes catalyze the formation of *cis* double bonds from the condensation reaction, whereas E-type enzymes catalyze *trans* double bond formation [3]. *cis*-Prenyltransferases (*cis*-PT, Z-type enzymes) are classically classified according to their product chain length into short-chain (C_{15}), medium-chain (C_{50-55}), and long-chain (C_{70-120}) [4]. DHDDS (dehydrodolichyl diphosphate synthase) is a eukaryotic long-chain *cis*-PT that catalyzes chain elongation of farnesyl diphosphate (FPP, an allylic diphosphate) *via* multiple condensations with isopentenyl diphosphate (IPP) [1,5,6]. This results in the formation of dehydrodolichyl diphosphate, a C_{55-100}polyprenyl diphosphate serving as a precursor for dolichylpyrophosphate, the glycosyl carrier molecule involved in N-linked protein glycosylation [1]. Among Ashkenazi Jews, a missense non-conservative mutation (K42E) in DHDDS results in autosomal recessive retinitis pigmentosa [7,8]. Therefore, the present protocol was developed in order to acquire purified DHDDS suitable for mechanistic studies.

Escherichia coli is considered the most convenient and cost-effective host for recombinant protein expression, and is therefore also the most frequently used host. However, when one attempts to heterologously overexpress proteins in *E. coli*, protein-specific considerations should be made. Obtaining properly folded, active recombinant proteins from *E. coli*, is not a simple matter due to the distinct properties of different proteins. Numerous approaches have been developed to overcome these hurdles. Here, the usage of protein fusion, optimized culture conditions and codon-optimization were used to allow the overexpression and purification of functionally active human DHDDS in *E. coli*. Of note, a previous attempt to overexpress yeast *cis*-PT without protein fusion was unsuccessful due to complete insolubility even in the presence of detergent [12]. The described protocol is simple, cost-effective, time sparing and allows one to obtain DHDDS preparations suitable for mechanistic studies. Given the homology of *cis*-PT among different species, we suggest that this protocol may be applied for other eukaryotic *cis*-PT as well.

Protocol

1 . Cloning of *cis*-PT for Overexpression in *E. coli*

1. Obtain pET-32b expression vector, designed for cloning and high-level expression of protein sequences fused with the 109aa thioredoxin (TRX) protein [17], and *E. coli* codon-optimized [18] coding sequence of full-length *cis*-PT.
2. Take care to have a TEV-protease (tobacco etch virus protease) cleavage site (ENLYFQ/G, where "/" indicates the cleavage point) [9] followed by a short flexible linker (SGSGSG, to enhance cleavage site accessibility) upstream of the *cis*-PT sequence (**Figure 1A**). Also, add suitable 5' and 3' restriction sites for cloning.
3. Clone the synthesized DNA construct into the pET-32b vector using standard ligation techniques [10].

2 . Overexpression of Human DHDDS in *E. coli*

1. Transform *E. coli* T7 express competent cells with the TRX-fusion DHDDS construct according to the manufacturer's instructions.
2. Plate the transformed cells according to the manufacturer's instructions on LB-agar under ampicillin selective conditions (200 mg/L).
3. Inoculate selected colonies into 100 mL of LB medium in 250 mL flasks starter cultures with 100 mg/L ampicillin and incubate overnight at 37 °C with constant shaking at 200 rpm.
4. Inoculate 80 mL of the culture into two 5 L flasks (40 mL per flask), each containing 1.5 L of 2x YT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) with 100 mg/L ampicillin.
5. Incubate the culture at 37 °C with constant shaking at 180 rpm until reaching OD_{600nm} = 0.5.
6. Lower the temperature to 16 °C and induce the cells by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Continue incubating under the same condition for 16-20 h for protein expression.
7. Harvest the cells by centrifugation (10,000 x *g* for 10 min).
   Note: The protocol can be paused here; the cells pellet should be kept at -80°C until purification.

3 . Purification of Human DHDDS

1. Resuspend the cells in buffer A (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10 mM β-mercaptoethanol), 1 µg/mL DNase I, and a protease inhibitor mixture.
2. Homogenize the cells using a glass-Teflon homogenizer.
3. Disrupt the cells using a microfluidizer or equivalent at 12,000 - 15,000 psi [11].
4. Centrifuge the cell lysate at 40,000 x g for 45 min at 4 °C. Recover the supernatant, containing the soluble fraction.
5. Load the supernatant onto a 5 - 10 mL cobalt immobilized-metal affinity chromatography (Co^{2+}-IMAC)[12] column with 10 mM imidazole, followed by thorough washing with buffer A and 10 mM imidazole to reduce nonspecific protein binding.
6. Elute the overexpressed proteins with buffer A supplemented with 250 mM imidazole.
7. Remove imidazole using a 53 mL preparative desalting column equilibrated with buffer A.
8. Add 6xHis-tagged TEV protease (1 mg TEV protease per 50 mg protein) to the eluted proteins to remove their 6xHis-tagged TRX fusion protein at 4 °C overnight.
9. Load the cleaved proteins onto a Co^{2+}-IMAC column, equilibrated with buffer A supplemented with 5 mM imidazole, to remove the cleaved 6x His-tagged TRX fusion protein and TEV protease.
10. Collect the flow-through and concentrate to 3 - 4 mL using a 30 kDa molecular weight cut-off centrifugal filter.
11. Load the concentrated protein onto a superdex-200 size-exclusion chromatography column equilibrated with buffer A for final purification. The protein elutes as a dimer (~76 kDa).
12. Select relevant fractions by comparing the elution profile to a column calibration curve.
13. Assess the purity of the preparation using SDS-PAGE.
14. Determine protein concentration needed for downstream experimentation and flash-freeze aliquots of purified, concentrated proteins in liquid nitrogen and store at -80 °C until use.

4 . Analytical Size-exclusion Chromatography (SEC)

1. To ensure the ability of the protein to endure freeze-thaw cycles, thaw an aliquot of the frozen proteins and centrifuge at 21,000 x *g* for 10 min to remove insoluble aggregates. Collect the supernatant.
2. Load the proteins onto a superdex-200 analytical column pre-equilibrated with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% Triton X-100, 10 mM β-mercaptoethanol, using an ultra-performance liquid chromatography system [13,14].
3. Monitor tryptophan fluorescence (λ_{ex} = 280 nm, λ_{em} = 350 nm) to detect the proteins' eluting elution profile. Be sure to have a valid calibration curve for molecular weight assessment - such curves are available *via* the SEC column manufacturers.

5 . Enzyme Kinetics - Time-dependent Activity [15,16,17]

1. To ensure the activity of the purified protein, mix 5 µM of purified DHDDS with 10 µM FPP and 50 µM ^{14}C-IPP to initiate the reaction in buffer A with 0.5 mM $MgCl_2$ at 22 - 30 °C.
   Note: The exact reaction conditions may vary with preparations of *cis*-PTs different from DHDDS. Moreover, the activity of DHDDS also depends on the temperature and [Mg^{2+}].
   Caution: ^{14}C-IPP is radioactive and should be used according to local radiation safety regulations.

2. Withdraw 15 µL samples from the reaction at 0, 2, 4 and 6 h, following immediate quenching of the reaction by the addition of 15 µL buffer A supplemented 20 mM EDTA (to a final concentration of 10 mM EDTA).
3. Add 1 mL H_2O-saturated 1-butanol and vortex thoroughly to extract the reaction products.
   Note: The protocol can be stopped here and the samples can be kept for later reading.
4. Add scintillation cocktail to the samples and, using a scintillation counter, quantitate reaction products in the butanol phase encompassing ^{14}C, together with the radioactivity of 15 µL from the reaction mixture, representing the total radioactivity.
5. Subtract the background readings measured using the 0 h samples from each time point and calculate the percent of ^{14}C-IPP utilized.
6. Calculate the net ^{14}C-IPP incorporation at each time point by calculating the percent utilized from the total ^{14}C-IPP concentrations, and plot the results as a function of time. An increase in net ^{14}C-IPP incorporation with time is expected.

Representative Results

General overview of the construct used here and the purification process are shown in **Figure 1**. The samples obtained at each purification step are shown in **Figure 2**. This SDS-PAGE analysis shows the stepwise purification of DHDDS, resulting in a highly purified product. **Figure 3** shows the results of analytical SEC of the purified enzyme, revealing that the protein is only observed as a homodimer. **Figure 4** shows a representative time-dependent activity assay. ^{14}C-IPP incorporation clearly rises over 6 h, verifying that the purified enzyme is functional.

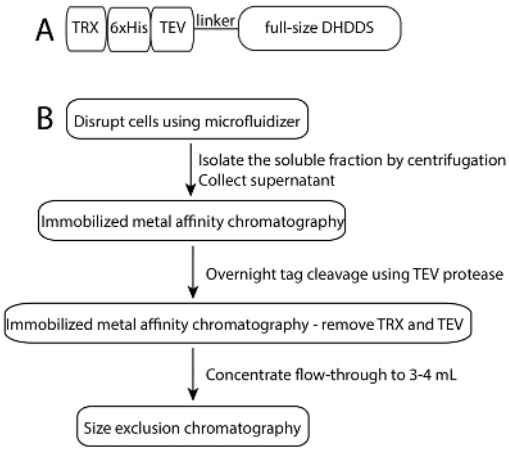

Figure 1: Human DHDDS Cloning and Purification. (**A**) The construct used in this study. (**B**) Outline of the human DHDDS purification protocol described here. Please click here to view a larger version of this figure.

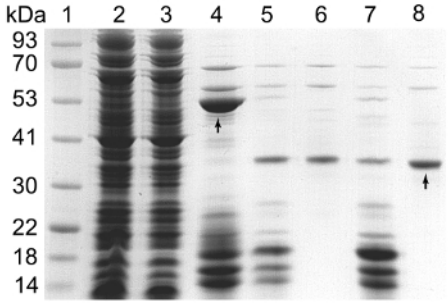

Figure 2: Purification of Human DHDDS. SDS-PAGE analysis of human DHDDS affinity purification steps. Lane 1, molecular-weight marker (kDa); lane 2, crude extract; lane 3, Co^{2+}-IMAC flow-through. Arrow indicates the TRX-DHDDS fusion protein; lane 4, Co^{2+}-IMAC eluate; lane 5, protein-TEV protease mixture following overnight incubation; lane 6, Co^{2+}-IMAC flow-through following TEV cleavage; lane 7, Co^{2+}-IMAC eluate following TEV cleavage; lane 8, purified human DHDDS (indicated by an arrow) following size-exclusion chromatography. Please click here to view a larger version of this figure.

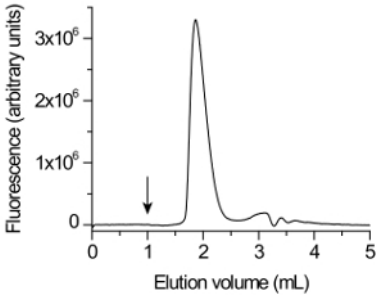

Figure 3: Analytical SEC of Purified Human DHDDS. Tryptophan fluorescence was monitored as described in the protocol. According to the calibration curve of this column, DHDDS forms a homodimer (77.4 kDa). The arrow indicates the void volume. Please click here to view a larger version of this figure.

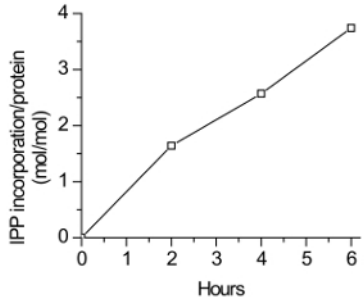

Figure 4: Time-dependent Activity of Purified Human DHDDS. *In vitro* activity of purified human DHDDS in the reaction with 10 µM FPP and 50 µM ^{14}C-IPP as substrates is expressed as IPP incorporation per protein (mol/mol). Please click here to view a larger version of this figure.

Discussion

The protocol described here for purification of functional human DHDDS in *E. coli* cells is simple and efficient, allowing one to overexpress and purify the protein in 3 - 4 days once a suitable construct is available. Such protocols for protein purification are of special significance given the breakthroughs in genome sequencing, which provided plethora of information regarding the genetics of many diseases [18], thereby requiring the development of high-throughput methods to characterize pathogenic mechanisms at the protein level [19].

To overcome common pitfalls in heterologous protein overexpression and purification, several measures were taken here, which are critical to successfully obtain soluble and functional proteins. First, DHDDS was overexpressed as a TRX-fusion. TRX facilitates the fusion protein folding and increases the fusion protein solubility, preventing inclusion body formation [20]. Next, to increase protein yield, the DNA construct was codon optimized for expression in *E. coli* [21]. Finally, to further reduce the likelihood of inclusion body formation, expression of the protein was induced at 16 °C to slow down the protein synthesis rate, likely assisting protein folding [22].

Taking into consideration the homology of *cis*-PT among different species, we suggest that this protocol may be applied for other eukaryotic *cis*-PT as well [1]. Using the current protocol as a starting point, and given the general guidelines critical for DHDDS expression, this method can be modified for optimal expression of different *cis*-PTs. For example, one may attempt different fusion partners (such as maltose-binding protein) or further optimize the culture conditions for the specific protein to be studied.

With their biomedical and biotechnological significance [1,7,8], future usage of the described protocol to obtain large quantities of purified functional DHDDS, together with other *cis*-PT, allowing the pursuit of their structural and functional characterization. For example, further molecular studies of DHDDS will resolve the mechanisms underlying DHDDS-related *retinitis pigmentosa*, potentially leading to discovery and development of novel therapeutic approaches. In addition, as prokaryotic *cis*-PTs are involved in bacterial wall synthesis, this group of enzymes potentially forms novel targets for new antibacterial agents. Indeed, thorough characterization of the eukaryotic and prokaryotic enzymes studies may allow the rational development of antimicrobial drugs selective for prokaryotic *cis*-PT. Finally, as natural rubber is synthesized by members of the *cis*-PT family, the approach described here may find future use in natural rubber industrial synthesis.

Disclosures

The authors have nothing to disclose.

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