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Refolding and Characterization of a Yeast Dehydrodolichyl Diphosphate Synthase Overexpressed in *Escherichia coli*

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Dehydrodolichyl diphosphate synthase (DDPPs) catalyzes the sequential condensation of isopentenyl diphosphate with farnesyl diphosphate to synthesize long-chain dehydrodolichyl diphosphate, which serves as a precursor of glycosyl carrier in glycoprotein biosynthesis in eukaryotes. To perform kinetic and structural studies of DDPPs, we have expressed yeast DDPPs using *Escherichia coli* as the host cell. Thioredoxin and His tag were utilized to increase the solubility of the recombinant protein and facilitate its purification using Ni–nitrilotriacetic acid (NTA) column. The protein was overexpressed in *E. coli* but mostly existed in pellet in the absence of detergent. The low quantity of soluble DDPPs was purified using Ni–NTA, Mono Q anion-exchange, and size-column chromatographies. The protein in the pellet was solubilized with 7 M urea and purified using Ni–NTA under denaturing condition. The protein refolding was achieved via the stepwise dialysis to remove the denaturant in the presence of 6 mM β-mercaptoethanol. Detergent *n*-octyl-β-D-glucopyranoside and Triton X-100 increased the solubility of the DDPPs so that refolding can be performed at higher protein concentration. Alternatively, on-column refolding was carried out in a single step to obtain the active protein in large quantities. β-Mercaptoethanol and Triton were both required in this quick refolding process. The kinetic studies indicated that the soluble and refolded DDPPs have comparable activities ($k_{cat} = 2 \times 10^{-4}$ s^{-1}). Unlike its bacterial homologue, undecaprenyl diphosphate synthase, yeast DDPPs activity was not enhanced by Triton.

Prenyltransferases are a group of enzymes which catalyze the chain elongation of allylic diphosphate via multiple condensations using isopentenyl diphosphate (IPP)[2] (1). *Z*- and *E*-type enzymes have been identified to catalyze the formation of *cis* and *trans* double bonds from the condensation reactions, respectively (2). The *Z*-type prenyltransferases mediate the synthesis of long-chain polyprenyl diphosphate compounds such as dehydrodolichyl diphosphate (DDPP), while the *E*-type enzymes catalyze the shorter products ranging from C_{10} to C_{50} (3). An exception is a short-chain (C_{15}) *Z*-type farnesyl diphosphate synthase (FPPs) recently identified from *Mycobacterium tuberculosis* (4). Two major *Z*-type enzymes have been discovered, which include undecaprenyl diphosphate synthase (UPPs) in prokaryotes and dehydrodolichyl diphosphate synthase (DDPPs) in eukaryotes. UPPs synthesizes a C_{55} long-chain polymer undecaprenyl diphosphate (UPP) by successive addition of eight molecules of IPP to farnesyl diphosphate (FPP). UPP in the bacterial cell membrane undergoes dephosphorylation and links with lipid II for its transport to outer membrane compartments, where the cell wall is made. UPPs encoding gene was discovered in *Micococcus luteus* and *Escherichia coli*, and many other UPPs homologues were identified from the genomic database (5–7). Its homologue in eukaryotes

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[2] Abbreviations used: IPP, isopentenyl diphosphate; DDPP, dehydrodolichyl diphosphate; DDPPs, dehydrodolichyl diphosphate synthase; UPPs, undecaprenyl diphosphate synthase; UPP, undecaprenyl diphosphate; FPPs, farnesyl diphosphate synthase; FPP, farnesyl diphosphate; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni–NTA, nickel–nitrilotriacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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is DDPPs that is involved in dolichol synthesis. DDPPs enzyme activity was previously found to be primarily membrane bound in the yeast (8, 9). Recently, the gene (Rer2) encoding DDPPs was identified from yeast mutants which were deficient in the dolichol synthesis and mislocalized an endoplasmic reticulum protein indicating the essential role of the enzyme in protein glycosylation (10, 11). As shown in Fig. 1, DDPPs catalyzes the IPP condensation reaction in the same stereochemical way (*cis*) as UPPs but results in a variety of different chain lengths. In yeast strain *Saccharomyces cerevisiae*, C_{60}-C_{75} polymers were identified as *in vitro* products of DDPPs enzyme, which were approximately two isoprene units shorter than endogenous dolichyl phosphate (9).

Compared to the *cis*-prenyltransferases, a larger number of *E*-type polyprenyl diphosphate synthases have been found in nature. Farnesyl diphosphate synthase (FPPs) is best studied among them. The crystal structure of FPPs has been solved and provided insight of the *E*-type prenyltransferase reaction mechanism (12). Other *E*-type enzymes share sequence similarity with FPPs and also possess two common DDXXD motifs which are important in substrate binding and catalysis as evident from site-directed mutagenesis and crystallographic studies (13–15). As shown in crystal structure of FPPs, the two motifs are located in the immediate vicinity of the proposed active site (13). There, the active-site aspartate side chains chelate a Mg^{2+} ion that is required for enzyme activity and substrates are bound through the interaction of the diphosphate moiety with Mg^{2+}. However, the *Z*-type enzymes including UPPs and DDPPs show no sequence homology to the *E*-type enzymes and lack the Asp-rich motif. To better understand *Z*-type prenyltransferases, we have expressed and purified *E. coli* UPPs for mechanistic studies and performed the site-directed mutagenesis to examine the roles of the conserved residues (16, 17). Since UPPs plays an essential role in bacterial cell wall biosynthesis, its specific inhibitor may become the lead compound for the development of new antibacterial agents. The ideal drug must not interfere with DDPPs reaction. Therefore, the structural and inhibition studies of DDPPs are crucial but large amount of DDPPs was not available. Since the gene (Rer2) coding for yeast DDPPs is known, we have chosen to overexpress it using *E. coli* and refolded it into active enzyme from pellet. We report here a convenient way to prepare highly pure DDPPs in large quantities as well as its characterization.

MATERIALS AND METHODS

Materials

The protein expression kit containing vector pET32LIC/Xa and competent cells *E. coli* JM109 and BL21 (DE3) was purchased from Novagene. *Taq* polymerase for PCR reaction was obtained from Gibco Life Technologies. The genomic DNA extraction kit, plasmid miniprep kit, and DNA elution kit were purchased from Qiagen. The yeast strain *S. cerevisiae* was obtained from the Culture Collection and Research Center of Taiwan. The culture medium containing yeast extract and tryptone peptone was purchased from Difco. Ni–NTA for the His-tagged protein purification was obtained from Qiagen. Thrombin cleavage capture kit containing biotinylated thrombin and streptavidin agarose was purchased from Novagen. The enzyme substrate [1-^{14}C]IPP (55 mCi/mmol) was obtained from Amersham Pharmacia Biotech and FPP was purchased from Sigma. Potato acidic phosphatase was product of Boehringer Mannheim. Reverse-phase TLC (RP-18 F_{254s}) was obtained from Merck. The detergent *n*-octyl-β-D-glucopyranoside was obtained from Sigma and Triton X-100 was from Calbiochem. All other reagents were of highest commercial grade.

DDPPs Expression

The culture medium containing 0.5% yeast extract, 1% tryptone peptone, and 2% glucose was utilized to grow yeast *S. cerevisiae* and its genomic DNAs was obtained using genomic DNA extraction kit. DDPPs gene was amplified by using polymerase chain reaction (PCR) method with forward primer 5′ ATGGAAACG-GATAGTGGT 3′ and backward primer 5′ GCAGACCT-TTCTTAATTCAAC 3′. The PCR was performed for 30 cycles of DNA denaturing at 94°C for 1 min, annealing at 38°C for 2 min, and polymerizing at 68°C for 40 s with a 5-min preheat at 94°C, and allowing final extension at 68°C for 7 min. The PCR product was subjected to electrophoresis and the band of correct size was sliced from the 0.8% agarose gel and then recovered by using DNA elution kit. Using the purified DDPPs gene as template, the second PCR was conducted to create sites for ligation with pET32Xa/LIC vector. The forward and backward primers were 5′ GGTATTGAGGGTCGCAT-GGAAACGGAT 3′ and 5′ AGAGGAGAGTTAGAGCCC-TTAATTCAACTT 3′, respectively. The purified PCR product was treated with T4 DNA polymerase in the presence of dGTP at 22°C for 30 min and mixed with the vector. The recombinant plasmid was then transformed into competent cell *E. coli* JM109 using a heat shock process. A single colony was selected from the overnight growth of transformed cells on an agar plate containing 100 μg/mL ampicillin. The DNA obtained from overnight culture of the single colony was completely sequenced using dideoxy chain termination method. The plasmid containing DDPPs gene with the same sequence as reported for the yeast *S. cerevisiae* DDPPs was finally transformed into the host *E. coli* BL21 for protein expression. For large-scale protein preparation,

a 5-mL overnight culture in LB medium containing 100 μg/mL ampicillin was used to inoculate each of the 500 mL fresh LB medium in a 2-L flask. The cells were grown at 30°C for 5 h before adding 1 mM IPTG for DDPPs induction. After 2 h of induction at 30°C, the cells were harvested by centrifugation.

Purification of Recombinant DDPPs

For the purification of recombinant DDPPs in absence of detergent, the 5 g of cell paste collected from 1 L of culture was suspended in 50 mL of lysis buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, and 6 mM β-mercaptoethanol. The following purification procedure was carried out at 4°C. The cells were disrupted at 12000 psi by using a French pressure cell press instrument (Sim-Aminco). The lysate was centrifuged at 10,000*g* to separate soluble proteins from pellet. The soluble lysate was loaded onto a 5-mL Ni–NTA column equilibrated with lysis buffer plus 5 mM imidazole. The column was exhaustively washed with the same buffer until the A_{280} absorbance reached the baseline. The buffer containing 30 mM imidazole was used to further wash the column and finally the DDPPs with His tag was eluted from the column using the buffer containing 300 mM imidazole. Since the purity of DDPPs was poor owing to its low quantity, the eluted 30-mL solution was dialyzed against 2 × 2 L 25 mM Tris (pH 7.5) and 6 mM β-mercaptoethanol to remove the salt for further purification using anion-exchange chromatography. The dialyzed sample was applied to a Mono Q column (5 mm × 5 cm, Amersham Pharmacia Biotech) and eluted with 20 column vol of NaCl gradient (0–0.5 M) in the same buffer. The fractions containing DDPPs according to SDS–PAGE analysis were pooled and concentrated and Superdex G-75 column chromatography (10 mm × 30 cm, Amersham Pharmacia Biotech) was used to achieve high purity. The elution buffer was 25 mM Tris (pH 7.5), 150 mM NaCl, and 6 mM β-mercaptoethanol.

In the purification of recombinant DDPPs soluble in 0.1% Triton X-100, a 10-mL Ni–NTA column was used. The wash and elution buffers for the Ni–NTA column chromatography were as described above and also included 0.1% Triton X-100.

Refolding of DDPPs from Inclusion Bodies

The pellet collected by centrifugation of the disrupted cells in the absence of detergent was stirred overnight with 50 mL of 25 mM Tris (pH 7.5), 150 mM NaCl, and 7 M urea to dissolve DDPPs. The solution with denatured protein was loaded in a 20-mL Ni–NTA column and washed with the same buffer containing 5 mM imidazole. Protein was then eluted with a 25 mM Tris (pH 7.5), 150 mM NaCl, 7 M urea, and 300 mM imidazole buffer. The purified recombinant protein was diluted to 0.1 mg/mL with the elution buffer and refolded via stepwise dialysis against 10 vol of buffer [25 mM Tris (pH 7.5), 150 mM NaCl, and 6 mM β-mercaptoethanol] each time until the urea concentration was negligible. The refolding was also performed in the presence of detergent (0.1% Triton X-100 or 0.05% *n*-octyl-β-D-glucopyranoside) with ~1 mg/mL protein.

Alternatively, the protein was refolded on the Ni–NTA column. The protein sample in urea was loaded onto a 20-mL Ni–NTA column. After loading, the column was washed with 5 column vol of buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 6 mM β-mercaptoethanol, 0.1% Triton X-100, and 5 mM imidazole. The bound protein was eluted with solution of 300 mM imidazole in 25 mM Tris (pH 7.5), 150 mM NaCl, 6 mM β-mercaptoethanol, and 0.1% Triton X-100.

Tag Cleavage Using Thrombin

The purified DDPPs (soluble, stepwise refolded, or on-column refolded) was subjected to thrombin protease cleavage to remove the thioredoxin and His tag located in the N-terminal region of the fusion protein. The thioredoxin fusion vector contains a FXa and a thrombin recognition sequences positioned at the junction between the thioredoxin and the N-terminus of the DDPPs. Since FXa was inhibited by Triton and the cleavage reaction proceeded slowly, thrombin (2 units) was used per milligram of DDPPs at 4°C for 15 h to complete tag removal. The biotinylated thrombin was captured using streptavidin–agarose and centrifugation was carried out to remove the thrombin from the solution according to manufacturer's instruction. The solution was loaded onto a 10-mL Ni–NTA column and the buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 6 mM β-mercaptoethanol was utilized to elute the untagged protein.

Kinetic Constant Measurements[3]

The 5 μM DDPPs was added to initiate the enzyme reaction in the presence of various concentrations of FPP (5–50 μM) and [^{14}C]IPP (10–100 μM) in a buffer of 100 mM Hepes (pH 7.5), 0.5 mM $MgCl_2$, 50 mM KCl, and 6 mM β-mercaptoethanol at pH 7.5 and 25°C in the absence or presence of 0.1% Triton X-100. Within 10% substrate consumption, a portion (40 μL) of the reaction mixture was withdrawn every hour for 4 h and mixed with 10 mM EDTA (final concentration) to terminate the reaction. The solution was mixed with an equal volume of 1-butanol and was vortexed to extract polyprenyl products. The products with ^{14}C label in butanol phase were quantitated using a Beckman scintillation counter. The kinetic constants were obtained by

[3] The DDPPs steady-state k_{cat} measured here was based on IPP consumption.

fitting data (initial rate vs substrate concentration) to Michaelis–Menten equation using computer program KaleidaGraph (Synergy software).

Product Analysis

The 200-μL reaction mixture containing 5 μM DDPPs, 5 μM FPP, 200 μM [^{14}C]IPP, 0.5 mM $MgCl_2$, and 50 mM KCl in 100 mM Hepes buffer (pH 7.5) was incubated at 25°C for 65 h to ensure complete reaction. The enzyme reaction was terminated with 10 mM EDTA and the products were extracted with an equal volume of 1-butanol. The butanol phase collected in an Eppendorf tube was dried under N_2. To the Eppendorf tube, a total volume of 100 μL solution containing 20% propanol, 4.4 units/mL acidic phosphatase, 0.1% Triton X-100, and 50 mM sodium acetate (pH 4.7) was added to hydrolyze the products according to the procedure of Fujii *et al.* (18). The polyprenols were extracted with *n*-hexane and analyzed by reverse-phase TLC using the mobile phase acetone/water (19/1). The distribution of radiolabeled products on TLC was examined using a BAS-1500 bioimaging analyzer (Fujifilm). For comparison, the *E. coli* UPPs reaction products C_{55}-C_{60} and C_{55}-C_{75} were generated in the presence and absence of 0.1% Triton X-100, respectively, using 5 μM FPP and 50 μM [^{14}C]IPP as substrates in buffer containing 100 mM Hepes (pH 7.5), 0.5 mM $MgCl_2$, and 50 mM KCl at 25°C. The 0.2 μM UPPs was employed in the 65-h reaction and the enzyme products were extracted with 1-butanol, hydrolyzed to alcohols, and analyzed by TLC as described above.

RESULTS AND DISCUSSION

The expression level of yeast DDPPs as a thioredoxin fusion protein in *E. coli* BL21 was 75–100 mg/L culture. When disrupting the cells in the presence of 0.1% Triton X-100, approximately 25% of the total DDPPs protein

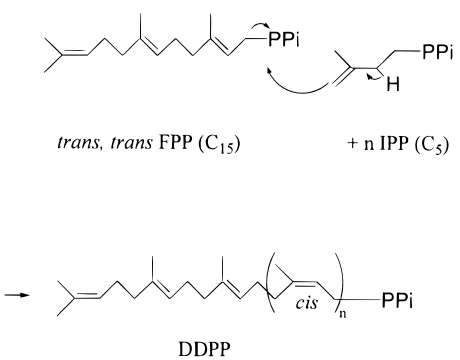

FIG. 1. The chain elongation of farnesyl diphosphate with isopentenyl diphosphate catalyzed by dehydrodolichyl diphosphate synthase. The condensation reactions lead to the formation of *cis* double bonds.

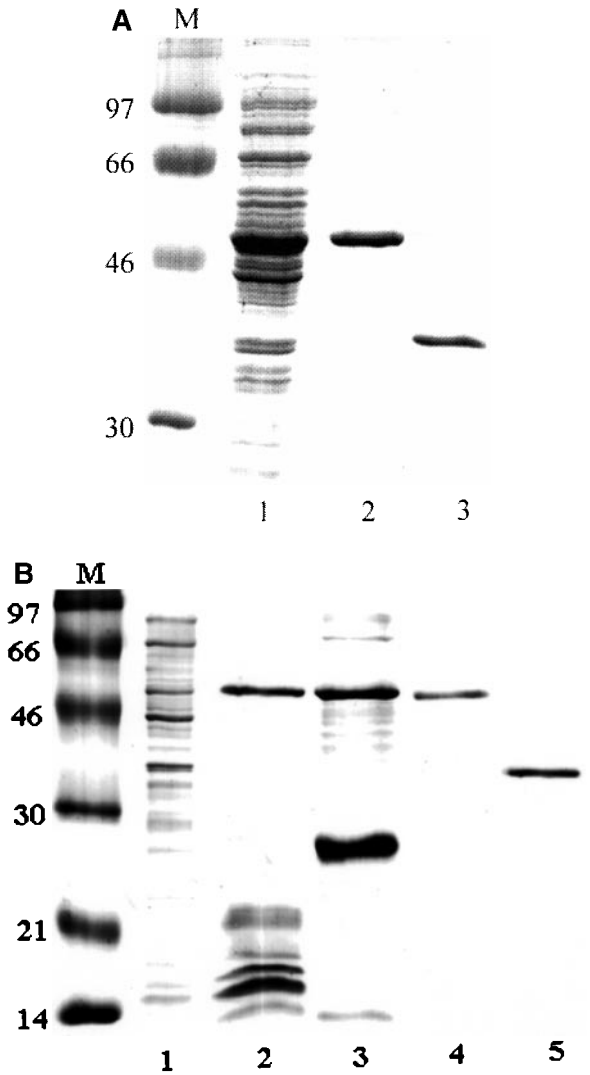

FIG. 2. Purification of recombinant DDPPs from *E. coli* cell lysate in the presence of 0.1% Triton X-100 (A) and in the absence of Triton (B). SDS–PAGE stained with Coomassie blue was utilized to analyze purity of enzyme at each purification stage. In (A), the protein in cell lysate, after Ni–NTA column and after tag cleavage, is shown in lanes 1–3, respectively. In (B), lanes 1–4 represent cell lysate, DDPPs eluted from Ni–NTA with 300 mM imidazole buffer, the enzyme after Mono Q anion-exchange column, and after Superdex G-75 size-exclusion column, respectively. Lane 5 is the DDPPs after thrombin cleavage to remove thioredoxin and His tag.

was soluble and the rest of the protein was in the pellet. The soluble DDPPs was purified using an Ni–NTA column in the presence of 0.1% Triton X-100 and the tag was cleaved using thrombin. About 14 mg of purified protein was obtained from 1 L culture. The protein in cell lysate, after Ni–NTA column, and after tag cleavage is shown in Fig. 1A, lanes 1–3, respectively. In contrast, the DDPPs expressed in pET28 vector without thioredoxin was completely insoluble even in the presence of Triton (data not shown) indicating the advantage of

TABLE 1

Purity and Yield in Each Step of the Soluble and Inclusion Body Purified Recombinant DDPPs from 1 L *E. coli* BL21 Cell Paste

Purification step	Total protein (mg)	DDPPs (mg)	Purification fold	Recovery yield (%)
Cell lysate[a]	130	1.4[b]	1	100
Ni–NTA column[a]	8	1.2[b]	15	86
Mono Q[a]	2.7	0.8[b]	30	57
Superdex G-75[a]	0.6	0.5[b]	93	36
Tag cleavage[a]	0.1	0.1	99	7
Pellet in 7 M urea[c]	110	88[b]	1	100
Ni–NTA column[c]	78	74[b]	1.2	84
Refolding and tag cleavage[c]	46	46	1.2	52

[a] Steps in purification of soluble DDPPs.
[b] The amount of recombinant DDPPs was estimated from the protein band intensity in SDS–PAGE gels after scanning and imagine analysis. The unexpressed cell lysate was used as control to calculate the quantity of DDPPs in the cell lysate.
[c] Steps in purifying DDPPs from inclusion bodies.

thioredoxin in enhancement of protein solubility. In order to prepare detergent-free soluble DDPPs for comparison of activity with the DDPPs in Triton (Triton could activate or inhibit the enzyme, see below), we prepared the cell lysate in the absence of Triton. Under this condition, it was estimated that only 1% of the total thioredoxin fusion DDPPs was soluble. The low quantity of soluble DDPPs was partially purified using Ni–NTA in the absence of detergent. This protein was eluted from Ni–NTA by 300 mM imidazole buffer and dialyzed and was further purified using a Mono Q anion-exchange column and a Superdex G-75 size-exclusion column. With this purity, the tag could be successfully removed using thrombin. The purity of soluble DDPPs at each stage of purification process is shown in the SDS–PAGE (Fig. 2B). The protein in cell lysate, after Ni–NTA column, Mono Q column and size-column chromatographies is shown in Fig. 2B, lanes 1–4, respectively. The recombinant DDPPs without the tag is shown in lane 5. Approximately 0.1 mg of recombinant protein as shown in lane 5 was obtained from a 1-L culture. Table 1 summarizes the yield and purity of recombinant DDPPs in each step of the purification procedure.

In the absence of Triton, the recombinant DDPPs was almost insoluble. We then developed a refolding process to prepare active DDPPs from inclusion bodies. The DDPPs in the pellet without detergent was solubilized in a buffer containing 7 M urea. The denatured protein was purified using Ni–NTA and protein refolding was subsequently performed via stepwise dialysis using four different conditions. These included (i) buffer [20 mM Tris (pH 7.5) and 150 mM NaCl] only, (ii) buffer plus 6 mM β-mercaptoethanol, (iii) buffer with 6 mM β-mercaptoethanol and 0.1% Triton X-100, and (iv) buffer containing 6 mM β-mercaptoethanol and 0.05% (1.7 mM) *n*-octyl-β-D-glucopyranoside. Detergents *n*-octyl-β-D-glucopyranoside and Triton X-100 increase yeast DDPPs solubility which was primarily membrane associated, but are inhibitory above their critical micelle concentrations (8, 9). In the presence of β-mercaptoethanol, refolding was successful and the refolded DDPPs showed enzyme activity. In contrast, without β-mercaptoethanol, the protein was inactive after removal of 7 M urea by dialysis. The detergents *n*-octyl-β-D-glucopyranoside and Triton X-100 increased the solubility of refolded protein so that the refolding could be performed at about 1 mg/mL protein concentration without any precipitation rather than 0.1 mg/mL in absence of detergent. The enzyme refolded under the conditions with β-mercaptoethanol and in the presence of *n*-octyl-β-D-glucopyranoside or Triton X-100 showed similar activities as purified recombinant DDPPs from the soluble cell lysate ($k_{cat} = 2 \times 10^{-4}$ s^{-1}). Although the recombinant DDPPs refolded in the buffer without β-mercaptoethanol failed to show any activity, the soluble DDPPs purified in the presence or absence of β-mercaptoethanol exhibited almost no difference in activity. This indicated that as long as the enzyme folded correctly, the reducing agent was not required for enzyme activity and the reducing agent was absolutely needed during the refolding of DDPPs. The SDS–PAGE analysis of the DDPPs purification from pellet and refolding through stepwise dialysis is shown in Fig. 3A. Pellet contained a large fraction of recombinant DDPPs (lane 1) that can be easily purified using Ni–NTA (lane 2). The protein after refolding and tag cleavage is shown in lanes 3 and 4, respectively. The purity and yield of DDPPs purified from inclusion bodies are also summarized in Table 1.

On-column refolding was performed in a single-step preparation of DDPPs without the need of time-consuming stepwise dialysis. This method was reported previously for convenient preparation of eukaryotic proteins from *E. coli*-based expression systems (19). The recombinant DDPPs with His tag was bound to Ni–NTA

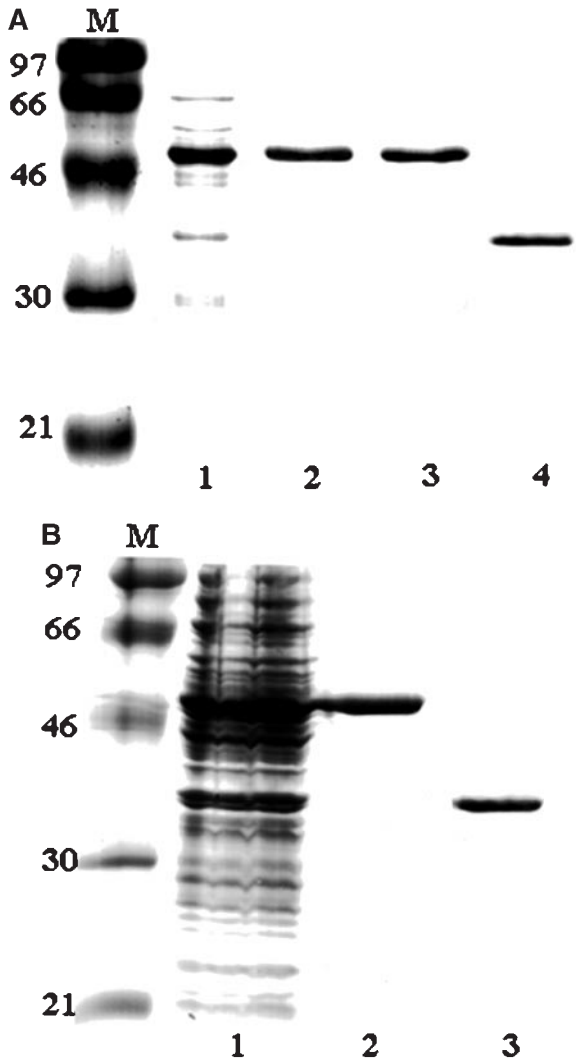

FIG. 3. DDPPs purification and refolding from pellet of *E. coli* via (A) stepwise dialysis and (B) on-column refolding. In (A), the SDS–PAGE shows the DDPPs in pellet (lane 1), eluted from Ni–NTA with 300 mm imidazole–urea buffer (lane 2), refolded through stepwise dialysis to remove urea (lane 3), and DDPPs without tag (lane 4). In (B), the protein in the pellet, refolded on the Ni–NTA column and eluted by 300 mM imidazole buffer containing 6 mM β-mercaptoethanol and 0.1% Triton X-100 and the thrombin cleaved DDPPs is shown in lanes 1–3, respectively, of the SDS–PAGE gel stained with Coomassie blue.

TABLE 2

Kinetic Constants of Recombinant DDPPs and UPPs in the Absence and Presence of 0.1% Triton X-100

Enzyme/condition	k_{cat} (s^{-1})
DDPPs	2.7×10^{-4}
DDPPs/0.1% Triton	2×10^{-4}
UPPs	0.013[a]
UPPs/0.1% Triton	2.5[a]

[a] Data taken from Ref. (16).

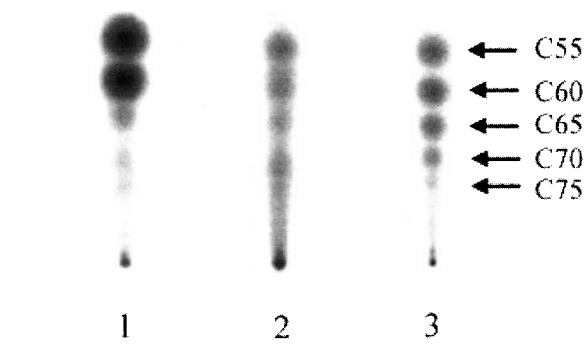

FIG. 4. Final products of recombinant DDPPs (5 μM) catalyzed condensation reactions of isopentenyl diphosphate (200 μM) with farnesyl diphosphate (5 μM). Serving as references, lanes 1 and 2 represent the products C_{55}-C_{60} and C_{55}-C_{75} generated by *E. coli* UPPs in the presence and absence of Triton X-100, respectively. The major products of recombinant yeast DDPPs as shown in lane 3 were C_{55}-C_{75} polymers in the absence of Triton.

column under denaturing conditions. The refolding of protein was accomplished by omitting the denaturant from the wash and elution buffer. The activity of the DDPPs refolded in the presence of both β-mercaptoethanol and 0.1% Triton X-100 also showed similar activity. Without either one of the two agents, the refolding failed and the protein was inactive. This was in contrast to the stepwise dialysis in which only β-mercaptoethanol was required for correct folding and Triton acted to increase protein solubility. Apparently, Triton facilitated the rapid folding in the process of on-column refolding. Nonionic detergents assist the refolding of guanidinium chloride-denatured Rhodance via their interaction with refolding intermediate (20). Figure 3B displays the SDS–PAGE analysis of purification and the on-column refolding of recombinant DDPPs. Lane 1 shows the recombinant DDPPs in pellet. The refolded DDPPs on Ni–NTA column was eluted by 300 mM imidazole (lane 2) and treated with thrombin to yield protein without tag (lane 3). The purification of DDPPs by on-column refolding showed similar fold of purification and yield as stepwise refolding (see Table 1).

To confirm that the recombinant DDPPs after refolding was active, kinetic constants were determined. The IPP K_m value was 27 μM and the k_{cat} value was 2×10^{-4} s^{-1}. The K_m value of FPP could not be measured because it was lower than the enzyme concentration (5 μM) used in the assay. Compared to the specific activity of 50–65 pmol DDPP synthesized/h/mg reported for the native DDPPs prepared from *S. cerevisiae* (9), our recombinant DDPPs was approximately 20-fold more active.[3] However, the recombinant DDPPs activity measured under steady-state condition was much lower than that of bacterial UPPs in the presence of Triton (see Table 2). Previously, we have identified that the product release was slow in the absence of Triton and Triton

increased the UPPs steady-state activity by accelerating the product release so that chemistry (IPP condensation) became the rate-limiting step (16). Under the single turnover condition with UPPs in excess of FPP, the enzyme activity 2.5 s^{-1} was much faster than 0.013 s^{-1} measured under the steady-state condition in absence of Triton where the slow product release limited the reaction rate. In the presence of Triton, the steady-state rate of UPPs was much higher and equal to the rate constant (2.5 s^{-1}) of IPP condensation. However, the 0.1% Triton X-100 has shown no stimulation effect on the recombinant DDPPs activity (Table 2).

Previously, the dolichols isolated from many species have a variety of chain lengths (7–9). Since the recombinant DDPPs after refolding was active, its product distribution was further examined. As shown in Fig. 4, the enzyme products contained different number of isoprene units, which were determined by comparing to the UPPs products with known chain lengths. The major product of UPPs was C_{55} polymer in the presence of Triton X-100 as shown in lane 1 (C_{60} was also produced after extended incubation time). Without Triton, UPPs generated C_{55}-C_{75} polyprenyl diphosphates (lane 2) as reported previously (16). For recombinant DDPPs catalysis in the absence of Triton, the large polymers C_{55}-C_{75} were seen in the TLC imagining data consistent with C_{60}-C_{75} observed for the native enzyme (9).

CONCLUSION

In summary, we have successfully refolded the recombinant DDPPs into an active enzyme from pellet, which showed similar activity to the soluble enzyme and yielded long-chain products. Reducing agent is essential for the refolding but not for the activity. Nonionic detergent with sugar head groups such as Triton X-100 and *n*-octyl-β-D-glucopyranoside assist in protein solubility during the refolding via a stepwise dialysis. In contrast, Triton X-100 is required for the rapid refolding of DDPPs while the protein was retained on the Ni–NTA column. Our process in purification of soluble DDPPs as a thioredoxin fusion protein in the presence of 0.1% Triton X-100 or refolding the recombinant DDPPs from the pellet provides a convenient way to prepare large quantities of enzyme routinely needed for structural studies. The protein without detergent could also be obtained in small quantity from the soluble lysate or through refolding. The expression and purification strategies developed here may be also useful to prepare recombinant DDPPs enzymes from other eukaryotic species for *in vitro* study.

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