Biochemical and Biophysical Research Communications 442 (2013) 105–111

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

In vitro and *in vivo* characterization of a novel insect decaprenyl diphosphate synthase: A two-major step catalytic mechanism is proposed

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ARTICLE INFO

Article history:
Received 25 October 2013
Available online 15 November 2013

Keywords:
Polyprenyl diphosphate synthase
Decaprenyl diphosphate synthase
In vitro and *in vivo* enzymatic assay
Ubiquinone
Coenzyme Q

ABSTRACT

Medium- and long-chain polyprenyl diphosphate synthases (PDDSs) catalyze the synthesis of the side-chain prenyl tails of ubiquinones, which play critical physiological roles in all organisms. This class of enzymes has been extensively studied in bacteria, yeast, plants and mammals, but very little information about such enzymes is available in insects. Here we cloned the cDNAs encoding the two subunits of an aphid long-chain PDDS (designated as AgDPPS1 and AgDPPS2). AgDPPS1 and AgDPPS2 had an open reading frame of 1230 bp and 1275 bp, with a calculated isoelectric point of 8.13 and 6.28, respectively. Sequence alignment and phylogenetic analysis showed that the enzyme was a candidate decaprenyl diphosphate (DPP) synthase with two heterologous subunits. Recombinant expression and *in vitro* enzymatic assay revealed that the two subunits were essential for the activity of the enzyme that catalyzed the formation of a major intermediate product geranylgeranyl diphosphate. *In vivo* analysis of ubiquinone (UQ) by expressing the insect enzyme in *Escherichia coli* identified UQ-10. Our data suggested that the insect enzyme is a novel DPP synthase with a two-major step catalytic mechanism, which catalyzes the formation of DPP as the final product, with geranylgeranyl diphosphate as the major intermediate product. This is the first characterization of an insect long-chain DPPS that synthesizes the side-chain of coenzyme Q-10.

1. Introduction

Polyprenyl diphosphate synthases (PDDSs), composed of a large family of prenyltransferases, catalyze the synthesis of prenyl diphosphates by condensing isopentenyl diphosphate (IPP) with allylic acceptors [1]. These enzymes can be classified into *cis*(*Z*)- and *trans*(*E*)-types according to the stereochemistry of reaction products synthesized, but the latter is the most common type, which synthesizes products up to C_{50} in length. *Trans*-PDDSs are further divided into short-chain (C_{10}–C_{20}), medium-chain (C_{30}–C_{35}), and long-chain (C_{40}–C_{50}) groups [2]. Geranyl diphosphate (GPP) synthase, farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase are short-chain PDDSs; heptaprenyl diphosphate (HepPP) synthase and hexaprenyl diphosphate (HexPP) synthase are medium-chain PDDSs, while octaprenyl diphosphate (OPP) synthase, solanesyl diphosphate (SPP) synthase and decaprenyl diphosphate (DPP) synthase (DPPS) are long-chain PDDSs.

Medium- and long-chain PDDSs are responsible for the synthesis of the prenyl side-chain tail of ubiquinone (UQ) [3]. Ubiquinone, also known as coenzyme Q (CoQ), localizes to the plasma membrane in prokaryotes or the mitochondrial membrane in eukaryotes, and has multiple functions [4]. The length of the prenyl side chain of ubiquinone varies between organisms [5]. For example, humans and *Schizosaccharomyces pombe* have UQ-10 [6,7], rats and *Arabidopsis thaliana* have UQ-9 [7,8], *Escherichia coli* has UQ-8 [9,10] and *Saccharomyces cerevisiae* has UQ-6 [11]. Expression of a heterologous PDDS gene in *E. coli* generated the same type of ubiquinone as that of the donor organisms, indicating that the length of the side chain of ubiquinone is determined by PDDS [3,12]. Medium- and long-chain PDDSs have been extensively studied in bacteria [9,10,13,14], yeast

Abbreviations: CoQ, coenzyme Q; DMAPP, dimethylallyl diphosphate; DPP, decaprenyl diphosphate; DPPS, decaprenyl diphosphate synthase; DTT, DL-1,4-dithiothreitol; FARM, the first aspartate-rich motif; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl diphosphate synthase; GPP, geranyl diphosphate; GST, glutathione S-transferase; HepPP, heptaprenyl diphosphate; HepPPS, heptaprenyl diphosphate synthase; HexPP, hexaprenyl diphosphate; HexPPS, hexaprenyl diphosphate synthase; IPP, isopentenyl diphosphate; IPTG, isopropyl thio-β-D-galactoside; OPP, octaprenyl diphosphate; OPPS, octaprenyl diphosphate synthase; PDDS, polyprenyl diphosphate synthase; SARM, the second aspartate-rich motif; SPP, solanesyl diphosphate; SPPS, solanesyl diphosphate synthase; UQ, ubiquinone.

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http://dx.doi.org/10.1016/j.bbrc.2013.11.025

[6,11], plants [8,15] and mammals [8]. Unfortunately, up to now, there is very little information available concerning insect counterparts.

Analysis of PDDS amino acid sequences derived from different organisms identified several conserved motifs [16]. Mutagenesis within and around the aspartate-rich motifs suggested that the highly conserved amino acid residues were involved in substrate binding and catalysis in PDDSs [17]. All short-chain PDDSs examined to date function as homodimers, except for the GPP synthase from spearmint, which functions as a heterotetramer [18]. In contrast, the medium-chain PDDSs are composed of two distinct dissociable subunits, with one subunit having a sequence similarity to other PDDSs and the other sharing little sequence similarity to known PDDSs [19]. Like short-chain PDDSs, the long-chain PDDSs from bacteria and fission yeast consist of two identical subunits tightly associated with each other [9,10]. However, the two DPPS subunits in humans and yeast were heterologous, and each of the subunits alone had no activity [7]. It is intriguing to know the features of an insect long-chain PDDS.

In the present study, we cloned the cDNAs encoding the two subunits of a long-chain PDDS from the cotton aphid, *Aphis gossypii*. Sequence alignment and phylogenetic analysis suggested that it was a candidate DPPS. *In vitro* and *in vivo* enzymatic assays revealed that the two heterologous subunits were essential for the function of the insect enzyme that catalyzed the formation of DPP as the final product, but with geranylgeranyl diphosphate as the major intermediate product. This is the first characterization of an insect long-chain DPPS that synthesizes the side-chain of UQ-10.

2. Materials and methods

2.1. Insect

The cotton aphid *A. gossypii* was derived from a single aphid collected from the host plant *Gossypium hirsutum* in China, and maintained by asexual reproduction at 18 °C with a light period of 16L:8D and RH 65–75%.

2.2. Cloning of the cDNA encoding the insect long-chain PDDS

Total RNA was extracted from whole-body insects using Trizol (Invitrogen). First-strand cDNA was synthesized from total RNA at 42 °C using oligo(dT)$_{18}$ primer (Clontech). The primers used for amplifying the PDDS cDNA were designed based on *Acyrthosiphon pisum* predicted DPPS mRNA sequences (GenBank acc. nos. XM_001947162 and XM_001945747). They were the forward primer DPPS1f (5′-CGCGCGGGATCCATGGCTTCGCGTATAACGTC-3′) and the reverse primer DPPS1r (5′-CGCGCGAAGCTTCTATTTCATTTACTA-3′) for subunit-1 cDNA, and DPPS2f (5′-CGCGCGGGATCCATGAACACCACACCAGCAGC-3′) and DPPS2r (5′-CGCGCGAAGCTTTCATTCGCCTCCCATTGCTA-3′) for subunit-2 cDNA. A *Bam*HI site and a *Hin*dIII site were incorporated in the forward and reverse primers, respectively. PCR was performed by pre-denaturing at 94 °C for 3 min, followed by 32 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min) and final extension at 72 °C for 10 min. The PCR products were subcloned and sequenced.

2.3. Sequence alignment and phylogenetic analysis

Homologous medium- and long-chain PDDSs (DPPS, SPPS and OPPS) and outgroup protein sequences from different organisms were retrieved from GenBank (AgDPPS1, *A. gossypii*, KC431243; AgDPPS2, *A. gossypii*, KC431244; ApDPPS1, *A. pisum*, XP_001947197; ApDPPS2, *A. pisum*, XP_001945782; AeDPPS1, *Acromyrmex echinatior*, EGI60261; AeDPPS2, *A. echinatior*, EGI70981; MrDPPS1, *Megachile rotundata*, XP_003704149; MrDPPS2, *M. rotundata*, XP_003707019; AfDPPS1, *Apis florea*, XP_003694345; AfDPPS2, *A. florea*, XP_003691655; NvDPPS1, *Nasonia vitripennis*, XP_001602352; NvDPPS2, *N. vitripennis*, XP_001605067), vertebrates (HsDPPS1, *Homo sapiens*, NP_055132; HsDPPS2, *H. sapiens*, NP_065114; PpDPPS1, *Pan paniscus*, XP_003821457; PpDPPS2, *P. paniscus*, XP_003824382; MamDPPS1, *Macaca mulatta*, XP_001102166; MamDPPS2, *M. mulatta*, NP_001253840; BtDPPS1, *Bos taurus*, DAA23563; BtDPPS2, *B. taurus*, NP_001093855; MumSPPS1, *Mus musculus*, BAE48219; MumSPPS2, *M. musculus* BAE48218; GgDPPS1, *Gallus gallus*, XP_418592; GgDPPS2, *G. gallus*, XP_419804; AcDPPS1, *Anolis carolinensis*, XP_003222159; AcDPPS2, *A. carolinensis* XP_003215679; DrDPPS1, *Danio rerio*, NP_001017656; DrDPPS2, *D. rerio*, NP_001002351; XtDPPS1, *Xenopus tropicalis*, NP_001072229; XtDPPS2, *X. tropicalis*, NP_001008170), plants (AtSPPS1, *A. thaliana*, NP_177972; AtSPPS2, *A. thaliana*, NP_173148), yeast (SpDPPS1, *Schizosaccharomyces pombe*, NP_595276; SpDPPS2, *S. pombe*, NP_594427; ScHexPPS, *S. cerevisiae*, P18900), bacteria (EcOPPS (ispB), *E. coli*, NP_417654; AgtDPPS, *Agrobacterium tumefaciens*, AAP56240; GoDPPS, *Gluconobacter oxydans*, BAA32241; RcDPPS (RcddsA), *Rhodobacter capsulatus*, ABB04465), and aligned using ClustalX [20], with the scoring matrix of Gonnet, open gap penalty and extending gap penalty of 10, and end gap and separation gap penalties of 0.05, respectively. The sequence features were analyzed based on sequence alignment. Phylogenetic analysis was performed using neighbor-joining statistical method on MEGA5 [21]. The basic parameters used for phylogenetic tree-building were as followed: substitution model, Dayhoff model; rates and patterns, uniform rates and homogeneous; gap/missing data treatment, complete deletion; test of phylogeny, bootstrap method (1000 bootstrap replications). *A. gossypii* FPP synthase (AgFPPS, GQ324701) and *A. gossypii* GGPPS (AgGGPPS, KF220654) were used as outgroups.

2.4. Recombinant protein expression in E. coli and purification

AgDPPS1 and AgDPPS2 cDNAs and the expression vector pET-42a(+) (Novagen, USA) were digested by *Bam*HI and *Hin*dIII (4 h at 37 °C), respectively. The cDNAs were then subcloned into pET-42a(+) with a GST tag. The constructs pET42a-AgDPPS1 and pET42a-AgDPPS2 were transformed into *E. coli* BL21 (DE3). Recombinant expression and protein purification were performed as described [22]. The final supernatant was fully mixed with GST resin (Novagen), and the column was washed and eluted according to the manufacturer's instructions. The solution was then concentrated using Amicon Ultra-15 Centrifugal Filter Unit (Millipore) and resuspended with the buffer (50 mM Tris–HCl, pH7.0, 50 mM $MgCl_2$, 1 mM DTT) for Factor Xa cleavage as described (Ma et al. [22]). The purified protein with GST-tag cut was finally resuspended in the storage buffer (50 mM Tris–HCl, pH7.0, 50 mM $MgCl_2$, 5 mM DTT and 10% glycerol) for enzymatic reaction and product identification.

2.5. Removal of GST tag from the fusion protein

The concentrated GST-tagged fusion proteins were treated with Factor Xa protease (Novagen) to cleave the N-terminal GST-tag at Xa site according to the manufacturer's instructions. Briefly, the sample was incubated with 2 U Factor Xa at 21 °C for 4 h. After the cleavage reaction, the cleaved target protein was collected and purified by affinity chromatography using GST-Bind resin. The GST-tag was bound to the resin, and the flow-through was collected, which was then concentrated using Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, USA) and finally resuspended in the storage buffer (50 mM Tris–HCl, pH7.0, 50 mM $MgCl_2$, 5 mM DTT and 10% glycerol) for enzymatic reaction

and product identification. All samples were checked for impurity by SDS–PAGE and protein concentrations were determined using the Bradford assay [23].

2.6. In vitro enzymatic reaction and product identification

The purified recombinant enzymes (AgDPPS1 and AgDPPS2) (3.0 μM), combined or individually, were incubated with four enzyme-substrate combinations in 50 μl of assay buffer (50 mM Tris–HCl, pH7.0, 50 mM $MgCl_2$ and 2 mM DTT) as followed: AgDPPS1+AgDPPS2 and FPP:IPP (20/80 μM); AgDPPS1+AgDPPS2 and DMAPP:IPP (30/30 μM); AgDPPS1 and FPP:IPP (20/80 μM), and AgDPPS2 and FPP:IPP (60/60 μM). All reactions were performed at 37 °C for 30 min, and samples were further incubated overnight in the presence of 20 U shrimp alkaline phosphatase (TaKaRa) to hydrolyze the products into extractable forms, which was then extracted with 150 μl *n*-hexane on ice and analyzed by gas chromatography–mass spectrometry (GC–MS).

The procedure for GC–MS analysis was performed as described [24]. The GC oven temperature was programmed with an initial hold time of 1 min, at a rate of 6 °C/min from 60 °C to 270 °C (hold 10 min). The masses were scanned over an *m*/*z* range of 15–550 amu.

2.7. Co-expression of AgDPPS1 and/or AgDPPS2 in E. coli

Sequence alignment and phylogenetic analysis indicated that the *A. gossypii* PDDS is a DPPS, but *in vitro* enzymatic analysis

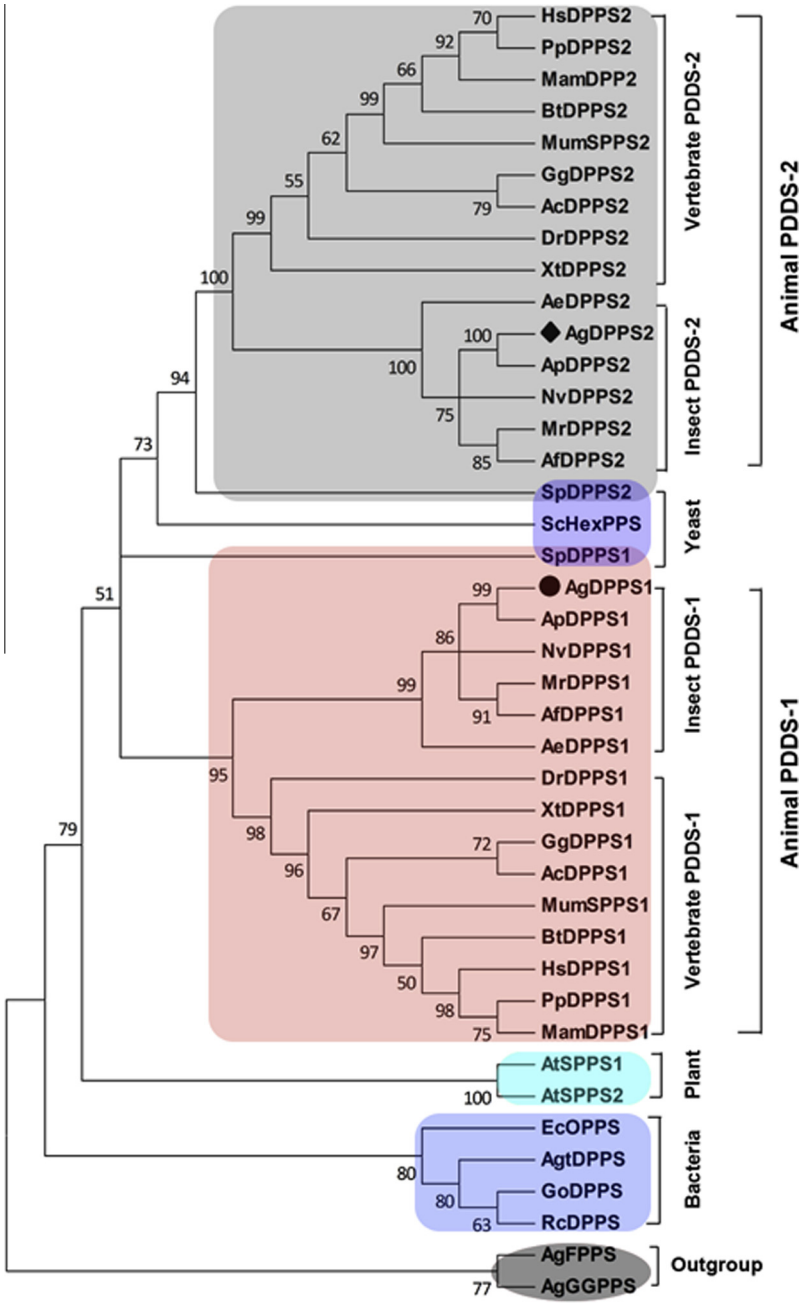

Fig. 1. A neighbor-joining phylogenetic tree of long-chain PDDSs reconstructed by using the MEGA5 package (Tamura et al. [21]). The branch lengths of the lines indicate the evolution distances, and numbers represent the tree confidence calculated by bootstrap analysis with 1000 replicates. The abbreviations and accession numbers are detailed in "Section 2".

and product identification by GC–MS detected only GGPP. This prompted us to further characterize the insect enzyme *in vivo* by expressing it in *E. coli* and identifying the ubiquinone species by high-performance liquid chromatography (HPLC).

The full-length cDNAs encoding AgDPPS1 and AgDPPS2 were amplified by PCR using the same protocol as described in Section 2.2. The *Bam*HI and *Hin*dIII sites were incorporated into the forward and reverse primers, respectively. The AgDPPS1 cDNA was fused with the vector pUC119 cut with *Bam*HI and *Hin*dIII, and AgDPPS2 cDNA with the vector pSTV28 according to standard protocols [25].

The constructs pUC119-AgDPPS1 and pSTV28-AgDPPS2 were then transformed into *E. coli* DH5α, and heterologously expressed under the control of the lac promoter. Positive transformants were grown in LB medium containing 50 μg/mL ampicillin and 50 μg/mL chloramphenicol at 37 °C and 250 rpm. The start culture (2 mL) was used to inoculate 100 mL of the same medium. The culture was allowed to grow to OD_{600} of ~0.80 and then shifted to 25 °C, and IPTG was added to a final concentration of 0.1 mM. After induction for 16 h, bacterial cells were pelleted by centrifugation (6000 rpm, 10 min) and washed with 0.9% NaCl (m/v).

2.8. Extraction and measurement of ubiquinone

Ubiquinone was extracted by the method of Lenaz [26]. Briefly, the cell suspension was taken into a round-bottom flask and stirred under reflux for 1 h at 90 °C. The mix was then placed in ice bath, with 14 mL of *n*-hexane added. After the thermal agitation, the upper liquid was drawn to another round-bottom flask, with 20 mL H_2O added. A second thermal agitation was carried out, and the upper layer was transferred to a small beaker for spontaneous evaporation. The residue was dissolved in 1 mL ethanol. The samples were centrifuged to remove insoluble material.

The analysis of ubiquinones was carried out by HPLC on a C18 reserve-phase column (ZORBAX SB-C18; 4.6 mm × 150 mm; Agilent) with ethanol as the mobile phase at a flow rate of 1 mL/min. Ubiquinones were detected at 275 nm and 30 °C [26].

3. Results

3.1. A. gossypii long-chain PDDS sequence

The full-length cDNAs encoding the two subunits of the *A. gossypii* long-chain PDDS (designated as AgDPPS1 and AgDPPS2) have been deposited in GenBank (accession nos. KC431243 and KC431244). The cDNA of AgDPPS1 contained an open reading frame of 1230 bp, encoding 409 amino acids, and the open reading frame of AgDPPS2 cDNA was 1275 bp, encoding 424 amino acids. The theoretical isoelectric points/molecular weight of AgDPPS1 and AgDPPS2 were 8.13/46.15 kDa and 6.28/46.74 kDa, respectively. Sequence comparison showed that the identity/similarity of the amino acid sequences of AgDPPS1 and AgDPPS2 were only 16%/34%, while the identity/similarity between AgDPPS1 and *A. pisum* DPPS1 were 98%/99%.

Sequence alignment found six conserved motifs (from I to V), *i.e.*, Motif I (GKxxR), Motif II (DDxxD$(x)_4$RRG), Motif III (GExxQ), Motif IV (Y$(x)_6$KT), Motif V (FQLxDDxxD) and Motif VI (K$(x)_4$DxxxGxxTxPxL), in all DPPS-1s examined, including insect, vertebrate, yeast and plant DPPS-1s, and bacterial DPPSs. The two characteristic conserved motifs in most known PDDSs, the first aspartate-rich motif (FARM) and the second aspartate-rich motif (SARM), appeared in Motif II and V, respectively (Supplementary Fig. 1A). We included the long-chain PDDS other than DPPS in our analysis, including the mouse SPPS (MumSPPS) and *E. coli* OPPS (EcOPPS). It was revealed that animal SPPS was highly similar to animal DPPSs, with only slight similarity to plant SPPS and bacterial DPPSs.

In contrast, all animal DPPS-2s had no significant sequence similarity with the PDDSs known to date (Suppl. Fig. 1B). Interestingly, the plant SPPS-2 (AtSPPS2) was highly similar to plant SPPS-1 (AtDPPS1), containing the conserved motifs, including the FARM and SARM, suggesting that the different subunits of plant SPPSs might be duplicated from the same ancestral gene, which is different from animal DPPSs where the two different subunits appeared to be heterologous.

3.2. Phylogenetic analysis

A neighbor-joining evolutionary tree was reconstructed with long-chain PDDS sequences from different taxa, which showed that all animal DPPS-1s and DPPS-2s formed two separate large groups with high bootstrap scores (100% and 95%, respectively), next to yeast medium- and long-chain PDDSs, including *S. pombe* DPPS1 (SpDPPS1), *S. pombe* DPPS2 (SpDPPS2) and *S. cerevisiae* HexPPS (ScHexPPS) (Fig. 1). Animal DPPS-1s and DPPS-2s were further divided into two subgroups (insect and vertebrate), respectively. Interestingly, the *M. musculus* SPPS1 and SPPS2 (MumSPPS1 and MumSPPS2) were accordingly grouped in animal DPPS1 and DPPS2 subgroups, but separated from plant SPPS (AtSPPS). In contrast to animal DPPS-1s and DPPS-2s, plant SPPS-1 and SPPS-2 (AtSPPS1 and AtSPPS2) were grouped in the same branch, while all bacterial long-chain PDDSs, including DPPSs and OPPS (AgtDPPS, GoDPPS,

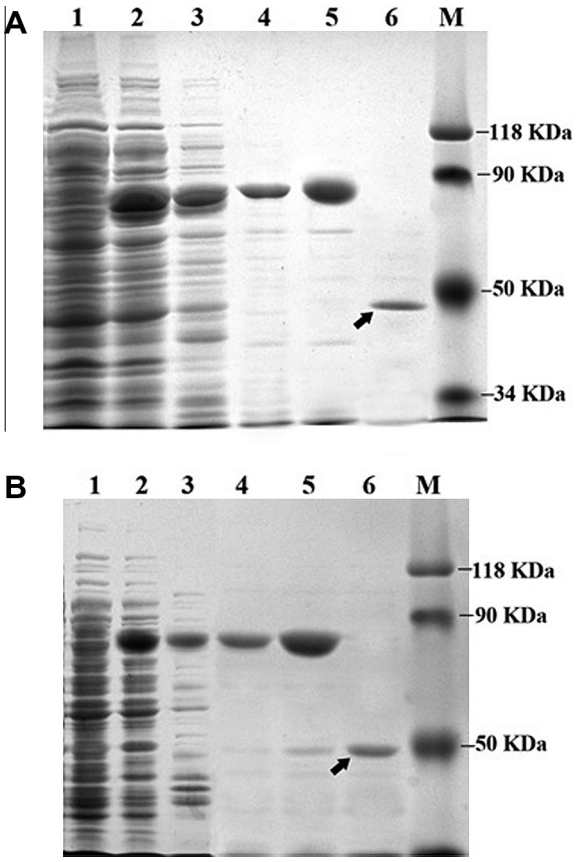

Fig. 2. SDS–PAGE of AgDPPS1 (A) and AgDPPS2 (B) at different stages of purification. Lane 1, cell-free homogenate before induction; lane 2, cell-free homogenate after induction with 0.1 mM IPTG; lane 3, supernatant after sonication and centrifugation; lane 4, purified GST-tagged enzyme; lane 5, concentrated GST-tagged enzyme; lane 6, enzyme with GST-tag cut. M, molecular-mass marker (118 kDa, 90 kDa, 50 kDa and 34 kDa).

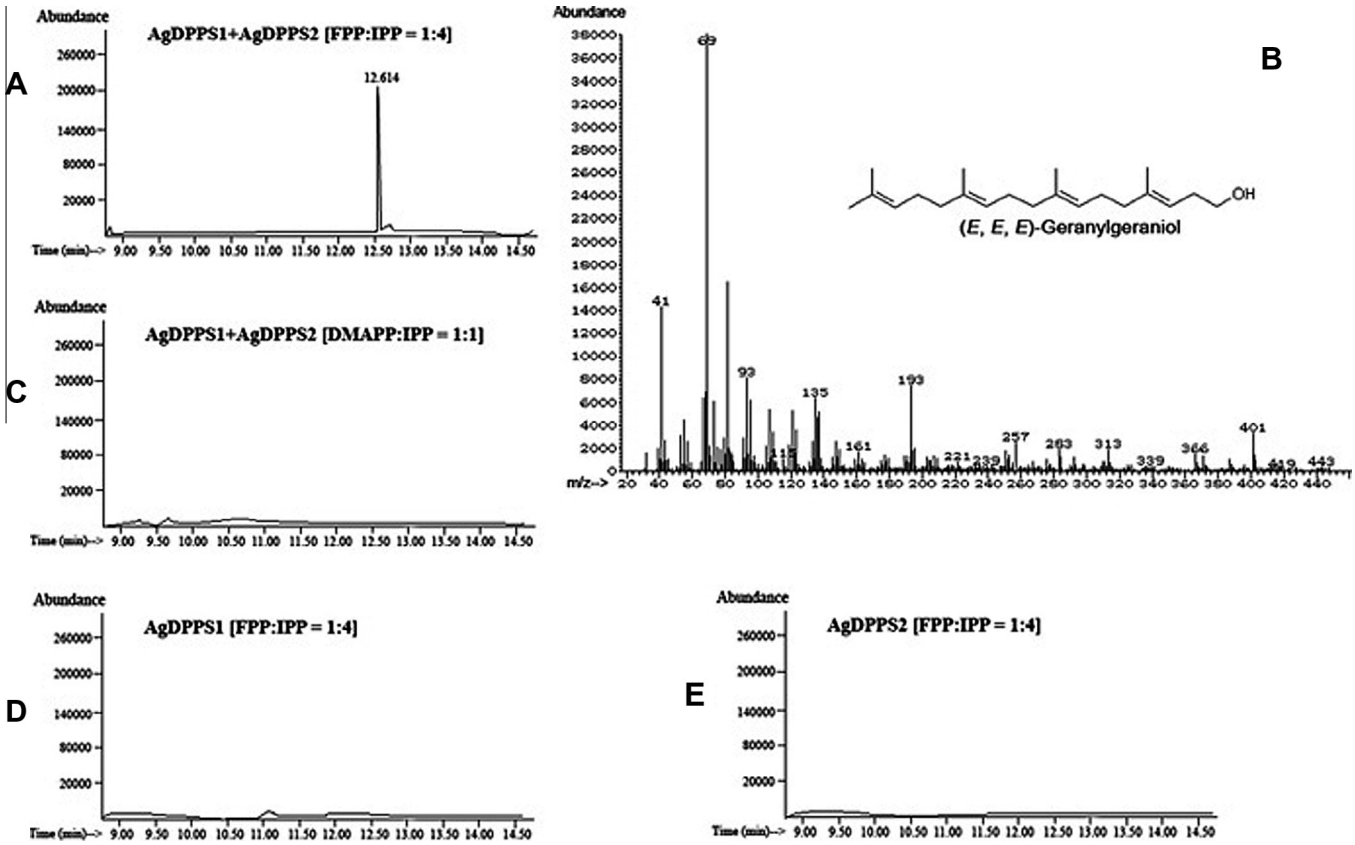

Fig. 3. Product identification by GC–MS. (A) AgDPPS1+AgDPPS2 and FPP+IPP; (B) mass-spectrum of the product; (C) AgDPPS1+AgDPPS2 and DMAPP+IPP; (D) AgDPPS1 and FPP+IPP, and (E) AgDPPS2 and FPP+IPP.

RcDPS and EcOPPS) were placed in another major group (Fig. 1). It was shown that the *A. gossypii* FPPS (AgFPPS) and *A. gossypii* GGPPS (AgGGPPS) formed an outgroup at the base of the phylogenetic tree. Our data suggest that the two subunits of animal long-chain PDDSs might be heterologous, while the two subunits of plant SPPS (AtSPPS1 and AtSPPS2) might be homologous. The protein tree also indicated that the short-chain PDDSs and medium- and long-chain PDDSs might have different origins.

3.3. Over-expression and purification of recombinant protein

The constructs pET42a-AgDPPS1 and pET42a-AgDPPS2 were transformed into *E. coli* BL21 (DE3), and the full-length cDNAs encoding AgDPPS1 and AgDPPS2 were over-expressed as GST-tagged fusion proteins (Fig. 2). The fusion proteins were serially purified by using GST resin columns as described in Section 2. The highly purified fusion proteins were then concentrated, and treated with Factor Xa that cut the GST-tag from the fusion proteins as confirmed by SDS–PAGE. The purified AgDPPS1 and AgDPPS2 had apparent molecular masses of ~46 kDa and ~47 kDa, respectively, which were in accordance with the theoretical molecular weights of AgDPPS1 and AgDPPS2 (Fig. 2). The recombinant enzymes without the GST-tag were used for enzymatic reaction.

3.4. In vitro enzymatic reaction and product identification by GC–MS

Four enzymatic reactions with different enzyme-substrate combinations were conducted, and the prenyl diphosphate products were then dephosphorylated by alkaline phosphatase treatment. The extractable alcohol form of prenyl diphosphate product was extracted by hexane and identified by GC–MS. The results showed that a peak with a retention time of 12.614 was detected by GC in the reaction with AgDPPS1+AgDPPS2 and FPP+IPP (Fig. 3A). This product was identified as (*E*, *E*, *E*)-geranylgeraniol, the extractable alcohol form of GGPP (Fig. 3B). Neither geranylgeraniol nor other relevant products were detected in the other three enzymatic reactions, including AgDPPS1+AgDPPS2 and DMAPP+IPP (Fig. 3C), AgDPPS1 and FPP+IPP (Fig. 3D), and AgDPPS2 and FPP+IPP (Fig. 3E). It was surprising that only GGPP (C_{20}) but not DPP (C_{50}, the expected product) was generated by *A. gossypii* DPPS. Our data suggested that AgDPPS1 and AgDPPS2 were two essential subunits for the enzymatic activity of *A. gossypii* DPPS, and *A. gossypii* DPPS accepted FPP but not DMAPP as the allylic co-substrate.

3.5. Detection of ubiquinone by HPLC

It was unexpected that *A. gossypii* DPPS catalyzed the formation of GGPP but not DPP as demonstrated by GC–MS analysis. Therefore, an *in vivo* enzymatic activity analysis was conducted by expressing AgDPPS1 and/or AgDPPS2 in *E. coli*. The results of HPLC analysis showed that the endogenous ubiquinone of *E. coli* (*i.e.*, UQ-8) was detected in both the wide-type DH5α (Fig. 4A) and all treatments, including DH5α harboring pUC119-AgDPPS1 (Fig. 4B), DH5α harboring pSTV28-AgDPPS2 (Fig. 4C), and DH5α harboring pUC119-AgDPPS1 plus pSTV28-AgDPPS2 (Fig. 4D). However, UQ-10 (the expected ubiquinone product of *A. gossypii* DPPS) was detected only in DH5α harboring pUC119-AgDPPS1 plus pSTV28-AgDPPS2 (Fig. 4D), suggesting that AgDPPS could catalyze the formation of DPP, the side-chain of UQ-10, and that both AgDPPS1

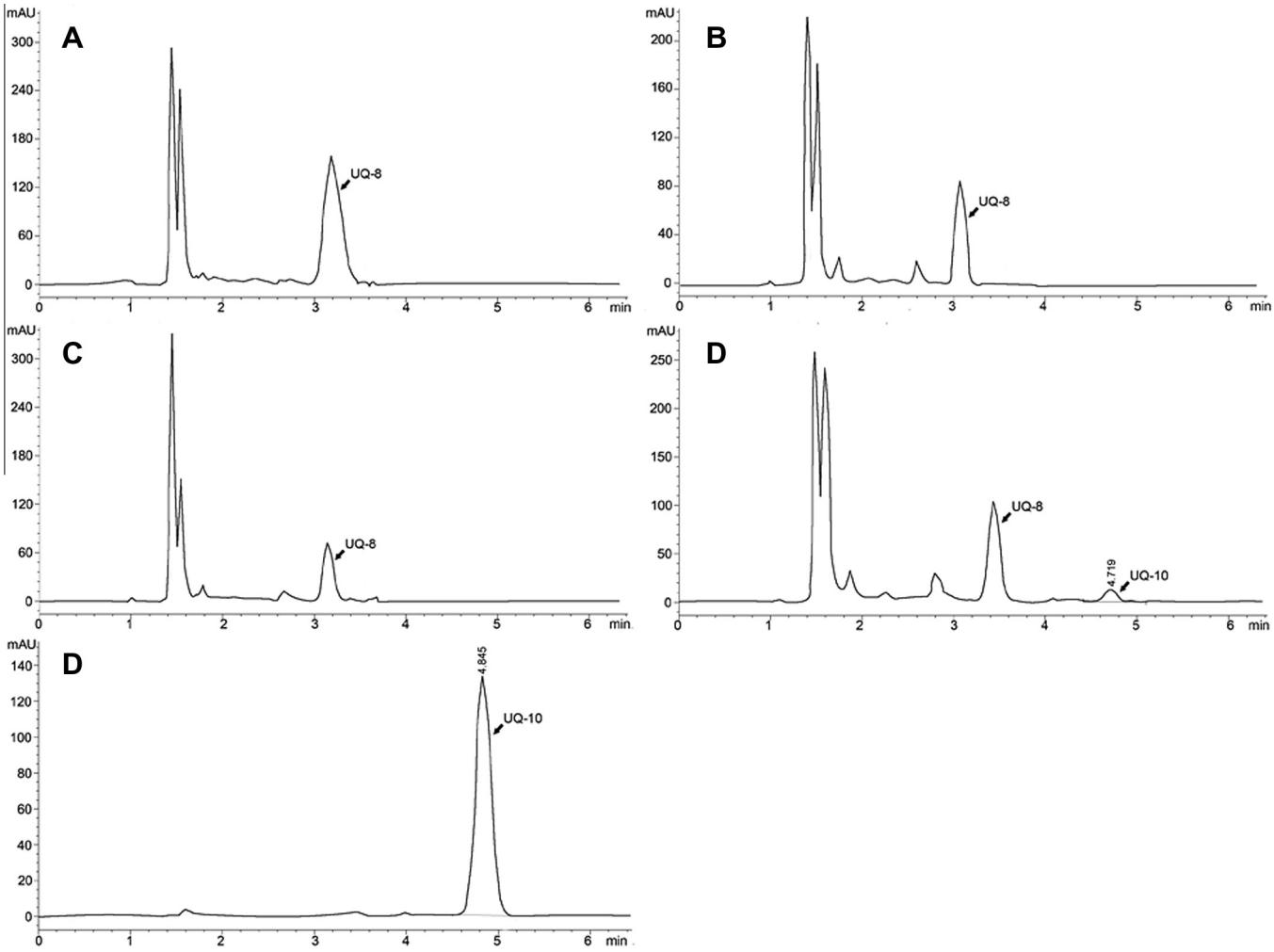

Fig. 4. Detection of ubiquinone by HPLC. Ubiquinones were extracted from *E. coli* wide-type DH5α (A), DH5α harboring pUC119-AgDPPS1 (B), DH5α harboring pSTV28-AgDPPS2 (C), and DH5α harboring pUC119-AgDPPS1 plus pSTV28-AgDPPS2 (D). Standard ubiquinone-10 (UQ-10) is also shown (E).

and AgDPPS2 were essential for the function of *A. gossypii* DPPS, which supported the results obtained by GC–MS analysis.

4. Discussion

We have cloned a novel insect DPPS, supported by sequence alignment and phylogenetic analysis, and verified by *in vitro* and *in vivo* enzymatic analysis. *In vitro* assay showed that GGPP was formed, and *in vivo* heterologous expression demonstrated that UQ-10 was produced, suggesting that the enzyme could catalyze the formation of DPP. Our data showed that the two heterologous subunits were essential for the function of the enzyme, which is a typical DPPS with a two-major step catalytic mechanism.

AgDPPS1 shared only 16% identity with AgDPPS2. While AgDPPS1 had the conserved motifs characterized by all PDDSs known today, AgDPPS2 had no sequence similarity to PDDSs known to date. Furthermore, AgDPPS1 and AgDPPS2 were phylogenetically placed in two different subgroups, which formed a large animal branch, separated from the long-chain PDDSs from other taxa. These facts suggest that animal, plant and bacterial long-chain PDDSs might have different evolutionary origins, and the two subunits of animal long-chain PDDSs appear to be heterologous [7], in contrast to the homologous subunits of plant SPPS [27].

Our *in vitro* enzymatic analysis showed that AgDPPS accepted FPP but not DMAPP as the allylic cosubstrate in the condensation reaction leading to the formation of prenyl diphosphates, which is consistent with previous studies [2]. Our studies verified that the two subunits were essential for the function of *A. gossypii* DPPS, since neither AgDPPS1 nor AgDPPS2 alone showed catalytic activity. This supports the results obtained in the mammal DPPSs, where the two subunits function as heterotetramers, and each subunit alone had no activity [7].

In vitro analysis of recombinant enzyme detected only GGPP but not the expected product DPP by GC–MS. Here the GGPP should be considered as an intermediate product, as it is technically difficult to detect the long-chain prenyl diphosphates (C_{40}–C_{50}) by gas chromatography. Indeed, heterologous expression of AgDPPS1 plus AgDPPS2 in *E. coli* produced UQ-10 together with UQ-8. UQ-8 is known to be the endogenous ubiquinone of *E. coli*, so it is certain that UQ-10 detected in the heterologous expression system must be synthesized by the *A. gossypii* DPPS. It has been extensively demonstrated that expression of a heterologous PDDS gene in *E. coli* generated the same type of ubiquinone as that of the donor organisms [3,12].

In conclusion, AgDPPS is a novel decaprenyl diphosphate synthase with two essential heterologous subunits. This insect long-chain PDDS is responsible for the biosynthesis of the side-chain

of UQ-10 in *A. gossypii*. A major intermediate product (GGPP) is produced during the consecutive condensation reaction starting from FPP and IPP, leading to the formation of the final product (DPP) by adding six more IPPs. The two-major step catalytic mechanism might be a biochemical feature of insect DPPSs. This is the first insect DPPS that has been functionally characterized *in vitro* and *in vivo*.

Acknowledgment

This work is supported by the National Science Foundation of China (Grant Nos. 31171845, 31371940 and 31071748).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.025.

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