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Cloning, expression and characterization of an insect geranylgeranyl diphosphate synthase from *Choristoneura fumiferana*

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ABSTRACT

Geranylgeranyl diphosphate synthase (GGPPS) catalyzes the condensation of the non-allylic diphosphate, isopentenyl diphosphate (IPP; C_5), with allylic diphosphates to generate the C_{20} prenyl chain (GGPP) used for protein prenylation and diterpenoid biosynthesis. Here, we cloned the cDNA of a GGPPS from the spruce budworm, *Choristoneura fumiferana*, and characterized the corresponding recombinant protein (r*Cf*GGPPS). As shown for other type-III GGPPSs, r*Cf*GGPPS preferred farnesyl diphosphate (FPP; C_{15}) over other allylic substrates for coupling with IPP. Unexpectedly, r*Cf*GGPPS displayed inhibition by its FPP substrate at low IPP concentration, suggesting the existence of a mechanism that may regulate intracellular FPP pools. r*Cf*GGPPS was also inhibited by its product, GGPP, in a competitive manner with respect to FPP, as reported for human and bovine brain GGPPSs. A homology model of *Cf*GGPPS was prepared and compared to human and yeast GGPPSs. Consistent with its enzymological properties, *Cf*GGPPS displayed a larger active site cavity that can accommodate the binding of FPP and GGPP in the region normally occupied by IPP and the allylic isoprenoid tail, and the binding of GGPP in an alternate orientation seen for GGPP binding to the human protein. To begin exploring the role of *Cf*GGPPS in protein prenylation, its transcripts were quantified by qPCR in whole insects, along with those of other genes involved in this pathway. *Cf*GGPPS was expressed throughout insect development and the abundance of its transcripts covaried with that of other prenylation-related genes. Our qPCR results suggest that geranylgeranylation is the predominant form of prenylation in whole *C. fumiferana*.
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1. Introduction

Some proteins undergo a post-translational modification known as prenylation, which induces their association to membranes or their interaction with other proteins. Prenylation is essential for many gene products including Ras proteins (Takai et al., 1992), G-proteins (Yamane et al., 1990), phosphatases (Cates et al., 1996) and kinases (Inglese et al., 1992), all of which have been identified in various organisms, mostly in non-insect systems. In insects, *Drosophila melanogaster* provides a good example of protein prenylation where it was shown to be essential for the non-retinal phosphodiesterase, PDE5/6, inducing its localization to the membrane where it plays an important role in cyclic guanosine monophosphate (cGMP) transport in Malpighian tubules (Day et al., 2008). In protein prenylation reactions, either a farnesyl diphosphate (FPP; C_{15}) or a geranylgeranyl diphosphate (GGPP; C_{20}) moiety is transferred to a carboxyl-terminal cysteine by a protein farnesyl-transferase (PFTase) or a protein geranylgeranyl-transferase (PGGTase type I or type II), respectively (Clarke, 1992; Maurer-Stroh et al., 2003). FPP is known to be provided by farnesyl diphosphate synthase (FPPS), while GGPP is generated by geranylgeranyl diphosphate synthase (GGPPS) (Reed and Rilling, 1975). Both enzymes are classified as short-chain *trans*-prenyltransferases and catalyze the fundamental chain elongation reaction between allylic diphosphates and the non-allylic diphosphate, isopentenyl diphosphate (IPP). FPPS is the most extensively studied *trans*-prenyltransferase, including in insects, and constitutes a potential biorational target site for insecticide development because of its pivotal role in the biosynthesis of juvenile hormone (Cusson et al.,

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2012) and its unique structural and catalytic features in certain groups of insects (Cusson et al., 2006; Vandermoten et al., 2009b).

GGPPS has received less attention than FPPS, particularly in insects. This enzyme has been well characterized in prokaryotes, plants, yeast and mammals (Artz et al., 2011; Kavanagh et al., 2006a; Takaya et al., 2003; Ye et al., 2007), but no insect GGPPS has been examined for its enzymatic properties, although sequences encoding this enzyme were reported for different insects, including *D. melanogaster* (Lai et al., 1998) and the termite *Nasutitermes takasagoensis* (Hojo et al., 2007), and many others have been deposited in Genbank. Alignment of deduced amino acid sequences for many GGPPSs revealed several conserved regions, including the first and second aspartate-rich motifs (FARM and SARM, respectively), the former of which is known in FPPS to bind the allylic substrate via the divalent cations Mg^{2+} or Mn^{2+} (Gabelli et al., 2006). The region encompassing the FARM and the five amino acids upstream from the FARM is known as the product chain-length determination (CLD) region (Wang and Ohnuma, 1999). Based on variations in the composition of the CLD region, GGPPSs are currently identified as belonging to one of three distinct categories: type I (archaea), type II (plants and eubacteria) and type III (fungus, insects and mammals) (Hemmi et al., 2003; Ling et al., 2007).

Although GGPPS from all organisms produces the same final GGPP product, kinetic studies indicate that type I and type II GGPPSs preferentially catalyze the condensation of dimethylallyl diphosphate (DMAPP; C_5) or geranyl diphosphate (GPP; C_{10}) with IPP. However, type III GGPPSs appear to use FPP as their principal allylic substrate (Sagami et al., 1994).

Current knowledge on the structure of GGPPSs is based on information obtained from X-ray crystallographic studies. GGPPS crystal structures from the bacterium *Thermus thermophilus* (PDB code 1WMW), the archaea *Pyrococcus horikoshii* (PDB code 1WY0) and the yeast *Saccharomyces cerevisiae* (Chang et al., 2006) exhibit a dimeric quaternary structure, a characteristic common to most *trans*-prenyltransferases for which X-ray structures are available. However, the crystal structure of human GGPPS unexpectedly showed hexameric (Kavanagh et al., 2006a) or octameric arrangements (Miyagi et al., 2007). It was suggested that these multimeric structures are limited to mammalian and, potentially insect GGPPSs, given the high primary sequence identity between human and *Drosophila* GGPPSs (57%) (Kavanagh et al., 2006a). Several additional GGPPS crystal structures have since been reported in the literature (e.g., Guo et al., 2007; Chen et al., 2008).

The synthesis of GGPP is likely to be important in insects since it was suggested that in most eukaryotes a greater proportion of the prenylated proteins are geranylgeranylated, as opposed to being farnesylated (Epstein et al., 1991; Moriya et al., 2010). In addition, GGPPS seems to play another role in some insects, providing the GGPP precursor essential for the synthesis of diterpenoid (C_{20}) compounds (Hojo et al., 2007).

Here, we report on the cloning of a GGPPS cDNA from the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae), and on the biochemical properties of the purified recombinant protein heterologously expressed in *Escherichia coli*. This first detailed characterization of an insect GGPPS indicates that *Cf*GGPPS displays many biochemical features similar to those of mammalian GGPPSs and also some unique features that we explored further through the analysis of homology models. To begin assessing the role of GGPPS in protein prenylation in *C. fumiferana*, we examined the transcriptional pattern of *Cf*GGPPS in whole insects at different developmental stages and we compared the transcript abundance of GGPPS with that of the PFTase and PGGTases I and II involved in protein prenylation.

2. Materials and methods

2.1. Cloning and sequencing of a C. fumiferana GGPPS cDNA

The *Cf*GGPPS cDNA sequence was obtained by PCR screening of a *C. fumiferana* Malpighian tubules cDNA library generated using the method of Matz (2000). Briefly, 300 ng of total RNA extracted (Qiagen, RNeasy Mini Kit) from Malpighian tubules of a *C. fumiferana* 6th instar larva was reverse transcribed using an oligo-dT primer (Table S1), followed by the synthesis of the second cDNA strand and ligation of an adapter (Adp2, produced by the annealing of both Adp-2 upper and lower; Table S1). The library was then amplified with 0.7 U of the Advantage 2 polymerase mix (Clontech), using 0.1 μM of an adapter-specific primer (Adp2-DAP; Table S1) in conjunction with 0.2 μM of the same oligo-dT primer and 0.3 mM of each dNTP. The cycling conditions consisted of an initial heating step at 94 °C for 1 min followed by 20 cycles of 95 °C, 30 s; 55 °C, 1 min; 72 °C, 4 min. Using the cDNA library as template, a partial *Cf*GGPPS cDNA sequence (two overlapping fragments) was obtained by amplification with degenerate primers designed against highly conserved regions of animal GGPPSs (P1-F/P1-R and P2-F/P2-R; Table S1). After an initial 3 min heating step at 94 °C, 0.5 U of the Taq DNA polymerase (Invitrogen) was added to the reaction mix and the cycling conditions began with 10 cycles of 94 °C, 45 s; 45 °C, 45 s; 72 °C, 2 min, followed by 30 more cycles at 94 °C, 45 s; 48 °C, 45 s; 72 °C, 2 min. Amplicons were purified from a 1% agarose gel using the Geneclean II kit (Q-BIOgene), then cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions, and sequenced. From this partial sequence, specific *Cf*GGPPS cDNA primers, SP-1 and SP-2 (Table S1), were designed to amplify the remaining 5′ and 3′ cDNA-ends, respectively, in conjunction with the oligo-dT primer and a proximal adapter primer (Adp2-PAP; Table S1). The cDNA library was used as a template with 1 U of the Platinum Taq DNA polymerase High Fidelity (Invitrogen) according to the manufacturer's instructions. Cycling conditions for this last amplification reaction started with a heating step at 94 °C for 1 min, followed by 35 cycles of 94 °C, 30 s; 52 °C, 30 min; 68 °C, 2 min, and a last extension step at 68 °C for 5 min. Amplicons were purified, cloned and sequenced as described above. Using the same amplification conditions, the entire *Cf*GGPPS coding region was finally cloned into the pET28a(+) expression vector (Novagen) by introducing 5′ and 3′ restriction sites with specific primers carrying respectively a NdeI and a XhoI extension (Table S1). After gel purification, the *Cf*GGPPS amplicon was digested with NdeI and XhoI, and cloned into the same restriction sites of the vector to generate a fusion protein with a His tag at the N-terminus. The integrity of the construction was confirmed by sequencing.

2.2. CfGGPPS expression and purification

The expression of r*Cf*GGPPS in the *E. coli* Rosetta strain (DE3) was induced under the control of the T7 promoter (Vandermoten et al., 2008). Induction was initiated with 1 mM isopropyl-β-D-thiogalactoside (IPTG), at 15 °C for 18 h. Bacterial protein homogenates were extracted after cell lysis by sonication (Branson sonicator, 80 W, 20% power) for 4 min (alternating 5 s on; 5 s off) on ice in the buffer 25 mM Tris–HCl (pH 8) containing 5 mM $MgCl_2$ and 1 mM of β-mercaptoethanol (BME). After a centrifugation step (16,000 *g*; 15 min), the soluble fraction was recovered. The recombinant GGPPS was purified from this fraction by Co^{2+} metal affinity chromatography using TALON Metal Affinity Resin (Clontech), with an ascending stepwise gradient of imidazole, as previously described (Sen et al., 2007a; Vandermoten et al., 2008). The eluted protein was desalted through a PD-10 column (Amersham

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