Substrate specificities of farnesyl diphosphate synthases with respect to cyclic substrate homologs

Tohru Musashi, Hiroshi Kanno, Jun Kawakami,
Saki Yokota*, Norimasa Ohya*, and Masahiko Nagaki

Graduate School of Science and Technology, Hirosaki University,
3 Bunkyo-cho, Hirosaki, Aomori 036-8561, Japan

*Department of Material and Biological Chemistry, Faculty of Science, Yamagata
University, Kojirakawa-machi, Yamagata 990-8560, Japan

Fax: 81-172-39-3947, e-mail: nagaki@cc.hirosaki-u.ac.jp

We investigated substrate specificities of farnesyl diphosphate synthases (FPSs) derived from porcine liver and *Bacillus stearothermophilus* by examining the reactivity of cyclopentylideneethyl diphosphate with several 3-alkyl homologs of isopentenyl diphosphate. Reaction of cyclopentylideneethyl diphosphate with isopentenyl diphosphate using porcine liver or bacterial enzyme gave 10-cyclopentyliden-3,7-dimethyldeca-2,6-dinenyl diphosphate as a double condensation product, with relative yields of 40.9% for the porcine liver enzyme and 15.9% for the bacterial enzyme. Reaction of cyclohexylideneethyl diphosphate with 3-ethylbut-3-enyl diphosphate using the bacterial enzyme gave 10-cyclohexyliden-3,7-diethyldeca-2,6-dinenyl diphosphate (yield: 24.6%).

Key words: farnesyl diphosphate synthase, substrate specificity, cyclopentylideneethyl diphosphate, 3-alkylisopentenyl diphosphate

1. INTRODUCTION

A large number of structurally diverse isoprenoids occur in nature, and all of these biosynthetic precursors are constructed by the activity of prenyltransferase [1-3]. These enzymes can be divided into two major families: *E*- and *Z*-prenyl chain elongating enzymes, each of which may be further classified into short- (*E*-I, *Z*-I), medium- (*E*-II, *Z*-II), and long-chain prenyl elongating enzymes (*E*-III, *Z*-III), as shown in Scheme 1 and Table 1 [4-10].

Scheme 1. Classification of prenyltransferase.

Farnesyl diphosphate synthase (FPS; *E*-I group) [EC 2. 5. 1. 10] is widely distributed among living beings, from higher animals down to bacteria, and catalyzes the consecutive condensation reactions of two molecules of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) via geranyl diphosphate (GPP) to yield the final product, (all-*E*)-farnesyl diphosphate (FPP) as shown in Scheme 2.

Table 1. Classification of prenyltransferase.

Group	P-transferase	Product	Structure	Activated factor
<i>E</i> -prenyl chain elongation				
<i>E</i> -I	GPS, FPS	C ₁₀ , C ₁₅	homodimer	
<i>E</i> -II	HexPS, HepPS	C ₃₀ , C ₃₅	heterodimer	
<i>E</i> -III	OctPS, SPS	C ₄₀ , C ₄₅	homodimer	Carrier protein
<i>Z</i> -prenyl chain elongation				
<i>Z</i> -I	FPS	C ₁₅	homodimer	
<i>Z</i> -II	DPS, UPS	C ₅₀ , C ₅₅	homodimer	Lipid (Triton X-100)
<i>Z</i> -III	DeDolPS, rubberS	C ₇₅₋₁₀₀ , C _{>500}	homodimer	

GPS: geranyl diphosphate synthase, HexPS: hexaprenyl diphosphate synthase, HepPS: heptaprenyl diphosphate synthase, OctPS: octaprenyl diphosphate synthase, SPS: solanesyl diphosphate synthase, DPS: decaprenyl diphosphate synthase, UPS: undecaprenyl diphosphate synthase, DeDolPS: Dehydrodolichyl diphosphate synthase, rubberS: rubber synthase

Scheme 2. The FPS reaction.

Koyama et al. succeeded in cloning, sequencing, and overexpressing genetic *Bacillus stearothermophilus* FPS in *Escherichia coli*. [11] The motif (DD**D), which is rich in aspartate (D), is known to be an active site of this enzyme, and tyrosine (Y81) in five upper streams of the first aspartate rich motif (FARM) is believed to be important in product chain length determination [11].

Several studies have investigated the substrate specificities of FPS with allylic and homoallylic substrate homologs [12–16]. We recently reported on the reactivities of several allylic substrate homologs which have a hydrophilic group at the ω -position, using *B. stearothermophilus* and porcine liver FPSs [17–19].

Here, we describe the substrate specificities of FPSs with respect to cyclic substrate homologs and isopentenyl diphosphate homologs having an alkyl group at the 3-position.

2. EXPERIMENT

2.1 Analysis

The prenyl alcohols produced by alkaline phosphatase treatment of the products from enzymatic reactions were measured by high-performance liquid chromatography (HPLC). Relative yields of FPS reactions were then calculated relative to those of FPP derived from the standard enzymatic reaction with IPP and DMAPP. Our materials were similar to those used in previous experiments [16–19]; our HPLC apparatus was a Hitachi L-6000 (Hitachi, Tokyo, Japan) equipped with a Hitachi LaChrom L-7420 UV-VIS detector and a ChromatoDAQ II (ULVAC, Inc., Chigasaki, Japan). We also used a LichroCART column (Merck-Japan, Tokyo, Japan) with a hexane:2-propanol (40:1) eluent.

Reaction product identification was carried out using liquid chromatography-mass spectrometry (LC-MS; Hitachi NanoFrontier LD). The HPLC analyses were performed on a Hitachi ELITE LaChrom HPLC system equipped with a pumping system (L-2100), a column oven (L-2300), and an ultraviolet-visible spectroscopy detector coupled with EZChromElite Windows XP software (Agilent Technologies Inc., Hachioji, Japan). The samples were separated on an ODS column (GL Science Inertsil ODS-3, 33 $\times$ 2.1 mm) with eluents A (0.1% formic acid in acetonitrile)

and B (0.1% formic acid in distilled water) at a flow rate of 0.2 mL/min. Gradient elution started with 70% acetonitrile (30% water) and reached 100% acetonitrile in 12 min. The LC effluent was introduced into the electrospray ion (ESI) source. Mass spectra were acquired using a Hitachi NanoFrontier LD spectrometer with an ESI source. Nitrogen was used as the sheath and auxiliary gas, with helium as the collision gas. The ESI MS spectra were acquired in positive ion modes. For full scan MS analysis, the spectra were recorded in the range of m/z 100–2000.

Product identification was carried out by gas chromatography-mass spectrometry (GC-MS) using a JMS-AM II 50 type GCG Mass spectrometer connected with an HP 5890 series II gas chromatograph equipped with ultra-alloy-1 (S). The column temperature was programmed from 90 to 280 $^{\circ}$ C with a linear gradient of temperature at a rate of 15 $^{\circ}$ C/min, and was then held at 280 $^{\circ}$ C for 3 min.

The infrared spectra were recorded in KBr. The nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNMGX 270 FT NMR spectrometer using tetramethylsilane (TMS) as an internal standard in CDCl_3 .

2.2 Chemicals

Synthesis of 3-alkylbut-3-enylIPP (3-alkylIPP) homologs and cyclic substrate homologs

3-EthylIPP, 3-propylIPP, and 3-butylIPP were synthesized according to our previously reported method [20, 21]. 3-EtIPP was also prepared by another method, as follows: 2-(3-chloromethylbut-3-enoyloxy)-tetrahydro-2H-pyran was obtained by selenium oxidation [22] and chlorination [23] of isopentenyl tetrahydropyranyl ether (IOTHP) was methylated by a Grignard reaction, yielding 3-ethylbut-3-en-1-ol (3-EtIOH) by deprotection of the THP group. The ^1H - (CDCl_3 , TMS) of the alcohol was δ 1.05 (3H, d $J=7$ Hz), 1.64 (OH), 2.06 (2H, q $J=7$ Hz), 2.32 (2H, t $J=6$ Hz), and 3.71 (2H, t $J=6$ Hz). The ^{13}C - as determined by NMR was δ 12.2, 28.6, 39.4, 60.4, 110.4, and 147.8. The alcohol was converted to the corresponding diphosphate according to the method described by Daviesson et al. [24]

The cyclic substrate homolog cyclopentylideneethyl diphosphate (CpPP) was synthesized using the same method we previously reported when synthesizing cyclohexylideneethyl dihydrophosphate (ChPP) using the Reformansky reaction [16].

2.3 Purification of porcine liver and *B. stearothermophilus* FPSs

Purification of FPS from porcine liver was carried out in accordance with the method of Dorsey et al. [25–27].

B. stearothermophilus FPS was purified as we previously reported [10,11,16]. The crude-free extracts of *E. coli* JM109 cells harboring the

expression plasmid pEX 11 for the FPS gene from *B. stearothermophilus* ATCC10149 were heat-treated and purified using two column chromatographic procedures [11,28].

2.4 Conditions of the enzymatic reaction

The incubation mixture for the porcine liver FPS reaction contained 100 μmol of Tris-HCl buffer (pH 7.5), 50 μmol of 2-mercaptoethanol, 10 μmol of MgCl_2 , 100 μmol of iodoacetamide, 2 μmol of KCl, 1 μmol of each substrate homolog (IPP, 3-alkylIPP, CpPP or ChPP), and FPS (approximately 25 mg) in a total volume of 1 mL. After incubation for 3 h at 37 $^\circ\text{C}$, the reaction mixture was treated with alkaline phosphatase for 5 h, extracted with pentane, and analyzed using HPLC and GC-MS. The *B. stearothermophilus* FPS reaction was conducted under the same conditions with an incubation temperature of 55 $^\circ\text{C}$, pH of 8.5, and 50 mmol of NH_4Cl instead of iodoacetamide.

Relative reactivity was based on the amount of product (100%) which hydrolyzed the reaction between the natural substrates GPP and IPP [16-19].

To obtain sufficient product for NMR spectroscopic analysis, the reaction volume was increased 52 times the total volume mentioned above, and the products were analyzed after purification by separated HPLC.

2.5 Simulation of the enzymatic reaction mechanism by molecular docking

We constructed a structural model of the FPS obtained from *B. stearothermophilus* based on the crystal structure of the FPS obtained from *E. coli* [29] using SWISS-MODEL (<http://swissmodel.expasy.org/>). Enzyme-substrate docking was carried out using the docking software GOLD (Genetic Optimisation for Ligand Docking, Astex Therapeutics Ltd., Cambridge, UK) [30]. The diphosphate group of the substrate combined with the DD**D portion of the FARM of FPS, thereby allowing determination mechanism by which the product was obtained.

3. RESULTS AND DISCUSSION

To clarify the reactivities of allylic substrates having cyclopentylideneethyl- or cyclohexylideneethyl groups and 3-alkylIPP homologs, we examined substrate specificities of FPSs derived from porcine liver and *B. stearothermophilus*.

Scheme 3. FPS reaction of CpPP (or ChPP) with 3-alkylIPP homologs.

3.1. Reaction of CpPP with IPP using FPS

As shown in Scheme 3, the hydrolysate derived from the reaction of CpPP ($m=1$) with IPP ($\text{R}=\text{CH}_3$) using porcine liver FPS gave an HPLC peak at 17.8 min and was then analyzed by GC-MS. The mass spectrum showed a molecular ion $[\text{M}]^+$ at m/z 248 (relative intensity: 0.33%), corresponding to $\text{C}_{17}\text{H}_{28}\text{O}$, and fragment ions at m/z 230 $[\text{M} - 18]^+$ (23.8%), 162 (18.7%), 135 (17.2%), and 95 (base peak). These data indicated that the alcohol had a structure of 10-cyclopentyliden-3,7-dimethyldeca-2,6-dien-1-ol ($n=2$), suggesting that chain elongation stopped at the second stage of condensation. The relative yield was 40.9%.

Similar reaction of CpPP with IPP using *B. stearothermophilus* FPS resulted in a condensation product (relative yield: 15.9%) with two molecules of IPP.

3.2 Reaction of CpPP with 3-alkylIPP using FPS

Reaction of CpPP with 3-ethylIPP ($\text{R}=\text{C}_2\text{H}_5$) using porcine liver FPS produced an alcohol after phosphatase treatment, which gave an HPLC peak at 15.7 min. Liquid chromatography-mass spectrometry (LC-MS) showed a molecular ion $[\text{M}]^+$ at m/z 274.2420 (calculated for $\text{C}_{19}\text{H}_{32}\text{O}$: 274.2453). Mass spectrum analysis showed a molecular ion $[\text{M}]^+$ at m/z 276 (relative intensity: 0.11%), corresponding to $\text{C}_{19}\text{H}_{32}\text{O}$, and fragment ions at m/z 258 $[\text{M} - 18]^+$ (15.7%), 176 (10.3%), 163 (15.2%), and 95 (base peak). These data indicated that the alcohol had a structure of 10-cyclopentyliden-3,7-diethyldeca-2,6-dien-1-ol ($n=2$). The relative yield was 37.8%.

Similar reaction of GPP with ethylIPP using *B. stearothermophilus* FPS resulted in a double condensation product (relative yield: 10.7%).

To determine the product structure using ^1H NMR spectra, we examined the reaction at 52-fold scale following HPLC purification. Proton NMR (CDCl_3 , TMS) showed δ 0.96 (3H, t, $J=7.5$ Hz), 0.99 (3H, t, $J=7.7$), 1.59-1.66 (4H, m), 2.00-2.10 (4H, m), 2.11 (4H, m), 2.17 (2H, t, $J=7.2$), 2.11 (2H, t, $J=6.7$), 4.17 (2H, t, $J=5.8$), and 5.08-5.38 (3H, m), indicating that the alcohol had a structure of 10-cyclohexyliden-3,7-diethyldeca-2,6-dien-1-ol.

We prepared a SWISS-MODEL of *B. stearothermophilus* FPS (Figure 1). In addition, docking imaging of product formation in FPS is shown in Figure 2 using simulation software. Given that the margin between Y81 and the product measured only approximately 6 $\text{\AA}$, we determined that the product was discharged promptly in this stage.

Reaction of CpPP with propylIPP ($\text{R}=\text{C}_3\text{H}_7$) using *B. stearothermophilus* FPS produced an alcohol after hydrolysis with alkaline phosphatase. The product exhibited a small peak at 15.0 min on GC, with GC-MS showing a molecular ion $[\text{M}]^+$ at m/z 304 (relative intensity: 1.00%), corresponding to $\text{C}_{21}\text{H}_{36}\text{O}$, and

fragment ions at m/z 286 $[M - 18]^+$ (5.1%), 190 (8.1%), 95 (58.5%), and 55 (base peak), indicating that the product had a structure of 10-cyclopentyliden-3,7-dipropyldeca-2,6-dien-1-ol ($n=2$). However, product quantity was insufficient for detection by HPLC.

ButylIPP ($R=C_4H_9$) was not accepted as a substrate of any bacterial or mammalian enzyme.

Figure 1. Overall image of FPS (monomer portion is shown despite being a homodimer).

Figure 2. Docking image of FPS and product, cyclohexyliden-3,7-diethyldeca-2,6-dienyl diphosphate (FPS was expanded with focusing on the portion from FARM to Y81).

3.3. Reaction of ChPP with 3-alkylIPP by use of FPS

We recently reported on the reaction of ChPP ($m=2$) and IPP [18]. Reaction of ChPP with ethylIPP ($R=C_2H_5$) using *B. stearotheophilus* FPS produced a double condensation product (Scheme 3), and the alcohol obtained following alkaline phosphatase treatment of this product showed an HPLC retention time at 17.3 min. LC-MS showed a main peak at m/z 290.2610 (calculated for $C_{20}H_{34}O$:290.2609), and GC-mass spectrometry showed a molecular ion $[M]^+$ at m/z 290 (relative intensity: 0.08%), corresponding to $C_{20}H_{34}O$, and fragment ions at m/z 272 $[M - 18]^+$ (7.8%), 243 (20.7%), 191 (2.5%), 109 (32.1%), and 67 (base peak), indicative of a structure of 10-cyclohexyliden-3,7-diethyldeca-2,6-dien-1-ol ($n=2$), and thus named. The relative yield was 24.6%. This finding indicates that the reaction of ChPP with ethylIPP using FPS proceeds in a manner similar to that observed between the natural substrates GPP and IPP.

The alcohols derived from the FPS reaction of ChPP with 3-propylIPP ($R=C_3H_7$) showed two peaks on HPLC at retention times of 17.2 (4.7% yield) and 15.9 min (4.8% yield), which were subsequently subjected to GC-MS analysis. Mass spectrum analysis of the former showed a molecular ion at m/z 222 (relative intensity: 0.2%), corresponding to $C_{15}H_{26}O$, and main fragment ions at m/z 204 $[M - 18]^+$ (14.7%), 161 (22.7%), 109 (21.0%), and 79 (base peak). Given these findings, this alcohol's structure was determined to be 6-cyclohexyliden-3-propylhex-2-en-1-ol ($n=1$). Mass spectrum analysis of the latter showed a molecular ion at m/z 318 (relative intensity: 0.1%), corresponding to $C_{22}H_{38}O$, with fragment ions observed at m/z 300 $[M - 18]^+$ (4.0%), 257 (13.1%), 205 (1.9%), 109 (33.1%), and 67 (base peak), indicating a structure of 10-cyclohexyliden-3,6-dipropyldeca-2,6-dien-1-ol ($n=2$). These products may therefore be defined as 6-cyclohexyliden-3-propylhex-2-en-1-ol and 10-cyclohexyliden-3,6-dipropyldeca-2,6-dien-1-ol, respectively, provided a similar enzymatic reaction manner is maintained.

Ultimately, however, neither ChPP ($m=2$) nor butylIPP ($R=C_4H_9$) were found to be acceptable as a substrate for bacterial or mammalian FPSs. Extreme steric hindrance may have hampered the substrate homologs from being taken into the pocket of enzyme.

4. CONCLUSIONS

We investigated substrate specificities of FPSs by examining the reactivity of CpPP (or ChPP) with 3-alkylIPP. Reaction of CpPP (or ChPP) with ethylIPP using *B. stearotheophilus* FPS gave 10-cyclopentyliden-3,7-dimethyldeca-2,6-dienyl diphosphate (CpPP: 10.7% yield; ChPP: 24.6% yield).

5. ACKNOWLEDGMENTS

This work was supported in part by a grant from the Eno Science Foundation (to M.N.).

6. REFERENCES

- [1] T. W. Goodwin (Ed.), "Aspects of Terpenoid Chemistry and Biochemistry", Academic Press (1971).
- [2] T. Koyama, and K. Ogura, *Comprehensive Natural Product Chemistry* Vol. 2. p 69, Pergamon, Oxford (1999).
- [3] K. Ogura, and T. Koyama, *Chem. Rev.*, **98** (1998) 1263. [4] H. Rembold, H. Hagenguth, J. Rascher, *Anal. Biochem.*, **101**, 356-263 (1980).
- [4] C. Burke, and R. Croteau, *Arch. Biochem. Biophys.*, **405** (2002) 130.
- [5] A. Koike-Takeshita, T. Koyama, S. Obata, and K. Ogura, *J. Biol. Chem.*, **270** (1995) 18396.
- [6] R. Tonhosolo, F. L. D7Alexandri, F. A. Genta, G. Wunderlich, F. C. Gozzo, M. N. Erberlin, V. J. Peres, E. A. Kimura, and A. M. Katzin, *Biochem. J.*, **392** (2005) 117.
- [7] M. C. Schulbach, P. J. Brennan, and C. D. Crick, *J. Biol. Chem.*, **275** (2000) 22876.
- [8] D. Kaur, P. J. Brennan, and D. C. Crick, *J. Bacteriol.*, **186** (2004) 7564.
- [9] T. Koyama, A. Saito, K. Ogura, S. Seto, *J. Am. Chem. Soc.*, **102** (1980) 3614.
- [10] M. Nagaki, M. Nakada, T. Musashi, J. Kawakami, T. Endo, Y. Maki, T. Koyama, *J. Mol. Catal. B, Enzymatic*, **17** (2002) 81.
- [11] T. Koyama, S. Obata, M. Osabe, A. Takeshita, K. Yokoyama, M. Uchida, T. Nishino, K. Ogura, *J. Biochem.*, **113**, 355 (1993).
- [12] G. Popják, P. W. Holloway, J. M. Baron, *Biochem. J.*, **111** (1969) 325.
- [13] T. Nishino, K. Ogura, S. Seto, *J. Am. Chem. Soc.*, **94** (1972) 6849.
- [14] V. J. Davisson, T. A. Meal, C. D. Poulter, *J. Am. Chem. Soc.*, **115** (1993) 1235.
- [15] T. Koyama, A. Saito, K. Ogura, S. Seto, *J. Am. Chem. Soc.*, **102** (1980) 3614.
- [16] M. Nagaki, M. Nakada, T. Musashi, J. Kawakami, N. Ohya, M. Kurihara, Y. Maki, T. Nishino, T. Koyama, *Biosci. Biotechnol. Biochem.*, **71** (2007) 1657.
- [17] M. Nagaki, M. Nakada, T. Musashi, Jun Kawakami, T. Endo, Y. Maki, T. Koyama, *J. Mol. Catal. B, Enzymatic*, **59** (2009) 225.
- [18] M. Nagaki, H. Kanno, T. Musashi, R. Shimizu, Y. Maki, H. Sagami, T. Koyama, *J. Mol. Catal. B, Enzymatic*, **60** (2009) 186.
- [19] M. Nagaki, K. Miyata, J. Kawakami, R. Miyamoto, Y. Maki, H. Sagami, T. Koyama, *Trans. Mater. Res. Soc. Jpn.*, **34** (2009) 553.
- [20] M. Nagaki, S. Sato, Y. Maki, T. Nishino, T. Koyama, *J. Mol. Catal. B: Enzymatic*, **9**, 33 (2000).
- [21] K. Ogura, T. Koyama, S. Seto, *J. Chem. Soc. Chem. Commun.*, **1972**, 881.
- [22] J. M. Coxon, E. Dansted, M. P. Hartshorn, *Org. synth., coll. vol. 6*, 946 (1988).
- [23] E. J. Corey, C. U. Kim, M. Takeda, *Tetrahedron Lett.*, **42**, 4339 (1972).
- [24] V. J. Davisson, A. B. Woodside, C. D. Poulter, *Methods Enzymol.*, **110**, 133 (1985).
- [25] J. K. Dorsey, J. A. Dorsey, J. W. Porter, *J. Biol. Chem.*, **241** (1966) 5353.
- [26] P. W. Holloway, G. Popják, *Biochem. J.*, **104**, 57 (1967).
- [27] K. Kishi, H. Sagami, K. Ogura, *J. Biochem.*, **93**, 1635 (1983).
- [28] M. Nagaki, H. Yamamoto, A. Takahashi, Y. Miki, J. Ishibashi, T. Nishino, T. Koyama, *J. Mol. Catal. B: Enzymatic*, **17**, 81 (2002).
- [29] D. J. Hosfield, Y. Zhang, D. R. Dougan, A. Broun, L. W. Tari, R.V. Swason, J. Finn, *J. Biochem. Chem.*, **279** (10) 8526 (2004).
- [30] M. L. Verdonk, J. C. Cole, M. J. Hartshorn, C. W. Murray, R. D. Taylor, *Proteins*, **52**, 609 (2003).

(Received January 18, 2010; Accepted March 11, 2010)