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Functional Analysis of Eubacterial *ent*-Copalyl Diphosphate Synthase and Pimara-9(11),15-Diene Synthase with Unique Primary Sequences

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We have previously cloned a DNA fragment that contained four ORFs and was confirmed to participate in viguiepinol {3-hydroxypimara-9(11),15-diene} biosynthesis by a heterologous expression experiment, from *Streptomyces* sp. strain KO-3988. Of the four ORFs, ORF2 and ORF4 were confirmed to encode an *ent*-CDP synthase and a GGDP synthase, respectively, by experiments using recombinant enzymes. In this study, ORF3, that did not show similarities with any other known proteins was expressed in *Escherichia coli* and used for functional analysis. The purified ORF3 product clearly converted *ent*-CDP into PMD. Since ORF2 and ORF3 are the first examples of enzymes with these biosynthetic functions from prokaryotes, enzymatic properties of both enzymes were investigated. ORF2 is likely to be a dimer and requires a divalent cation such as Mg^{2+} and Zn^{2+} for its activity. The optimum pH and temperature were 5.5 and 35°C. The *K*m value was calculated to be $13.7 \pm 1.0\,\mu M$ for GGDP and the *k*cat value was 3.3×10^{-2}/sec. ORF3 is likely to be a monomer and also requires a divalent cation. The optimum pH and temperature were 7.0 and 30°C. The *K*m value for *ent*-CDP was estimated to be $2.6 \pm 0.2\,\mu M$ and the *k*cat value was 1.4×10^{-3}/sec.

Key words: *ent*-copalyl diphosphate synthase, diterpene, isoprenoid cyclase, pimara-9(11),15-diene synthase, *Streptomyces*.

Abbreviations: CDP, copalyl diphosphate; FDP, farnesyl diphosphate; GGDP, geranylgeranyl diphosphate; MBP, maltose binding protein; ORF, open reading frame; PMD, pimara-9(11),15-diene; TDP, terpentedienol diphosphate.

INTRODUCTION

Isoprenoids are the largest single family of compounds found in nature with over 24,000 known examples (*1, 2*). All isoprenoids are biosynthesized from a common C_5-unit precursor, isopentenyl diphosphate. The successive condensation of isopentenyl diphosphate with dimethylallyl diphosphate gives rise to geranyl diphosphate (C_{10}), farnesyl diphosphate (FDP, C_{15}) and geranylgeranyl diphosphate (GGDP, C_{20}), which are generally cyclized by organism-specific cyclases to produce the parent skeletons of monoterpenes (C_{10}) (*3*), sesquiterpenes (C_{15}) (*4*) and diterpenes (C_{20}) (*5*). Therefore, the first and key enzymes leading to diversity of isoprenoids are isoprenoid cyclases.

These isoprenoid cyclases are classified into two major types with respect to their modes of cyclization (*3–6*). Type-A cyclization is initiated by ionization of polyprenyl diphosphate to an allylic carbocation. The other type of cyclization (type-B) is initiated by protonation at the terminal-double bond of polyprenyl diphosphate. Although these cyclases use common substrates, such as GGDP and FDP, they can make a variety of basic skeletons specific for each cyclase, that is, an initiation reaction either by ionization or protonation, followed by a series of cyclizations and rearrangements prior to deprotonation of the final carbocation.

Until now, dozens of isoprenoid cyclase genes have been cloned mainly from eukaryotes. Among type-B cyclases, triterpene cyclases, such as squalene-hopene cyclases, β-amyrin synthases, and oxidosqualene-lanosterol cyclases, have been cloned and characterized in detail (*7–9*). Other than triterpene cyclases, some genes encoding diterpene cyclases, which catalyzed a conversion of GGDP into *ent*-CDP or *syn*-CDP in gibberellin (*10–17*) and phytoalexins (*15, 18, 19*) biosynthesis, have been cloned.

As for type-A cyclases, Croteau and co-workers extensively studied reaction mechanisms of monoterpene, sesquiterpene and diterpene cyclases from grand fir (*Abies grandis*) (*20, 21*). They also cloned and characterized a gene encoding taxadiene synthase (C_{20}) that catalyzes the committed step of biosynthesis of taxol, an anticancer drug isolated from yew (*22*). Besides *A. grandis*, dozens of isoprenoid cyclase genes have been cloned from plants such as Norway spruce, maize, pine, ginkgo, snapdragon, grapevine, lotus and tobacco (*20, 21*). Moreover, *ent*-kaurene synthases that convert *ent*-CDP into *ent*-kaurene in gibberellin biosynthesis were cloned

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from several plants (*23, 24*). Very recently, a series of diterpene cyclases genes essential for biosynthesis of phytoalexins in rice, such as *ent*-cassa-12,15-diene synthase, *syn*-pimara-7,15-diene synthase, *ent*-sandaracopimaradiene synthase and stemar-13-ene synthase gene, have been cloned (*25–29*). Furthermore, unique genes encoding bi-functional diterpene cyclases, which have distinct catalytic domains essential for type-A and type-B cyclizations, have been cloned from *A. grandis* (*30*) and fungi (*31*).

In contrast to these eukaryotic isoprenoid cyclases, only limited studies on prokaryotic isoprenoid cyclases have been reported so far (*32*). Cane *et al*. have cloned type-A sesquiterpene cyclase genes, such as pentalenene synthase (*33*), germacradienol and germacrene D synthase (*34*), and (+)-*epi*-isozizaene synthase (*35*) from *Streptomyces* strains. We have also cloned a gene encoding TDP synthase, which is a type-B eubacterial diterpene cyclase essential for production of terpentecin and with a low similarity to eukaryotic diterpene cyclases (<30% identity), for the first time of prokaryotic origin (*36, 37*). The enzyme could be easily expressed in a soluble form in *E. coli* and its enzymatic character was studied with a recombinant enzyme (*36, 37*). We also showed that an ORF, Rv3377c, in *Mycobacterium tuberculosis*, which had 37% identity to the TDP synthase, catalyzed a conversion of GGDP into tuberculosinol diphosohate (type-B cyclase) (*38*). Moreover, we have recently cloned a DNA fragment containing four ORFs from *Streptomyces* sp. strain KO-3988 that was confirmed to participate in viguiepinol {3-hydroxypimara-9(11),15-diene} biosynthesis by a heterologous expression experiment (*39*). Among the ORFs, ORF2 was also confirmed to be an *ent*-CDP synthase (type-B) by an enzyme assay using recombinant enzyme (*40*). Very interestingly, the protein most homologous to the *ent*-CDP synthase was not *ent*-CDP synthases found in eukaryotes, but TDP synthase (36% identity over 499 amino acids) that catalyzes the conversion of GGDP into TDP as described above. Moreover, ORF3, located at just downstream of the *ent*-CDP synthase gene (ORF2) and suggested to participate in viguiepinol biosynthesis, did not show similarities with any other proteins in various databases (*39, 40*). These results suggested that eubacterial diterpene cyclases have unique primary sequences different from eukaryotes. In this study, we therefore investigated first the function of the ORF3 product and confirmed it to be a type-A diterpene cyclase catalyzing the conversion of *ent*-CDP into PMD for the first time of an enzyme with this biosynthetic function.

We also investigated in detail the enzymatic properties of the *ent*-CDP synthase and the PMD synthase using recombinant enzymes.

MATERIALS AND METHODS

Materials—GGDP was purchased from Sigma–Aldrich and further purified by column chromatography to remove impurities such as Mg^{2+}. The purification procedure was described previously (*37*). The other chemicals were all analytical grade. Five plasmids, pET21 (*C*-terminal His-Tagged, Novagen), pCAL-n-FLAG (*N*-terminal FLAG tagged, Stratagene), pGEX-6P-1 (*N*-terminal glutathione S-transferase-fused, GE Healthcare), pMAL-c2X and pMAL-p2X (*N*-terminal MBP-fused, New England Biolabs), were used for expression of the recombinant ORF3.

DNA Isolation and Manipulation—Plasmids from *E. coli* were prepared using a Qiagen plasmid kit (Qiagen). All restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo (Osaka, Japan). Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research, Inc., San Diego, CA, USA). Other general procedures were performed as described by Maniatis *et al*. (*41*).

Preparation of Recombinant Enzymes—To overproduce the *ent*-CDP synthase as an N-terminal His-tagged protein, an *E. coli* M15 transformant harbouring pFQ-DC1, which had been previously constructed by using an expression vector, pQE30 (*40*), was used. N-Terminal six-His tagged fusion proteins of *ent*-CDP synthase was purified by a Ni-nitrilotriacetic acid agarose column and used for enzyme assays and kinetic studies. To overproduce the PMD synthase as an *N*-terminal MBP-fused protein, the gene was amplified by the following 5′ and 3′ primers, 5′-CGGAATTCACCC GGCCCCTTGGCGACCTCTCCCTC-3′ and 5′-ACCAAG CTTCATGTCGGAGGGCAGGCCGGA-3′. The amplified PCR product was digested with *Eco*RI and *Hin*dIII, separated by agarose gel electrophoresis and purified with a Gel Extraction Kit (QIAGEN). After sequence confirmation, the fragment was inserted into the same sites of pMAL-c2X. In the resulting plasmid pMAL-c2X-PMDS, the recombinant protein was expressed as an N-terminal MBP-fused protein. Expression and purification conditions for the recombinant enzyme were essentially the same as those described in the manufacturer's protocols. In brief, *E. coli* TB1 (NEB) harbouring pMAL-c2X-PMDS was grown at 37°C in Luria Broth medium with ampicillin (100 μg/ml) and D-glucose (0.2%). Expression of the recombinant protein was induced by adding 1 mM IPTG when the optical density at 600 nm reached about 0.8. The cultivation was continued for an additional 16 h at 18°C. The recombinant protein was purified with amylose affinity column chromatography (NEB) and fused MBP was cleaved with Factor Xa (NEB). After elution of the recombinant PMD synthase, purity was analyzed by an SDS-PAGE on 12.5% gels. Protein concentration was determined by a protein-dye standard assay (Bio-Rad) using bovine serum albumin as a standard. The apparent molecular mass was estimated by gel filtration using a $G3000SW_{XL}$ (7.8 mm × 300 mm) column (TOSOH, Japan) that had been equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl.

Enzyme Assay of the ent-CDP Synthase—A standard reaction mixture (500 μl) containing 50 mM citrate buffer (pH 5.5), 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1% (w/v) Tween-80, 20% (v/v) glycerol, 30 μM GGDP and 5 μg/ml of the purified *ent*-CDP synthase, was incubated at 35°C for 10 min. The condition was confirmed to fit the steady-state kinetic parameters. After incubation, 100 μl of 0.5 M EDTA (pH 8.0) was added to terminate the reaction and reaction products were analyzed by reversed-phase

HPLC using an ion-pair reagent. Analytical conditions were the same as those for TDP (*40*). For kinetic assays, triplicate enzyme assay sets were performed at each substrate concentration and the Hanes–Woolf plot, substrate concentration [(S)]/velocity versus (S), were used for the estimation of kinetic constants.

Enzyme Assay of the PMD Synthase—The reaction mixture (5 ml) contained 50 mM Tris–HCl (pH 6.8), 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1% (w/v) Tween-80, 20% (v/v) glycerol, 2 μM *ent*-CDP (GGDP, or FDP) and 10 μg/ml of the purified PMD synthase. After incubation of the reaction mixture at 30°C for 5 min, 1 ml of 0.5 M EDTA (pH 8.0) was added to terminate the reaction. The reaction product was extracted with chloroform and the organic layer was evaporated to dryness under reduced pressure. The dried material was dissolved in a small volume of ethanol and analyzed by a reverse phase HPLC. Analytical conditions were described previously (*39*). Triplicate enzyme assay sets were performed at each substrate concentration, and Hanes–Woolf plot was used to estimate kinetic constants.

RESULTS

Functional Analysis of the ORF3 Product—We have previously cloned a 5-kb DNA fragment containing four ORFs (ORF1 to ORF4) from *Streptomyces* sp. strain KO-3988 (*39, 40*). The DNA fragment presumably conferred viguiepinol {3-hydroxypimara-9(11),15-diene} productivity upon *Streptomyces lividans* TK23 (*39*). Moreover, ORF2 and ORF4 were confirmed to encode an *ent*-CDP synthase and a GGDP synthase, respectively (*40*). Considering that ORF1 had significant similarity to a variety of P450 enzymes catalyzing hydroxylation reactions, ORF3 was strongly suggested to be an enzyme catalyzing the conversion of *ent*-CDP into PMD (Fig. 1), although ORF3 showed no similarities with any other enzymes, including eukaryotic diterpene cyclases that have been confirmed to catalyze the formation of pimaradiene type compounds from *ent*-CDP or *syn*-CDP (*23–29*). To examine the possibility that ORF3 has the estimated enzyme activity, we tried to express a recombinant ORF3 in *E. coli* as an N-terminal His-tagged protein. However, the expressed proteins strongly formed inclusion bodies even after several attempts to increase the amount of soluble enzyme by decreasing the amount of IPTG added for induction, and by prolonging cultivation at low temperature. Therefore, in this study, we tried to express the ORF3 product as a C-terminal His-tagged, N-terminal FLAG tagged, N-terminal GST-fused or N-terminal MBP-fused recombinant protein. Among the expression vectors tested so far, we were able to express small amounts of recombinant ORF3 as a soluble form when MBP was fused to its N-terminus with pMAL-c2X (Fig. 2). By cultivating the transformant at low temperature, the soluble recombinant form slightly increased and was used for further analyses after purification.

To verify that ORF3 could convert *ent*-CDP into PMD, we incubated *ent*-CDP with the purified ORF3 product or the heat-denatured ORF3 product under standard reaction conditions, and the reaction mixture was analyzed by reversed-phase HPLC. When *ent*-CDP was incubated with the active ORF3 product, a compound was specifically formed (Fig. 3). The compound was purified and its structure was determined by mass spectrum and NMR spectral analyses. Eventually, the compound was confirmed to be PMD, which had been also produced by *S. lividans* transformant harboring the 5-kb DNA fragment together with viguiepinol (*39*).

Characterization of the ent-CDP Synthase—Since there are few reports on characterization of *ent*-CDP synthases

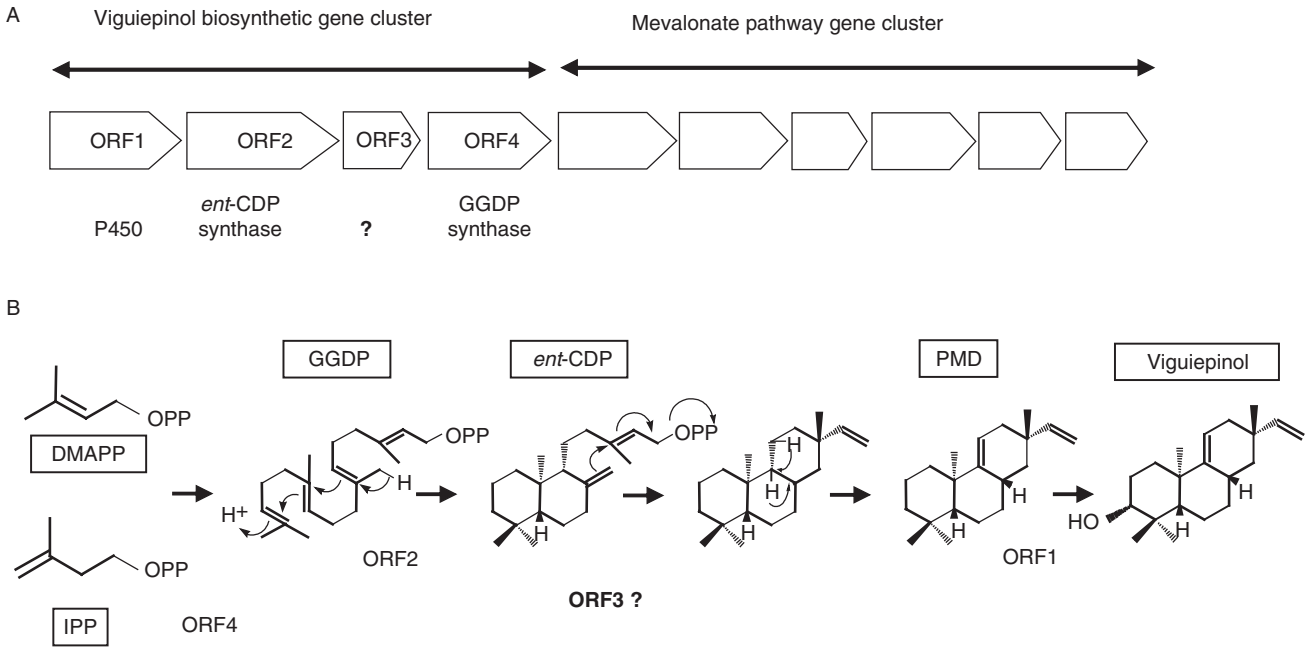

Fig. 1. **Gene cluster (A) and putative pathway (B) for viguiepinol biosynthesis**.

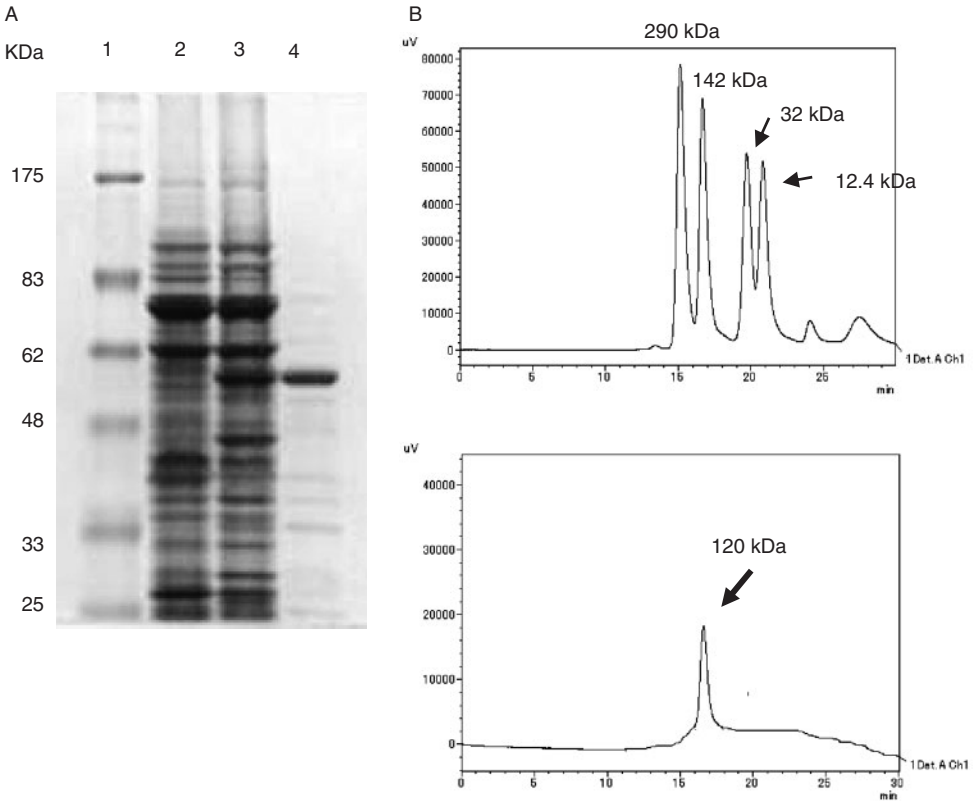

Fig. 2. **Electrophoresis (A) and gel filtration chromatography (B) of the overproduced and purified *ent*-CDP synthase.** (A): Molecular mass marker (lane 1), total soluble proteins from the *E. coli* harboring pQE30 (lane 2), pFQ-DC1 (lane 3) and purified *ent*-CDP synthase (lane 4), were analyzed on an SDS-PAGE (12.5%). Proteins were stained with Coomassie brilliant blue R-250. (B): Elution profile of the standard proteins (upper) and the purified *ent*-CDP synthase (lower) are shown. Molecular weight standards [glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142.0 kDa), myokinase (32.0 kDa), chyochrome c (12.4 kDa)] were used.

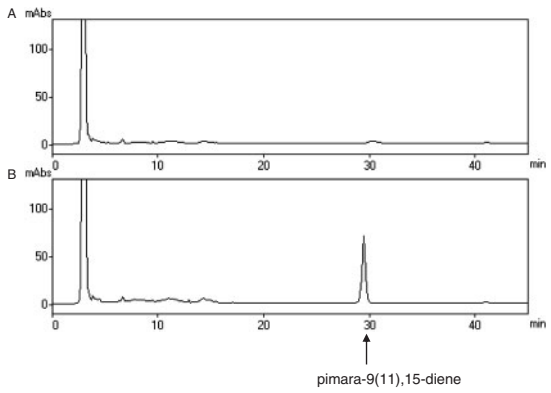

Fig. 3. **HPLC analysis of the product generated by the recombinant PMD synthase with *ent*-CDP as the substrate.** Reaction products generated by denatured PMD synthase (A) and active PMD synthase (B) in the presence of *ent*-CDP were analyzed by HPLC. The reaction product was purified and confirmed to be pimara-9(11),15-diene by NMR analysis.

from eukaryotes and the ORF2 product was the first example of an *ent*-CDP synthase from prokaryotes, enzymatic properties of the *ent*-CDP synthase (ORF2) were investigated using a recombinant enzyme. We have previously reported that the TDP synthase, which was the most homologous enzyme to the *ent*-CDP synthase, formed a homodimer (*37*). Therefore, we first investigated whether the *ent*-CDP synthase also formed a dimer structure. The assay mixture containing the His-tagged *ent*-CDP synthase with a calculated molecular mass of 55 kDa was subjected to gel filtration. As shown in Fig. 2, one major peak corresponding to a molecular mass of 120 kDa was detected and no protein with a molecular mass of 55 kDa corresponding to the molecular mass of the monomer was eluted. The peak was fractionated and confirmed to contain the *ent*-CDP synthase by SDS-PAGE analysis (data not shown), suggesting that the *ent*-CDP synthase forms a homodimer.

We next investigated the effect of metal ions on enzyme activity because many isoprenoid cyclases analyzed so far required Mg^{2+} (*3–5*). The *ent*-CDP synthase was dialyzed with 50 mM Tris-HCl (pH 6.0) containing 10 mM EDTA for 24 h and then diluted 5 times with the same buffer without EDTA. Thereafter, 1 mM and 10 mM of divalent cations were added and the enzyme activities were assayed. No formation of *ent*-CDP was detected in the absence of Mg^{2+}. The enzyme activity of the *ent*-CDP synthase was highest with Mg^{2+} at a concentration of 1 mM. Relative activities of the other divalent cations

(1 mM) were as follows: Zn^{2+}, 82%; Co^{2+}, 69%; Mn^{2+}, 63%; Ni^{2+}, 32%. No or negligible activity (<1%) was detected with Fe^{2+}, Ca^{2+}, and Cu^{2+}.

The optimum pH was measured in several buffers at various pH values (final concentration of 0.05 M): citrate buffer, pH 5.0–7.5; potassium phosphate buffer, pH 5.0–7.0; MES (Good's buffer) buffer, pH 5.5–7.0; Tris–HCl, pH 6.5–9.0. The *ent*-CDP synthase showed a high activity at a broad pH range with each of the buffers. Though maximum activity was observed at pH 5.5 (citrate buffer), more than 90% of the relative activity was observed between pH 5 and 8. However, the activity was rapidly lost with decreasing pH. The effect of temperature on enzyme activity was also investigated over a range of 20–80°C in 0.05 M citrate buffer, pH 5.5. The enzyme activity was maximal at 35°C and was detected after incubation at 35°C in 0.05 M citrate buffer, pH 5.5 for 1 h. Under optimum pH and temperature conditions, 5 mM 2-mercaptoethanol and 0.1% Tween–80 were found to be required for full activity of the *ent*-CDP synthase.

The kinetic properties of the *ent*-CDP synthase were studied under the optimum reaction conditions. The *K*m value and *k*cat value were calculated to be 13.7 ± 1.0 μM for GGDP and 3.3×10^{-2}/s, respectively. The enzyme activity was inhibited by GGDP at a concentration of more than 30 μM, as had been reported for other isoprenoid cyclases (*3–5*). These enzymatic properties of the *ent*-CDP synthase were summarized in Table 1.

Characterization of the PMD Synthase—As described earlier, ORF3 that had no similarity to any other proteins was confirmed to be a PMD synthase. Therefore, we next investigated the enzymatic properties of this unique diterpene cyclase. We first investigated the quaternary structure of the PMD synthase. The assay mixture containing the recombinant PMD synthase was

Table 1. **Kinetic properties of *ent*-CDP synthase and PMD synthase.**

Enzyme	Substrates	Optimum		Required Metals	*K*m (μM)	*k*cat (s^{-1})
		pH	Temp (°C)			
ent-CDP synthase	GGDP	5.5	35	$Mg^{2+} > Zn^{2+} > Co^{2+}$	13.7 ± 1.0	3.3×10^{-2}
PMD synthase	*ent*-CDP	7.0	30	$Mg^{2+} > Co^{2+}$, Zn^{2+}	2.6 ± 0.2	1.4×10^{-3}

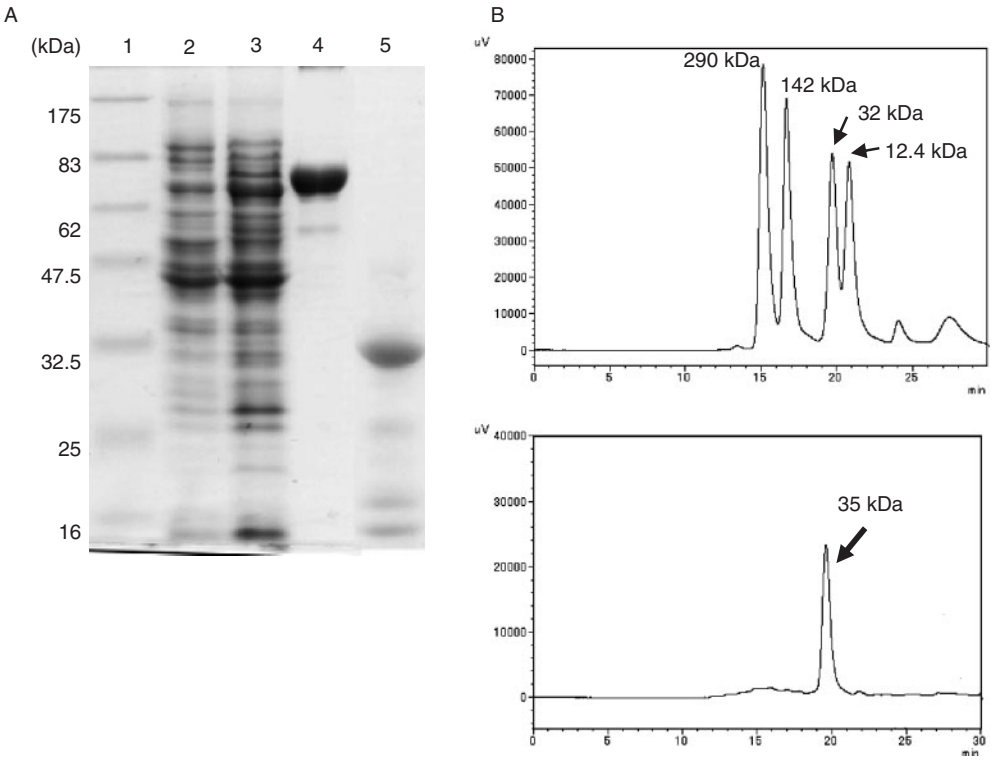

Fig. 4. **Electrophoresis (A) and gel filtration chromatography (B) of the overproduced and purified PMD synthase.** (A): Molecular mass marker (lane 1), total soluble proteins from the *E. coli* harboring pMAL-c2X (lane 2), pMAL-c2X-PMDS (lane 3), purified *N*-terminal MBP-fused PMD synthase (lane 4) and purified PMD synthase (lane 5) were analyzed on an SDS-PAGE (12.5%). Proteins were stained with Coomassie brilliant blue R-250. (B): Elution profile of the standard proteins (upper) and the purified PMD synthase (lower) are shown. Molecular weight standards were the same as those shown in Fig. 2.

subjected to gel filtration. As shown in Fig. 4, a single peak with a molecular mass of 35 kDa that corresponded to the molecular mass of the monomer was eluted. The peak was fractionated and confirmed to contain the PMD synthase by SDS-PAGE analysis (data not shown), suggesting that the PMD synthase can form a monomer.

Next, the effect of metal ions on the activity of the PMD synthase was studied. The metal ion-free PMD synthase that was prepared by the same method as the *ent*-CDP synthase, was used for the enzyme assay with *ent*-CDP as a substrate in the presence or absence of various metal ions. The PMD synthase absolutely required Mg^{2+} for its activity as had been reported for other isoprenoid cyclases, and the optimum concentration was 1 mM. Enzyme activity was also detected with 1 mM of Co^{2+}, Zn^{2+} and Ni^{2+}. When the enzyme activity measured with 1 mM of Mg^{2+} was taken as 100%, the relative activities of Co^{2+}, Zn^{2+} and Ni^{2+} were 100%, 59% and 57%, respectively. No or a very weak activity (<1%) was detected with other divalent metal ions such as Mn^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+}.

Effect of pH and temperature on the enzyme activity of PMD synthase was also investigated with the same buffers as those used for characterization of the *ent*-CDP synthase. The PMD synthase was active at a pH range of 6.0–8.0 and the optimum pH was 7.0 with Tris–HCl buffer. The optimum temperature for enzyme activity was 30°C in 0.05 M Tris–HCl buffer, pH 7.0. For maximum activity, the same additives that were effective for the *ent*-CDP synthase activity were also essential. The *K*m value and *k*cat value were calculated to be $2.6 \pm 0.2\,\mu M$ and was 1.4×10^{-3}/s, respectively. The properties of the PMD synthase were summarized in Table 1.

Substrate Specificity of PMD Synthase—Though the PMD synthase did not show significant similarities to any other proteins, we had previously found that the enzyme possessed a very weak similarity to Cyc2 that was confirmed to convert TDP to terpentetriene in terpentecin biosynthesis (15% identity) (*39*). Since the Cyc2 was previously demonstrated to react with GGDP and FDP besides the intrinsic substrate TDP (*37*), we examined if the PMD synthase might also react with GGDP, FDP and TDP, but no products were detected.

DISCUSSION

In this study, we investigated the function of the ORF3 product and confirmed it to be a type-A diterpene cyclase catalyzing the conversion of *ent*-CDP into PMD for the first time of an enzyme with this biosynthetic function. We also investigated in detail the enzymatic properties of the *ent*-CDP synthase and the PMD synthase using recombinant enzymes. To date, several *ent*-CDP synthase genes have been cloned from plants and fungi (*10–19, 31*). However, only a few *ent*-CDP synthases were characterized by (partially) purified native enzymes and recombinant enzymes (*31, 42–44*). To the best of our knowledge, this is the first report on the characterization of a prokaryotic *ent*-CDP synthase. Although the *ent*-CDP synthase characterized in this study only had a low amino acid similarity to eukaryotic *ent*-CDP synthases (~27% identity), some enzymatic properties such as the *K*m value for GGDP, *k*cat value, suitable concentration and kind of divalent cations and concentration of substrate inhibition were almost the same as those of eukaryotic enzymes, which had been previously reported (*31, 42–44*). These results suggested that some specific amino acid residues responsible for the catalytic reaction would be conserved in both prokaryotic and eukaryotic enzymes. To estimate these residues, we aligned the *ent*-CDP synthase with two *ent*-CDP synthases of rice (Fig. 5). We could detect several conserved regions, including the putative substrate binding region. These amino acid residues might be important for catalysis. To identify the exact catalytic sites, however, analyses of the crystal structure of the enzyme and characterization of mutated enzymes obtained by site-directed mutagenesis would be necessary.

The protein that showed the highest homology to the *ent*-CDP synthase was the TDP synthase (36% identity over 499 amino acids) that catalyzes the conversion of GGDP into TDP in terpentecin biosynthesis (*37*). Both enzymes showed similar enzymatic properties such as substrate specificity, *K*m and *k*cat values, suitable concentration of divalent cations, etc (*37*). However, in contrast to the *ent*-CDP synthase that catalyzed a series of cyclizations prior to deprotonation of the final carbocation, the TDP synthase accompanied rearrangements of methyl group and hydride shifts to neutralize the final carbocation after a series of cyclizations (*37*). Therefore, chimera enzymes constructed by the *ent*-CDP synthase and the TDP synthase and mutated enzymes generated by site-directed mutagenesis might be useful to understand the unique methyl transfer reaction. These studies are now in progress and will be reported in the near future.

The ORF3 that had no similarities with any other proteins in various databases was clearly confirmed to be a PMD synthase. To the best of our knowledge, this is the first report on a PMD synthase catalyzing the conversion of *ent*-CDP into PMD. To date, several genes encoding diterpene cyclases that accepted *ent*- or *syn*-CDP as a substrate have been cloned from plants and fungi (*23–29*) and the enzymes from fungi were reported to be multifunctional and could directly convert GGDP into *ent*-kaurene (*31*). On the other hand, plant enzymes such as *ent*-cassa-12,15-dienesynthase, *syn*-pimara-7,15-dienesynthase(*26, 28*), *ent*-sandaracopimaradiene synthase (*25*), and stemar-13-ene synthase gene (*27*), were confirmed to be monofunctional enzymes that use *ent*-CDP or *syn*-CDP as an intrinsic substrates in a similar manner to the PMD synthase. Very interestingly, all these plant enzymes consisted of >750 amino acid residues and their primary sequences had >40% homology with each other. In contrast, the PMD synthase was composed of only 295 amino acids and had no homology with any other proteins. These findings suggest that eubacterial and eukaryotic diterpene cyclase genes have independently evolved with each other. Therefore, it would be very interesting to examine if the PMD synthase has a similar tertiary structure to plant cyclases that accept *ent*-CDP as a substrate,

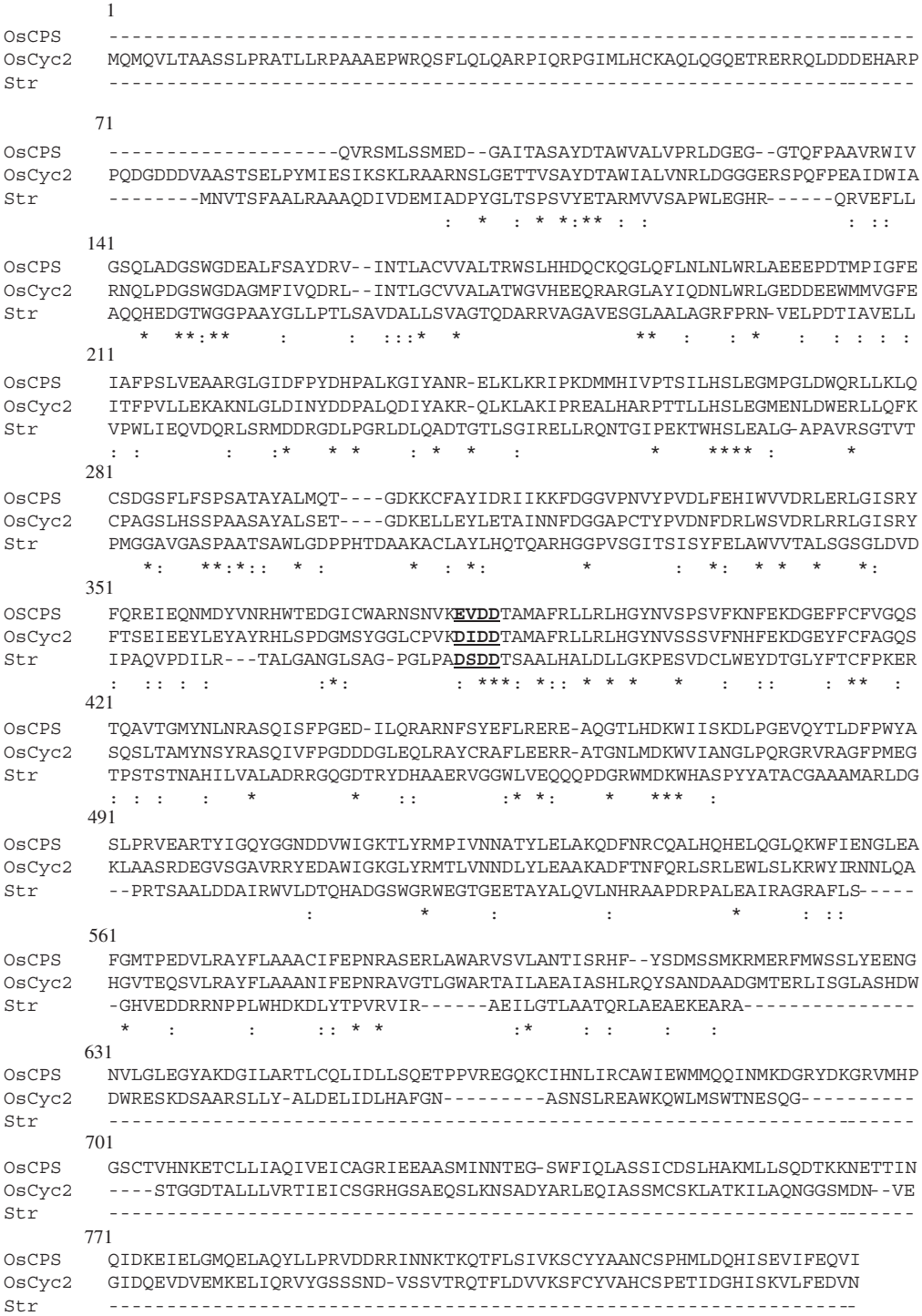

Fig. 5. **Alignment of *ent*-CDP synthases. Multiple alignment of the ORF2 product with *ent*-CDP synthases cloned from rice is shown.** Identical and similar amino acid residues are indicated by asterisks (∗) and colons (:). Abbreviations: OsCPS, *ent*-CDP synthase (AB126932); OsCyc2, *ent*-CDP synthase (AB066271); Str, the ORF2 product. The numbers on the left are the residue numbers for OsCyc2. The amino acid residues known to be putative GGDP binding sites are underlined.

though there are no reports on tertiary structures of plant diterpene cyclases. However, very recently, several genes encoding diterpene cyclases that accept *ent*-CDP as a substrate have been cloned from rice and their recombinant enzymes have been successfully expressed in *E. coli* (*25, 26, 29*). Therefore, we will be able to compare the tertiary structures of eubacterial and eukaryotic diterpene cyclases in the near future.

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