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Efficient *in vitro* synthesis of *cis*-polyisoprenes using a thermostable *cis*-prenyltransferase from a hyperthermophilic archaeon *Thermococcus kodakaraensis*

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ABSTRACT

The *Tk-idsB* encoding *cis*-prenyltransferase which catalyzes consecutive *cis*-condensation of isopentenyl diphosphate to allylic diphosphate was isolated from a hyperthermophilic archaeon *Thermococcus kodakaraensis*, and enzymatic characteristics of the recombinant *Tk*-IdsB were examined. *Tk*-IdsB was not fully denatured even at 90 °C and preferably utilizes both C_{10} and C_{15} allylic diphosphates to yield mainly the C_{60}–C_{65} products. Based on structural models, single alanine-substitution mutants at Glu68, Lys109, or Leu113 were constructed, showing that all the three produced longer chains (C_{65}–C_{70}) than the wild-type and the substitution at 109 (K109A) was the most effective. *Tk*-IdsB was applied to an organic-aqueous dual-phase system and more than 90% of the products were recovered from the organic phase when 1-butanol or 1-pentanol was overlaid. When 1-octanol was overlaid, 70% of the products were obtained from the upper organic phase. The product distributions were changed depending on the hydrophobicity of organic solvents used. *Tk*-IdsB was then immobilized onto silica beads to make *Tk*-IdsB more tolerant, showing that half-life of enzyme at 80 °C was prolonged by immobilization. When the immobilized *Tk*-IdsB was applied in the organic-aqueous dual-phase system, immobilized *Tk*-IdsB catalyzed consecutive condensation more efficiently than the unimmobilized one.

1. Introduction

cis-Prenyltransferase (*cis*-isoprenyl diphosphate synthase) catalyzes the consecutive condensation of isopentenyl diphosphate (IPP, C_5) with a short-chain *trans*-allylic diphosphate by *cis*-configuration to produce linear *cis*-polyisoprenes (Fig. 1A). The product chain-lengths are widely different (C_{15}–$C_{>100}$) among *cis*-prenyltransferases, according to which it is proposed that they are classified into three subgroups, *i.e.*, short (C_{15}), medium (C_{50}–C_{55}), and long ($C_{>70}$)-chain *cis*-prenyltransferases (Takahashi and Koyama, 2006).

Medium-chain *cis*-prenyltransferases are mainly derived from bacteria, whose reaction products are converted to a monophosphate form and then utilized as a sugar-carrier lipid across the membrane in the cell-wall biosynthesis (Takahashi and Koyama, 2006). On the other hand, the eukaryotic enzymes belong to long-chain *cis*-prenyltransferases, and like the case of bacteria, their reaction products are utilized as a sugar-carrier lipid mainly acting on the endoplasmic reticulum (ER) and Golgi membranes for the biosynthesis of glycoproteins, however, different from the bacterial sugar-carrier lipid, the eukaryotic one acts in the monophosphorylated form of that α-terminal double-bound is saturated, *i.e.*, dolichol phosphate (Liang et al., 2002). As commonly known, natural rubber is a highly condensed huge polymer molecule ($C_{>10,000}$), but its biosynthesis still remains unclear, while the molecular cloning and characterization of *cis*-prenyltransferases from rubber tree, *Hevea brasiliensis* were conducted (Asawatreratanakul et al., 2003). As for archaea, their sugar-carrier lipid is a dolichol phos-

Abbreviations: CD, circular dichroism; DMAPP, dimethylallyl diphosphate; ER, endoplasmic reticulum; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; UPP, undecaprenyl diphosphate.

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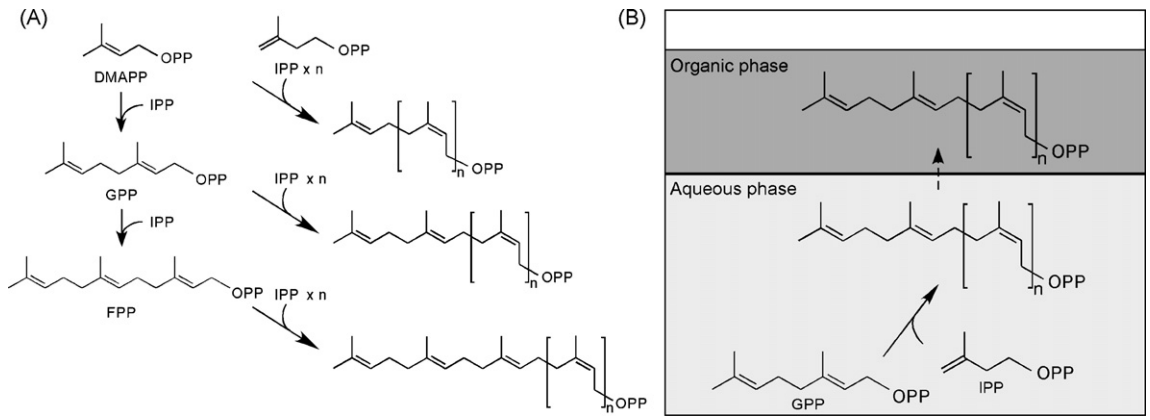

Fig. 1. Synthetic process of *cis*-polyisoprenes. (A) Biosynthetic pathway of *cis*-polyisoprenes in Archaea. (B) Concept of the organic-aqueous dual-phase system. The light- and dark-shaded areas indicate the aqueous and organic phase, respectively. An arrow indicates consecutive condensation by *Tk*-IdsB. OPP, diphosphate moiety.

phate type, as well as eukaryotes, and it is also assumed to be utilized for the glycoprotein biosynthesis (Kuntz et al., 1997). So far, from a thermoacidophilic archaeon, *Sulfolobus acidocaldarius*, *cis*-prenyltransferase was cloned, and found that it mainly produces the C_{50}–C_{55} *cis*-polyisoprenes (Hemmi et al., 2001). The mechanism of the product chain-length determination of *cis*-prenyltransferase was elucidated to some extent but the exact mechanisms have been unclear. The product chain-length is predominantly determined by the structure, charge or polarity of amino acid residues of a hydrophobic cleft in the enzyme, into which the elongating *cis*-polyprenyl chain is accommodated, and that change of the cleft's property by mutation altered the length of the final product together with the product distribution (Kharel et al., 2006; Ko et al., 2001).

In the present study, we examined the enzymatic characteristics of *cis*-prenyltransferase (*Tk*-IdsB) from a hyperthermophilic archaeon *Thermococcus kodakaraensis* which grows optimally at 85 °C, and determined amino acids involved in chain-length determination. The obtained *Tk*-IdsB was then applied to an organic-aqueous dual-phase system to increase its *cis*-condensation activity (Fig. 1B). The condensation process should be inhibited due to hydrophobic interaction between the enzyme and hydrocarbon of the longer products *in vitro*. The hydrophobic chain of the elongating product does not readily protrude into aqueous phase and it tends to interact with the enzyme. To achieve the efficient *in vitro* synthesis, the organic-aqueous dual-phase system was expected to be effective, because an organic solvent pool allows high amounts of hydrophobic products to be solubilized (Fujiwara et al., 2008). In addition, we attempted to improve the stability of *Tk*-IdsB by immobilization on silica beads by the carbodiimide coupling method, and the immobilized *Tk*-IdsB has been applied to the dual-phase system.

2. Materials and methods

2.1. Microorganisms and materials

T. kodakaraensis was cultivated as described previously (Morikawa et al., 1994). *Escherichia coli* TG1 and a pUC19 plasmid (Novagen) were used for the gene manipulation. *E. coli* BL21-CodonPlus (DE3)-RIL (STRATAGENE), and a pET21a plasmid (Novagen) were used for the expression of the recombinant protein. [4-^{14}C]IPP was purchased from NEM^{TM} Life Science Products. Non-labeled IPP, dimethylallyl diphosphate (DMAPP, C_5), (all-*trans*)-geranyl diphosphate (GPP, C_{10}), (all-*trans*)-farnesyl diphosphate (FPP, C_{15}), and acid phosphatase from potato were products of Sigma. A pre-coated reverse-phase TLC plate (RP-18) was purchased from Merck.

2.2. Cloning of Tk-IdsB

The whole genome of *T. kodakaraensis* KOD1 has been deposited in DDBJ/EMBL/GenBank databases under the project accession no. AP006878. An open reading frame (TK1173) was identified as an ortholog of *cis*-prenyltransferase and was termed *Tk-idsB* that stands for *T. kodakaraensis cis*-isoprenyl diphosphate synthase. The 881 bp of *Tk-idsB* was amplified by PCR using the *T. kodakaraensis* genomic DNA (200 ng) as the template and a pair of specific primers (0.3 μM), TK1173-NdeI-Fw (5′-GAG TGA GGG CAT ATG CTA TAC AGA TTG G-3′) and TK1173-BamHI-Rv (5′-GTA CAC CTT CTT GGA TCC TGG ACT CAG-3′); newly introduced NdeI and BamHI sites are underlined, respectively. The reaction was performed for 25 cycles of 15 s at 93 °C, 30 s at 60 °C and 1 min at 68 °C. The obtained fragment was digested with BamHI and NdeI, and then ligated into pET21a vector to yield the expression plasmid, pET21a-Tk-idsB.

2.3. Expression and purification of Tk-IdsB

E. coli BL21-CodonPlus(DE3)-RIL cells harboring pET21a-Tk-idsB were grown at 37 °C in 31 of 2× YT medium (1.6% Bacto Tryptone [Becton, Dickinson and Co.], 1.0% Bacto Yeast Extract [Becton, Dickinson and Co.], 0.5% NaCl, pH 7.0) supplemented with ampicillin (50 μg l^{-1}) until the optical density at 660 nm reached 0.4. Then, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and cultivated at 37 °C for 4 h. The cells were harvested by centrifugation (8000 × *g*, 10 min, 4 °C) and then disrupted by sonication in buffer 1 (50 mM Tris–HCl, 150 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1% TritonX-100, pH 8.5). After centrifugation (25,000 × *g*, 40 min, 4 °C), the supernatant was heated for 10 min at 60 °C, and the denatured proteins were excluded by centrifugation (25,000 × *g*, 40 min, 4 °C). The remaining thermostable proteins were subjected to the $(NH_4)_2SO_4$ precipitation, and the 15–40% saturated fraction was recovered by centrifugation (25,000 × *g*, 40 min, 4 °C), and dialyzed against the buffer 1. The sample was then applied to a Q sepharose Fast Flow column (GE healthcare) equilibrated with buffer 1 and the flow-through fraction was recovered, concentrated by ultrafiltration with an Amicon Ultra-15 (MILLIPORE) and subjected to gel filtration using a Superdex 200 HR 10/30 column (GE healthcare) at 0.5 ml min^{-1} with buffer 1. The homogeneity of *Tk*-IdsB was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue (CBB)

staining. The protein concentration was determined with the Coomassie Plus Protein Assay Reagent Kit (Pierce, Illinois, USA) according to the manufacturer's instructions using bovine serum albumin as a standard. The purified *Tk*-IdsB (228 mg) was obtained from 3 l culture of *E. coli* cells.

2.4. CD spectroscopic analysis

Circular dichroism (CD) spectroscopic experiments were performed on a J-820 CD spectropolarimeter (JASCO) at 25 °C. Far-UV CD spectra were measured using a 1.0 cm pathlength-quartz cuvette at 0.05 nm steps over the wavelength range of 200–260 nm with a scan speed of 50 nm min^{-1}. Thermal denaturation curve of the purified enzyme was determined by monitoring the change in the CD value (molecular ellipticity [θ]) at 222 nm using solution containing the *Tk*-IdsB protein at 2.2 μM of tetrameric protein in H_2O in the cuvette, increasing the temperature at a rate of 1.0 °C min^{-1}. The mean residue ellipticity, θ [measured in ° cm^2 $dmol^{-1}$] was calculated using an average amino acid molecular weight of 110.

2.5. Measurement of enzymatic activity and product analysis

The enzymatic activity was determined by measuring the amount of [4-^{14}C]IPP incorporated into the products extractable by 1-butanol. The standard reaction mixture, in a total volume of 200 μl, contained 100 mM Tris–HCl buffer (pH 8.0), 1 mM $MgCl_2$, 50 μM [4-^{14}C]IPP (370 GBq mol^{-1}), 50 μM allylic diphosphate (DMAPP, GPP, or FPP), and the purified *Tk*-IdsB (20 ng). The reaction was carried out at 65 °C for 10 min and then terminated by adding 200 μl of cold NaCl-saturated aqueous solution (Fujiwara et al., 2004). The products were extracted by 1 ml of 1-butanol saturated with the NaCl-saturated aqueous solution, and the radioactivity of the 1-butanol extract was measured by an LS6500 Multi-Purpose Scintillation Counter (BECKMAN COULTER). For kinetic studies, the concentration of allylic diphosphate (DMAPP/GPP/FPP) or [4-^{14}C]IPP was varied, while its counter substrate of [4-^{14}C]IPP or DMAPP/GPP/FPP was kept at excess concentration. To analyze the products, the extracted polyprenyl diphosphates were hydrolyzed to the corresponding alcohols with potato acid phosphatase as reported previously (Fujiwara et al., 2004) and analyzed by TLC on a reverse-phase RP-18 plate with a solvent system of acetone–H_2O (19:1 or 29:1).

2.6. Enzymatic reaction in a dual-phase system

The reaction was carried out at 65 °C in a 1-cm glass cuvette (4 ml size, 1 cm × 1 cm × 4 cm) with magnetic stirring according to the reported procedure with slight modifications (Fujiwara et al., 2008). One milliliter of 1-butanol, 1-pentanol or 1-octanol was overlaid to 2 ml of the aqueous phase. After the reaction, the reaction mixture was mixed with 200 μl of cold NaCl-saturated aqueous solution to terminate the reaction. The reaction products obtained from both the phases were separately dephosphorylated and then analyzed by TLC as described above.

2.7. Site-directed mutagenesis into Tk-IdsB

Three kinds of *Tk*-IdsB point-mutants (*Tk*-IdsB-E68A, -K109A, and -L113A) were constructed, in which Glu68, Lys109, and Leu113 was replaced with alanine, respectively, by the PCR method as follows. PCR was carried out with 20 ng of pET21a-Tk-IdsB DNA as the template and 0.3 μM of 5′-phosphorylated primers (E68A-Fw [5′-AGA AGC TCG cgG AAA TCC TTG AG-3′]/E68A-Rev [5′-TTG ATC CGA ATA GGT GGC CGT AC-3′], K109A-Fw [5′-TTC GAG GAG gcG TTC AAG GAA C-3′]/K109A-Rev [5′-GAG GTT CAT GAG GGC CTC AAC TTC-3′], or L113A-Fw [5′-GTT CAA GGA Agc CGT TCA GGA TGA AAG G-3′]/L113A-Rev [5′-TTC TCC TCG AAG AGG TTC ATG AGG-3′]). Mismatch bases are indicated by small letters. The reaction was performed with KOD Plus polymerase (Toyobo) according to the manufacturer's instructions for 25 cycles of 15 s at 93 °C, 30 s at 60 °C and 1 min at 68 °C. The linear PCR products corresponding to the whole region of pET21a-Tk-idsB were self-circularized to yield plasmids, pET21a-Tk-idsB-E68A, -K109A, and -L113A, respectively.

2.8. Immobilization of Tk-IdsB

Carboxyl microparticles (Bangs Laboratories Inc.) were used for covalent coupling of the *Tk*-IdsB protein by activating the carboxyl groups with water-soluble carbodiimide. The carbodiimide reacts with the carboxyl group of the particle to create an active ester that is reactive toward primary amines on the protein. After suspending the microspheres with 170 μl of coupling buffer (50 mM MES–NaOH, pH 5.2), 20 μl of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (200 mg ml^{-1}, PIERCE) was added. After gently mixing, 200 μl of the *Tk*-IdsB solution (8.1 mg ml^{-1}) was added into the mixture, and gently mixed again. After incubation for 1 h at room temperature, the microspheres were recovered by centrifugation (15,000 × *g*, 10 min, 4 °C). The pellet was washed with 400 μl of wash solution (10 mM Tris–HCl, 0.05% bovine serum albumin, pH 8.0) three times and stored at 4 °C. The amount of the protein bound on the particle was estimated by quantifying the unbound protein in the wash solution.

3. Results and discussion

3.1. Structural analysis of Tk-IdsB

Tk-IdsB was purified as described in Section 2. When the purified enzyme was subjected to SDS-PAGE, the single protein band corresponding to a molecular mass of 30 kDa was confirmed (Fig. 2A). By gel filtration, molecular mass of the native form of *Tk*-IdsB was estimated to be 120 kDa, which suggests that it forms a tetramer (data not shown), whereas other *cis*-prenyltransferases so far reported are a dimmer enzyme (Fujihashi et al., 2001; Hemmi et al., 2001; Ko et al., 2001). Thermostability of *Tk*-IdsB protein was evaluated by CD spectrum analysis. The far-UV CD spectra of *Tk*-IdsB at 20 °C and 90 °C are shown in Fig. 2B. Both the spectra at 20 °C and 90 °C showed minimal values at 222 nm, which is typical of a protein containing α-helices, as predicted by the crystal structures of the other *cis*-prenyltransferases, *E. coli* and *M. luteus* undecaprenyl diphosphate (UPP, C_{55}) synthases (Fujihashi et al., 2001; Ko et al., 2001). However, minimal value at 208 nm as another typical pattern of the α-helical structure was not detected due to unexpected noise. Since the conformation of the enzyme seemed to be changed when the temperature was increased from 20 °C to 90 °C, the thermal denaturation curve was obtained by monitoring the change in the CD value at 222 nm in the range of 20–96 °C (Fig. 2C). As a result, the CD value at 222 nm started to increase at 90 °C but did not reach a constant value even at 96 °C, suggesting that *Tk*-IdsB was not completely denatured even at 96 °C. *Tk*-IdsB appears fairly thermostable applicable to an aqueous-organic dual-phase system for the efficient *in vitro* synthesis of *cis*-polyisoprenes.

3.2. Enzyme characteristics of Tk-IdsB

Tk-IdsB was incubated with 50 μM allylic diphosphate (DMAPP, GPP, or FPP) and 50 μM [4-^{14}C]IPP. When the incubation was carried out at 65 °C for 10 min, *Tk*-IdsB condensed [4-^{14}C]IPP with all the allylic diphosphates tested, showing that the activity for DMAPP was lower than those for GPP and FPP. Maximum activity was achieved under the condition of pH 8.5 at 60–70 °C in the

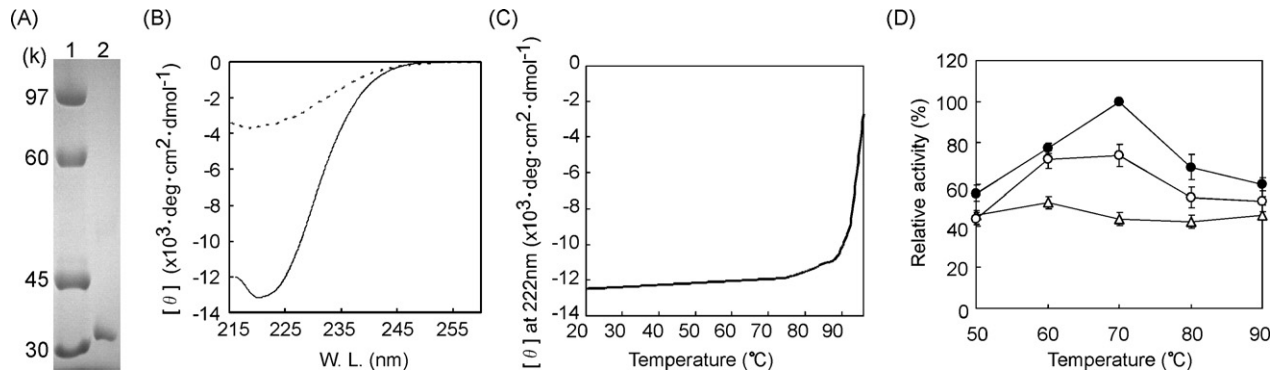

Fig. 2. Enzymatic characteristics of *Tk*-IdsB. (A) SDS-PAGE with CBB staining. Lane 1, molecular mass markers; Lane 2, purified *Tk*-IdsB. (B) Far-UV CD spectra of *Tk*-IdsB at 20 °C and 90 °C. CD spectra were monitored after incubating *Tk*-IdsB at respective temperatures for 24 h. Solid and broken lines indicate spectra at 20 °C and 90 °C, respectively. (C) Thermal denaturation curves of *Tk*-IdsB. The change of CD value was monitored at 222 nm. The temperature increased at a rate of 1 °C min^{-1} from 20 °C to 96 °C. (D) Effects of temperature on enzymatic activity. Closed triangle, closed circle, and open circle indicate activities using DMAPP, GPP, and FPP as an allylic diphosphate, respectively. Enzyme activities are obtained from triplicate experiments and relative activities are shown.

case of any allylic diphosphate used (Fig. 2D). Half-life of *Tk*-IdsB at 80 °C was 46 min (Fig. 4C).

The kinetic parameters of *Tk*-IdsB were determined with [4-^{14}C]IPP and DMAPP, GPP, or FPP as substrates at the reaction temperature of 65 °C. The results are summarized in Table 1. The K_m values for the allylic diphosphates decreased with the extension of the chain-length of the allylic diphosphate as in the case of *trans*-prenyltransferase from *T. kodakaraensis* (Fujiwara et al., 2004). The k_0 values for GPP and FPP were almost comparable, but the k_0 value for DMAPP was much lower than those for GPP and FPP. Consequently, the k_0 K_m^{-1} value for FPP was 2.5-fold and 1.8-fold higher than those for GPP and DMAPP, respectively. On the other hand, the K_m and k_0 values for IPP varied depending on the counter substrates (DMAPP, GPP, or FPP). *Tk*-IdsB showed the lowest K_m and the highest k_0 values for IPP when GPP and FPP were used as the counter substrate, respectively. Consequently, the k_0 K_m^{-1} value for IPP was the highest when GPP was used, *i.e.*, 1.5-fold and 32-fold higher than those for IPP with FPP and DMAPP, respectively. These results indicate that *Tk*-IdsB preferably utilizes both GPP and FPP as the starting substrate, but not DMAPP. The k_0 K_m^{-1} value of *Tk*-IdsB (1500 s^{-1} μM^{-1}) was the highest among those of *cis*-prenyltransferases so far characterized (0.61 s^{-1} μM^{-1} for *E. coli* UPP synthase, 62 s^{-1} μM^{-1} for *Mycobacterium tuberculosis* UPP synthase) (Ko et al., 2001; Schulbach et al., 2001), indicating *Tk*-IdsB possesses a favorable characteristic for the efficient *cis*-polyisoprenes production, as well as its high thermostability.

The reaction products obtained by the incubation of *Tk*-IdsB with [4-^{14}C]IPP and GPP at 65 °C were dephosphorylated and then analyzed by reverse-phase TLC, which revealed that *Tk*-IdsB produces *cis*-polyprenyl diphosphates ranging from C_{15} to C_{90} (>C_{75}, 11.5%; C_{65}–C_{70}, 60.7%; C_{55}–C_{60}, 17.4%; C_{45}–C_{50}, 3.3%; C_{30}–C_{40}, 3.9%; <C_{25}, 3.2%), mainly yields the C_{60}–C_{65} products (Fig. 4A), slightly longer than those obtained from the other bacterial and archaeal enzymes (*E. coli* UPP synthase [C_{55}], *M. luteus* UPP synthase [C_{55}], and *S. acidocaldarius cis*-prenyltransferase [C_{50}–C_{55}]) (Hemmi et al., 2001; Kharel et al., 2006; Ko et al., 2001).

3.3. Alteration of product distribution by site-directed mutagenesis

Previous studies showed that hydrophobic *cis*-polyprenyl chain elongating during the condensation reaction is accommodated along by a hydrophobic cleft in the enzyme (Ko et al., 2001). In *E. coli* UPP synthase, Leu137, which forms the bottom portion of the inner hydrophobic cleft with the other bulky amino acid residues, is crucial for the product-length determination to act as a steric hindrance to the chain elongation, and that alanine-substitution of Leu137 caused the formation of the C_{70-75} *cis*-polyisoprene rather than the C_{55} (Ko et al., 2001). In the structure of *E. coli* UPP synthase (PDB ID: 1UEH), we found a vent-like architecture leading to the hydrophobic cleft for the product accommodation as shown in Fig. 3. The model structures of *Tk*-IdsB and *cis*-prenyltransferases from *Saccharomyces cerevisiae* (Srt1p), which gives mainly C_{90}–C_{100} products, were constructed by SWISS-MODEL (Arnold et al., 2006) using *E. coli* UPP synthase (PDB ID: 1UEH) as an available template. Based on the models, the vent-like architectures are well conserved in all the three enzymes (Fig. 3). The sizes of these vents appeared to be proportional to the product-chain length, *i.e.*, Srt1p (ca. 8.96 Å in diameter, C_{90}–C_{100}) > *Tk*-IdsB (ca. 8.56 Å in diameter, C_{60}–C_{65}) > UPP synthase (ca. 8.40 Å in diameter, C_{55}). Therefore, we speculated that the elongating polyisoprene-chain would extrude through the vent out of the enzyme and that the efficiency of the extrusion would depend on the vent size. According to this speculation, expansion of the vent of *Tk*-IdsB should cause the extension of the product length. To test this, we introduced the single alanine-substitution into the Glu68, Lys109, and Leu113 to create *Tk*-IdsB-E68A, -K109A and -L113A, respectively, in which the vent size was expected to be enlarged.

All the *Tk*-IdsB mutants were purified and their product distributions were examined. The product analysis by reverse-phase TLC showed that the product distributions of all the mutants were shifted to longer region to give the C_{65}–C_{70} as the main products (Fig. 4A). Notably, *Tk*-IdsB-K109A also increased the products longer

Table 1
Kinetic parameters of *Tk*-IdsB.

Substrate	Counter-substrate	V_{max} (nmol min^{-1} μg^{-1})	K_m (μM)	k_0[a] (s^{-1})	k_0 K_m^{-1} (s^{-1} μM^{-1})
DMAPP	150 μM IPP	1.30 ± 0.10	9.40 ± 1.10	194 ± 76	565
GPP	50 μM IPP	3.85 ± 0.35	1.75 ± 0.17	8340 ± 670	415
FPP	100 μM IPP	5.18 ± 0.26	0.96 ± 0.07	9800 ± 480	1020
IPP	100 μM DMAPP	1.89 ± 0.09	111 ± 3.70	3580 ± 170	32.2
IPP	50 μM GPP	4.90 ± 0.34	5.88 ± 0.33	7420 ± 650	1500
IPP	6 μM FPP	7.44 ± 0.31	13.20 ± 0.38	14900 ± 57	1010

[a] k_0 values were calculated on the basis that *Tk*-IdsB forms a tetramer.

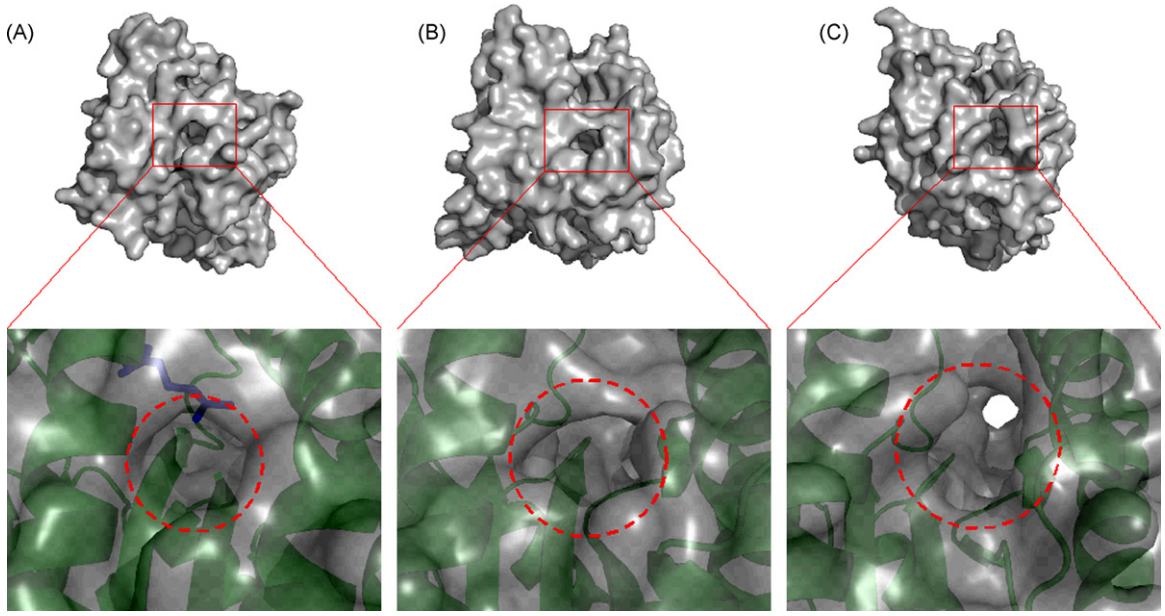

Fig. 3. Vent-like architectures leading to the hydrophobic cleft. The model structures of *Tk*-IdsB and *cis*-prenyltransferases from *S. cerevisiae* (Srt1p), which gives mainly C_{90}–C_{100} products, were constructed by SWISS-MODEL using *E. coli* UPP synthase (PDB ID: 1UEH) as an available template. The circled areas indicate estimated vent-like regions. (A) *E. coli* UPP synthase; (B) *Tk*-IdsB; (C) Srt1p.

than the C_{70} (Fig. 4A). These results indicate that the three single-mutations introduced affected the product distribution of *Tk*-IdsB, especially the alanine-substitution to Lys109 was the most effective for the product-length extension.

3.4. Enzymatic reaction in a dual-phase system

Thermostable enzymes generally show tolerance to various denaturants, such as organic solvents, because their unfolding rates are generally very low. Thus, *Tk*-IdsB was expected to have tolerance to various organic solvents, and it was applied to the dual-liquid phase reaction system for *cis*-polyisoprene production. We tested the organic solvent (1-butanol, 1-pentanol, or 1-octanol) overlaid on the aqueous reaction mixture and their effect on the product distribution was examined. *Tk*-IdsB was incubated with 50 μM GPP and 50 μM [4-^{14}C]IPP at 65 °C in the single aqueous system or the dual-phase system, and the condensation activity was monitored. The reaction products were analyzed by reverse-phase TLC. As a result, the C_{15}–C_{90} products were given from all the reaction systems tested, but the product distributions were different between

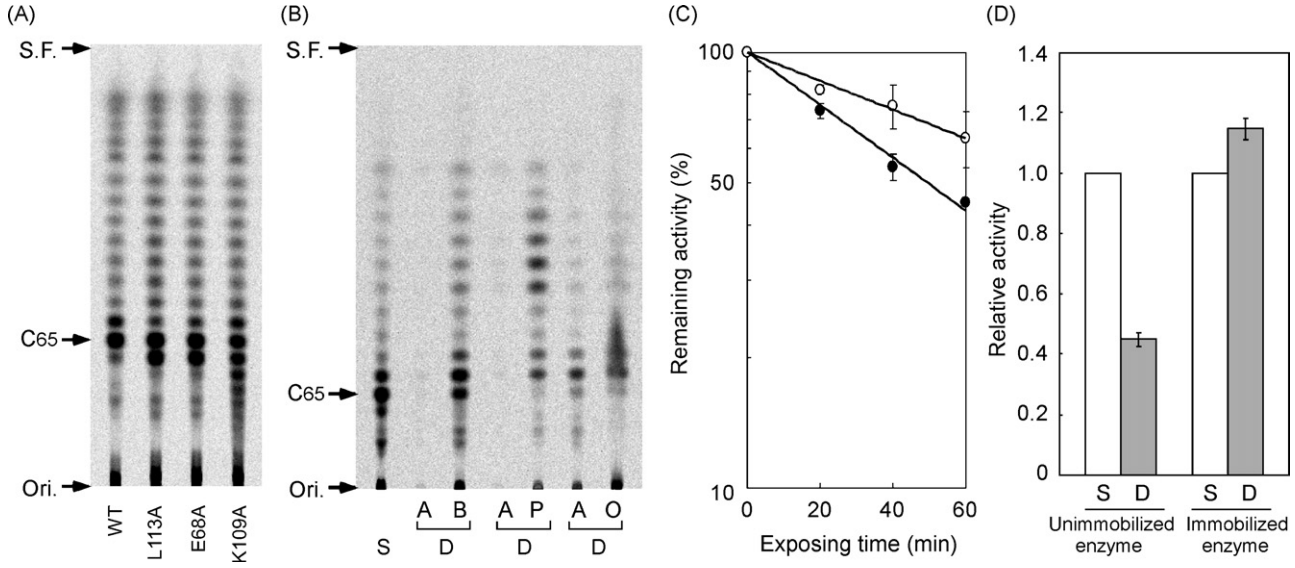

Fig. 4. TLC autoradiochromatograms of alcohol derivatives obtained by hydrolysis of the *cis*-polyisoprene products, and effect of the dual-phase reaction on the product distributions. (A) Product distributions of the wild-type *Tk*-IdsB and the single alanine-substitution mutants. The enzymatic reaction with [4-^{14}C]IPP and GPP was carried out at 65 °C and the obtained reaction products were analyzed by TLC. RP-18 plate was developed using acetone–H_2O (29:1) as the mobile phase. Ori., origin; S.F., solvent front. (B) Product distributions of *Tk*-IdsB in the dual-phase system. RP-18 plate was developed using acetone–H_2O (19:1) as the mobile phase. S, samples reacted in the single-phase aqueous condition; D, samples reacted in the dual-phase condition. A, samples obtained from the aqueous phase; B, samples obtained from the 1-butanol phase; P, samples obtained from the 1-pentanol phase; O, samples obtained from the 1-octanol phase. (C) Remaining activity after heat treatment. Heat treatment was carried out at 80 °C in 50 mM Tris–HCl buffer (pH 8.0). Open circle, remaining activity of the immobilized *Tk*-IdsB; closed circle, remaining activity of the unimmobilized form. (D) Relative values of the enzymatic activities in immobilized and unimmobilized *Tk*-IdsB. The values normalized to the activity in the single-phase reaction are shown. S, total activity obtained from the single aqueous phase; D, total activity obtained from the dual-phase containing 1-butanol.

the single-phase and the dual-phase depending on the organic solvents used (Fig. 4B). It is noteworthy that more than 90% of the products were recovered from the upper phase of dual-phase system containing 1-butanol or 1-pentanol. When 1-octanol was overlaid, 70% of the products were obtained from the upper organic phase. When 1-octanol and 1-pentanol were used as the upper phase, the main products became the C_{55}–C_{60} (52.4% of total upper phase product) and the C_{30}–C_{40} (49.6% of total upper phase product), respectively, while in the case of 1-butanol, the C_{60}–C_{65} were mainly produced as well as those obtained by the single-phase system. Besides 1-octanol and 1-pentanol, 1-hexanol and 1-heptanol were also examined. When 1-hexanol or 1-heptanol was overlaid, distribution patterns were similar to the case 1-pentanol overlaid (data not shown). It appeared that the amount of the intermediates increased when the organic solvent with relatively high hydrophobicity was used, probably because the moderately hydrophobic intermediates were released from the enzyme and recovered into the organic solvent even on the elongation proceeding.

3.5. Stabilization of Tk-IdsB by immobilization on beads

To make *Tk*-IdsB more tolerant, immobilization of *Tk*-IdsB on silica beads was performed by the carbodiimide coupling method as described in Section 2. The amount of protein bound on the particle was estimated to be 0.87 mg from 1.62 mg of *Tk*-IdsB. About 54% of *Tk*-IdsB was successfully immobilized on silica beads however the specific activity of the immobilized enzyme was decreased. The specific activity of free *Tk*-IdsB (1.89 nmol min^{-1} μg^{-1}) was decreased to 2.10×10^{-2} nmol min^{-1} μg^{-1}. Thermostability was then evaluated by measuring the remaining activity when heat treatment was carried out at 80 °C. Half-life of the enzymatic activity of the immobilized *Tk*-IdsB was 156 min, while the free enzyme showed a half-life of 46 min, indicating that the immobilization improved the stability of *Tk*-IdsB (Fig. 4C). When the free *Tk*-IdsB was applied in the dual-phase reaction containing 1-butanol, the enzymatic activity was decreased in comparison with the case in the single aqueous phase reaction. In contrast, when the immobilized *Tk*-IdsB was applied in the dual-phase system, higher condensation activity was detected (Fig. 4D). No significant difference was observed in the product distributions between the immobilized and unimmobilized forms (data not shown).

In this study, we revealed that *Tk*-IdsB is highly thermostable and possesses the high reaction activity of *cis*-condensation, and that its stability could be reinforced by immobilizing on beads. We also demonstrated that it is possible to change the product distribution of *Tk*-IdsB by introducing the site-directed mutagenesis based on the structural information, or by choosing overlaid organic solvents of the dual-phase system. The obtained *cis*-polyisoprenes with various chain-lengths are expected to be promising materials in chemical and pharmaceutical fields for developing useful compounds, such as a new functional polymer molecule.

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