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Biochemical and Biophysical Research Communications 365 (2008) 118–123
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Efficient synthesis of *trans*-polyisoprene compounds using two thermostable enzymes in an organic–aqueous dual-liquid phase system

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Received 18 October 2007
Available online 30 October 2007

Abstract

The *trans*-polyisoprene compounds are synthesized by *trans*-isoprenyl diphosphate synthase (IDS) with consecutive condensation of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP). The *in vitro* condensation by IDS does not proceed efficiently by hydrophobic interaction between IDS and the hydrocarbon of longer products. In the present study, the enzymatic synthesis of *trans*-polyisoprenyl diphosphates was attempted in an organic–aqueous dual-liquid phase system with thermostable enzymes obtained from *Thermococcus kodakaraensis*. The conversion from DMAPP to a longer-chain product was achieved in a dual-liquid phase system, and more than 80% of the products were recovered in the organic phase. When the mutant IDS-Y81S, in which Tyr81 is replaced with Ser, was used in the dual-phase system, productivity was enhanced about four times and the ratio of the longer-chain products was increased. Co-incubation of IPP isomerase from *T. kodakaraensis* with IDS or IDS-Y81S enabled the direct synthesis of polyisoprenyl diphosphates from IPPs.

Keywords: Archaea; Isopentenyl diphosphate isomerase; *Thermococcus kodakaraensis*; *trans*-Isoprenyl diphosphate synthase; Dual-phase reaction system

trans-Isoprenyl diphosphates are utilized as the precursors of most isoprene compounds. Two enzymes play an important role for their biosynthesis *in vivo* [1,2]. Isopentenyl diphosphate isomerase (IDI) is the first enzyme that catalyzes the interconversion of isopentenyl diphosphate (IPP, C_5) and dimethylallyl diphosphate (DMAPP, C_5). The second enzyme is *trans*-isoprenyl diphosphate synthase (IDS), which is involved in the consecutive condensation of IPP with allylic diphosphate in the *trans*-configuration. The process is summarized in Fig. 1A. Archaea contain various C_{15}, C_{20}, and more highly condensed isoprene compounds, such as squalene, its derivatives [3], carotenoids [4], isoprenylquinones [5], and isoprenylated proteins [6]. These isoprene compounds are derived from linear *trans*-isoprenyl diphosphates with various chain lengths. Geranylgeranyl diphosphate (GGPP, C_{20},) is a precursor for the core membrane lipids and carotenoids, and farnesyl diphosphate FPP (C_{15}) is a precursor for squalene and its derivatives. These linear compounds are synthesized by *trans*-isoprenyl diphosphate synthase (IDS).

Abbreviations: GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; TLC, thin-layer chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; MOPS, 3-(*N*-morpholine) propane sulfonic acid; FARM, first aspartate-rich motif.

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doi:10.1016/j.bbrc.2007.10.133

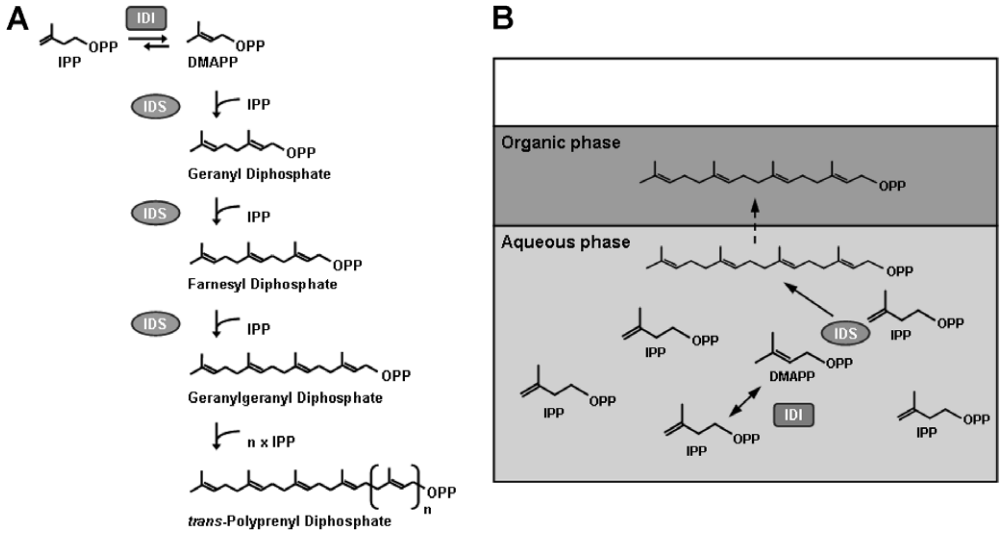

Fig. 1. Synthesis process of polyisoprenyl diphosphates. (A) Biosynthetic pathway in archaea. (B) Concept of the organic–aqueous dual-phase system. The light- and dark-shaded areas indicate the aqueous and organic phases, respectively. Double-headed and single-headed arrows indicate isomerization by IDI and consecutive condensation by IDS, respectively.

When the consecutive *trans*-condensation is performed *in vitro*, the condensation process is inhibited due to hydrophobic interaction between IDS and hydrocarbon of the longer products. The hydrophobic chain of the elongating product does not readily protrude into aqueous phase and it tends to interact with the enzyme. To achieve an efficient *in vitro* synthesis, the use of an organic–aqueous two-liquid phase system is expected as shown in Fig. 1B, since an organic solvent pool allows high amounts of hydrophobic products to be solubilized. However, this approach has not been successfully implemented because most enzymes are not stable in organic solvents. Profound losses in activity often occur due to the direct exposure of the enzyme to the organic phase or to organic–aqueous interfaces. A commonly tried approach to improve the enzyme instability in the organic–aqueous phase is their appropriate immobilization [7,8]. However, thermostable enzymes from hyperthermophiles generally show tolerance to various denaturants, such as organic solvents, because their unfolding rates are generally very low [9]. The increased number of hydrogen bonds and hydrogen bonding networks [10], hydrophobic interactions in the protein interior [11], density of side-chain packing [12], ion-pairing [13,14], ion-pair networks and their location between α helices [15], and secondary structure content [16] contribute to their high stability. In the present study, we performed an enzymatic synthesis of polyisoprenyl diphosphates in an organic–aqueous dual-liquid phase using the thermostable IDS obtained from the hyperthermophilic archaeon *Thermococcus kodakaraensis* and we also combined the IDI obtained from *T. kodakaraensis* into the system. *T. kodakaraensis* is a sulfur-reducing hyperthermophilic archaeon that shows optimum growth at 85 °C [17–19]. Both IDI and IDS from *T. kodakaraensis* showed the highest thermostability among examined enzymes so far reported [20,21]. The enzymes are hence expected to be suitable tools to develop the dual-phase system.

Materials and methods

Expression and purification of IDI. Construction of pET21-Tk-idi, an expression plasmid for isopentenyl diphosphate isomerase (IDI) of *T. kodakaraensis*, was described before [20]. The recombinant IDI was purified according to a reported procedure with slight modifications [20]. *Escherichia coli* BL21(DE3) cells harboring pET21-Tk-idi were induced for overexpression with 1 mM isopropyl-β-D-thiogalactopyranoside at the mid-exponential growth and incubated for 5 h at 37 °C. Cells were harvested by centrifugation (8500*g*, 10 min) and disrupted by sonication in buffer 1 (50 mM sodium phosphate, pH 7.0). After centrifugation (27,000*g*, 20 min), the supernatant was heated at 90 °C for 10 min, and denatured proteins were excluded by centrifugation (27,000*g*, 20 min). The supernatant was applied to ammonium sulfate precipitation at 70% saturation and the resulting precipitate was dissolved in buffer 1. The sample was applied to a BIOASSIST Q column (Tosoh) equilibrated with buffer 1 and fractionated with a linear gradient of 100–800 mM NaCl. Gel filtration was then performed with Superdex 200 HR 10/30 column (GE Healthcare) at 0.4 ml/min. The homogeneity of IDI was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with silver staining. Protein concentrations were measured by the method of Bradford with bovine serum albumin as a standard [22].

Expression and purification of IDS and IDS-Y81S. Construction of pET8-Tk-idsA, an expression plasmid for *trans*-isoprenyl diphosphate synthase of *T. kodakaraensis*, was described before [21]. Overexpression and purification was carried out according to the procedure for the IDI expression as well. Obtained cells were disrupted by sonication in buffer 2 (50 mM sodium phosphate, 10 mM 2-mercaptoethanol, 50 mM NaCl, pH 6.0). After centrifugation (27,000*g*, 20 min), the supernatant was heated at 90 °C for 15 min and denatured proteins were excluded by centrifugation (27,000*g*, 20 min). The supernatant was applied to ammonium sulfate precipitation at 70% saturation and the resulting precipitate was dissolved in buffer 2. The sample was applied to a Q Sepharose Fast Flow column (GE Healthcare) equilibrated with buffer 2 and fractionated with a gradient of 150–250 mM NaCl. Final polishing was achieved by gel filtration (Superdex 200 HR 10/30 column GE Healthcare) at 0.4 ml/min. As for

IDS-Y81S, plasmid pET8-Tk-idsA-Y81S was used instead of pET8-Tk-idsA. Other procedures were carried out as for the IDS purification.

Circular dichroism (CD) spectroscopic analysis. CD spectroscopic experiments were performed on a J-820 CD spectropolarimeter (JASCO) at 20 °C. Far-UV CD spectra in the 200–260 nm region were measured using a 1.0 cm pathlength-quartz cuvette at 0.05 nm steps over the wavelength range designated with a scan speed of 50 nm min^{-1}. The experiments were performed with 5 μM of the protein in a buffer 3 (50 mM sodium phosphate, pH 6.0). All samples were preincubated at room temperature for 30 min before each scan. Thermal denaturation curve of the purified enzyme was determined by monitoring the change in the CD value at 222 nm using solution containing protein at 5 μM in buffer 3 in the cuvette as the temperature increased at a rate of 1.0 °C min^{-1}.

Measurement of enzymatic activity in a single-phase reactor. The enzymatic activity of *trans*-isoprenyl diphosphate synthase was measured by determination of the amount of [4-^{14}C]IPP incorporated into polyisoprenyl diphosphates as described before [21]. The standard assay was performed in a total volume of 2 ml containing a 100 mM 3-(*N*-morpholine) propane sulfonic acid (MOPS) buffer, pH 8.0, 5 mM $MgCl_2$, 50 μM [4-^{14}C]IPP (37 GBq · mol^{-1}), a 50 μM DMAPP, and 10–20 ng of the purified enzyme. The reaction was carried out at 65 °C in a 1-cm glass cuvette (4 ml size, 1 cm × 1 cm × 4 cm). After the reaction, the reaction mixture was mixed with 200 μl of H_2O saturated with NaCl and cooled down on ice to terminate the reaction and then samples (200 μl) were taken. The reaction products were then extracted with 1 ml of 1-butanol saturated with NaCl–saturated H_2O, and the radioactivity in the 1-butanol extract was measured by the dpm mode with an LS 6500 Multi-Purpose Scintillation Counter (Beckman-Coulter) and a Clear-sol I scintillation cocktail (Nacalai Tesque). When IDI was included with IDS, 10–20 ng of purified IDI, 10 μM FMN, and 5 mM NADPH were added to the above-mentioned reaction mixture instead of DMAPP.

Measurement of enzymatic activity from a dual-phase reactor. The reaction was carried out at 65 °C in a 1-cm glass cuvette (4 ml size, 1 cm × 1 cm × 4 cm). Two milliliters of the aqueous phase of the reaction mixture was the same as mentioned above, and 1 ml of 1-butanol was overlaid as an organic phase. Co-incubation experiment was also carried out with the combination of IDI and IDS/ IDS-Y81S as mentioned above. After the reaction, the reaction mixture was mixed with 200 μl of H_2O saturated with NaCl. Samples (200 μl) of the aqueous and organic phases were taken from lower and upper phases, respectively. The reaction products of the aqueous phase were then extracted with 1 ml of 1-butanol saturated with NaCl–saturated H_2O, and the radioactivity in the 1-butanol extract was measured. The sample (100 μl) from an organic phase was also taken from the upper phase and mixed with 900 μl of 1-butanol saturated with NaCl–saturated H_2O, and then the radioactivity was measured.

Product analysis by thin-layer chromatography (TLC). To analyze the products, the extracted polyisoprenyl diphosphates were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to the method reported previously [21]. The alcohols were extracted with *n*-pentane and analyzed by TLC on a reverse-phase RP-18 plate with a solvent system of acetone–H_2O (9:1). The positions of authentic standards were visualized with iodine vapor, and the absolute radioactivity of the spots was detected with a Bio-image analyzer BAS1500 (Fuji). The product distributions were determined based on the molar ratios of the products that were obtained by division of the absolute radioactivity of each spot by the number of the IPPs incorporated into the corresponding polyisoprenyl alcohol.

Results and discussion

Stability of IDS and IDS-Y81S in an organic–aqueous dual-liquid phase reactor

Tk-idsA encoding IDS from *T. kodakaraensis* was heterologously expressed in *E. coli* cells, and the recombinant form of IDS was purified to homogeneity. The obtained IDS possessed almost equal specific activity (47 nmol min^{-1} μg^{-1} at 90 °C) to that of previously reported [21]. The melting temperature (T_m), change of enthalpy (ΔH_m), and heat capacity change (ΔC_p) of IDS have been reported to be 91.0 °C, 307.6 kJ mol^{-1}, and 8.5 kJ mol^{-1} K^{-1}, respectively [21]. The T_m value indicates that IDS is extremely thermostable, which is quite reasonable in view of the optimal growth temperature (85 °C) of *T. kodakaraensis*. It is noteworthy that IDS showed the highest thermostability among the *trans*-isoprenyl diphosphate synthases so far reported. We performed further studies on thermostability in an organic–aqueous dual-liquid phase by CD analysis. Various organic solvents were used to separate polyisoprene compounds from an aqueous solution. Since 1-butanol was the most suitable to extract polyisoprene compounds longer than the C_{10} length, it was chosen as an organic solvent to test availability for a dual-phase system. The far-UV CD spectra of the purified IDS are shown in Fig. 2. The spectrum at 20 °C showed minimal values at 208 nm and 222 nm, the typical pattern of an α-helical protein. The thermal denaturation curve was obtained in the absence of 1-butanol by monitoring the change in the CD value in a 1 cm cuvette (4 ml size) at 222 nm. The CD value at 222 nm started to increase at 90 °C but did not reach a constant value even at 98 °C (data not shown). A typical denaturation pattern was not observed at the condition (temperature-rising rate, 0.5 °C/min). One milliliter of 1-butanol was laid on the aqueous solution in the cuvette (4 ml size), and the CD value of IDS in the aqueous phase was monitored by mixing two solutions with a magnetic stir (600 rpm). The value in the presence of 1-butanol started to increase at 64 °C, indicating that the enzyme started denaturation at that temperature. Next, the thermal stability was examined by monitoring the time course of the CD value at 65 °C. The enzyme containing solution was kept at 65 °C, and the change of the CD value at 222 nm was monitored. No significant increase was observed within 1000 s, indicating that IDS maintained the native structure until 1000 s and started to denature gradually after that. The obtained results indicated that T_m is apparently higher than 60 °C and IDS maintains the structure at least for 10 min under the 1-butanol-mixed condition. Investigations on thermostability were also performed for mutant IDS, in which Tyr81 is replaced with Ser. Previous studies on the chain-length regulation of the final product have revealed that, in archaeal GGPP synthase, the aromatic residue at the fifth position before the first aspartate-rich motif (FARM) acts as a steric hindrance to terminate the chain elongation and defines the final product length as C_{20} [23,24]. Elimination of the bulky side-chain and elevation of the hydrophilicity at the position allow the enzyme to produce a longer-chain products (>C_{20}). IDS-Y81S has been reported to produce longer-chain products [21]. The CD spectra of IDS-Y81S are shown in Fig. 2. The spectrum at 20 °C also showed a typical pattern of an α-helical protein. The thermal denaturation curve was obtained as well

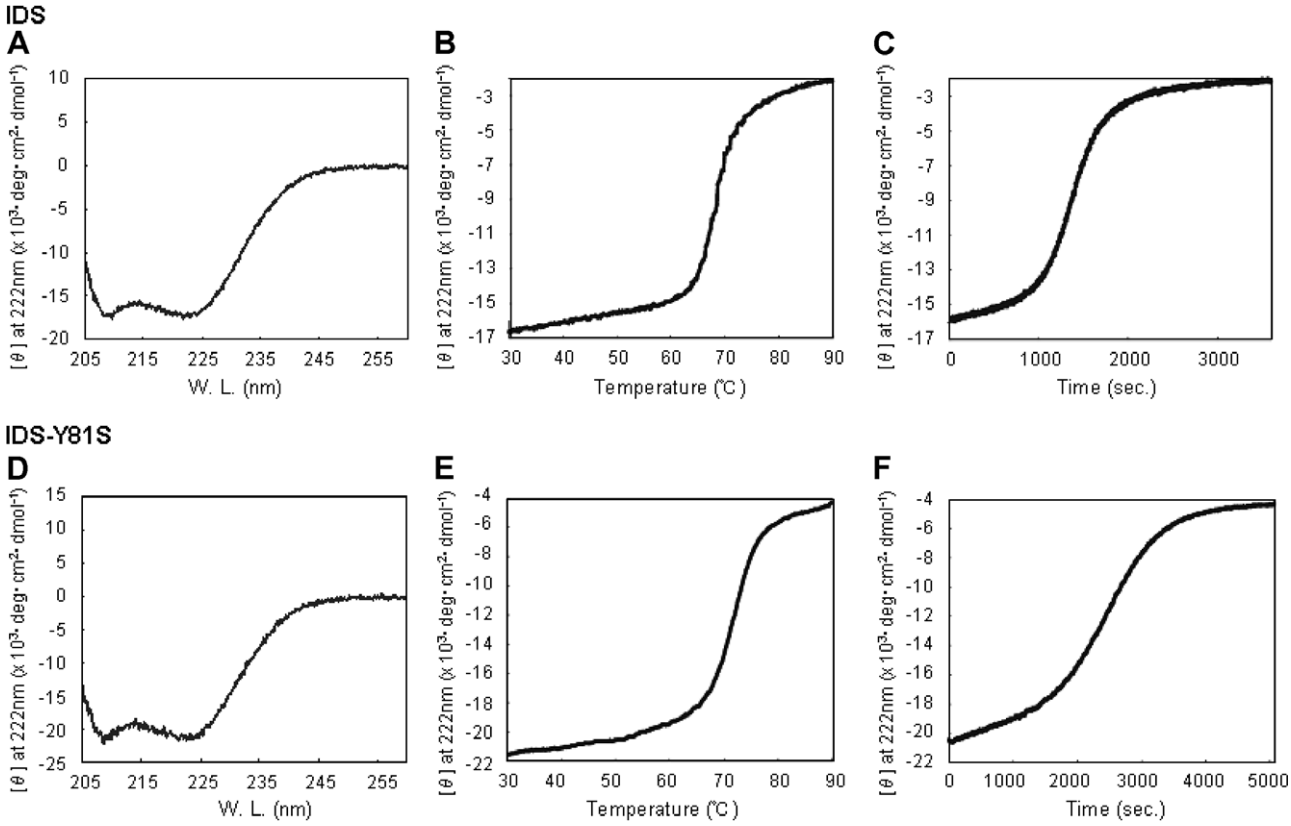

Fig. 2. CD spectral analyses of IDS and IDS-Y81S. (A,D) Far-UV CD spectra collected in a 50 mM sodium phosphate buffer (pH 6.0). (B,E) Thermal denaturation curves of the protein in the presence of 1-butanol. The change of CD value was monitored at 222 nm. The temperature increased at a rate of 1 °C/min from 30 °C to 90 °C. (C,F) Thermal denaturation of the protein in the presence of 1-butanol at 65 °C. The change of CD value was monitored at 222 nm.

in the presence of 1-butanol. As shown in Fig. 2, the CD value at 222 nm started to increase at 64 °C, indicating that the enzyme starts denaturation at that temperature. When the solution was kept at 65 °C and the change of the CD value at 222 nm was monitored, no significant increase was observed within 1500 s, indicating that IDS-Y81S maintained the native structure for 1500 s. IDS-Y81S possesses higher stability than wild-type IDS.

Enzymatic activity of IDS and IDS-Y81S in a dual-phase reactor

Based on the results of the stability experiment, we devised a dual-phase reactor system, in which 1-butanol were overlaid on the normal aqueous reaction mixture, and compared the enzymatic activity of IDS in both the systems. The activity in the single-phase system was first examined. IDS was incubated with 50 μM DMAPP and 50 μM [4-^{14}C]IPP, and the condensation activity was monitored. When the incubation was carried out at 65 °C for 10 min, the maximum activity was achieved under the condition of pH 8.0 (100 mM MOPS buffer) and 5 mM $MgCl_2$. The reaction products were dephosphorylated and then analyzed by reverse-phase TLC (Fig. 3). Farnesol (C_{15}) and geranylgeraniol (C_{20}) were mainly detected by TLC analysis, indicating that IDS gave FPP and GGPP as the main products as reported previously [21]. When the reaction was performed in the dual-phase system, most products were obtained from the organic phase (Fig. 3). This result indicated that the produced FPP and GGPP were transferred to the organic phase during the enzymatic reaction. The total amount of the products from both the organic and aqueous phases was almost equal to that of the single-phase reactor, as shown in Fig. 3.

When the enzymatic reaction of IDS-Y81S was carried out at 65 °C with 50 μM DMAPP and 50 μM [4-^{14}C]IPP in the single-phase system, it gave the C_{25} product as the main product in the long-chain region ($>C_{20}$). Small amounts of C_{35} and C_{40} products were also observed (Fig. 3). This result was consistent with that obtained in the previous study [21]. However, in the dual-phase reactor system, the product distribution shifted to longer-chain region as observed in the pattern of TLC (Fig. 3). It is noteworthy that longer products were mainly obtained from the organic phase. In the aqueous single-phase reaction, the consecutive condensation is inhibits due to the insolubility of the longer products in aqueous solution and the unfavorable hydrophobic interaction between the enzyme and the product's hydrocarbon chain. An organic solvent pool is considered to allow high amounts of hydrophobic products to be solubilized, especially the longer-chain isoprene compounds. We quantified the distribution ratio of the products, as summarized in Table 1. Bioconversion from DMAPP and

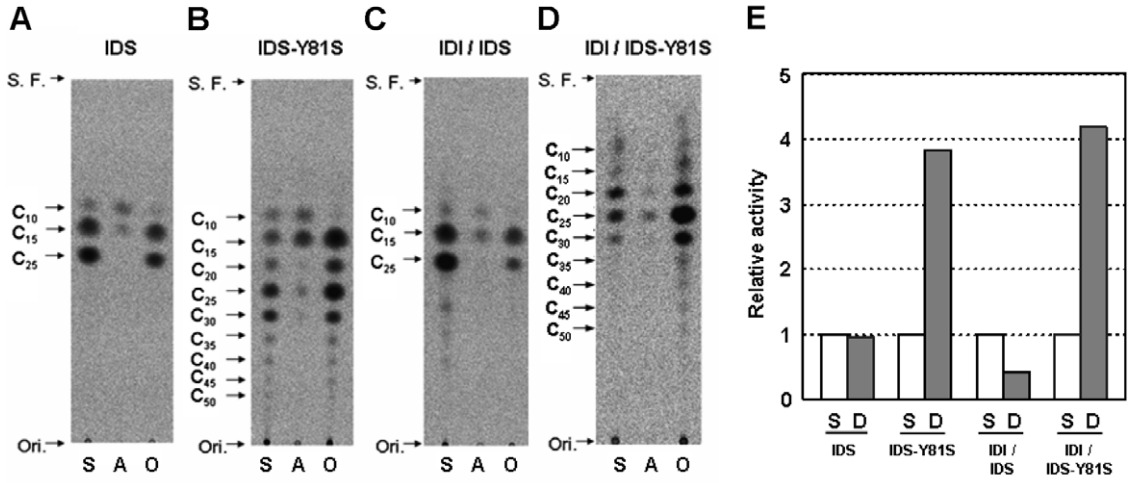

Fig. 3. Effect of the dual-phase reaction on the product distributions. (A) TLC patterns and product distributions obtained by IDS. (B) TLC patterns and product distributions obtained by IDS-Y81S. (C) TLC patterns and distributions obtained by enzymes IDI/IDS. (D) TLC patterns and distributions obtained by enzymes IDI/IDS-Y81S. TLC autoradiochromatograms of prenyl alcohols were obtained after enzymatic hydrolysis. S, samples reacted in a single aqueous condition; A, samples obtained from the aqueous phase of the dual-phase condition; O, samples obtained from the organic phase of the dual-phase condition; Ori., origin; S.F., solvent front (E) Relative values of the enzymatic activities. The values normalized to the activity in a single-phase reaction are shown. All analyses were conducted in triplicate and the averages were taken. S, total activity obtained from the single aqueous phase; D, total activity obtained from aqueous and organic phases.

Table 1
Product distributions in single or dual-phase reaction (%)

Enzyme	Reaction condition	Fraction	C_{10}	C_{15}	C_{20}	C_{25}	C_{30}	C_{35}	C_{40}	C_{45}	C_{50}	Total
IDS	S		9.4	36.7	53.9	—	—	—	—	—	—	100.0
	D	A	22.6	5.3	—	—	—	—	—	—	—	27.9
		O	2.7	37.8	31.6	—	—	—	—	—	—	72.1
IDS-Y81S	S		5.6	14.8	8.2	36.5	29.8	3.6	1.5	—	—	100.0
	D	A	3.2	8.7	0.4	0.9	—	—	—	—	—	13.2
		O	0.5	23.1	10.4	40.7	10.6	1.2	0.3	—	—	86.8
IDI/IDS	S		13.0	78.9	8.1	—	—	—	—	—	—	100.0
	D	A	17.9	22.4	0.6	—	—	—	—	—	—	40.9
		O	1.9	40.2	17.0	—	—	—	—	—	—	59.1
IDI/IDS-Y81S	S		13.0	6.0	27.8	40.2	12.9	0.1	—	—	—	100.0
	D	A	—	—	—	0.3	—	—	—	—	—	0.3
		O	1.9	2.7	15.3	66.8	9.3	2.0	0.8	0.6	0.3	99.7

S, Single phase; D, Dual phase; A, Aqueous phase; O, Organic phase; —, not detectable.

IPPs to FPP and GGPP was achieved using IDS in the single-phase system and its efficiency was not enhanced in the dual-phase system. However, more than 70% of the products were recovered in the organic phase. In the dual-phase system, IDS-Y81S showed about four times higher yield of the polyisoprene products than that in the conventional single-phase system, and over 80% of the products were recovered in the organic phase of the two-phase system. The effect of organic solvents on IDS-Y81S activity seems more significant than that on the wild-type activity.

Polyisoprene production with the combination of IDI and IDS/IDS-Y81S

IDI is an important enzyme that catalyzes the interconversion of IPP and DMAPP. IDI functions for supplying both the electrophilic primer substrate and the condensation substrate for the synthesis of *trans*-isoprenyl diphosphates, the universal precursors for polyisoprene compounds. The thermal stability of IDI from *T. kodakaraensis* was examined and reported [20]. The melting temperature (T_m), change of enthalpy (ΔH_m), and heat capacity change (ΔC_p) of IDI were 88.0 °C, 444 kJ mol^{-1}, and 13.2 kJ mol^{-1} K^{-1}, respectively, indicating that IDI is fairly thermostable, as are IDS and IDS-Y81S. We utilized IDI in combination with IDS to achieve a simple polyisoprene production from IPPs. IDI was obtained as a recombinant form according to the reported procedure with slight modifications [20]. Bioconversion directly from IPPs to FPP and GGPP in the absence of DMAPP was achieved using IDI and IDS in a single-phase system as shown in Fig. 3. When IDS was replaced with IDS-Y81S,

condensation occurred as well. The enzyme activity was remarkably enhanced in the dual-phase system. It is noteworthy that IDS-Y81S and IDI produced more than four times the amount of polyisoprenes, and about 80% of the product was recovered in 1-butanol of the dual system, as shown in Fig. 3 and Table 1. However, the product ratio of longer-chain products ($>C_{35}$) decreased in comparison with the case in the absence of IDI. The decrease of longer-chain products would be caused by the shortage of available IPP molecules in the reactor. In the presence of IDI, IPP is converted to DMAPP by IDI, resulting in a decline of the IPP concentration. This decline might have an effect on the condensation efficiency.

As reported above, the use of an organic–aqueous two-liquid phase system improved the unfavorable hydrophobic interaction between *trans*-isoprenyl diphosphate synthase and the hydrocarbon of the longer products. The organic pool allows the hydrophobic products to be solubilized [7]. Besides organic solvents, some additives such as surfactants are expected to enhance the condensation because they are known to be functional to decline the hydrophobic interaction [25]. Adding surfactants to the dual system would enable the system to be more efficient by declining unfavorable hydrophobic interactions. Further experiments are in progress to prove this idea.

Acknowledgments

This research work was supported by a research grants from The Takeda Foundation and JST (Japan Science and Technology Agency, Research for Promoting Technological Seeds).

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