Truncated Type II isopentenyl diphosphate isomerase from hyperthermophilic Achaeon *Thermococcus kodakaraensis* implicates the necessity of its N-terminal amino acid residues in protein thermostability

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Abstract: The enzyme isopentenyl diphosphate isomerase (IDI, EC 5.3.3.2) interconverts isopentenyl diphosphate and dimethylallyl diphosphate. We had previously cloned *Tk-idi* gene encoding the thermostable *Tk*-IDI enzyme from *Thermococcus kodakaraensis* KOD1. Four putative start codons were found on *Tk-idi* gene at 123, 213, 297 and 321 positions downstream of the first start codon. In the present work four mutants were obtained by deleting 123, 213, 297 and 321 nucleotides from the 5'-end of *Tk-idi* gene to obtain *Tk-idim, Tk-idim1, Tk-idim2, and Tk-idim3*, respectively. When we tried to express these truncated genes in *Escherichia coli* only *Tk-idim* was expressed in the active form. The product, *Tk*-IDIM, was purified and characterized. The molecular mass of the enzyme, estimated by gel filtration chromatography, was 300 kDa which indicated that the truncated enzyme retained the octameric form. The removal of 41 N-terminal amino acids did not exhibit a significant effect on the enzyme activity however, the thermostability of the enzyme decreased. The decrease in thermostability of *Tk*-IDIM correlated well with the results of circular dichroism (CD) analysis and structural modeling.

Keywords: Type II IPP-isomerase, isoprenoids, archaea, mutation, circular dichroism, thermostability, structural modeling.

INTRODUCTION

Natural products have been playing the key role for the discovery and development of therapeutics used to treat diseases such as pain, cardiovascular disease and cancer (Newman, 2000). Among natural products isoprenoids or terpenoids are important biomolecules in living organisms and are abundantly present in nature with more than 50,000 known products (Chang and Keasling, 2006; Christianson, 2007). Isoprenoids are found in all organisms; among them are many essential biological molecules, such as dolichols (Matsuoka *et al*., 1991), ubiquinone or menaquinone in bacteria (Ashby and Edwards, 1990), sterols (Popjak, 1970), carotenoids and triterpenes in plants (Goodwin, 1971), and prenylated proteins (Clarke, 1992). Isoprenoids perform unconfined essential functions. Isoprenoids serve as structural components of cellular and organelle membranes, electron transport, hormone-based signaling, regulation of transcription and post-translation, meiosis, apoptosis, glycoprotein biosynthesis, and protein degradation. They regulate reproductive cycles, mediate cellular redox chemistry, serve as protective agents against damage by sunlight, attract mates, and transport sugars during glycoprotein biosynthesis (Hahn *et al*., 1999). All isoprenoids are produced by condensation of a five carbon intermediate (Cane, 1999). Two pathways are known for the synthesis of isopentenyl diphosphate and dimethylallyl diphosphate, one is well established mevalonate pathway used by eukaryotes, archaea and a few gram positive bacteria (Kuzuyama, 2002), and the other is 2-C-methyl-D-erythritol 4-phosphate or 1-deoxy-D-xylulose-5-phosphate pathway (Altincicek, *et al*., 2001a; Altincicek, *et al*., 2001b; Campos, *et al*., 2001). Methyl-D-erythritol 4-phosphate pathway is used by many eubacteria, green algae, and chloroplast of higher plants (Takagi *et al*., 2004).

There are two types of IDIs (type I IDI and type II IDI), the type I IDI is found in many organisms such as *Rhodobacter capsulatus* (Hahn *et al*., 1996a), *Escherichia coli* (Hahn *et al*., 1999), *Saccharomyces cerevisiae* (Anderson *et al*., 1989) and humans (Hahn *et al*., 1996b). This enzyme needs only a divalent cation Mg^{2+} for its enzymatic activity. The type II IDI (Fig. 1), is found in some gram-positive bacteria such as *Streptomyces* sp. Strain CL190 (Kaneda *et al*., 2001) *Staphylococcus aureus* (Laupitz *et al*., 2004), cyanobacteria *Synechocystis* sp. strain PCC 6803 (Barkley *et al*., 2004a), archaea *Methanothermobacter thermautotrophicus,* (Barkley *et al*., 2004b) and *Sulfolobus shibatae* (Yamashita *et al*., 2004b). The amino acid sequence similarity is not found between type I and type II *idi* gene products, and the enzyme strictly requires both FMN and NADPH to exhibit activity (Kaneda *et al*., 2001; Laupitz *et al*., 2004).

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Thermococcus kodakaraensis KOD1, a sulfur-reducing hyperthermophilic archaeon, was isolated from a solfatara in Kodakara Island, Kagoshima, Japan (Morikawa *et al*., 1994). We previously cloned *Tk-idi* encoding the type II IDI product and described in detail the thermodynamic and enzymatic characteristics of the enzyme (Siddiqui *et al*., 2005). Four putative start codons were found on *Tk-idi* gene. In order to know if one of these four codons is an active start codon, we deleted 123, 213, 297 and 321 bases from *Tk-idi* gene. This deletion caused the removal of 41, 71, 91 and 107 N-terminal amino acids from IDI. The 41 amino acids short mutant IDIM was found active. The enzyme characteristics of IDIM were studied with especial concentration on the role of 41 N-terminal amino acids on protein stability.

MATERIALS AND METHODS

Materials

The hyperthermophilic archaeon strain *T. kodakaraensis* KOD1 was cultivated according to previously reported method (Morikawa *et al*., 1994). Overexpression of the mutant genes was carried out using *Escherichia coli* strain BL21(DE3) (Stratagene) and pET-21a vector (Novagen). BL21 (DE3) cells were cultivated at 37°C in NZCYM medium. Ampicillin was used at a final concentration of 50 mg L^{-1}.

Genetic manipulations

Recombinant genetic manipulations were performed according to previously reported methods (Sambrook *et al*., 2001). All the enzymes used in the experimental work were purchased from Takara Shuzu Co, Kyoto, Japan. Sarcosyl and CsCl equilibrium density gradient ultra centrifugation methods (Imanaka *et al*., 1981) were used for the preparation and purification of chromosomal DNA from *T. kodakaraensis* KOD1. Wizard Miniprep DNA purification kit (Promega, Madison, USA) was used for Small scale plasmid DNA preparation from *E. coli* and for large scale plasmid DNA preparation Qiagin plasmid Maxi kit (Qiagen Inc, Chatsworth, Calif) was used.

Cloning of Tk-idim, Tk-idim1, Tk-idim2, Tk-idim3 genes

Based on the DNA sequence of *Tk-idi* the forward primers IPP-F, IPP-F1, IPP-F2, IPP-F3 and a reverse IPP-R primer (Table 1) were designed for the amplification of *Tk-idim, Tk-idim1, Tk-idim2, Tk-idim3* genes. The amplification of genes was performed by polymerase chain reaction (PCR) using KOD1 DNA polymerase (Toyobo, Japan) as follows: 3 min at 98°C; 30 s at 94°C, 30 s at 55°C and 1 min at 74°C (30 cycles) in a thermal cycler (Gene Amp PCR System 24000, Perkin Elmer, Foster, Calif). The resulting amplified PCR products for *Tk-idim, Tk-idim1, Tk-idim2, Tk-idim3* genes were digested with *Nde*I and *Bam*HI (forward primers possess the *Nde*I while reverse IPP-R1 contained *Bam*HI site as underlined in table 1) and the plasmid pET-21a was also digested with *Nde*I and *Bam*HI. The digested vector and PCR products were ligated to obtain pIDIM, pIDIM1, pIDIM2 and pIDIM3 plasmids.

DNA sequencing

To know the correct amplification by PCR, all the restriction fragments cloned into pET-21a vector were sequenced by the dideoxy chain termination method (Sanger *et al*., 1977) using ALF Express (Pharmacia, Uppsala, Sweden) sequencer.

Genes expression and purification of Tk-IDIM protein

For the expression of genes the constructed plasmids pIDIM, pIDIM1, pIDIM2 and pIDIM3 were introduced into *E.coli* BL21(DE3) cells. Heterologous expression of the genes was induced with the addition of 0.5 mM isopropyl-ß-D-thioglactopyranoside when optical density (660 nm) of the culture reached at 0.5. After 5 hours of post induction the 5 liter culture medium was centrifuged at 9,000 × g for 10 min. The cells were resuspended in buffer A consisting of 50 mM phosphate at pH 7.0 and lyzed by sonication. The sonicated sample was centrifuged at 26,000 × g for 20 min and supernatant fraction was used for ammonium sulfate precipitation (70%) and kept overnight at 4°C. The pallet after centrifugation was again dissolved in 25 mL of buffer A, dialyzed overnight at 4°C against buffer A. The dialyzed solution was centrifuged and filtered using 0.22 µm MILLEX GS and applied to HiTrapQ column (GE Healthcare). *Tk*-IDIM was eluted by applying linear gradient of NaCl in buffer A. The active fractions were pooled and dialyzed against buffer A, and then applied to column BIOASSIST Q (Tosoh, Tokyo, Japan). *Tk*-IDIM was eluted by applying the gradient of 0.2 to 1.0. M NaCl in buffer A. Active fractions from the BIOASSIST Q column were combined and concentrated by using micro con centrifuge devices (Millipore Co, Bedford, USA).

Molecular mass determination and measurement of enzyme activity

To determine the molecular mass of purified *Tk*-IDIM, the gel filtration column superdex 200 (GE Healthcare) was used. The column had been equilibrated with buffer B. The standard proteins used for the determination of molecular mass were: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and BSA (66 kDa). The enzymatic activity of *Tk*-IDIM was measured based on the acid-lability of DMAPP according to the previously reported method (Kaneda *et al*., 2001).

Circular dichroism (CD) analysis

To study the unfolding profiles of *Tk*-IDIM at different temperatures, various concentrations of Gdn-HCl were used. The Gdn-HCl induced denaturation profiles were measured on an automatic spectropolarimeter JASCO J-820 (Japan Spectroscopic Company) with CD ellipticity at 222 nm. For the equilibrium measurement, various

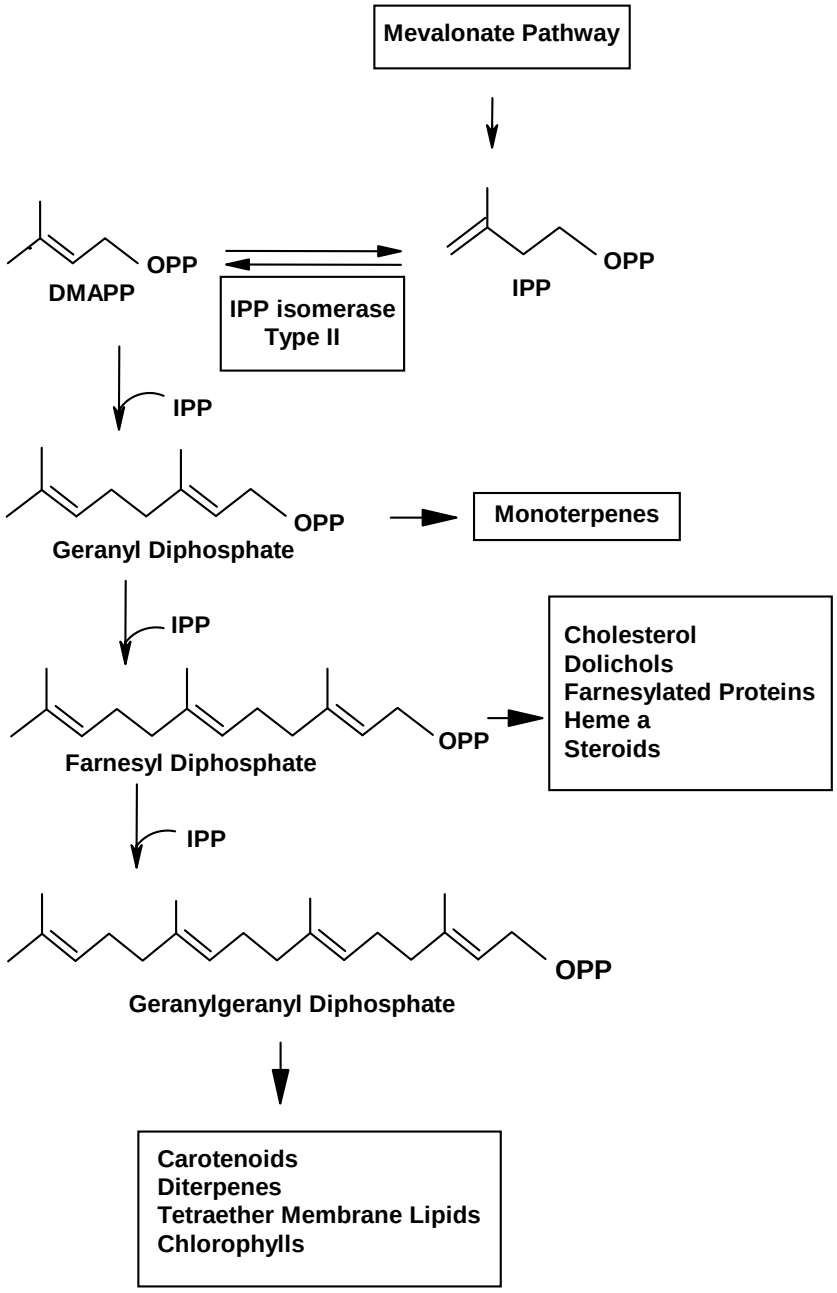

Fig. 1: Isoprenoid biosynthetic via mevalonate (MVA) pathway. IPP is converted to its isomer DMAPP by the action of IPP isomerase type II. DMAPP acts as primer substrate to synthesize linear short chain *trans*-prenyl diphosphate. OPP stands for diphosphate moiety.

concentrations of Gdn-HCl ranging 0.5 to 7 M were prepared in 50 mM sodium phosphate buffer (pH 7.0). *Tk*-IDIM solution (40 µL) containing 0.1 mg of the protein was mixed with 460 µL of above prepared buffer in a total volume of 500 µL. The samples were incubated at temperatures 40, 50, 60, 70, 80 and 85°C. After three days of incubation the samples were used for CD analysis at the same temperature which was used for the incubation i.e. if the incubation was performed at temperature 40°C, the CD data were collected at 40°C. Analysis of the experimental data was done by a two-state folding mechanism. During the unfolding process at a certain temperature the equilibrium constant (K_{app}) was determined using the following Equation 1:

$$K_{app} = f_U / f_N = (\theta_N - \theta) / (\theta - \theta_U) \quad (1)$$

In equation 1, f_N represents the fractions of the native and f_U denaturant states, whereas θ_N is the value of 100% native and θ_U 100% denaturant state, and θ measured intensity. During the unfolding process the

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idi                                                    idim
MGEFDREELT IIRKFEHIEH CLKRNVQAHV TNGFEDVHFV HMSLPEIDKD EIDLSVEFLG    60
1                idim1                                 2 idim2  idim3
RKFDYPIFIA GMTGGTKGSQ LAGRINKTLA KAAQELNIPM GVGSQRAMIR KPETWESYYV   120
           3                                    4        5
RDVAPDVFLV GNLGAPQFSE TIRERYGLEE ALKAVETIQA DALAIHMNPL QESVQPEGDT   180

QYRGVLKALA ELKAEFPYPI IAKETGAGVS MEVAVRLESI GIDAIDVGGL GGTSWSGVEY   240

YRAKDEIGKD LALRFWDWGI KTAISVAEVR YATELPIIAT GGMRDGIAMA KALAMGATFA   300

GVALPLLKPA VKGDVEGVIK ILRRYIEEIR NAMFLVGARN VEELRKVPLV ITGFTREWLE   360

QRIDLPSYLR NRGI                                                    374
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Fig. 2: Deduced amino acid sequence of *Tk*-IDI (accession no. AB109220). The bolds "**M"** with below numbering 2, 3, 4 and 5 indicate the four putative starting methionines downstream of the first methionine.

Table 1: List of primers used in this study

Primer name	Nucleotide sequence	Primer length
IPP-F	5'-CTTCGTCCATATGAGCCTGCCCGAGAT-3'	27
IPP-F1	5'-CATCGCTCATATGACCG GCGGAACGAA-3'	27
IPP-F2	5'-AGCTCAACATTCATATGGGCGTTGGCA-3'	27
IPP-F3	5'-GTCAGAGGCATATGATAAGGAAGCCC G-3'	27
IPP-R	5'-GAGGGATCCCGCAAAGGCGGA AATCAGATGCC-3'	32

Underline sequences are the recognition sites for *Nde*I and *Bam*HI.

conformational free-energy change (ΔG) is related to the equilibrium constant by the equation $\Delta G = -RT \ln K_{app,}$ where R represents gas constant and T absolute temperature in Kelvin. A two-state unfolding model can assume a simple linear dependence of stability between the denaturant concentration [Gdn-HCl] and ΔG, as represented in the following Equation 2:

$$\Delta G = \Delta G_0 - m\,[\text{Gdn-HCl}] \qquad (2)$$

Where ΔG_0 is the ΔG in the unfolding-free state and m is proportionality constant that represents the slope of the linear line between [Gdn-HCl] and ΔG.

The ΔG_0 values were obtained at different temperatures according to the above mentioned procedure, and the temperature dependence of the ΔG_0 value was obtained according to the previously reported method (Ohnuma *et al*., 1997). α-helical contents were determined by the following equation.

$$XH = \{[\theta]_{208}-(4000)\}/\{-33000(-4000)\} \times 100$$

Prediction of secondary structure and 3D modeling

The three dimensional (3D) model figures of *Tk*-IDIM were constructed on the basis of standard homology modeling protocols. The query sequences were submitted to Swiss Model Server (http://swissmodel. expasy. org//SWISS-MODEL.html) and homologue structures were obtained from ExPdb (modified PDB database). The alignment of *Tk*-IDIM was done with the template and submitted to the Swiss Model Server. The resulting protein model was examined and evaluated using verify 3D server (Arnold *et al*., 2006; Schwede *et al*., 2003).

RESULTS

Cloning and expression of mutant genes

Tk-idi gene contained 1122 nucleotides which encoded 374 amino acids. Four putative start codons at 123, 213, 297 and 321 nucleotides downstream of the first start codon were found and the products resulting from these start codons contained 333, 303, 275 and 267 amino acids, respectively (Fig. 2).The N-terminally truncated genes including *Tk-idim* (123 nt truncated), *Tk-idim1*(213 nt truncated), *Tk-idim2* (297 nt truncated) and *Tk-idim3* (321 nt truncated), were cloned in pET-21a vector and sequenced. When we tried to express these genes in *E. coli* only *Tk-idim* could be expressed in enzymatically active form. Other truncated genes either could not express or their gene products were inactive. Therefore, they were not investigated further. The expression of *Tk-idim* resulted in the production of 36 kDa enzymatically active protein, *Tk*-IDIM, corresponding to 333 amino acids.

Purification of Tk-IDIM

Purification of *Tk-idim* product after ion exchange and gel

filtration chromatographies resulted in a single band when analyzed by SDS-PAGE (fig. 3). Throughout the purification process, the colour of protein solution was yellow indicating that *Tk*-IDIM was also a flavoprotein. When the purified protein was applied on a gel filtration chromatography column Superdex 200 HR the active peak corresponded to a molecular mass of 300 kDa. The molecular mass, 36,547 Da, as predicted from the deduced amino acid sequence of the protein indicated that *Tk*-IDIM existed in an octameric from.

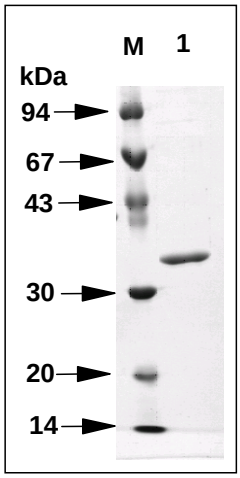

Fig. 3: CBB stained 0.1% SDS-15% PAGE. Lane: 1: purified Tk-IDIM. M, molecular weight markers (Phosphorylase b, 94,000 Da; Albumin, 67,000 Da; Ovalbumin, 43,000 Da; Carbonic anhydrase, 30,000 Da; Trypsin inhibitor, 20,100 Da; α-lactalbumin; 14,178 Da).

Enzymatic activity of Tk-IDIM

The activity of *Tk*-IDIM was detected by using optimum conditions as previously reported (Siddiqui *et al*., 2005), in a 100 mM MOPS buffer (pH 8.0) containing 5 mM $MgCl_2$, 100 µM [4-^{14}C]IPP, 5 mM NADPH, and 10 µM FMN. The dependence of the activity on the reaction temperature was tested under the optimum conditions. When the reaction was performed at various temperatures, maximum activity of *Tk*-IDIM was observed at 75°C.

CD spectral studies of Tk-IDIM

In order to compare the thermostability of *Tk*-IDIM and *Tk*-IDI, CD spectrum analysis was performed under similar conditions. Both the proteins exhibited a similar spectrum. Far-UV CD spectra (200 to 260 nm) of *Tk*-IDIM at temperatures 40, 50, 60, 70, 80 and 85°C are collectively shown in Fig. 4A. A 41% α-helical content of *Tk*-IDIM was calculated by measuring the intensity of the ellipticity [θ] at 208 nm at 40°C. When temperature was increased, the two unique peaks were declined indicating the change in protein conformation with the increase in temperature. In order to know the thermal stability, a thermal denaturation curve was obtained by plotting the change of ellipticity [θ] value at 222 nm with temperature (data not shown). It was observed that the [θ] value increased continuously with the increase in temperature and even at 80°C it did not attain a constant value. The thermal unfolding of *Tk*-IDIM was not fully reversible under these conditions.

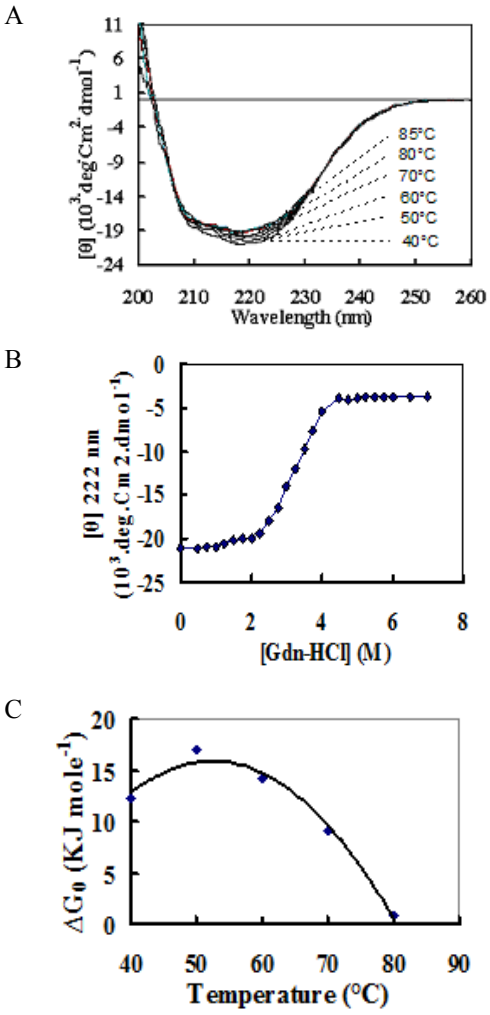

Fig. 4: CD spectra analysis of *Tk*-IDIM. (A) Far-UV CD spectra collected at temperatures 40, 50, 60, 70, 80 and 85°C in 50 mM sodium phosphate (pH 7.0). (B) Gdn-HCl titration curves of *Tk*-IDIM at 40 °C in 50 mM sodium phosphate (pH 7.0). (C) Dependence of the ΔG_0 on temperature. The ΔG_0 values were determined by the two-state folding model. A fitting quadratic curve of ΔG_0 values against temperatures indicated that the ΔG_{0max} value was 17 kJ mol^{-1} at 50°C at the vertex and the *T*m value was 80°C at the horizontal intersection.

To fully characterize the thermal unfolding, the thermodynamic parameters were obtained by applying the alternating chemical denaturation procedure. For chemical denaturation guanidine hydrochloride (Gdn-HCl) was

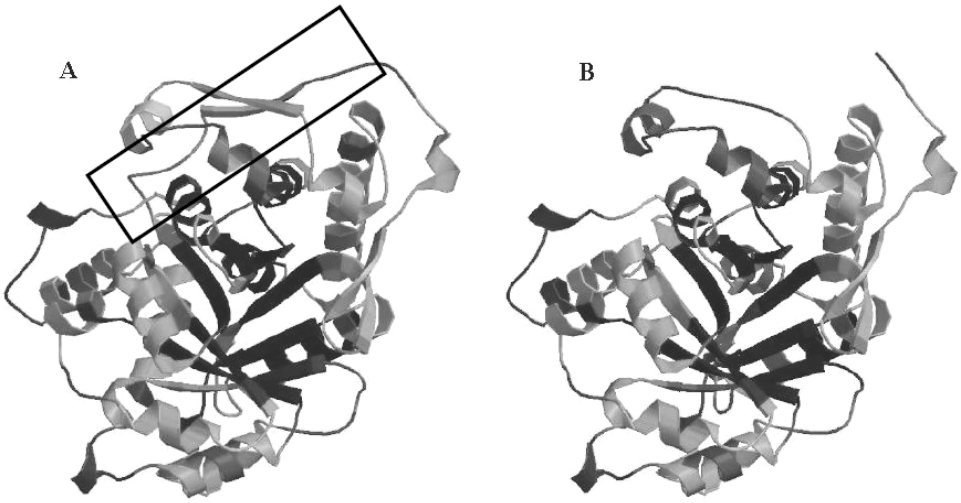

Fig. 5: Ribbon diagram illustrating the structural model of (A) *Tk*-IDI and (B) *Tk*-IDIM. The position of 41 N-terminal amino acid residues in *Tk*-IDI is marked by a box.

selected as a denaturant. It has been reported previously that the proteins when incubated with various concentrations of Gdn-HCl for three days at different temperatures gave a typical unfolded spectrum due to the decrease in far-UV CD intensity (Fujiwara *et al*., 2004). By using this method *Tk*-IDIM was also incubated at different temperatures (40 to 85°C) in 1 to 7 M Gdn-HCl for three days. Unfolding of *Tk*-IDIM was measured by recording CD spectra at a wavelength of 222 nm because [θ] value shorter than 215 nm could not be clearly detected due to noises. The denaturation profile of *Tk*-IDIM at 40°C (fig. 4B) was according to the two-state folding model. The profiles obtained at other temperatures also agreed with this model (data not shown). The conformational free-energy change values (ΔG_0) in the unfolding-free state at various temperatures were then calculated (fig. 4C). The fitting quadratic curve of ΔG_0 values against temperatures gave the maximum free-energy change ($\Delta G_{0\max}$) of 17 kJ mol^{-1} at 50°C, and the melting temperature (*Tm*) of 80°C. The heat capacity change (ΔC_p) was calculated to be 13.8 kJ mol^{-1} K^{-1}. Protein folding equilibrium thermodynamics requires the measurement of the free energy change ΔG_0 between the native and unfolded states as a function of temperature. Three different models have been postulated to explain the thermostabilization on the basis of theoretical thermodynamics, i.e., high stability model, flattened model and shifted model (Shiraki *et al*., 2001; Nojima *et al*., 1977). In the high stability model, high conformational stability leads to high thermal stability which directly relates to increase in both $\Delta G_{0\ \max}$ and *T*m values. The $\Delta G_{0\ \max}$ value of *Tk*-IDI was 21 kJ mol^{-1} at 60°C and *T*m value was 88 °C (Siddiqui *et al*., 2005) as compared to *Tk*-IDIM values of 17 kJ mol^{-1} and 80°C, respectively. This decrease in $\Delta G_{0\ \max}$ and *T*m values of *Tk*-IDIM support the involvement of N–terminal amino acids in protein stability. In the flattened model low heat capacity change accompanying protein unfolding (ΔC_p) is a result of the low dependence of $\Delta G_{0\ \max}$ on temperature. The ΔC_p of Tk-IDIM was 13.8 kJ mol^{-1} K^{-1} compared to 13.2 kJ mol^{-1} K^{-1} of Tk-IDI. This may contribute to decrease in thermostability of Tk-IDIM. In the shifted model, the $\Delta G_{0\ \max}$ vs. temperature curve shifts to a higher temperature for protein stability. The $\Delta G_{0\ \max}$ value of Tk-IDIM shifted to 50 °C as compared to 60°C of Tk-IDI (fig. 4C).

DISCUSSION

The enzymes from hyperthermophiles are usually extremely thermostable (Ozawa *et al*., 2012). *Tk-idi* gene from *T. kodakaraensis*, which encodes type II isopentenyl diphosphate isomerase was previously cloned, and its enzymatic and structural characteristics were reported (Siddiqui *et al*., 2005). Four putative start codons at 123, 213, 297 and 321 nucleotides downstream of the first start codon were found. In order to know the role of N-terminal amino acids on enzymatic activity and protein thermostability, 123, 213, 297 and 321 nucleotides were deleted from *Tk-idi* gene to obtain *Tk-idim, Tk-idim1, Tk-idim2, Tk-idim3*. When the expression of these genes was examined in *E. coli* we found that only *Tk-idim* could be expressed in enzymatically active form. Other truncated genes either could not express or their gene products were inactive probably due to distortion of the secondary and tertiary structures.

When the optimal temperature for the enzyme activity

was examined, highest activity of *Tk*-IDIM was observed at 75°C whereas *Tk*-IDI had exhibited highest activity at 80°C. Similarly half-life of *Tk*-IDIM was 30 min at 70°C compared to 60 min of Tk-IDI at the same temperature. The decrease in thermostability and optimal temperature for enzyme activity of *Tk*-IDIM suggested the involvement of N-terminal amino acids of *Tk*-IDI in protein thermostability.

Sequence analysis of *Pyrococcus furiosus* IPP isomerase revealed the presence of additional charged residues in the protein as compared to those found in IPP isomerases from mesophile origin. It was suggested that these additional charged residues might contribute to thermal stability of the protein by increasing the number of salt-bridges thereby decreasing flexibility of N-terminal segment (Dutoit *et al*., 2008). Similarly, the presence of charged residues was also observed in case of *Tk*-IDI (fig. 2). This could also be a possible explanation for the decrease in thermal stability of *Tk*-IDIM (which lacks 41 N-terminal residues of *Tk*-IDI and, hence, is devoid of several charged residues). Moreover, structural modeling of *P. furiosus* IPP isomerase showed that the N-terminal segment was important in substrate stabilization by ion-pair interactions and protecting it from hydrolysis by bulk solvent (Dutoit *et al*., 2008). Structural modeling for *Tk*-IDI (Fig. 5A) showed that the N-terminal segment forms a cover over IPP which is not present in the case of *Tk*-IDIM (Fig. 5B) thus decreasing its stability.

In conclusion, when 41 N-terminal amino acids were removed from Tk-IDI the truncated protein retained the octameric form as well as the enzyme activity. However, the thermostability of the enzyme decreased indicating that the N-terminal deleted amino acids has a role in the thermostability of Tk-IDI.

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REFERENCES

Altincicek BB, Kollas AK, Eberl M, Wiesner J, Sanderbrand S, Hintz M, Beck E and Jomaa H (2001a). *LytB*, a novel gene of the 2-*C*-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis in *Escherichia coli*. *FEBS Lett*., **499**: 37-40.

Altincicek B, Kollas AK, Sanderbrand S, Wiesner J, Hintz M, Beck E and Jomaa H (2001b). *GcpE* is involved in the 2-*C*-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis in *Escherichia coli*. *J. Bacteriol*., **183**: 2411-2416.

Anderson MS, Mehlbacher M, Street IP, Proffitt J and Poulter CD (1989). Isopentenyl diphosphate: dimethylallyl diphosphate isomerase. An improved purification of the enzyme and isolation of the gene from *Saccharomyces cerevisiae*. *J. Biol. Chem*., **264**: 19169-19175.

Arnold K, Bordoli L, Kopp J and Schwede T (2006). The SWISS-MODEL Workspace: A web-based environment for protein structure homology modeling. *Bioinformatics*., **22**: 195-201

Ashby MN and Edwards PA (1990). Elucidation of two yeast coenzyme Q mutants. Characterization of the structural gene encoding hexaprenyl pyrophosphate synthetase. *J. Biol. Chem*., **26**: 13157-13164.

Barkley SJ, Desai SB and Poulter CD (2004a). Type II isopentenyl diphosphate isomerase from *Synechocystis* sp. Strain PCC 6803. *J. Bacteriol*., **186**: 8156-8158.

Barkley SJ, Cornish RM and Poulter CD (2004b). Identification of an archaeal type II isopentenyl diphosphate isomerase in *Methanothermobacter thermautotrophicus*. *J. Bacteriol*., **186**: 1811-1817.

Cane DE (1999). Isoprenoid biosynthesis overview. In: Barton DHR and Nakanishi K editors. Comprehensive Natural Product Chemistry, Elsevier, Oxford, pp.1-484.

Christianson DW (2007). Chemistry. Roots of biosynthetic diversity. *Science*., **316**: 60-61.

Campos N, Rodriguez-Concepcion M, Seemann M, Rohmer M and Boronat A (2001). Identification of *gcpE* as a novel gene of the 2-*C*-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis *Escherichia coli*. *FEBS Lett*., **488**: 170-173.

Chang MCY and Keasling JD (2006). Production of isoprenoid pharmaceuticals by engineered microbes. *Nat. Chem. Biol*., **2**: 674-681.

Clarke S (1992). Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu. Rev. Biochem*., **61**: 355-386.

Dutoit R, Ruyck J, Durisotti V, Legrain C, Jacobs E and Wouters J (2008). Overexpression, physicochemical characterization, and modeling of a hyperthermophilic *Pyrococcus furiosus* type 2 IPP isomerase. *Proteins*., **71**: 1699-1707.

Fujiwara S, Yamanaka A, Hirooka K, Kobayashi A, Imanaka T and Fukusaki E (2004). Temperature-dependant modulation of farnesyl diphosphate/geranyl diphosphate synthase from hyperthermophilic archaea. *Biochem. Biophys. Res. Commun*., **325**: 1066-1074.

Goodwin TW (1971). Biosynthesis of Carotenoids and Plant Triterpenes. *Biochem. J*., **123**: 293–329.

Hahn FM, Baker JA and Poulter CD (1996a). Open reading frame 176 in the photosynthesis gene cluster of *Rhodobacter capsulatus* encodes *idi*, a gene for isopentenyl diphosphate isomerase. *J. Bacteriol*., **178**: 619-624.

Hahn FM, Xuan JW, Chambers AF and Poulter CD (1996b). Isopentenyl diphosphate:dimethylallyl diphosphate isomerase: Overproduction, purification and characterization. *Arch. Biochem. Biophys*., **332**: 30-34.

Hahn FM, Hurlburt AP and Poulter CD (1999). Escherichia coli open reading frame 696 is *idi*, a nonessential gene encoding isopentenyl diphosphate isomerase. *J. Bacteriol*., **181**: 4499-4504

Imanaka T, Tanaka T, Tsunekawa H and Aiba S (1981). Cloning of the genes for penicillinase, *penP* and *penI*, of *Bacillus licheniformis* in some vector plasmids and their expression in *Escherichia coli*, *Bacillus subtilis*, and *Bacillus licheniformis*. *J. Bacteriol*., 147: 776-786.

Kaneda K, Kuzuyama T, Takagi M, Hayakawa Y and Seto H (2001). An unusual isopentenyl diphosphate isomerase found in the mevalonate pathway gene cluster from *Streptomyces* sp. Strain CL190. *Proc. Natl. Acad. Sci, USA*., **98**: 932-937.

Kuzuyama T (2002). Mevalonate and non-mevalonate pathways for the biosynthesis of isoprene units. *Biosci. Biotechnol. Biochem*., **66**: 1619-1627.

Laupitz R, Hecht S, Amslinger S, Zepeck F, Kaiser J, Richter G, Schramek N, Steinbacher S, Huber R, Arigoni D, Bacher A, Eisenreich W and Rohdich F (2004). Biochemical characterization of *Bacillus subtilis* type II isopentenyl diphosphate isomerase, and phylogenetic distribution of isoprenoid biosynthetic pathways. *Eur. J. Biochem*., **271**: 2658-2669.

Matsuoka S, Sagami H, Kurisak, A and Agura K (1991). Variable product specificity of microsomal dehydrodolichyl diphosphate synthase from rat liver. *J. Biol. Chem*., **266**: 3464-3468.

Morikawa M, Izawa Y, Rshid N, Hoaki T and Imanaka T (1994). Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. *Appl. Environ. Microbiol*., **60**: 4559-4566.

Newman, DJ, Cragg, GM and Snader, KM (2000). The influence of natural products upon drug discovery (Antiquity to late 1999). *Nat. Prod. Rep*., **17**: 215-234.

Nojima H, Ikai A, Oshima T and Noda H (1977). Reversible thermal unfolding of thermostable phosphoglycerate kinase thermostability associated with mean zero enthalpy change. *J. Mol. Biol*., **116**: 429-442.

Ohnuma S, Hirooka K, Ohto C and Nishino T (1997). Conversion from archaeal geranylgeranyl diphosphate synthase to farnesyl diphosphate synthase. Two amino acids before the first aspartate-rich motif solely determine eukaryotic farnesyl diphosphate synthase activity. *J. Biol. Chem*., **272**: 5192-5198.

Ozawa Y, Siddiqui MA, Takahashi Y, Urushiyama A, Ohmori D, Yamakura F, Arisaka F and Imai T (2012). Indolepyruvate ferredoxin oxidoreductase: An oxygen-sensitive iron sulfur enzyme from the hyper-thermophilic archaeon *Thermococcus profundus*. *J. Biosci. Bioeng*., **114**: 23-27.

Popjak G (1970). Natural Substances Formed Biologically from Mevalonic Acid. *In:* Goodwin TW (editors). Biochemical Society Symposium No.29, Academic Press, New York, pp.17-37.

Sambrook J, Fritsch EF and Maniatis T (1989). Molecular cloning – a laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger F, Nicklen S and Coulson AR (1977). DNA Sequencing with chain terminating inhibitor. *Proc. Natl. Sci, USA*., **74**: 5463-5467.

Schwede T, Kopp J, Guex N and Peitsch MC (2003). SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Research*., **31**: 3381-3385.

Shiraki K, Nishikori S, Fujiwara S, Hashimoto H, Kai Y, Takagi M and Imanaka T (2001). Comparative analysis of the conformational stability of a hyperthermophilic protein and its mesophilic counterpart. *Eur. J. Biochem*., **268**: 4144-4150.

Siddiqui MA, Yamanaka A, Hirooka K, Bamaba T, Kobayashi A, Imanaka T, Fukusaki E and Fujiwara, S (2005). Enzymatic and structural characterization of type II isopentenyl diphosphate isomerase from hyperthermophilic archaeon *Thermococcus kodakaraencis*. *Biochem. Biophys. Res. Commun*., **331**: 1127-1136.

Takagi M, Kaneda K, Shimizu T, Hayakawa Y, Seto H and Kuzuyama T (2004). *Bacillus subtilis ypgA* gene is *fni*, a nonessential gene encoding Type 2 isopentenyl diphosphate isomerase. *Biosci. Biotechnol. Biochem*., **68**: 132-137.

Unno H, Yamashita S, Ikeda Y, Sekiguchi S, Yoshida N, Yoshimura T, Kusunoki M, Nakayama T, Nishino T and Hemmi H (2009). New role of flavin as a general acid-base catalyst with no redox function in type 2 isopentenyl-diphosphate isomerase. *J. Biol. Chem*., **284**: 9160-9167.

Yamashita S, Hemmi H, Ikeda Y, Nakayama T and Nishino T (2004). Type 2 isopentenyl diphosphate isomerase from a thermoacidophilic archaeon *Sulfolobus shibatae*. *Eur. J. Biochem*., **271**: 1087-1093.