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Molecular cloning and characterization of TK1111, a cupin-type phosphoglucose isomerase from *Thermococcus kodakarensis*

[Bir Cupin-tip fosfoglukoz izomeraz olan TK1111'in *Thermococcus kodakarensis*'den moleküler klonlanması ve karakterizasyonu]*

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ABSTRACT

Aim: To characterize, TK1111, a cupin-type phosphoglucose isomerase from a hyperthermophilic archaeon *Thermococcus kodakarensis*.

Methods: The genome of *T. kodakarensis* was searched in order to find an open reading frame annotated as glucose-6-phosphate isomerase. The structural gene was cloned, expressed in *Escherichia coli* and the purified gene product was characterized. Effect of metal ions on the restoration of enzyme activity was examined.

Results: Genome search of *T. kodakarensis* revealed the presence of an open reading frame, TK1111, annotated as glucose-6-phosphate isomerase. TK1111 exhibited high homology with archaeal PGIs related to the cupin-superfamily instead of well-known family of PGI enzymes. The gene encoding TK1111 consisted of 567 nucleotides corresponding to a polypeptide of 189 amino acids. The structural gene was cloned, expressed in *Escherichia coli* and the purified gene product was characterized. Recombinant TK1111 displayed highest activity (43 U/mg) at 90 °C and pH 6.0. To our knowledge this is the only PGI from cupin-superfamily exhibiting highest activity in the acidic pH. TK1111 catalyzed the conversion of fructose 6-phosphate to glucose 6-phosphate following Michaelis-Menten kinetics with a K_m and V_{max} values of 2.2 mM and 12.5 µmol min^{-1} mg^{-1}, respectively at 37 °C. Addition of Zn^{2+} ions in the assay mixture remarkably enhanced the enzyme activity in contrast to the PGI from *Thermococcus litoralis*, another member of the same genus exhibiting 75% identity to TK1111. The enzyme activity was completely diminished when TK1111 was heated at 70 °C in the presence of EDTA. Complete restoration of the enzyme activity was observed when TK1111 was incubated at room temperature in the presence of Zn^{2+}.

Conclusion: TK1111 from *T. kodakaraensis* is a novel phosphoglucose isomerase that belongs to cupin-type isomerases. To our knowledge this is the only PGI from cupin-family exhibiting highest activity in the acidic pH. Enzyme activity was remarkably enhanced in the presence of Zn^{2+} in contrast to other members of the family.

Key Words: Phosphoglucose isomerase, metalloenzyme, cupin-family, thermostability, *Thermococcus kodakarensis*, hyperthermophile, archaeon

Conflict of Interest: There is no conflict of interest between the authors.

ÖZET

Amaç: Hipertermofilik bir archeon olan *Thermococcus kodakarensis*'den, bir cupin-tip fosfoglukoz izomeraz olan TK1111'in karakterizasyounun yapılması.

Yöntemler: *T. kodakarensis*'in genomu glukoz-6-fosfat izomeraz için bir açık çerçeve bulmak amacıyla araştırılmıştır. Yapısal gen klonlanarak, *Escherichia coli*'de eksprese edilmiş ve saflaştırılan gen ürünü karakterize edilmiştir. Metal iyonlarının enzim aktivitesinin yenilenmesi üzerine etkileri incelenmiştir.

Bulgular: *T. kodakarensis*'in genom araştırması, glukoz-6-fosfat izomeraz için tanımlanan bir açık çerçeve, TK1111, varlığını ortaya çıkartmıştır. TK1111, iyi bilinen PGI enzim aileleri yerine, cupin-süperailesiyle ilişkili olan archeal PGIlarla yüksek homoloji göstermiştir. TK1111'ü kodlayan gen, 189 amino asitlik bir polipeptidle ilişkili 567 nükleotid içermektedir. Yapısal gen klonlanarak, *Escherichia coli*'de eksprese edilmiş ve saflaştırılan gen ürünü karakterize edilmiştir. Rekombinant TK1111 en yüksek aktiviteyi (43 U/mg) 90 °C ve pH 6.0'da göstermiştir. Bilgilerimize dayanarak cupin-süperailesindeki PGI'lar arasında, en yüksek aktivitesini asidik pH'da gösteren tek enzimdir. TK1111, Michealis-Menten kinetiğini takip ederek, fruktoz-6-fosfatın glukoz-6-fosfata çevrimini katalizler ve 37 °C'de sırasıyla 2.2 mM ve 12.5 µmol min^{-1} mg^{-1} K_m ve V_{max} değerlerine sahiptir. Reaksiyon ortamına Zn^{2+} iyonlarının eklenmesi, aynı genusun başka bir üyesi olan ve TK1111'e %75 benzerlik gösteren, *Thermococcus litoralis*'den elde edilen PGI'nın aksine, enzim aktivitesini belirgin biçimde arttırmıştır. TK1111, EDTA varlığında 70 °C'ye ısıtıldığında enzim aktivitesi tamamen azalmıştır. TK1111 oda sıcaklığında ve Zn^{2+} varlığında inkübe ediliğinde enzim aktivitesinin tamamen geri kazanılmıştır.

Sonuç: *T. kodakaraensis*'den elde edilen TK1111, cupin-tip izomerazlara dahil olan yeni bir fosfoglukoz izomerazdır. Bilgilerimize göre cupin-ailesi içinde, en yüksek aktivitesini asidik pH'da gösteren tek PGI'dır. Ailenin diğer üyelerinin aksine, enzim aktivitesi Zn^{2+} varlığında belirgin biçimde artış göstermiştir.

Anahtar Kelimeler: Fosfoglukoz izomeraz, metaloenzim, cupin-ailesi, termostabilite, *Thermococcus kodakarensis*, hipertermofil, archaeon

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Phosphoglucose isomerase (PGI; EC 5.3.1.9) catalyses the reversible isomerization of glucose-6-phosphate (G6P), an aldose, and fructose-6-phosphate (F6P), a ketose. PGI is an important player of sugar metabolism in living organisms. Majority of the PGIs belong to the PGI superfamily, consisting of the PGI family and the bifunctional phosphoglucose/phosphomannose isomerase (PGI/PMI) family [1]. PGIs belonging to the PGI superfamily are found in all the three domains of life and are extensively studied. Bifunctional PGIs/PMIs are mainly found in the crenarchaeotal branch of archaea [2]. A third and a novel type of PGI has been found in euryarchaeotal branch of archaea [1, 3, 4] and it belongs to the cupin-family comprising a group of functionally diverse proteins that contain a central domain forming a small barrel called "cupin." Most of the cupin enzymes are metallo enzymes having two conserved sequences(consensus, $G(X)_{5-8}H(X)H(X)_{3-6}E(X)_6G$, motif I; and $G(X)_5P(X)_4H(X)_3N$, motif II)The PGIs belonging to this group have been characterized from archaea including *Archaeoglobus fulgidus* [5], *Methanosarcina mazei* [5], *Pyrococcus furiosus* [1, 3], and *Thermococcus litoralis* [4] as well as from bacteria including *Salmonella enterica* and *Ensiferm eliloti* [5]. Determination of active-site residues of PGI from *P. furiosus* has been accomplished by docking of monosaccharides onto the structure models of the enzyme [6].

Thermococcus kodakarensis is a hyperthermophilic euryarchaeon isolated from a solfatara at a wharf on Kodakara Island, Kagoshima, Japan [7, 8]. The strain is a strict anaerobe and it can grow heterotrophically on starch, pyruvate, amino acids and peptides. Whole genome of *T. kodakarensis* has been sequenced and reported [9]. The genome sequence revealed the presence of an open reading frame exhibiting high homology to the cupin-type PGIs. We describe here cloning of the PGI gene (TK1111) from *T. kodakarensis*, and characterization of the gene product produced in *Escherichia coli*.

Materials and Methods

Materials

Restriction enzymes, DNA and protein markers, TA cloning and DNA extraction kits, *Taq* DNA polymerase (recombinant), and pTZ57R/T cloning vector were purchased from Thermo Scintific (Thermo Scintific, MA, USA). Expression vectors pET-21a(+) was obtained from Stratagene (Stratagene, La Jolla, CA, USA), *E. coli* DH5α and BL21 Codon Plus cells, were purchased from Novagen (Novagen, Madison, WI, USA). Primers were synthesized from e-oligos (Gene Link, NY, USA). All other chemicals were purchased from Sigma (Sigma-Aldrich, Taufkirchen, Germany).

Homology comparison and phylogenetic tree construction

Database homology search was performed by Basic Local Alignment Search Tool (BLAST) program [10]. Multiple sequence alignment of TK1111 homologs was performed and a neighbor-joining tree of the aligned sequences was generated with ClustalW [11] provided at: (http://clustalw.ddbj.nig.ac.jp/).

Cloning of TK1111

The structural gene, TK1111, encoding a homolog of cupin-type PGI, was amplified by polymerase chain reaction (PCR) using genomic DNA of *T. kodakarensis* as a template and a sequence specific set of forward (5-GGTGATCATATGGAGTACAAGCGCCC) and reverse (5-GGATTTCGGGCTACTCCTTCCAGC) primers. *Nde*I restriction enzyme site was introduced in the forward primer (underlined sequence). PCR amplified TK1111 gene was inserted in cloning vector pTZ57R/T using T_4 DNA ligase and the resulting plasmid was named as pTZ-TK1111. TK-1111 gene was cut from pTZ-TK1111 using *Nde*I (introduced in the forward primer) and *Hind*III (present in the multicloning site of pTZ57R/T) restriction enzymes and ligated in pET-21a at the corresponding sites. The resulting plasmid, pET-TK1111, was used to transform *E. coli* BL21-CodonPlus(DE3)-RIL for production of TK1111 enzyme.

Production in E. coli and purification of recombinant TK1111

E. coli cells harbouring pET-TK1111 were grown to early log phase and expression of the gene was induced with 0.2 mM isopropyl-thio-β-D-galactoside (IPTG). The cells were harvested by centrifugation at 7,000 × *g* for 5 min at 4 °C, resuspended in 50 mM Tris-HCl (pH 8.0), and disrupted by sonication at 4 °C. The supernatant after centrifugation was heated at 85 °C for 25 min. The heat labile proteins from the host cells were denatured and removed by centrifugation at 15,000 × g for 20 min at 4 °C. The heat-stable recombinant TK1111 in the supernatant was further purified using ÄKTA Purifier chromatography system (GE Healthcare, Uppsala, Sweden). The heat-treated cell extract was applied to anion-exchange Resource Q (6 mL) column (GE Healthcare) and recombinant TK1111 was eluted with a linear gradient of 0 to 1 M NaCl. Analysis of the purified TK1111 was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Measurement of protein concentration

The concentration of proteins in solution was determined by Bradford method [12] with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, CA, USA) using bovine serum albumin as a standard. The standard curve was plotted using 2 to 20 μg of bovine serum albumin in 1 mL of Bio-Rad protein assay reagent. The incubations

were performed at room temperature for 5 min and the absorbance was measured at 595 nm.

Gel electrophoresis

DNA samples were electrophoresed on 0.8% agarose gel followed by ethidium bromide staining and visualized under the UV light. Protein samples were analyzed by polyacrylamide gel electrophoresis (PAGE) containing 0.1% sodium dodecyl sulphate (SDS) followed by staining with Coomassie brilliant blue (CBB).

TK1111 enzyme activity assay

The formation of glucose 6-phosphate from fructose 6-phosphate was determined by measuring the formation of NADPH at 340 nm. The assay mixture contained 50 mM sodium phosphate pH 7.0, 10 mM $MgCl_2$, 0.5 mM NADP, 3 mM fructose 6-phosphate, and 1.25 units of D-glucose-6-phosphate dehydrogenase. One unit of activity was defined as the amount of enzyme required to convert 1 μmol of fructose 6-phosphate to glucose 6-phosphate per min. The routine enzyme assays were performed at 50 °C unless mentioned otherwise. At this temperature the coupling enzymes remained active, and TK1111 was sufficiently active to measure its enzyme activity. The coupling enzymes were present in excess, to ensure that the detected NADPH absorbance at 340 nm (€ = 6300 M^{-1} cm^{-1}) corresponds to the PGI activity of TK1111.

Effect of temperature and pH on TK1111 activity

The temperature optimum was determined in the direction of glucose 6-phosphate formation. Purified recombinant TK1111 was incubated in an assay mixture containing 50 mM sodium phosphate buffer, pH 7.0. The assay mixture was incubated in a water bath at temperatures ranging from 30 to 100 °C, pre-heated for 2 min, and the enzyme reaction was started by adding 3 mM fructose 6-phosphate. After 2 min of incubation the reaction was stopped by transferring the assay mixture on ice/ethanol, and the amount of glucose 6-phosphate formed was determined at room temperature by measuring the reduction of NADP (340 nm) with glucose-6-phosphate dehydrogenase. Corrections were made for the chemical isomerization of fructose 6-phosphate in the absence of TK1111.

Optimum pH for TK1111 enzyme activity was determined at 50 °C in 50 mM buffers over the pH range 5.0–9.5. Except for buffer and temperature, assay conditions were identical to analyze the temperature optimum.

Kinetic parameters

Kinetic parameters were determined at 37 °C and pH 6.0 with standard assay mixtures containing various concentrations of fructose-6-phosphate. For determination of the K_m and V_{max} values, the concentration of the substrate ranged from 0.05 to 5 mM.

Results and Discussion

Cloning TK1111 gene

Polymerase chain reaction for the amplification of TK1111 gene, using the set of primers given in the materials and methods section as priming strands and genomic DNA of *T. kodakarensis* as template, resulted in the amplification of 0.6 kb DNA fragment (data not shown) exactly matching the size of TK1111 gene. The amplified gene fragment was inserted in pTZ57R/T. When the resulting ligation mixture was used to transform *E. coli* DH5α competent cells, a total of 17 white and 6 blue colonies appeared on the selection plates. White colonies were further screened for the presence of TK1111 gene by polymerase chain reaction (data not shown). Plasmid DNA was isolated from one of the positive clones and digested with *Nde*I and *Hind*III which resulted in the liberation of a 0.6 kb DNA fragment from the vector (data not shown). Nucleotide sequence of the insert was determined and the results of DNA sequencing confirmed that the cloned fragment encodes TK1111.

Comparison of TK1111 sequence

TK1111 gene consisted of 567 nucleotides encoding a protein of 189 amino acids with a calculated molecular mass of 24,675 Da and a pI of 5.1. Amino acid sequence comparison demonstrated that TK1111 exhibits a high degree of similarity to PGIs of the cupin-family. Most cupin-family enzymes are metalloenzymes with two conserved sequences (consensus, G(X)5-8H(X)H(X)3-6E(X)6G, motif I; and G(X)5P(X)4H(X)3N, motif II). These motifs were conserved in TK1111.

When we constructed a phylogenetic tree based on the amino acid sequences of various PGIs, TK1111 clustered with PGIs from family Thermococcaceae of archaeal kingdom Euryarchaeota (Fig. 1). Eight active site residues of the cupin-family PGIs (T^{63}, G^{79}, H^{80}, H^{82}, E^{93}, Y^{95}, H^{136} and Y^{160}; *A. fulgidus* numbering) [13] were completely conserved in TK1111. Amino acid sequence comparison showed that TK1111 displayed highest homology of 92% (identity) with uncharacterized PGI originating from *Thermococcus zilligii* (accession no. WP_010478801). Among the characterized PGIs, TK1111 displayed highest homology of 82% with PGI from *P. furiosus* (accession no. NP_577925). Interestingly, TK1111 displayed relatively lower homology (75%) to PGI characterized from *T. litoralis* (accession no. WP_004067944), a member of the same genus as *T. kodakarensis* from which TK1111 was originated [4]. Amino acids involved in metal ion binding (H^{88}, H^{90}, E^{97}, and H^{136}; *P. furiosus* numbering) were completely conserved in TK1111. Similarly, amino acids involved in the formation of two small networks, a 3-residue (K^{80}, E^{175}, K^{180}) and a 4-residue (D^{94}, R^{95}, D^{155}, K^{92}) network, for ion pair formation in *P. furiosus* PGI [14] were completely conserved in TK1111. The 3-residue network

is missing in *T. litoralis* PGI as K^{80} and K^{180} are replaced by N^{81} and V^{181} (*T. litoralis* numbering), respectively (Fig. 2). Glutamate at position 45 (E^{45}, *P. furiosus* numbering), present in PGIs from *T. litoralis and P. furiosus,* is missing in TK1111. Apart from the amino acids common in the characterized PGIs from family Thermococcaceae, TK1111 shared amino acids G^{20}, E^{42}, L^{43}, K^{45}, E^{46}, K^{80}, F^{84}, F^{89}, A^{91}, W^{119}, V^{127}, V^{139}, E^{144}, I^{151}, I^{173}, E^{178}, K^{180}, V^{182}, R^{186}, and K^{188} (TK1111 numbering) with PGI from *P. furiosus* while these amino acids were replaced by N^{20}, K^{42}, M^{43}, E^{45}, G^{46}, N^{80}, M^{84}, Y^{89}, S^{91}, F^{119}, I^{127}, I^{139}, K^{144}, L^{151}, V^{173}, K^{178}, V^{180}, K^{182}, K^{186}, and R^{188} in PGI from *T. litoralis* (Fig. 2). On the other hand E^{39}, I^{57}, E^{116}, and T^{141} were identical in TK1111 and *T. litoralis* where as they were replaced by R^{39}, V^{57}, D^{116}, and I^{141} in *P. furiosus*. These sequence replacements indicate the possibility of TK1111 being the prototype of PGIs in family Thermococcaceae.

Expression of TK1111 gene in E. coli

In order to analyze the function of TK1111, the encoding gene was cloned and expressed in *E. coli*. SDS-PAGE analysis of soluble and insoluble fractions of the cells containing pET-21a or pET-TK1111 revealed the presence of an additional band, corresponding to approximately 24 kDa, in the soluble fraction of the cells containing pET-TK1111 which corresponded to the calculated molecular mass of TK1111 gene product. Production of TK1111 was more than 30% of the host proteins. Heat treatment at 85 °C for 25 min resulted in the precipitation of the heat-labile host proteins leaving TK1111 in the supernatant. Purification of TK1111 was achieved to apparent homogeneity by ammonium sulphate precipitation and ion exchange chromatography (Fig. 3).

Characterization of TK1111

Enzyme activity assay at various temperatures and pH showed that TK1111 displayed highest activity at 90 °C (Fig. 4A) and pH 6.0 (Fig. 4B). To our knowledge TK1111 is the only PGI belonging to cupin-superfamily exhibiting highest enzyme activity in acidic pH. The extremely high temperature for highest enzyme activity and thermostability of TK1111 are in accordance with its function under the hyperthermophilic growth conditions of *T. kodakarensis* [7, 8]. Thermostability experiments demonstrated that TK1111 exhibits a half-life of 40 min at 90 °C (data not shown) whereas PGI from *P. furiosus* has been reported highly thermostable with a half-life of 7.9 h at 95 °C [15].

When we examined the effect of divalent metal ions on TK1111 enzyme activity we found that addition of Zn^{2+} in the assay mixture remarkably enhanced the enzyme activity. There was more than 3-fold increase in enzyme activity in the presence of 200 μM $ZnCl_2$. Addition of

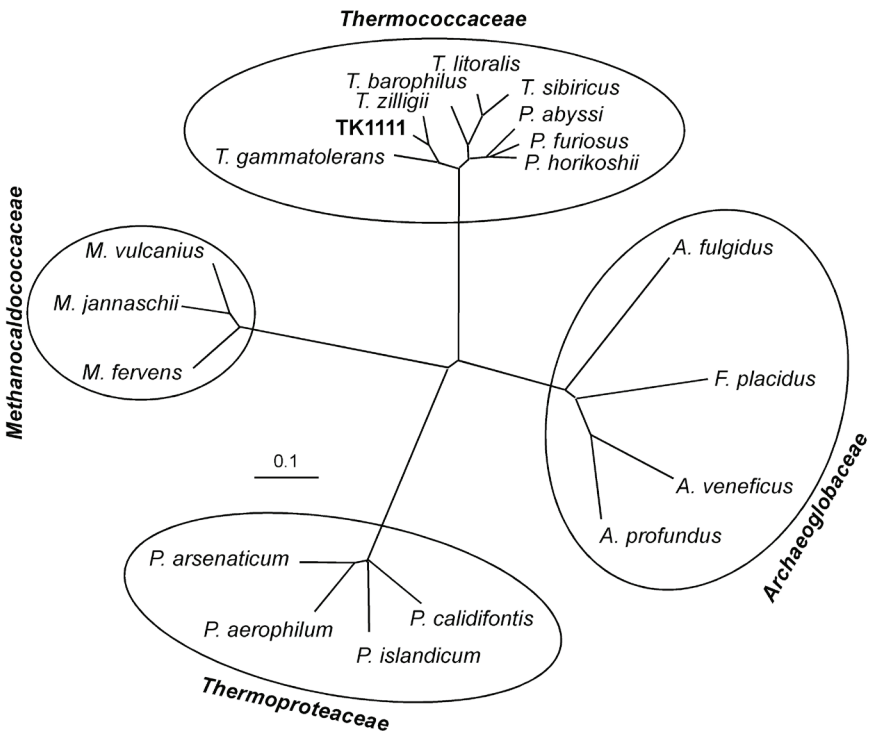

Fig. 1. Phylogenetic analysis of TK1111 and its archaeal homologs. The tree was constructed by ClustalW provided by DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/). Segments responding to an evolutionary distance of 0.1 are shown. Accession numbers of the PGI sequences are: *A. fulgidus*, WP_010878991; *A. profundus*, YP_003400960; *A. veneficus,* YP_004342097; *F. placidus*, YP_003435171; *M. fervens*, YP_003128819; *M. jannaschii*, AAB99624; *M. vulcanius*, YP_003247125; *P. aerophilum*, WP_011008072; *P. arsenaticum*, WP_011901579; *P. calidifontis*, WP_011849291; *P. islandicum*, YP_931140; *P. abyssi*, WP_010868864; *P. furiosus*, NP_577925; *P. horikoshii*, NP_143784; *T. barophilus*, YP_004070204; *T. gammatolerans*, YP_002959444; *T. litoralis*, WP_004067944; *T. sibiricus*, YP_002994223; *T. zilligii*, WP_010478801; TK1111, YP_183524.

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T.litoralis     MKYKEPFGVKLDFETGIIENAKKSVRRLSDMKGYFIDEEAWKKMVEEGDPVVYEVYAIEQ   60
P.furiosus       MYKEPFGVKVDFETGIIEGAKKSVRRLSDMEGYFVDERAWKELVEKEDPVVYEVYAVEQ   59
TK1111          MEYKRPIGLDIDLETGVIPGAKKLVRRLSDLKGYFLDEEAYNELL-KEDPVVYEVYAIEQ   59
                  ** * *   * *** *  *** ******  *** ** *        ********* **

                                          ●  ●      ●
                          *               ** *      *  *
T.litoralis     EEKEGDLNFATTVLYPGKVGNEFFMTKGHYHSKIDRAEVYFALKGKGGMLLQTPEGEARF  120
P.furiosus      EEKEGDLNFATTVLYPGKVGKEFFFTKGHFHAKLDRAEVYVALKGKGGMLLQTPEGDAKW  119
TK1111          EEKEGDLNFATTVLYPGKVGKEFFFTKGHFHAKPDRAEIYYGIKGKGGMLLQTPEGEAEW  119
                ******************* *** **** * * **** *   ************* *
                                                  Motif I

                             ●
                             *               *
T.litoralis     IEMEPGTIVYVPPYWAHRTINTGDKPFIFLALYPADAGHDYGTIAEKGFSKIVVEENGKV  180
P.furiosus      ISMEPGTVVYVPPYWAHRTVNIGDEPFIFLAIYPADAGHDYGTIAEKGFSKIVIEENGEV  179
TK1111          IPMGPGTVVYVPPYWAHRTVNTGNEPFIFLAIYPADAGHDYGSIKEKGFSKIVIEEDGEV  179
                * * *** *********** * *  ****** ********** * ******** ** * *
                        Motif II

T.litoralis     VVKDNPKWRM                                                   190
P.furiosus      KVVDNPRWKK                                                   189
TK1111          KVVDNPRWKE                                                   189
                 * *** *
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Fig. 2. Multiple sequence alignment of TK1111 and other two characterized PGIs from family Thermococcaceae. Metal ion binding motif I and II, conserved in cupin-family PGIs, are shown in bold. Active site residues found in the cupin-family PGIs are shown by asterisks at the top. Amino acids involved in metal binding in *P. furiosus* PGI are shown with filled circles at the top. Amino acids identical in all the three sequences are shown by asterisks at the bottom.

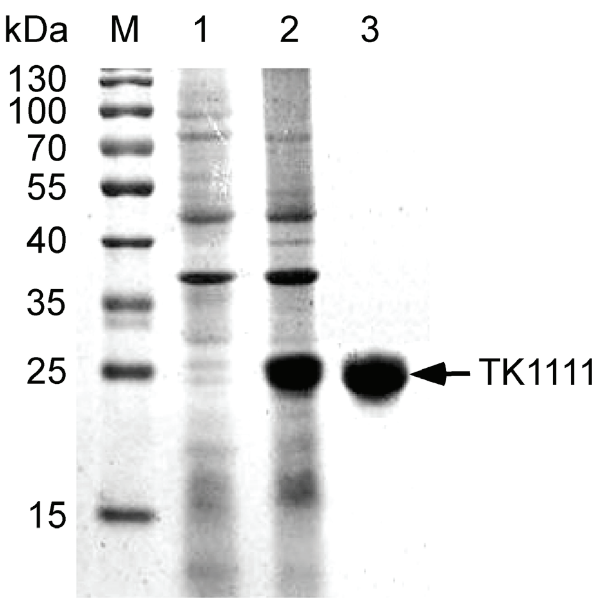

Fig. 3. Coomassie brilliant blue stained 14% SDS-PAGE demonstrating production and purification of recombinant TK1111. Lane M, protein marker (#SM0661, Thermo Scientific); lane 1, lysate of cells containing pET-21a plasmid; lane 2, lysate of cells containing pET-TK1111 plasmid; lane 3, purified TK1111.

Mn^{2+} and Fe^{2+} also enhanced the enzyme activity but to a lesser extent. Enhancement of enzyme activity in the presence of Zn^{2+} is in contrast with PGI from *T. litoralis* where Zn^{2+} is reported to inhibit the enzyme activity [4]. The enzyme activity of PGI from *P. furiosus*, another member of the same family, was not affected in the presence of Zn^{2+}. However, the enzyme activity was remarkably enhanced in the presence of Mn^{2+} [14]. The variable preference of the three PGIs from family Thermococcaceae towards metal ions may be attributed to the variability in the amino acid sequence in motif 1 and 2. It has been shown that the two histidine residues (H^{88} and H^{90}, *P. furiosus* numbering) and a glutamate residue (E^{97}) in motif 1 together with the histidine residue (H^{136}) in motif 2 act as ligands for the binding of an active-site metal ion, such as Fe^{2+}, Mn^{2+}, or Zn^{2+} [14, 16]. These residues are completely conserved in all the three characterized PGIs from family Thermococcaceae. However, residues after E^{97} in motif 1 are quite different in all the three PGIs which may contribute to metal preference (Fig. 2).

Presence of EDTA in the reaction mixture or measurement of enzyme activity after dialysis against 5 mM EDTA reduced the enzyme activity of TK1111. The enzyme activity was completely diminished when TK1111 was heated at 70 °C in the presence of 10 mM EDTA. Complete restoration of the enzyme activity was observed when TK1111, after EDTA treatment and extensive dialysis, was incubated at room temperature in the presence of Zn^{2+}. Mn^{2+} and Fe^{2+} were also able to restore the activity but to a lesser extent (Fig. 5).

Kinetic parameters

TK1111 displayed a specific activity of 43 U/mg under optimal conditions, 90 °C and pH 6.0, when fructose

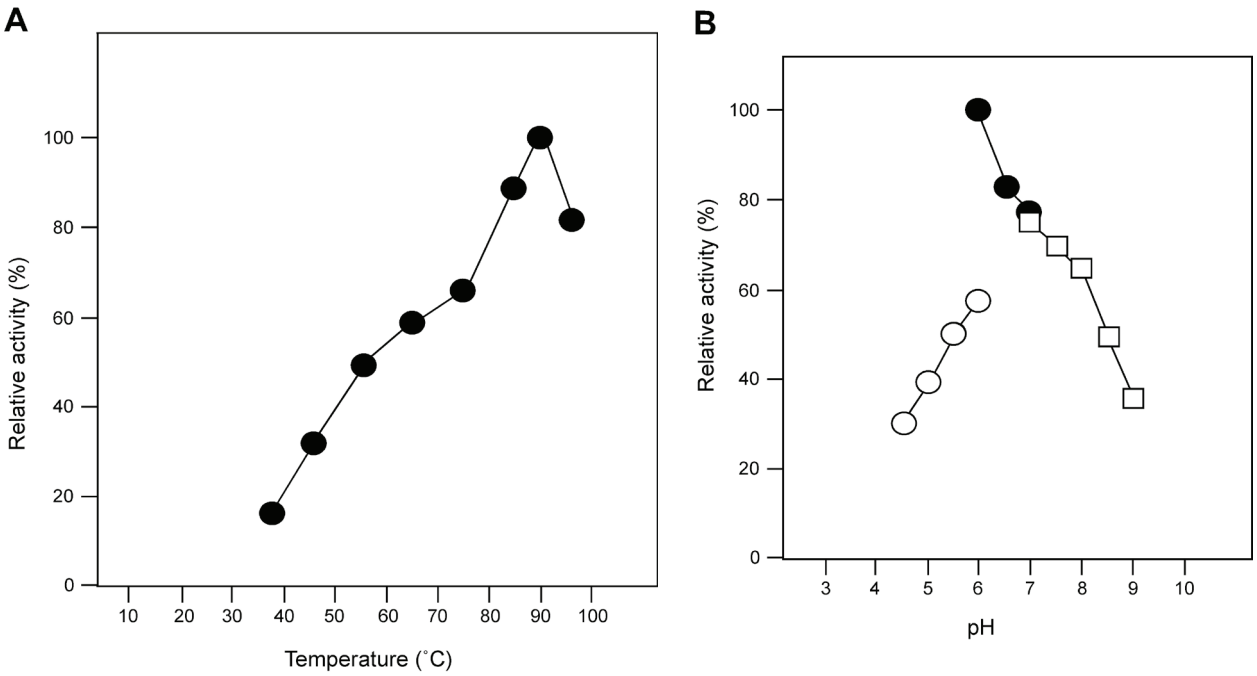

Fig. 4. Effect of temperature and pH on TK1111 enzyme activity. A) Optimal temperature of the enzyme activity. Assays were performed at various temperatures ranging from 37 to 95 ºC under standard conditions. B) Optimal pH for TK1111 enzyme activity. Assays were conducted in various buffers at 50 ºC. Buffers used were: acetate buffer, open circles; phosphate buffer, closed circles; Tris-Cl buffer, open squares.

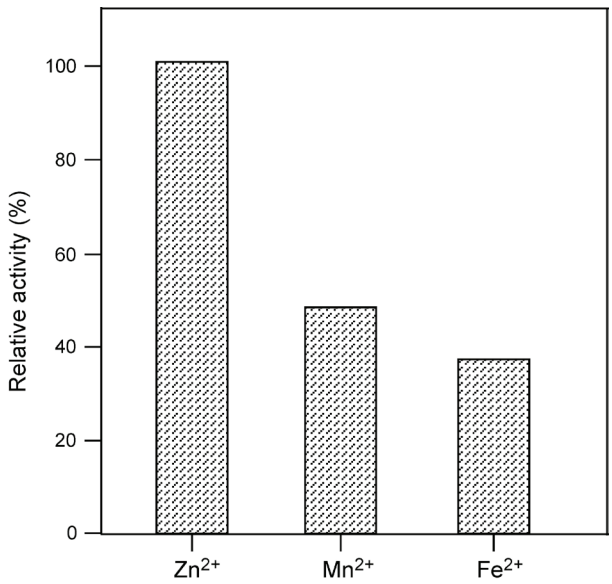

Fig. 5. Effect of metal ions on the restoration of TK1111 enzyme activity. TK1111 was heated at 70 ºC in the presence of 10 mM EDTA for 10 min and dialyzed extensively to remove the EDTA bound metal ions. Enzyme activity was measured after addition of 200 µM $ZnCl_2$ or $MnCl_2$ or $FeCl_2$.

6-phosphate was used as substrate. The enzyme activity was 6-times lower at 37 °C which is quite similar with PGI from *P. furiosus* [3]. K_m and V_{max} values of TK1111 towards fructose 6-phosphate, determined from Lineweaver-Burke plot, were 2.2 mM and 12.5 µmol min^{-1} mg^{-1}, respectively at 37 °C. k_{cat}, calculated on the basis of monomeric form of TK1111, was found to be 5 s^{-1} and k_{cat}/K_m value was 2.27 mM^{-1} s^{-1} at 37 °C. k_{cat} value at 90 °C was 28 s^{-1}. Kinetic data cannot be compared with other members of the family Thermococcaceae as analyses were performed at different temperatures.

In summary, TK1111 belongs to the cupin-family of PGIs and is the only PGI from this family that exhibits highest activity in the acidic pH. Enzyme activity of TK1111 was remarkably enhanced in the presence of Zn^{2+} which is in contrast to the other PGIs characterized from this family. Further characterization, particularly elucidation and stereochemistry of the reaction catalyzed by TK1111, is in progress.

Conflict of Interest: There is no conflict of interest between the authors.

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