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Highly Thermostable L-Threonine Dehydrogenase from the Hyperthermophilic Archaeon *Thermococcus kodakaraensis*

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L-Threonine dehydrogenase, a key enzyme in the L-threonine metabolism, catalyses the NAD^+-dependent conversion of L-threonine to 2-amino-3-ketobutyrate, that non-enzymically decarboxylates to aminoacetone. A search of the genome sequence of hyperthermophilic archaeon, *Thermococcus kodakaraensis* revealed the presence of a closely related orthologue (TK0916) of archaeal and bacterial L-threonine dehydrogenase genes. Expression in *Escherichia coli*, purification and characterization of the TK0916 gene product revealed that this gene actually coded for a protein with high levels of L-threonine dehydrogenase activity (7.26 U mg^{-1}). The enzyme exhibited highest activity at pH 12 and 90°C. The K_m values for L-threonine and NAD^+ at 50°C were 1.6 mM and 0.028 mM, respectively. The enzyme activity was dependent on divalent cations. The half-life of the enzyme was more than 2 h at 85°C and 24 min in boiling water. This is the most thermostable threonine dehydrogenase exhibiting optimal activity at the highest pH (12) reported to date. This is the first report on the characterization of a TDH from genus *Thermococcus*.

Key words: hyperthermophilic archaea, thermostable L-threonine dehydrogenase, *Thermococcus kodakaraensis*.

Abbreviations:

L-Threonine is an indispensable amino acid and L-threonine dehydrogenase (TDH; EC 1.1.1.103) is a key enzyme involved in threonine metabolism in both prokaryotes and eukaryotes. Under normal conditions, threonine is synthesized by microbes from oxaloacetate via aspartate, aspartate-4-phosphate, aspartate semialdehyde, homoserine and homoserine phosphate, which is finally converted to threonine in a reaction catalysed by threonine synthase. Threonine degradation occurs by two major pathways. Threonine is either converted by TDH to 2-amino-3-ketobutyrate which by the action of 2-amino-3-ketobutyrate CoA ligase produces glycine and acetyl CoA. Alternatively, L-serine/threonine dehydratase (EC 4.2.1.16) converts threonine to NH_4^+ and 2-ketobutyrate and the latter is further metabolized by way of acetyl CoA. TDH is recognized as the major route for threonine utilization in both prokaryotes (*1*, *2*) and eukaryotes (*3*). This pathway accounts for 87% of the threonine catabolized in rat liver (*4*), and L-threonine dehydrogenase activity is the only threonine catabolic reaction that is detected in chicken liver extracts (*5*). The reaction catalysed by L-threonine dehydrogenase makes it possible for some strains of *Escherichia coli* and *Pseudomonas aeruginosa* to utilize threonine as the sole carbon and energy sources (*6–8*).

TDH has been reported from all the three kingdoms of life i.e., eucarya, bacteria and archaea. It has been identified in *E. coli* K-12 (*1*), a psychrophilic bacterium *Cytophaga* sp. KUC-1 (*9*), *Clostridium sticklandii* (*10*), chicken liver mitochondria (*5*), goat liver mitochondria (*11*), porcine liver mitochondria (*12*) and hyperthermophilic archaea *Pyrococcus horikoshii* OT3 (*13–15*) and *Pyrococcus furiosus* (*16*). However, only the enzymes from *E. coli*, *Cytophaga* sp. and *P. horikoshii* have been extensively characterized, and their primary structures have been determined (*9*, *13–15*, *17*). *Thermococcus kodakaraensis* KOD1 is a hyperthermophilic archaeon isolated from a solfatara on Kodakara Island, Kagoshima, Japan (*18*, *19*). The strain is an obligate anaerobe and grows optimally at 85°C. The complete genome sequence of *T. kodakaraensis* has been determined and annotated (*20*). We are interested in the metabolism involving the interconversion of threonine and glycine in *T. kodakaraensis*. An orthologue search revealed that *T. kodakaraensis* contains DNA sequences corresponding to L-threonine dehydrogenase and 2-amino-3-ketobutyrate CoA ligase. We have crystallized the gene product and reported preliminary crystallographic analysis of threonine dehydrogenase (*21*). In the present study, we have examined the enzymatic properties of the gene product, revealing that the enzyme is true L-threonine dehydrogenase in this archaeon.

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MATERIALS AND METHODS

Materials—*T. kodakaraensis* KOD1 was isolated from a solfataric hot spring at a wharf in Kodakara Island, Kagoshima, Japan (*18*, *19*). *E. coli* strain DH5α was used for subcloning of the gene fragments and DNA manipulations. *E. coli* strain BL21 (DE3) CodonPlus-RIL (Stratagene, La Jolla, CA, USA) was used as a host and pET-8c vector (Novagen, Madison, WI, USA) was employed for gene expression. The plasmid DNA pTZ57R/T and restriction enzymes were purchased from Fermentas International Inc. (830 Harrington Court, Burlington, ON L7N 3N4, Canada).

Analyses of Threonine Dehydrogenase Gene from T. kodakaraensis—The complete sequence of the *T. kodakaraensis* genome has been reported (*20*). The TDH homologous gene (*Tk-tdh*) was found by using the BLAST search in the GenBank databases. DNA sequencing was performed using DTCS quick start master mix kit and a Beckman-Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA, USA). Open reading frame search and molecular mass calculations were performed using DNASIS software (Hitachi Software, Yokohama, Japan). Multiple alignment and phylogenetic analysis was performed using the ClustalW program (*22*) provided by DNA Data Bank of Japan (DDBJ). Search for orthologue genes in various genomes was performed with CMR blast at http://tigrblast.tigr.org/cmr-blast/.

Cloning of Tk-tdh—The following set of two oligonucleotide primers was used for amplification of the *Tk-tdh* gene by polymerase chain reaction (PCR). The forward primer, 5′-GAA**CCATGG**CCGAGAAAATGCAG GCT-3′, was designed on the basis of the N-terminal amino-acid sequence of *Tk*-TDH, and a unique *Nco*I restriction site (shown in bold) was introduced. The reverse primer, 5′-GCA**GAATTC**ACCGTGGGAAGTTGG TATCTC-3′, was designed on the basis of the C-terminal amino-acid sequence and a unique *Eco*RI restriction site (shown in bold) was introduced. *Tk-tdh* gene was amplified by PCR using the genomic DNA of *T. kodakaraensis* as a template. The PCR amplified fragment was purified and 350 ng of this ligated with 155 ng of pTZ57R/T cloning vector using T_4 DNA ligase (Fermentas). The resulting plasmid pTZ-*tdh* was used to transform *E. coli* DH5α competent cells. Positive clones were screened by blue-white colony screening and further by colony PCR. The positive clones were further confirmed by isolating and digesting the recombinant plasmid pTZ-*tdh* with specific restriction enzymes. The single cleavage of the recombinant plasmid was performed with *Bam*H1 while double digestion was carried out with *Bam*H1 and *Eco*R1. After confirming the sequence, the *Nco*I–*Eco*RI restriction fragment was inserted into the pET-8c expression vector at the corresponding sites. The resulting plasmid was named as pET-*tdh*.

Expression of Tk-tdh Gene in E. coli—The plasmid pET-*tdh* was used to transform *E. coli* strain BL21(DE3)CodonPlus-RIL which were grown overnight at 37°C in LB medium (tryptone 1%, yeast extract 0.5%, NaCl 0.5%; pH 7.0) containing ampicillin (50 μg/ml). The culture was inoculated (1%) into fresh LB medium (1 l) containing ampicillin (50 μg/ml) and the cultivation was continued until OD_{660} reached 0.5. Heterologous expression of the archaeal gene was induced by the addition 0.2 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for another 4 h at 37°C.

Purification and Molecular Mass Determination—Cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and washed with 50 mM Tris–HCl (pH 8.0). The cell pellet was resuspended in 30 ml of the same buffer, and the cells were then disrupted by sonication in ice water. Soluble and insoluble fractions were separated by centrifugation (15,000 × *g* for 30 min at 4°C). The soluble fraction containing the recombinant *Tk*-TDH was incubated at 85°C for 20 min and centrifuged at 15,000 × *g* for 30 min at 4°C to remove heat-labile proteins from the host *E. coli*. All purification steps were performed at room temperature with columns purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK) unless mentioned otherwise. *Tk*-TDH was precipitated with 70% ammonium sulfate, dissolved in 10 ml of 50 mM Tris–HCl (pH 8.0) and dialysed against 500 ml of 50 mM Tris–HCl buffer pH 8.0 for 4 h with three changes of the same buffer. A 5 ml of the soluble fraction was loaded onto an anion-exchange column (Resource Q) which was equilibrated with 50 mM Tris–HCl buffer pH 8.0. Proteins were eluted with a linear gradient of 0–1.0 M sodium chloride in 50 mM Tris–HCl buffer pH 8.0. Fractions with TDH activity (3.5 ml containing 28 mg protein) were pooled and dialysed against 500 ml of 50 mM Tris–HCl buffer pH 8.0 containing 2 M ammonium sulfate for 4 h with three changes of the same buffer. The dialysed sample was applied to a hydrophobic column (Resource ISO) equilibrated with 50 mM Tris–HCl buffer pH 8.0 containing 2 M ammonium sulfate. The bound proteins were eluted with a linear gradient of 2.0 to 0 M ammonium sulfate in 50 mM Tris–HCl buffer pH 8.0. The protein concentration was determined by the method of Bradford (*23*) using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc. Hercules, CA. USA). Bovine serum albumin was used as the standard.

Apparent molecular mass of the purified protein was estimated by gel filtration on a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 150 mM sodium chloride. The void volume was determined with blue dextran (2000 kDa), and a standard calibration curve was obtained using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and albumin (67 kDa) (Amersham Pharmacia Biotech).

The apparent homogeneity of the protein was examined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (*24*).

Enzyme Assay—The TDH activity was spectrophotometrically assayed by using a Shimadzu UV-160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with a thermostat. The standard reaction mixture was composed of 100 mM glycine/NaOH buffer (pH 12.0), 25 mM L-threonine, 2.5 mM NAD^+, 0.1 mM $ZnCl_2$ and enzyme in a final volume of 1.0 ml. The reaction mixture was incubated at 50°C for 5 min, and the

change in absorbance due to NADH formation was monitored at 340 nm ($\varepsilon = 6.2\,mM^{-1}\,cm^{-1}$). One unit of enzyme is defined as the amount catalysing the formation of 1 μmol NADH per min in the L-threonine oxidation.

Stability, Temperature and pH Optima—To determine the pH stability, the enzyme in buffers of various pH values was incubated at 50°C for 20 min, and the remaining activities were then assayed. The buffers (100 mM each) were an acetate buffer (pH 3.5–6.5), MOPS/NaOH buffer (pH 6.5–8.0), Tris–HCl buffer (pH 8.0–9.0) and glycine/NaOH buffer (pH 9.0–12.5). The optimal pH of the enzyme reaction was determined by running the standard assay at 50°C using 100 mM each of sodium phosphate (pH 6.5–8.0), Tris–HCl buffer (pH 7.5–10) and glycine/NaOH buffer (pH 9–12.5). To determine the thermostability, the enzyme solutions in 10 mM MOPS/NaOH buffer (pH 7.0) were incubated at different temperatures, and the residual activity was determined by the standard assay method. The optimal temperature of the reaction was determined by running the standard assay at temperatures from 35°C to 95°C.

Metal Ion Requirement and Substrate Specificity—To determine the metal ion requirements, the enzyme solution was heated in boiling water for 5 min in the presence of 1 mM EDTA and then passed through PD-10 desalting column (Amersham Biosciences) in order to remove the metal ions and EDTA. The effect of various divalent metal ions on the reactivation of the enzyme activity was examined by incubating the enzyme with 0.1 mM of $MgCl_2$, $CaCl_2$, $ZnCl_2$, $NiCl_2$, $CoCl_2$, $CuCl_2$ or $MnCl_2$ for 5 min at 50°C before assay. The assay was performed as described above.

L-Threonine and L-serine were examined as substrates for *Tk*-TDH activity. The reaction mixture was composed of 100 mM glycine/NaOH (pH 12.0), 25 mM of each substrate, 2.5 mM NAD^+, 0.1 mM $ZnCl_2$ and enzyme in a final volume of 1.0 ml. The assay was performed as describe above.

RESULTS AND DISCUSSION

Phylogenetic and Structural Analysis of Tk-TDH—In the genome sequence of *T. kodakaraensis* a gene coding for L-threonine dehydrogenase (*Tk*-TDH) was found. *Tk*-TDH was composed of 350 amino acids with a calculated molecular weight of 38,101 Da. *Tk*-TDH belongs to the cluster of orthologous group of proteins 1063 and displays high similarity with TDHs from archaea and bacteria. The highest similarity (88% identity) was found with enzymes from *P. furiosus, P. horikoshii* and *P. abyssi* (Table 1). *P. furiosus* and *P. horikoshii* are the only two TDHs characterized from hyperthermophilic archaea. The crystal structure of TDH from *P. horikoshii* has been determined (*25*, *26*). Amino-acid residues Cys^{97}, Cys^{100}, Cys^{103} and Cys^{111} of TDH from *P. horikoshii* coordinate with Zn^{2+} to establish tetrahedral geometry which has been invoked to confer hyperthermostability to the enzyme (*26*). These four residues (Cys^{97}, Cys^{100}, Cys^{103} and Cys^{111}) are conserved in *Tk*-TDH and may also be involved in the binding of the structural zinc. But the motif, ^{97}CGKCYACKHNRYHVC^{111} (*T. kodakaraensis* sequence and numbering), in which these cysteine residues are present shows four differences between *Tk*-TDH and the corresponding enzymes from other hyperthermophiles (Fig. 1). Metal dependent alcohol dehydrogenases have been extensively studied, from biochemical and structural angles, and are known to contain two zinc atoms per subunit of the enzyme. One of these is the structural zinc and the other at the active site involved in catalysis (*27*, *28*). The chemical analysis and electron density of the *P. horikoshii* TDH shows only the structural zinc mentioned above. However, the comparison of the crystal structure of *P. horikoshii* TDH with those of alcohol dehydrogenases shows the presence, at the equivalent position of, Cys^{42}, His^{67}, Glu^{68} and Glu^{152}, which could be involved in the binding of a low-affinity catalytic zinc. These residues are conserved in *Tk*-TDH at identical positions. In TDH from *P. horikoshii*, Glu^{92}, His^{94}, Glu^{152} and Arg^{294} have been identified to be involved in substrate binding, these residues are conserved in *Tk*-TDH at the equivalent positions. In TDH from *P. horikoshii* the highly conserved sequence motif, GxGxxG (residues 175–180) together with Glu^{199} and Arg^{204}—constituting the Rossmann fold for the binding of NAD(H)—were found near the active site cleft. These amino acids, GAGPLG (175–180) and Glu^{199} plus Arg^{204} are conserved in *Tk*-TDH and could also be assigned the coenzyme binding role (Fig. 1).

Tk-TDH displayed medium homology with its bacterial counterparts (*Bacillus licheniformis* 52%; *Thermus thermophilus* 51%; *Xanthomonas oryzae* 45%; *E. coli* 43% and *Salmonella typhimurium* 42%) whereas a very low similarity was found with eukaryal counterparts (Table 1). These TDHs belong to alcohol dehydrogenase superfamily that can be divided into three groups, zinc-dependent long-chain (~350 amino acids), zinc-independent short-chain (~250 amino acids) and iron-activated (~385 amino acids) (*29*). *Tk*-TDH sequence was compared

Table 1. ***Tk-tdh* orthologue genes found in other organisms.**

Source	Accession number	Identity (%)
Pyrococcus furiosus	AAL81115	87.64
Pyrococcus horikoshii	BAA29746	87.37
Pyrococcus abyssi	CAB50292	87.64
Thermoanaerobacter ethanolicus	ZP_00778486	51.72
Bacillus licheniformis	AAU23458	51.16
Thermus thermophilus	YP_004176	50.44
Xanthomonas oryzae	BAE70235	45.00
Yersinia pseudotuberculosis	YP_068606	43.11
Escherichia coli	P07913	42.82
Salmonella typhimurium	NP_462608	41.94
Streptomyces coelicolor	CAB71246	41.81
Canis familiaris	XP_534558	14.00
Aedes aegypti	EAT45305	13.14
Sus scrofa	NP_999169	12.57
Danio rerio	AAH63962	12.57
Mus musculus	NP_067455	12.00
Gallus gallus	XP_420039	11.71
Drosophila melanogaster	AAF51607	11.17
Takifugu rubripes	AAL89661	11.14

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P.furiosus          MSEKMVAIMKTKPEYGAELVEVDVPKPGPGEVLIKILATSICGTDLHIYEWNEWAQTRIR   60
P.horikoshii        MSEKMVAIMKTKPGYGAELVEVDVPKPGPGEVLIKVLATSICGTDLHIYEWNEWAQSRIK   60
T.kodakaraensis     MAEKMQAIMKTKPAYGAELVEVDVPKPGPGEVLIKVLATSICGTDLHIYEWNEWAQSRIK   60
                    * *** ******* ********************* ******************* **

P.furiosus          PPQIMGHEVAGEVVEVGPGVEGIEVGDYVSVETHIVCGKCYACKRGQYHVCQNTKIFGVD   120
P.horikoshii        PPQIMGHEVAGEVVEIGPGVEGIEVGDYVSVETHIVCGKCYACRRGQYHVCQNTKIFGVD   120
T.kodakaraensis     PPQIMGHEVAGEVVEVGPGVEDLQVGDYISVETHIVCGKCYACKHNRYHVCQNTKIFGVD   120
                    *************** *****    **** **************    **************

P.furiosus          TDGVFAEYAVVPAQNVWKNPKNIPPEYATLQEPLGNAVDTVLAGPIAGKSVLITGAGPLG   180
P.horikoshii        TDGVFAEYAVVPAQNIWKNPKSIPPEYATLQEPLGNAVDTVLAGPISGKSVLITGAGPLG   180
T.kodakaraensis     MDGVFAHYAIVPAKNAWKNPKDMPPEYAALQEPLGNAVDTVLAGPIAGRSTLITGAGPLG   180
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P.furiosus          LLGIAVAKASGAYPVIVSEPSEFRRNLAKKVGADYVINPFEEDVVKEVMDITDGNGVDVF   240
P.horikoshii        LLGIAVAKASGAYPVIVSEPSDFRRELAKKVGADYVINPFEEDVVKEVMDITDGNGVDVF   240
T.kodakaraensis     LLGIAVAKASGAYPVIVSEPSEFRRKLAKKVGADYVVNPFEEDPVKFVMDITDGAGVEVF   240
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P.furiosus          LEFSGAPKALEQGLQAVTPAGRVSLLGLFPGKVSIDFNNLIIFKALTVYGITGRHLWETW   300
P.horikoshii        LEFSGAPKALEQGLQAVTPAGRVSLLGLYPGKVTIDFNNLIIFKALTIYGITGRHLWETW   300
T.kodakaraensis     LEFSGAPKALEQGLKAVTPGGRVSLLGLFPREVTIDFNNLIIFKALEVHGITGRHLWETW   300
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P.furiosus          YTVSRLLQSGKLNIDPIITHKYKGFDKYEEAFELMRAGKTGKVVFMLK--             348
P.horikoshii        YTVSRLLQSGKLNLDPIITHKYKGFDKYEEAFELMRAGKTGKVVFMLK--             348
T.kodakaraensis     YTVSSLIQSGKLNLDPIITHKYKGFDKFEEAFELMRAGKTGKVVFFPHKG             350
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Fig. 1. **Primary structure comparison of the hyperthermophilic TDHs.** The asterisks beneath the sequences indicate the identical amino-acid residues in all the three proteins. The organism names are indicated on the left, while the residue numbers on the right. NAD^+-binding motif GXGXXG is shown in the box. The multiple-sequence alignment was performed by using the ClustalW program provided by DNA data bank of Japan (DDBJ).

with TDHs sequences available in various databases and an unrooted phylogenetic tree of threonine dehydrogenases from various sources (archaea, bacteria and eukaryotes) was constructed with the neighbourhood joining method (Fig. 2). Phylogenetic tree demonstrated two major groups. One comprising of archaeal and bacterial TDHs with 340–350 amino-acid residues and the other consisting of eukaryotic TDHs having 368–403 amino acids. The analysis revealed that *Tk*-TDH was clustered with threonine dehydrogenases from hyperthermophilic archaea. Several archaeal proteins are more similar to the eukaryotic proteins in their primary structure (*30–32*). Archaeal threonine dehydrogenases displayed more similarity to bacterial counterparts instead of eukaryotic ones indicating a distant relationship between the archaeal and eukaryal threonine dehydrogenases.

Production in E. coli and Purification of Recombinant Tk-TDH—Induction with IPTG resulted in the expression of *Tk-tdh* in *E. coli*. Disruption of cells and analysis of the cell lysate revealed that *Tk*-TDH was produced in soluble form. Heat treatment of the soluble fraction removed most of the host proteins and provided about 80% pure recombinant *Tk*-TDH protein (Fig. 3). In addition to partial purification, heat treatment doubled the enzyme activity of *Tk*-TDH (data not shown) indicating that it has a role in proper folding of the recombinant enzyme. It has been reported that high temperature plays an important role for proper folding and oligomerization of glutamate dehydrogenase from *T. kodakaraensis* (*33*). Further purification was achieved by anion exchange and hydrophobic interaction column chromatographies. Anion exchange column chromatography removed nucleic acids as well as certain proteins, and the hydrophobic interaction column chromatography provided the purified recombinant protein. The purified enzyme showed a single band on 12% SDS–PAGE (Fig. 3) with a molecular weight of 38 kDa which is in fair agreement with the molecular weight calculated from amino-acid sequence (38,101 Da). The subunit composition of the purified enzyme was investigated by gel filtration chromatography. The molecular mass of *Tk*-TDH was estimated to be 154 kDa by this technique which shows that the enzyme exists in a homotetrameric form similar to threonine dehydrogenases from *E. coli*, *Cytophaga* sp., *C. sticklandii*, *P. horikoshii* and *P. furiosus* (*1*, *9*, *10*, *13*, *16*). It is known that the porcine liver enzyme exists in a dimeric form (*12*) while those from goat liver and chicken liver are monomeric (*5*, *11*).

Temperature and pH Profile of Tk-TDH—The effect of temperature on the enzyme activity was examined from 35°C to 95°C. The enzyme showed high thermostability with a maximum activity at 90°C (Fig. 4A). The enzyme showed a half life of 2 h at 85°C and 24 min in boiling water (Fig. 4C). The thermostability of L-threonine dehydrogenase has been reported for enzymes from *Staphylococcus aureus* (*34*), *Cytophaga* sp. (*9*), *P. horikoshii* (*13*) and *P. furiosus* (*16*). The first two enzymes were rapidly inactivated above 50°C whereas the enzymes from *P. horikoshii* and *P. furiosus* exhibited optimum activity at 70°C and 100°C, respectively. The half-life of TDH from *P. furiosus* was 36 min at 90°C

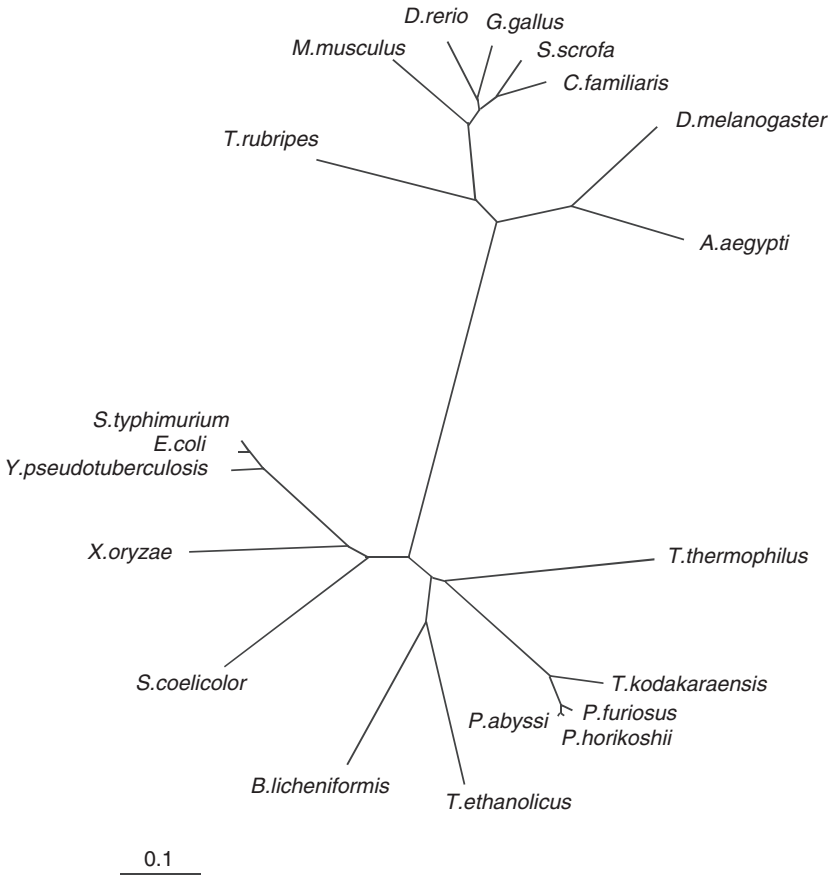

Fig. 2. **Phylogenetic tree for threonine dehydrogenases (TDHs) constructed by the neighbour joining method.** The tree was constructed by the CLUSTAL W programme provided by DDBJ. Segments corresponding to an evolutionary distance of 0.1 are shown. Each name at the terminus represents the species from which the protein originated.

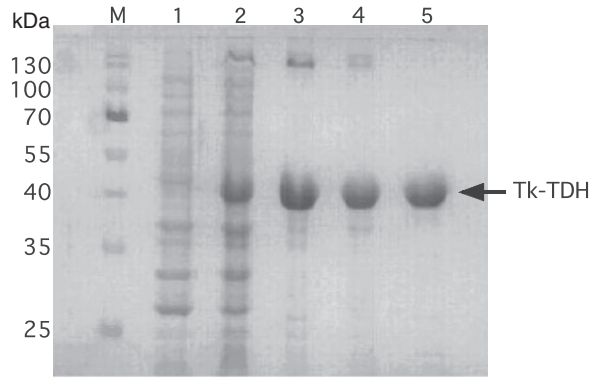

Fig. 3. **SDS–PAGE of purified recombinant *Tk*-TDH.** Lane M, molecular mass marker; lane 1, cell lysate from host cells containing the pET-8c vector only; lane 2, cell lysate from host cells containing the *Tk-tdh* gene; lane 3, soluble fraction after heat treatment; lane 4, eluate after anion exchange column chromatography with Res Q; lane 5, eluate after hydrophobic interaction column chromatography with Res ISO.

and 11 min at 100°C. Thermostability comparison shows that *Tk*-TDH is the most thermostable enzyme among the L-threonine dehydrogenases described to date. An Arrhenius plot was constructed and is shown in Fig. 4B. The enzyme displayed a linear increase in activity from 45 to 90°C. The activation energy was calculated to be 62 kJ/mol.

The enzyme was active over a wide pH range from 6.5 to 12.5 with an optimum pH of 12 at 50°C. The enzyme showed relative activities of 81%, 87%, 91% and 95% at pH 10.5, 11, 11.5 and 12.5, respectively (Fig. 5). L-Threonine dehydrogenases from *P. horikoshii, P. furiosus, E. coli, Cytophaga* sp. and chicken liver have optimum pH values of 10, 10, 10.3, 9.5 and 8.7, respectively. This shows that TDHs from hyperthermophilic archaea exhibit higher pH optima than enzymes from animals and mesophilic organisms. TDH from *T. kodakaraensis* is highly thermostable among the TDHs characterized to-date with the highest optimum pH and works in the widest pH range having ~13% enzyme activity at neutral pH compared to 100% at pH 12.

Effect of Metal Ions on the Activity of Tk-TDH—TDH from animals, mesophilic bacteria as well as hyperthermophiles are known to be metalloproteins requiring zinc for catalysis. TDH from *P. furiosus* (*16*) and *P. horikoshii* (*13*, *15*) are reported to be inactivated when the enzymes were dialysed against 0.1 mM EDTA. In contrast, *Tk*-TDH retained 65% of its original activity after dialysis at 4°C against a buffer containing 1mM EDTA. These results suggest that the binding of Zn^{2+} to the *T. kodakaraensis* enzyme is much stronger than that to

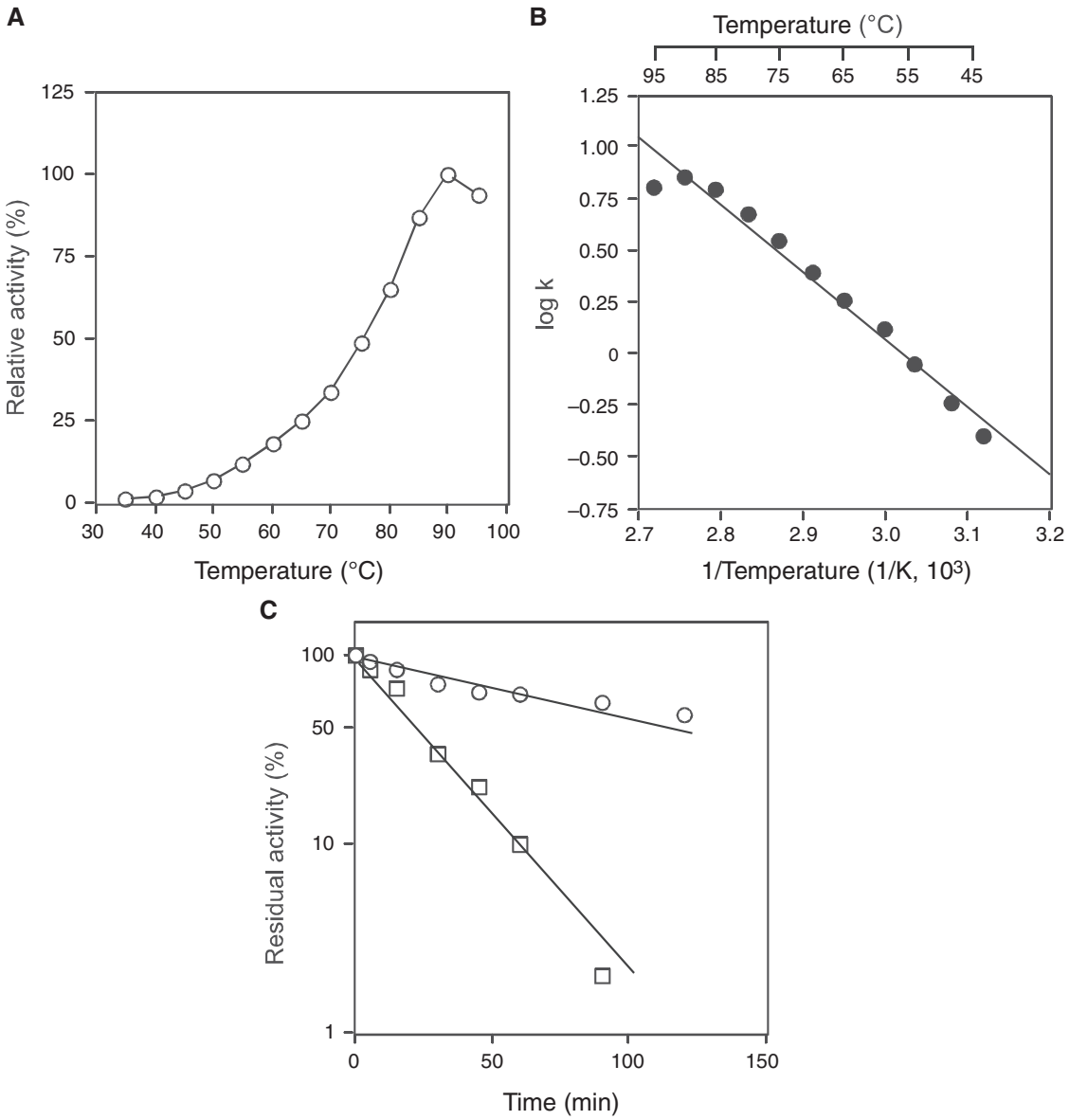

Fig. 4. **Effect of temperature on the enzyme activity and stability of *Tk*-TDH.** (A) Temperature profile of *Tk*-TDH activity. TDH activity was examined at pH 10 at various temperatures in the presence of 0.1 mM $ZnCl_2$ at 50°C as described in MATERIALS AND METHODS section. (B) Arrhenius plot (log *k* versus reciprocal absolute temperature) of *Tk*-TDH. The rate constant *k* is expressed in mol l^{-1} min^{-1} and the temperature in kelvin. (C) Effect of temperature on the stability. *Tk*-TDH was heated in MOPS/NaOH buffer (pH 7.0) at 85°C (circles) and 100°C (squares) for various times and the residual activity was examined at 50°C.

P. horikoshii and *P. furiosus* enzymes. The four zinc-binding cysteine residues are conserved in all the three hyperthermophilic threonine dehydrogenases, while the domain, from residue 97 to 111, containing the cysteines, shows one difference between the enzymes from *P. horikoshii* and *P. furiosus* but four when compared with *Tk*-TDH. This difference may have some role in the tight binding of Zn^{2+} in *Tk*-TDH. The activity was, however, lost when *Tk*-TDH was heated at 60°C for 40 min and in boiling water for 5 min in the presence of 1 mM EDTA. There was no decrease in enzyme activity when the protein was heated at 60°C for 40 min in the absence of EDTA. The apoenzyme thus obtained regained 75% of the original activity when reconstituted with 0.1 mM Zn^{2+} (Fig. 6A). Cu^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+} and Mg^{2+} could replace Zn^{2+} with a 63%, 54%, 40%, 38%, 30% and 27% reactivation, respectively. These results indicate that *Tk*-TDH activity is metal ion dependent similar to TDHs from *P. horiloshii*, *P. furiosus* and *E. coli*. It should though be noted that L-threonine dehydrogenase from *Cytophaga* sp. does not require any metal ion for its activity (*9*).

As the inactivated *Tk*-TDH could regain only 75% of its original activity with the addition of 0.1 mM Zn^{2+} we therefore examined the effect of various concentrations of Zn^{2+} on the reactivation of *Tk*-TDH and found that

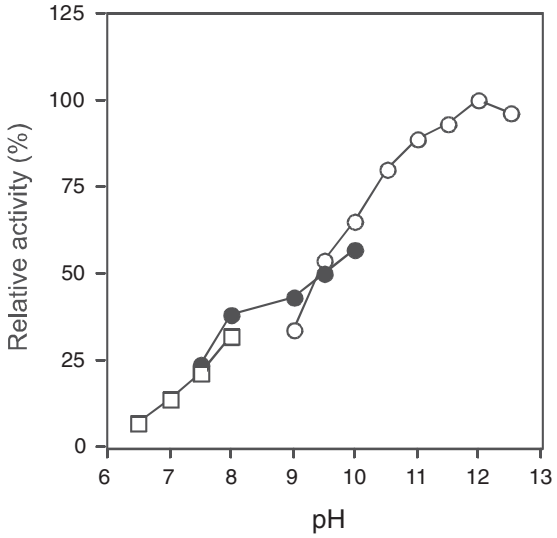

Fig. 5. **Effect of pH on TDH activity of *Tk*-TDH.** Effect of pH was studied using the buffers described in the MATERIALS AND METHODS section. Activity was examined in the presence of 0.1 mM $ZnCl_2$ at 50°C. Symbols: squares, sodium phosphate buffer; colsed circles, Tris-HCl buffer; open circles, glycine/NaOH buffer.

0.05 mM Zn^{2+} exhibited a highest reactivation (100%) of *Tk*-TDH (Fig. 6B). We have experienced a slight precipitation of the enzyme at higher concentrations of Zn^{2+} which might have been responsible for the reduction in the enzyme activity.

Substrate Specificity of Tk-TDH—The enzyme activities, using L-threonine and L-serine as substrates for *Tk*-TDH, were examined. The enzyme showed activity towards L-threonine (7.26 U/mg) and L-serine (0.98 U/mg) like those from *P. horikoshii and E. coli*. L-Threonine dehydrogenases from *Cytophaga* sp., *C. sticklandii* and chicken liver do not utilize L-serine as substrate (*5*, *9*, *10*).

K_m Values for L-Threonine and NAD$^+$—The K_m values for L-threonine and NAD^+ were determined to be 1.6 mM and 0.028 mM respectively at 50°C. K_m values for L-threonine and NAD^+ from *P. horikoshii* are 0.2 mM and 0.024 mM at 50°C; from *P. furiosus* are 1.5 mM and 0.055 mM; from *E. coli* are 1.43 mM and 0.19 mM; from *C. sticklandii* are 18 mM and 0.1 mM; from goat liver are 5.5 mM and 1 mM, from chicken liver are 8.4 mM and 0.98 mM, from rat liver are 10.6 mM and 0.08 mM and porcine liver are 5 mM and 0.1 mM. Among the three kingdoms of life the archaeal and bacterial TDHs seem to have higher affinity towards the substrate compared to their eucaryal counterparts.

At present we cannot predict the fate of intermediate 2-amino-3-ketobutyrate formed by the enzymatic oxidation of threonine in *T. kodakaraensis*. A homologue of 2-amino-3-ketobutyrate CoA ligase has been found on the genome of *T. kodakaraensis* and there is a probability that 2-amino-3-ketobutyrate may be converted into glycine in the cell by 2-amino-3-ketobutyrate CoA ligase. However, 2-amino-3-ketobutyrate is a thermolabile compound and it is interesting to know how it survives at high temperature. There is a possibility that this unstable intermediate is directly transferred from the active site of one enzyme to that of the other, rather than the transfer occurring via the solution.

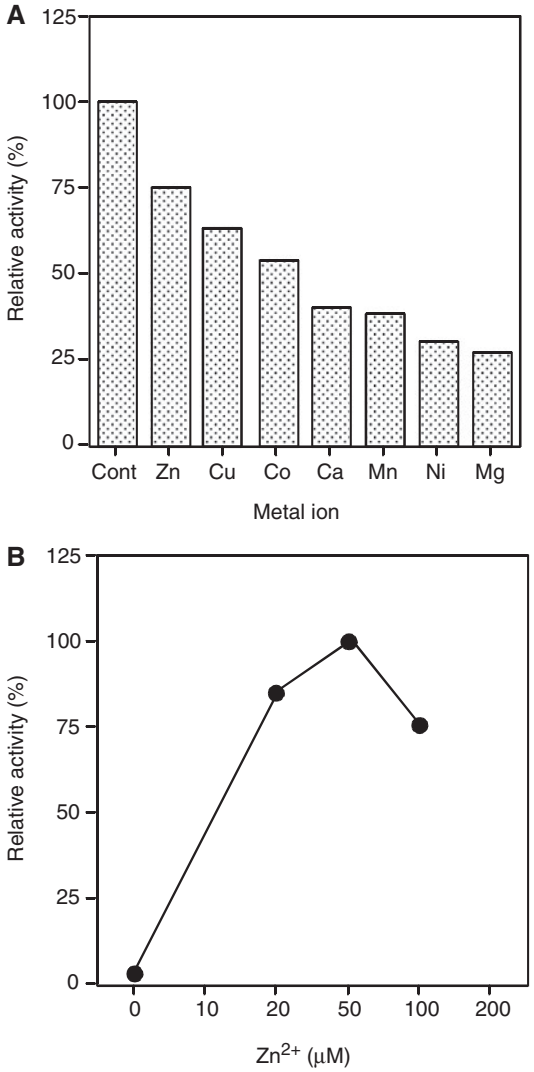

Fig. 6. **Effect of metal cations on the reactivation of *Tk*-TDH.** (A) Effect of various metal cations. The enzyme was heated in boiling water in the presence of 1 mM EDTA for 5 min. The inactivated enzyme was reactivated with the addition of various metal cations. A chloride salt of each metal cation was used and the activity was examined at 50°C. All the salts were added at a final concentration of 0.1 mM. (B) Effect of various concentrations of $ZnCl_2$ on the reactivation of *Tk*-TDH.

CONFLICT OF INTEREST

None declared.

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