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Gene Cloning and Characterization of TK1392, an NADH oxidase from *Thermococcus kodakaraensis* with a distinct C-terminal domain

[Farklı C-terminalli *Thermococcus kodakaraensis*'e ait TK1392, NADH Oksidaz'ın Genetik Klonlanması ve Karakterizasyonu]

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ABSTRACT

Aim: To clone NADH oxidase from *Thermococcus kodakaraensis* and express in *Escherichia coli*, purify the gene product and characterize the enzyme.

Methods: The gene encoding an NADH oxidase was cloned and expressed using *E. coli* expression system. The recombinant protein was purified by heat treatment and ion exchange column chromatography, and characterized.

Results: Nicotinamide adenine dinucleotide oxidase homologs have been found in the genomes of hyperthermophilic archea, including three in *Thermococcus kodakaraensis* KOD1 which have been designated as TK0304, TK1299 and TK1392. We have characterized TK1392 gene and its product. TK1392 gene consisted of 1239 nucleotides, corresponding to a polypeptide of 413 amino acids with a calculated molecular weight of 45,244 Da and an isoelectric point of 8.73. The amino acid sequence of TK1392 protein was compared with its homologs from other microorganisms and a phylogenetic tree was constructed. To examine the molecular properties of NADH oxidase, the structural gene encoding TK1392 was cloned, expressed and the gene product was characterized. Molecular weight of the recombinant protein was 45 kDa when determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and 90 kDa when analyzed by gel filtration chromatography. The enzyme exhibited a pH and temperature optima of 7.0 and 75°C, respectively. The recombinant enzyme displayed a K_m value of 35±3 μM toward NADH and a specific activity of 19.3 U mg^{-1}.

Conclusion: Recombinant TK1392 is a true NADH oxidase and exists in a dimeric form. The enzyme is thermostable and possesses high NADH oxidase activity.

The authors state that there is no conflict of interest.

Key words: *Thermococcus kodakaraensis*; NADH oxidase; flavoenzyme; cloning; thermostable; hyperthermophile.

ÖZET

Amaç: Çalışmanın amacı, *Thermococcus kodakaraensis*'den bir NADH oksidazın klonlanması ve *Escherichia coli* de ekspresyonu sonrası gen ürününün saflaştırılması ve ürün enziminin karakterize edilmesidir.

Metotlar: NADH oksidaz geni klonlamış ve *E. coli* ekspresyon sisteminde eksprese edilmiştir. Rekombinant protein, ısı muamelesi ve iyon-değiştirici kolon kromatografisi vasıtasıyla saflaştırılmış ve karakterize edilmiştir.

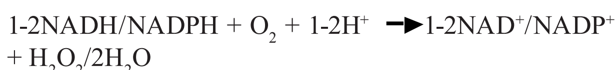
Bulgular: Nikotinamit adenine dinükleotit oksidaz homologları, hipertermofilik arkea genomları içinde bulunmaktadır. Bu genomlar, TK0304, TK1299 ve TK1392 şeklinde tasarlanmış üç sekans halinde *Thermococcus kodakaraensis* KOD1 içinde bulunmaktadırlar. Biz bu çalışmada, TK1392 genini ve ürününü karakterize etmiş bulunmaktayız. TK1392 geni 1239 nükleotitden meydana gelmekte ve bu sekans, 413 amino asit zincirinden oluşan, molekül ağırlığı 45,244 Da olan bir polipeptit zincirini oluşturmaktadır. Proteinin izoelektrik noktası 8.73 bulunmuştur. TK1392 proteininin amino asit sekansı, diğer mikroorganizmalarla ve oluşturulmuş bir filogenetik ağaç içindeki homologlarıyla karşılaştırılmıştır. NADH oksidazın moleküler özelliklerinin araştırılması için TK1392 geni klonlanmış ve gen ürünü eksprese edilmiştir. Rekombinant proteinin molekül ağırlığı SDS-PAGE ile 45 kDa ve jel filtrasyon kromatografisi ile 90 kDa olarak bulunmuştur. Enzimin optimum pH'sı 7.0 ve optimum aktivite derecesi 75°C olarak bulunmuştur. Rekombinant enzimin NADH'a karşı K_m değeri 35±3 μM ve spesifik aktivitesi 19.3 U mg^{-1} olarak bulunmuştur.

Sonuçlar: Rekombinant TK1392, gerçek bir NADH oksidazdır ve dimerik formda bulunmaktadır. Enzim termostabildir ve yüksek bir NADH oksidaz aktivitesi göstermektedir.

Anahtar Kelimeler: *Thermococcus kodakaraensis*; NADH oksidaz; flavoenzim; klonlama; termostabil; hipertermofil.

Introduction

Maintaining the cellular redox balance is a basic requirement for living cells to sustain metabolism and growth. The intracellular redox state is mainly dependent on the intracellular concentration ratios of oxidized and reduced form of nicotinamide adenine dinucleotide. NADH/NADPH oxidase, a flavoenzyme, catalyzes the oxidation of NADH/NADPH, with the consumption of oxygen, to NAD^+/$NADP^+$ and hydrogen peroxide/water.



$$1\text{-}2NADH/NADPH + O_2 + 1\text{-}2H^+ \rightarrow 1\text{-}2NAD^+/NADP^+ + H_2O_2/2H_2O$$

They are believed to play an important role in oxidative stress defense and in the regeneration of oxidized pyridine nucleotides. Homologs of NADH/NADPH oxidase have been found in several archaea including *Pyrococcus horikoshii* [1], *Pyrococcus* furiosus [2], *Thermococcus profundus* [3], *Archaeoglobus fulgidus* [4], and *Methanocaldococcus jannaschii* [5]. This was surprising because most of these organisms are strict anaerobes, a class of organisms that have not been expected to possess NADH oxidases.

Thermococcus kodakaraensis KOD1 is a hyperthermophilic archaeon isolated from a solfatara on Kodakara Island, Kagoshima, Japan [6, 7]. The strain is a strict anaerobe and grows optimally at 85°C on a variety of organic substrates including starch, pyruvate, amino acids, and peptides [7, 8]. The genome sequence of *T. kodakaraensis* has been determined and annotated [9]. Like the genomes of other hyperthermophilic archaea, *T. kodakaraensis* genome harbors more than one, in fact three (TK0304, TK1299, and TK1392) open reading frames that are annotated as NADH oxidase genes. In the present study, we have examined the enzymatic properties of the gene product of TK1392, revealing that it is a true NADH/NADPH oxidase in this archaeon.

Materials and Methods

Chemicals

Chemicals used in this study were purchased from Sigma (St. Louis, Mo., USA) and were of analytical grade. Restriction endonucleases, DNA extraction kit, ligation kit, protein standards and DNA markers were obtained from Fermentas Life Sciences (Hanover, MD, USA).

Sequence Manipulation

Amino acid sequence analysis, molecular mass and pI calculations were performed by using DNASIS software (Hitachi Software, Tokyo, Japan). Database homology searches were executed by using the Basic Local Alignment Search Tool (BLAST) program [10]. Multiple sequence alignment and phylogenetic analyses were performed by using CLUSTAL W program [11] provided by DNA Data Bank of Japan (DDBJ) at their web site (http://clustalw.ddbj.nig.ac.jp/top-e.html). The matrix type used for multiple sequence alignment was "Gonnet".

Primer Designing

The genome sequence of *T. kodakaraensis* is available under the accession number NC_006624. The gene sequence of TK1392 was obtained from GenBank and restriction map of the gene was constructed using software WEBcutter (http://rna.lundberg.gu.se/cutter2/). Primers for polymerase chain reaction (PCR) were designed using Primer 3.0 (http://primer3.sourceforge.net/) and FastPCR (http://www.biocenter.helsinki.fi/bi/Programs/) softwares. An *Nde*I site was introduced to the 5'-end of the gene in order to clone it in pET-21a expression vector. Melting temperature, GC contents, 3'-end complementarity and potential hairpin formation of the selected primers were checked by Oligonucleotide Properties Calculator available at http://www.unc.edu/~cail/biotool/oligo/.

Cloning of TK1392 gene

For cloning of TK1392 gene, forward (TK1392-N 5-CGATGCCGCAGATACATATGCTCTC) and reverse (TK1392-C 5-GCGGTATATCATGCTCTCACCACCTC) primers were designed based on the DNA sequence of the gene. TK1392 gene was amplified by PCR using TK1392-N and TK1392-C primers as priming strands and genomic DNA of *T. kodakaraensis* as template. The PCR reaction mixture (50 µL) contained: 1 X *Taq* DNA polymerase buffer, 2 mM $MgCl_2$, 2.5 µM dNTPs, 1 pmol of each primer, 0.5 µg template DNA and 5 U *Taq* DNA polymerase. The PCR conditions were: initial denaturation step at 95°C for 3 min; followed by 30 cycles of: denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72°C for 50 s; and a final extension step at 72°C for 10 min. Agarose gel electrophoresis of the PCR amplified product was carried out and the amplified product was eluted from the gel. The purified PCR product was ligated in pTZ57R/T using Rapid DNA Ligation Kit (Fermentas) and *E. coli* DH5α cells were transformed by using the standard calcium chloride method [12]. Transformants were selected by blue white screening. The resulting plasmid was named as pTZ-TK1392. Plasmid DNA was isolated from white transformants and cut with restriction enzymes in order to confirm the size of the insert. The presence of TK1392 gene was confirmed by DNA sequencing using CEQ800 Beckman Coulter sequencing system (Beckman, CA, USA).

Expression of TK1392 gene

For expression, the gene fragment was cleaved from pTZ-TK1392 plasmid utilizing the *Nde*I and *Hin*dIII restriction enzymes and ligated in pET-21a expression vector digested with the same two restriction enzymes. The resulting plasmid was named as pET-TK1392. This recombinant plasmid was used for the expression of TK1392 gene in *E. coli* BL21 (DE3). Cells carrying recombinant vector were grown overnight at 37°C in LB medium containing ampicillin (100 µg/mL). The precul-

ture was inoculated (1%) into fresh LB medium containing ampicillin and cultivation was continued until the optical density at 660 nm reached 0.4. The gene expression was induced with 0.2 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation was continued for another 4.5 h at 37°C. Cells were harvested by centrifugation at 5,000 × g for 10 min and washed with 50 mM Tris-HCl buffer (pH 8.0). The cell pellet was resuspended in the same buffer, and the cells were disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation at 12,000 × g for 15 min. The soluble fraction was heated at 80 °C for 30 min and centrifuged. The resulting supernatant was subjected to anion exchange chromatography by using Akta Purifier FPLC system (GE Healthcare, NJ, USA). The anion exchange column (Resource Q) was equilibrated with 50 mM Tris-HCl pH 8.0 and the crude enzyme preparation was loaded onto the column. Proteins were eluted by a linear gradient of 0 to 1 M NaCl in 50 mM Tris-HCl pH 8.0. Fractions containing TK1392 were collected and dialyzed against 50 mM Tris-HCl pH 8.0. Protein pattern analysis was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Protein contents of the samples were estimated by the Bradford assay using bovine serum albumin as a standard. Absorbance was taken at 595 nm after incubating the samples with Bradford reagent for 10 min at room temperature [13].

Molecular mass of the purified protein was determined by gel filtration chromatography using Superdex 200 10/300 GL column (GE Healthcare). A standard curve between molecular mass and elution volume was made by using ferritin (440 kDa), lactate dehydrogenase (140 kDa), human serum albumin (69 kDa), xylanase (38 kDa), myoglobin (17 kDa) and α–lactalbumin (14 kDa) as standard markers.

Enzyme activity assay

NADH oxidase activity of TK1392 was assayed spectrophotometrically by using a Shimadzu UV-160A spectrophotometer equipped with a thermostat. The standard assay mixture consisted of 50 mM Tris-HCl pH 7.5, 0.4 mM NADH and TK1392 reconstituted with 100 μM FAD in a final volume of 1.0 mL. The reaction mixture was incubated at 60°C. The oxidation of NADH was monitored at 340 nm by recording the decrease in absorbance at 340 nm for 5 min. A control experiment contained all the contents except for TK1392. The decrease in absorbance in the control was subtracted from the experimental value. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol NADH in 1 min. The activity was expressed as a percentage of the maximum value.

Effect of temperature and pH on the enzyme activity

Optimal temperature for TK1392 enzyme activity was determined by incubating the standard reaction mixture at various temperatures ranging from 50°C to 90°C in 50 mM Tris-HCl pH 8.0. In order to determine the thermostability of TK1392, the recombinant enzyme, in 50 mM Tris-HCl buffer (pH 8.0), was incubated at 75, 80, 85 and 90°C for various intervals of time, and the residual activity was determined by the standard assay method at 60°C.

Optimum pH for the NADH oxidase activity of recombinant TK1392 was determined by incubating the assay mixture at various pH at 60 °C using 50 mM each of sodium acetate buffer (pH 4.0-6.0), sodium phosphate buffer (6.0-7.0), Tris-HCl buffer (pH 7.0-8.5).

K_m and specific activity calculations

Kinetic parameters of the enzyme toward NADH were determined at 75°C and pH 7.0 by plotting initial velocities towards various substrate concentrations (0, 5, 10, 25, 50, 100, 200 μM). A control experiment without the addition of enzyme was used at each concentration and non-enzymatic rate of thermal decomposition of NADH was subtracted from the experiment before plotting the graph between initial velocities and the substrate concentrations. The kinetic constants were calculated using Lineweaver-Burk plot.

For specific activity calculation the reaction conditions were the same as described above except for the substrate concentration which was 100 μM.

Results and Discussion

Cloning of TK1392 gene

In order to clone the gene encoding TK1392, a set of forward (TK1392-N) and reverse (TK1392-C) primers, designed on the basis of 5'- and 3'-end of the gene, was used to amplify the gene by PCR which resulted in the amplification of a 1.2 kb DNA fragment (Fig. 1) exactly matching the size of TK1392 gene.

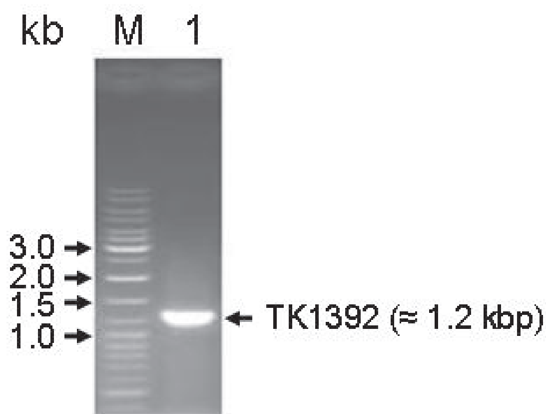


Fig.1. Ethidium bromide stained 1% agarose gel demonstrating PCR amplified TK1392 gene. Lane M, molecular weight standards (#SM1163; Fermentas); lane 1, 1.2 kb PCR amplified TK1392 gene.

PCR amplified TK1392 gene fragment was purified from agarose gel and inserted in pTZ57R/T using T_4 DNA ligase. When the resulting plasmid pTZ-TK1392 was used to transform *E. coli* DH5α competent cells a total of 45 white and 14 blue colonies appeared on the selection plates. White colonies were further screened for the presence of TK1392 gene by colony PCR (data not shown).

Plasmid DNA was isolated from one of the positive clones and digested with *Nde*I and *Hin*dIII which resulted in the liberation of a 1.2 kb DNA fragment from the vector (data not shown). Expression vector, pET-21a, was also digested with the same two restriction enzymes. Expression vector pET-21a and TK1392 were purified from the gel and ligated using T_4 DNA ligase. The resulting plasmid, pET-TK1392, was used to transform *E. coli* DH5α competent cells. Positive clones were selected by colony PCR and restriction enzyme digestion analysis (data not shown). One of the positive clones was subjected to DNA sequencing.

Sequence analysis and construction of phylogenetic tree

TK1392 gene consisted of 1,239 nucleotides and encoded a protein of 413 amino acids with a calculated molecular mass of 45,244 Da and a pI of 8.73. Sequence analysis indicated that amino acid residues with non polar side chains, involved in hydrophobic interactions, are present abundantly in TK1392 (Table 1).

Table 1. Amino acid composition of TK1392.

Amino acid	Count	Mol %
Ala	38	9.2
Arg	27	6.5
Asn	13	3.1
Asp	17	3.9
Cys	1	0.2
Gln	7	1.7
Glu	36	8.7
Gly	41	9.9
His	5	1.2
Ile	30	7.3
Leu	42	10.2
Lys	27	6.5
Met	8	1.9
Phe	11	2.7
Pro	19	4.6
Ser	14	3.4
Thr	9	2.2
Trp	3	0.7
Tyr	15	3.6
Val	51	12.3

NADH oxidases in the family *Thermococcaceae* have been characterized from two organisms, *P. furiosus* and *T. profundus* [2, 3]. When multiple sequence alignment of TK1392 was performed, using the amino acid sequence of these two proteins and the other two homologs of NADH oxidase (TK0304 and TK1299) from *T.kodkaraensis*, we could identify the nucleotide binding domains (Fig. 2). However, archaeal specific NADH oxidase amino acids identified by Case & co-workers [5] were quite different in TK1392 compared to these sequences in *T. profundus*, *P. furiosus*, TK0304 and TK1299, where they were completely conserved (Fig. 2). In fact the whole C-terminal region is quite distinct from the other four NADH oxidases. A phylogenetic tree was constructed by comparing the amino acid sequence of TK1392 and NADH oxidases from archaea and bacteria. In the phylogenetic tree TK1392 clustered with other uncharacterized archaeal NADH oxidases (Fig. 3). NADH oxidases that have been characterized were quite far from TK1392 in the phylogenetic tree. Among the NADH oxidases that have been characterized, TK1392 displayed a highest homology of 46% (identity) with that originated from *Thermotoga maritima* (Table 2). TK1392, surprisingly, displayed only a 29% identity to NADH oxidase from *T. profundus* that has recently been characterized [3].

Production in E. coli and purification of TK1392

In order to examine whether the TK1392 gene product exhibits the NADH oxidase activity, the encoding gene was overexpressed by utilizing the T7 promoter expression system in *E. coli*. Heterologous gene expression by induction with 0.2 mM IPTG at 37°C resulted in the production of 45 kDa TK1392 protein (Fig. 4). When the soluble fraction containing the recombinant TK1392 protein was incubated at 80°C for 20 min most of the proteins from *E. coli* were precipitated and removed by centrifugation (Fig. 4). Soluble fraction after heat treatment was further purified by ion exchange chromatography. Recombinant TK1392 did bind to the anion exchange column (Resource Q) at pH 8.0 and eluted when NaCl concentration was 0.4 M (data not shown). Analysis of the sample after anion exchange chromatography demonstrated a single protein band on SDS-PAGE (Fig. 5). When the recombinant TK1392 was analyzed for molecular mass determination by gel filtration chromatography using Superdex 200 10/300 GL column, it eluted at a retention volume of 13.6 corresponding to a molecular mass of 90 kDa (Fig. 6) indicating that recombinant TK1392 exists in a dimeric form. Similar to TK1392, NADH oxidases from *P. furiosus* [2], *T. maritima* [14], *Thermotoga hypogea* [15] and *M. jannaschii* [5] have been reported to exist in dimeric forms. However, an NADH oxidase from *T. profundus* has been reported to be a hexamer [3].

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                     FAD-binding                           Active-site
                      domain I                                 Cys

P.furious       ---MRIVVIGSGTAGSNFALFMRKLDRKAEITVIGKEETMQ--YSPCALPHVISGVIEKP   55
TK0304          ---MKIVVVGSGTAGSNFALFMRKLDRKAEITVIGKEPTMQ--YSPCALPHVVSGTIEKP   55
T.profundus     MERKRVVIIGGGAAGMSAASRVKRLKPEWDVKVFEATEWVS--HAPCGIPYVVEGISPK-   57
TK1299          MERKTVVVIGGGAAGMSTASRVKRLKPEWDVKVFEATEWVS--HAPCGIPYVVEGISPK-   57
TK1392          MLSYDVVVIGGGPAGMAAAAKAKELG--LNVLLLDENDYLGGILPQCIHPGFGLHYFKEE   58
                      *  * * **   *     *                     *  *

P.furious       EDVIVFPNEFYEKQR-IKLLLNTEAKKIDRE-------RKVVVTDKGE--IPYDKLVIAT  105
TK0304          EDIIVFPNEFYEKQK-INLMLNTEAKAIDRE-------RKVVVTDKGE--VPYDKLVLAV  105
T.profundus     EKLMHYPPEVFIKKRGIDLHMKAEVIEVEQG-------RVRVREPDGEHTYEWDYLVFAN  110
TK1299          EKLMHYPPEVFIKKRGIDLHLKAEVIEVEQG-------RVRVREEDGEKTYEWDYLVFAN  110
TK1392          LTGPEFASRLAKRLVELGVEYRTAARVLEIKNYSDLEKVVIFTSPAGVYQVWAKAIIYAA  118
                                                              *           *
                                                         NAD-binding
                                                           Domain

P.furious       GSKAFVPPIKGVENEGVFTLKSLEDVRKIKEFIKKRNPKNAVVIGAGLIGLEGAEAFAKL  165
TK0304          GSKAFIPPIKGVENEGVFTLKSLDDVRRIKAYIAERKPKKAVVIGAGLIGLEGAEAFAKL  165
T.profundus     GASPQVPAIEGCHLEGVFTADLPPDAVAITEYMEKHDVKNVAVIGTGYIAIEMAEAFVER  170
TK1299          GASPQVPAIEGIDLPGVFTADLPPDAVAITEYLEKNPVENVVVIGTGYIAIEMAEAFVER  170
TK1392          GARERHAFEIGIVGDRVAGIYTAGEAQTLMDIYGVLPGKEVVIVGSGDVGLIMARRFALE  178
                *         *     *                           * *      *  *

P.furious       GMKVTVVELLEHLLPTMLDKDIAKIVEENMRKYGVDFKFGVGVDEIIG-DPVEKVKVGEE  224
TK0304          GMEVLIVELMDRLMPTMLDKDTAKLVQAEMEKYGVSFRFGVGVSEIIG-SPVRAVKIGDE  224
T.profundus     GKNVTLIGRSERVLRKTFDKEITEVVEGKLREN-LNLRLEELTMRFEGDGRVEKVITDAG  229
TK1299          GKNVTLIGRSERVLRKTFDKEITDIVEEKLRNH-LNLRLEEVTLRIEGKERVERVVTDAG  229
TK1392          GAKVKAVVEL-MPYPGGLARNVMILRDFNIPLYLSHKVVEVRGKGRVQRVRVVKVDENFN  237
                *  *                                               *  *
                                                                FAD-binding
                                                                 domain II

P.furious       EID-------ADIVLVATGVRANVELAKEAGLEVNRG---IVVNEYLQTSDPDIYAIGDC  274
TK0304          EVP-------ADLVLVATGVRANTDLAKQAGLEVNRG---IVVNEHLQTSDPEVYAIGDC  274
T.profundus     EYP-------ADLVIVATGIKPNTELARQLGVRVGETGA-IWTNDKMQTSVENVYAAGDV  281
TK1299          EYP-------ADLVIVATGIKPNTELARGLGVRIGETGA-IWTNDRMQTSVENVYAAGDV  281
TK1392          EIPGSEFWIEVDTLVISAGLIPSVKKLKKIGVEIDPATGGPVVNDRLETSVPGIFVAGNS  297
                *          *      *           *            *    **       *

P.furious       AEVIDAVTGKRTLSQLGTSAVRMAKVAAENIAGRNVKFRPVFNTAITEIFDLEIGAFGIT  334
TK0304          AEVIDAVTGKRTLSQLGTSAVRMAKVAAEHIAGKDVSFRPVFNTAITELFGLEIGTFGIT  334
T.profundus     AETKRMITGRRVWMPLAPAGNKMGYVAGSNIAGKEVHFPGVLGTSITKFLDLEIGKTGLT  341
TK1299          AETKHLITGRRVWMPLAPAGNKMGYVAGSNIAGKEIHFPGVLGTSITKFLDLEIGKTGLT  341
TK1392          LLINDLVD------YVAEQGELAAKSAKEFIENGGIESR--------KWVKVEKGQN---  340

                                          *   *                     * *
                                       □□□□                              □

P.furious       EERAKKEEIEVVVGKFRGSTKPEYYPGGKPIVVKLIFRKEDRRLIGAQIVGGERVWGRIM  394
TK0304          EERAKKEDIEVAVGKFKGSTKPEYYPGGKPITVKLIFRKSDRKLIGGQIVGGERVWGRIM  394
T.profundus     EAEAIKEGYDVRTAFIKAGTKPHYYPGSRTIWLKGVVDNETNKLLGVQAVGAE-ILPRID  400
TK1299          EAEAMKEGYDVRTAFIKAGTRPHYYPGSKTIWLKGVVDNETNRLLGVQAVGGD-ILPRID  400
TK1392          ----------------VRLIAPHYLGGDRDVYLYLRVARPMENVELRIPEIGKK-L-RLP  384

                                     * *  *
                                     □□□□     □

P.furious       TLSALAQKGATVEDVVYLETAYAPPISPTIDPITIAAEMAMRKL-                 438
TK0304          TLSALAQKGATVEDVAYLETAYAPPISPTIDPITVAAEMAQRKLR                 439
T.profundus     TAAAMLTAGFTTKDAFFTDLAYAPPFAPVWDPLIVLARVLKF---                 442
TK1299          TAAAMITAGFTTKDVFFTDLAYAPPFAPVWDPLIVLARVLKF---                 442
TK1392          VVKPAEMIRLKLRAEEIQKASERLTVEVVRA--------------                 413
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Fig. 2. Multiple sequence alignment of TK1392. Alignment was performed with CLUSTAL W. Archaeal specific NADH oxidase amino acids (5) are shown in bold with close circle at the top. Identical sequences are shown by asterisks at the bottom.

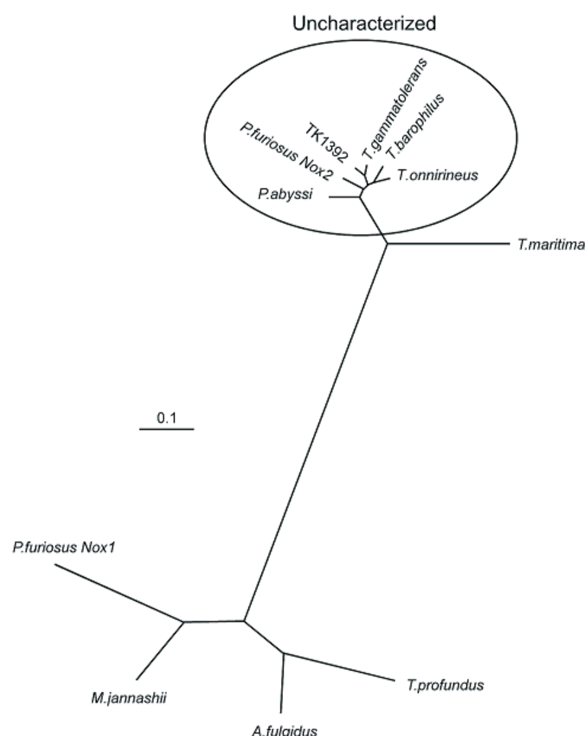


Fig.3. Phylogenetic tree constructed on the basis of amino acid sequence information. Calculations were performed by ClustalW program provided by DNA Data Bank of Japan. Segments corresponding to an evolutionary distance of 0.1 are shown.

100% at 75 Effect of temperature and pH on the enzyme activity of TK1392

When we examined the enzyme activity of TK1392 at various temperatures we found that the activity increased with the increase in temperature from 50 to 75°C. Highest activity was observed when the reaction mixture was incubated at 75°C (Fig. 7A). The enzyme activity started decreasing beyond 75°C and only 27% activity could be detected at 90°C compared 100% at 75°C. The optimal temperature for TK1392 enzyme activity (75°C) is a little lower than the optimal growth temperature of the strain (85°C) which is not unusual. NADH oxidases from several hyperthermophiles have lower optimal temperature for their activities compared to optimal growth temperatures of these hyperthermophiles. NADH oxidase from *T. maritima* and *T. hypogea* (optimal growth temperature of 90°C) showed highest activity at 80°C [14] and 85°C [15], respectively. Similarly NADH oxidases from *T. profundus* (optimal growth temperature 80°C) and *P. furiosus* (optimal growth temperature 100°C) displayed optimum activities at 70°C [2] and 85°C [3], respectively.

Thermostability of TK1392 was examined at 75 (the optimal temperature for enzyme activity), 80, 85 (the optimal growth temperature of the micro-organism) and 90°C. The enzyme was quite stable at 75°C displaying more than 90% of enzyme activity even after incubation

Table 2. Amino acid sequence comparison of TK1392 and related homologs.

Organism	Accession number	% Identity
Thermococcus gammatolerans	ACS34294	90
Thermococcus onnurineus	ACJ15689	82
Thermococcus barophilus	EDY40282	80
Pyrococcus furiosus	AAL82130	78
Pyrococcus abyssi	NP_125963	75
Aciduliprofundum boonei	ZP_04875406	65
Desulfurococcus kamchatkensis	YP_002428573	60
Staphylothermus marinus	YP_001040280	55
Dictyoglomus thermophilum	YP_002251539	50
Eubacterium dolichum	ZP_02076883	47
Thermoanaerobacter pseudethanolicus	YP_001664539	46
Halothermothrix orenii	YP_002508563	46
Thermotoga maritima	NP_229233	46
Fusobacterium nucleatum	ZP_00143540	44
Clostridium acetobutylicum	NP_347952	44
Syntrophus aciditrophicus	YP_460764	42
Pyrococcus horikoshii	BAD 77802	34
Archeoglobus fulgidus	NP_069111	29
Thermococcus profundus	NP_579261	29
Thermus thermophilus	YP_005524	26
Sulfolobus solfataricus	CAB 57297	25

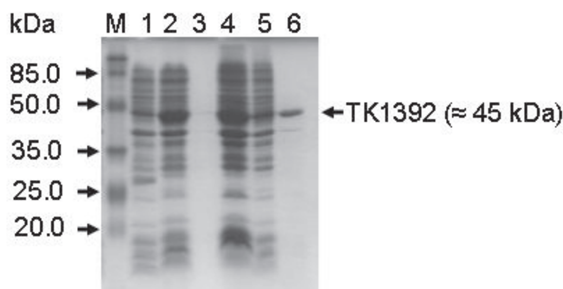


Fig. 4. Coomassie brilliant blue stained 12% SDS-PAGE showing production of TK1392. Lane M, protein marker (#SM0441, Fermentas); Lane 1: cells carrying pET- 21a vector; Lane 2: cells carrying pET-TK1392; Lane 3, insoluble fraction of the sample in lane 2; Lane 4, soluble fraction of the sample in lane 2; Lane 5, insoluble fraction after heat treatment of sample in lane 4; Lane 6, soluble fraction after heat treatment of sample in lane 4.

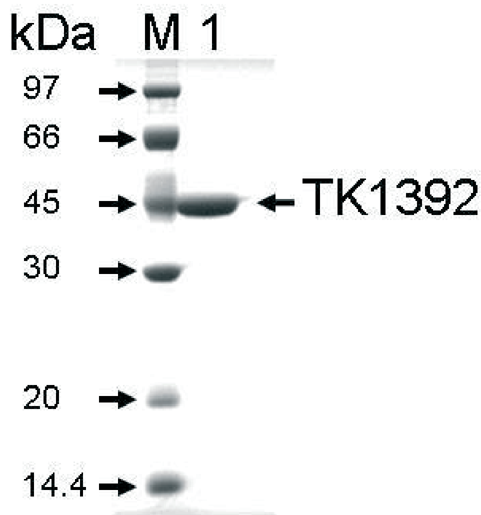


Fig. 5. Coomassie brilliant blue stained 12% SDS-PAGE showing purified recombinant TK1392. Lane M, protein marker (# 17-0446-01, GE Healthcare); lane 1: purified recombinant Tk1392.

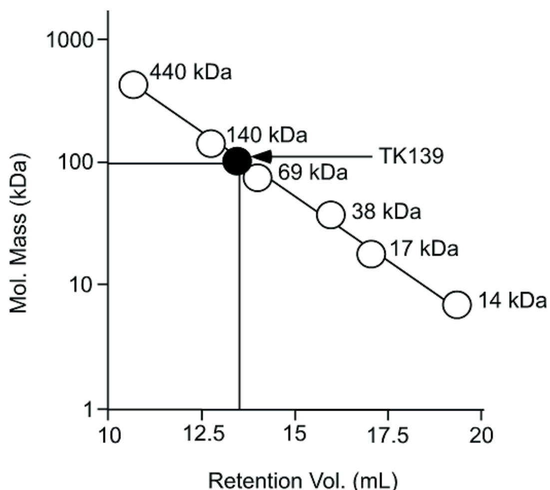
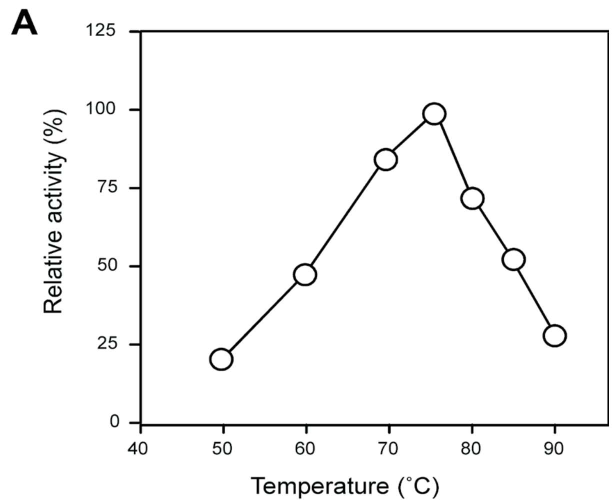
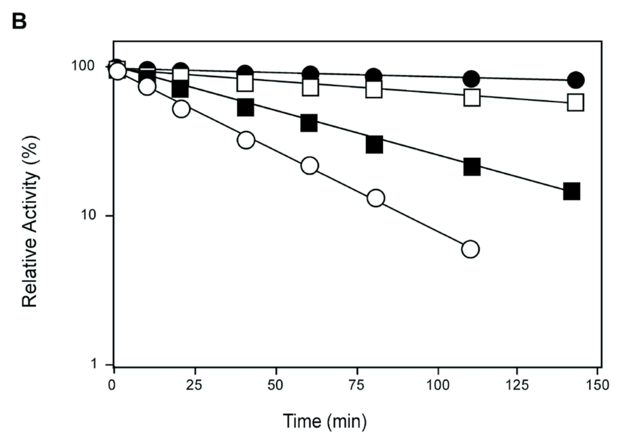


Fig. 6. Molecular mass determination of TK1392 by gel filtration column chromatography. Symbols used are: open circles, elution volume of standards; filled circle, elution volume of TK1392.





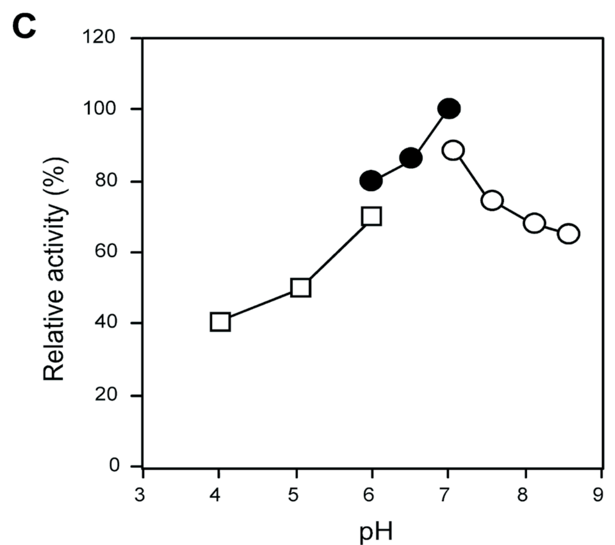


Fig. 7. Effect of temperature and pH on the enzyme activity of recombinant TK1392. (A) Effect of temperature. The enzyme activity was examined at various temperatures at pH 8.0. (B) Effect of temperature on the stability. TK1392 was heated in Tris-HCl buffer (pH 8.0) at 75 °C (*close circles*), 80 °C (*open squares*), 85 °C (*close squares*) and 90 °C (*open circles*) for various time intervals and the residual activity was examined at 60 °C. (C) Activity as a function of pH. Activity was examined at various pH and 60 °C. Following buffers were used: sodium acetate buffer (*open squares*), sodium phosphate buffer (*closed circles*), and Tris-HCl buffer (*open circles*).

Table 3. Purification of recombinant TK1392 produced in *E. coli*.

Step	Total protein (mg)	Activity (U)	Specific activity (U/mg)	Recovery (%)	Purification-fold
Cell free extract	374	195	0.52	100	__
Heat treatment	14.4	205	14.24	105	27
Anion exchange chromatography	7.2	139	19.3	71	37

of 150 min. The half-life of the enzyme was 50 min at 85°C and 20 min at 90°C (Fig. 7B).

The effect of pH on the enzyme activity was examined by measuring the enzyme activity at different pH in various buffers. The enzyme displayed its activity in a wide pH range. Highest activity was found at pH 7.0 in sodium phosphate buffer (Fig. 7C) which indicated that NADH oxidase functioned optimally under physiological conditions of *T. kodakaraensis*. This is also true for NADH oxidases from acidophilic archaea including *Acidianus ambivalens* and *Sulfolobus solfataricus* which have optimal activity at highly acidic pH similar to physiological conditions of these microorganisms [16, 17].

TK1392 displayed a specific enzyme activity of 19.3 U mg^{-1}. The specific activity of TK1392 was higher than the NADH oxidases from other archaeal species. For example NADH oxidases (NOXA-I and NOXB-1) from *A. fulgidus* have specific activities of 5.8 U mg^{-1} and 4.1 U mg^{-1}, respectively [4]. Similarly NADH oxidases from *T. profundus*, *P. furiosus* and *Methanococcus jannaschii* have been reported to have specific activities of 7 U mg^{-1} [3], 2 U mg^{-1} [2] and 16 U mg^{-1} [5], respectively. On the other hand NADH oxidases from bacterial origin displayed higher specific activities. For insistent, NADH oxidase from *Lactobacillus brevis* displayed a specific activity of 116 U mg^{-1} [18] and *T. martima* NADH oxidase exhibited a V_{max} of 230 ± 14 [14]. A plausible justification can be that above mentioned archaea thrive at high temperature in the absence of oxygen; therefore, NADH oxidases from these microorganisms do not have high specific enzyme activities.

Recombinant TK1392 displayed a K_m value of 35±3 µM toward NADH. Most of the NADH oxidases characterized from hyperthermophiles displayed a low K_m value. NADH from *T. maritima* [14] *P. furiosus* [2] and *T. profundus* [3] exhibited K_m values of 3, 4 and 53 µM, respectively. The low K_m values reflect their high affinity toward NADH.

Role of NADH oxidase in *T. kodakaraensis* is not very clear. *T. kodakaraensis* is a strict anaerobe and it is unlikely that NADH oxidase acts in the classic sense to transfer electrons to O_2 for the purpose of regenerating NAD^+. It may instead play a protective role against oxygen stress by direct reduction of O_2 to water. Higher enzyme activities of TK1392 and NADH oxidases from other hyperthermophilic archaeal strains at temperatures lower than their optimal growth temperatures further support this hypothesis as the dissolved oxygen concentrations are higher at low temperatures. More studies are required to determine the physiological role of NADH oxidase in this strictly anaerobic microorganism.

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