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TK1656, a thermostable L-asparaginase from *Thermococcus kodakaraensis*, exhibiting highest ever reported enzyme activity

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Two L-asparaginase homologs, TK1656 and TK2246, have been found in the genome of *Thermococcus kodakaraensis*. The gene encoding TK1656 consists of 984 nucleotides corresponding to a polypeptide of 328 amino acids. To examine the properties of TK1656, the structural gene was cloned, expressed in *Escherichia coli* and the purified gene product was characterized. TK1656 exhibited high asparaginase activity (2350 U mg^{-1}) but no glutaminase activity. The enzyme also displayed the D-asparaginase activity but 50% to that of L-asparaginase. The highest activity was observed at 85°C and pH 9.5. TK1656 catalyzed the conversion of L-asparagine to L-aspartatic acid and ammonia following Michaelis–Menten kinetics with a K_m and V_{max} values of 5.5 mM and 3300 μmol min^{-1} mg^{-1}, respectively. The activation energy from the linear Arrhenius plot was found to be 58 kJ mol^{-1}. Unfolding studies suggested that urea could not induce complete unfolding and inactivation of TK1656 even at a concentration 8 M; however, in the presence of 4 M guanidine hydrochloride enzyme structure was unfolded with complete loss of enzyme activity.

[**Key words:** *Thermococcus kodakaraensis*; L-Asparaginase; Structural stability; Thermal stability; Unfolding]

L-Asparaginases (L-asparagine-amidohydrolase, EC 3.5.1.1) catalyze the hydrolysis of L-asparagine to L-aspartic acid and ammonia. Their ability to hydrolyze L-asparagine in blood plasma makes them potent candidates for the treatment of leukemia since tumor cells, unable to synthesize L-asparagine, are selectively killed by deprivation of this amino acid (1). L-asparaginases have also been used in making diagnostic biosensors as the amount of ammonia produced by the action of the enzyme directly correlates to the level of L-asparagine in the blood (2).

Several microbial genera, from bacterial, archeal and eukaryotic sources, have been screened for L-asparaginases and a number of reports are available in the literature on the production of this enzyme (3–7). The intrinsic glutaminase activity of L-asparaginases, which could result in serious side effects, restricts their clinical applications. Although the enzymes from *Escherichia coli* and *Erwinia carotovora* possess strong preference to asparagine over glutamine, however, their low stability requires multiple dose administration for effective treatment (8). Furthermore, it has been reported that fried and baked food contains a significant amount of acrylamide, a carcinogen, formed by the reaction of asparagine with reducing sugars (9). A pretreatment with L-asparaginase before frying or baking prevents acrylamide formation (10). In this regard, L-asparaginases from fungal and bacterial sources are being used in baking industries. These enzymes are thermolabile and work at a narrow pH range. Therefore, there is a need to identify and characterize an asparaginase that is free of glutaminase activity and active over a wide range of temperature and pH.

Thermococcus kodakaraensis KOD1 is a hyperthermophilic archaeon isolated from a solfatara on the shore of Kodakara Island in Kagoshima, Japan (11,12). Several thermostable and novel enzymes have been characterized from this archaeon (13–16). It is a model hyperthermophilic organism whose whole genome has been sequenced and reported (17). The genome sequence revealed the presence of two L-asparaginase homologs, TK1656 and TK2246. In the present study we describe cloning and properties of TK1656, a highly active L-asparaginase with no glutaminase activity.

MATERIALS AND METHODS

Materials All chemicals used in the experiments were of analytical grade purchased either from Sigma–Aldrich Co. or Fluka Chemical Corp. or Thermo Fisher Scientific Inc. DNA polymerase, restriction enzymes, cloning vectors, and DNA purification and transformation kits were purchased from Thermo Fisher Scientific Inc. Gene specific primers were commercially synthesized from Gene Link, Inc. *E. coli* DH5α cells were used for cloning purposes and BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA, USA) cells were employed for the expression of TK1656 gene that was cloned in pET-21a vector (Novagen, Madison, WI, USA). *E. coli* cultures were grown in Luria–Bertani (LB) broth with or without 100 μg/mL ampicillin.

Cloning, expression and purification of TK1656 TK1656 gene (accession No. NC_006624) was amplified by polymerase chain reaction using sequence specific forward (5′-CATATGAAACTTCTGGTTCTCGGCACGG) and reverse (5′-TTAACTCC CAGTGATTTCGCCC) primers as priming strands and genomic DNA of *T. kodakaraensis* as template. Site for restriction enzyme *Nde*I was introduced in the forward primer (underlined sequence). PCR amplified TK1656 gene was purified from the gel by DNA purification kit and ligated in cloning vector pTZ57 R/T using T4 DNA ligase. The

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resulting plasmid construct was named as pTZ-Tk1656. TK1656 gene was liberated from pTZ-Tk1656 using *Nde*I (introduced in the forward primer) and *Hind*III (present in the multicloning site of pTZ57 R/T) restriction enzymes and cloned in pET-21a at the corresponding sites. The resulting plasmid, pET-*Tk1656*, was used to transform *E. coli* BL21-CodonPlus(DE3)-RIL for expression of *TK1656*. Expression of the gene was induced either by 0.5 mM isopropyl-thio-β-D-galactoside (IPTG) or 10 mM lactose.

Unless mentioned otherwise, all steps in the purification procedure were carried out at room temperature, using 50 mM Tris-Cl buffer (pH 8.0). *E. coli* BL21-CodonPlus(DE3)-RIL cells (about 2 g wet weight) suspended in 30 mL buffer were disrupted by sonication. After centrifugation at 14,000 ×*g* for 20 min, the supernatant containing TK1656 was heat treated at 80°C for 20 min. The heat labile host proteins were removed by centrifugation at 14,000 ×*g* for 20 min. Supernatant, containing thermostable TK1656, was applied to a Resource Q column (GE Healthcare, UK) which was equilibrated with the same buffer using an AKTA Explorer (GE Healthcare) chromatography system. TK1656 was eluted with a linear gradient of 0–1 M NaCl.

Determination of molecular mass and quaternary structure of TK1656 The subunit molecular mass of recombinant TK1656 was determined by SDS-PAGE as well as by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). For MALDI-TOF analysis, 2 μg of TK1656 was mixed with 20 μL of matrix-B (5 mg sinapinic acid dissolved in 1 mL of 30% acetonitrile containing 0.1% trifluoroacetic acid) and 5 μL of this mixture was spotted on stainless steel mass spectrometric plate and allowed to dry for 30 min. The mass spectrum of the purified enzyme was recorded by using Bruker Autoflex MALDI-TOF mass spectrophotometer (Bruker Daltonics Inc., MA, USA).

Native molecular mass of TK1656 was determined by Superdex 200 10/300 GL gel filtration column (GE Healthcare) equilibrated with 150 mM NaCl in 50 mM phosphate buffer (pH 7.0). The calibration curve was obtained with the standard proteins including ferritin (440 kDa), phosphorylase B (195 kDa), lactate dehydrogenase (140 kDa), malate dehydrogenase (70 kDa), and myoglobulin (17 kDa).

Enzyme assay L-asparaginase activity of TK1656 was measured by estimating the amount of ammonia produced during the reaction. Enzyme assay mixture composed of 100 μL of 50 mM Tris–Cl buffer, 50 μL of 40 mM L-asparagine or D-asparagine or L-glutamine or D-glutamine, 5–50 μL of enzyme solution and 0–45 μL of water to make final assay volume 200 μL. The reaction was allowed to proceed for 10 min and stopped by adding 50 μL of 15% trichloroacetic acid (TCA). Reaction mixture was centrifuged at 14,000 ×*g* and 100 μL of clear supernatant was added to 800 μL of water and 100 μL of Nessler's reagent. Optical density was measured at 425 nm in a Shimadzu Biospec-1601 DNA/Protein/Enzyme Analyzer. The experiments were performed in triplicates and repeated for at least two times to ensure the reproducibility. One unit (U) of L-asparaginase activity was defined as the amount of enzyme liberating 1 μmol of NH_3 in 1 min under the conditions mentioned above.

Effect of temperature, pH and divalent metal ions Effect of temperature and pH was determined by measuring the enzyme activity at various temperatures (30–95°C) while keeping the pH constant or varying the pH values (pH 4–10) and keeping the temperature unchanged.

Effect of divalent metal ions on the enzyme activity was analyzed in the presence of 0.1 mM or 1 mM of each metal ions examined. Chloride salts of Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} were used.

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P.horikoshii    ----------------------MRILILG MGGT IAS---VKGERGYESALSVSKILKLA 34
P.furiosus      ----------------------MKILLIG MGGT IAS---VKGENGYEASLSVKEVLDIA 34
TK1656          ----------------------MKLLVLG TGGT IAS---AKTEMGYKAALSADDILQLA 34
E.coli-II       MEFFKKTALAALVMGFSGAALALPNITILA TGGT IAGGGDSATKSNYTAGKVGVENLVNA 60
                                          *****          *         *  *

P.horikoshii    GIS--SEAKIEARDLMNVDSTLIQPSDWERLAKEIEKEVWEYDGIVIT HGTDT MAYSASM 92
P.furiosus      GIK--DCEDCDFLDLKNVDSTLIQPEDWVDLAETLYKNVKKYDGIIVT HGTDT LAYTSSM 92
TK1656          GIRREDGAKIETRDILNLDSTLIQPEDWVTIGRAVFEAFDEYDGIVIT HGTDT LAYTSSA 94
E.coli-II       VPQLKDIANVKGEQVVNIGSQDMNDDVWLTLAKKINTDCDKTDGFVIT HGTDT MEETAYF 120
                           *  *       *              **   ******

P.horikoshii    LSFMLRNPPIPIVLTGSMLPITEKNSDAPFNLRTALEFVK---LGIRGIYIAFNGKVMLG 149
P.furiosus      ISFMLRNPPIPIVFTGSMIPATEENSDAPLNLQTAIKFAT---SGIRGVYVAFNGKVMLG 149
TK1656          LSFMIRNPPIPVVLTGSMLPITEPNSDAPRNLRTALTFAR---KGFPGIYVAFMDKIMLG 151
E.coli-II       LDLTVKCD-KPVVMVGAMRPSTSMSADGPFNLYNAVVTAADKASANRGVLVVMNDTVLDG 179
                        *  * * * *    * * **  *            *           *

P.horikoshii    VRASKIRSMGFDAFESINYPNVAEIKDDKLRILHIPDFYGDEF----FSDIKYEPKVLVI 205
P.furiosus      VRTSKVRTMSRDAFESINYPIIAELRGEDLVVNFIPKFNNGEV----TLDLRHDPKVLVI 205
TK1656          TRVSKVHSLGLNAFQSINYPDIAYVKGDEVLVRHKPRIGNGEP----LFDPELDPNVVHI 207
E.coli-II       RDVTKTNTTDVATFKSVNYGPLGYIHNGKIDYQRTPARKHTSDTPFDVSKLNELPKVGIV 239
                    *        * * **               *                  * *

P.horikoshii    KLIPGLSGDIVREALRLGYKGIILEGYGVGGIPYRGTDLFEVVSSISKRIPVVLTTQAIY 265
P.furiosus      KLIPGLSGDIFRAAVELGYRGIVIEGYGAGGIPYRGSDLLQTIEELSKEIPIVMTTQAMY 265
TK1656          RLTPGLSPEVLRAVAR-ATDGIVLEGYGAGGIPYRGRNLLEVVSETAREKPVVMTTQALY 266
E.coli-II       YNYANASDLPAKALVDAGYDGIVSAGVGNGNLYKT---VFDTLATAAKNGTAVVRSSRVP 296
                      *             **   * * *                     *

P.horikoshii    DGVDLQRYKVGRIALEAGVIPAGDMTKEATITKLMWILGHTKNIEEVKQLMGKNITGELT 325
P.furiosus      DGVDLTRYKVGRLALRAGVIPAGDMTKEATVTKLMWILGHTNNVEEIKVLMRKNLVGELR 325
TK1656          GGVDLTRYEVGRRALEAGVIPAGDMTKEATLTKLMWALGHTRDLEEIRKIMERNIAGEIT 326
E.coli-II       TGATTQDAEVD--DAKYGFVASGTLNPQKARVLLQLALTQTKDPQQIQQIFNQY------ 348
                 *       *       *    *          *   *  *

P.horikoshii    RVS                                                          328
P.furiosus      D--                                                          326
TK1656          GS-                                                          328
E.coli-II       ---                                                          348
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FIG. 1. Amino acid sequence comparison of TK1656 with its homologs that have been characterized from family Thermococcacae and *E. coli*. Asterisks below the sequence show identical amino acids. The names at the left hand side indicate the organism from which the sequence originated. The conserved asparaginase active site signature 1 and asparaginase active site signature 2 (http://prosite.expacy.org/PDOC00132) are shown in bold. Gaps are shown by dashes. The sequence accession numbers are: YP_184069, TK1656; NP_142084, *P. horikoshii* OT3; NP_579776, *P. furiosus* DSM 3638; and YP_006121249, *E. coli.*