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Cloning and Expression of the Gene for Inorganic Pyrophosphatase of *Thermus caldophilus* GK24 and Properties of the Enzyme

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Abstract The gene (*ppaT*) encoding *Thermus caldophilus* GK24 pyrophosphatase (*Tca* pyrophosphatase) was cloned and sequenced. The gene was found to contain an open reading frame encoding 175 amino acids with a calculated mass of 19,155 Da. The *ppaT* gene was expressed under the control of the *tac* promoter in *Escherichia coli*. The recombinant *Tca* pyrophosphatase was purified 21.4-fold with 56% yield and specific activity of 25.7 U mg^{-1}, following a combination of heating (to denature the *E. coli* proteins) and one step of DEAE-Sephacel column chromatography. The native enzyme was found to have an approximate molecular mass of 110,000 Da and consisted of six subunits. The enzyme exhibited maximal activity at pH of 8.0-8.5 and was stable at 80-90°C. A divalent cation was absolutely required for the enzyme activity, with Mg^{2+} being the most effective.

Key words: *ppaT* gene, gene expression, pyrophosphatase, *Thermus caldophilus* GK24, *Tca* pyrophosphatase

Inorganic pyrophosphatase (EC 3.6.1.1; pyrophosphatase), which hydrolyzes inorganic pyrophosphate into inorganic phosphate, is an essential enzyme [2], providing a thermodynamic pull for many biosynthetic reactions [7]. Soluble pyrophosphatases have already been quite extensively characterized from various prokaryotic and eukaryotic sources. The best-studied soluble pyrophosphatases are those from *Escherichia coli* and *Saccharomyces cerevisiae*. *E. coli* pyrophosphatase is a homohexamer with 175 amino acids [10], whereas *S. cerevisiae* pyrophosphatase is a homodimer of 286 amino acids [6]. Alignment of the sequences of these pyrophosphatases indicates that the active site residues are very well conserved, even though the overall level of sequence similarity is low [3, 9].

Thermostable pyrophosphatases are commonly used in cycle sequencing methods, when using thermostable DNA polymerase in PCR [18, 21]. In order to prevent termination peak dropouts due to pyrophosphorolysis, sequencing enzyme mixtures are formulated with pyrophosphatase. The enzyme is also suitable for a long-range PCR. The addition of a pyrophosphatase catalyzes the removal of pyrophosphate, and the equilibrium of the extension reaction is thus shifted more favorably to forward DNA polymerization. As regards biotechnological applications, a number of thermostable pyrophosphatases have been isolated and characterized from *Thermus aquaticus* [22], *Thermus thermophilus* HB8 [17], *Sulfolobus acidocaldarius* [14], and *Aquifex aeolicus* [5]. Although pyrophosphatases have been studied in many species, there has been no previous report of a thermostable pyrophosphatase from the extreme bacterium *T. caldophilus* GK24. This organism was isolated from the Kawamata Hot Spring, Tochigi-ken, Japan [19] and can grow well within a temperature range of 70-75°C with a maximum at 82°C.

Accordingly, the current paper reports on the cloning and nucleotide sequencing of the *ppaT* gene, its expression in *E. coli*, and the purification and properties of recombinant *Tca* pyrophosphatase.

Materials and Methods

Bacterial Strains

E. coli MV1184 was used as the host for the plasmid preparations and gene expression. The *T. caldophilus* GK24 [19] and *T. thermophilus* HB8 (ATCC No. 27624) cells were prepared as described by Yoo *et al.* [24]. The chromosomal DNAs of *T. caldophilus* GK24 and *T.*

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thermophilus HB8 were isolated by the method of Marmur [13].

Identification of *ppaT* Gene and Preparation of Genomic Library

Most of the methods used for the molecular cloning and hybridization were based on those of Sambrook *et al.* [16]. To clone the *ppaT* gene, the ^{32}P-labeled structural gene coding for *T. thermophilus* HB8 (*Tth*) pyrophosphatase was used as a hybridization probe. The amplification of the *Tth* pyrophosphatase gene [17] was performed according to Saiki *et al.* [15] using *T. thermophilus* genomic DNA. The amplified gene was labeled with [α-^{32}P] dCTP using a Random Primer DNA Labeling Kit (Takara, Shiga, Japan).

The 1.5-kb *Bam*HI and 0.8-kb *Sac*I fragments of *T. caldophilus* GK24 genomic DNA, which hybridized with the probe, were separately ligated at the *Bam*HI and *Sac*I sites of pBluescript SK, respectively, and *E. coli* MV1184 was then transformed with the plasmids. Positive clones that hybridized with the probe were identified by screening a genomic library.

DNA Sequencing and Computer-Assisted Analysis

The restriction DNA fragments being sequenced were cloned into the appropriate restriction sites of pBluescript SK+/– vectors. The DNA sequencing reactions were performed using an ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster, U.S.A.) and run on an Applied Biosystem 373 automated DNA sequencer. The sequence data was analyzed using DNASIS.

Construction of Expression Plasmid pTCPP

Based on the DNA sequence of the *ppaT* gene, two primers were synthesized. The 5' (N-terminal) primer sequence was 5'-NNNNGAATTCATGGCGAACCTGAAGAGCCTT-3, while the 3' (C-terminal) primer sequence was 5'-NNNN-GTCGACTAGCCCTTGTAGCGGGCG-3', which created the underlined unique *Eco*RI and *Sal*I sites, respectively, at each end of the amplified DNA fragment. The DNA amplification was performed using 2.5 units of *Taq* DNA polymerase in a 50 µl reaction mixture containing PCR reaction buffer, 2.5 pmol of the primers, 0.2 mM each of dNTP, and 0.1 µg *T. caldophilus* GK24 genomic DNA, as described by Saiki *et al.* [15]. The amplified 0.53-kb DNA fragment was isolated from a low-melting-temperature agarose gel. The fragment was ligated into the expression vector pJR [8] that had been digested with *Eco*RI and *Sal*I, giving a fusion that used the *tac* promoter. The ligation reaction was then transformed into *E. coli* MV1184. Clones exhibiting a high *Tca* pyrophosphatase activity were selected and designated as plasmid pTCPP.

Purification of Expressed *Tca* Pyrophosphatase in *E. coli*

Ten ml of an overnight culture of *E. coli* MV1184 harboring the recombinant plasmid pTCPP grown in an L-broth containing ampicillin was transferred to 1 l of the same medium and cultured at 37°C. When the A_{600} of the culture was about 0.8, the culture was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM and then incubated at 37°C for 6 h. The cells were collected by centrifugation, suspended in 20 ml of buffer A [10 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$] containing 1 mM PMSF, then disrupted by sonication. The disrupted cells were centrifuged at 20,000 rpm, at 4°C for 20 min, to remove any *E. coli* cell debris. The sonicated extract was incubated at 80°C for 50 min. After centrifugation, the supernatant was used as the source of crude enzymes. The nucleic acids in the supernatant were precipitated by the addition of 1% streptomycin sulfate at room temperature with stirring for 60 min, then the precipitate was removed by centrifugation. The supernatant was dialyzed against buffer A, then applied to a DEAE-Sephacel column (1.5× 11.3 cm) that had been equilibrated with buffer A. The adsorbed protein was eluted by a linear gradient of NaCl (0–0.5 M) in 120 ml of buffer A. The major peak of *Tca* pyrophosphatase activity was eluted at approximately 0.25 M NaCl concentration. The major fractions (total 27 ml) showing *Tca* pyrophosphatase activity were pooled and dialyzed against buffer A.

***Tca* Pyrophosphatase Activity Assays**

The inorganic pyrophosphatase activity was assayed at 80°C for 30 min in the following reaction mixture; 0.5 ml of 50 mM Tris-HCl (pH 8.0) buffer containing 1 mM $MgCl_2$, 0.02 ml of sodium pyrophosphate, and 0.1 ml of the enzyme sample. Sodium pyrophosphate was dissolved in water at a final concentration of 1.7 mM and used as substrate. The reaction was terminated by the addition of 0.1 ml of 1.0 M citric acid. The inorganic phosphate liberated was determined according to the method of Heinonen and Lahti [4, 11]. One unit of activity was defined as the amount of enzyme that liberated 1 µmol of inorganic pyrophosphate in 30 min at 80°C.

Protein Analysis and Determination of Molecular Mass

The protein concentration was determined by the procedure of Lowry *et al.* [12], using bovine serum albumin as the standard protein. An electrophoretic analysis was performed by SDS-PAGE according to the method of Weber *et al.* [23], with a 4% (w/v) acrylamide stacking gel and 12% (w/v) acrylamide separating gel.

The molecular mass of the native *Tca* pyrophosphatase was determined using a Biologic Workstation (Bio-Rad, Hercules, U.S.A.) on a Bio-Prep SE-100/17 column (0.8× 30 cm) equilibrated with buffer A containing 100 mM NaCl. The molecular mass in the native state was estimated using the Bio-Rad size exclusion standard (catalog number 151-1901). The *Tca* pyrophosphatase molecular mass was

calculated by plotting the log versus the elution volume/void volume ratio [1].

RESULTS AND DISCUSSION

Molecular Cloning and Deduced Amino Acid Sequence of the Gene Encoding *Tca* Pyrophosphatase

To clone the *ppaT* gene, the ^{32}P-labeled structural gene coding for *Tth* pyrophosphatase was used as a hybridization probe. A positive clone (pTBIP), which hybridized with the probe, was identified by screening a genomic library containing the 1.5 kb *Bam*HI DNA fragment, while a positive clone (pTSIP), which hybridized with the probe, was also identified by screening a genomic library containing the 0.8 kb *Sac*I DNA fragmemt.

The restriction maps of the 1.5 kb *Bam*HI and 0.8 kb *Sac*I DNA fragments are presented in Fig. 1A. Each enzyme site in the restriction maps was used for the subcloning and DNA sequencing of the cloned DNA fragments. The position of the *Tca* pyrophosphatase gene

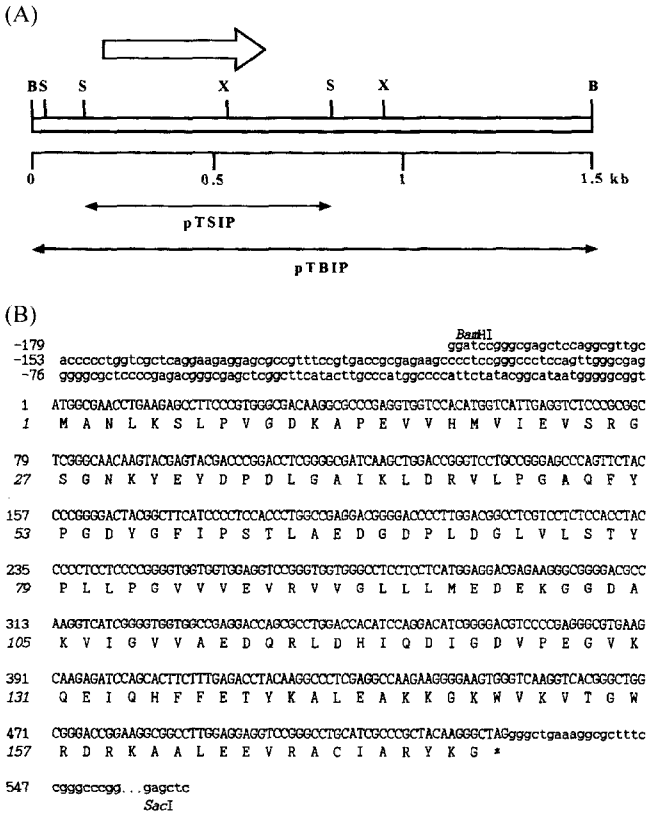

Fig. 1. Structure of the *ppaT* gene of *T. caldophilus* GK24 and its nucleotide sequence.
(A) Restriction map of the *ppaT* gene and positions of cloned DNA fragments in plasmid pTBIP (1.5 kb *Bam*HI) and pTSIP (0.8 kb *Sac*I). Open arrow indicates the coding region of the *ppaT* gene. The restriction enzyme sites are shown: B, *Bam*HI; S, *Sac*I; X, *Xho*I. (B) Nucleotide and deduced amino acid sequences of the *ppaT* gene. Asterisk indicates the stop codon.

in the cloned fragments is indicated by the open arrow. Figure 1B shows the nucleotide sequence of the cloned DNA fragments and deduced amino acid sequence of *Tca* pyrophosphatase, which was comprised of 175 amino acid residues and had a molecular mass of 19,155 Da.

The whole amino acid sequence of *Tca* pyrophosphatase showed 99% identity with *Tth* pyrophosphatase [17], and 45% with *Aquifex aeolicus* (*Aae*) pyrophosphatase (GenBank Accession No. AE000745) and *E. coli* pyrophosphatase [10] (results not shown). In the case of *Tca* pyrophosphatase and *Tth* pyrophosphatase, the two genes revealed a 90% homology to each other at the DNA level. The identified amino acids involved in the active site of *E. coli* pyrophosphatase [9] were identical to those of *Tca* pyrophosphatase, *Tth* pyrophosphatase, and *Aae* pyrophosphatase, thus indicating their importance in the catalytic mechanism or integrity of pyrophosphatase and also suggesting a common evolutionary origin.

Purification of *Tca* Pyrophosphatase from *E. coli*

Tca pyrophosphatase was purified by combining heating (to denature the *E. coli* proteins) and one step of DEAE-Sephacel column chromatography. The purification of the enzyme is summarized in Table 1. The specific activity of the purified enzyme was 21.4-fold higher than that of the sonicated extract, and recovery was approximately 56% from the sonicated extract. The purity of the enzyme was monitored by SDS-PAGE (Fig. 2). Most proteins derived from *E. coli* were almost completely removed by the heat treatment, while the nucleic acids and certain contaminating proteins were removed by the DEAE-Sephacel column chromatography. A single protein band corresponding to a molecular mass of 23,000 Da was obtained by SDS-PAGE. This molecular mass was somewhat different from the 19,155 Da calculated from the deduced amino acid sequence. The molecular mass of the native enzyme was also determined to be 110,000 Da by gel filtration on a column of Bio-Prep SE-100/17 (results not shown). These results suggested that the enzyme with *Tca* pyrophosphatase activity was a hexamer in its native conformation. The native conformation of *Tca* pyrophosphatase was similar to those of *E. coli* pyrophosphatase [10] and *Tth* pyrophosphatase [20], yet different from those of *T. aquaticus* (*Taq*) pyrophosphatase, *Aae* pyrophosphatase, and *Saccharomyces*

Table 1. Purification summary of *Tca* pyrophosphatase expressed in *E. coli*.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg^{-1})	Recovery (%)
Sonicated extract	251.0	301	1.2	100
Heat-treatment	26.0	200	7.7	66
DEAE-Sephacel	6.6	170	25.7	56

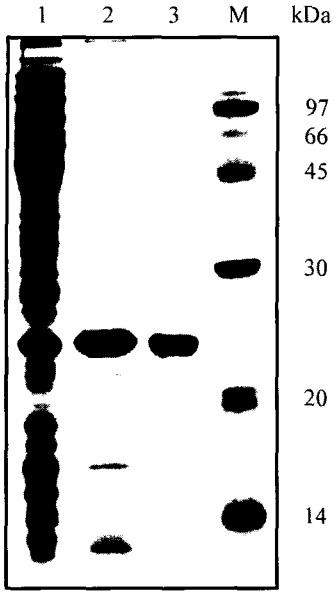

Fig. 2. SDS-PAGE analysis of *Tca* pyrophosphatase.
Lane 1, sonicated extract of induced cell (*E. coli* MV1184/pTCPP); lane 2, the same sample heated at 80°C for 50 min and cleared by centrifugation; lane 3, purified by DEAE-Sephacel column chromatography; lane M, low-molecular-mass markers.

cerevisiae pyrophosphatase; for example, *Taq* pyrophosphatase [22] and *Aae* pyrophosphatase [5] are homotetramers, while *S. cerevisiae* pyrophosphatase is a homodimer [6].

Properties of Purified *Tca* Pyrophosphatase

The effect of temperature on *Tca* pyrophosphatase activity was determined at a range of 55–100°C. The optimum temperature was found to be approximately 80°C (Fig. 3A), therefore, the optimum temperature for *Tca* pyrophosphatase was similar to those for *Taq* pyrophosphatase [22] and *Tth* pyrophosphatase [17]. The thermostability of the enzyme was tested at two different temperatures: 80°C and 90°C. The enzyme was comparatively stable at 80°C; however, at temperatures above 90°C the thermostability of the enzyme decreased slowly (Fig. 3B). The half-life at 90°C was 6 h. Thr relationship between *Tca* pyrophosphatase activity and pH was determined using buffers of 50 mM MES (pH 5.5–6.5), 50 mM Tris-HCl (pH 7.0–9.0), and 50 mM Glycine-NaOH (pH 9.0–10.0). The optimum pH for the activity was found to be in the range of 8.0–8.5 in Tris-HCl (Fig. 3C). Thus, the optimum pH of *Tca* pyrophosphatase was similar to those of *Taq* pyrophosphatase (pH 8.3) [22] and *Tth* pyrophosphatase (pH 7.8) [17]. At pH below 6.0 and above 9.0, the activity decreased drastically.

Table 2. Effect of various substances on *Tca* pyrophosphatase.

Substance	Relative activity (%)
Control	100
$CaCl_2$	28
$CoCl_2$	315
$CuCl_2$	311
$MgCl_2$	371
$MnCl_2$	298
$ZnCl_2$	304
EDTA	24
DTT	167

The activities were measured at 80°C in 50 mM Tris-HCl (pH 8.0). Each substance was tested at a concentration of 1 mM in the presence of 1.7 mM sodium pyrophosphate.

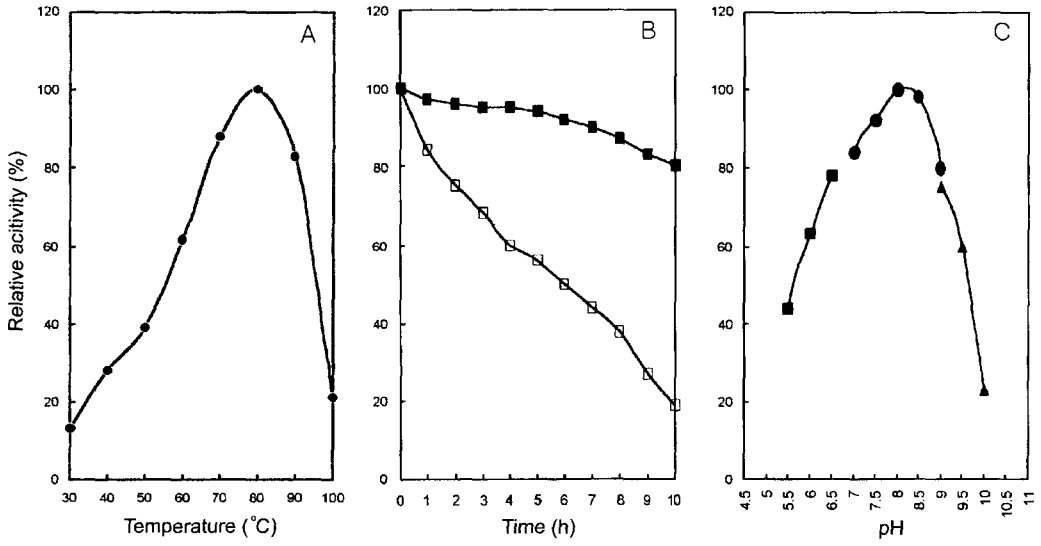

Fig. 3. Properties of *Tca* pyrophosphatase.
The enzyme activity was assayed at the indicated pH and temperature in the presence of 1 mM $MgCl_2$. A: Effects of temperature on *Tca* pyrophosphatase activity in 50 mM Tris-HCl (pH 8.0). B: Thermostability of *Tca* pyrophosphatase in 50 mM Tris-HCl (pH 8.0): 80°C (■) and 90°C (□). C: Effect of pH on *Tca* pyrophosphatase activity: 50 mM MES (■), 50 mM Tris-HCl (●), and 50 mM GlycineNaOH (▲).

The effects of various compounds at 1 mM concentration were tested on the hydrolysis of inorganic pyrophosphate. The activity of *Tca* pyrophosphatase was activated by Mg^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+}, yet inhibited by Ca^{2+} and EDTA (Table 2). This dependence of the catalytic activity on Mg^{2+} has already been observed in the majority of pyrophosphatases investigated [5, 10, 14, 17, 22]. It should also be noted that divalent metal ions were necessary for catalysis.

In conclusion, *Tca* pyrophosphatase from *Thermus caldophilus* GK24 was cloned and characterized. The present study, therefore, provides an excellent model for gene expression studies of thermostable pyrophosphatases and simple purification of such an enzyme in an *E. coli* system. These enzymes are well suited for cycle sequencing and long-range PCRs using thermostable DNA polymerase.

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