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Cloning, Analysis, and Expression of the Gene for Thermostable Polyphosphate Kinase of *Thermus caldophilus* GK24 and Properties of the Recombinant Enzyme

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Abstract The gene encoding *Thermus caldophilus* GK24 polyphosphate kinase (*Tca* PPK) was cloned and sequenced. The gene contains an open reading frame encoding 608 amino acids with a calculated molecular mass of 69,850 Da. The deduced amino acid sequence of *Tca* PPK showed a 40% homology to *Escherichia coli* PPK, and 39% to *Klebsiella aerogenes* PPK. The *Tca ppk* gene was expressed under the control of the T7*lac* promoter on pET-22b(+) in *E. coli* and its enzyme was purified about 70-fold with 36% yield, following heating and HiTrap chelating HP column chromatography. The native enzyme was found to have an approximate molecular mass of 580,000 Da and consisted of eight subunits. The optimum pH and temperature of the enzyme were 5.5 and 70°C, respectively. A divalent cation was required for the enzyme activity, with Mg^{2+} being the most effective.

Key words: Polyphosphate kinase, *Thermus caldophilus* GK24, *Tca* PPK, gene cloning, gene expression

Inorganic polyphosphate (poly P) is a linear polymer of orthophosphate residues linked by high-energy phosphoanhydride bonds [9]. The enzyme responsible for the synthesis of poly P in bacteria is polyphosphate kinase (PPK), which catalyzes the reversible transfer of the terminal phosphate of ATP to form a long chain poly P [7]. The activities of PPK include, in addition to the synthesis of poly P from ATP, the conversion of ADP back to ATP in an apparent reversal of the reaction, and the conversion of other nucleotides, especially GDP to GTP [8].

The best-studied PPK is that from *Escherichia coli*. The *Escherichia coli ppk* gene [3] contains an open reading frame of 2,064 bp which translates to a protein of 688 amino acid residues with a calculated molecular mass of 80,278 Da. Seven base pairs downstream from the carboxyl terminus of PPK is the gene (*ppx*) for exopolyphosphatase (PPX). The two genes, *ppk* and *ppx*, constitute an operon; interruption of *ppk* results in the loss of *ppx* expression. The promoter region contains two putative PhoB boxes, the regulatory factor in the Pho regulon, which responds to low inorganic phosphate levels in the medium [4].

Most remarkably, recent determinations of the DNA sequences of diverse bacterial genomes have revealed a high degree of PPK conservation [8]. These include some of the major pathogens including *Pseudomonas aeruginosa*, *Salmonella dublin*, *Neisseria meningitides*, and *Mycobacterium tuberculosis* [8]. However, a search of other microbial sequences has shown no significant PPK homologies. These include *Bacillus subtilis*, *Streptococcus pyogenes*, *Thermotoga martima*, and *Saccharomyces cerevisiae* [8]. The absence of PPK in these species and the assured presence of poly P in all cells argue for a pathway of poly P synthesis other than PPK. Indeed, such an auxiliary activity has been detected in extracts of *ppk* mutants of several bacterial species and has been purified from *Klebsiella pneumoniae* [8].

PPKs produced by thermophiles are of interest from a biotechnological perspective and are investigated not only in scientific areas like thermostability, but also for application in enzymatic ATP-regenerating systems [13]. Thermostable PPKs have also been recently applied in a practical synthesis of the oligosaccharide, *N*-acetyllactosamine [14]. Although PPKs and their genes have been studied in *E. coli* and pathogenic bacteria, there are no reports of thermostable PPK from genus *Thermus*. *T. caldophilus* GK24 was isolated from the Kawamata Hot Spring, Japan and can grow well within a temperature range of 70–75°C with a maximum at 82°C [5].

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Accordingly, in the current paper, we reports on the cloning and nucleotide sequence of the *ppk* gene from *T. caldophilus* GK24, its expression in *E. coli*, and the purification and properties of recombinant *Tca* PPK.

MATERIALS AND METHODS

Strains and Reagents

T. caldophilus GK24 cells were prepared as described previously [5, 22]. *E. coli* BL21 was used as the host for the plasmid preparations and gene expression. [γ-^{32}P]ATP (3 Ci mmol^{-1}) and HiTrap chelating HP column were purchased from Amersham Bioscience. Other reagents were obtained from Sigma.

Preparation of DNA Libraries from *T. caldophilus* GK24

Chromosomal DNA was isolated from *T. caldophilus* GK24 as described previously [12]. A genomic DNA library for *T. caldophilus* GK24 was constructed in pWE15 cosmid vector (Stratagene, Cambridge, U.K.). DNA fragments were partially digested with *Sau*3AI, and 35- to 40-kb fragments were fractionated by sucrose density gradient centrifugation and ligated to the *Bam*HI site of the pWE15 cosmid vector. The mixture was packaged according to the manufacturer's instructions, and *E. coli* was infected with the packaged mixture. Screening for gene clusters related to phosphate metabolism was carried out as described by Sambrook *et al.* [18] using the *T. caldophilus* GK24 (*Tca*) alkaline phosphatase gene [15] randomly labeled with ^{32}P. A positive clone (named cos #250), which hybridized with the probe, was identified by screening genomic libraries.

DNA Sequencing and Computer-Assisted Analysis

The restriction fragments being sequenced were cloned into the appropriate restriction sites of pBluescript SK+/– vectors. The DNA sequencing was performed with a DNA sequencer ABI PRISM 377 using BigDye Terminator Cycle Sequencing Ready Reaction Kits (PE Biosystems, Foster City, U.S.A.). The sequence data were analyzed using DNASIS and PROSIS. The Basic Local Alignment Search Tool (BLAST) search system was used for the sequence comparisons.

Construction of the pTCPPK for Expression of *Tca ppk* Gene

Based on the DNA sequence of the *Tca ppk* gene, two primers were synthesized. The 5' primer, PPK-N, 5'-GGG-GCATATGCACCTCCTTCCC-3', containing a unique *Nde*I site (underlined) includes an ATG starting site of translation, followed by the sequence encoding the first five amino acids of the NH_2-terminal sequence. The 3' primer, PPK-C, 5'-NNNNGTCGACTAGCTCCAGGCGCTGGGCG-3', containing a unique *Sal*I site (underlined) matches the region encoding the last six amino acids of the carboxyl-terminal sequence. This 3' primer makes the protein message in frame with the sequence of the hexahistidine tag present in the pET-22b(+) vector (Novagen, Madison, U.S.A.). The DNA amplification was performed using 2.5 units of *Taq* DNA polymerase in a 50 µl reaction mixture of a PCR reaction buffer, 2.5 pmol of the primers, 0.2 mM each of dNTP, and 0.1 µg of *T. caldophilus* GK24 genomic DNA, as described by Saiki *et al.* [17]. A predicted 1.827 kb fragment containing the *Tca ppk* gene was digested with *Nde*I and *Sal*I, purified from 0.8% low-melting-agarose gel, and ligated into an expression vector pET-22b(+) that had been digested with the same enzymes. The ligate was transformed into *E. coli* BL21. Clones with the correct construction of the expression vector for the *ppk* gene were selected and designated plasmid pTCPPK.

Expression and Purification of the Hexahistidine-Tagged *Tca* PPK in *E. coli*

An overnight culture of 50 ml of *E. coli* BL21 harboring the recombinant plasmid pTCPPK grown in an L-broth containing 50 µg ml^{-1} ampicillin was transferred to 2 liter of the same medium. When the A_{600} of the culture was about 0.8, the cultures were induced by the addition of IPTG to a final concentration of 0.2 mM and then the cells were grown for an additional 6 h. The cells were collected by centrifugation, suspended in buffer A [10 mM sodium phosphate buffer (pH 6.0), 1 mM $MgCl_2$, 1 mM PMSF], and disrupted by sonication. Sonication was performed three times on ice for 1 min with 1-min intervals for cooling. The sonicate was incubated at 80°C for 30 min. After centrifugation, the supernatant was dialyzed against buffer B [20 mM sodium phosphate (pH 7.4), 0.5 M NaCl], then applied to a HiTrap chelating HP column (Amersham Bioscience) packed with 5 ml of chelating Sepharose that had been equilibrated with buffer B. The elution of adsorbed protein was performed with a linear gradient of imidazole (0–0.5 M) in 120 ml of buffer C [20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 0.5 M imidazole]. The fractions (total 15 ml) showing PPK activity were pooled and dialyzed against buffer D [10 mM MES buffer (pH 5.5), 1 mM $MgCl_2$, 1 mM PMSF]. The enzyme fraction with PPK activity was then assayed and analyzed. Protein concentration was determined by the procedure of Lowry *et al.* [11] with bovine serum albumin as a standard protein.

Enzyme Assay for PPK

The PPK activity was determined by measuring the production of acid-insoluble [^{32}P] poly P as described previously with minor modifications [2]. The reaction mixture (20 µl) contained 50 mM MES buffer (pH 5.5), 40 mM ammonium sulfate, 4 mM $MgCl_2$, 2 mM [γ-^{32}P]ATP (3 Ci mmol^{-1}), and enzyme solution. After incubation for 10 min at 70°C, the

reaction was stopped by the addition of 4 µl of 7% $HClO_4$ and 20 µl of 2 mg ml^{-1} bovine serum albumin, and the mixture was incubated on ice for 10 min. The mixture was spotted on a Whatman DE-81 filter paper disk (23 mm), dried on a heat block and then washed with a solution of 0.1 M pyrophosphate followed by ethanol. The amount of [γ-^{32}P]ATP incorporation was measured on the dried filter paper disk with a Beckman LS 6500 scintillation system. One unit of enzyme is defined as the amount incorporating 1 pmol of phosphate into acid-insoluble poly P per min.

Determination of Molecular Mass and Electrophoretic Analysis

The molecular mass of native PPK was determined by Biologic Workstation (Bio-Rad) on the Bio-Prep SE-1000/17 column (0.8×30 cm) equilibrated with buffer A containing 200 mM NaCl. The molecular mass in native state was estimated using the Bio-Rad size exclusion standard (Bio-Rad, catalog number 151–1901). The Biologic Workstation was kept at a constant flow rate of 0.5 ml/min at room temperature during elution. The PPK molecular mass was calculated by plotting the log versus the elution volume/void volume ratio [1].

RESULTS AND DISCUSSION

Molecular Cloning of the *Tca ppk* Gene

To search the gene cluster related to polyphosphate metabolism, the labeled structural gene that codes for *Tca* alkaline phosphatase was used as a hybridization probe [15]. Colony hybridization of the cosmid libraries of *T. caldophilus* GK24 genomic DNA, performed at a high stringency, revealed a cosmid clone (cos #250) showing a prominent reaction with the probe (results not shown). Accordingly, two samples of cos #250 clone including

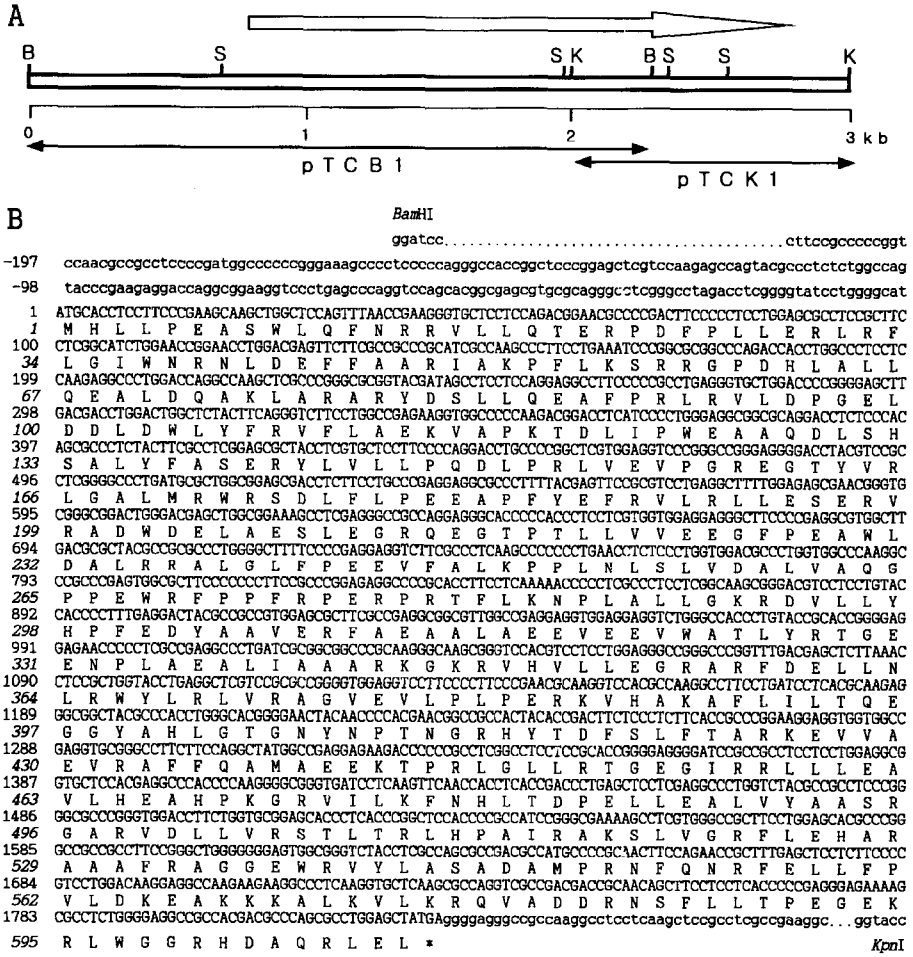

Fig. 1. Structure of *ppk* gene of *T. caldophilus* GK24 and its nucleotide sequence.
(A) Restriction map of *Tca ppk* gene and positions of the cloned DNA fragments in plasmid pTCB1 (2.3 kb *Bam*HI) and pTCK1 (0.9 kb *Kpn*I). Open arrow indicates the coding region of the *Tca ppk* gene. The restriction enzyme sites are shown: B, *Bam*HI; K, *Kpn*I; S, *Sac*I. (B) The nucleotide and deduced amino acid sequence of the *Tca ppk* gene. Numbers on the left refer to the nucleotide and amino acid sequence. Asterisk indicates the stop codon. The nucleotide sequence of the *Tca ppk* gene was deposited in GenBank with accession number AY126478.

about 40 kb of *T. caldophilus* GK24 genomic DNA were separately digested with *Bam*HI and *Kpn*I, followed by separation by a low-melting-agarose gel electrophoresis, and the DNA fragments were separately collected from each region. Each *Bam*HI fragment and *Kpn*I fragment were separately ligated at the *Bam*HI site and *Kpn*I site in the multiple cloning site of pBluescript SK-, respectively, and then *E. coli* strain MV1184 was transformed with the plasmids. The above plasmids including *Bam*HI fragments and *Kpn*I fragments were used for partial sequencing analysis. Sequence data were submitted for analysis of sequence homology against the nonredundant GenBank database using BLASTX. The sequences corresponding to about 2.3 kb *Bam*HI fragment (clone pTCB1) and about 0.9 kb *Kpn*I fragment (clone pTCK1) were found to be homologous to PPK from *E. coli*.

Nucleotide Sequence of the *Tca ppk* Gene and Its Deduced Amino Acid Sequence

The restriction maps of the 2.3 kb *Bam*HI fragment and 0.9 kb *Kpn*I fragment are presented in Fig. 1a. Each enzyme site in the restriction map was used for the subcloning and DNA sequencing of the cloned DNA fragments. A search of the nucleotide sequence of the right region for the 2.3 kb *Bam*HI fragment (clone pTCB1) showed high homology to that of genes encoding microbial PPK. However, a search of the nucleotide sequence of the left region for the 2.3 kb *Bam*HI fragment (clone pTCB1) showed high homology to that of genes encoding microbial PPX. PPX removes poly P to maintain the dynamic balance of the poly P level in *E. coli* cells. Accordingly, the *ppk* and *ppx* in genus *Thermus* exist in two adjacent genes, such as *E. coli* [4] or *Pseudomonas aeruginosa* 8830 [23]. The position of the *Tca ppk* gene in the cloned fragments is indicated by the open arrow. Figure 1b shows the partial nucleotide sequence of the cloned DNA fragments and deduced amino acid sequence of *Tca* PPK, which was comprised of 608 amino acid residues with a calculated molecular mass of 69,850 Da. This gene encoding *Tca* PPK is slightly smaller than that of *Escherichia coli* PPK, which translates to a protein of 688 amino acid residues with a calculated molecular mass of 80,278 Da [3]. The

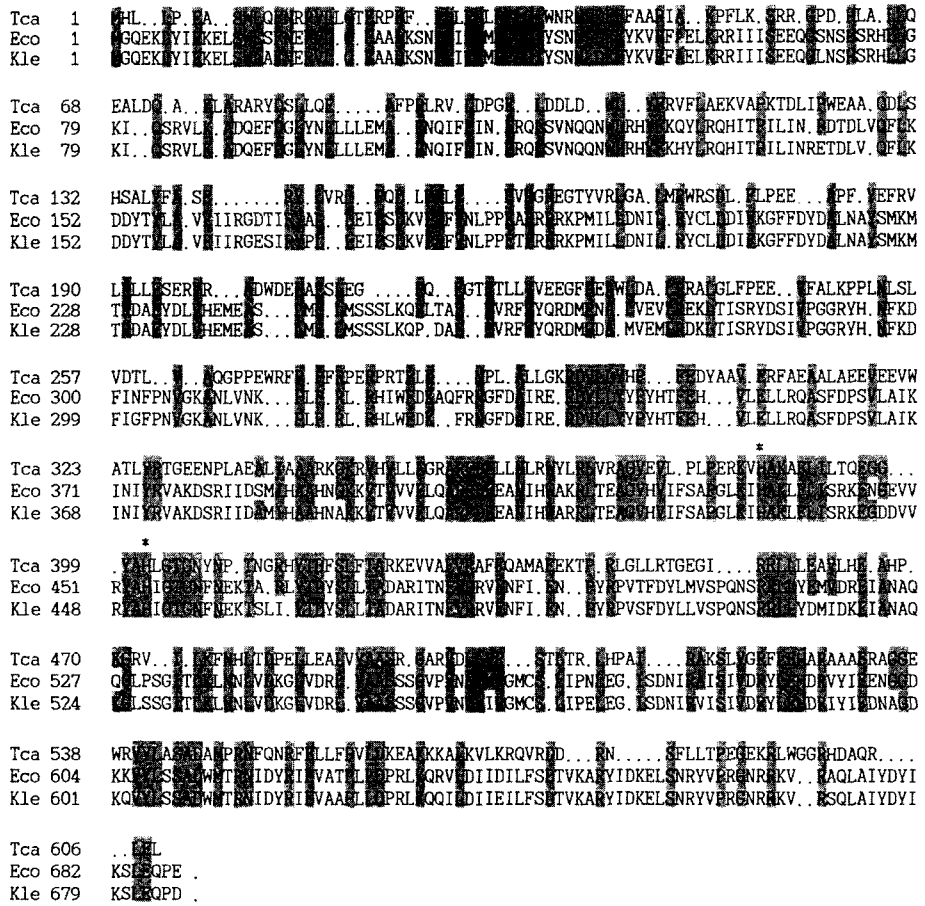

Fig. 2. Comparison of the amino acid sequence of *Tca* PPK with those of other PPKs.
The sequence of *Tca* PPK (Tca) is shown in comparison with those of *E. coli* PPK (Eco) [3] and *Klebsiella aerogenes* PPK (Kle) [6]. Identical amino acids between *Tca* PPK and others are indicated by dark-shaded boxes. (*) Amino acids considered to be involved in the catalytic action of *E. coli* PPK [10].

promoter-like sequence in the -35 region and the -10 region, which can function in *E. coli*, was not found in the upstream flanking region of the *Tca ppk* gene. The potential ribosome binding sequence, which was homologous to the consensus Shine-Dalgarno sequence [19], was not found. The G+C content of the coding region for the *Tca ppk* gene was 67% (results not shown). The codon usage in *Tca* PPK was heavily biased towards the use of G and C in the third position (93.6% G+C), as would be expected for *Thermus* species with G+C rich DNA [10].

The *ppk* gene has been also cloned and sequenced in several species, such as *E. coli* [3], *Acinetobacter* sp. strain ADP1 [21], *Klebsiella aerogenes* [6], and *Pseudomonas aeruginosa* 8830 [23]. The deduced amino acid sequence of *Tca* PPK exhibited a 40% identity with *E. coli* PPK [3], and 39% with *Klebsiella aerogenes* PPK [6] (Fig. 2). Two histidine residues (His435 and His454) necessary for PPK activity in *E. coli* [10] (His386 and His401 in the *Tca* PPK amino acid sequence) are strictly conserved throughout the family (Fig. 2). It has been suggested that the two histidine residues of *Tca* PPK play a crucial role for PPK activity in the phosphorylation, converting the γ-phosphate of ATP into poly P chains.

Construction of the Recombinant Vector Producing *Tca* PPK

The pET-22b(+) vector has a very strong T7*lac* promoter, a ribosome binding site, and a His-tag cluster, which was used to accelerate the protein purification. The *Tca ppk* gene was amplified through the PCR by using primers PPK-N and PPK-C. The amplified 1.827 kb DNA fragment was ligated into an expression vector pET-22b(+) using *Nde*I and *Sal*I sites to obtain pTCPPK. The obtained genetic construct retained the open reading frame, and the target protein contained 15 additional amino acid residues at the C-terminus, including a cluster of 6 histidine residues to allow purification of the recombinant protein by metal affinity chromatography.

Purification of the *Tca* PPK

The *E. coli* BL21 harboring pTCPPK was cultured in the presence of IPTG and about 6.9 g of wet cell paste was obtained from 2 liter of culture. His-tagged *Tca* PPK was expressed in a soluble form. The purity of *Tca* PPK was examined by SDS-PAGE (Fig. 3). It was found that this *Tca* PPK is thermostable, therefore, the cell lysate was heat treated at 80°C for 30 min before the chromatography step.

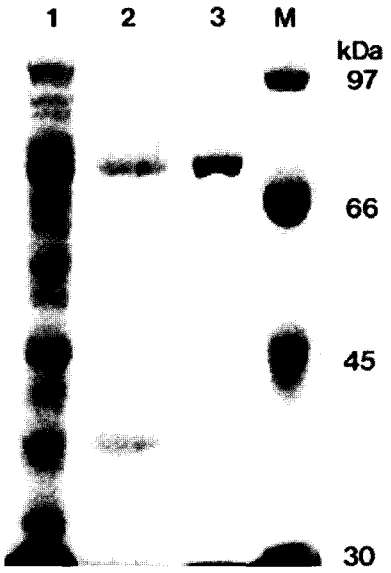

Fig. 3. SDS-PAGE analysis of *Tca* PPK.
The electrophoresis was performed on a vertical gel containing polyacrylamide using a Mighty Small Kit II system (Hoffer Scientific Instruments). Lane 1, sonicated extract of induced culture (*E. coli* BL21/pTCPPK); lane 2, the same sample heated at 80 °C for 30 min and cleared by centrifugation; lane 3, purified by HiTrap chelating HP column chromatographyl; lane M, low-molecular-mass markers (molecular masses are indicated at the right).

The heat denaturation step resulted in the precipitation of the majority of proteins originating from *E. coli* (Fig. 3, lane 2). Several *E. coli* proteins still remained soluble after the heating step. For the final purification, the clarified supernatant was chromatographed on a HiTrap chelating HP column. The recombinant enzyme was eluted from the column as a single peak (Fig. 3, lane 3). A band corresponding to a 71,000 Da protein was observed in SDS-PAGE, and this molecular mass showed a good agreement with 69,850 Da calculated from the deduced amino acid sequence.

The recombinant *Tca* PPK was readily purified to homogeniety in two steps. The purification of the enzyme is summarized in Table 2. The specific activity of the purified enzyme sample was over 70 times higher than that of the crude extracts, and the recovery of the enzyme was about 36% on the basis of the activity in the crude extracts.

The molecular mass of native enzyme was also determined to be 580,000 Da by gel filtration on a column of Bio-Prep SE-1000/17 (results not shown). These results suggest that the enzyme with *Tca* PPK activity is an octamer in its native state. The native state of *Tca* PPK was different from

Table 1. Purification of *Tca* PPK expressed in *E. coli*.

Purification steps	Total protein (mg)	Total activity (units×10^{-2})	Specific activity (units×10^{-4} mg^{-1})	Recovery (%)
Sonicated extract	772	14.1	1.83	100
Heat-treatment	28	5.5	19.6	39
HiTrap chelating HP column	4	5.1	129	36

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

Substance (1 mM)	Relative activity (%)
Control	100
$CaCl_2$	42.8
$CoCl_2$	364.5
$CuCl_2$	58.7
EDTA	23.6
$MgCl_2$	370.9
$MnCl_2$	88.7
$ZnCl_2$	98.6

Activities were measured at 70°C in 50 mM MES (pH 5.5).

those of *E. coli* PPK and *Propionibacterium shermanii* PPK: for example, *E. coli* PPK is a homotetrameric protein that is associated with the outer membrane consisting of four subunits [2], while *P. shermanii* PPK is a monomer [16].

Characterization of the Recombinant *Tca* PPK

The effect of temperature on *Tca* PPK activity was determined within a range of 40–90°C. The optimum temperature was found to be approx. 70°C (Fig. 4A). The thermostability of the enzyme was tested at two different temperatures: 70°C and 80°C (Fig. 4B). The enzyme was comparatively stable at 70°C, however, at temperatures above 80°C the thermostability of the enzyme decreased slowly. The dependence of *Tca* PPK activity on the pH was determined between pH 4.0–9.0. The buffers used were 50 mM sodium citrate (pH 4.0–5.0), 50 mM MES (pH 5.0–7.0), 50 mM HEPES (pH 7.0–8.0), and 50 mM glycine-NaOH (pH 8.0–9.0). The optimum pH for *Tca* PPK activity was observed at 5.5 (Fig. 4B), indicating that the optimum pH for *Tca* PPK activity is slightly different from that observed in *E. coli* PPK [2] and *Neisseria meningitides* PPK [20]. Optimum pH for *E. coli* PPK and *N. meningitides* PPK was found to be between 6.2 and 7.2.

The effects of various compounds (1 mM concentration) were tested on the synthesis of the polyphosphate. The activity of *Tca* PPK was activated by Mg^{2+} and Co^{2+}, but inhibited by Ca^{2+}, Cu^{2+}, Mn^{2+}, and EDTA (Table 2). This dependence of the catalytic activity on Mg^{2+} has already been observed in the majority of PPKs [2, 20].

In conclusion, the gene encoding *Tca* PPK was for the first time cloned from the genus *Thermus*, and its recombinant enzyme was characterized. The present study, therefore, provides a model for gene expression studies of thermostable PPKs and simple purification of such an enzyme in a *E. coli* system for industrial application. PPK, including PPX, would also be one of the major enzymes related to the polyphosphate metabolism in genus *Thermus*. We are presently screening the complete gene encoding PPX as a hybridization probe using the 2.3 kb *Bam*HI fragment.

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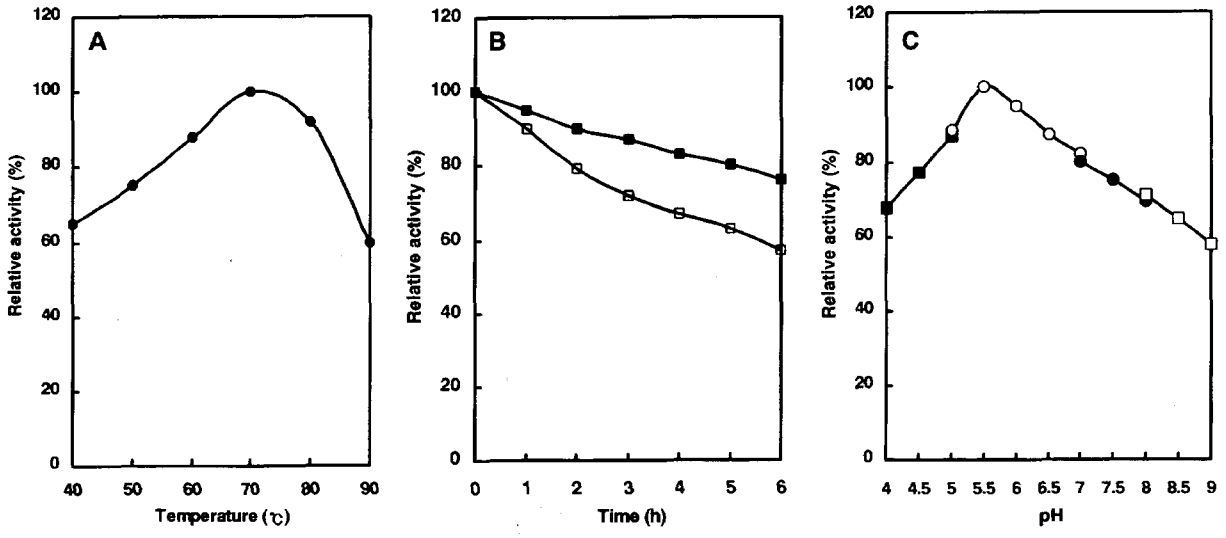

Fig. 4. Properties of recombinant *Tca* PPK.

The enzyme activity was assayed at the indicated pH and temperature in 1 mM $MgCl_2$. A: Effects of temperature on *Tca* PPK activity in 50 mM MES (pH 5.5). B: Thermostability of *Tca* PPK in 50 mM MES (pH 5.5) at 70°C (■) and 80°C (□). C: Effect of pH on *Tca* PPK activity in 50 mM Sodium citrate (■), 50 mM MES (○), 50 mM HEPES (●), and 50 mM Glycine-NaOH (□).

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