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Cloning, Expression, and Characterization of a Hyperalkaline Phosphatase from the Thermophilic Bacterium *Thermus* sp. T351

CHOI, JEONG JIN, JONG WOO PARK, HYEKYUNG SHIM, SUKCHAN LEE, MOOSIK KWON, JOO-SUNG YANG, HEON HWANG[1], AND SUK-TAE KWON*

Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, Korea
[1]Department of Biomechatronic Engineering, Sungkyunkwan University, Suwon 440-746, Korea

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Abstract The gene encoding *Thermus* sp. T351 alkaline phosphatase (T351 APase) was cloned and sequenced. The gene consisted of 1,503 bp coding for a protein with 500 amino acid residues including a signal peptide. The deduced amino acid sequence of T351 APase showed relatively low similarity to other *Thermus* APases. The T351 APase gene was expressed under the control of the T7*lac* promoter on the expression vector pET-22b(+) in *Escherichia coli* BL21(DE3). The expressed enzyme was purified by heat treatment, and UNO™ Q and HiTrap™ Heparin HP column chromatographies. The purified enzyme exhibited high activity at extremely alkaline pHs, reaching a maximum at pH 12.0. The optimum temperature of the enzyme was 80°C, and the half-life at 85°C was approximately 103 min. The enzyme activity was found to be dependent on metal ions: the addition of Mg^{2+} and Co^{2+} increased the activity, whereas EDTA inhibited it. With *p*-nitrophenyl phosphate as the substrate, T351 APase had a Michaelis constant (K_m) of 3.9×10^{-5} M. The enzyme catalyzed the hydrolysis of a wide variety of phosphorylated compounds.

Key words: Alkaline phosphatase, sequence analysis, thermostable enzyme, *Thermus* sp. T351

Alkaline phosphatase (APase, EC 3.1.3.1) is a nonspecific phosphomonoesterase that catalyzes the hydrolysis of phosphate esters, being most active at alkaline pH [6, 28]. The enzymes are present in various organisms, ranging from prokaryotes to eukaryotes, with the exception of some higher plants. APase is the most essential enzyme for the survival of organisms under inorganic phosphate starvation, and functions in phosphate metabolism and transport mechanisms [20, 38].

*Corresponding author
Phone: 82-31-290-7863; Fax: 82-31-290-7870;
E-mail: stkwon@yurim.skku.ac.kr

Escherichia coli APase has been most extensively studied [5, 6, 28]. The active *E. coli* APase is a homodimeric protein of 94,000 Da, in which each of the mature monomers contains 449 amino acid residues and has one catalytic site [1, 2]. The enzyme is synthesized as a precursor monomer with a signal peptide at the amino-terminal end. Upon translocation to the periplasmic space, the signal peptide is removed, and two of the resulting mature monomers dimerize to give the active enzyme. An important feature in the catalytic site of *E. coli* APase is the presence of three metal ions, two atoms of Zn^{2+} and one atom of Mg^{2+}, and these metal ions play a key role in the enzyme activity [13]. Based on the X-ray crystal structure of *E. coli* APase, a reaction mechanism involving metal ion-assisted catalysis has been described [13, 33].

Thermostable enzymes have been the subject of considerable interest for both academic research and industrial applications. *E. coli* APase and calf intestine APase have been most commonly used in the application fields of APase, but their low catalytic activity under high temperature has restricted their further applications; for these reasons, thermostable APase is now receiving much attention [9]. To date, a number of thermostable APases have been identified and characterized from (hyper)thermophilic eubacteria and archaebacteria, including *Bacillus stearothermophilus* [21], *Thermus* species [9, 26, 37], *Thermotoga* species [8, 36], and *Pyrococcus abyssi* [38]. Each enzyme has somewhat different characteristics in regard to optimum pH and temperature, metal ion dependency, substrate specificity, and so on.

APases are valuable in practical applications. The enzymes have been mainly used in molecular cloning to remove 5'-phosphates from linearized vector for preventing self-ligation [19]. The enzymes are also suited for probing, blotting, nonradioactive detection techniques, and sequencing systems [19]. In particular, the 5'-recessive termini of linearized vector

can easily be dephosphorylated by thermostable APase at high temperature; oligonucleotides conjugated directly to thermostable APase can be used as DNA probes for hybridization at high temperature. In addition to applications in molecular biology, APases have good utility in immunology and diagnostics, such as a reporter in enzyme immunoassays and as a biochemical marker in quantitative measurements of disease [27, 35]. The increasing number of applications that employ APase-based technique has generated an increasing demand for various APases, including thermostable APases.

Extremely thermophilic bacteria belonging to the genus *Thermus* can routinely be grown at temperatures of 70–75°C. A few enzymes have been identified and characterized from *Thermus* sp. T351; however, no information is available on the sequence and characteristics of a thermostable APase from this organism. In this study, we describe the cloning, sequence analysis, and expression of the gene encoding *Thermus* sp. T351 APase (T351 APase). We also report the purification and properties of the enzyme showing high activity at extremely alkaline pHs.

MATERIALS AND METHODS

Strains and Culture Conditions

Thermus sp. T351 (ATCC 31674) was obtained from the American Type Culture Collection. The *Thermus* sp. T351 cells were grown in ATCC medium 461 at 75°C for 18 h with vigorous shaking.

E. coli MV1184 was used for plasmid propagation and nucleotide sequencing. *E. coli* BL21(DE3), which harbours the T7 RNA polymerase gene under the control of a chromosomal *lac*UV5 gene [34], was used for gene expression. The *E. coli* cells were grown in Luria-Bertani (LB) medium with appropriate antibiotics at 37°C with vigorous shaking.

Genomic DNA Isolation, Hybridization, and DNA Walking PCR

The genomic DNA of *Thermus* sp. T351 was isolated by the method of Marmur [18] with slight modifications. The probe used for the DNA-DNA hybridization to detect the T351 APase gene was prepared from the expression plasmid pEAP1 [16], containing the *T. caldophilus* GK24 (*Tca*) APase gene (GenBank accession number AF168770) [26], and was labeled with [α-^{32}P]dCTP by random priming. DNA hybridization was performed by the agarose gel membrane hybridization method [32]. *Thermus* sp. T351 genomic DNAs digested with three restriction enzymes (BamHI, HindIII, or KpnI) were fractionated on 0.7% agarose gel, and the probe was hybridized at 53°C to the fractionated genomic DNA on the agarose gel membrane. Colony hybridization was done according to the standard procedures [10], using the aforementioned ^{32}P-labeled probe. DNA inserts of positive clones were characterized following plasmid DNA extraction.

DNA walking PCR was performed using DNA Walking *SpeedUp*™ Premix Kit (Seegene, Korea) to clone the residual region of the T351 APase gene, which had not been obtained from the hybridized DNA. The experiment was conducted according to the supplier's manual with three target-specific primers, TSP1 (5'-GGCGAAGAAGGGC-TTCAG-3'), TSP2 (5'-CAGCCCCCCGTTCACCGT-3'), and TSP3 (5'-CGCGCTGGACTCGGTGAC-3'). A 0.6-kb fragment was amplified by the consecutive PCR, and was then purified from 2% low-melting agarose gel.

DNA Sequencing and Sequence Analysis

The nucleotide sequences of the hybridized DNA and purified PCR product were determined using an Applied Biosystems 3730 DNA sequencer (U.S.A.). The obtained sequences were compared with known proteins in the database, using the BLAST sequence comparison program. Nucleotide and deduced amino acid sequence analyses were performed by using the DNASIS (Hitachi Software Engineering, Japan) and PCGENE (Intelligenetics, U.S.A.) softwares. The MultAlin program [7] was used to conduct multiple sequence alignment between functionally related proteins.

Construction of Expression Plasmid

Most of the methods used for plasmid construction were predicated on those described by Sambrook *et al.* [29]. For the expression of T351 APase, the APase gene was amplified by direct PCR of the *Thermus* sp. T351 genomic DNA. On the basis of the nucleotide sequence of the T351 APase gene, two primers were synthesized: the 5' (N-terminal) primer YAPNN, 5'-AGGTGAACATATGAAGCG-GAGGGATCTTCTG-3', added a unique NdeI site (underlined), which has the translation initiation codon, ATG; and the 3' (C-terminal) primer YAPCS, 5'-TCCAGTCGACCTAAG-CCAGGGCGTCTTGC-3', which matches the C-terminal sequence including the stop codon, added a unique SalI site (underlined). The amplified fragment containing the T351 APase gene was digested with NdeI and SalI, purified from 1% low-melting agarose gel, and ligated into the expression vector pET-22b(+) (Novagen, U.S.A.) that had been digested with the same enzymes. *E. coli* BL21(DE3) was transformed with the ligate by electroporation [12]. Clones with the correct construct were selected by the restriction enzyme analysis of plasmid minipreps.

Purification of the Expressed Enzyme

A 20 ml of overnight culture of *E. coli* BL21(DE3) harboring the relevant expression plasmid, which was grown in LB broth containing 100 μg/ml ampicillin, was transferred to 2 l of the same medium. This culture was grown at 37°C

until an A_{600} of 0.8 was reached. Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was grown for another 6 h [4, 16]. The cells were harvested by centrifugation and resuspended in buffer A (20 mM Tris-HCl, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The resuspended cells were disrupted by sonication and centrifuged at 35,000 ×*g*, 4°C for 20 min to remove *E. coli* cell walls and insoluble debris. The nucleic acids in the sonicated extract were precipitated by 1% streptomycin sulfate at room temperature for 30 min, and were removed by centrifugation at 35,000 ×*g*, 4°C for 20 min. The majority of heat-labile *E. coli* proteins were eliminated by heat treatment at 80°C for 30 min. After removal of the denatured proteins by centrifugation at 35,000 ×*g*, 4°C for 20 min, the supernatant was dialyzed against buffer A, and was then applied onto a UNO™ Q column (Bio-Rad Laboratories, U.S.A.) preequilibrated with buffer A. The column was washed with 10 column volumes of buffer A, and protein was eluted with a linear gradient of 0–1 M NaCl prepared in buffer A. Major fractions containing the desired protein were pooled and dialyzed against buffer A. T351 APase was further purified by a HiTrap™ Heparin HP column (Amersham Biosciences, Sweden). After loading of the sample, the column was washed with 10 column volumes of buffer A, and was developed with a linear gradient of 0–1 M NaCl prepared in buffer A. The purified enzyme was desalted by dialysis against buffer A.

Protein concentration was determined by the method of Lowry *et al.* [17] with bovine serum albumin (BSA) as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, as previously described by Laemmli [15], with 10% polyacrylamide gel.

APase Activity Assay

The APase activity of the purified enzyme was measured as described by Lee *et al.* [16]. The basic reaction mixture (1 ml) contained 50 mM Glycine-NaOH (pH 11.0), 1 mM $MgCl_2$, 1 mM *p*-nitrophenyl phosphate (*p*NPP), and enzyme solution. This mixture was incubated at 75°C for 10 min, and the reaction was terminated by the addition of 0.2 ml of 2 N NaOH. Enzyme blanks were equally assayed along with the corresponding enzyme-contained samples, and were used as a control for each reaction in spectrophotometry. The extent of hydrolysis was determined from the absorbance of the liberated *p*-nitrophenol (*p*NP) at 410 nm using an extinction coefficient of 1.77×10^4 M^{-1} cm^{-1} [25]. One unit of T351 APase is defined as the amount of enzyme required to liberate 1 μmol of *p*NP from *p*NPP in 1 min under the optimal reaction condition.

Kinetic Parameter

The Michaelis constant (K_m) of T351 APase for *p*NPP was measured at 80°C in the optimal buffer composed of 50 mM Glycine-NaOH (pH 12.0) and 2 mM $MgCl_2$. The K_m value was determined from the Lineweaver-Burk plot.

Substrate Specificity

The hydrolysis of various substrates, including nonchromogenic ones, by T351 APase was carried out at 80°C for 10 min in 50 mM Glycine-NaOH (pH 12.0) buffer containing 2 mM $MgCl_2$. The substrates used were as follows: *p*NPP, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), AMP, CMP, GMP, UMP, ATP, dAMP, α-naphthyl acid phosphate, bis(*p*-nitrophenyl) phosphate, and glycerophosphate. The inorganic phosphate released was measured by the procedure of Chen *et al.* [3].

Results and Discussion

Cloning, Sequencing, and Sequence Analysis of the T351 APase Gene

To clone the T351 APase gene, the structural gene coding for *Tca* APase was used as a hybridization probe. Agarose gel membrane hybridization of the restriction enzyme digests of the *Thermus* sp. T351 genomic DNA revealed an approximately 7.0-kb BamHI fragment and an approximately 2.8-kb KpnI fragment, which hybridized with the ^{32}P-labeled probe (data not shown). The region containing the 2.8-kb KpnI fragment was excised from a gel and ligated into pBluescript SK-, and then *E. coli* MV1184 was transformed with the ligate. After colony hybridization, the insert in the plasmid extracted from the positive clone was subcloned and sequenced in both strands; however, the KpnI fragment did not contain the 5'-end sequence of the T351 APase gene. Upon DNA walking PCR, a 0.6-kb fragment was amplified, which contained the 5' end and upstream flanking region of the T351 APase gene. The T351 APase gene consisted of 1,503 bp coding for a protein with 500 amino acid residues (data not shown). The nucleotide sequence of the T351 APase gene was deposited in GenBank with accession number DQ148966.

A potential ribosome-binding sequence was found on the upstream flanking region of the T351 APase gene. The GGAG sequence at 12-base upstream from the open reading frame was homologous to the consensus ribosome-binding sequence [31]. However, potential bacterial promoter sequences were not found on the upstream of the ribosome-binding sequence. The G+C content of the T351 APase gene was 69.7%. The G+C content in the third position of codons was even higher, at 94.0%, than 70.1% and 45.1% in the first and second positions, respectively, of codons. Most protein-encoding genes from (hyper)thermophilic eubacteria and archaebacteria have shown that G and C were preferentially used in the third position of codons [4, 11, 26, 30].

The amino acid sequence of T351 APase was deduced from its nucleotide sequence (refer to Fig. 1). The N-

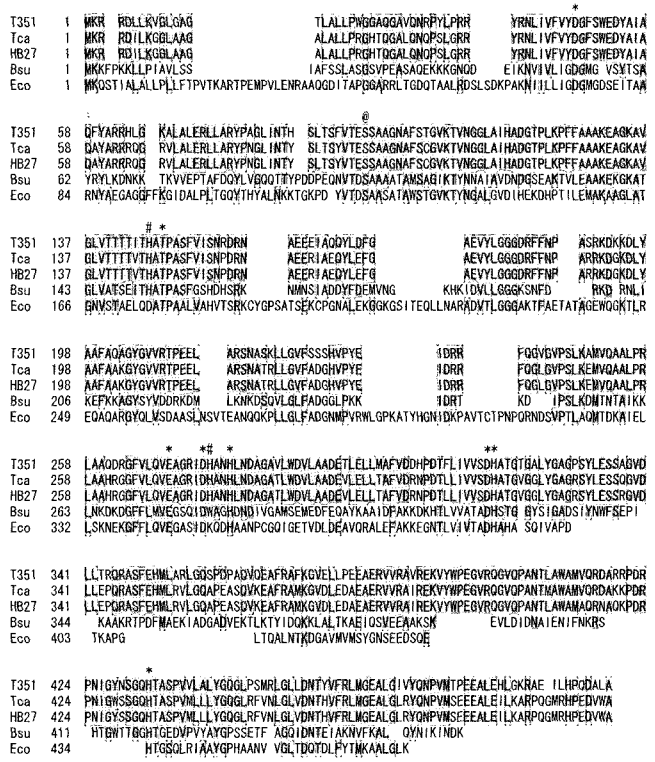

Fig. 1. Amino acid sequence alignment of APases.
The sequence of T351 APase (T351) is shown as compared with those of *Tca* APase (Tca, GenBank accession number AAF13361), *T. thermophilus* HB27 APase (HB27, YP_006007), *B. subtilis* APase III (Bsu, P19405), and *E. coli* APase (Eco, CAA28257). Identical amino acids between T351 APase and others are indicated by stippled boxes. @, phosphorylation site; *, metal ligands of M1 and M2 sites, and primary metal ligands of M3 site; #, secondary metal ligands of M3 site.

terminal end of T351 APase was revealed as a domain with a high hydrophobicity, suggesting that it is a signal peptide. The common structure of signal peptides has been described as a positively charged n-region, followed by a hydrophobic h-region and a neutral polar c-region [24]. In accordance with the common structure, the most probable cleavage site in the precursor T351 APase is between A27 and V28; therefore, the putative mature form of the precursor protein is comprised of 473 amino acid residues.

Similarity Analysis of the Amino Acid Sequence of T351 APase

The deduced amino acid sequence of T351 APase was aligned and compared with those of other APases (Fig. 1). Although the primary structure of T351 APase showed somewhat high similarity to those of *Thermus* APases, the similarity was relatively lower than those observed among other *Thermus* APases. T351 APase exhibited 81.0%, 81.0%, 80.8%, and 77.8% similarities to *Tca* APase (GenBank accession number AAF13361), *T. thermophilus* HB27 APase (YP_006007), *T. thermophilus* HB8 APase (YP_145306), and *Thermus* sp. FD3041 APase (AAC31201), respectively, whereas similarities of minimum 93.0% were shown among the other APases, except T351 APase (Table 1). T351 APase also exhibited 26.8% similarity to *B. subtilis* APase III (P19405) and 22.6% to *E. coli* APase (CAA28257).

Amino acid sequence alignment revealed that T351 APase contains well-defined conserved catalytic sites (Fig. 1). These catalytic sites include the amino acid residue S95, which has been shown to be incorporated within the phosphoseryl intermediate, in addition to the residues involved in the coordination of three metal ions [13, 22, 33]. The metal ligands in T351 APase are as follows: D275, H279, and H433 for the M1 metal-binding site; H318, D317, and D47 for the M2 metal-binding site; and E270, T148, H146, and H276 for the M3 metal-binding site. No difference was observed in the ligands of the M1 and M2 sites, and the primary ligands of the M3 site between T351 APase and *E. coli* APase; however, the secondary ligands, H146 and H276, of the M3 site in T351 APase differed from the corresponding residues in *E. coli* APase. These amino acid substitutions have also been shown in *P. abyssi* (*Pab*) APase [38] and mammalian APases [22].

Expression and Purification of T351 APase

The T351 APase gene was amplified and inserted into the NdeI and SalI sites of pET-22b(+). The resulting expression plasmid was designated pEYAP. *E. coli* BL21(DE3) harboring pEYAP was cultured in a 2-l fermentor, and the harvested cells (10.5 g) were initially sonicated and treated with streptomycin sulfate. We then utilized the thermophilic property of T351 APase, and eliminated most *E. coli* proteins by heating at 80°C for 30 min and centrifuging to remove all of

Table 1. Percent sequence similarity of APases from the genus *Thermus*.

Source	*T.* sp. T351	*T. caldophilus* GK24 (AAF13361[a])	*T. thermophilus* HB27 (YP_006007[a])	*T. thermophilus* HB8 (YP_145306[a])	*T.* sp. FD3041 (AAC31201[a])
T. sp. T351	100.0	81.0	81.0	80.8	77.8
T. caldophilus GK24		100.0	98.8	99.6	93.4
T. thermophilus HB27			100.0	98.8	93.0
T. thermophilus HB8				100.0	93.2
T. sp. FD3041					100.0

[a]GenBank accession number of APase.

Table 2. Purification scheme of T351 APase expressed in *E. coli*.

Purification step	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Recovery (%)
Sonicated extract	444.0	667.3	1.5	100.0
Heat treatment	24.2	475.4	19.6	71.2
UNO™ Q column	7.8	211.8	27.2	31.7
HiTrap™ Heparin HP column	5.5	169.4	30.8	25.4

[a]The APase activity was assayed at 80°C for 10 min in the reaction mixture containing 50 mM Glycine-NaOH (pH 12.0) and 2 mM $MgCl_2$.

the denatured proteins. Several *E. coli* proteins still remained soluble after the heating step. The soluble supernatant from the heating step was then chromatographed on a UNO™ Q column and a HiTrap™ Heparin HP column. The purification of the enzyme is summarized in Table 2. The specific activity of the purified enzyme was determined to be 30.8 U/mg, and the recovery was approximately 25.4%, based on the total activity of the sonicated extract. The purification of the enzyme was monitored by SDS-PAGE (Fig. 2). The SDS-PAGE revealed a single protein band with a molecular mass of 52,000 Da, which was in good agreement with the sum of the molecular mass, 54,407 Da, of T351 APase calculated from the deduced amino acid sequence.

Properties of T351 APase

The dependence of the T351 APase activity on pH was determined in the pH range of 7.0–12.0. The buffers used were 50 mM Tris-HCl (pH 7.0–9.5) and 50 mM Glycine-NaOH (pH 9.0–12.0). The enzyme activity was not observed below pH 9.5; its activity increased from pH 10.0, reaching a maximum at pH 12.0 (Fig. 3A). Moreover, the enzyme showed an approximately 90% activity at pH 13.0 in 50 mM

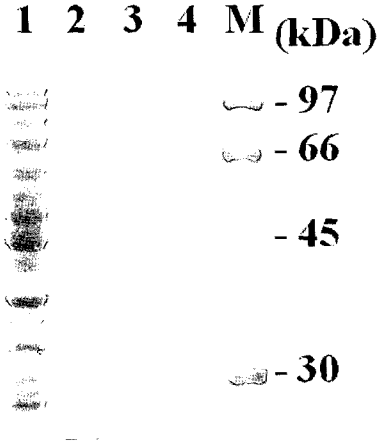

Fig. 2. SDS-PAGE analysis of T351 APase.
Electrophoresis was performed on a vertical gel of 10% polyacrylamide. Lane 1, sonicated extract; lane 2, heat treatment; lane 3, UNO™ Q column chromatography; lane 4, HiTrap™ Heparin HP column chromatography; lane M, low-molecular-mass markers (molecular masses are indicated at the right).

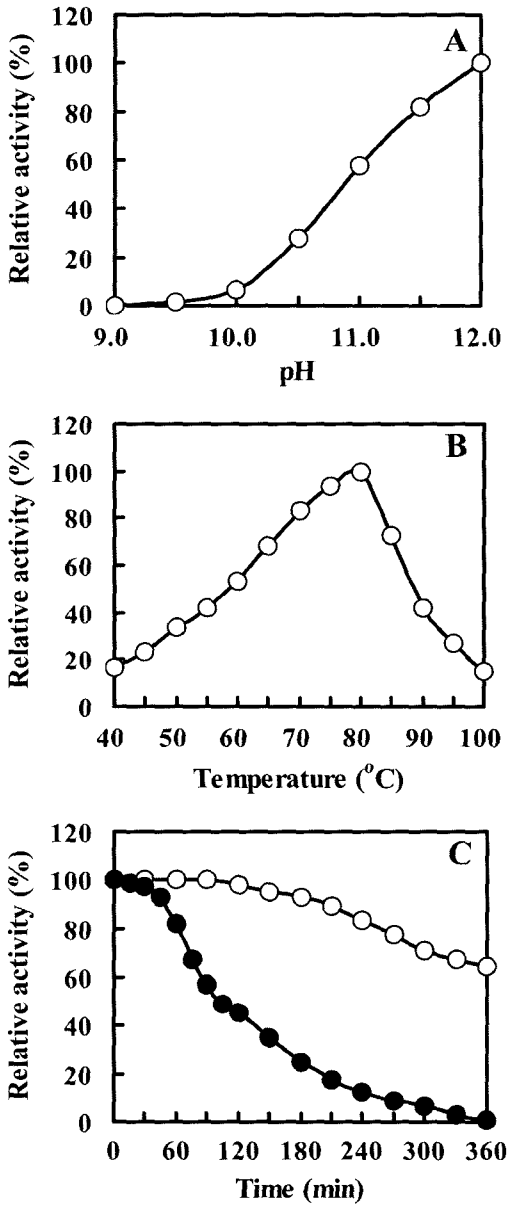

Fig. 3. Effects of pH and temperature on the T351 APase activity.
A. Dependence of the T351 APase activity on pH. **B.** Dependence of the T351 APase activity on temperature. **C.** Thermostability of T351 APase. The purified T351 APase (20 μg/ml) was incubated at 75°C (○) and 85°C (●) in 20 mM Tris-HCl (pH 8.0) and 50 mM NaCl. Aliquots of the mixture were removed at intervals up to 6 h and quenched on ice. The residual activity of the quenched samples was measured in the basic reaction mixture.

hydroxide-chloride buffer, although the buffer does not have good buffering power (data not shown). T351 APase is the most alkaline APase characterized, thus we named it hyperalkaline phosphatase. In contrast, almost all of the APases from prokaryotes and eukaryotes have an optimum pH in the range of 8.0–11.0 [9, 16, 22, 38]. Therefore, it is necessary to determine how T351 APase has the higher activity at extremely alkaline pH values. The pH stability of T351 APase was examined by incubating the enzyme solution at different pH values for 16 h at 25°C and by measuring the residual activity under the basic reaction condition. The results indicated that T351 APase was considerably stable at pH 7.0–12.0 (data not shown).

The dependence of the T351 APase activity on temperature was determined in the range of 40–100°C. The maximal activity was observed at 80°C (Fig. 3B). The thermostability of T351 APase was tested by measuring the decrease in activity after preincubation at two different temperatures: 75°C and 85°C. The enzyme was fairly stable at 75°C and was comparatively stable at 85°C. The half-life of the enzyme at 85°C was approximately 103 min (Fig. 3C).

The effects of metal ions on the T351 APase activity were examined with 1 mM each of $CaCl_2$, $CoCl_2$, $CuSO_4$, $MgCl_2$, $MnCl_2$, or $ZnSO_4$. The enzyme was found to be activated by Mg^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} (Fig. 4A). Among those, the most effective metal ion was Mg^{2+}, which increased the enzyme activity by about 10-fold, compared with when no metal ion was added; Zn^{2+} and Mn^{2+} slightly increased the enzyme activity. In *E. coli* APase, replacement of D153 and K328 (mature *E. coli* APase sequence numbering), the secondary ligands of the M3 site, with histidine resulted in the conversion of an Mg^{2+}-binding site into a Zn^{2+}-binding site [22, 23]. The affinity between the Mg^{2+} and M3 sites became lower for the mutant *E. coli* APases, and the mutant enzymes were activated by the addition of Mg^{2+}. The residues D153 and K328 in *E. coli* APase correspond to the residues H146 and H276 in T351 APase. Both are similar to corresponding residues found in *Tca* APase [16], *Pab* APase [38], and mammalian APases [22]. These appear to be consistent with T351 APase behavior towards Mg^{2+}. The T351 APase activity assay was performed in a variety of $MgCl_2$ concentrations. The optimal Mg^{2+} concentration was 2 mM (Fig. 4B). The effect of EDTA on the T351 APase activity was examined, and the enzyme activity was completely inhibited by EDTA (data not shown). The enzyme activity of the EDTA-inhibited T351 APase was reversed by the addition of Mg^{2+}, as expected. The above results indicate that T351 APase activity strongly depends on metal ions and the enzyme is a metalloenzyme.

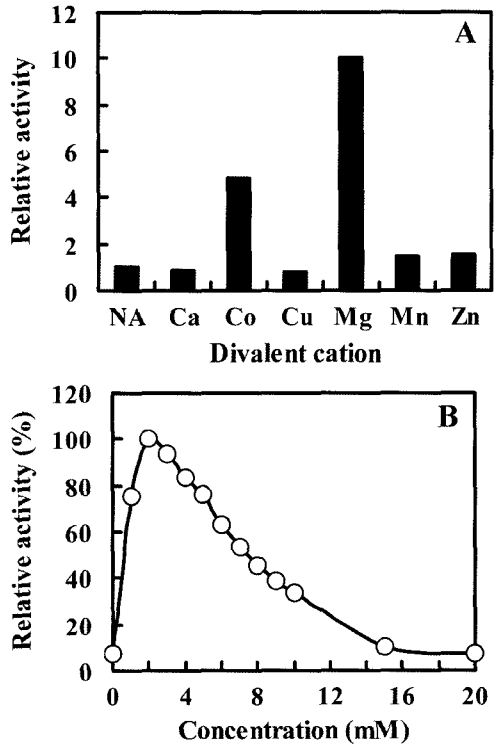

Fig. 4. Effects of divalent cations on the T351 APase activity.
A. Effects of various divalent cations. The enzyme activity when no divalent cation was added (relative activity=1) was arbitrarily selected as the standard for comparison. NA, no addition. **B.** Effects of various Mg^{2+} concentrations.

Michaelis Constant of T351 APase

Michaelian kinetic parameter was calculated using Lineweaver-Burk representation. The initial rate of hydrolysis was obtained in the optimal reaction condition using *p*NPP concentration ranging from 0.001 mM to 0.5 mM. The K_m value of T351 APase for *p*NPP was 0.039 mM (data not shown), which was similar to that of *Tca* APase (0.036 mM) [14].

Substrate Specificity of T351 APase

The relative rate of release of inorganic phosphate by T351 APase was measured for different phosphorylated substrates.

Table 3. Relative activity of T351 APase on various substrates.

Substrate	Relative activity (%)
p-Nitrophenyl phosphate (*p*NPP)	100.0[a]
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	50.3
Adenosine 5'-monophosphate (AMP)	306.1
Cytidine 5'-monophosphate (CMP)	343.8
Guanosine 5'-monophosphate (GMP)	358.8
Uridine 5'-monophosphate (UMP)	388.6
Adenosine 5'-triphosphate (ATP)	257.4
2'-Deoxyadenosine 5'-monophosphate (dAMP)	113.3
α-Naphthyl acid phosphate	88.6
Bis(*p*-nitrophenyl) phosphate	6.1
Glycerophosphate	248.7

[a]*p*NPP was arbitrarily selected as the standard for comparison.

The enzyme exhibited higher rates of hydrolysis for nucleoside monophosphates, ATP, and glycerophosphate (Table 3). Lower activity was shown towards BCIP and α-naphthyl acid phosphate. Reactivity with bis(*p*-nitrophenyl) phosphate, a diester substrate, was negligible.

In summary, we have cloned, expressed, and characterized a hyperalkaline phosphatase from the thermophilic bacterium *Thermus* sp. T351. T351 APase had some differences in properties, especially higher activity at extremely alkaline pH values, compared with other APases from prokaryotic and eukaryotic sources. Therefore, the enzyme could conceivably have a good utility in the APase-catalyzing reactions that proceed well at higher pHs or use certain substrate stable at higher pHs. We are currently conducting experiments to utilize T351 APase in various application fields, based on the results described in this paper.

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