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# A Moderately Thermostable Alkaline Phosphatase from *Geobacillus thermodenitrificans* T2: Cloning, Expression and Biochemical Characterization

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**Abstract** A gene-encoding alkaline phosphatase (AP) from thermophilic *Geobacillus thermodenitrificans* T2, termed *Gtd* AP, was cloned and sequenced. The deduced *Gtd* AP protein comprises 424 amino acids and shares a low homology with other known AP (<35% identity), while it exhibits the conservation of the active site and structure element of *Escherichia coli* AP. The *Gtd* AP protein, without a predicted signal peptide of 30 amino acids, was successfully overexpressed in *E. coli* and purified as a hexa-His-tagged fusion protein. The pH and temperature optima for purified enzyme are 9.0 and 65 °C, respectively. The enzyme retained a high activity at 45–60 °C, while it could be quickly inactivated by a heat treatment at 80 °C for 15 min, exhibiting a half-life of 8 min at 70 °C. The $K_m$ and $V_{max}$ for *p*NPP were determined to be 31.5 μM and 430 μM/min at optimal conditions. A divalent cation is essential, with a combination of $Mg^{2+}$ and $Co^{2+}$ or $Zn^{2+}$ preferred. The enzyme was strongly inhibited by 10 mM ethylenediaminetetraacetic acid (EDTA) and vanadate but highly resistant to urea and dithiothreitol. The properties of *Gtd* AP make it suitable for application in molecular cloning or amplification.

**Keywords** Alkaline phosphatase · Cloning · Characterization · *Geobacillus* · Thermostable

## Introduction

Alkaline phosphatases (EC 3.1.3.1) (APs) catalyze the nonspecific hydrolysis of phosphomonoesters, optimally active at alkaline pH. These enzymes are ubiquitous in nature and play a vital role in phosphate metabolism and transportation [1]. AP from *Escherichia coli* is the most extensively studied so far and has been served as a basis for comparison to enzymes with similar homology. *E. coli* AP is a homodimeric metalloenzyme and its catalytic site comprises a serine residue and two $Zn^{2+}$ and one $Mg^{2+}$ per

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monomer. Detailed catalytic mechanisms of APs have been deduced from structure of *E. coli* enzyme, which involves a double in-line displacement and the participation of all three metal ions [2–4].

APs have many biotechnological applications, such as molecular biology, immunoassay, and clinical diagnostics [5–7]. Previously, APs from diversified sources, with widely differing thermal profiles, have been isolated and characterized. In general, APs are very stable enzymes. For example, *E. coli* AP remains activity at 80 °C [8] and that from *Pyrococcus abyssi* even at 105 °C [9]. However, these highly stable APs are difficult to be eliminated at the end of dephosphorylation reaction of DNA cloning and amplification. Although several thermal sensitive APs from psychrophilic or mesophilic organisms such as cold water shrimp, Antarctic bacterium TAB5 and calf intestinal are now available commercially and used in many molecular biology assays, their further applications are restricted due to low thermal resistance and shelf lives of these thermoliable APs. Thus moderately thermostable APs are attractive, since they are stable at room temperature and relatively more convenient, compared with highly stable APs, to be destroyed by heat treatment at the end of reactions. Furthermore, variants of APs from extremophilic organisms could serve as ideal model molecular for investigations of enzyme evolution, protein thermal adaptation mechanism as well as metal-dependent catalysis.

*Geobacillus* is a genus of moderately thermophilic bacilli with a high 16S rRNA sequence similarity (98.5–99.2%), which have attracted industrial interests for their potential application in biotechnology as an important source of thermostable enzymes [10]. Up to now, several thermostable enzymes such as thermostable L-arabinose isomerase [11], lipase [12], α-amylase, and α-glucosidase [13] from *Geobacillus* spp. have been characterized, whereas no AP in them was investigated.

In this study, we describe the cloning of a gene coding for thermostable AP (named *Gtd* AP) from a thermophilic Gram-positive bacterium, *Geobacillus thermodenitrificans* T2. The recombinant *Gtd* AP was overexpressed and purified in *E. coli* and its biochemical properties were characterized. The recombinant enzyme was stable at temperature below 60 °C, while it could be quickly inactivated by a heat treatment at 80 °C for 15 min. As a moderately thermostable AP, *Gtd* AP might be of significant biotechnological interest and scientific research value.

## Materials and Methods

### Materials

Enzymes used in vector construction were from New England Biolabs. All the chemicals were purchased from Sigma (Sigma-Aldrich) unless otherwise specified.

### Construction of Genomic Libraries and Screening for Thermostable Phosphatase Positive Clones

Genomic DNA from thermophilic bacterial was prepared with the phenol-chloroform extraction [14] and partially digested with *Sau3A I*. The DNA fragments >2 kb were recovered from agarose DNA gel and spliced into *Bam*HI-digested, dephosphorylated pUC19 plasmid. *E. coli* DH 5α were transformed with the ligation mixture and plated on LB plates containing 100 μg/mL ampicillin and 0.1-mM isopropyl-D-thiogalactopyranoside (IPTG) to form about $10^3$ colonies/10-cm dish. Then all colonies were lifted onto

nitrocellulose filter papers and the filters were placed, colony side up, in a 10-cm plate and incubated with 3 ml buffer (0.1 M Tris–HCl, pH 8.5, 1% Triton X-100) at 70 °C for 5 min. Ten microliters of 0.6 M *p*-nitrophenyl phosphate (*pNPP*) (Merk, USA) was supplemented and incubated an additional 5 min at 70 °C. Colonies producing thermostable phosphatase would hydrolyze *p*NPP, releasing *p*NP that is yellow in color. By corresponding to the original plates, putative positive colonies were found and picked. Plasmids (termed pTP) from these colonies were prepared and sequenced using M13 forward/reverse sequencing primers by submission to DNA sequencing facility of Unit Gene, Inc (Shanghai, China).

## Sequence Analysis

Homology searches were performed with BLAST program at the NCBI web server (http://www.ncbi.nlm.nih.gov/BLAST). SignalP 3.0 Server was used to predict probably signal peptide and the cleavage sites (http://www.cbs.dtu.dk/services/SignalP). Multiple sequences alignments were performed by GeneDoc programmer (http://www.psc.edu/biomed/genedoc).

## Recombinant Protein Expression

According to the sequencing result, three polymerase chain reaction (PCR) primers were designed to amplify the *Gtd* AP gene corresponding region from plasmid pTP (two forward primers: F-1, 5′-CTAGCTAGCTTCAAATCGAAACGCTGCGC-3′; F-2, 5′-CTAGC TAGCGCACCGTCCAAGCCCGCAA-3′, and a reverse primer: R, 5′-CCGCTCGA GAAATCCATGGCTTTCGTTGT-3′). The full-length coding sequence of *Gtd* AP was amplified using *Pfu* DNA polymerase with primers F-1 and R and *Gtd* AP without a deduced signal peptide sequence was amplified with primers F-2 and R, respectively. Each of the obtained fragments with expected size was gel-purified and cloned into the *Nhe* I and *Xho* I sites, respectively, of pET-28b (Novagen, USA). The fidelity of inserting fragments in pET vectors was confirmed by sequencing. *E. coli* Rosetta (DE3) pLyS harboring the constructed expression plasmids were grown in 1 L Luria-Bertani (LB) medium containing kanamycin (100 mg $L^{-1}$) and chloramphenicol (34 mg $L^{-1}$) until $OD_{600}$ 0.6–0.8. After induction for 10 h at 30 °C with 0.5-mM IPTG, cells were harvested by centrifugation.

## Purification of *Gtd* AP Fusion Protein

The harvested cell pellet was suspended in a standard buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole pH 8.0) and ultrasonicated on ice. The lysate was centrifuged at 14,000×*g* for 20 min at 4 °C. The supernatant was used for purification procedure with Ni-NTA Superflow chromatography according to the manufacturer's protocol (QIAGEN). Finally, the bound enzyme was completely eluted with an elution buffer (250 mM imidazole, 300 mM NaCl, 50 mM $NaH_2PO_4$, pH 8.0). Active fractions were pooled and phosphate buffer were replaced with 50 mM Tris–HCl buffer (pH 8.5) by ultrafiltration (Amicon-Ultra-15 column; Millipore, USA).

## Protein Determination

The protein samples were separated in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined using bicinchoninic acid (BCA) protein assay (Pierce) with bovine serum albumin (BSA) as the protein

standard. Protein purity was determined by high-performance liquid chromatography (HPLC) analysis using reverse phase column system (C-18 column, Agilent) and the chromatogram was analyzed by UNICORN V3.20 software.

### Enzyme Activity Assay

A spectrophotometric assay was used to determine the AP activity [15]. The standard assay was carried out in 0.5 ml of reaction mixture containing 0.1 M Tris–HCl buffer (pH 8.0), 2.4 mM *p*NPP, and 2.0 μg enzyme (enzymes used were preactivated by 5 mM $Co^{2+}$ and $Mg^{2+}$ ions, except as otherwise indicated). After incubation at 65 °C for 5 min, the reaction was terminated by adding equal volume of 2.0 M NaOH, and the released *p*NP ($\varepsilon$= 18,380 $M^{-1}$ $cm^{-1}$) was measured at 405 nm using a spectrophotometer (Hitachi Co., Japan). One unit of enzyme activity is defined as 1 μmol *p*NPP hydrolyzed per minute.

### Temperature, pH, Thermostability Profiles, and Kinetic Parameters

The optimal pH of enzyme activity was investigated in 0.1 M Tri–HCl (pH 7.0–9.0) and 0.5 M diethanolamine (pH 9.0–13.0) buffer at 65 °C. All of the pH buffers were calibrated at high temperature. Temperature effect of activated *Gtd* AP was determined by standard assay from 25 to 100 °C. Thermostability of the enzyme was investigated by the standard assay after preincubating enzymes at indicated temperatures for various times. Kinetic parameters, $K_m$ and $V_{max}$, were determined from data obtained by determining the initial rate of *p*NPP hydrolysis.

### The Effect of Metal Ions and Inhibitors on Enzyme Activity

To examine the effects of metal ions and inhibitors, enzyme was preincubated in the absence or presence of various divalent ions (final concentration 5 mM) and inhibitors at 4 °C for 1 h, and then assayed the activity by standard procedure. All of the examined metal ions were in their chloride form.

## Results and Discussion

### Characterization of Thermophilic Strain T2

Thermophilic bacteria were isolated from a hot spring in the People's Republic of China. Microscopic inspection revealed that strain T2 was a rod-shaped *Bacillus*, 0.4–0.8 μm wide, 4.0–8.0 μm long, which grow optimally at around 65 °C. And 16S *rRNA* gene of bacterium was amplified [16] and compared with known sequences from NCBI GenBank database. The partial sequence of *16S rRNA* gene from strain T2 (GenBank Accession No. EF570295) exhibited a high level of homology (>99.2% sequence identity) with those of *G. thermodenitrificans* strains. On the basis of phenotype and *16S rRNA* gene sequence analysis, the strain T2 was assigned to *G. thermodenitrificans* T2.

### Cloning and Sequence Analysis of *G. thermodenitrificans* T-2 AP

Through activity-based screening of thermophilic strain T2 genomic library, one clone with apparent phosphatase activity to *p*NPP, a general phosphatase substrate, were detected from

approximately 6,000 transformed colonies. According to the result of sequencing, the recombinant plasmid of this clone showed an insert of 1,778 bp. Meanwhile, it demonstrated a significant ORF of 1,275 bp encoding a polypeptide of 424 amino acids. This ORF was preceded at a spacing of 4 bp by a potential ribosome-binding sequence (5′-GGAG-3′), which was homologous to the consensus Shine–Dalgarno sequence [17]. The *E. coli* promoter-like sequences, in the −35 and the −10 regions, were not found on the upstream region of the ORF. In the 3′-noncoding flanking region of the gene, there was no potential transcriptional termination sequence forming a stem-and-loop structure. BLASTP results, however, showed that the encoding protein from this ORF was homologous to other known alkaline phosphatases. Therefore, this ORF was suggested to be an alkaline phosphatase gene of *G. thermodenitrificans* T-2 (termed *Gtd* AP, GenBank Accession No. EU239359).

Most other bacterial APs are periplasmic proteins. *E. coli* AP and *Bacillus subtilis* AP III have 21 and 32 amino acids signal peptides, respectively [18, 19]. Thus, *Gtd* AP was subjected to the SignalP program designated for protein sequences from gram-positive bacteria for signal peptides prediction [20]. The program located an N-terminal signal sequence consisting of 30 amino acids, which would be cleaved between Ala-30 and Ala-31. Accordingly, the mature *Gtd* AP protein was deduced to be composed of 394 amino acids, resulting in an estimated molecular mass of 42,149 Da and *p*I 6.04.

A multiple amino acid sequence alignment was performed using the *Gtd* AP sequence and a selection of APs (Fig. 1). As seen from the alignment, *Gtd* AP only has a low-sequence identity with previous characterized thermophilic *Thermotoga maritima* AP (33.6% identity), *Thermotoga neapolitana* AP (32.6 identity), mesophilic *Bacillus subtilis* AP IV (32.2% identity), and *E. coli* AP (28.2% identity). However, almost all of the residues with key roles in *E. coli* AP were well conserved in *G. thermodenitrificans* T-2 enzyme and the aligned homologs. In *E. coli* mature AP, Ser102 and Arg166, which are implicated in the direct interaction with substrates are conserved in all case. The active site of *E. coli* AP contains two $Zn^{2+}$ ions and one $Mg^{2+}$ ion, the residues interacting with $Zn^{2+}$ I (Asp-327, His-331, and His-412), and the residues interacting with $Zn^{2+}$ II (Asp-51, Asp-369, and His-370) in *E. coli* AP are highly conserved in the compared sequences (Fig. 1). The only variation occurred at amino acid Lys-328, an indirect ligand to $Mg^{2+}$ in the *E. coli* AP, which is replaced by a Glu in *Gtd* AP. This residue is found to be His or Trp in several known eukaryotic and bacterial APs [19, 21, 22]. In general, based on the strictly conservative active sites, the catalytic mechanism was proposed to be similar to *E. coli* AP in *G. thermodenitrificans* T-2 enzyme. However, an altered metal ion binding in *E. coli* AP has been verified by a His or Trp substitution in the position 328 [23, 24]. Although Glu substitution in *Gtd* AP is long enough to interact with the $Mg^{2+}$, the positional change of the carboxylate may distort the metal-binding site and thereby may reduce the affinity of this site for $Mg^{2+}$ in *Gtd* AP. Moreover, APs from *E. coli* as well as most other organisms exist in dimeric or multimeric structure [2, 25]. Higher order quaternary structure in *E. coli* AP has been demonstrated to provide thermal stability for the enzyme [26]. However, the residues of *Gtd* AP corresponding to *E. coli* AP dimmer interface, in contrast with highly conserved residues in active sites, are far less conserved in the compared sequences. These variations at interface residues may influence the overall folding stability of AP variants. In addition, four Cys residues are also found in *E. coli* APs and all of them form interchain disulfide bond [2]. The formation of disulfide bonds in APs has been shown to be a factor responsible for their stability [27, 28]. However, no Cys residue is present in the deduced mature *Gtd* AP.

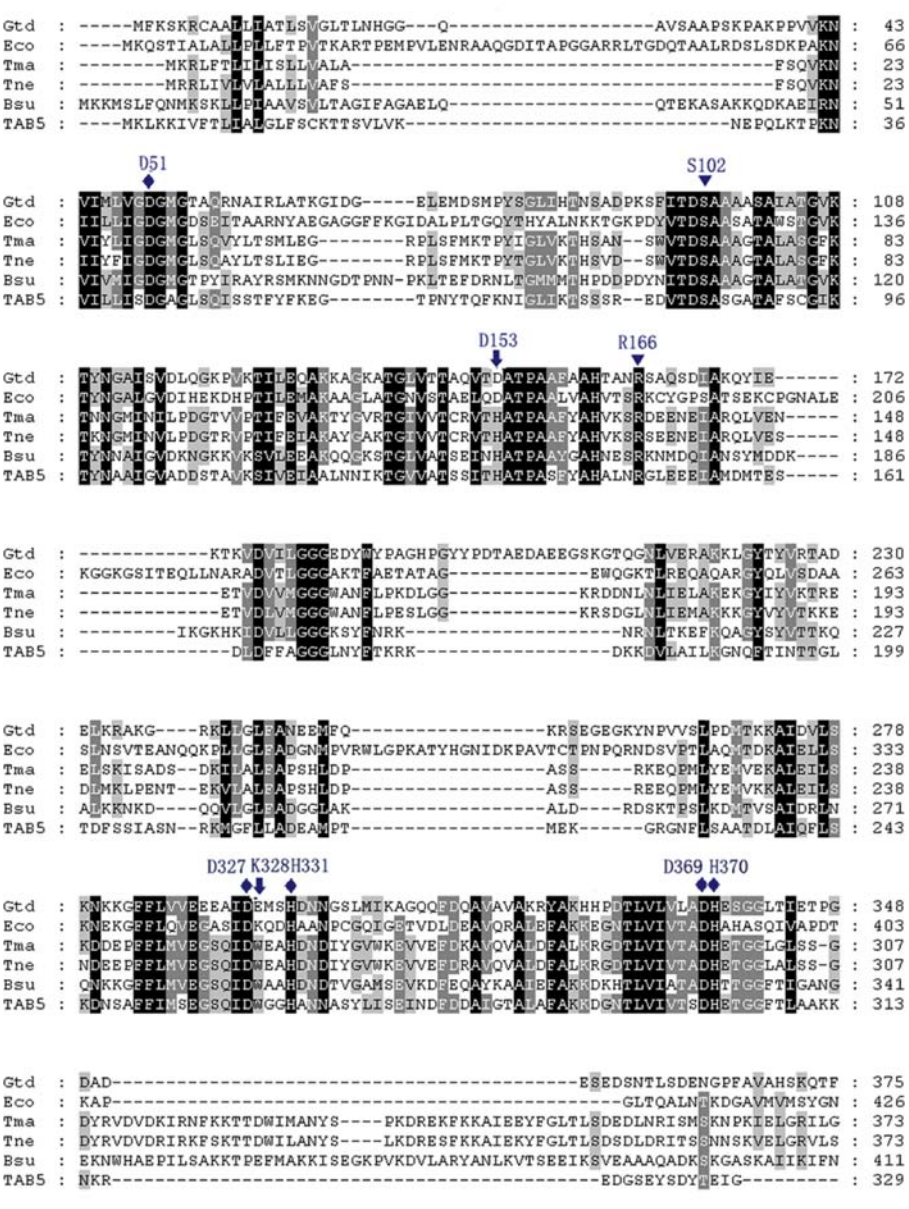
D51
S102
D153
R166
D327 K328H331
D369 H370
H412

**Fig. 1** Sequence alignment of AP precursors from *G. thermodenitrificans T2* (Gtd), *E. coli* (Eco; Accession No. AAA83893), *T. maritima* (Tma; Accession No. AAD35249), *T. neapolitana* (Tne; Accession No. AAX98659), *B. subtilis* isoform IV (Bsu; Accession No. AAA18323), Antarctic bacteria TAB5(TAB5; Accession No. CAB82508). Residues that are coordinated directly to the metal ions are indicated with (♦), residues forming the salt-link important for phosphate binding (D153, K328) are indicated with (⬇), and the phosphorylation site (S102 and R166) is indicated with (▼). Numbering of amino acid residues involved in the active site corresponds to that of the mature AP sequence from *E. coli*. Similar and identical amino acid residues that occur in at least 50% of the AP sequences are shown on gray and black backgrounds, respectively

## Expression and Purification of *G. thermodenitrificans* T-2 AP

To identify the deduced N-terminal signal sequence and further characterize *Gtd* AP, the encoding sequence of *Gtd* AP with and without the deduced N-terminal signal peptide (30 amino acids) were amplified by PCR and cloned into pET28b vectors.

The cultivation of *E. coli* Rosetta (DE3) pLyS cells harboring the recombinant plasmid containing the *Gtd* AP gene with and without the putative signal sequence under control of the strong bacteriophage T7 promoter led to different expression effects. For the *Gtd* AP with the putative signal sequence, detectable but relatively small amounts of the *Gtd* AP protein were produced. The quantity of protein isolated was not enough to allow detection of a possible cleavage of the signal sequence. For the overexpression of the recombinant *Gtd* AP without the putative signal sequence, under the optimal condition, the enzyme was produced in soluble form in *E. coli* cell and accounts for more than 20% of the total cellular protein (Fig. 2, lane1).

The cytosolic fraction was collected and used for purification of the recombinant protein. The recombinant *Gtd* AP was completely eluted from the column with 250 mM imidazole (Fig. 2, lane 3). Approximately 27 mg recombinant enzyme was obtained from 1-L bacterial culture (Table 1). HPLC analysis demonstrated the purity of the recovered protein was approximately 97% (data not shown). After the recombinant *Gtd* AP was activated by

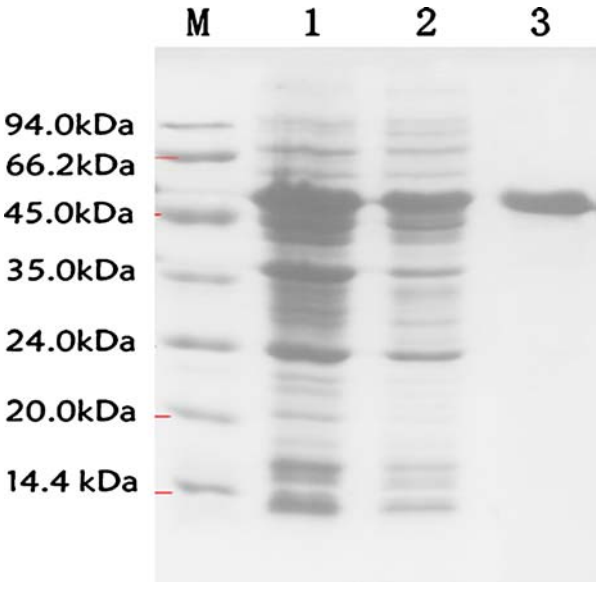


**Fig. 2** Analysis of recombinant *Gtb* AP expression and purification on 12% SDS-PAGE; lane *M*, molecular weight marker; lane *1*, total protein of induced bacteria; lane *2*, supernatant of induced bacteria; lane *3*, purified recombinant *Gtd* AP protein

**Table 1** Purification of recombinant *Gtd* AP from *E. coli* cells.

| | Total proteins(mg)[a] | Total activity(U)[b] | Special activity(U/mg) | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| Lysate supernatant | 1,229.2 | 38,965.6 | 31.7 | 100 | 1 |
| Ni-chelation | 27.2 | 26,713.1 | 982.1 | 68.5 | 30.9 |

[a] Total protein was determined by BCA assay (Pierce) with BSA as a standard.

[b] Activity unit expressed in μmol/min with *p*NPP as substrate.

the complement metals (a combination of $Co^{2+}$ with $Mg^{2+}$), *Gtd* AP showed a higher specific activity of 982.1 U/mg under optimal conditions, which is comparable to *E. coli* AP optimal value 600 U/mg [8]. High specific activity made *Gtd* AP attractive for further practical application.

## Activity of the *G. thermodenitrificans* T-2 AP

The effect of temperature and pH were determined by using the enzyme activated by metal ions. The recombinant *Gtd* AP had the optimal activity at pH 9.0, exhibiting at least 53% of the maximal activity at pH 7.0 (Fig. 3a). This clearly distinguished *Gtd* AP from other thermostable APs, which only retained about 8–20% of their maximal activity at the neutral pH [29, 30]. As shown in Fig. 3b, the enzyme activity of recombinant *Gtd* AP increased from 25 ° to 65 °C, reaching the maximal at around 65 °C. Moreover, the enzyme also exhibited at broad high activity (more than 62% maximal activity) at temperatures ranging from 45 ° to 65 °C, which is consistent with the temperature for the survival of *Geobacillus* species. The purified recombinant *Gtd* AP was found to retain a relatively high activity after incubation of 60 °C for 1 h. This result showed that this enzyme was more stable than AP from shrimp [31], calf intestine [32], *Bacillus stearothermophilus* [33], and most psychrophiles [8, 34–38]. These thermal sensitive APs significantly lost activity after the incubation at 60 °C. Further analysis showed that *Gtd* AP for total thermal inactivation was only 15 min by a heat treatment of 80 °C and the half-life value at 70 °C was 8 min (Fig. 3**c**), which is less stable than its counterparts in various thermophiles [9, 22, 29, 30, 39–42]. For example, *Thermus caldophilus* retained 80% activity after incubation at 80 °C for 12 h [39] and even *E. coli* AP exhibited a half-life value at 80 °C more than 6 h [8]. The thermal stability of recombinant *Gtd* AP, together with its broad high activity between 45 °and 60 °C, and its quick inactivation by an appropriate heat treatment suggested that the recombinant *Gtd* AP might be a good candidate for practical applications.

Values of kinetic constants were determined on the basis of the Lineweaver–Burk plots. Under the optimal conditions, the recombinant *Gtd* AP hydrolyzed an artificial substrate *p*NPP with a $K_m$ of 31.5 μM and $V_{max}$ of 430 μM/min. A similar $K_m$ was found in APs from mesophilic *E. coli* [43] and thermophilic *Meiothermus ruber* [41], which ranges from 21 to 55 μM.

## Metal Ion Requirements for the Enzyme Activity

Since the known APs are metalloenzymes, the effect of 5 mM of various metal ions on *Gtd* AP activity was evaluated as shown in Fig. 4. Surprisingly, negligible activity was detected in the absence of exogenous metal ions in the reaction mixture, and similar results have been observed for the recombinant AP from *Meiothermus ruber* [41]. Howeve, the crude enzyme extract, supernatant, without exogenous metal ions supplemented, was activated.

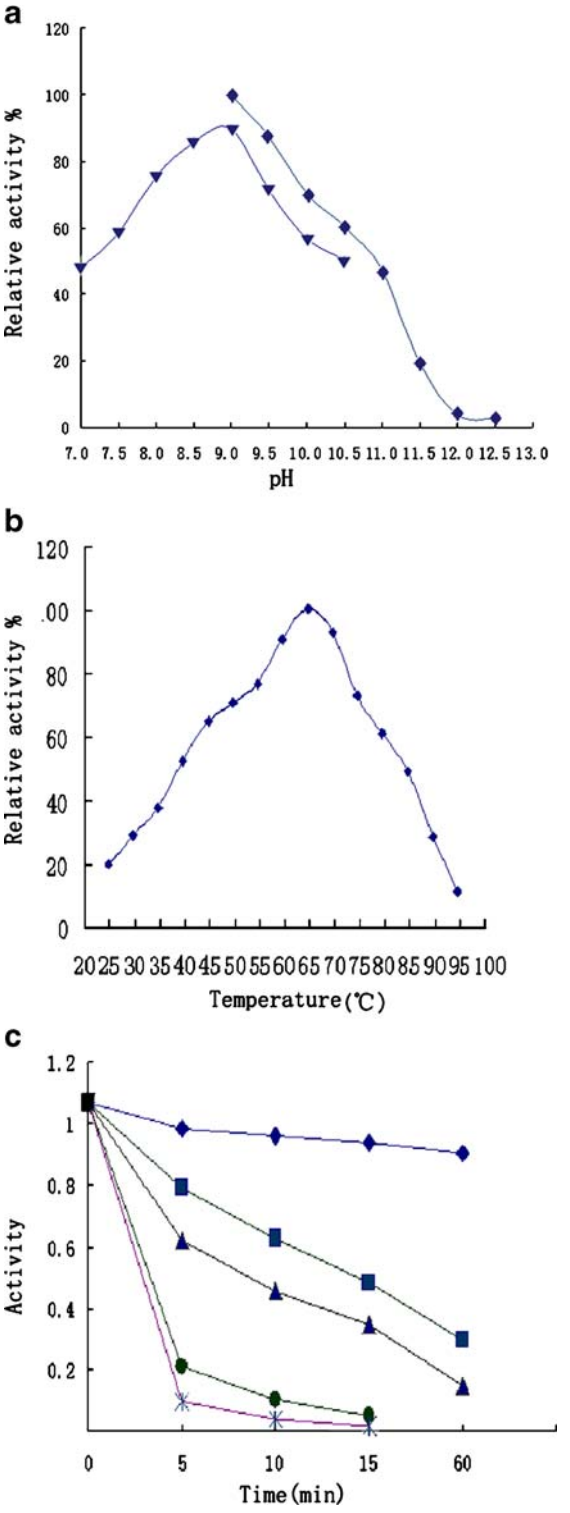


**Fig. 3** Properties of recombinant *Gtb* AP. **a** Effect pH on the activity. The activity was examined at 65 °C in 0.1 M Tris–HCl (▼) and 0.5 M diethanolamine buffer (♦). **b** Temperature effect. The activity was measured in 0.1 M Tris–HCl buffer (pH 8) at different temperatures. Maximal enzyme activities observed were set as 100% relative activity. **c** Thermostability. The residual activity was measured by standard assay after incubation of enzyme at 60 °C (♦), 65 °C (■), 70 °C (7), 75 °C (●), 80 °C (✻) for indicated time. Each data point in above figures represents an average of three determinations

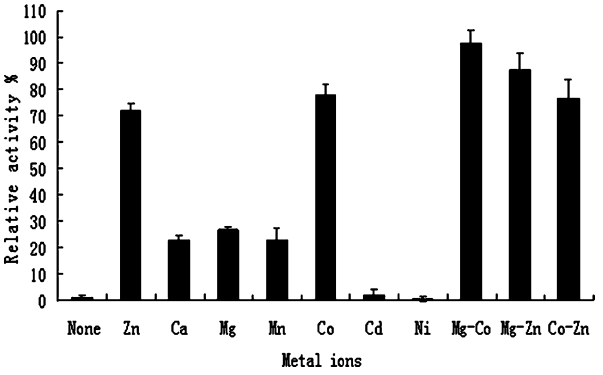


**Fig. 4** Effect of various metal ions on recombinant *Gtd* AP activity. The enzyme was incubated in the absence or presence of indicated metal ions (final concentration 5 mM) and then assayed for activity by the standard method. Maximal enzyme activities observed were set as 100% relative activity

One plausible explanation for this is that the metal ions are poorly bounded in the active center of *Gtd* AP so that they are easily lost during the purification process.

In contrast with typical $Zn^{2+}$- and $Mg^{2+}$-activated APs, the optimum complement to *Gtd* AP was a combination of $Co^{2+}$ with $Mg^{2+}$, which most significantly activated native enzyme compared with other added ions. Furthermore, the effect of $Co^{2+}$ alone was slightly less than that of the addition of $Co^{2+}$ in combination with $Mg^{2+}$. Similar results are also found in other thermostable AP from *Thermus yunnanensis* [42] and *Thermotoga maritime* [22]. For these known $Co^{2+}$-activated APs, $Zn^{2+}$ usually has a weak or inhibited function to enzyme activity. It is interesting that *Gtd* AP could be activated by both $Co^{2+}$ and $Zn^{2+}$ at a similar extent. This feature also distinguishes *Gtd* AP from others. A less activated effect was found for the addition of $Ca^{2+}$, $Mg^{2+}$ or $Mn^{2+}$ in recombinant *Gtd* AP. No effect on the activity was observed when $Ni^{2+}$ or $Cd^{2+}$ was added.

## The Inhibitors

The effects of several chemicals on the activity of *Gtd* AP are characterized in Table 2. Almost complete inactivation of the enzyme was observed with ionic detergent 1% SDS, while non-ionic detergent, 1% Triton X-100, enhanced about 143% enzyme activity. Inorganic phosphate (10 mM) acted as a potent inhibitor and reduced the activity of the *Gtd* AP enzyme by 77%. Likewise, vanadate, a classical alkaline phosphatase inhibitor, strongly

**Table 2** Effect of various chemicals on recombinant *Gtd* AP activity. [a]

| Chemicals | Concentration | Relative activity (%) |
|---|---|---|
| SDS | 1% | 5.4±0.8 |
| Triton X-100 | 1% | 143.9±6.3 |
| Inorganic phosphate | 1 mM | 75.2±2.4 |
| | 10 mM | 22.8±1.12 |
| Molybdate | 1 mM | 83.2±4.3 |
| | 10 mM | 103.0±6.0 |
| Vanadate | 1 mM | 32.8±1.5 |
| | 10 mM | 10.9±1.2 |
| Urea | 1 M | 111.8±1.7 |
| | 2 M | 83.8±2.8 |
| DTT | 1 mM | 87.3±6.7 |
| EDTA | 2 mM | 20.9±2.1 |
| | 10 mM | 1.2±0.4 |

[a] The purified enzyme was incubated with the listed chemicals at 4 °C for 1 h and the remaining activity was then determined. Activity is expressed as a percentage of that of the control with no incubation. Data represent mean±standard deviation.

inhibited the *Gtd* AP enzyme. These observations agree with the data reported for other bacterial alkaline phosphatases. A slight decrease in activity was observed with 2 M urea. Resistance to denaturing agents is characteristic of some thermostable enzymes and may be advantageous for application of these proteins in harsh conditions. Thiol-reducing dithiothreitol (DTT; 1 mM) has been reported to triple the activity of *Thermus caldophilus* AP [39] and to nearly completely inactivate the *Pyrococcus abyssi* enzyme [9]. However, the dithiothreitol only slightly affected the activity of the *Gtd* AP enzyme, possibly because no any Cys is presented in *Gtd* AP. As it is natural for a metalloenzyme, *Gtd* AP was inhibited about 98.8% of activity by 10 mM ethylenediaminetetraacetic acid (EDTA).

## Conclusion

The present work reported cloning, sequencing, and biochemical characterization of a moderately thermostable AP from *Geobacillus thermodenitrificans*. The temperature activity and thermostability of *Gtd* AP may therefore provide a basis for thermostable enzyme utilization at moderate temperature such as 45–60 °C. On the other hand, the high yields of soluble recombinant *Gtd* AP in *E. coli* made the bulk production possible and easy, which meets the demands for reagent enzyme.

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