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Expression and Characterization of Recombinant Thermostable Alkaline Phosphatase from a Novel Thermophilic Bacterium *Thermus thermophilus* XM

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Abstract A gene (*tap*) encoding a thermostable alkaline phosphatase from the thermophilic bacterium *Thermus thermophilus* XM was cloned and sequenced. It is 1506 bp long and encodes a protein of 501 amino acid residues with a calculated molecular mass of 54.7 kDa. Comparison of the deduced amino acid sequence with other alkaline phosphatases showed that the regions in the vicinity of the phosphorylation site and metal binding sites are highly conserved. The recombinant thermostable alkaline phosphatase was expressed as a His_6-tagged fusion protein in *Escherichia coli* and its enzymatic properties were characterized after purification. The pH and temperature optima for the recombinant thermostable alkaline phosphatases activity were pH 12 and 75 ºC. As expected, the enzyme displayed high thermostability, retaining more than 50% activity after incubating for 6 h at 80 ºC. Its catalytic function was accelerated in the presence of 0.1 mM Co^{2+}, Fe^{2+}, Mg^{2+}, or Mn^{2+} but was strongly inhibited by 2.0 mM Fe^{2+}. Under optimal conditions, the Michaelis constant (K_m) for cleavage of *p*-nitrophenyl-phosphate was 0.034 mM. Although it has much in common with other alkaline phosphatases, the recombinant thermostable alkaline phosphatase possesses some unique features, such as high optimal pH and good thermostability.

Keywords gene expression; *Thermus thermophilus* XM; thermostable alkaline phosphatase

Alkaline phosphatases (APases; EC 3.1.3.1) exist widely in nature from microorganisms to mammals, and play an important role in fundamental biochemical processes, especially phosphate transportation and metabolism [1–3]. They can be applied extensively in diagnostics, immunology, and molecular biology as sensitive biological markers in processes such as enzyme-linked immunosorbent assay, Western blotting analysis, nucleic acid hybridization, and *in situ* hybridization [4–6]. APase from *Escherichia coli* has been extensively studied in terms of biosynthesis, structure, and catalytic mechanism [7–13]. *E. coli* APase is located in the periplasmic space as a homodimer and each monomer comprises 449 amino acid residues and contains two Zn^{2+} and one Mg^{2+}.

At present, *E. coli* APase and calf intestine APase are the most commonly used, but their inherently low thermal resistance and shelf-lives have restricted their further applications under some special circumstances, for example, high temperature and high pH. Compared with common APase, thermostable APase (TAPase) has many beneficial qualities, such as high thermostability, high reaction rates, and excellent resistance to denaturation or microbial contamination [14]. Due to these advantages, there has been increasing attention to TAPase from thermophilic bacteria. So far, a number of TAPases have been isolated and their corresponding genes have been cloned and characterized from various sources, such as *Thermus* species, *Thermotoga neapolitana*, *Meiothermus ruber*, *Bacillus stearothermophilusi*, and *Pyrococcus abyssi* [15–25].

In this study, we cloned and sequenced a *tap* gene from *T. thermophilus* XM, discussed the characteristics of the deduced primary structure of the enzyme, expressed it in *E. coli* and provided a preliminary characterization of the recombinant TAPase (rTAPase).

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Materials and Methods

Identification of strain

A thermophilic bacterium, whose optimal growth temperature is in the range of 70–75 ºC in Luria Bertani (LB) medium, was isolated from a hot spring at Dongfu beach (Xiamen, China). After incubation in LB medium for 24 h at 75 ºC with shaking at 150 rpm, the bacterial cells were harvested and the genomic DNA was purified using the Wizard genomic DNA purification kit (Promega, Madison, USA). To identify the isolate, the 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR product was cloned into pMD-18T vector (TaKaRa, Dalian, China) and sequenced. The nucleotide sequence homology was analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast).

Cloning of the *tap* gene

Based on the complete coding region of the TAPase genes from the genus *Thermus*, the following specific primers were synthesized: tapN (5′-GGGGGATCCAAGCGAAGGGACATCCTG-3′, sense primer, *Bam*HI restriction site underlined); and tapC (5′-GGGAAGCTTTTAGGCCCAGACGTCCTC-3′, antisense primer, *Hin*dIII restriction site underlined). The amplified fragment of 1506 bp was obtained by PCR with genomic DNA of *T. thermophilus* XM as the template, cloned into pQE30 vector (Qiagen, Hilden, Germany), and further confirmed by sequencing. The 25 μl PCR mixture consisted of 100 ng genomic DNA of *T. thermophilus* XM, 0.4 μM each primer, 200 μM each dNTP, 2.5 mM Mg^{2+}, 1.25 U *LA-Taq* DNA polymerase (TaKaRa), and the buffer supplied by the manufacturer. The reaction was carried out for 25 cycles as follows: denaturation at 94 ºC for 30 s; annealing at 58 ºC for 30 s; and extension at 72 ºC for 2 min.

Sequence homology searches were carried out using the BLAST program. Sequence analysis and alignment were carried out with DNAMAN software (version 5.1; Lynnon BioSoft, Vaudreuil, Canada).

Expression and purification of rTAPase

After incubation at 37 ºC overnight, *E. coli* XL-Blue containing the recombinant plasmid pQE30-tap and the control (vector only) were inoculated into new media at a ratio of 1:100. When the A_{600} was 0.6, the cultures were induced with 0.5 mM isopropyl-β-*D*-thiogalactoside for an additional 6 h at 37 ºC. Cells were harvested by centrifugation, then suspended in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. After sonication, the insoluble debris was removed by centrifugation at 18,000 *g* for 20 min and the supernatant was heated for 20 min at 70 ºC. The resulting precipitate was again removed by centrifugation. Subsequently, the His_6-tagged rTAPase in the supernatant fraction was purified using Ni-NTA metal-affinity chromatography according to the instructions of the QIAexpressionist system (Qiagen, Valencia, USA). The purified rTAPase was dialyzed against the buffer containing 10 mM Tris-HCl, pH 8.0, 50% glycerol, and the protein concentration was determined using the CB protein assay kit (Calbiochem, La Jolla, USA).

Gel electrophoresis and Western blot analysis

Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [26] then transferred onto a polyvinylidene fluoride membrane (GE Healthcare, Princeton, USA) by semi-dry blotting at a constant current of 0.5 mA/cm^2 for 1.5 h. The membrane was immersed in blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3% bovine serum albumin, and 0.05% Tween-20) at room temperature for 1 h, followed by incubation with mouse anti-His antibody (GE Healthcare; diluted 1:3000) in blocking buffer at 4 ºC overnight. Subsequently, a secondary antibody, alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Promega) was added at a dilution of 1:7500 in blocking buffer at room temperature for 1 h, then the immunoblot signals were detected using NBT-BCIP substrate (Roche, Mannheim, Germany) in detection solution (50 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5).

Measurement of rTAPase activity

The enzymatic reaction was carried out at 75 ºC for 10 min in 1.0 ml appropriate buffer containing 6 mM *p*-nitrophenyl-phosphate disodium salt hexahydrate (*p*NPP; Amresco, Solon, USA) and 1.5 μg of rTAPase, and terminated on ice. The activity of rTAPase was measured spectrophotometrically at 410 nm. To correct the rate of non-enzymatic hydrolysis of *p*NPP, the reaction mixture without enzyme was used as a reagent blank. One unit of activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from *p*NPP in 1 min under the above assay conditions.

Characterization of rTAPase

The rTAPase was characterized by optimum pH, optimum temperature, thermostability, and the effect of

metal ions on its activity. Unless otherwise indicated, the following experiments were carried out using the reaction conditions described in the above section and the data were presented as the mean values of at least three independent experiments.

To assess the effect of pH, rTAPase activity was measured at various pH values ranging from 7.5 to 13.0. Tris-HCl buffer (100 mM) was used in the pH range 7.5–9.0, and 100 mM diethanolamine buffer was used in the range 8.5–13.0.

The optimum temperature was measured by carrying out the rTAPase activity assay at temperatures ranging from 40 ºC to 95 ºC at intervals of 5 ºC and at pH 12.0. To determine the thermostability, the enzyme was incubated for 6 h at different temperatures (50 ºC, 65 ºC, 80 ºC, and 95 ºC). Samples were taken out every 30 min and the residual activity was then measured as described above.

To test the effect of metal ions, rTAPase activity was determined by measuring the residual activity after incubation of the enzyme solutions with various metal ions such as $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $MgCl_2$, $MnSO_4$, and $ZnSO_4$ at 0.1 mM or 2.0 mM concentration.

Michaelis constant (K_m) of rTAPase

The K_m value of rTAPase was determined under optimum conditions using different concentrations of *p*NPP from 0 to 6 mM. A typical plot was obtained when v was plotted against $v/[S]$ (v, initial rate; $[S]$, substrate concentration), according to the method of Eadie-Hofstee and the K_m value was estimated by linear regression from it.

Results

Cloning and sequencing analysis of the *tap* gene

At present, analysis based on the 16S rRNA gene sequence is considered to be a standard in bacterial identification. For the thermophilic bacterium from Xiamen, its 16S rRNA gene was amplified and sequenced (GenBank accession No. DQ647385). BLAST analysis revealed that the strain had 99% similarity with *T. thermophilus* HB8 (GenBank accession No. AP008226). Based on sequence homology, the strain was considered to be a *T. thermophilus* sp., so it was named *T. thermophilus* XM.

A DNA fragment (approximately 1.5 kb) was amplified by PCR using *tap* gene primers and sequenced. Sequence analysis revealed that the gene consisted of 1506 bp (GenBank accession No. DQ645419) and was predicted to code a polypeptide of 501 amino acids with a calculated molecular mass of 54.7 kDa and isoelectric point of 8.7. The result from the DNA BLAST search showed that the *tap* gene shared over 93% sequence identity with other *tap* genes from the genus *Thermus*. In particular, it had the highest identity with *T. thermophilus* HB8 (99%).

Comparison of amino acid sequences of TAPase and other bacterial APase

When the deduced amino acid sequence of the gene was compared with other APases from thermophilic, mesophilic, and psychrophilic bacteria, TAPase had the highest identity with that from genus *Thermus*, such as *T. thermophilus* HB27 (99.6%) and *T. caldophilus* (98.4%), followed by *M. ruber* APase (64.3%). Identity was lower with *Thermotoga neapolitana* APase (27.3%), *Bacillus subtilis* APase (24.2%), Antarctic psychrophilic bacterium TAB5 APase (20.6%), and *E. coli* APase (18.2%). Although the whole sequence homology is not high between different APases, the alignment analysis showed that their active site regions involved in metal ion coordination, phosphorylation, and phosphate binding are highly conserved (**Fig. 1**), except that the residues D153 and K328 (*E. coli* numbering) are replaced by histidine (compared with *E. coli* APase).

Expression and purification of rTAPase

The *tap* gene was cloned into a His_6-tagged expression vector pQE30, then the recombinant plasmid pQE30-tap and empty vector pQE30 were transformed into XL-Blue. After induction with isopropyl-β-*D*-thiogalactoside, total cellular proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue. A protein an with apparent molecular mass of approximately 55 kDa, consistent with the predicted 54.7 kDa molecular mass of TAPase, was observed in XL-Blue cells transformed with pQE30-tap [**Fig. 2(A)**, lane 1] but absent from empty vector pQE30 [**Fig. 2(A)**, lane 2]. The His_6-tagged protein was purified by Ni-agarose chromatography and its concentration was determined to be 0.15 mg/ml. The purified product contained two bands of molecular masses 55 and 43 kDa, the slower migrating band being more abundant than the faster migrating band [**Fig. 2(A)**, lane 3]. Western blot analysis revealed that the 55 kDa protein was recognized specifically by anti-His antibody, indicating that it was indeed rTAPase [**Fig. 2(B)**].

Properties of rTAPase

Generally, the pH value of reaction solution can

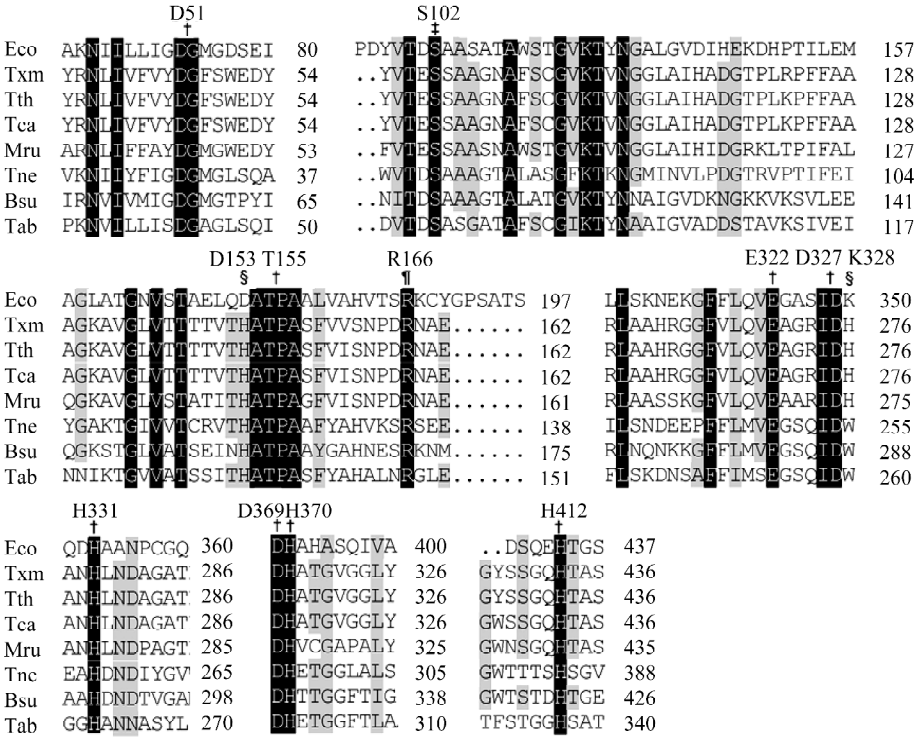

Fig. 1 Multiple alignments of the deduced amino acid of recombinant thermostable alkaline phosphatase from *Thermus thermophilus* XM with several representative alkaline phosphatases from thermophilic, mesophilic, and psychrophilic bacterium

Identical and similar residues in more than 75% of the sequences are shaded black and gray, respectively. *Escherichia coli* APase residue numbering was used. † metal ion coordination site (Zn1, Zn2 and Mg primary ligand); ‡ phosphorylation site; § metal ion coordination site (Mg secondary ligand); ¶ phosphate binding site. Bsu, *Bacillus subtilis* (GenBank accession No. BAA19698); Eco, *E. coli* (GenBank accession No. ZP00721282); Mru, *Meiothermus ruber* (GenBank accession No. AAN65628); Tab, Antarctic psychrophilic bacterium TAB5 (GenBank accession No. CAB82508); Tca, *Thermus caldophilus* (GenBank accession No. AAF13361); Tne, *Thermotoga neapolitana* (GenBank accession No. AAX98659); Tth, *Thermus thermophilus* HB27 (GenBank accession No. AAS82354); Txm, *Thermus thermophilus* XM.

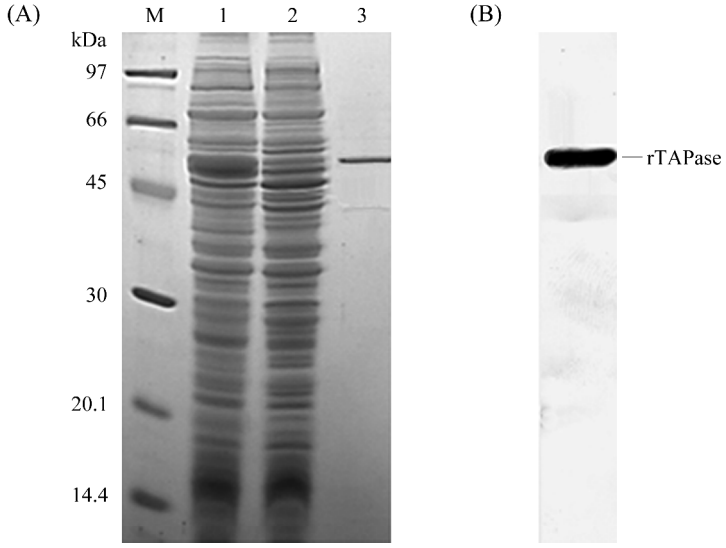

Fig. 2 SDS-PADE and Western blot analysis of rTAPase

(A) Coomassie Brilliant Blue-stained 10 % sodium dodecyl sulfate-polyacrilamide gel of purification of recombinant thermostable alkaline phosphatase (rTAPase). M, protein marker; 1, recombinant cell lysis (containing pQE30-tap); 2, control (pQE30 only); 3, purified recombinant protein. (B) Western blot analysis of rTAPase. Purified recombinant protein was separated by sodium dodecyl sulfate-polyacrilamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes, followed by incubation with anti-His antibody (diluted 1:3000) then alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G.

significantly affect enzyme activity. Like other APases, the rTAPase was activated under alkaline conditions. **Fig. 3** shows the pH profile of purified rTAPase activity. The optimal pH range of the enzyme was found to be 11.5–12.5, and maximal activity occurred at pH 12.0. It was nearly inactive below pH 7.5.

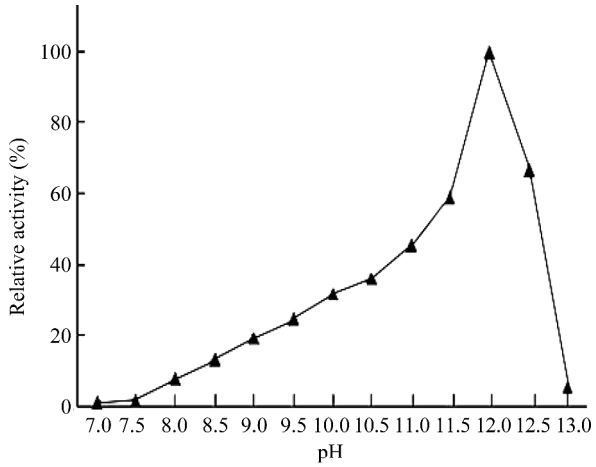

Fig. 3 Relative activity of recombinant thermostable alkaline phosphatase from *Thermus thermophilus* XM at various pH values from 7.0 to 13.0 at 75 ºC

For pH 7.0–9.0, 100 mM Tris-HCl buffer was used; and for pH 8.5–13.0, 100 mM diethanolamine buffer was used.

The purified rTAPase had its maximal enzymatic activity at 75–80 ºC, and approximately 50% of the activity was retained at temperatures above 40 ºC and below 85 ºC, indicating that the enzyme is capable of functioning over a broad temperature range (**Fig. 4**). In order to better estimate the effect of temperature on enzyme activity,

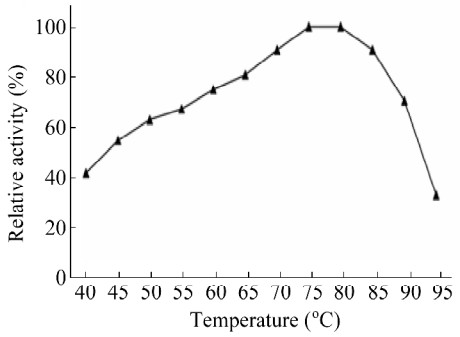

Fig. 4 Relative activity of recombinant thermostable alkaline phosphatase from *Thermus thermophilus* XM at various temperatures ranging from 40 ºC to 95 ºC at pH 12.0

thermostability was determined by incubating the rTAPase at temperatures ranging from 50 ºC to 95 ºC for different time points. As shown in **Fig. 5**, the rTAPase can be characterized as a moderately thermostable enzyme: over 50% of the activity was retained even after 6 h at 80 ºC. However, the activity was sharply decreased after treatment for 1.5 h at 95 ºC.

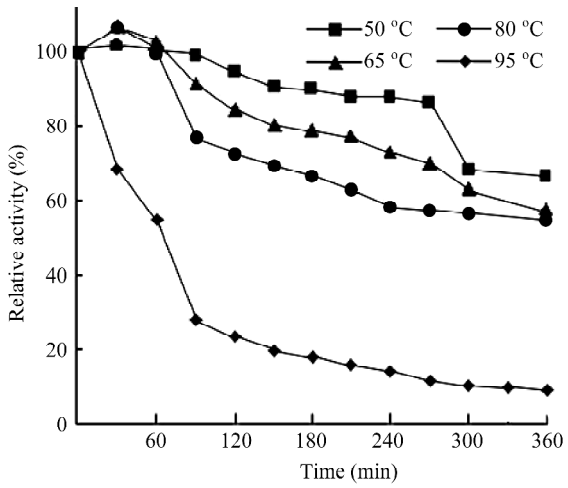

Fig. 5 Thermostability of recombinant thermostable alkaline phosphatase from *Thermus thermophilus* XM

The enzyme solutions were incubated at the indicated temperatures, then the residual enzyme activity was assayed spectrophotometrically at 410 nm using pNPP as chromogenic substrate.

As APases are metalloenzymes [2], metal ions as co-factors might play important roles in enzymatic reactions. The effects of some divalent metal ions on the rTAPase activity are shown in **Table 1**. Mg^{2+} is the most optimal for enzymatic reaction activity, but Zn^{2+}, Ca^{2+}, and Cu^{2+} show an inhibitory effect. Co^{2+}, Fe^{2+}, and Mn^{2+} (0.1 mM) can also significantly enhance the activity of rTAPase, especially Co^{2+}, however, there is a strong inhibition in the presence of 2 mM Fe^{2+}. As expected, the enzymatic activity of rTAPase was strongly influenced in the presence of the chelating agent EDTA (data not shown).

The K_m value of the rTAPase for *p*NPP was estimated to be 0.034±0.009 mM (**Fig. 6**), similar to those previously reported in other bacteria, such as *T. caldophilus* APase (0.036 mM) [20], *M. ruber* APase (0.055 mM) [23], and *E. coli* APase (ranging from 0.021 mM to 0.040 mM) [28,29], suggesting that the initial velocity of the reaction catalyzed by APases from both thermophilic and mesophilic bacteria is close.

Table 1 Effect of metal ions on the activity of recombinant thermostable alkaline phosphatase from *Thermus thermophilus* XM

Metal ions	Relative activity (%) †	
	0.1 mM	2.0 mM
Ca^{2+}	79.1	84.1
Co^{2+}	243.4	74.7
Cu^{2+}	96.4	70.5
Fe^{2+}	125.0	11.3
Mg^{2+}	127.8	157.4
Mn^{2+}	130.9	67.8
Zn^{2+}	77.2	48.6

† Relative to the activity with no metal ions (100%).

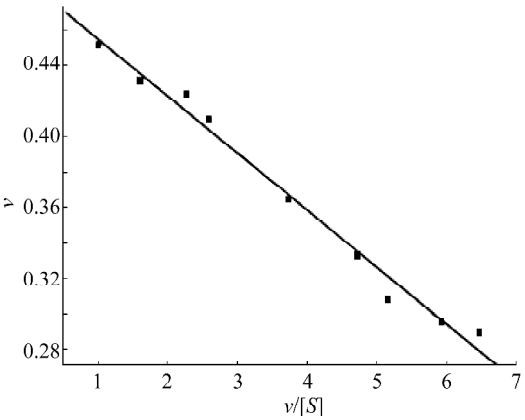

Fig. 6 Determination of the Michaelis constant (K_m) of recombinant thermostable alkaline phosphatase from *Thermus thermophilus* XM for *p*-nitrophenyl-phosphate disodium salt hexahydrate

The enzyme (1.5 μg/ml) was measured at 75 ºC, pH 12.0 with various substrate concentrations from 0 to 6 mM. v was plotted against $v/[S]$ (v, initial rate; $[S]$, substrate concentration) according to the method of Eadie-Hofstee and the K_m value was estimated by linear regression from it.

Discussion

APases from thermophilic microorganisms are becoming more and more interesting in diagnostics, immunology and molecular biology such as non-radioactive labeling, due to their inherent advantages which are more resistant to thermal denaturation during preparation or labelling procedures, easier to transport and store, and longer half-life of enzyme activity.

In this study, a *tap* gene was cloned from *T. thermophilus* XM. Although the sequence homology is very low between different APases, the amino acid residues in the active site were highly conserved (**Fig. 1**). All ligands to Zn1, Zn2 and Mg, S102 and R166 are absolutely conserved in all APases. In *E. coli* APase, Zn1 is coordinated by two imidazole bases of H331 and H412, as well as one carboxyl of D327. Zn2 is bound by two carboxyls of D51 and D369 along with one imidazole base of H370. Mg is bound by residues D51, T155, and E322 [12]. However, the secondary ligands of the Mg ion, that is, D153 and K328, are replaced by histidine in *T. thermophilus* XM APase (**Fig. 1**). D153 is a part of the Mg ion binding site, and K328 is important for phosphate binding in *E. coli* APase. The same substitutions were found in *T. thermophilus* HB27, *T. caldophilus*, *M. ruber*, and mammalian APases. Previous studies showed that the replacement of D153 resulted in an enhancement of enzymatic activity when the Zn^{2+} bound in this site is replaced by Mg^{2+} [27].

The characterization studies showed that rTAPase is one of the most characterized alkaline APases so far identified. By analogy with the mammalian APase, which have higher pH optima than the *E. coli* APase, we can speculate that the substitution of amino acid residues corresponding to D153 and K328 in rTAPase by histidine is responsible for the more alkaline activity profile of the rTAPase [30]. However, the reason for alkaline phosphatase's resistance to extreme pH remains unclear. In fact, we are trying to reveal the inherent mechanism of this resistance to extreme alkaline in another experiment.

APase is a metalloenzyme, and different metal ions could have different influences on rTAPase activity. Many studies showed that the same metal ion has different effects (enhancer or inhibitor) on the activity of APases from different sources, for example, Zn^{2+} could be an inhibitor for *M. ruber* APase but be an enhancer for *Thermotoga neapolitana* APase; Co^{2+} could enhance the activity of *T. thermophilus* XM APase but inhibit the activity of *M. ruber* APase [22–25]. We hypothesize that the phenomenon might be the result of the differences between the active sites conformation of different APases. As shown in **Fig. 1**, although almost all metal coordinating amino acid residues in the active sites are conserved, the residues close to metal binding sites are variable from different sources. It suggested that these varieties might result in some conformational changes to affect the binding affinity for different metal ions.

In summary, a *tap* gene from *T. thermophilus* XM was successfully cloned and expressed in *E. coli*. The recombinant enzyme shared common characteristics of alkaline phosphatase and displayed good thermostability.

In addition, it also revealed a unique property in that it showing a strong competence for high alkaline conditions compared with other APases. These characteristics suggest that the enzyme has potential value in experimental science. For instance, it could be applied in nucleic acid hybridization and *in situ* hybridization under high temperature and alkaline environments instead of APases from other sources.

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