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Purification and Characterization of a Thermostable Alkaline Phosphatase Produced by *Thermus caldophilus* GK24

You-Jin Kim, Tae-Shin Park, Hyun-Kyu Kim and Suk-Tae Kwon*

Department of Genetic Engineering, Sung Kyun Kwan University, Suwon 440-746, Korea
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Abstract : The thermophilic and thermostable alkaline phosphatase was purified to near homogeneity from the osmotic lysis of *Thermus caldophilus* GK24. The purified enzyme had an apparent molecular mass of 108,000 Da and consisted of two subunits of 54,000 Da. Isoelectric-focusing analysis of the purified enzyme showed a pI of 7.3. The enzyme contained two Cys residues, and its amino acids composition was quite different from that of *Thermus aquaticus* YT-1 alkaline phosphatase and *Escherichia coli* alkaline phosphatase. The optimum pH and temperature of the enzyme were 11.0-11.5 and 80°C, respectively. The enzyme was stable in the pH range of 9.0-12.0 at 25°C for 36 h, and the half-life at 80°C (pH 11.0) was 6 h. The enzyme was activated by $MgCl_2$ and inhibited by EDTA. With *p*-nitrophenyl phosphate (*p*NPP) as the substrate, the enzyme had a Michaelis constant (K_m) of 3.6×10^{-5} M. The enzyme preferentially hydrolyzed the phosphomonoester bond of AMP in ribonucleotides and glycerophosphate.
Key words : *Thermus caldophilus* GK24, thermostable alkaline phosphatase

Alkaline phosphatase (APase) is a non-specific phosphomonoesterase that functions through a phosphoseryl intermediate to produce a free inorganic phosphate or to transfer the phosphoryl group to other alcohols (Coleman and Gettins, 1983). APase is widely distributed in nature. It is a dimeric metalloenzyme with two Zn^{2+} and one Mg^{2+} in each active site region (Kim and Wyckoff, 1991). It is found in both prokaryotes and eukaryotes. Many physiological functions of the enzyme have been postulated, including transport of substances such as fatty acid choline and calcium (Norman *et al.*, 1970; Pekarthy *et al.*, 1972; Koyama *et al.*, 1983), nutritional supply of phosphate (Baren and Levinthal, 1960) and bone formation (Matsuzawa and Anderson, 1971).

The *E. coli* APase has been studied most extensively by a variety of physicochemical techniques (Kim and Wyckoff, 1991). The enzyme is involved in the acquisition of phosphate from esters when free inorganic phosphate is depleted (Torriani, 1960). The enzyme is a dimer (*Mr*=94,000) with 449 amino acid residues per monomer in each of the two chemically identical subunits and is located in the periplasmic space with neither a carbohydrate nor a fatty acid tail (Bradshow *et al.*, 1981; Chang *et al.*, 1986).

Extremely thermophilic bacteria belonging to the genus *Thermus* can be routinely grown at temperatures of 70-75°C, and some species can grow at 85°C (Wiegel, 1986). The enzymes of thermophilic bacteria are generally heat stable, and the stability of these proteins is interesting for protein chemistry. Besides their scientific interest, these heat-stable enzymes are valuable because of biotechnological applications. Thus, a number of highly thermostable enzymes have been isolated and characterized from *Thermus* bacteria (Ulrich *et al.*, 1972; Chien *et al.*, 1976; Takahash *et al.*, 1984; Matsuzawa *el al.*, 1988; Takase and Horikoshi, 1988;). However, there is only one known APase from *Thermus* bacteria. The enzyme has been isolated from *T. aquaticus* YT-1, has an approximate molecular weight of 143,000 Da, and consists of three subunits, each with a molecular weight of 51,000 Da (Yeh and Trela, 1976).

In our work we have found thermophilic APase from *T. caldophilus* GK24. Here we report the purification and properties of *T. caldophilus* GK24 (*Tca*) APase compared with those of other APases.

Materials and Methods

Strain and culture conditions

T. caldophilus GK24 cells (Taguchi *et al.*, 1982) were initially grown at 75°C, pH 7.6, in 0.4% polypeptone, 0.2% yeast extract and a basal salt (Ramaly and Hixon, 1970). For the induction of *Tca* APase, 1% of the full growth culture was inoculated into an induction medi-

*To whom correspondence should be addressed.
Tel : 82-331-290-7863, Fax : 82-331-290-7870
E-mail : stkwon@yurim.skku.ac.kr

um consisting of 0.2% bactotryptone, 0.5% glucose, 0.3 % sodium glutamate, 20 mM Tris/HCl (pH 8.0) and a basal salt (sodium phosphate was omitted), and then incubation was carried out at 72℃ for about 18 h with shaking (Kim *et al.*, 1995).

APase assays

Enzyme activity was assayed with *p*NPP (Sigma, St. Louis, USA). The assay mixture contained 0.8 ml of 0.1 M Glycine/NaOH buffer, pH 11.0, 1 mM $MgCl_2$, 0.1 ml of 10 mM *p*NPP in water, and 0.1 ml of an enzyme sample. The reaction was performed at 80℃ and terminated by the addition of 0.2 ml of 2 M NaOH. The extent of hydrolysis was determined from the absorbance of the liberated *p*-nitrophenol at 410 nm using an extinction coefficient of 1.77×10^4 M^{-1} cm^{-1} (Onish *et al.*, 1979). A unit of activity was defined as that amount of enzyme which liberated 1 μmol of *p*-nitrophenol in 1 min at 80℃.

The hydrolysis of non-chromogenic substrates by APase was carried out in 0.04 M sodium barbitol buffer (at various pH values) containing 1 mM $MgCl_2$. The inorganic phosphate released was measured by the procedure of Chen *et al.* (1956) as described by Ames (1966).

Preparation of the affinity chromatography adsorbent

The affinity adsorbent material was prepared by coupling the diazonium salt of 4-(*p*-aminophenylazo) phenylarsonic acid (Aldrich Chemical) to a tyraminyl-Sepharose derivative obtained by the reaction of tyramine with cyanogen bromide-activated sepharose 4B (Brenna, 1975).

Purification of APase

Step 1. Preparation of crude extracts: The cells were harvested by centrifugation, then washed two times with 200 ml of cold 10 mM Tris/HCl, pH 8.0. Cells were subject to osmotic shock (Neu and Heppel, 1965) by the following standard procedure: Cells (60 g, wet weight) were suspended in 200 ml of 20% sucrose, 10 mM Tris/HCl, pH 8.0. Lysozyme was added to 0.1 mg/ml, and the cell suspension was incubated at room temperature for 15 min in the presence of 1 mM EDTA and then for a further 10 min in the presence of 10 mM $MgSO_4$. The mixture was centrifuged for 20 min at 12,000×g. The supernatant fluid was collected, and the pellet was rapidly mixed with 200 ml of cold 10 mM Tris/HCl (pH 8.0). The suspension was mixed in an ice bath for 15 min and centrifuged, and the supernatant fluid was collected.

The pellet was also mixed with 100 ml of cold 10 mM Tris/HCl (pH 8.0) and centrifuged, and the supernatent fluid was collected.

Step 2. Treatment with streptomycin sulfate and ammonium sulfate precipitation: Solid streptomycin sulfate was slowly added to the crude extract solution with gentle stirring at room temperature to give a 1% streptomycin sulfate solution. The mixture was then stirred for 30 min and centrifuged at 13,200 g for 20 min. The supernatant was brought to 70% saturation by the slow addition of powdered ammonium sulfate. After 10 h, the crude proteins were precipitated by centrifugation (13,200 g, 30 min, 4℃). The pellet was dissolved in 100 ml standard buffer (10 mM Tris/HCl buffer, pH 7.4, containing 1 mM $MgCl_2$), and dialyzed four times for 6 h against the same buffer.

Step 3. DEAE-cellulose chromatography: After dialysis, the enzyme solution was applied to a column of DEAE-cellulose DE32 which had been previously equilibrated with the standard buffer (Piggot *et al.*, 1972). Most of the applied enzyme activity was eluted without retention on washing of the column with the standard buffer. This step was essential for removing most proteins for the following purification step.

Step 4. Affinity chromatography on 4(4-aminophenylazo) phenylarsonic acid-Sepharose 4B: The breakthrough fraction (nonabsorbed alkaline phosphatase) obtained above was applied to a column of the affinity gel (Cuattrecasas, 1970) equilibrated with the standard buffer. The column was washed with equilibrated buffer and with equilibrated buffer containing 1 M KCl.

Finally, APase was eluted with a linear gradient of 0-2.0 M guanidine-HCl in the standard buffer containing 1 M KCl. The enzyme fractions were dialyzed against the standard buffer. The purified APase was stored in the frozen state at -20℃ until use. The purified enzyme preparation migrated as a single protein band on the SDS-PAGE.

Protein determination

Protein was determined by the procedure of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Electrophoretic analysis

Polyacrylamide gel electrophoresis under non-denaturing conditions was based on the procedure of Davis on continuous 7% polyacrylamide gels at 15℃ (1964). The samples were made 10% in glycerol and 20 μl aliquots were layered on the gels. After an electrophoretic run, one gel was stained for protein with Coomassie brilliant blue G-250 (Holbrook and Leaver, 1976) and one for APase activity. APase activity was detected by the simultaneous capture method (Gabriel, 1971). The activity staining mixture contained 50 mM Tris/HCl pH 11.0, containing 1 mM $MgCl_2$, 10 mM α-

napthylphosphate and 1 mg/ml fast red TR. The gel was incubated in the mixture at 80°C for 20 min and then rinsed in water. Gels were stored in 7% acetic acid.

Determination of Molecular Weight

The molecular weight of native APase was determined by gel filteration (Andrew, 1965) through a 1.2×70 cm column of Sephacryl HR S 200. The column was equilibrated and eluted with 50 mM Tris/HCl buffer, pH 8.0, containing 0.1 M NaCl and 1 mM $MgCl_2$. The elution volume (*Ve*) of each fraction was determined from the absorbance at 280 nm and the enzyme activity. The void volume (*Vo*) was determined by dextran-blue exclusion. The *Tca* APase molecular mass was calculated by plotting the log (molecular weight) versus the *Ve*/*Vo* ratio.

Subunit studies were performed using SDS-PAGE according to the method of Weber and Osborn (1969). Samples were incubated at 100°C for 5 min in 10 mM Tris/HCl pH 8.0, 1% SDS and 1% 2-mercaptoethanol to obtain complete denaturation of the enzyme. The electrophoretic run was performed on 12% polyacrylamide vertical gels. Proteins were stained in SDS gels with Coomassie briliant blue R250 (Hol- brook and Leaver, 1976). Gels were stored in 7% acetic acid.

Determination of pI by isoelectric focusing

Analytical isoelectric focusing was carried out in the pH range 3.5-9.5 on a polyacrylamide gel slab at 10°C using Ampholyte (Pharmacia) according to the method of Bollag and Edelstein (1991).

Amino acid analysis

Amino acid analysis was performed as described by Spackman *et al.* (1957) on a Waters model HPLC PICO TAG system amino acid analyzer. Lyophilized samples of *Tca* APase were hydrolyzed at 110°C in 6 M HCl for 24, 48 and 72 h. The cysteine content was determined after a 24 h hydrolysis at 110°C as cysteic acid, as described by Moore (1963).

The tryptophan content was determined after a 24 h hydrolysis at 110°C in 3 M mercaptoethanesulfonic acid by the method of Penke *et al.* (1974). Evaluation of the free thiols of APase was performed by reaction with 5,5'-dithio-bis (2-nitrobenzoic acid) according to Ellman (1959).

pH profile

The dependence of APase activity on the pH was determined between pH 4.0-12.0, using *p*NPP as a substrate. The buffers used were: 50 mM sodium citrate in the pH range of 4.0-7.0, 50 mM Tris/HCl in the pH range of 7.0-9.0 and 50 mM glycine/NaOH in a pH range of 9.0-12.5. The pH values were adjusted at room temperature. The APase assays were carried out at 80°C in the presence of 2.8 mM *p*NPP.

Optimal temperature and thermostability

The dependence of APase activity on temperature was studied under standard assay conditions over 40-100°C. Enzyme thermostability was studied at 80°C in a protein concentration of 10 μg/ml. The purified enzyme was incubated in 10 mM glycine/NaOH buffer (pH 11.0) and Tris/HCl (pH 9.0) in sealed Eppendorf tubes for 0-24 h at 80°C. At each desired time, aliquots of the preincubated enzyme were withdrawn from the incubation mixtures and assayed at 80°C in standard conditions.

Kinetic constants

The kinetic constants of *Tca* APase for *p*NPP were measured at 80°C in glycine/NaOH buffer pH 11.0. All the enzyme determinations were done in duplicate and the respective kinetic parameters were evaluated from Lineweaver-Burk plots (Lineweaver and Burk, 1934).

Results and Discussion

Induction and purification of *Tca* APase

The APase of *T. caldophilus* GK24 is produced when the cells are starved of phosphate (Kim *et al.*, 1995). The highest specific activity is obtained when the induction medium is completely devoid of phosphate. When the cells are starved of phosphate, the production of the enzyme starts and continues at a constant rate for at least 18 h (data not shown).

The purification of the enzyme is summarized in Table 1. The specific activity of the purified enzyme sample was more than 16.6 times that of the ammonium sulfate precipitate, and the recovery of the enzyme was about 8.7% on the basis of the APase activity in the crude extract.

A *Tca* APase from *T. caldophilus* GK24 was purified to homogeneity in four steps including DEAE-cellulose (Piggot *et al.*, 1972) and affinity chromatography (Onish

Table 1. Purification scheme for *Tca* APase from *T. caldophilus* GK24. The purification was started with 60 g wet cells

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Crude extract	4,500	1,155,644	257	100
Streptomycin/ Ammonium sulfate	672	367,892	548	31.8
DEAE column	152	157,002	1,033	13.6
Affinity column	11	100,101	9,100	8.7

et al., 1979). Most of the proteins of crude enzyme were adsorbed at pH 7.4 to the DEAE-cellulose resin, but *Tca* APase was not adsorbed to the DEAE-cellulose resin. Thus, most of the applied enzyme activity was easily obtained without retention on washing of the DEAE-cellulose column with standard buffer.

Tca APase was adsorbed tightly at neutral pH to the affinity resin and most of the proteins adsorbed were washed with the standard buffer containing 1 M KCl, but *Tca* APase was not eluted with the same buffer. The elution of *Tca* APase was achieved with the standard buffer containing 1.5 M guanidine-HCl and 1 M KCl. Buffers containing a high concentration of phosphate or of salt were not effective, as described also for the purification of yeast APase (Onish *et al.*, 1979) and calf intestine APase (Brenna *et al.*, 1975) with the same affinity adsorbent.

The purified sample of *Tca* APase appeared to be homogeneous by gel electrophoresis under nondenaturing conditions (Fig. 1A). A single protein band was evident when the gel was stained with Coomassie blue, and this band was coincident with APase activity when the gel was stained with activity staining mixture. A single protein band corresponding to a molecular mass of 54,000 Da was obtained by polyacrylamide gel electrophoresis under denaturing conditions (Fig. 1B).

The molecular mass of native APase was estimated by gel filteration on a Sephacryl HR S 200 column. The value obtained was 108,000 Da (Fig. 2). These

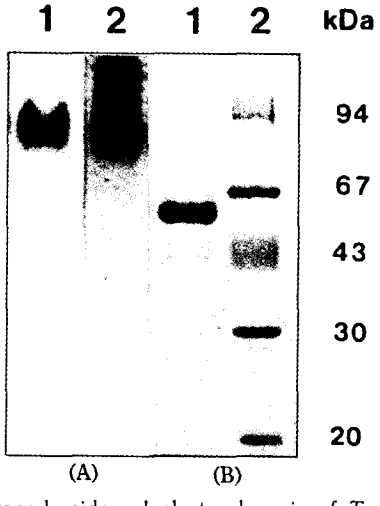

Fig. 1. Polyacrylamide gel electrophoresis of *Tca* APase. (A) A sample of APase was subjected to electrophoresis under nondenaturing conditions as described in Materials and Methods. Lane 1 was stained with Coomassie brilliant blue and the parallel lane 2 for APase activity. (B) A sample of APase was subjected to electrophoresis under denaturing conditions (SDS-PAGE), After an electrophoretic run, the gel was stained with Coomassie brilliant blue. Lane 1, purified enzyme; lane 2, molecular markers.

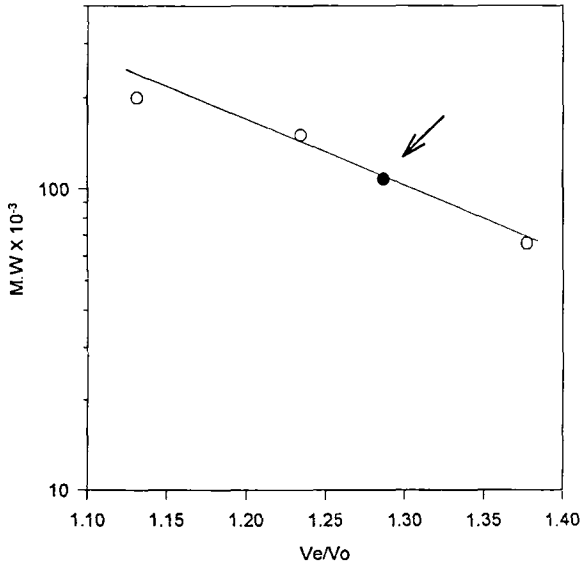

Fig. 2. Determination of the molecular weight of *Tca* APase by gel filtration on Sephacryl HR S 200. (●) *Tca* APase; (○) molecular mass markers (β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; albumin, 66 kDa; Carbonic anhydrase, 29 kDa).

results suggest that *Tca* APase in its native conformation is a dimer consisting of two identical subunits.

The approximate molecular weight of this enzyme was lower than that reported for *Taq* APase (143,000 Da) (Yeh and Trela, 1976). The apparent subunit mass of this enzyme was a little higher than those reported for *Taq* (Yeh and Trela, 1976) and *E. coli* APases (Bradshaw *et al.*, 1981).

Isoelectrofocusing of the purified enzyme showed a band corresponding to pI 7.3 when the plates were stained for protein and activity.

Amino acid composition of *Tca* APase

The amino acid composition of *Tca* APase is shown in Table 2, compared with those of *Taq* (Yeh and Trela, 1976) and *E. coli* APases (Bradshaw *et al.*, 1981). The number of residues of each amino acid was calculated on the basis of a monomer molecular mass of 54 kDa. The amino acid composition of two enzymes which belong to the genus *Thermus* are quite a different. For instance, the content of the lysine, methione and tyrosine is considerably lower in the *Tca* APase than in *Taq* APase.

Tca APase has low Lys and high Arg contents compared with the *E. coli* APase. This is another characteristic of *Tca* APase, which is consistent with the correlation of amino acid substitutions observed in thermophilic and methophilic alkaline protease (Matsuzawa *et al.*, 1988).

Tca APase contains two Cys residues. Since free Cys

Table 2. Amino acid composition of *Tca* APase in comparison with those of several APases. The amino acid composition of *Tca* APase was calculated on the basis of a *Mr* of 54,000 (see the text)

Amino acid APase	*Tca* APase		*Taq* APase	*E. coli*
	found	integral		
	residues/ molecule			
Lys	12.1	12	20	28
His	11.6	12	10	10
Arg	47.8	48	31	13
Trp	8.7	9	10	3
Asx	44.4	44	42	48
Thr	19.8	20	22	40
Ser	10.8	11	24	22
Glx	55.1	55	49	46
Pro	31.2	31	23	21
Gly	51.7	52	43	45
Ala	64.3	64	58	64
Val	41.0	41	38	23
Met	2.9	3	15	8
Ile	12.4	12	15	16
Leu	50.5	51	44	39
Tyr	8.4	8	19	11
Phe	16.3	16	20	8
Cys	1.9	2	2	4
Total	490.90	491	485	449

residues were detected with the method of Ellman (1959) using 5.5'-dithio-bis(2-nitrobenzoic acid) and the treatment of *Tca* APase with 2-mercaptoethanol and DTT was activated for activity of the enzyme, the Cys residues of *Tca* APase seem to form a sulfhydryl group.

Apparently, the NH_2-terminal residue was blocked because repeated cycles of automated Edman degradation with a sequencer produced no detectable phenylthiohydant-conjugated amino acids.

Effect of pH on the activity and stability of *Tca* APase

The change in activity as a function of pH was determined by the procedure described in Materials and Methods. The results are shown in Fig. 3. The *Tca* APase exhibited maximum activity toward *p*NPP hydrolysis in glycine/NaOH buffer at pH 11.0-11.5. This result was different from that reported on the *Taq* APase purified from *T. aquaticus* YT-1 (Yeh and Trela, 1976). *Taq* APase for *p*NPP hydrolysis was maximal when assayed in Tris/HCl buffer at pH 9.2. The pH stability of the enzyme was determined by incubating the enzyme solution at different pH values for 36 h at 25°C and then assayed at pH 11.0. The results indicated that enzymes are relatively stable at pH 9.0-12.0 in the presence of 1 mM $MgCl_2$ (data not shown).

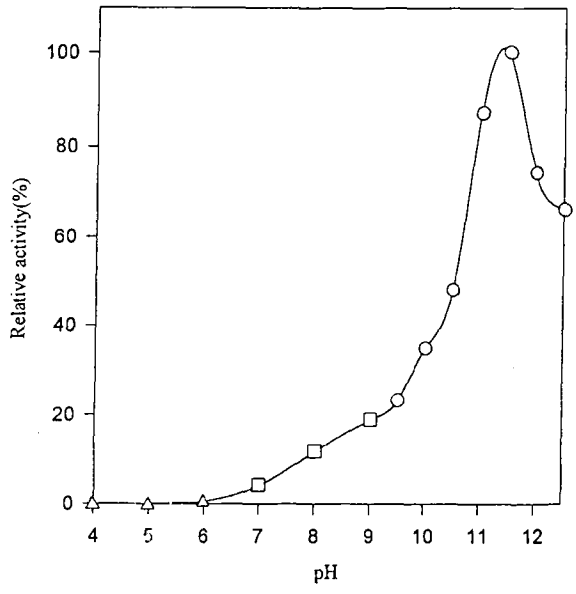

Fig. 3. Effect of pH on the APase activity of *Tca* APase. The reaction was carried out in the following buffers (50 mM concentration) in the presence of 1 mM $MgCl_2$: Sodium citrate (△), Tris/HCl (□), Glycine/NaOH (○), at 80°C. pH values shown are those at 25°C.

Effect of temperature on the activity and stability of *Tca* APase

The effect of temperature on the *Tca* APase activity was examined in the presence of $MgCl_2$ (Fig. 4). The temperature at which the maximal activity was ob-

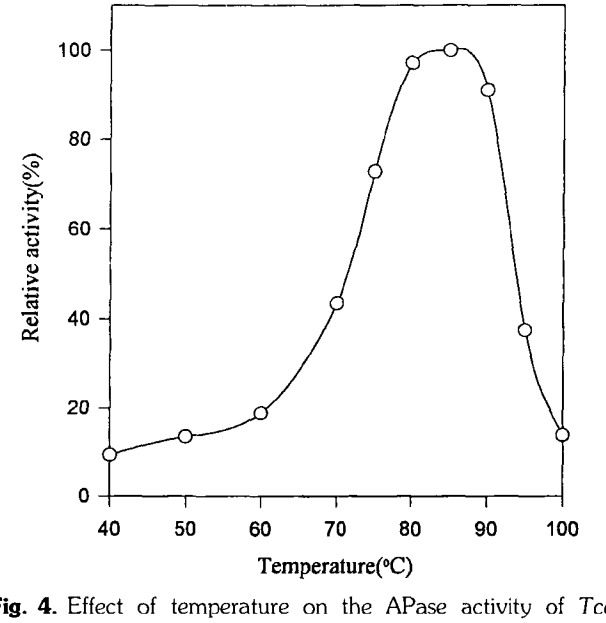

Fig. 4. Effect of temperature on the APase activity of *Tca* APase. The enzyme activity was assayed at the indicated temperature in the presence of 1 mM $MgCl_2$.

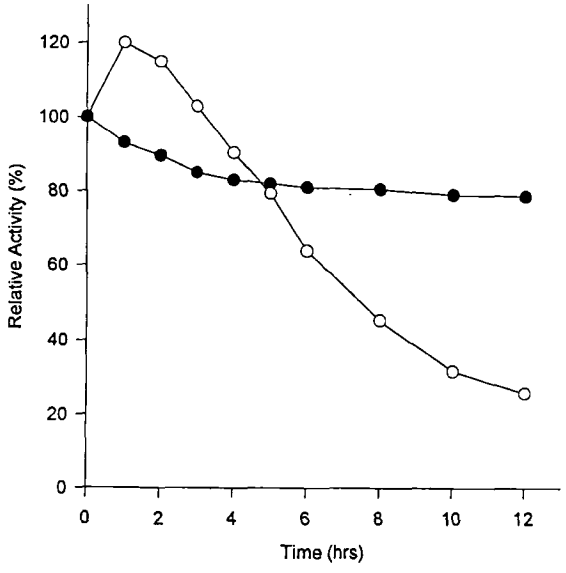

Fig. 5. The heat stability of *Tca* APase. The enzyme was dissolved at a concentration of 10 μg protein/ml in the following buffers (50 mM concentration) in the presence of 1 mM $MgCl_2$: Glycine/NaOH at pH 11.0 (○), Tris/HCl at pH 9.0 (●), After incubation at 80°C for the indicated times, the remaining activity was assayed as described under Materials and Methods.

tained was 80-85°C.

Fig. 5 shows the thermostability of *Tca* APase at 80°C. The enzyme was fairly stable at pH 9.0 in Tris/HCl buffer solution at 80°C. However, the enzyme was unstable at pH 11.0 in glycine/NaOH buffer at the same temperature. The half-life at pH 11.0 and pH 9.0 (80°C) was 6 and 72 h, respectively.

Hydrolytic activity of *Tca* APase toward various substrates

The hydrolytic activity of the enzyme towards ten kinds of substrates was examined. The results are shown in Table 3. The purified *Tca* APase shows higher rates of hydrolysis for the nucleotide monophosphates. The enzyme preferentially hydrolyzed the phosphomonoester bond of glycerophosphate. Lower reactivity was shown towards α-naphthyl acid phosphate and 5-bromo-4-chloro-3-indolyl phosphte (BCIP). Reactivity with tris (*p*-nitrophenyl) phosphate and bis (*p*-nitrophenyl) phosphate was negligible.

Effect of EDTA, DTT and divalent cations on catalytic activity

The effect of EDTA and DTT on the activity of the enzyme was examined by assaying enzyme samples in the presence of EDTA and DTT at various concentrations. *Tca* APase was inhibited by 2 mM EDTA, but the enzyme was activated 3 times by 1 mM DTT (data not shown).

Table 3. Relative activity of *Tca* APase on various substrates. The activity of hydrolysis was determined in 50 mM glycine/NaOH buffer at pH 11.0. Reaction mixtures contained 3.0 mM substrate and were incubated at 80°C. Hydrolysis rate was measured by the amount of inorganic phosphate formed

Substrate	Relative hydrolysis rate
*p*NPP	1.0
BCIP	0.35
AMP	1.47
UMP	1.01
GMP	1.09
CMP	0.83
ATP	1.09
dAMP	1.09
TMP *p*-nitrophenyl ester	0.02
α-naphthyl acid phosphate	0.35
Bis(*p*-nitrophenyl) phosphate	0.02
Tris(*p*-nitrophenyl)phosphate	0
Glycerophosphate	1.54

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

The effects of metal ions on the activity of the enzyme were examined by assaying enzyme samples in the presence of various metal ions at the indicated concentration. The results are shown in Table 4. Activation of Tca APase is observed with Co^{2+}, Mg^{2+} and Ca^{2+}, whereas Zn^{2+} is inhibitory. Manganese ions have little effect on activity.

The 2 mM EDTA-inhibited enzyme can be reversed by adding Mg^{2+} ions but not by adding zinc or calcium ions. The Zn^{2+} ion is an essential metal ion for most of the other APase (Kim and Wyckoff, 1991) but not essential for *Tca* APase. Thus, *Tca* APase is very unusual in its requirement for Mg^{2+} and not Zn^{2+}. Similar results were observed in studies of *Haloarcula marismortui* APase (Goldman *et al.*, 1990). The optimal Mg^{2+} concentration for enzymatic activity is 1 mM (data not shown).

Table 4. Effect of divalent cations of *Tca* APase

Divalent cations	Relative activity (intact enzyme) (1 mM)
$MgCl_2$	190.0
$CaCl_2$	5.2
$CuSO_4$	99.5
$MnCl_2$	83.2
$ZnSO_4$	31.0
$CoCl_2$	8.0
None[a]	100.0

The enzyme (0.1 g) was added to an assay mixture containing 50 mM glycine/ NaOH, pH 11.0, 2.8 mM *p*NPP. $MgCl_2$ was omitted from the assay mixture, and the indicated divalent cations were added.

[a]Activity detected in the absence of metal ions.

Effect of phosphate on catalytic properties

The *K*m value of *Tca* APase for *p*NPP was determined from Lineweaver-Burk plots (Lineweaver and Burk, 1934). Measurements were made at 80°C in 0.1 M glycine/ NaOH buffer, pH 11.0. The presence of phosphate in the assay affects the Km of reaction but not the *V*max. The *K*m of *p*NPP in the absence of phosphate is 0.036 mM. This value increases to 0.41 mM at 1 mM phosphate concentration. This mode of inhibition is typical of competitive inhibition.

Acknowledgements

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