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Cloning, Expression and Characterization of Alkaline Phosphatase from a Thermophilic Bacterium *Geobacillus caldoxylosilyticus* TK4

[*Geobacillus caldoxylosilyticus* TK4 Termofilik Bakterisinden Alkalin Fosfatazın Klonlanması, Ekspresyonu ve Karakterizasyonu]

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ABSTRACT

Aim: To clone the gene for alkaline phosphatase (AP) from a novel thermophilic *Geobacillus caldoxylosilyticus* TK4 strain and to express and characterize this enzyme.

Methods: The gene was cloned into pET28a(+) vector and expressed in *Escherichia coli* BL21(DE3)pLysS. The recombinant protein was purified by using nickel affinity chromatography and characterized.

Results: The gene was 1410 bp long and contained highly conserved regions in the vicinity of the phosphorylation and metal binding sites. The recombinant AP had pH and temperature optima of 9.5 and 50 ºC, respectively, and was ~100% active at between 10 and 30 °C for 2 h. and after 12 h incubation at pH 9.5 and 4°C. It was less active in presence of 1 mM Hg^{2+}, Cd^{2+} and Al^{3+}, and was completely inhibited by 1 mM EDTA. Its K_m and V_{max} values, using *p*-nitrophenyl phosphate as substrate were 87 µM and 0.049 U mg $protein^{-1}$, respectively.

Conclusion: This recombinant AP shared characteristics of other APs such as conserved amino acid residues, electrophoretic behaviour, pH- and temperature optima, K_m and V_{max} values and could have some clinical and molecular biological applications.

Key Words: Alkaline phosphatase, Gene expression, *Geobacillus caldoxylosilyticus,* Cloning, Thermophilic

ÖZET

Amaç: *Geobacillus caldoxylosilyticus* TK4 termofilik bakterisinden elde edilen alkalin fosfataz (AP) kodlayan genin klonlanması, ekspresyonu ve bu enzimin karakterizasyonudur.

Yöntem: *G. caldoxylosilyticus* TK4 termofilik bakterisinden AP kodlayan gen pET28a(+) vektörüne klonlanıp *E. coli* BL21(DE3)pLysS'de ekspres edildi. Rekombinant protein nikel affinite kromatografisiyle saflaştırıldı ve saf proteinin karakterizasyonu gerçekleştirildi.

Bulgular: AP kodlayan gen 1410 bç uzunluğundadır ve fosforilasyon ve metal bağlanma bölgelerinin oldukça korunduğu gözlenmiştir. Rekombinant AP'nin optimum pH ve sıcaklığı sırasıyla 9,5 ve 50 ºC'dir. Enzim, 10 ve 30°C aralığındaki sıcaklıklarda, 2 saat süreyle ve pH 9,5'ta 4 ºC'de 12 saat inkübasyondan sonra ~%100 aktiftir. 1 mM Hg^{2+}, Cd^{2+} ve Al^{3+} varlığında AP aktivitesi azalırken, 1 mM EDTA ile tamamen inhibe olmaktadır. Enzimin K_m ve V_{maks} değerleri *p*-nitrofenil fosfat kullanılarak sırasıyla 87 µM ve 0.049 U mg $protein^{-1}$ olarak belirlendi.

Sonuçlar: *G. caldoxylosilyticus* TK4'ten elde edilen rekombinant AP'ın, diğer AP'larla korunmuş aminoasit birimleri, elektroforetik davranış, pH ve sıcaklık optimumu, K_m ve V_{maks} değerleri bakımından benzer karakteristik özelliklere sahip olduğu ve bazı klinik ve moleküler biyoloji uygulamalarında kullanılabileceği sonucuna varılmıştır.

Anahtar Kelimeler: Alkalin fosfataz, Gen ekspresyonu, *Geobacillus caldoxylosilyticus*, Klonlama, Termofilik

Introduction

Alkaline phosphatase (AP, orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) belongs to a group of enzymes that are widely distributed in nature from microorganisms to mammals and important in phosphate transport and metabolism. These enzymes are applied extensively in diagnostics, immunology, and molecular biology as they are sensitive markers in enzyme-linked immunosorbent assay, Western blotting analysis, nucleic acid hybridization, and in situ hybridization [1].

The structure and catalytic properties of *Escherichia coli* AP and of many mammalian APs have been studied extensively [2-5]. These phosphatases show great structural and functional diversity in subunit size, metal ion requirements and substrate specificities [6-9].

At present, *E.coli* AP and calf intestinal AP are the most commonly used, but their inherently low thermal resistance and shelf-lives have restrict their application under conditions of high temperature and high pH. Thermostable APs have many beneficial qualities, such as high thermostability, high reaction rates, and excellent resistance to denaturation or microbial contamination [1]. Because of these advantages, there has been increasing attention to AP from thermophilic bacteria. [1,10,11]

We have cloned the AP gene from *Geobacillus caldoxylosilyticus* TK4 growing at 40-75 °C obtained from Kestanbol thermal spring in Canakkale-Turkey [3], expressed it in *E. coli* and characterized the recombinant enzyme.

Materials and Methods

Materials

All chemicals and restriction enzymes used were purchased from Sigma (St. Louis, MO, USA) and Fermentas (Maryland, USA) or Promega (Madison, USA), respectively. *G. caldoxylosilyticus* TK4 has been deposited in the National Collections of Industrial Food and Marine Bacteria under the number 14283 [12]. *E. coli* JM101 and *E. coli* BL21(DE3)pLysS were used as the host for cloning and gene expression, respectively. The plasmid vectors for construction of genomic DNA library, cloning of PCR products and gene expression were pUC18, pGEM-T Easy and pET-28a(+), respectively. *G. caldoxylosilyticus* TK4 was grown aerobically at 60 ºC in Luria Bertani (LB) medium. All the *E. coli* strains containing recombinant plasmids were cultured in LB at 37 ºC which were supplemented with 50 µg mL^{-1} ampicillin or kanamycin when needed. Standard molecular cloning techniques were employed throughout the study [13].

Genomic DNA library construction

Genomic DNA was isolated from an overnight culture of *G. caldoxylosilyticus* TK4 by using Wizard Genomic DNA Purification Kit (Promega) and partially digested with *Eco*R I. DNA fragments were ligated into pUC18 previously digested with the *Eco*R I, and the ligation products were transformed into *E. coli* JM101. Recombinant vectors were identified by blue/white colony screening. Only 50 recombinant vectors containing approximately 2–3 kbp DNA fragments were isolated using Wizard Plus SV Minipreps DNA Purification Systems (Promega). They were sequenced by Macrogen Inc. (Seoul, Korea). Sequence alignments were performed using the online blastx search engine at the National Center for Biotechnology Information (NCBI).

Inverse PCR

Two primers (forward: Ap F1 5′-ATACCGGGGAAGACGTTCC-3′ and reverse: Ap R1 5′-ATTCGTCACATAGCTGTATCCG-3′) were designed from the known sequence of the *G. caldoxylosilyticus* TK4 AP (*Gca*TK4AP) gene [14]. Inverse PCR was performed to determine the missing DNA sequence of the gene in the 5′ end [14]. To clone this part of gene, genomic DNA was digested with *Bam*H I and self-ligated overnight at 23 °C using T4 DNA ligase. After ligation, the DNA was precipitated with ethanol and the DNA pellet was resuspended in 30 µL nuclease free water. PCR was performed using 2-5 µL of the suspension in presence of Ap F1 and Ap R1 primers using the following program: 95 °C 2 min; 36 cycles of 94 °C 1 min, 53 °C 1 min, 72 °C 2 min; and at 72 °C 3 min. The inverse PCR product was purified with Wizard SV Gel and PCR Clean-Up System (Promega), and cloned into the pGEM-T Easy vector (Promega). After the isolation of plasmids, they were sequenced [13].

Gene cloning and sequence analysis

The entire nucleotide sequence of the gene was determined from the determined DNA sequence. Protein identity and amino acid sequence searches were performed by using the BLAST tool at the NCBI website and conserved amino acids were determined using ClustalW program. The 1410-bp that encodes AP was amplified from *G. caldoxylosilyticus* TK4 genomic DNA by PCR with the forward primer Ap F2 5′-G<u>GCTAGC</u>TTAAAATTGTTCAGAAAAAAATCTTACC-3′ which introduces an *Nhe* I site just before the start codon, and the reverse primer Ap R2 5′-<u>GGATCC</u>GTCAACTACTTATCTTCAATGAC-3′, which provides a *Bam*H I site and an additional stop codon immediately upstream of the gene stop codon, using the following program: 95 °C 2 min; 36 cycles of 94 °C 1 min, 53 °C 1 min, 72 °C 2 min; and at 72 °C 5 min. The PCR product was purified with Wizard SV Gel and PCR Clean-Up System, double digested with *Nhe* I and *Bam*H I and then ligated with pET-28a(+) which had been digested with the same enzymes.

Gene expression

The ligation product was transformed into *E. coli* BL21(DE3) pLysS and then the transformed cells were grown in LB medium containing 50 µg mL^{-1} kanamycin. After incubation with shaking at 37 ºC until the A_{600} reached 0.8–1.0, induction was carried out by addition of isopropyl thio-β-*D*-galactoside (IPTG) at a final concentration of 1 mM and incubating for 6 h at 37 ºC. The expression product contained 6xHis-tag at its N-terminal.

Enzyme purification

The induced cells were collected by centrifugation (10,000xg, at 4 ºC for 20 min), resuspended in 200 mM sodium glycine buffer (pH 9.5, containing 0.5 mg mL^{-1} lysozyme), incubated at 37 ºC for 30 min and disrupted by sonication with cooling on ice. Cell debris was removed by centrifugation (13,500xg, at 4 ºC for 10 min). *E. coli* proteins were partially removed by incubating the supernatant at 70 ºC for 10 min followed by centrifugation (13,500xg, at 4 ºC for 10 min). The recombinant protein was purified with MagneHis Protein Purification System (Promega) containing paramagnetic precharged nickel particles [1]. Protein concentration was determined using the Lowry method [15].

Enzyme assay

AP activity was measured spectrophotometrically at 410 nm with p-nitrophenyl phosphate (*p*NPP) as substrate. A typical

assay contained 200 mM sodium glycine buffer (pH 9.5), and appropriate amounts of purified enzyme and the substrate in a final volume of 1 mL [1,16]. Reactions were carried out at optimum temperature (50 °C) for 15 min and stopped by addition of 50 µL of 4 M NaOH. To compensate for non-enzymatic hydrolysis of *p*NPP, a reaction mixture lacking enzyme served as a reagent blank. One unit of activity was defined as the amount of enzyme that produced 1 µmol pNP in 1 min. Specific activity was expressed as units mg^{-1} of protein.

SDS polyacrylamide gel electrophoresis and activity staining

SDS polyacrylamide gel (12% acrylamide concentration) electrophoresis was performed as described by Laemmli [17]. A nondenaturating gel (12% acrylamide) was used to analyze the native form of the enzyme. Staining specific for AP activity was used to detect AP in nondenaturing gels [7]. After electrophoretic migration, the gel was incubated in 50 mM NaOH-glycine (pH 9.5) buffer that contained 0.5 mM $CoCl_2$ and 2.5 mM 4-methylumbelliferylphosphate as substrate, for 20 min at 60 °C. Active bands were visualized under UV light.

Effect of pH and temperature on the activity of GcaTK4AP

Activity of purified AP as a function of pH was assayed at 55 ºC, using the buffers (200 mM); sodium acetate (pH 3.0 to 6.5), phosphate (pH 6.0 to 8.0), Tris-HCl (pH 6.5 to 9.5) and glycine-NaOH (pH 8.5 to 11.0).

Optimum temperature was determined at optimum pH (i.e., 200 mM glycine-NaOH buffer, pH 9.5) value and the enzyme activities were measured at 10 ºC increments between 10–90 ºC.

For both the above, activity was expressed as a percentage of the maximum [18].

Enzyme kinetics

Kinetic parameters of purified AP were determined from the rate of hydrolysis of concentrations of *p*NPP from 0.01 to 1 mM as described under enzyme assay. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined from the Lineweaver–Burk plot [19]. Effect of protein concentration on the AP activity was investigated in a concentration range of 0.10-200 µg mL^{-1}. The activity was assayed under the same conditions [20].

Enzyme pH- and thermal- stability

Purified enzyme was incubated at 4 ºC, for 12, 24 or 48 h, in the buffers: phosphate (pH 7.0-7.5), Tris-HCl (pH 8.0-8.5) and glycine-NaOH (pH 9.0-11.0) before activity was assayed as described above.

For thermostability, an aliquot of enzyme solution in an ependorf tube was incubated for 2 h at 10 ºC intervals between 10 and 70 ºC. Samples taken every 30 min were brought to room temperature and the activity assayed as described above.

In both the above, a control with non-incubated enzyme was used to determine maximal activity [20].

Effect of metal ions and EDTA on enzyme activity

Chloride salt solutions of K^+, Na^+, Li^+, Ni^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Cr^{3+} and Al^{3+}, and EDTA solution were added to the standard reaction mixture separately that the final concentration of each adjusted to 1 mM. Activity assayed in absence of any of these metal ions or EDTA represented the maximal activity [21].

Results and Discussion

We constructed a genomic library of *G. caldoxylosilyticus* TK4 with pUC18 plasmid and *E. coli* JM101 as host. The analyzed sequences revealed that the 786 bp fragment of Clone 17 shared ~73% similarity with AP gene from *Exiguobacterium sibiricum* 255-15. However, ~600 bp of the gene in the 5' end was missing. Inverse PCR with Ap F1 and Ap R1 yielded a product of ~1250 bp. After purification, the product was cloned in the pGEM-T Easy vector and sequenced to reveal the missing part of the gene. The AP gene was amplified from *G. caldoxylosilyticus* TK4 genomic DNA by PCR.

Sequence analysis revealed that the *Gca*TK4AP gene consisted of 1410 bp coding for a polypeptide of 470 amino acid, with a calculated molecular mass of 51.59 kDa. The molecular mass of the 6xHis-tagged AP was calculated to be 54.043 kDa. The sequence of *Gca*TK4AP is available in the GenBank database under the accession no. FJ799980.

The deduced protein shared 69%, 67%, 67%, 67%, 38% and 24% similarity with APs from *E. sibiricum* 255-15 (GenBank accession no. YP_001815175), *Bacillus cereus* ATCC 14579 (GenBank accession no. AAP11256), *Bacillus weihenstephanensis* KBAB4 (GenBank accession no. ABY45355), *Bacillus thuringiensis* str. Al Hakam (GenBank accession no. ABK87157), *Shewanella sp.* SIB1 (GenBank accession no. BAD90663) and *Thermus thermophilus* HB27 (GenBank accession no. AAS82354), respectively.

The sequence alignment showed that *Gca*TK4AP had numerous conserved residues (Fig. 1) (painted as gray). In *E. coli* AP, Mg is bound by residues D51, T155, and E322 with D153 forming part of the Mg^{2+}-ion binding site, K328 being important for phosphate binding [22]. However, D153 and K328, were replaced by histidine and tryptophan, respectively, in *Gca*TK4AP. The same substitutions occur in APs of *E. sibiricum* 255-15, *B. cereus* ATCC 14579, *Antarctic psychrophilic bacterium* TAB5 [23] (GenBank accession no. Y18016), *T. neapolitana* [24] (GenBank accession no. AAX98659). Recombinant protein was successfully expressed in *E. coli* BL21(DE3)pLysS under control of T7 RNA polymerase promoter with 6xHis-tag in the N-terminal.

While the specific AP activity of crude extract, induced for 6 h, was 0.003 U/mg protein, it was 0.186 U/mg protein for purified enzyme. Thus, the expressed recombinant protein was purified ~62-fold from the induced crude extract, with the one-step purification procedure using nickel affinity chromatography.

SDS polyacrylamide gel electrophoresis and activity staining

APs are classically described as being homodimeric, which is the case of those from *E. coli* and mammals [10]. However, many monomeric [25, 26] and oligomeric [8] forms have been described. The molecular weight of our polypeptide determined to be ~50 kDa by SDS polyacrylamide gel electrophoresis (Fig. 2A) was consistent with that calculated from predicted amino acid sequence (51.59 kDa). Molecular mass of thermostable monomeric recombinant AP from *Thermus thermophilus* XM was reported to be 55 kDa [1]. The thermostable AP from *Thermus yunnanensis* sp. nov. contained a single polypeptide of molecular mass ~52 kDa on SDS-PAGE analysis and appeared to be a 104 kDa homodimer in solution [27]. We saw only one band on nondenaturating PAGE with activity staining (Fig. 2B). The band at lane 1 showed that *Gca*TK4AP

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                                            *
E. sibiricum 255-155            IRN---VIFLIGDGMG-VSYTSAHRYLKNDPSTPLAEKTAFDQYLVGQQMTYPEDPQQN- 92
G. caldoxylosilyticus TK4       VKN----FM--GDGMG-VSYTSAYRYLKDNPNTKFVERTEFDKYLVGQQMTYPKDPKKN- 92
B. cereus ATTC 14579             IKN---VIVLIGDGMG-PSYMTAHRYMKDNPKTFEMESTEFDKHLVGTQKTYPEDEHEN- 98
E. coli W3110                   DKPAKNIILLIGDGMG-DSEITAARNYAEGAGGFFKGIDALP--LTGQYTHYALNKKTGK 117
T. thermophilus HB27            YRN---LIVFVYDGFSWEDYAIAQAYARRRQGRVLALERLLARYPNGLINTYSLTSYVT- 93
                                      Θ
E. sibiricum 255-155            ---VTDSASAATAMSSG-VKTYNAA-IAVDNDKSEVKTVLEAAKERGKSTGLVATSE-IT 146
G. caldoxylosilyticus TK4       ----TDSASAATAMAAG--KTYNAA--SVDNDGSEVKTVLEAAKAAGKATGLVATSE--T 142
B. cereus ATTC 14579             ---ITDSASAATAMSAG-IKTYNAA-IAVDNDKAEVKTVLEQAKEKGKSTGLVATSE-IT 152
E. coli W3110                   PDYVTDSAASATAWSTG-VKTYNGA-LGVDIHEKDHPTILEMAKAAGLATGNVSTAE-LQ 174
T. thermophilus HB27            -----ESSAAGNAFSCG-VKTVNGG-LAIHADGTPLKPFFAAAKEAGKAVGLVTTTT-VT 145

                                θ *          △
E. sibiricum 255-155            HATPASFGAHDENRKNMNA-IADDYFKER-VNGNHK-----------IDVLLGGGKSNF- 192
G. caldoxylosilyticus TK4       HATPASFGAHDENRQNMNA--ANDYYDEL--NSEHK-----------VDVLLGGGTDLF- 186
B. cereus ATTC 14579             HATPAAFGAHDISRKNMDA-IANDYFDEK-IKGKHK-----------VDVMLGGGVKNF- 198
E. coli W3110                   DATPAALVAHVTSRKCYGPSATSEKCPGNALEKGGKGSITEQLLNARADVTLGGGAKTFA 234
T. thermophilus HB27            HATPASFVISNPDRNAEER-IAEQYLEFG------------------AEVYLGGDRFFN- 185


E. sibiricum 255-155            ------VRPDVDLTKSFKKDGYSYVTNLDQL----QTDQNKQVLGLFADGGLPKR-I--- 238
G. caldoxylosilyticus TK4       -------RKDRNLVEEFKKDGYSYVTNRNEL----LKDQNEQVLGLFAPRGLPKM----- 230
B. cereus ATTC 14579             ------VRKDRNLTEEFKKSGYSYVTDRDQL----LNDKNDQILGLFAPGGLDKM-I--- 244
E. coli W3110                   ETATAGEWQGKTLREQAQARGYQLVSDAASLNSVTEANQQKPLLGLFADGNMPVRWLGPK 294
T. thermophilus HB27            ---PARRKDGKDLYAAFAAKGYGVVRTPEEL----ARSNATRLLGVFADGHVPYEID--- 235

                                                                                   *     *$
E. sibiricum 255-155            ------------------DRENT--VPSLEKMTNSA-IKRLDSNKKGFFLMVEGSQ-IDW 276
G. caldoxylosilyticus TK4       ------------------DRTED---PSLEEMTKSA--ERLSKDKDGFFLMVEGSQ--DW 265
B. cereus ATTC 14579             ------------------DRNEK--TPSLEEMTNAA-IERLNKNKNGFFLMVEGSQ-IDW 282
E. coli W3110                   ATYHGNIDKPAVTCTPNPQRNDS--VPTLAQMTDKA-IELLSKNEKGFFLQVEGAS-IDK 350
T. thermophilus HB27            ------------------RRFQGLGVPSLKEMVQAA-LPRLAAHRGGFVLQVEAGR-IDH 275

                                  *                                        **
E. sibiricum 255-155            AGHDND-IVGAMSEMEDFERAFKAA-IAFAKKDKHTLVVATADHSTGGYS-IGADG-IYN 332
G. caldoxylosilyticus TK4       AGHDND--VSAMSEMEDFEKAFKAA--EFAKKDKHTLVVATADHSTGGYS--GADG--YN 317
B. cereus ATTC 14579             AGHDND-IVGAMSEMEDFEKAFKAA-IEFAKKDKNTLVVATADHATGGLS-LGANG-EYN 338
E. coli W3110                   QDHAAN-PCGQIGETVDLDEAVQRA-LEFAKKEGNTLVIVTADHAHASQI---------- 398
T. thermophilus HB27            ANHLND-AGATLWDVLAADEVLELL-TAFVDRNPDTLLIVVSDHATGVGGLYGAGRSYLE 333

E. sibiricum 255-155            WFAEP-IKAAKKTPDFMAEK-IINGADVRQTLTTYIDQKKLALTEDEIGSVIRAA----- 385
G. caldoxylosilyticus TK4       WFGAP--KAAKRTPDFMAEE--ANGADVEETLKKY----DLELTAEE--QSVKDA----- 362
B. cereus ATTC 14579             FKVEP-IKAAKRTPDFMANE-IAKGANVEETLKKY---IDLQLTPEEIQAVNDIA----- 388
E. coli W3110                   --VAP----DTKAPG--------------------------------------------- 407
T. thermophilus HB27            SSRGVDLLEPQRASFEHMLRVLGQAPEASQVKEAFRAMKGVDLEDAEAERVVRAIREKVY 393

                                                                                        *
E. sibiricum 255-155            ----ESQKLLDIDNAIEAIFNE---------RSHTGWTTGGHTGEDVPVYAFGPAKERFA 432
G. caldoxylosilyticus TK4       ----AEQGKADKDKGVKRVDDA--EH--FDKRSHTGWTTSAHTGEDVPVYAYGPGSERFA 414
B. cereus ATTC 14579             ----PSKDVTKIDNAIEDIFNK---------RSVTGWTTGGHTGEDVNVYAFGPGKYLFS 435
E. coli W3110                   ----LTQALNTKDGAVMVMSYG-----------NSEEDSQEHTGSQLRIAAYGPHAANVV 452
T. thermophilus HB27            WPEGVRQGVQPANTLAWAMAQRN---AQKPDRPNIGYSSGQHTASPVMLLLYGQGLRFVN 450
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Figure 1. Sequence alignment *Gca*TK4AP, *Exiguobacterium sibiricum* 255-15 AP, *Bacillus cereus* ATCC 14579 AP, *E. coli* W3110 AP (GenBank accession no. AP_001034), and *Thermus thermophilus* HB27 AP. The amino acid residues forming the three metal binding sites are denoted by asteriks (*). D153 and K328 are also denoted by θ and $, respectively.

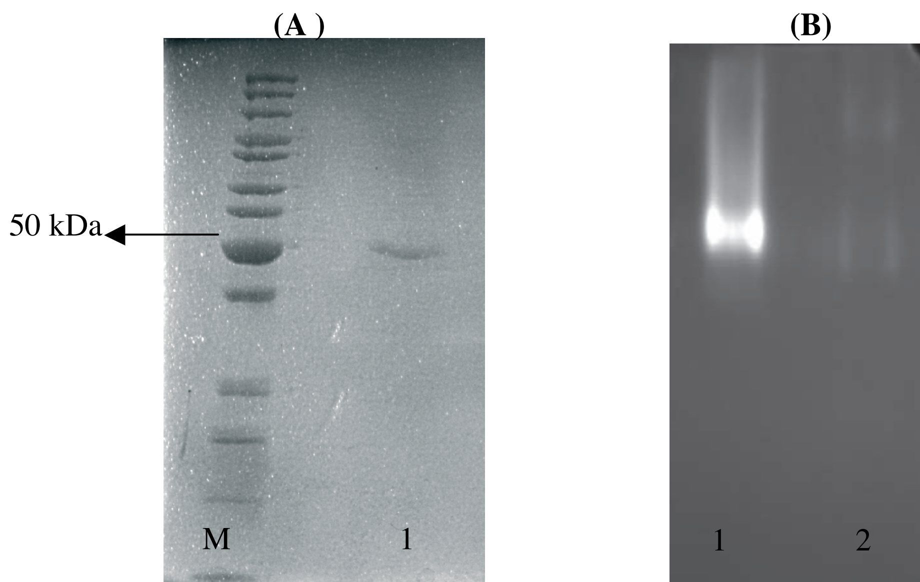

Figure 2. SDS and Non-denaturing PAGE showing purified recombinant *Gca*TK4AP. **(A)**. SDS-PAGE, Lane M, molecular weight markers, Lane 1, The purified recombinant enzyme containing 6xHis-tagged; **(B)**. Activity satining, Lane 1, The purified recombinant enzyme containing 6xHis-tagged, Lane 2, *E. coli* BL21(DE3)pLysS proteins.

was overproduced under the control of T7 RNA polymerase promoter in *E. coli* and the recombinant protein expressed with a N-terminal 6xHis-tag was active and purified effectively with MagneHis Protein Purification Systems. A single activity band was observed in the culture supernatant of an Antarctic psychrotolerant *Bacillus* sp. P9 in native PAGE, indicating the production of only one type of AP by the bacteria [28]. Two extracellular APs purified from psychrophilic *Arthrobacter* strain also had a single activity band on nondenaturating PAGE with different migration patterns [26].

Effect of pH and temperature on enzyme activity

The pH activity curves of the phosphatases differ. Like other APs, the *Gca*TK4AP was activated under alkaline conditions, the highest activity being at pH 9.5 (Fig. 3A). *Chryseobacterium meningosepticum* AP activity was studied between pH 7.0 and pH 9.5 (sodium glycine buffer), and optimal activity was at pH 8.5 [29]. An extracellular AP purified from *Arthrobacter* strain had activity between pH 7.0 and 11.0 with a peak of maximal activity around pH 9.5. In contrast, the other extracellular AP had a optima at pH 9.0 (sodium glycine buffer) and little activity at pH 10.0 [16].

The recombinant AP had maximum activity at 50 °C (Fig. 3B). Optimum temperatures of the AP from an aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1 [11], a recombinant thermostable AP from a novel thermophilic bacterium *T. thermophilus* XM [1] and a highly thermostable AP from the *P. abyssi* with optimum growth temperature 100 °C [7], were 95, 75 and 70 °C, respectively. However, optimum temperatures of AP from *Pinctada fucata* [30], thermolabile APs from a Psychrotrophic bacterium [23] and *Bacillus* sp. P9 [28] were 45, 50 and 40 °C, respectively. *Gca*TK4AP retained about 20% of its optimum activity at 10 °C indicating that the purified enzyme had a characteristic between hyperthermophilic and thermolabile APs.

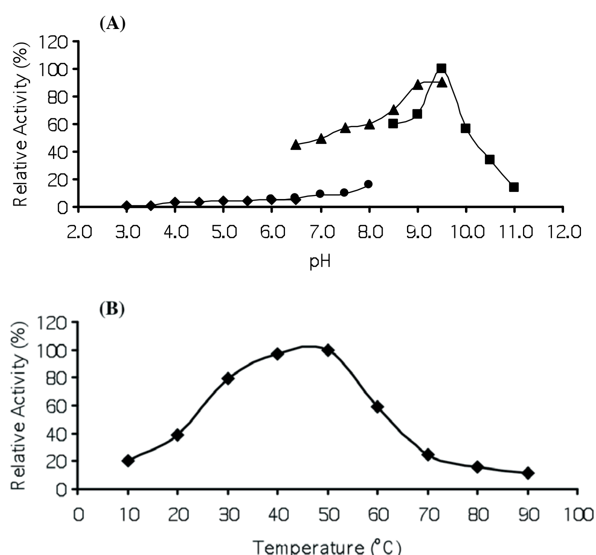

Figure 3. (A). pH- activity profile of *Gca*TK4AP. Assays were performed in 200 mM of different buffer systems at indicated pH for 15 min. Sodium acetate buffer (pH 3.0-6.5) (♦), Phosphate buffer (pH 6.0-8.0) (●), Tris-HCl buffer (pH 6.5-9.5) (▲) and Glycine-NaOH buffer (pH 8.5-11.0) (■) were used. **(B)**. Temperature–activity profile of *Gca*TK4AP. The activities were determined in 200 mM Glycine-NaOH buffer (pH 9.5) for 15 min at different temperatures from 10 to 90 °C.

Enzyme kinetics

Optimum substrate concentration was 250 µM (data not shown). The K_m and V_{max} were 87 µM and 0.049 U mg protein^{-1}, respectively (Fig. 4). K_m values, determined in presence of *p*NPP as a substrate, vary widely thus those for *P. fucata* [30], psychrophilic *Arthrobacter* isolate [26], *C. meningosepticum* [29] and recombinant *T. thermophilus* XM [1] APs were 2.86 mM, 89 µM, 115 µM and 0.034 mM, respectively. Maximum reaction velocities of AP from *C. meningosepticum* [29], *P. fucata* [30] and an *Arthrobacter* isolate [26] were 71 µmol min^{-1}, 9.09 µmol min^{-1} and 6 U mg protein^{-1}, respectively. Activity of our recombinant enzyme increased up to a final protein concentration of 100 µg mL^{-1} and it remained constant after this value. It was reported by Suzuki et al. (2005) that 50-100 µg mL^{-1} of final protein concentration for AP had been used in the reaction mixtures.

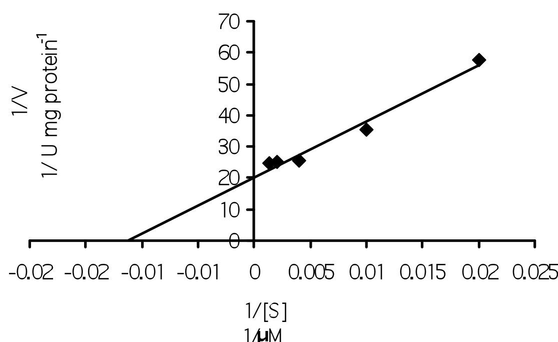

Figure 4. Lineweaver–Burk plot of *Gca*TK4AP. Enzyme kinetic parameters were obtained by measuring the rate of *p*NPP hydrolysis at various substrate concentrations ranging from 0.01 to 1 mM in the standard reaction mixture (at 50 ºC in 200 mM sodium glycine buffer, pH 9.5). K_m and V_{max} values were determined from the Lineweaver–Burk plot using the Microsoft Excel software.

pH- and thermal-stability of the enzyme

The pH-stability profile (Fig. 5A) showed that activity was fully retained at pH 9.5 after 12 h incubation but gradually decreased after this time. AP from a marine bacterium retained its activity after incubation for 15 min at pH values from 6.0 to 11.0 [10]. The other AP from *Vibrio* sp. was most stable at pH 8.0-9.0 while its activity decreased below pH 6.0 after 24 h incubation [25].

The thermal stability profile for the recombinant AP (Fig. 5B) showed that almost full activity was retained after incubation at 10 to 30 °C for 2 h. ~50% of original activity was lost after incubation for 30 min at 50 °C and almost full inactivation occurred at 60 °C and 70 °C. The moderately thermostable AP from *Geobacillus thermodenitrificans* T2 was inactivated at 80 °C for 15 min, and had a half-life of 8 min at 70 °C [31]. *P. abyssi* AP had half-lives of 18 and 5 h at 100 and 105 °C, respectively. *T. neapolitana* AP had a half-life of ~4 h at 90 °C in presence of $CoCl_2$ [7]. Incubation of *C. meningosepticum* AP for 10 min at 50 °C or 60 °C caused 40 and 60% inactivation, respectively and at 100 °C for 2 min caused total loss of the enzyme activity [29]. When *Bacillus* sp. P9 AP was incubated at 60 °C, it lost 62% of its original activity within 10 min and had virtually no activity after 50 min [28].

AP is used to dephosphorylate nucleic acids in molecular biology, as a reporter enzyme for secreted proteins, and as an indicator of activity in research and diagnostic kits. New phosphatases with higher activity at lower temperatures could have

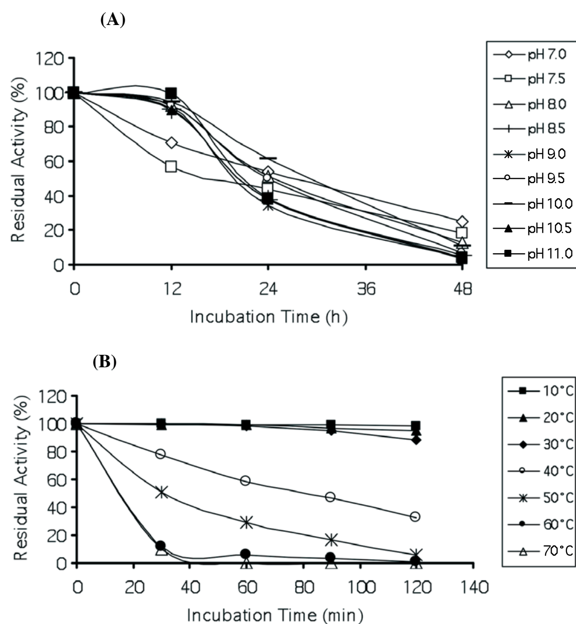

Figure 5. (A). pH stability profile of *Gca*TK4AP. Assays were performed in using the following buffers (200 mM); Phosphate buffer (pH 7.0-7.5), Tris-HCl buffer (pH 8.0-8.5) and Glycine-NaOH buffer (pH 9.0-11.0) **(B)** Thermal stability profile of *Gca*TK4AP. The activities were determined in 200 mM Glycine-NaOH buffer (pH 9.5)

advantages in these applications. In addition, a heat-labile phosphatase would be useful in applications in which the activity must be removed before proceeding to the next reaction. For example, an easily heat-inactivated AP could eliminate the need for the phenol extraction and ethanol precipitation steps after dephosphorylation of linearized plasmid DNA during the formation of recombinant DNA molecules. TK4 AP has an activity at low temperatures and heat-lability and could be advantageous for molecular biological applications.

Effect of metal ions and EDTA on enzyme activity

APs are usually considered to be Zn(II)- and Mg(II)- dependent enzymes [22] but show diversity with respect to metal ion requirements [16]. As expected the activity of *Gca*TK4AP was strongly affected by presence of EDTA (1 mM final concentration). Most of the metal ions we tested did not have much effect on the AP activity, but Hg^{2+}, Cd^{2+} and Al^{3+}, decreased the activity by 85, 55 and 18%, respectively (Fig. 6). AP from the *Cobetia marina* did not require divalent cations for activity [10]. Zn^{2+}, Ca^{2+}, and Cu^{2+} inhibited recombinant *T. thermophilus* XM AP while 2 mM Mg^{2+}, 0.1 mM Mn^{2+} or Co^{2+} activated it [1].

In conclusion**,** we have cloned the *Gca*TK4AP gene and expressed it in *E. coli*. The recombinant enyzme shares characteristics of APs in terms of conserved amino acid residues, electrophoretic behaviour of the enzyme, pH- and temperature optima, K_m and V_{max} values etc. The enzyme may be of use in some clinical and molecular biological applications.

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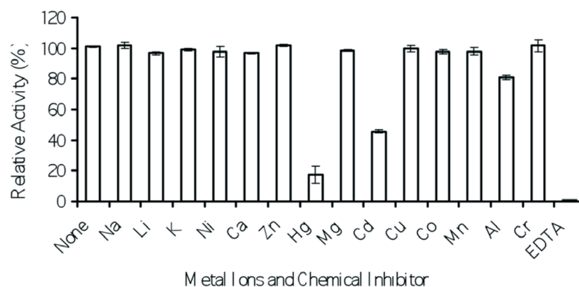

Figure 6. Effect of some metal ions and EDTA on the of *Gca*TK4AP. The effect of metal ions and EDTA on the enzyme activity was separately investigated by adding chloride salt solutions of metal ions and EDTA solution directly to the standard reaction mixture in a final concentration of 1mM.

References

[1] Li J, Xu L, Yang F. (2007) Expression and characterization of recombinant thermostable alkaline phosphatase from a novel thermophilic bacterium *Thermus thermophilus* XM. Acta Bioch Biophy Sin. 39:844-850.

[2] Kim EE, Wyckoff HW. (1991) Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. J Mol Biol. 218:449–464.

[3] Karamyshev AL, Karamysheva ZN, Kajava AV, Ksenzenko VN, Nesmeyanova MA. (1998) Processing of *Escherichia coli* alkaline phosphatase: role of the primary structure of the signal peptide cleavage region. J Mol Biol. 277:859–870.

[4] Besman M, Coleman JE. (1985) Isozymes of bovine intestinal alkaline phosphatase. J Biol Chem. 260:11190–11193.

[5] Bortolato M, Besson F, Roux B. (1999) Role of metal ions on the secondary and quaternary structure of alkaline phosphatase from bovine intestinal mucosa. Proteins. 37:310–318.

[6] Vincent JB, MW Crowder, Averill BA. (1992) Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions.Trends Biochem Sci. 17:105–110.

[7] Fitt PS, Peterkin PI. (1976) Isolation and properties of a small manganese-ion-stimulated bacterial alkaline phosphatase. Biochem J. 157:161–167.

[8] Goldman S, Hecht K, Eisenberg H, Mevarech M. (1990). Extracellular Ca^{+2}-dependent inducible alkaline phosphatase from the extremely halophilic archeabacterium *Haloarcula marismortui*. J Bacteriol. 172:7065–7070.

[9] Yeh MF, Trela JM. (1976) Purification and characterization of a repressible alkaline phosphatase from *Thermus aquaticus*. J Biol Chem. 251:3434–3439.

[10] Zappa S, Rolland JL, Flament D, Gueguen Y, Boudrant J, Dietrich J. (2001) Characterization of a highly thermostable alkaline phosphatase from the euryarchaeon *Pyrococcus abyssi*. Appl Environ Microb. 67:4504−4511.

[11] Helianti I, Okubo T, Morita Y, Tamiya E. (2007) Characterization of thermostable native alkaline phosphatase from an aerobic hyperthermophilic archaeon, *Aeropyrum pernix* K1. Appl Microbiol Biot. 74:107-112.

[12] Canakcı S, Belduz AO, Saha BC, Yasar A, Ayaz FA, Yayli N. (2007) Purification and characterization of a thermostable α-L-arabinofuranosidase from *Geobacillus caldoxylosilyticus* TK4. Appl Microbiol Biot. 75:813-820.

[13] Sambrook J, Fritsch EF, Maniatis T. (1989) Molecular cloning. A laboratory manual, 2nd edn. Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[14] Ertunga NS, Colak A, Belduz AO, Canakcı S, Karaoglu H, Sandalli C. (2007) Cloning, expression, purification and character-

ization of fructose-1,6-bisphosphate aldolase from *Anoxybacillus gonensis* G2. J Biochem.141:817-825.

[15] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951) Protein measurement with the folin phenol reagent. J Biol Chem.193:265-275.

[16] De Prada P, Loveland-Curtze J, Brenchley JE. (1996) Production of two extracellular alkaline phosphatases by a psychrophilic *Arthrobacter* strain. Appl Environ Microb. 62:3732-3738.

[17] Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680–685.

[18] Colak A, Sisik D, Saglam N, Guner S, Canakci S, Belduz AO. (2005) Characterization of a thermoalkalophilic esterase from a novel thermophilic bacterium, *Anoxybacillus gonensis* G2. Bioresource Technol. 96:625-631.

[19] Lineweaver H, Burk D. (1934) The determination of enzyme dissociation constant. J Am Chem Soc. 56:658–661.

[20] Yildirim M, Col M, Colak A, Güner S, Dülger S, Beldüz AO. (2005) Diphenolases from *Anoxybacillus kestanbolensis* strains K1 and $K4^T$. World J Microb Biot. 21:501-507.

[21] Colak A, Sahin E, Yildirim M, Sesli E. (2007) Polyphenol oxidase potentials of three wild mushroom species harvested from Liser High Plateau, Trabzon. Food Chem. 103:1426-1433.

[22] Kim EE, Wyckoff HW. (1990) Structure of alkaline phosphatases. Clin Chim Acta, 186:175-187.

[23] Suzuki Y, Mizutani Y, Tsuji T, Ohtani N, Takano K, Haruki M, Morikawa M, Kanaya S. (2005) Gene cloning, overproduction and characterization of thermolabile alkaline phosphatase from a psychrotrophic bacterium. Biosci Biotech Biochem. 69:364-373.

[24] Dong G, Zeikus JG. (1997) Purification and characterization of alkaline phosphatase from *Thermotoga neapolitana*. Enzyme Microb Tech. 21:335-340.

[25] Sharipova MR, Balaban NP, Mardanova AM, Nekhotyaeva NV, Dementyev AA, Vershinina OA, Garusov AV, Leshchinskaya IB. (1998) Isolation and properties of extracellular alkaline phosphatase from *Bacillus intermedius*. Biochemistry (Moscow). 63:1178-1182.

[26] Eguchi M. (1995) Alkaline phosphatase isozymes in insects and comparison with mammalian enzyme. *Comp Biochem PhysB*. 111:151-162.

[27] De Prada P, Brenchley JE. (1997) Purification and characterization of two extracellular alkaline phosphatases from a psychrophilic *Arthrobacter* isolate. *Appl Environ Microb,* 63:2928-2931.

[28] Dhaked RK, Alam SI, Dixit A, Singh L. (2005) Purification and characterization of thermo-labile alkaline phosphatase from an Antarctic psychrotolerant *Bacillus* sp. P9. Enzyme Microb Tech. 36: 855-861.

[29] Berlutti F, Passariello C, Selan L, Thaller MC, Rossolini GM. (2001) The *Chryseobacterium meningosepticum* PafA enzyme: prototype of a new enzyme family of prokaryotic phosphate-irrepressible alkaline phosphatases? Microbiology+. 147:2831-2839.

[30] Xiao R, Xie LP, Lin JY, Li CH, Chen QX, Zhou HM, Zhang RQ. (2002) Purification and enzymatic characterization of alkaline phosphatase from *Pinctada fucata*. J Mol Catal B-Enzym. 17:65-74.

[31] Zhang Y, Ji C, Zhang X, Yang Z, Peng J, Qui R, Xie Y, Mao Y. (2008) A Moderately Thermostable Alkaline Phosphatase from *Geobacillus thermodenitrificans* T2: Cloning, Expression and Biochemical Characterization. Appl Biochem Biotech. 151:81-92.