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High-Level Expression in *Escherichia coli* of Alkaline Phosphatase from *Thermus caldophilus* GK24 and Purification of the Recombinant Enzyme

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Abstract High-level expression of *Thermus caldophilus* GK24 alkaline phosphatase (*Tca* APase) was achieved in *Escherichia coli* using the pET-based expression plasmids, pEAP1 and pEAP2. In the case of plasmid pEAP2, the signal peptide region of *Tca* APase was replaced by the PelB leader peptide of expression vector pET-22b(+). Furthermore, the expression level was somewhat higher than that of plasmid pEAP1. A rapid purification procedure of *Tca* APase overproduced in *E. coli* was developed which involved heating to denature *E. coli* proteins followed by HiTrap Heparin HP column chromatography. Optimal temperature and pH and Mg^{2+} dependence of the recombinant *Tca* APase were similar to those of native enzyme isolated from *T. caldophilus* GK24.

Key words: Alkaline phosphatase, high-level expression, recombinant *Tca* alkaline phosphatase, *Thermus caldophilus* GK24, *Thermus caldophilus* GK24 alkaline phosphatase

Alkaline phosphatases (APases) catalyze the hydrolysis of phosphate monoesters, being most active at alkaline pH [2, 10]. The enzymes are present in various organisms, ranging from bacteria to mammals with the exception of some higher plants. *Escherichia coli* APase has been extensively studied. The enzyme, encoded by the *phoA* gene, is synthesized as a precursor monomer with a signal peptide at the amino-terminal end [2, 10]. Upon translocation to the periplasmic space, the signal peptide is removed and two of the resulting mature monomers dimerize to give the active enzyme [2, 3, 10]. The expression of *phoA* is induced under phosphate-limited conditions, allowing regulated expression [16]. The synthesis of APase is positively and negatively regulated by the *phoB* and *phoR* genes, respectively [18]. The enzyme, a dimeric metalloenzyme of 94,000 Da, consists of 449 amino acid residues per monomer and two atoms of zinc and one atom of magnesium at each active site [1, 4]. Based on the X-ray crystal structures of *E. coli* APase, a reaction mechanism involving metal ion assisted catalysis has been described [4, 13].

APases have been commonly used in molecular biology [7], such as to remove 5-phosphates from linearized vector to prevent self-ligation during cloning procedures or as a reporter in enzyme-immunoassays. The enzyme is suited for labeling primers and detecting PCR amplified products. In particular, oligonucleotides conjugated directly to thermostable APase can be used as DNA probes for hybridization at high temperature. Also, the 5'-recessive termini of linearized vector using the thermostable APase can be easily dephosphorylated at high temperature.

The properties of *Thermus caldophilus* GK24 (*Tca*) APase have been reported [5], and *Tca* APase is a homodimer of approximately 108,000 Da. The enzyme is stable at temperatures above 80°C, activated by Mg^{2+}, and inhibited by Zn^{2+}. In contrast, Zn^{2+} is essential for the activity of *E. coli* APase [1, 2, 7]. The gene encoding *Tca* APase was recently cloned into *E. coli* and the primary structure was deduced from its nucleotide sequence [9]. Like *E. coli* enzyme, *Tca* APase is expressed as a precursor monomer with an amino-terminal signal peptide. The 54,760 Da *Tca* APase precursor monomer is comprised of 501 amino acid residues. The alignment of amino acid sequences of *Tca* and *E. coli* APases revealed that the reduced activity of *Tca* APase in the presence of Zn^{2+} is likely due to differences in specific amino acid residues in the metal binding site [9]. The gene encoding *Tca* APase was poorly expressed in *E. coli* YK537 harboring pGAPB and pJRAP [9].

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In this article, we report the high-level expression of *Tca* APase in *E. coli* and a rapid purification procedure of the expressed enzyme. Moreover, we compare the properties of the recombinant enzyme with those of the native APase purified from *T. caldophilus* GK24.

MATERIALS AND METHODS

Strain, Enzymes, and Reagents

E. coli BL21 was used as the host for gene expression. Restriction enzymes and T4 DNA ligase were purchased from Takara (Japan). Oligodeoxyribonucleotides were synthesized using an automatic DNA synthesizer (Applied Biosystems, U.S.A.). Other reagents were obtained from Sigma (U.S.A.).

DNA Amplification by PCR

The DNA amplification by PCR was performed using 2.5 units of *Taq* DNA polymerase in a 50 µl reaction mixture containing PCR reaction buffer, 5 pmol of the primers, 0.2 mM each of dNTP, and 10 ng of plasmid pGAPB (harbors the gene encoding precursor *Tca* APase) [9], as described by Saiki *et al.* [11]. The reaction mixture was heated at 95°C for 5 min, followed by 25 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min.

Construction of the Expression Plasmids pEAP1 and pEAP2

Most of the methods used for molecular cloning were based on those of Sambrook and Russell [12]. Based on the nucleotide sequence of the gene for precursor *Tca* APase with the signal peptide sequence (GenBank accession No. AF168770) [9], primers were synthesized: the 5 (N-terminal) primer, AN1, 5'-NNNNCATATGAAGCGAAGGGACATCCTG-3', containing a unique *Nde*I site (underlined) includes an ATG starting site of translation, followed by the sequence encoding the first seven amino acids of the signal peptide sequence; and the 3' (C-terminal) primer, AC, 5'-NNNNGTCGACTTAGGCCCAGACGTCCTCG-3', containing a unique *Sal*I site (underlined) matches the C-terminal sequence including the stop codon. The PCR product was digested with *Nde*I and *Sal*I, purified from 0.8% low-melting-temperature agarose gel, and ligated into the expression vector pET-22b(+) (Novagen, U.S.A.) that had been digested with the same enzymes, giving a fusion that used the T7*lac* promoter from the vector. *E. coli* BL21 was transformed with the ligate by electroporation. The correct construction of the expression plasmid was identified by restriction enzyme analysis of plasmid minipreps. The resulting expression plasmid pEAP1 possessed the signal peptide sequence of *Tca* APase itself.

The signal peptide of *Tca* APase was replaced by the PelB leader for the construction of the expression plasmid pEAP2. The primer was synthesized on the basis of the sequence for mature *Tca* APase previously reported [9]. The mature *Tca* APase was comprised of the C-terminal 474 amino acid residues. The 5' (N-terminal) primer, AN2, 5'-NNNNGAATTCAATGCTGCAGAACCAGCCTTCCTTG-3', added a unique *Eco*RI site (underlined) and translation initiation codon, ATG, followed by the sequence coding the LeuGlnAsnGlnProSerLeu of the proposed N-terminal amino acid sequence of mature *Tca* APase [9]. The primer had a base of A added between the *Eco*RI site and ATG start codon for adjusting the reading frame with the *pelB* leader sequence. PCR for DNA amplification was performed using primers AN2 and AC as described above. The PCR product was digested with *Eco*RI and *Sal*I, purified from 0.8% low-melting-temperature agarose gel, and ligated into the expression vector pET-22b(+) that had been digested with the same enzymes, giving a fusion that used the PelB leader from the vector. *E. coli* BL21 was transformed with the ligate by electroporation. The correct construction of the expression plasmid, pEAP2, was confirmed by the method described above.

Expression of the *Tca* APase Gene in *E. coli*

For expression of the *Tca* APase gene under the control of T7*lac* promoter, a seed culture of *E. coli* BL21 harboring the recombinant plasmid was grown overnight at 37°C in L-broth containing 100 µg/ml ampicillin [14]. A 10 ml aliquot of seed culture was inoculated into 1 liter of the same medium and cultured at 37°C. When the A_{600} of the culture was about 0.8, the culture was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final 0.2 mM concentration and then incubated at 37°C for 6 h.

Preparation of Whole-Cell Suspension for Assay

A routine assay with whole-cell was carried out by a modification of the method of Ulrich *et al.* [15]. Samples of induced cells to be assayed were chilled immediately in an ice bath, and 1 ml aliquote from each of the ice-chilled samples (adjust the final $O.D._{600}$ to 5.0/ml) was centrifuged and resuspended in 1 ml of 50 mM Glycine-NaOH buffer (pH 11.0). These suspensions were treated with the same volume of a toluene:acetone (2:1) solution and incubated for 5 min, with agitation, at room temperature. After centrifugation, the treated samples were immediately assayed for enzyme activity as described below.

Tca APase Activity Assay

The APase activity was assayed with 1 mM *p*-nitrophenylphosphate (*p*NPP). The reaction mixture contained 0.8 ml of 50 mM Glycine-NaOH buffer (pH 11.0)/1 mM $MgCl_2$, 0.1 ml of 10 mM *p*NPP, and 0.1 ml of enzyme solution. The reaction was performed at 80°C and terminated by the addition of 0.2 ml of 2 N 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} [8]. One unit of APase is defined as the amount of enzyme to liberate 1 µmol of *p*-nitrophenol at 80°C in 1 min.

Purification of the Expressed *Tca* APase in *E. coli*

One liter of the induced cells was collected by centrifugation, resuspended in 10 ml of buffer A [10 mM Tris-HCl buffer (pH 7.4), 1 mM $MgCl_2$] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and then disrupted by sonication. Sonication was performed three times on ice for 1 min with 1-min intervals for cooling. The sonicated extract was incubated at 80°C for 30 min. After centrifugation, the supernatant was applied to a HiTrap Heparin HP column (5 ml) (Amersham Biosciences, Sweden) that had been equilibrated with buffer A. The column was washed with 50 ml of buffer A, and adsorbed protein was eluted with a linear gradient of KCl (0- 1.0 M) in 50 ml of buffer A. The fractions having *Tca* APase activity were pooled and dialyzed against buffer A. The dialysate was assayed and analyzed.

Protein Determination and Analytical Gel Electrophoresis

The protein concentration was determined by the procedure of Lowry *et al.* [6] with bovine serum albumin as a standard. SDS-PAGE was performed by the method of Weber *et al.* [17] with 5% (w/v) acrylamide stacking gel and 10% (w/v) acrylamide separating gel.

Results and Discussion

Construction of the Expression Plasmids pEAP1 and pEAP2

When used a *tac* promoter-based expression vector system the gene encoding *Tca* APase was poorly expressed in *E. coli* YK537 (0.18 units/ml of culture medium) [9]. For high-level expression, the pET expression vector system was chosen, which is one of the most powerful systems developed for the expression of recombinant proteins in *E. coli*. The construction procedures of plasmids pEAP1 and pEAP2 are schematically shown in Fig. 1B. The 1,506 bp DNA fragment containing the entire sequence for precursor *Tca* APase with the signal peptide sequence was amplified from pGAPB [9] by PCR using primers AN1 and AC (Fig. 1A). Both the DNA fragment and expression vector pET-22b(+) were digested with *Nde*I and *Sal*I, and the digested DNA fragment was then inserted into pET-22b(+) to yield the expression plasmid pEAP1. The resulting recombinant plasmid pEAP1 possessed the entire sequence for precursor *Tca* APase with the signal peptide sequence (Fig. 1B). The 1,425 bp DNA fragment containing the sequence for mature *Tca* APase was amplified from pGAPB [9] by PCR using

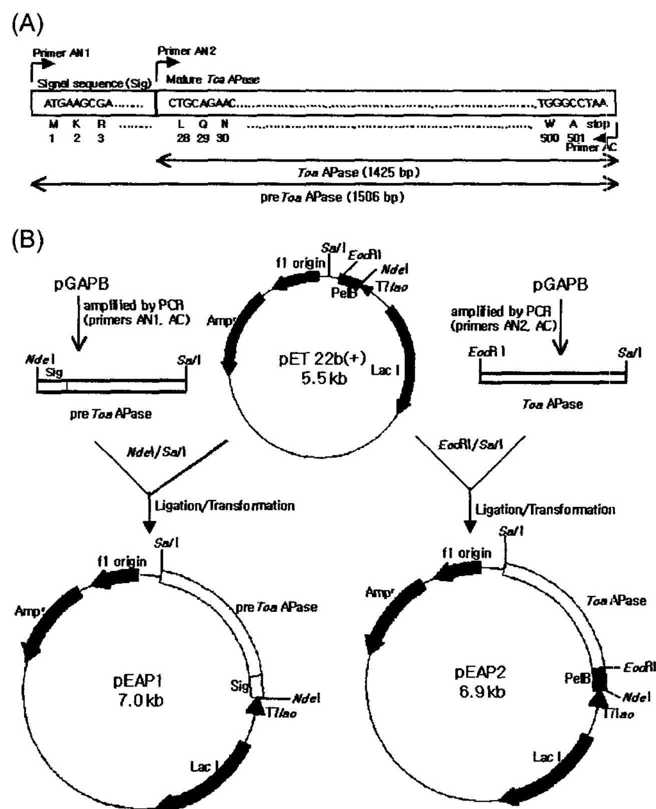

Fig. 1. Construction of the expression plasmids pEAP1 and pEAP2.

(A) Nucleotide and deduced amino acid sequences around the junction of signal peptide and N-terminal amino acid of *Tca* APase including C-terminal region. (B) Construction scheme for the expression plasmids pEAP1 and pEAP2. Plasmid pEAP1 was constructed by cloning the 1,506 bp *NdeI/SalI* fragment of precursor *Tca* APase gene (pre *Tca* APase) derived from PCR of plasmid pGAPB [9] into the same sites of pET-22b(+). Plasmid pEAP2 was constructed by cloning the 1,425 bp *Eco*RI/*Sal*I fragment of mature *Tca* APase gene (*Tca* APase) derived from PCR of plasmid pGAPB [9] into the same sites of pET-22b(+). The lengths of the DNA segments are not to scale. Amp^r, ampicillin resistance gene; LacI, *lac* repressor gene; PelB, PelB leader peptide of pET-22b(+); Sig, putative signal peptide of *Tca* APase; T7*lac*, T7*lac* promoter.

primers AN2 and AC (Fig. 1A). Both the DNA fragment and expression vector pET-22b(+) were digested with *Eco*RI and *Sal*I. The digested DNA fragment was then cloned into pET-22b(+) to yield the expression plasmid pEAP2. Thus, in the recombinant plasmid pEAP2, the signal peptide portion of precursor *Tca* APase was replaced by the PelB leader peptide (Fig. 1B).

Expression of the *Tca* APase Gene in *E. coli*

Tca APase was highly expressed in *E. coli* BL21 harboring pEAP1 and pEAP2 after induction with 0.2 mM IPTG. The expression level of *Tca* APase was tested by the hydrolysis of *p*NPP. The activity of *Tca* APase in *E. coli* BL21 harboring pEAP2 reached 2.5 units/ml (5 $O.D._{600}$/ml) of culture medium. The expression level in *E. coli* BL21

Table 1. Comparison of enzyme activity of recombinant *Tca* APases.

Species	*Tca* APase activity (units/ml of culture medium)
E. coli BL21 (control)	0.003
E. coli BL21/pEAP1	2.0
E. coli BL21/pEAP2	2.5

One unit of APase is defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol at 80°C in 1 min. The density of cells for the determination of unit was adjusted in $O.D._{600}$=5.0/ml.

harboring pEAP2 was 1.25-fold better than that in *E. coli* BL21 harboring pEAP1 (Table 1). Thus, the use of the *pelB* leader peptide sequence in the pET-22b(+) for the high-level expression of the *Tca* APase gene was somewhat better than that of the original signal peptide sequence. The leader peptide sequence appears to be influencing the efficiency of gene expression. The expression level of *Tca* APase in *E. coli* BL21 harboring pEAP2 was also roughly 13.9-fold higher than that in *E. coli* YK537 under the control of *tac* promoter (0.18 units/ml of culture medium) [9].

Purification of the Recombinant *Tca* APase in *E. coli*

The advantage of production of thermostable enzymes in the *E coli* system is its ease to purify the thermostable enzymes by heat treatment. Such a step was applied to the purification of *Tca* APase in *E. coli*. Therefore, the *E. coli* BL21 harboring pEAP2 was cultured in 1-l flasks containing 200 ml of L-broth medium, and about 6 g of wet cell paste was obtained from 1 l of culture. The harvested cells were first sonicated, and the recombinant *Tca* APase was then purified by combination of heating (to denature *E. coli* proteins) and one step of HiTrap Heparin HP column chromatography. The purification procedure of the enzyme is summarized in Table 2. The specific activity of the purified enzyme was approximately 30.7-fold higher than that of the sonicated extract, and recovery was approximately 50% on the basis of the sonicated extract. The purification of the enzyme was monitored by SDS-PAGE (Fig. 2). Most of the proteins originated from *E. coli* were removed by the heat treatment (Fig. 2), and nucleic acids and residual contaminating proteins were removed by HiTrap Heparin HP column chromatography, thus indicating that the above contaminants were easily removed from the *Tca* APase

Table 2. Purification summary of the recombinant *Tca* APase.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Recovery (%)
Sonicated extract	367	2,130	5.8	100
Heat treatment	45	1,810	40.2	85
HiTrap Heparin HP	6	1,070	178.3	50

The purification was started with 6 g wet weight of cells.

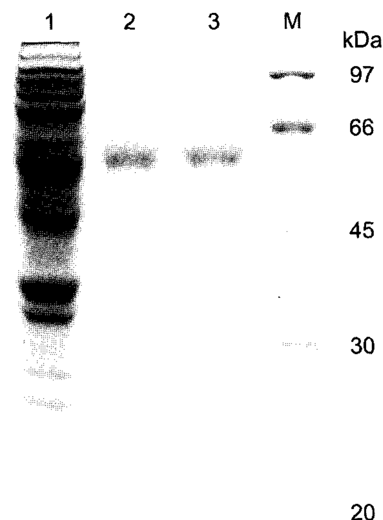

Fig. 2. SDS-PAGE analysis of the recombinant *Tca* APase.
The electrophoresis was performed on a vertical gel containing polyacrylamide using a Mighty Small Kit II system (Hoffer Scientific Instruments). Lane 1, sonicated extract of induced cells (*E. coli* BL21/pEAP2); lane 2, same sample heated at 80°C for 30 min and cleared by centrifugation; lane 3, HiTrap Heparin HP column chromatography pool; lane M, low-molecular-mass markers (molecular masses are indicated at the right).

preparation. When adsorbed protein was eluted with a linear gradient of KCl (0- 1.0 M) in buffer A, a single peak of *Tca* APase activity was eluted at an approximate KCl concentration of 0.3 M. SDS-PAGE analysis of the *Tca* APase activity pooled peak fractions revealed a single polypeptide with a molecular mass of approximately 56,000 Da, which correlates with the native *Tca* APase purified from *T. caldophilus* GK24 [5] (Fig. 2).

Properties of the Recombinant Enzyme

The recombinant *Tca* APase derived from pEAP2 has the PelB leader peptide from the expression vector pET-22b(+) instead of the original signal peptide of *Tca* APase. The effect of temperature on the recombinant *Tca* APase activity was determined within a range of 40- 95°C, and the maximal activity was observed at 85°C in 50 mM Glycine-NaOH (pH 11.0) (Fig. 3A); however, it decreased slightly at temperatures above 85°C. As such, the effect of temperature on the recombinant *Tca* APase was similar to that on the native *Tca* APase purified from *T. caldophilus* GK24 [5]. The effect of pH on the recombinant *Tca* APase activity was determined between pH 7.0- 12.0, using 50 mM Tris-HCl (pH 7.0- 9.0) and 50 mM Glycine-NaOH (pH 9.0- 12.0) buffers. The optimum pH for the *Tca* APase activity was observed at pH 11.5 in Glycine-NaOH (Fig. 3B), thus revealing that the pH profile of the recombinant

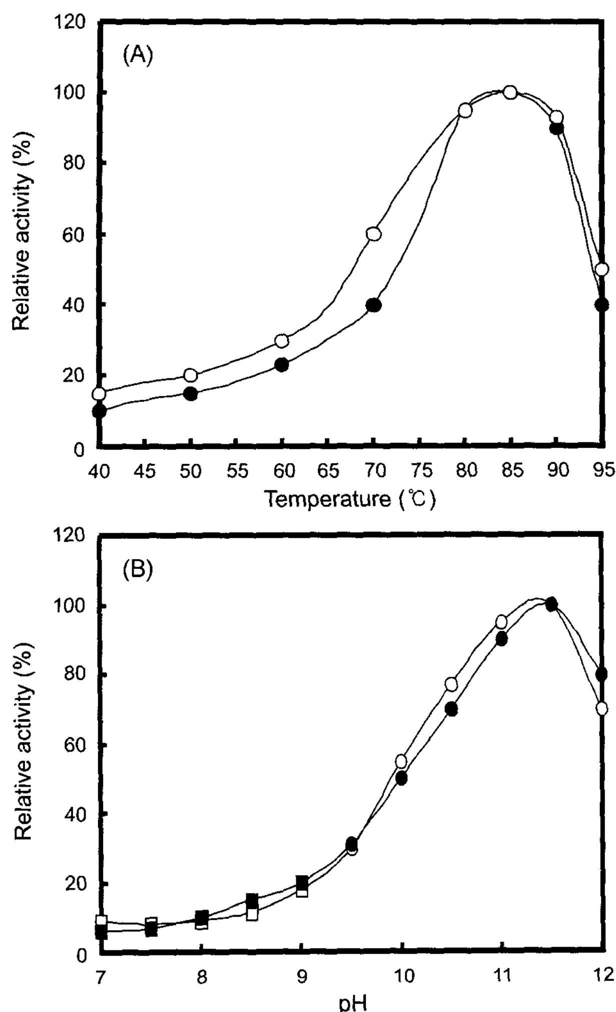

Fig. 3. Properties of the recombinant *Tca* APase derived from pEAP2.
The enzyme activity was assayed at the indicated temperature and pH in 1 mM $MgCl_2$. (A) Effects of temperature on the recombinant *Tca* APase (○) and native *Tca* APase (●) activities in 50 mM Glycine-NaOH buffer (pH 11.0). (B) Effects of pH on the recombinant *Tca* APase (○, □) and native *Tca* APase (●, ■) activities in 50 mM Tris-HCl (□, ■) and 50 mM Glycine-NaOH (○, ●).

Tca APase was similar to that of the native *Tca* APase [5]. At acid conditions below pH 6.0, the *Tca* APase activity decreased drastically (data not shown). The recombinant *Tca* APase activity was activated by Mg^{2+}; however, it was inhibited by Ca^{2+}, Co^{2+}, Zn^{2+}, and EDTA at 1 mM concentration (data not shown). This dependence of the catalytic activity on Mg^{2+} has already been observed in most of the APases investigated [2, 5, 10]. The above results thus indicate that properties of the recombinant enzyme and native *Tca* APase purified from *T. caldophilus* GK24 [5] were not significantly different from each other.

In conclusion, no significant differences in the properties of the recombinant and native *Tca* APases were found. Therefore, this study serves as a model for gene expression studies and purification of thermostable APase overproduced in *E. coli*. In addition, a large-scale production of the thermostable APase for industrial use may become practical.

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