

## Recombinant Thermostable AP Exonuclease from *Thermoanaerobacter tengcongensis*: Cloning, Expression, Purification, Properties and PCR Application

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### Abstract

Apurinic/aprimidinic (AP) sites in DNA are considered to be highly mutagenic and must be corrected to preserve genetic integrity, especially at high temperatures. The gene encoding a homologue of AP exonuclease was cloned from the thermophilic anaerobic bacterium *Thermoanaerobacter tengcongensis* and transformed into *Escherichia coli*. The protein product showed high identity (80%) to human Ape1 nuclease, whereas to *E. coli* exonuclease III – 78%. This is the first prokaryotic AP nuclease that exhibits such high identity to human Ape1 nuclease. The very high expression level (57% of total soluble proteins) of fully active and soluble His<sub>6</sub>-tagged *Tte* AP enzyme with His<sub>6</sub>-tag on C-terminal end was obtained in *Escherichia coli* Rosetta (DE3) pLysS. The active enzyme was purified up to 98% homogeneity in one chromatographic step using metal-affinity chromatography on Ni<sup>2+</sup>-IDA-Sepharose resin. The yield was 90 mg (14 000 kU) of pure His<sub>6</sub>-tagged *Tte* AP (153 kU/mg) from 1 liter of culture. The optimal conditions of *Tte* AP endo-, exonuclease and 3'-nuclease activity were investigated using fluorescein labeled dsDNA with inserted AP sites and ssDNA. Optimal *Tte* AP endonuclease activity was observed at 70–75°C, pH 8.0 and at low Mg<sup>2+</sup> concentration (0.5 mM). Higher Mg<sup>2+</sup> concentration (> 1 mM) enhanced 3'-5' exonuclease activity and at Mg<sup>2+</sup> concentration > 2.0 mM 3' nuclease activity was observed. Because of the endonuclease activity of *Tte* AP exonuclease, the enzyme was applied in PCR amplification of long DNA templates. *Tte* AP exonuclease eliminated AP-sites in DNA template and improved the efficiency of DNA amplification.

**Key words:** AP endonuclease, AP site, DNA damage, Polymerase Chain Reaction *Thermoanaerobacter tengcongensis*

### Introduction

Repair of DNA damages by base excision is one of the main strategies used by the cell to defend against exogenous or endogenous genotoxic stress. These damages have the potential to cause cell death or mutation. All organisms possess a panel of DNA repair mechanisms to repair damaged DNA and to maintain viability and genomic stability. Among these DNA repair mechanisms, base excision repair (BER) is one of the most highly conserved. Enzymes of the BER pathway act to remove the abasic fragment, fill the nucleotide gap, and seal the nick. AP endonucleases from *Escherichia coli* are classified into two main families termed exonuclease III (ExoIII or Xth) and endonuclease IV (EndoIV or Nfo).

The ExoIII protein of *E. coli* is a 3'-phosphatase capable of activating DNA substrates for polymerase-

mediated primer extension (Richardson and Kornberg, 1964), and has AP endonuclease, 3'-5' exonuclease and RNase H activity (Demple and Harrison, 1994; Wilson *et al.*, 1998). The 3'-phosphodiesterase and AP endonuclease activities of ExoIII play a major role in the BER pathway. Interestingly, *Neisseria meningitidis* possesses two AP endonucleases that have distinct activities in DNA repair: one is a typical Neisserial AP endonuclease (NApe), whereas the other is a specialised 3'-phosphodiesterase Neisserial exonuclease (NExo) (Carpeneter *et al.* 2007).

The human major abasic endonuclease is Ape1, an ExoIII homologue (Wilson and Barsky, 2001). Unlike ExoIII, the human enzyme has relatively poor 3'-5' exonuclease activity (Lebedeva *et al.*, 2003), but exhibits AP endonuclease activity (Seki *et al.*, 1992; Wilson *et al.*, 1995) as well as very selective 3'-phosphodiesterase activity (Suh *et al.*, 1997). Ape1 was found to be the

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major 3'-nuclease that can excise analogs of nucleotides (Chou *et al.*, 2000). The nuclease specificity of Ape1 and Ape2 was recently investigated (Hadi *et al.*, 2002). It was found that mutation at F226 and W280 of Ape1 increases 3'-5' exonuclease activity. Recently discovered yeast Eth1/Apn2 and human Ape2 proteins (Bennett, 1999; Hadi and Wilson, 2000; Johnson *et al.*, 1998; Unk *et al.*, 2000) exhibit high similarity within the core of nuclease domains of Ape1 and ExoIII, but show relatively poor AP endonuclease and 3'-repair activities (Bennett, 1999; Hadi and Wilson, 2000; Unk *et al.*, 2000; Unk *et al.*, 2001).

The thermophilic AP endonuclease from the archaeon *Archaeoglobus fulgidus* was described by Schmiedel *et al.* The enzyme shared 33% overall sequence identity and 55% similarity to ExoIII. It showed a strong Mg<sup>2+</sup> dependent nicking activity at AP-sites, nicking of undamaged double-stranded (ds) DNA and a weak exonucleolytic activity (Schmiedel *et al.*, 2009).

*Thermoanaerobacter tengcongensis* isolated from Chinese hot spring, a rod-shaped, Gram-negative, anaerobic bacterium that was recently described as an extremely thermophilic bacterium (Xue *et al.*, 2001). The complete genome sequence of *T. tengcongensis* clone MB4T (Bao *et al.*, 2002) consists of a 37.6% GC-content 2,689,441, base pair (bp) circular chromosome that contains 2808 predicted coding regions, 1,481 (52.7%), which have functional assignments, and 1,327 (47.3%) of which are of unknown function. Genome analysis also reveals that 30 genes have their homologues in the genomes of other thermophilic eubacteria and archaea.

The results of this study demonstrates that *Tte* AP exonuclease from *Thermoanaerobacter tengcongensis* exhibits high similarity to human Ape1 enzyme and exhibits 3'-nuclease activity. The *Tte* AP exonuclease can be efficiently produced in *E. coli* over-expression system and large amount of fully active protein can be purified using a single-step chromatography procedure with Ni<sup>2+</sup>-IDA-sepharose. We also report the application of the recombinant *Tte* AP exonuclease in PCR analysis.

## Experimental

### Materials and Methods

#### Bacterial strains, plasmids, enzymes, and reagents.

*Thermoanaerobacter tengcongensis* (DSM 15242) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. *E. coli* TOP10F', pUC57, were used for cloning of gene for *Tte* AP exonuclease. The Rosetta(DE3)pLysS strain and pET-30 LIC plasmid were applied for expression of His<sub>6</sub>-tagged *Tte* AP exonuclease. Ni<sup>2+</sup>-IDA-sepharose 6B was prepared as described by Porath *et al.* (Porath and Olin,

1985). Restriction and modification enzymes were purchased from NEB (USA) and Fermentas MBI (Lithuania). The reagents for PCR, IPTG, agarose and reagents for protein purification were purchased from Sigma (USA), oligonucleotides were purchased from MWG Biotech (Germany).

#### Phylogenetic analysis of AP-sites excision enzymes.

Based on the *T. tengcongensis* genomic DNA sequence (GenBank acc. No. NC003869) of the gene encoding potential AP endonuclease (ExoIII) was found (locus TTE2226) and translated into amino acid sequence. The comparison with other known eukaryotic and prokaryotic AP-sites excision enzymes was performed using program AlignX from Vector NTI Suite 7.1 software package (Invitrogen, Inc.). The phylogenetic tree was constructed using the same program regarding to Kimura's correction and the phylogenetic tree was built using the Neighbour Joining method (NJ) of Saitou and Nei.

#### Cloning of the gene encoding *Tte* AP exonuclease from *T. tengcongensis*.

Genomic DNA from *Thermoanaerobacter tengcongensis* was isolated using Genomic DNA Prep Kit (A&A Biotechnology, Poland). The specific primers were designed: *Tte*APE1NdeI, (5' GCG-GCGCATATGAAACTCGTATCGTGG 3') (containing *Nde*I recognition site), *Tte*APE1-XhoC-His (5'GCG-GCGCTCGAGTTTTATGAGGTCTTCTTTTAC-TATAAG 3') (containing *Xho*I recognition site). The bolded parts of primer sequences are complementary to the nucleotide sequences of the gene encoding *Tte* AP exonuclease, whereas 5' overhanging ends of primers contain recognition sites for restriction endonucleases (underlined), and are designated to facilitate cloning. The reaction solution consisted of: 50 ng of the *T. tengcongensis* genomic DNA, 2 µl (10 µM) of each primer, 5 µl (10 mM) dNTPs, 5 µl 10×PCR buffer (100 mM Tris-HCl, pH 8.9, 500 mM KCl, 25 mM MgCl<sub>2</sub>, 1% Triton X-100), 2 U of *Pwo* DNA polymerase (Dabrowski and Kur, 1998) and 30 cycles were performed with a temperature profile of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C. The amplified product was analyzed by electrophoresis on a 1% agarose gels stained with ethidium bromide. Specific PCR product was obtained (approximately 802 bp; 0.1 µg) and purified using Clean-Up Kit (A&A Biotechnology, Poland). The product was then phosphorylated using T4 polynucleotide kinase and then ligated with pUC57/*Sma*I dephosphorylated plasmid vector. The *E. coli* TOP10F' cells were transformed with the ligation mixture and colonies were assayed for the presence of the gene encoding *Tte* AP exonuclease by PCR amplification and restriction analysis. DNA fragments encoding *Tte* AP exonuclease gene were excised using *Nde*I and *Xho*I endonucleases and cloned into pET-30 LIC expression plasmid in *Nde*I and *Xho*I sites. After *E. coli* TOP10F' transformation, four clones were selected and sequenced. After

sequencing, one of the plasmids (pET30TteAPC) was chosen and used for His<sub>6</sub>-tagged *Tte* AP exonuclease expression and purification procedure.

**Purification of the *Tte* AP exonuclease.** Rosetta (DE3)pLysS strain transformed with pET30TteAPC plasmid was grown overnight at 37°C in 10 ml LB supplemented with 50 µg/ml chloramphenicol and 34 µg/ml kanamycin and 0.5% glucose. After 24 h, 500 ml LB containing 50 µg/ml chloramphenicol and 34 µg/ml kanamycin was inoculated and the culture was followed to an O.D. of 0.3 at 600 nm. IPTG was then added to the final concentration of 0.5 mM, and then culture was continued until cells reach the stationary phase. The cells were harvested by centrifugation and the pellet was resuspended in 10 ml of buffer A (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100). Cells were disrupted by fast freezing at -80°C, and then were homogenized for 5 min, and insoluble proteins were removed by centrifugation. The clarified supernatant was further applied directly onto a Ni<sup>2+</sup>-IDA-sepharose 6B column (10 ml of bed volume) pre-equilibrated with buffer A. The column was washed preliminary with 30 ml buffer A, and with 30 ml of buffer A containing 50 mM imidazole, and washed again with buffer A containing 80 mM. The *Tte* AP exonuclease was eluted using buffer A containing 0.5 M imidazole. The purity of the eluted protein was over 98%. Pure *Tte* AP exonuclease was dialyzed against storage buffer AG (20 mM Tris-HCl pH 8.0, 50 mM KCl, 50% glycerol) with PMSF (phenylmethylsulfonyl fluoride) and stored at -20°C until use.

***Tte* AP exonuclease activity assays.** For the analysis of properties of *Tte* AP exonuclease, a different 5' fluorescein labeled dsDNA and ssDNA were used. The AP endonuclease activity was assayed using 50 nt hybrid DNA combined from two synthetic oligonucleotides with inserted AP sites (AP-fluoro: Fluorescein-5' GCATAGACATAGATACAGATACAT<sup>AP</sup>CTAGAGACTAGGCTCGTAATCCCA G 3' and AP: 5'CTGGGATTACGAGCCTAGTCTC<sup>AP</sup>AGGATGTATCTGTATCTATGTCTATGC 3'). The 3'-5' exonuclease activity of *Tte* AP exonuclease was examined using a dsDNA hybrid consisted of three synthetic oligonucleotides forming nicked DNA (FluoroHyb1: Fluorescein-5' GCGGCGGCTGGCACC 3', Hyb 2: GCTGGCTCGGGTGGGCTGAGCTGCCTGGCGGCAC 3' and Hyb3 5'GTGCCGCCAGGCAGCTCAGGCCACCCGAGCCAGCGGTGCCAGGCGCCGC 3'). The 3'-5' exonuclease activity was also investigated using hybrid DNA combined from Hyb1 and Hyb3 oligonucleotides. The 3'-nuclease activity was analyzed using FluoroHyb1 oligonucleotide. All reactions were performed at 70°C for 30 min at 66 mM Tris-HCl (pH 8.0) buffer - 20 pmol of DNA and 100 U of *Tte* AP exonuclease were used. The thermostability of *Tte* AP

exonuclease was evaluated at different temperatures (from 37°C up to 95°C) using 10 pmol of AP-DNA and 200 U of enzyme and incubated for 15 min in 66 mM Tris-HCl (pH 8.0) buffer containing 0.5 mM Mg<sup>2+</sup>.

**Use of *Tte* AP exonuclease in PCR analysis.** Different amounts of the *E. coli* genomic DNA (300 to 0.1 ng) were depurinated by heating in 0.01 M sodium citrate buffer pH 5.1, 0.1 NaCl at 75°C for 60 min. Depurinated DNA samples were incubated in 66 mM Tris pH 8.0, 0.5 mM MgCl<sub>2</sub> with 30 U of *Tte* AP exonuclease was incubated at 70°C for 30 min, then the enzyme was denatured at 90°C for 5 min. Enzyme treated DNA samples were amplified. The length of DNA target was approximately 3000 bp. The PCR was carried out with cloned Pwo DNA polymerase 2.5 U/50 µl of reaction combined with Pwo dUTPase (Dabrowski and Ahring, 2003). PCR was performed using primer Sup (5' AGGCCTGATAAGCGCAGCGTATCAGG 3') and primer Fr3 (5' CCGATATGTGCGAAGGCTTACCGG 3') and 30 cycles with a temperature profile of 30 s at 94°C, 1 min at 65°C, and 3 min at 72°C. The presence of the PCR products was analyzed on 1% agarose gel.

## Results and Discussion

**Amino acid sequence analysis of *Tte* AP exonuclease.** The amino acid sequence of *Tte* AP exonuclease was compared with other BER enzymes using the AlignX program of Vector NTI suite 7 (Invitrogen). Multiple sequence alignment was performed and the phylogenetic tree of *Tte* AP exonuclease is shown in Fig. 1a. The comparison indicates that group of ExoIII proteins from *Clostridiaceae* including *T. tengcongensis* AP exonuclease shows higher identity to eukaryotic AP exonucleases (human, rat, mouse, fruit fly), than to prokaryotic ExoIII proteins. *Tte* AP exonuclease is similar to human APE1 in 80% whereas *E. coli* ExoIII is similar to human APE1 in 78%. As it was described previously (Hadi *et al.*, 2002), the comparison shows high conservation of these enzyme sequences. The high similarity between enzymes from the BER pathways is probably the consequence of their important function in DNA damage repair. Focused on *E. coli* ExoIII, *Tte* AP exonuclease and human APE1 proteins alignment is separately presented in Fig. 1b. The high similarity of *Tte* AP exonuclease to human Ape1 protein is clearly shown. This similarity is within the critical limits for AP site binding or catalysis where *E. coli* ExoIII contains different residue (Q112, L226). The analysis of predicted secondary structure also presents higher similarity of *Tte* AP exonuclease to Ape1 than *E. coli* ExoIII to APE1 protein. Highly inhibited AP endonuclease function was found in APE1 protein after substitution of residues F226A and W280I (Hadi *et al.*, 2002).

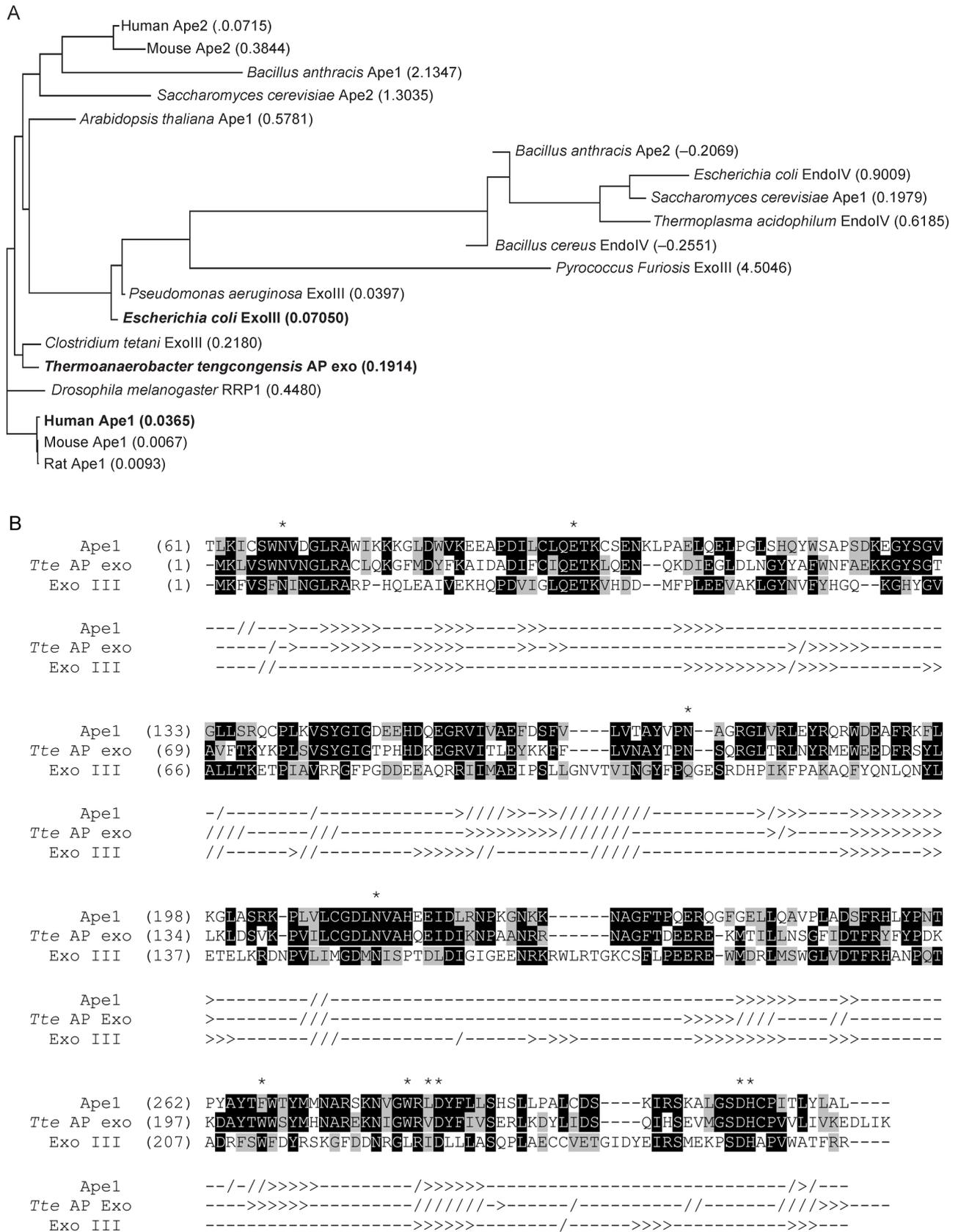


Fig. 1. The amino acid sequence alignment of the BER pathway enzymes. (A) Phylogenetic tree of the ExoIII family from different organisms. The calculated distance values in parenthesis is followed the molecule name displayed on the tree. (B) Alignment of the AP endonuclease domains of Ape1, *Tte* AP exonuclease and ExoIII. Residues conserved in two or more of sequences are highlighted in black (identical) or grey (similar). Residues of Ape1 that are critical for AP site binding of catalysis are indicated by an asterisk (\*). Location of secondary structure elements in Ape1, *Tte* AP exonuclease and ExoIII (as assessed by NNPREPDICTION (Kneller *et al.*, 1990; McClelland and Rumelhart, 1988)) are marked below the respective sequences:  $\beta$ -strands are represented as ' $\prime$ ', and  $\alpha$ -helices as '>'. '---' indicates gaps in the alignment.

The inhibition of *E. coli* ExoIII binding to AP site was observed when substitution W212S was carried out (Shida *et al.*, 1999). Based on these previous observations, the presence of aromatic residue of *Tte* AP exonuclease (W202) is required for efficient recognition of AP sites by ExoIII family.

**Expression of the recombinant His<sub>6</sub>-Tagged *Tte* AP exonucleases.** The recombinant His<sub>6</sub>-*Tte* AP exonuclease was produced in *E. coli* Rosetta(DE3)pLysS harbouring pET30*Tte*APC plasmid. The host for protein expression contains a modified pLysS plasmid (pRARE) with rarely used tRNA genes in *E. coli* and gene encoding T7 lysozyme, which strongly represses protein expression by T7 RNA polymerase binding and inhibition. Owing to a high copy in the *E. coli* cell of this plasmid the multiplication of tRNA is possible and helps to avoid the codon bias of *E. coli* and enhance the expression of heterologous proteins (Brinkmann *et al.*, 1989; Novy *et al.*, 2001; Seidel *et al.*, 1992). The selected clones were grown in LB medium supplemented with chloramphenicol, kanamycin and glucose. Glucose strongly inhibits protein expression from pET vector (Fig. 2, lane 3) in *E. coli* cells and keeps stable number of plasmid copies in the cell during the overnight growth. The induction of *Tte* AP exonuclease expression was initialized by IPTG addition at A<sub>600</sub> of 0.3 and culture was grown for 6 h at 37°C reaching the highest protein expression level (Fig. 2, lane 4).

**Purification of the recombinant *Tte* AP exonuclease.** The purity of enzyme was examined by SDS-PAGE (Fig. 2). Soluble His<sub>6</sub>-tagged *Tte* AP exonuclease was

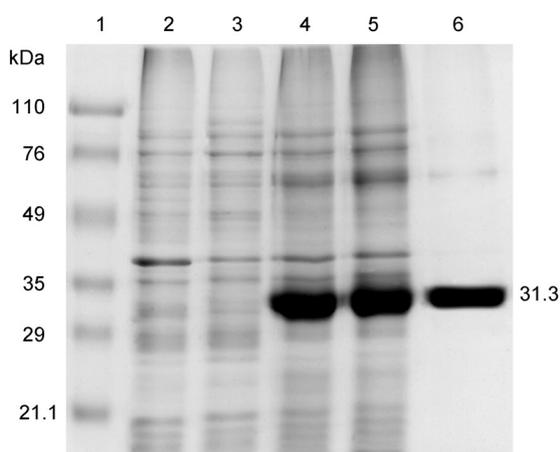


Fig. 2. SDS electrophoresis in 12% polyacrylamide gel of the fractions obtained by purification of recombinant *Tte* AP exonuclease from *E. coli* Rosetta(DE3)pLysS+ pET30*Tte*APC. Each loaded sample corresponds to 25 µl of culture. Lane 1, low molecular weight marker (Bio-Rad); lane 2, lysate from *E. coli* Rosetta(DE3)pLysS; lanes 3, lysate from *E. coli* Rosetta(DE3)pLysS+ pET30*Tte*APC after overnight culture supplemented with 0.5% of glucose; lanes 4, lysate after induced expression of *Tte* AP exonuclease; lanes 5, fraction of soluble proteins after expression of *Tte* AP exonuclease; lane 6, *Tte* AP exonuclease after purification on Ni<sup>2+</sup>-IDA-sepharose.

Table I  
Purification of *Tte* AP exonuclease from Rosetta(DE3)pLysS strains containing pET30*Tte*APC plasmid from 500 ml of culture.

Purification step	Protein [mg]	Activity of AP exonuclease [kU]	Specific activity [kU/mg]	Expression level <sup>a</sup> [%]
Fraction of soluble proteins	78	nd	nd	nd
Metal-affinity chromatography	45	7000	153	56

<sup>a</sup> Percentage of *Tte* AP exonuclease protein after purification in relation to total soluble proteins; nd – not determined

expressed in the cytosol (Fig. 2, lane 5). The recombinant enzyme was eluted from the column as a single peak (~98% purity) (Fig. 2, lane 6). Another band corresponding to 32 kDa was observed in SDS-PAGE of crude extract of *E. coli* Rosetta(DE3)pLysS with pET30*Tte*APC cultures after IPTG induction (Fig. 2, lane 4). This band was absent in the control crude extracts of *E. coli* Rosetta(DE3)pLysS culture (Fig. 2, lane 2) and in crude extracts of *E. coli* Rosetta(DE3)pLysS containing pET30*Tte*APC overnight culture in the presence of glucose, which strongly suppresses basal expression of proteins from the *lac* promoter. (Novy and Morris, 2001). Protein concentrations were estimated by the Bradford method for protein quantification (Bradford, 1976) using BSA as a standard (see Table I). The activity of recombinant *Tte* AP exonuclease was evaluated using fluorescein labeled dsDNA with inserted AP sites. Digested DNA was analyzed on agarose gel. To evaluate the activity of *Tte* AP exonuclease, control experiments were conducted with restriction enzyme *Exo*III (Fermentas MBI, Lithuania). We obtained 45 mg of pure His<sub>6</sub>-tagged *Tte* AP exonuclease exhibiting 153 kU/mg specific activity from 500 ml of cultured *E. coli* Rosetta(DE3)pLysS+ pET30*Tte*APC (Table I).

**AP endonuclease, 3'-5' exonuclease and 3'-nuclease activities of *Tte* AP exonuclease.** The evaluation of AP endonuclease activity of *Tte* AP exonuclease was performed using a synthetic DNA hybrid with two inserted AP sites (Fig. 3, Frame A). The DNA hybrid and products of the hybrid digestion were detected by fluorescein labeling the 5' end of the oligonucleotide. The products of digestion performed in buffers with different Mg<sup>2+</sup> concentration were analyzed on high-resolution NuSieve agarose (Fig. 3B). The effect of degradation is observed by change of mobility (band shift) of 5'-fluorescein labeled ssDNA or dsDNA due to DNA nicking or exonucleolytic degradation.

The *Tte* AP endonuclease activity is observed when Mg<sup>2+</sup> concentration is below 1 mM, while 3'-5' exonuclease of the AP exonuclease activity is exhibited when Mg<sup>2+</sup> concentration is 1.0 mM or higher. The

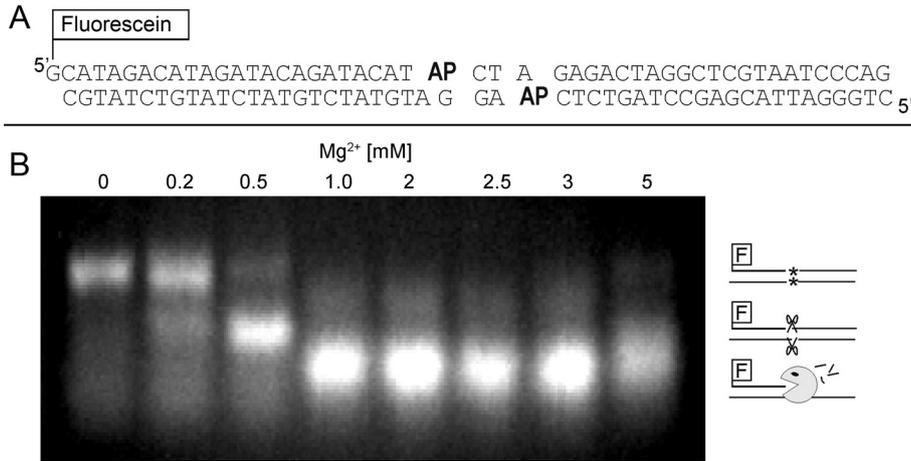


Fig. 3. AP endonuclease activity assay. (A) Structure of AP-DNA used in measurement of AP endonuclease activity. (B) The agarose gel electrophoresis analysis of products after digestion of 20 pmol of AP-DNA at indicated Mg<sup>2+</sup> concentrations using 100 U of *Tte* AP exonuclease.

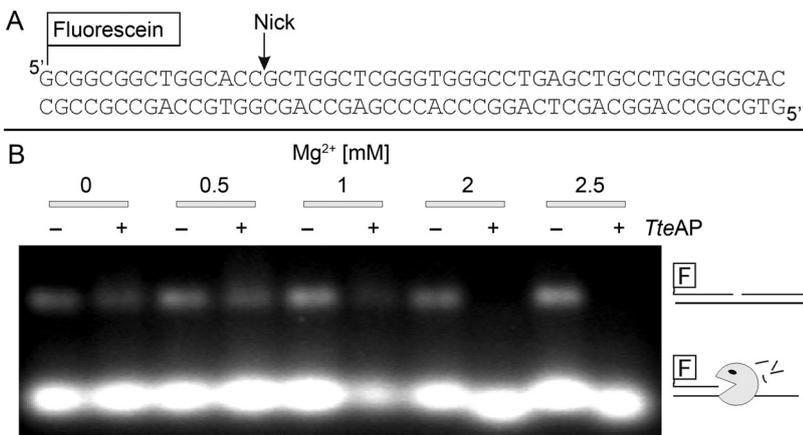


Fig. 4. 3'-5' exonuclease activity assay using nicked DNA. (A) Structure of hybrid DNA used in measurement of exonuclease activity. (B) The agarose gel electrophoresis analysis of products after digestion of 20 pmol of hybrid DNA at indicated Mg<sup>2+</sup> concentrations using 100 U of *Tte* AP exonuclease (+) and without enzyme (-).

3'-5' exonuclease activity of *Tte* AP exonuclease was confirmed in assay with nicked DNA hybrid used as a substrate (Fig. 4A). The nicked DNA was incubated with and without *Tte* AP exonuclease in buffers with different Mg<sup>2+</sup> concentration. The degradation of nicked DNA was exhibited when Mg<sup>2+</sup> concentration reached 1.0 mM (Fig. 4B). The 5'-3' exonuclease activity was also detected using fluorescein 5' labeled dsDNA with 5' cohesive end (Fig. 5A). The 3'-5' exonuclease activity was observed when Mg<sup>2+</sup> concentration was over 1.0 mM (Fig. 5B). The strong signal of small DNA fragment on Figure 4b and 5b is observed because of the overloading of fluorescein labeled primer (FluoroHyb1)

during hybridization. This overloading was necessary to obtain stable DNA hybridization during enzymatic reaction at high temperature. Similar influence of various Mg<sup>2+</sup> concentrations on exonucleolytic activities of *E. coli* ExoIII was also observed (Wilson *et al.*, 1998). Therefore ExoIII is applied for many molecular biology techniques: degradation of 3' end for unidirectional mutagenesis (Henikoff, 1984), generation of a single-stranded template for DNA sequencing (Guo and Wu, 1982), site directional mutagenesis (Vandeyar *et al.*, 1988), and many more.

Human Ap1 enzyme exhibits characteristic 3'-nuclease activity which is necessary in 3'mismatch repair

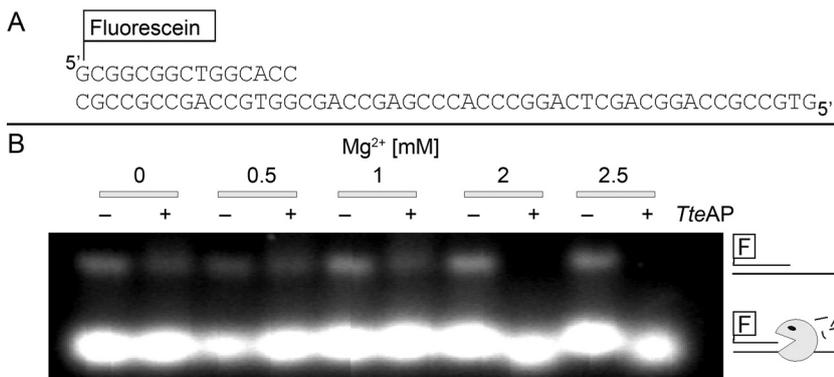


Fig. 5. 3'-5' exonuclease activity assay using DNA with 5' overhang. (A) Structure of hybrid DNA used in measurement of exonuclease activity. (B) The agarose gel electrophoresis analysis of products after digestion of 20 pmol of hybrid DNA at indicated Mg<sup>2+</sup> concentrations using 100 U of *Tte* AP exonuclease (+) and without enzyme (-).

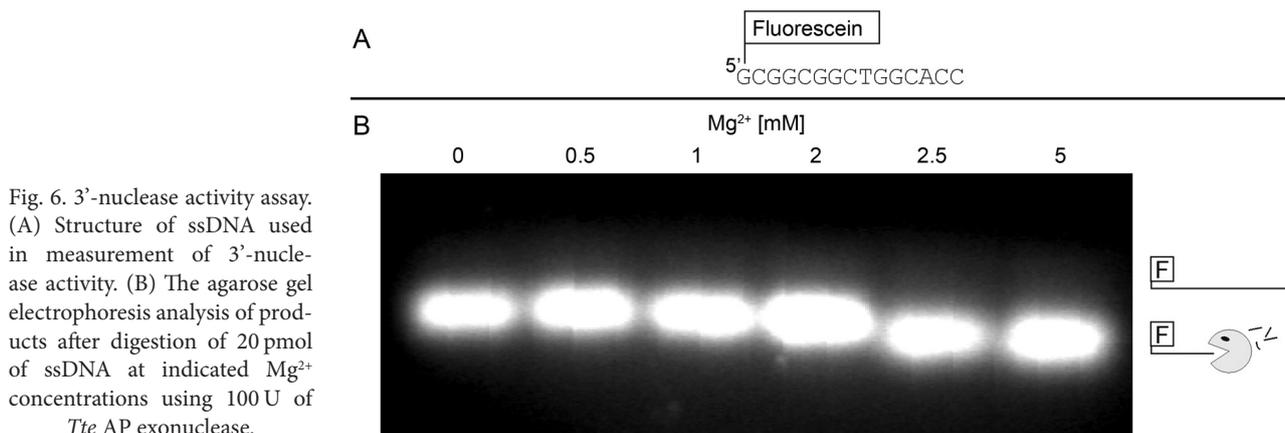


Fig. 6. 3'-nuclease activity assay. (A) Structure of ssDNA used in measurement of 3'-nuclease activity. (B) The agarose gel electrophoresis analysis of products after digestion of 20 pmol of ssDNA at indicated Mg<sup>2+</sup> concentrations using 100 U of *Tte* AP exonuclease.

(Hadi *et al.*, 2002), whereas this activity is absent in ExoIII enzyme. For the determination of 3'-nuclease activity from *Tte* AP exonuclease, the fluorescein-labeled 15 nt oligonucleotide was used (Fig. 6A). The oligonucleotide sequence was designed and examined using Vector NTI software and DNA secondary structure formation was not observed.

The oligonucleotide was incubated with the same amount of *Tte* AP exonuclease at different Mg<sup>2+</sup> concentration at 70°C for 30 min. The degradation of ssDNA was observed when Mg<sup>2+</sup> concentration was over 2.0 mM (Fig. 6B). The degradation of only a few nucleotides was observed due to fluorescein shielding of the 5' terminal binding sight of the enzyme. Function and endo- and exonuclease activities of *Tte* AP exonuclease are strongly depended on Mg<sup>2+</sup> concentration. The presence of a 3'-nuclease activity domain in prokaryotic BER pathway systems is unique. A 3'-nuclease activity is a consequence of inherent high identity of the amino acid sequence, and most likely the structure similarity to human Ape1 exonuclease.

**Thermostability of *Tte* AP exonuclease.** The optimal temperature of growth of *T. tengcongensis* is 70°C at pH 7.0–8.0. *Tte* AP exonuclease should be very stable in these conditions because it is responsible for repairing AP sites, which are generated very frequently at high temperatures. For examination of *Tte* AP exonuclease thermostability, the synthetic fluorescein-labeled hybrid DNA (50 bp), with inserted AP sites, was used (Fig. 3A). 200 U of enzyme were incubated at different temperatures with 10 pmoles of hybrid DNA for 15 min and analyzed on high-resolution NuSieve agarose gel

(Fig. 7). Inactivation of the enzyme was nearly complete at 84°C and above, and it is stable up to 80°C. The activity of the enzyme was also observed at 37°C, but that could be an effect of enzyme excess used in this assay.

**Enhancing PCR amplification using *Tte* AP exonuclease.** A number of modifications to the basic PCR method have been published in an attempt to increase the efficiency of amplification of long PCR products efficiency as well as the specificity of the reaction. The application of ExoIII protein of *E. coli* in long PCR reaction for elimination of AP sites has also been described (Fromenty *et al.*, 2000).

*Tte* AP exonuclease was employed for elimination of AP sites generated in *E. coli* genomic DNA. The enzyme indicated strong 3'-5' exonuclease and 3'-nuclease activity at 2.5 mM Mg<sup>2+</sup> (the Mg<sup>2+</sup> amount usually applied in PCR reaction). Its AP endonuclease activity was exhibited at 0.5 mM Mg<sup>2+</sup>. Therefore, to avoid degradation of template DNA and primers, genomic DNA was treated with *Tte* AP exonuclease at the presence of 0.5 mM Mg<sup>2+</sup>. Then, the enzyme was inactivated at 90°C for 5 min. PCR was performed with different amounts of genomic DNA and the products of reaction were analyzed on 1% agarose gel (Fig. 8). As previously described for ExoIII enzyme (Fromenty *et al.*, 2000), improved PCR efficiency is observed because of elimination of AP-sites, and inhibition of DNA polymerase is not observed. The inhibition of PCR is mainly observed when high amount of genomic DNA was used (300, 100 ng) (Fig. 8, lanes 2 and 3), and at a lower level of DNA (10 ng) (Fig. 8, lane 5). The high PCR inhibition effect was observed at DNA damage levels below 3 ng

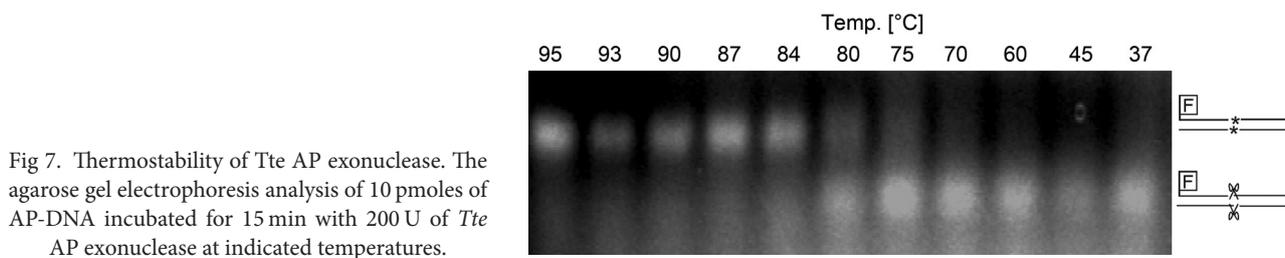


Fig 7. Thermostability of *Tte* AP exonuclease. The agarose gel electrophoresis analysis of 10 pmoles of AP-DNA incubated for 15 min with 200 U of *Tte* AP exonuclease at indicated temperatures.

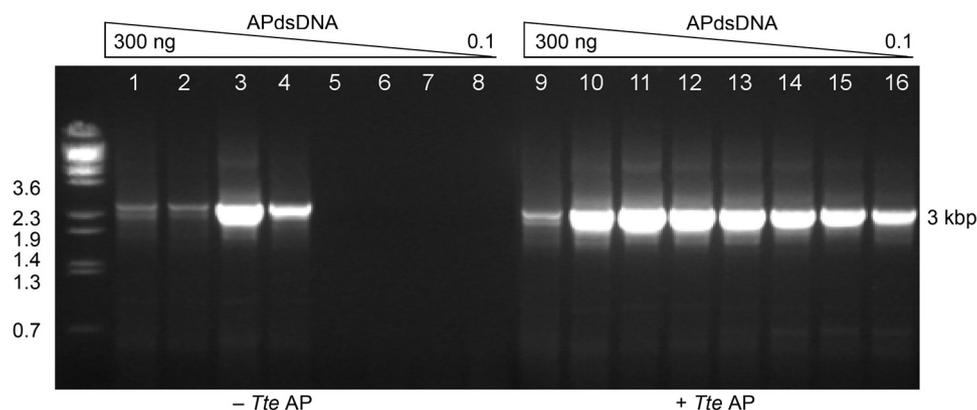


Fig. 8. Application of *Tte* AP exonuclease in PCR reaction. Analysis of PCR products after amplification using different amount of depurinated genomic DNA 300 ng (lanes 1, 9), 100 ng (lanes 2, 10), 33 ng (lanes 3, 11), 10 ng (lanes 4, 12), 3 ng (lanes 5, 13), 1 ng (lanes 6, 14), 0.3 ng (lanes 7, 15) and 0.1 ng (lanes 8, 16) after 30 U of *Tte* AP exonuclease pretreatment (+*Tte*AP) for 15 min (lanes 9–16) and without enzyme treatment (-*Tte*AP) (lanes 1–8).

(Fig. 8, lanes 6–9). The optimal amplification efficiency was obtained for 33 ng of genomic DNA, where the ratio of AP sites of genomic DNA to amount of used for PCR DNA polymerase permits for the efficient PCR. The application of 30 U *Tte* AP exonuclease eliminates AP sites before PCR (Fig. 8, lanes 11–16), but the ratio between number of AP sites and the amount of enzyme is still very high for repairing the highest concentration of genomic DNA (Fig. 8, lane 10).

The depurination effect of DNA is observed especially for samples stored for extended periods (historical samples) or/and stored in not optimal *i.e.* acidic conditions (Fromenty *et al.*, 2000). *Tte* AP exonuclease could be a useful tool in amplification of DNA from this kind of samples.

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