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Cloning, Expression, and Characterization of DNA Polymerase from Hyperthermophilic Bacterium *Aquifex pyrophilus*

CHOI, JEONG JIN AND SUK-TAE KWON*

Department of Genetic Engineering, Sungkyunkwan University, 300 Chunchun-Dong, Jangan-Ku, Suwon 440-746, Korea

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Abstract The gene encoding *Aquifex pyrophilus* (*Apy*) DNA polymerase was cloned and sequenced. The *Apy* DNA polymerase gene consists of 1,725 bp coding for a protein with 574 amino acid residues. The deduced amino acid sequence of *Apy* DNA polymerase showed a high sequence homology to *Escherichia coli* DNA polymerase I-like DNA polymerases. It was deduced by amino acid sequence alignment that *Apy* DNA polymerase, like the Klenow fragment, has only the two domains, the 3'→5' exonuclease domain and the 5'→3' polymerase domain, containing the characteristic motifs. The *Apy* DNA polymerase gene was expressed under the control of T7*lac* promoter on the expression vector pET-22b(+) in *E. coli*. The expressed enzyme was purified by heat treatment, and Cibacron blue 3GA and UNO™ Q column chromatographies. The optimum pH of the purified enzyme was 7.5, and the optimal concentrations of KCl and Mg^{2+} were 20 mM and 3 mM, respectively. *Apy* DNA polymerase contained a double strand-dependent 3'→5' proofreading exonuclease activity, but lacked any detectable 5'→3' exonuclease activity, which is consistent with its amino acid sequence. The somewhat lower thermostability of *Apy* DNA polymerase than the growth temperature of *A. pyrophilus* was analyzed by the comparison of amino acid composition and pressure effect.

Key words: *Aquifex pyrophilus*, DNA polymerase, exonuclease activity, hyperthermophile, sequence analysis, thermostability

DNA polymerases play leading roles in cellular DNA replication and repair. Beginning with the discovery and characterization of DNA polymerase I from *Escherichia coli* by Kornberg and colleagues in the 1950s [19], a variety of DNA polymerases have been isolated and identified from prokaryotic and eukaryotic sources. The amino acid sequences of these DNA polymerases have been aligned and partial homologous regions have been identified [2, 16].

Thermostable DNA polymerase was first isolated from the thermophilic bacterium *Thermus aquaticus* YT-1 and its properties have been reported [7]. Similar enzymes have been isolated from other *Thermus* strains, including *T. caldophilus* GK24 [29] and *T. filiformis* [18]. Thermostable DNA polymerases with an integrated 3'→5' exonuclease activity have also been isolated from hyperthermophilic archaebacteria *Thermococcus litoralis* [27] and *Pyrococcus furiosus* [25]. The increasing number of applications utilizing polymerase chain reaction (PCR) and isothermal amplification has generated increasing demands for thermostable DNA polymerases.

Hyperthermophiles are organisms that grow optimally at 80°C or higher temperatures. The thermostability of hyperthermophiles has raised considerable interest for both academic research and industrial applications. With maximal growth temperature near 95°C, the genus *Aquifex* is the most thermophilic bacteria known. *A. pyrophilus* is a marine, rod-shaped, Gram-negative bacterium isolated from a marine thermal deposit near a site of volcanic activity [15]. This organism is a microaerophile, growing chemolithoautotrophically by reducing oxygen and using hydrogen, thiosulfate, and sulfur as electron donors. *A. pyrophilus* is regarded as the most ancient bacterium because of its autotrophic nature and its deepest location in the eubacterial phylogenetic tree [4]. Several enzymes have been identified and characterized from *A. pyrophilus*; however, no information is available on the sequence and characteristics of a thermostable DNA polymerase from this organism.

In this paper, we present the entire nucleotide sequence of the *A. pyrophilus* (*Apy*) DNA polymerase gene and the deduced primary structure of its protein. We also report the expression, purification, and properties of *Apy* DNA polymerase. Finally, we discuss the somewhat lower

*Corresponding author
Phone: 82-31-290-7863; Fax: 82-31-290-7870;
E-mail: stkwon@yurim.skku.ac.kr

thermostability of *Apy* DNA polymerase compared to the growth temperature of *A. pyrophilus*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A. pyrophilus (DSM6858) was obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). *A. pyrophilus* cells were grown in a modified SME medium containing (per liter) 30 g of NaCl, 7 g of $MgSO_4 \cdot 7H_2O$, 5.5 g of $MgCl_2 \cdot 6H_2O$, 0.65 g of KCl, 2 g of $Na_2S_2O_3$, 0.1 g of NaBr, 2 g of $NaHCO_3$, 0.15 g of NH_4Cl, 0.15 g of K_2HPO_4, 0.5 g of $CaCl_2 \cdot 2H_2O$, and 10 ml of trace element solution [33]. The cells were inoculated into 20 ml of liquid medium in a 125-ml bottle that was filled with 300 kPa of hydrogen, carbon dioxide, and oxygen at a ratio of 79.5: 20:0.5 and grown at 85°C for 16 h with moderate shaking [15].

E. coli MV1184 was used for plasmid propagation and nucleotide sequencing [13]. *E. coli* BL21(DE3), which carries the T7 RNA polymerase gene under the control of a chromosomal *lac*UV5 gene [34], was used for gene expression. The *E. coli* cells were grown in LB medium with appropriate antibiotics at 37°C with vigorous shaking.

Genomic DNA Isolation and Hybridization

Genomic DNA of *A. pyrophilus* was isolated by the method of Marmur [26] with slight modifications. The probe used for the DNA-DNA hybridization to detect the *Apy* DNA polymerase gene was prepared from the expression plasmid pTAAP [6], containing the *A. aeolicus* (*Aae*) DNA polymerase gene (GenBank Accession Number AE000765) [12], and labeled with [α-^{32}P]dATP by random priming. DNA hybridization was performed by the agarose gel membrane hybridization method [32]. *A. pyrophilus* genomic DNAs digested with four restriction enzymes (*Eco*RI, *Hin*dIII, *Pst*I, or *Sac*I) were fractionated on a 0.7% agarose gel, and the probe was hybridized at 50°C to the fractionated genomic DNA on the agarose gel membrane. Colony hybridization was done according to the standard procedures using the previously mentioned ^{32}P-labeled probe. DNA inserts of positive clones were characterized following plasmid DNA extraction.

DNA Sequencing and Sequence Analysis

The restriction fragments to be sequenced were cloned into appropriate restriction sites of pBluescript SK- and pUC19 vectors. DNA sequences were determined by the dideoxy chain termination method. The obtained sequences were compared with known proteins in the database using the sequence comparison program, BLAST. Nucleotide and deduced amino acid sequence analyses were performed by using the DNASIS (Hitachi Software Engineering Co., Japan) and PCGENE (Intelligenetics Co., U.S.A.) softwares. The MultAlin program [11] was used for multiple sequence alignment between functionally related proteins.

Construction of Plasmid Expressing *Apy* DNA Polymerase

Most of the methods used for plasmid construction were based on those of Sambrook *et al.* [30]. For the expression of *Apy* DNA polymerase, the DNA polymerase gene was amplified by direct PCR of the *A. pyrophilus* genomic DNA. Based on the nucleotide sequence of the *Apy* DNA polymerase gene, two primers were synthesized: the 5' (*N*-terminal) primer, ApyPolNN, 5'-NNNNCATATGACCTTTG-AGTATATTACGG-3', and a unique *Nde*I site (underlined) added, which has the translation initiation codon, ATG; and the 3' (*C*-terminal) primer, ApyPolCS, 5'-NNNNGTC-GACTCAGTCCTTAATCCACCTCTC-3', which matches the *C*-terminal sequence including the stop codon, a unique *Sal*I site (underlined) added. The amplified 1.74 kb fragment containing the *Apy* DNA polymerase gene was digested with *Nde*I and *Sal*I, purified from 0.8% low-melting agarose gel, and ligated into the expression vector pET-22b(+) that had been digested with the same enzymes. *E. coli* BL21(DE3) was transformed with the ligate by electroporation. Clones with the correct construct were selected by restriction enzyme analysis of plasmid minipreps.

Purification of the Expressed *Apy* DNA Polymerase in *E. coli*

A 20 ml of overnight culture of *E. coli* BL21(DE3) harboring the expression plasmid grown in LB broth containing 100 μg/ml ampicillin was transferred to 2 l of the same medium [14, 22]. This culture was grown at 37°C to an A_{600} of 0.6, then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and allowed to grow for another 5 h. Cells were harvested by centrifugation at 6,000 rpm, 4°C, for 20 min. The cell pellet was resuspended in 10 mM Tris-HCl (pH 8.0) buffer containing 1 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF), and then disrupted by sonication. The disrupted cells were centrifuged at 20,000 rpm, 4°C for 20 min to remove *E. coli* cell walls and insoluble debris. The enzyme was purified as shown in Table 1.

Protein concentration was determined by the method of Lowry *et al.* [24] with bovine serum albumin (BSA) as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, as previously described by Laemmli [20], with 8% polyacrylamide gel.

DNA Polymerase Activity Assay

Apy DNA polymerase activity was measured as described by Choi *et al.* [10] with slight modifications. The basic reaction mixture (50 μl) contained 25 mM Mops-NaOH (pH 7.5), 40 mM KCl, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol,

Table 1. Purification summary of *Apy* DNA polymerase expressed in *E. coli*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Sonicated extract	1,176.4	264,877.4	225.2	100
Heat treatment	75.5	127,332.5	1,686.5	48.1
Cibacron blue 3GA	19.4	44,829.8	2,310.8	16.9
UNO™ Q	16.4	39,983.9	2,438	15.1

Purification was started with 16.4 g wet weight of cells. DNaseI was added to the sonicated extract at a final concentration of 0.5 μg/ml, and the mixture was incubated at 37°C for 30 min. The majority of the heat-labile *E. coli* proteins were precipitated by heat treatment at 80°C for 30 min. After removal of the denatured proteins by centrifugation at 20,000 rpm, 4°C for 20 min, the supernatant was dialyzed against buffer A [10 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$ and 50 mM KCl], then applied onto a Cibacron blue 3GA column preequilibrated with buffer A. Protein was eluted with a linear gradient of 50- 1,000 mM KCl prepared in buffer A. Major fractions showing DNA polymerase activity were pooled and dialyzed against buffer A. *Apy* DNA polymerase was further purified by a UNO™ Q column (Bio-Rad Laboratories Inc., U.S.A.). The column was developed with a linear gradient of 50- 1,000 mM KCl in buffer A. The purified *Apy* DNA polymerase was desalted by dialysis against buffer A and stored at 4°C.

100 μM each of dATP, dCTP and dGTP, 10 μM dTTP, 0.5 μCi of [*methyl*-^{3}H]thymidine 5'-triphosphate, 1.25 μg of activated calf thymus DNA, and enzyme solution. The mixture was incubated at 75°C for 10 min. The reaction was stopped on ice and an aliquot was spotted onto a DE81 filter paper disc (23 mm). The disc was dried on a heat block, and washed in 0.5 M sodium phosphate (pH 7.0) buffer for 10 min and in 70% ethanol for 5 min, then dried. Incorporated radioactivity of the dried filter paper disc was counted using a Beckman LS6500 scintillation counter. One unit of *Apy* DNA polymerase is defined as the amount of polymerase that incorporates 10 pmole of [^{3}H]TTP into an acid-insoluble product at 75°C in 10 min.

Exonuclease Activity Assay

To prepare the 3' end-labeled DNA substrate, pBluescript SK- DNA linearized by *Not*I was filled by Klenow fragment in the presence of [α-^{32}P]dCTP. To prepare the 5' end-labeled DNA substrate, pBluescript SK- DNA linearized by *Sma*I was phosphorylated by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. After the labeling, each DNA substrate was purified by gel filtration on a Sephadex G-25 column, followed by ethanol precipitation. For exonuclease activity assay, end-labeled DNA substrate was placed in 20 mM Mops-NaOH (pH 7.5), 20 mM KCl, 3 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 μg of BSA, and enzyme solution. The reaction mixture (50 μl) was incubated at 70°C in the presence or absence of dNTPs. The reaction was stopped on ice and precipitated by adding 1 ml of 5% trichloroacetic acid in the presence of BSA as a carrier. After centrifugation, the supernatant was withdrawn and its radioactivity was counted [10].

RESULTS AND DISCUSSION

Cloning, Sequencing, and Sequence Analysis of the *Apy* DNA Polymerase Gene

To clone the *Apy* DNA polymerase gene, the structural gene coding for *Aae* DNA polymerase was used as a hybridization probe. Agarose gel membrane hybridization of the restriction enzyme digests of *A. pyrophilus* genomic DNA revealed an approximately 2.15 kb *Hin*dIII fragment and an approximately 1.8 kb *Sac*I fragment which hybridized with the ^{32}P-labeled probe, respectively (data not shown). The regions containing the two fragments were excised from a gel and ligated into pBluescript SK-, respectively, and then *E. coli* MV1184 was transformed with the ligates. After colony hybridization, both ends of the inserts in the plasmids extracted from each positive clone were sequenced. Sequence comparison with already known DNA polymerase sequences demonstrated that the 2.15 kb *Hin*dIII fragment and the overlapping 1.8 kb *Sac*I fragment encode the complete gene of a new DNA-dependent DNA polymerase with a putative 3'→5' exonuclease activity.

Figure 1A shows a restriction enzyme map of the *Apy* DNA polymerase gene constructed from the two plasmids, pADPH containing the 2.15 kb *Hin*dIII fragment and pADPSI containing the 1.8 kb *Sac*I fragment. DNA fragments containing the gene and flanking regions were subcloned using each enzyme site of the restriction enzyme map and sequenced in both strands. The nucleotide sequence and deduced amino acid sequence of the *Apy* DNA polymerase gene are shown in Fig. 1B. The nucleotide sequence of the *Apy* DNA polymerase gene is also available from GenBank, Accession Number AF283724. The *Apy* DNA polymerase gene consists of 1,725 bp coding for a protein with 574 amino acid residues. The molecular mass of the protein derived from this amino acid sequence was 65,955 Da.

Consensus bacterial promoter sequences were found on the upstream flanking region of the *Apy* DNA polymerase gene. The TAAACT and TTGACA sequences at 16 and 56 bases upstream from the open reading frame (ORF) were homologous to the -10 (TATAAT) and -35 (TTGACA) promoter sequences (Fig. 1B). However, a potential ribosome-binding sequence was not found on the downstream of the putative promoter sites. Although a ribosome-binding sequence was not found in the cloned gene, the first methionine codon of the ORF was proposed as the

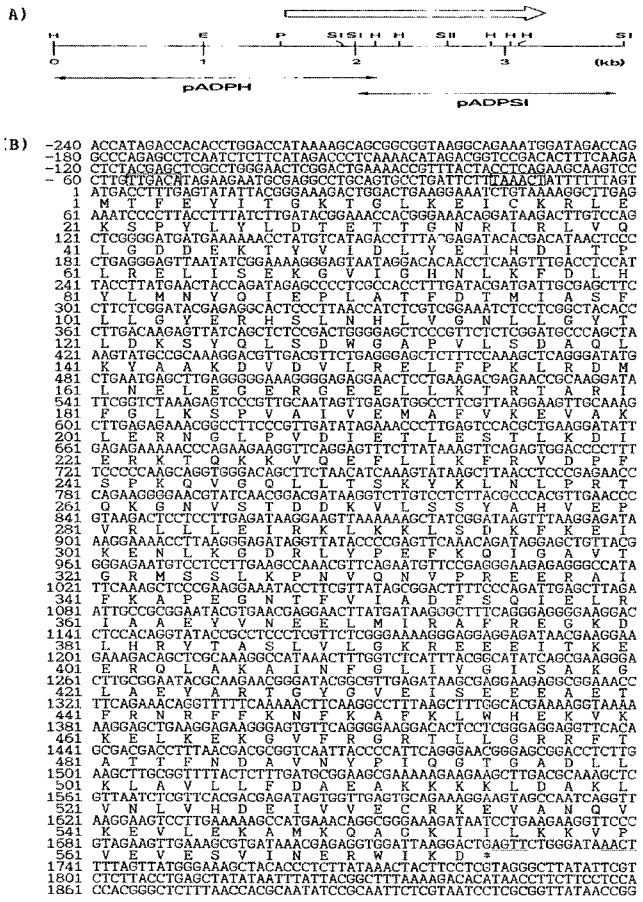

Fig. 1. Restriction enzyme map, and nucleotide and deduced amino acid sequences of the *Apy* DNA polymerase gene.
(A) Restriction enzyme map and positions of the cloned DNA fragments in plasmids pADPH and pADPSI. The restriction enzyme sites used for the subcloning and DNA sequencing of cloned DNA fragments are shown: E, *Eco*RI; H, *Hind*III; P, *Pst*I; SI, *Sac*I; SII, *Sac*II. The position of the *Apy* DNA polymerase gene in the cloned fragments is indicated by the open arrow. **(B)** Nucleotide and deduced amino acid sequences of the *Apy* DNA polymerase gene. Numbers in the margin refer to nucleotides (upper) and amino acid residues (lower). Asterisk indicates the stop codon. Potential promoter sequences are boxed, and the potential transcription termination palindrome is underlined.

initiation site of translation, because it was located downstream of the consensus promoter sequences. The sequence of the *Apy* DNA polymerase gene exhibited a short palindromic transcription terminator followed by runs of uridine residues (Fig. 1B). The low enthalpy of the predicted stem-loop structure suggests that a ρ-like factor is required for transcription termination.

The codon usage of the *Apy* DNA polymerase gene was similar to that of the *Aae* DNA polymerase gene [12] (data not shown). Only two codons, AGA and AGG, among six arginine codons were used in the DNA polymerase genes from *Aquifex*, whereas the two codons were not used at all in the *E. coli* DNA polymerase I gene [17]. The dominant use of AGA and AGG codons for arginine is consistent with the codon bias in random sequence analysis of genomic DNAs from hyperthermophilic bacteria [9]. The G+C content of the *Apy* DNA polymerase gene was 47.6%, slightly higher than approximately 40% G+C content in genomic DNA. The G+C content in the third position of codons was higher, at 59.0%, compared with 52.7% and 31.1% in the first and second positions of codons, respectively. In the case of amino acids using only two codons, the frequency of G or C in the third position of codons was even higher, at 66.8%, similar to the DNA polymerase genes from *Thermus* [18, 21].

Similarity Analysis of the Amino Acid Sequence of *Apy* DNA Polymerase

The deduced primary structure of *Apy* DNA polymerase was aligned and compared with those of *E. coli* DNA

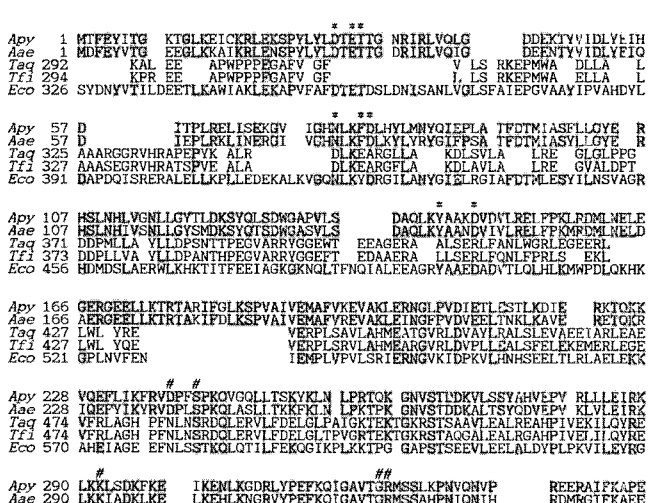

Fig. 2. Comparison of the amino acid sequence of *Apy* DNA polymerase with those of other *E. coli* DNA polymerase I-like DNA polymerases.
The sequence of *Apy* DNA polymerase (*Apy*) is shown as compared with those of *Aae* DNA polymerase (*Aae*) [12], *Taq* DNA polymerase (*Taq*) [21], *Tfi* DNA polymerase (*Tfi*) [18], and *E. coli* DNA polymerase I (*Eco*) [17]. Identical amino acids between *Apy* DNA polymerase and others are indicated by stippled boxes. Asterisks indicate the three highly conserved motifs, ExoI, ExoII and ExoIII, and sharps indicate the six highly conserved regions, 1, 2a, 2b, 3, 4, and 5.

polymerase I-like DNA polymerases, showing a high homology (Fig. 2). It was deduced by amino acid sequence alignment that *Apy* DNA polymerase, like the Klenow fragment, has only the two domains containing the characteristic motifs described in Ref. [2]: one is the 3'→5' exonuclease domain consisting of ExoI, II, and III motifs, which is responsible for proofreading activity, and the other is the 5'→3' polymerase domain consisting of six conserved regions, 1, 2a, 2b, 3, 4, and 5. The presence of highly conserved motifs and critical residues in *Apy* DNA polymerase implies that the enzyme has 3'→5' exonuclease activity and 5'→3' polymerase activity. The deduced amino acid sequence of *Apy* DNA polymerase was closely related to that of *Aae* DNA polymerase [12], sharing 78.0% of similarity. *Apy* DNA polymerase also showed 24.6% homology to *T. aquaticus* YT-1 (*Taq*) DNA polymerase [21], 24.2% to *T. filiformis* (*Tfi*) DNA polymerase [18], and 32.4% to *E. coli* DNA polymerase I [17].

Expression and Purification of *Apy* DNA Polymerase

The pET system is one of the most powerful systems developed for cloning and expression of recombinant proteins in *E. coli* [34]. The pET-22b(+) vector has a very strong and stringent T7*lac* promoter. The *Apy* DNA polymerase gene was amplified and inserted into the *Nde*I and *Sal*I sites of pET-22b(+). The resulting expression plasmid was named pEAPP (data not shown).

The culture of *E. coli* BL21(DE3) harboring pEAPP was carried out in a 2 l fermentor. *Apy* DNA polymerase was expressed as a soluble form in the cytosol. At first, the harvested cells (16.4 g) were sonicated and treated with DNaseI. We then exploited the thermophilic property of *Apy* DNA polymerase, and eliminated most *E. coli* proteins by heating at 80°C for 30 min and centrifuging to remove denatured proteins. Several *E. coli* proteins still remained soluble after the above heating step. The soluble supernatant from the heating step was then chromatographed on Cibacron blue 3GA and UNO™ Q columns. The purification of the enzyme is summarized in Table 1. The specific activity of the purified enzyme was more than 10.8-fold that of the sonicated extract, and recovery was approximately 15.1% on the basis of the total activity of the sonicated extract. The purification of the enzyme was monitored by SDS-PAGE (Fig. 3). SDS-PAGE revealed a single protein band with a molecular mass of 63,500 Da, which agrees with the sum of molecular mass, 65,955 Da, of *Apy* DNA polymerase calculated from the deduced amino acid sequence.

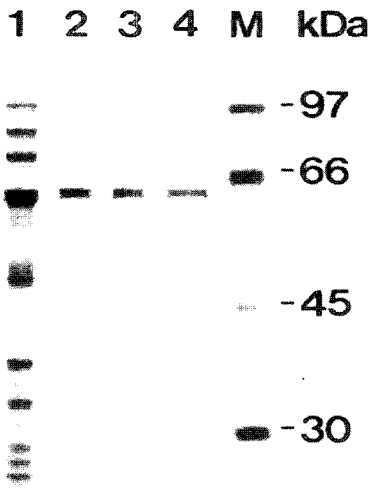

Fig. 3. SDS-PAGE analysis of *Apy* DNA polymerase.
Electrophoresis was performed on a vertical gel of 8% polyacrylamide. Lane 1, sonicated extract; lane 2, heat treatment; lane 3, Cibacron blue 3GA column chromatography; lane 4, UNO™ Q column chromatography; lane M, low-molecular-mass markers (molecular masses are indicated at the right).

Properties of *Apy* DNA Polymerase

The dependence of *Apy* DNA polymerase activity on pH was determined in the pH range of 6.0–10.0. The buffers used were 50 mM Mops-NaOH (pH 6.0–8.0), 50 mM Tris-HCl (pH 7.0–9.5), and 50 mM Glycine-NaOH (pH 9.0–10.0). The activity of the enzyme was highest at pH 7.5 in Mops-NaOH (Fig. 4A). The pH profile of *Apy* DNA polymerase was slightly shifted to alkaline pH, compared to that of *Aae* DNA polymerase [6]; however, at alkaline conditions above pH 8.5, *Apy* DNA polymerase activity decreased drastically. In contrast, other commercially available thermostable DNA polymerases, such as *Taq* DNA polymerase, *T. litoralis* (*Tli* or Vent) DNA polymerase, and *P. furiosus* (*Pfu*) DNA polymerase, have an optimal activity at alkaline pH values, generally in the range of 8.5–9.0. The optimal concentration of the buffer, Mops-NaOH at pH 7.5, was 5–30 mM (data not shown).

The effect of temperature on the *Apy* DNA polymerase activity was determined in the range of 40–90°C. Polymerization by *Apy* DNA polymerase occurred over a broad range of temperatures, reaching a maximum at 75°C on an activated calf thymus DNA template (Fig. 4B). This optimum appears to reflect denaturation of the template at higher temperatures, because the enzyme was stable even at temperatures above 80°C. The stability of *Apy* DNA polymerase was examined by measuring the decrease in activity after incubation at three different temperatures: 75°C, 85°C, and 95°C. The enzyme was fairly stable at 75°C and comparatively stable at 85°C; however, the thermostability of the enzyme decreased drastically at temperatures above 90°C (data not shown). The half-life in the presence of 0.02% BSA at 85°C was 6 h. Although the thermostability of *Apy* DNA polymerase was better than that of *Aae* DNA polymerase [6], the results on the thermostability of the enzyme were worse than we expected. This somewhat lower thermostability, compared to the

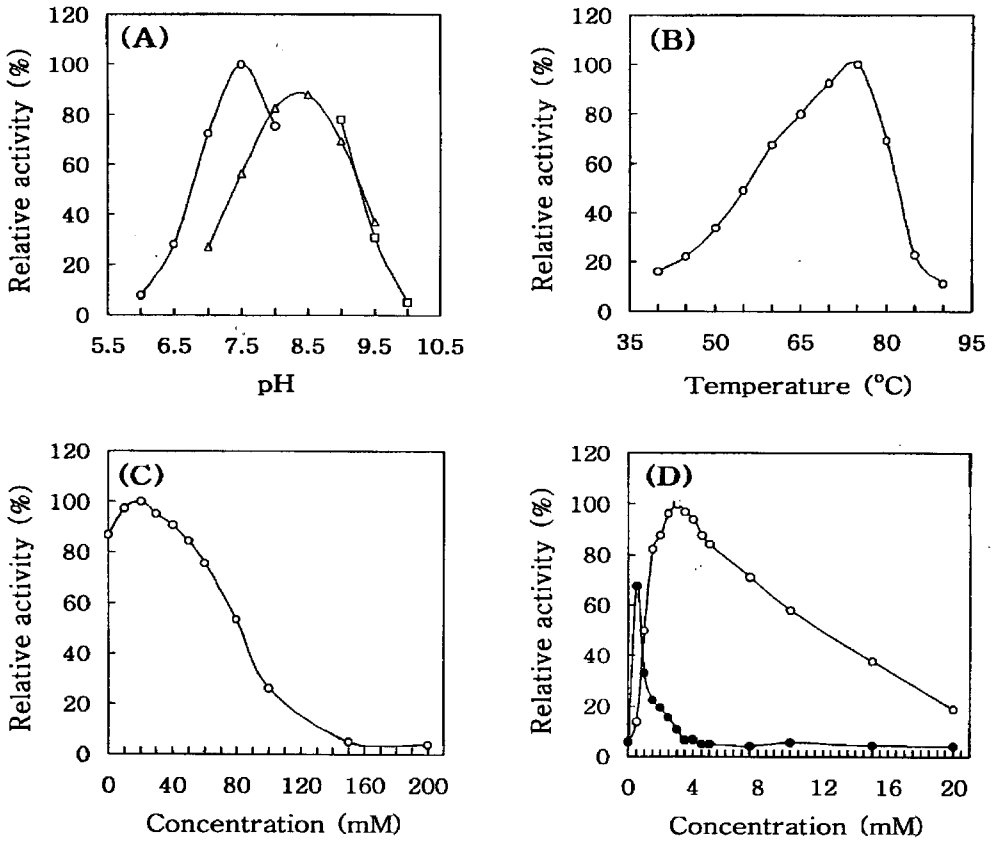

Fig. 4. Properties of *Apy* DNA polymerase.
(A) Effect of pH on the *Apy* DNA polymerase activity: Mops-NaOH (○), Tris-HCl (△), and Glycine-NaOH (□). **(B)** Effect of temperature on the *Apy* DNA polymerase activity. **(C)** Effect of KCl on the *Apy* DNA polymerase activity. **(D)** Effects of divalent cations, Mg^{2+} (○) and Mn^{2+} (●), on the *Apy* DNA polymerase activity.

growth temperature of *A. pyrophilus*, will be discussed at the end of this paper.

The results on the influence of KCl concentration on the *Apy* DNA polymerase activity are shown in Fig. 4C. Its optimal concentration was 20 mM, declining sharply at 60 mM or higher. Figure 4D shows the effects of different concentrations of Mg^{2+} and Mn^{2+} on the activity of *Apy* DNA polymerase. When each of the divalent salts, $MgCl_2$ and $MgSO_4$, was added to the reaction mixture, the activity of the enzyme gradually increased up to 3 mM in proportion to the concentration of salt. The optimal Mg^{2+} concentration was 3 mM and the optimal Mn^{2+} concentration was 0.5 mM. Divalent cations are necessary for the polymerization reaction. The activation of *Apy* DNA polymerase by Mg^{2+} ion is in agreement with the effects of this cation on other DNA polymerases. Most DNA-binding enzymes, including DNA-dependent DNA polymerases, prefer the presence of Mg^{2+} ions.

The effects of DTT (0-20 mM), EDTA (0-3 mM), $(NH_4)_2SO_4$ (0-100 mM), BSA (0-0.1%), and Triton X-100 (0-1%) on the activity of *Apy* DNA polymerase were examined by assaying enzyme samples in the presence of these reagents at various concentrations (data not shown). DTT had no significant influence on the enzyme activity. The enzyme was completely inhibited by 2 mM EDTA and 30 mM $(NH_4)_2SO_4$. *Apy* DNA polymerase was activated by 0.03% BSA and 0.2% Triton X-100, with 50% and 40% increases, respectively.

Exonuclease Activities of *Apy* DNA Polymerase

Incubation of DNA polymerase with linear DNA fragments in the absence of dNTPs led to degradation of the fragments, indicating the existence of an exonuclease activity in the preparation. This activity was checked by assaying the ^{32}P-labeled product released from an end-labeled DNA substrate: when the substrate was labeled on its 3' end, about 85% of the ^{32}P was released in 30 min; when the substrate was labeled on its 5' end, no ^{32}P was released (Fig. 5). These results demonstrate that *Apy* DNA polymerase possesses a high 3'→5' exonuclease activity, but no 5'→3' exonuclease activity, which is consistent with its deduced amino acid sequence. Organisms living at very high temperatures may be under unusually strong evolutionary pressure to maintain those genes that specify phenotypic characteristics critical

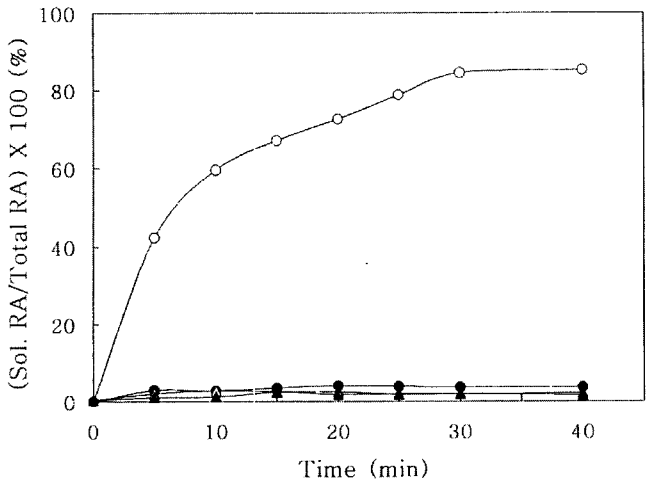

Fig. 5. Exonuclease activities of *Apy* DNA polymerase.
The 3'→5' exonuclease activity was assayed in the absence (○) or in the presence (●) of dNTPs. The 5'→3' exonuclease activity was assayed in the absence (△) or in the presence (▲) of dNTPs.

to life there: that is, beyond a certain point, survival of hyperthermophiles may not be furthered by additional genetic change. The existence of a DNA polymerase with a high 3'→5' proofreading exonuclease activity is, therefore, necessary and crucial for the survival of hyperthermophiles, although such a DNA polymerase is not helpful to rapid amplification of DNA.

Thermostability of *Apy* DNA Polymerase

We tried to establish the PCR conditions for *Apy* DNA polymerase, based on the results of enzyme assays, however, an amplified product was not obtained under various conditions. Similar results were also shown in *Aae* DNA polymerase (unpublished data). One should note the somewhat lower thermostability of DNA polymerases from *Aquifex*, compared to its growth temperature, as well as the higher exonuclease activity of these DNA polymerases. Although it is not clear whether the lower thermostability of the DNA polymerases reflects the nature of the enzymes *in vivo* or whether it reflects the difficulties of reproducing biological conditions *in vitro*, there are a few differences, compared with the higher thermostable proteins.

The thermostable property of proteins extracted from thermophiles would appear to be intrinsic to the molecule itself, because the proteins retain their thermal resistance after purification. Despite the absence of a general rule to achieve thermostability, a great many thermostable proteins are stabilized by an increase in hydrogen bonds, ionic bonds, and salt bridges, and better hydrophobic internal packing [31, 36]; it means that each thermostable protein adopts various strategies, and the adaptation to higher temperature is realized through the same electrostatic and hydrophobic interactions among the ordinary amino acid residues as those in proteins from mesophiles. It is, therefore, generally accepted that protein thermostability is basically governed by the amino acid sequence. The availability of complete genome sequences has allowed the analysis of a large enough data set to evaluate the influence of amino acid composition on the features of encoded proteins. The comparative analysis of amino acid composition encoded by six genomes, including two hyperthermophile genomes, revealed some important differences between the proteins from hyperthermophiles and those from mesophiles [12]. The proteins from hyperthermophiles have higher levels of charged residues (Arg, Asp, Glu, His, and Lys) on average than those from mesophiles, primarily at the expense of polar uncharged residues (Asn, Cys, Gln, Gly, Ser, Thr, and Tyr). Hydrophobic residues (Ala, Ile, Leu, Met, Phe, Pro, Trp, and Val) are distributed in similar ratios in both. These patterns have been confirmed on random sequence analysis of genomic DNA and individual proteins from hyperthermophiles [8, 9, 36]. However, in the present study, the higher levels of charged residues in *Apy* DNA polymerase, as well as *Aae* DNA polymerase, compared with *E. coli* DNA polymerase I were mainly compensated by the difference in the content of hydrophobic residues (Fig. 6). These results indicate that increased number of electrostatic interactions, such as hydrogen bond and ion pair, formed by charged amino acids may contribute to the comparative thermostability of *Apy* DNA polymerase, whereas the lower hydrophobic amino acid content may disturb the

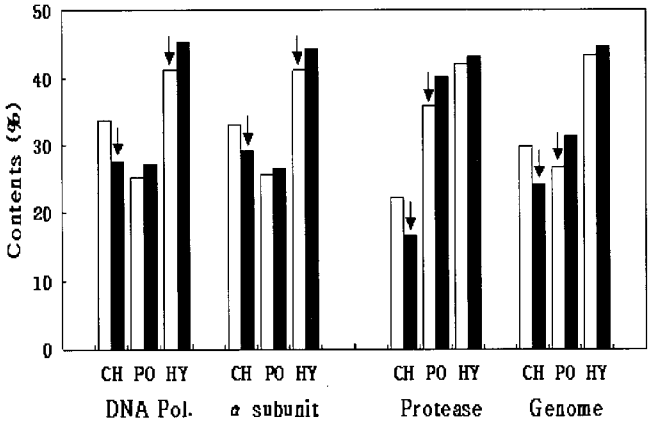

Fig. 6. Comparisons of the sum of percentages of various amino acid classes in individual proteins or genomes between hyperthermophile (open columns) and mesophile (closed columns).
DNA Pol., *Apy* DNA polymerase (open columns) and *E. coli* DNA polymerase I (closed columns); α subunit, *A. aeolicus* DNA polymerase III α subunit (open columns) and *E. coli* DNA polymerase III α subunit (closed columns); Protease, *A. pyrophilus* protease (open columns) and *Bacillus amyloliquefaciens* protease (closed columns); Genome, two hyperthermophile genomes (open columns) and four mesophile genomes (closed columns). CH, charged amino acids; PO, polar uncharged amino acids; HY, hydrophobic amino acids. The comparison of the amino acid contents between genomes from hyperthermophiles and mesophiles is from Deckert *et al.* [12].

formation of a stable internal structure of the enzyme, thus preventing further increase of thermostability. To confirm this possibility, we analyzed the amino acid compositions of *A. aeolicus* DNA polymerase III α-subunit [3], which has somewhat lower thermostability like DNA polymerases from *Aquifex*, and *A. pyrophilus* protease [8], which is one of the most thermostable proteases, together with the mesophilic counterparts. As expected, the patterns of amino acid contents of the *A. aeolicus* DNA polymerase IIIα subunit and *A. pyrophilus* protease were similar to those of DNA polymerases from *Aquifex* and highly thermostable proteins, respectively (Fig. 6).

Generally, elevated pressures lead not only to an upward shift of the maximal growth temperature mainly in hyperthermophiles [5], but also to an increase of thermostability in many thermophilic enzymes [23, 28]. Enhanced thermostability of DNA polymerase by pressure at stressful temperature has been demonstrated with the enzymes from three thermophilic organisms, *T. aquaticus*, *P. furiosus*, and *P.* strain ES4, regardless of their native pressure [35]. These reports suggest the importance of pressure for the extension of growth condition and the thermostability of proteins in hyperthermophiles, and also give a clue to explain the somewhat lower thermostability of *Apy* DNA polymerase. It is needed for the culture of *Aquifex* cells to maintain the pressure at 300 kPa; this fact suggests that the thermostability of DNA polymerases from *Aquifex* may be comparatively low under *in vitro* atmospheric condition. The most thermolabile property of DNA polymerase among the proteins from an organism was also shown in *Pyrococcus* [1]. The DNA polymerase could, therefore, be practically used as an ecologically relevant indicator of physical stabilization effects because of its relatively low stability and importance in cell reproduction.

In conclusion, we have cloned, expressed, and characterized a DNA polymerase from the hyperthermophilic bacterium *A. pyrophilus*. *Apy* DNA polymerase has some differences in properties, especially higher activity at neutral pHs, compared with commercially available DNA polymerases from other thermophilic bacteria, therefore, it could conceivably have a good utility in the DNA polymerase-catalyzing reactions that are proceeded well at neutral pHs or that use certain DNA, as a substrate, stable at neutral pHs. Although *Apy* DNA polymerase is a somewhat lower thermostable enzyme and less capable of withstanding temperatures used in cycled PCR, the enzyme can withstand temperatures up to 85°C and thus may also function well in high temperature isothermal amplification methods. The analysis on the lower thermostability of *Apy* DNA polymerase would facilitate the study on the determinants of thermostability that have became an important task in basic and applied research; the study on the lower thermostability of replication enzymes from the genus *Aquifex* would also help in the understanding of the *Aquifex* replication system and the explanation of the limitation at growth temperature of *Aquifex*, which have many highly thermostable enzymes, even though above the growth temperature.

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