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Characterization of the Family I inorganic pyrophosphatase from *Pyrococcus horikoshii* OT3

SUNG-JONG JEON,[1,2] and KAZUHIKO ISHIKAWA[3,4]

[1] *Department of Biotechnology and Bioengineering, Dong-Eui University, Busan, 614-714, Korea*

[2] *Department of Biomaterial Control, Dong-Eui University, Busan, 614-714, Korea*

[3] *Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST Kansai), 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan*

[4] *Corresponding author (kazu-ishikawa@aist.go.jp)*

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Summary A gene encoding for a putative Family inorganic pyrophosphatase (PPase, EC 3.6.1.1) from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 was cloned and the biochemical characteristics of the resulting recombinant protein were examined. The gene (Accession No. 1907) from *P. horikoshii* showed some identity with other Family I inorganic pyrophosphatases from archaea. The recombinant PPase from *P. horikoshii* (*Ph*PPase) has a molecular mass of 24.5 kDa, determined by SDS-PAGE. This enzyme specifically catalyzed the hydrolysis of pyrophosphate and was sensitive to NaF. The optimum temperature and pH for PPase activity were 70 °C and 7.5, respectively. The half-life of heat inactivation was about 50 min at 105 °C. The heat stability of *Ph*PPase was enhanced in the presence of Mg^{2+}. A divalent cation was absolutely required for enzyme activity, Mg^{2+} being most effective; Zn^{2+}, Co^{2+} and Mn^{2+} efficiently supported hydrolytic activity in a narrow range of concentrations (0.05–0.5 mM). The K_m for pyrophosphate and Mg^{2+} were 113 and 303 μM, respectively; and maximum velocity, V_{max}, was estimated at 930 U mg^{-1}.

Keywords: archaea, hyperthermophile.

Introduction

Inorganic pyrophosphatases (PPases, EC 3.6.1.1) are widely distributed in all living cells and catalyze the hydrolysis of pyrophosphate (PP_i) into two orthophosphates (P_i) (Chen et al. 1990, Lundin et al. 1991). In many organisms, the bulk of PPase activity is soluble, localized in the cytosol and serves mainly to remove the PP_i formed in biosynthetic reactions driven by nucleotide triphosphates (Kornberg 1962). Pyrophosphatases from the Bacteria, Archaea and Eukarya have been characterized. Bacterial and archaeal PPases exist as either homotetramers or homohexamers, with a subunit molecular mass of about 20 kDa. In contrast, eukaryotic PPases form mostly homodimers, with a subunit molecular mass of about 28–35 kDa. To date, the soluble PPases have been classified into Family I and Family II PPases (Shintani et al. 1998, Young et al. 1998). Family I PPases have a highly conserved active site structure formed by 14–16 amino acid residues and three to four Mg^{2+} ions and have similar catalytic properties (Harutyunyan et al. 1996, Heikinheimo et al. 1996, Baykov et al. 1999). Family II PPases are activated by Mn^{2+} and display 10–20 times greater activity than Family I PPases (Shintani et al. 1998, Parfenyev et al. 2001).

Enzymes derived from microorganisms growing at extreme temperatures are of biotechnological use as highly thermostable biocatalysts. Thermostable PPases are commonly used to suppress pyrophosphorolysis in DNA sequencing reactions with DNA polymerases (Tabor and Richardson 1990, Vander et al. 1997). Some extremely thermostable PPases have been isolated and characterized from archaea (*Sulfolobus* (Wakagi et al. 1992), *Methanobacterium* (van Alebeek et al. 1994), *Methanococcus* (Kuhn et al. 2000), *Thermus* (Satoh et al. 1998) and *Thermoplasma* (Richter and Schafer 1992)). However, no information is available on the biochemical properties of thermostable PPase from hyperthermophilic archaeon *Pyrococcus* spp.

In the genome database of the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3, we found the homologous PPase gene. The putative *Ph*PPase gene has been classified as a member of the Family I PPases (Young et al. 1998, Shintani et al. 1998), and the structure of *P. horikoshii* PPase has already been reported (Liu et al. 2004). In this study, we report on the cloning of *P. horikoshii* PPase and compare the properties of the recombinant *Ph*PPase with those of other microbial PPases.

Materials and methods

Materials

The DNA primers and substrates were prepared by FASMA (Kanagawa, Japan). The plasmid pET-21a was purchased from Novagen (Madison, WI, USA). The KOD DNA polymerase

was purchased from Toyobo (Osaka, Japan). We obtained ATP, ADP and AMP from Sigma (St. Louis, MO). Sodium tripolyphosphate (PPP_i), sodium pyrophosphate (PP_i), sodium phosphate (P_i), phosphoenolpyruvate (PEP) and *p*-nitrophenyl phosphate (pNP) were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

Enzyme construction and expression

Chromosomal DNA of *P. horikoshii* OT3 was prepared as reported previously (Gonzalez et al. 1998). The gene (*PH1907*) was amplified by PCR using the chromosomal DNA as the template and two primers, PP1 (forward): 5′-AGGGGGTA CATATGAACCCGTTCCACGACCT-3′; and PP2 (reverse): 5′-AATAAAGTCGACTCACTCCTTCTTGCCGA-3′, which was designed based on an open reading frame (ORF) sequence for a protein of 178 amino acids. Amplification by PCR was carried out with KOD DNA polymerase at 94 °C for 30 s, 55 °C for 2 s, 74 °C for 30 s for 30 cycles. An amplified 0.53-kbp DNA fragment was digested with restriction enzymes *Nde*I and *Sal*I (the *Nde*I and *Sal*I sites are underlined in primers PP1 and PP2, respectively) and inserted into the pET-21a vector. The nucleotide sequences of the inserted genes were confirmed with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Wellesley, MA). *Escherichia coli* BL21(DE3) cells containing the expression plasmid were grown in 2YT medium (1% yeast extract, 1.6% tryptone, 0.5% NaCl) containing ampicillin (100 μg ml^{-1}) to an OD_{600} of approximately 0.6. Gene expression was then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h at 37 °C.

Purification of the recombinant proteins

Escherichia coli cells containing the expressed recombinant enzyme were centrifuged and frozen at −70 °C. The thawed cells were then disrupted by sonication in buffer A (50 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 0.1 mM EDTA). The suspension of disrupted cells was centrifuged at 27,000 *g* for 30 min and the supernatant fraction was heat-treated at 85 °C for 30 min, followed by recentrifugation. The supernatant was loaded on a HiTrap Q column (Amersham Biosciences, Piscataway, NJ) equilibrated in buffer A, and the bound protein was eluted with a linear gradient of NaCl (0 to 1.0 M in the same buffer). The protein solution was concentrated using a centricon 10 filter from Amicon (Millipore, Bedford, MA) and dialyzed against buffer B (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 15 mM $MgCl_2$). The dialyzed solution was loaded on a HiPrep Sephacryl S-200 HR 26/60 column (Amersham Biosciences) and eluted with buffer B. The purity of the recombinant protein was assessed by SDS-PAGE. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Enzyme assay

In the standard assay, PPase activity was determined by the formation of inorganic phosphate at 60 °C in the reaction mixture containing 50 mM Tris-HCl (pH 7.5 at 60 °C), 5 mM $MgCl_2$ and 1 mM sodium phosphate. The reaction was started by the addition of enzyme, and 100-μl samples were taken at 30-s time intervals and put on ice. The phosphate present was determined by adding 1 ml of phosphate reagent (1% ammonium heptamolybdate (w/v), 0.83 M sulfuric acid, 8% iron (II) sulfate (w/v)) and the absorbance of the phosphomolybdate complex was measured at 660 nm after 30 min (van Alebeek et al. 1994). Activities were developed with 0.1 μmol of standard sodium phosphate. The activity towards other substrates such as sodium tripolyphosphate (PPP_i), phosphoenolpyruvate (PEP), *p*-nitrophenyl phosphate (pNP), ATP, ADP and AMP was determined by the same method as above except that PP_i was replaced by each substrate. One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μmol PP_i per min at 60 °C.

Results and discussion

Identification of inorganic pyrophosphatase gene

In the *Pyrococcus horikoshii* OT3 genome database (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ot3_ G1) (Kawarabayasi et al. 1998), we identified an ORF (Accession No. PH1907) encoding a putative inorganic pyrophosphatase (*Ph*PPase). The putative *Ph*PPase gene encodes a protein of 178 amino acids, with a predicted molecular mass of 20,833 Da and an isoelectric point of 4.81. From the data of sequence homology (Shintani et al. 1998, Young et al. 1998), *Ph*PPase is classified as a member of Family I PPase. The deduced amino acid sequence showed a high identity with other archaeal PPases from *Pyrococcus furiosus* (94%) (Robb et al. 2001), *Methanopyrus kandleri* (69%) (Slesarev et al. 2002), *Methanobacterium thermoautotrophicum* (61%) (Smith et al. 1997) and *Thermoplasma acidophilum* (54%) (Richter and Schafer 1992). Structural and multiple sequence aligments reveal a group of 14 conserved, mostly polar, amino acid residues (Kankare 1994). The 14 amino acids have been found to be identical in all other *Ph*PPase amino acid sequences.

The ORF (PH1907) was amplified by PCR from a *P. horikoshii* genomic DNA, cloned and sequenced to confirm the sequences in the database. The gene was expressed in *E. coli* cells, and the recombinant protein was purified as described in the Materials and methods. Expression and subsequent purification yielded 3.2 mg of *Ph*PPase from a 1-l culture.

Molecular properties

The molecular mass of the protein from PH1907 was estimated to be about 24.5 kDa by SDS-PAGE (Figure 1). This value is inconsistent with the size (20,833 Da) calculated from the amino acid sequence. This discrepance may be explained by the many positively charged amino acid residues of *Ph*PPase. Recently, a three-dimensional crystal structure of *Ph*PPase showed that the enzyme is an oligomeric hexamer (Liu et al. 2004).

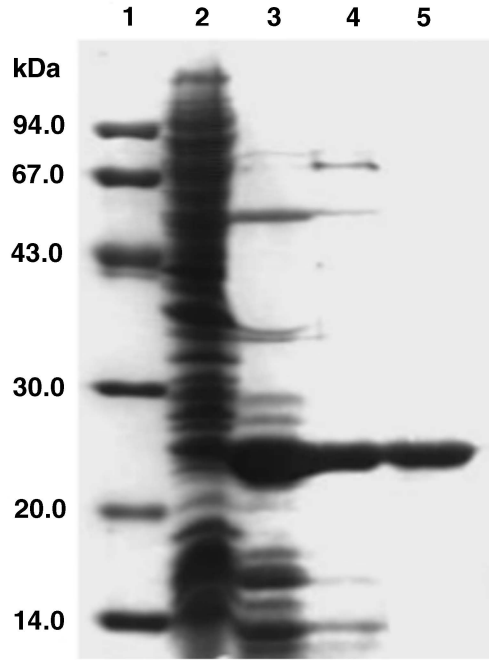

Figure 1. Purification of recombinant *Pyrococcus horikoshii* pyrophosphatase (*Ph*PPase). Lane 1, molecular mass markers; Lane 2, crude extract of induced cells; Lane 3, supernatant of crude extract after heat treatment at 85 °C for 30 min; Lane 4, HiTrap Q column peak fractions; and Lane 5, Sephacryl S-200 HR 26/60 column peak fractions. The gel was stained with Coomassie brilliant blue.

Catalytic properties

We measured the activity of the recombinant *Ph*PPase in the presence of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 1 mM sodium pyrophosphate. The recombinant *Ph*PPase showed maximal activity in the temperature range from 60 to 75 °C, with a temperature optimum of 70 °C (Figure 2a). The optimal pH for the *Ph*PPase was 7.5 at 60 °C. Heat inactivation of the enzyme was estimated by measuring the residual PPase activity after heat treatment at two different temperatures in the presence or absence of 5 mM Mg^{2+} (Figure 2b). The recombinant *Ph*PPase showed high thermostability, and the half-life of heat inactivation was about 50 min at 105 °C. The heat stability of *Ph*PPase was much higher than that reported previously for other thermostable PPases from *Aquifex aeolicus* (90 min at 95 °C) (Hoe et al. 2001) and *Sulfolobus acidocaldarius* (150 min at 95 °C) (Hansen et al. 1999). Furthermore, the heat stability of *Ph*PPase was enhanced by the addition of Mg^{2+} to the incubation mixture, as reported for the other PPases (Hachimori et al. 1979, Ichiba et al. 1988). The difference between the optimum temperature of *Ph*PPase and the optimum growth temperature of *P. horikoshii* suggests that *Ph*PPase requires co-factors for its thermophilicity in cells.

Divalent cation requirement

Family I PPase has shown strong metal cation-dependency, with Mg^{2+} conferring the highest efficiency (Cooperman 1982) and sensitivity to inhibition by Ca^{2+} (Yang and Wensel 1992). In contrast, Family II PPase prefers Mn^{2+} over Mg^{2+} (Parfenyev et al. 2001). *Pyrococcus horikoshii* pyrophosphatase is dependent on the presence of divalent cations for catalytic activity, with the highest activity in the presence of Mg^{2+}. Other cations (Zn^{2+} Co^{2+} Mn^{2+}) could efficiently replace Mg^{2+}, but the effectiveness of the latter cations is limited to a narrow range of concentrations (0.05–0.5 mM) (Figure 3). Neither Ni^{2+} nor Ca^{2+} could activate the enzyme for catalysis. These results support the conclusion that *Ph*PPase belongs to the Family I PPases. The enzyme responded sensitively to changes in the cation concentration of the reaction mixture. In particular, a drastic decrease in enzyme activity was observed at Zn^{2+}, Co^{2+} and Mn^{2+} concentrations above 0.5 mM (Figure 3). The inhibition at high cation concentrations is probably due to the presence of free cations in the assay (Celis and Romero 1987).

Substrate specificity

The substrate specificity of *Ph*PPase was investigated (Table 1) and found to be high with respect to PP_i. The release activities of inorganic phosphate (P_i) from PPP_i, ATP and ADP were determined to be 2.7, 5.9 and 2.9% of that from PP_i, respectively. Hydrolysis of AMP, PEP and pNP by *Ph*PPase was not observed.

The reaction rate of *Ph*PPase at different PP_i and Mg^{2+} concentrations did not follow normal Michaelis-Menten kinetics.

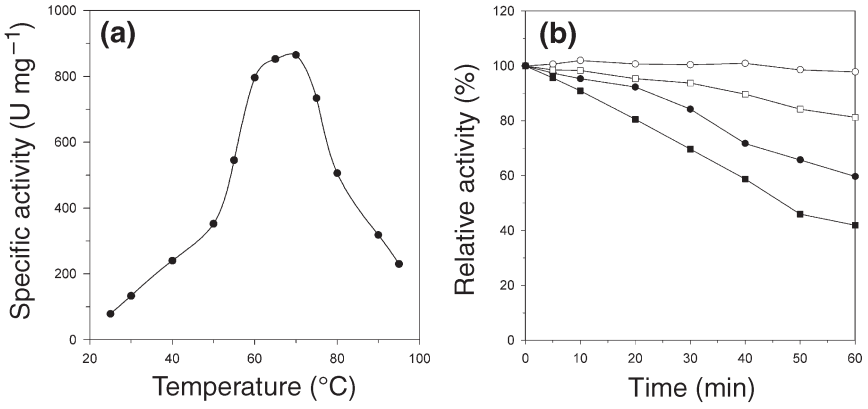

Figure 2. Thermophilicity and thermostability of *Pyrococcus horikoshii* pyrophosphatase (*Ph*PPase). (a) The PPase activity was determined at the indicated temperatures, as described in the Materials and methods. Negative control reactions in the absence of the enzyme were performed in parallel. (b) Enzyme (1 μM *Ph*PPase in 50mM Tris-HCl, pH 7.5) was incubated at 100 °C in the presence (○) or absence (●) of Mg^{2+} and at 105 °C in the presence (□) or absence (■) of Mg^{2+}, and the residual PPase activity of samples was measured at 60 °C.

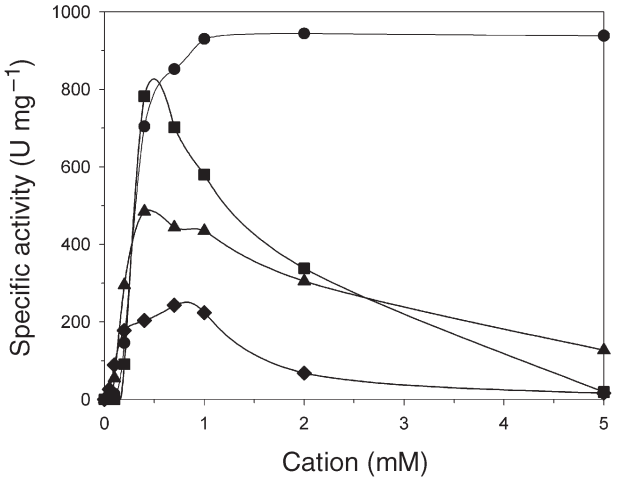

Figure 3. Effect of divalent cation concentration on pyrophosphate (PP_i) hydrolysis. Assay conditions were as follows: 50mM Tris-HCl, pH 7.5, 1 mM sodium pyrophosphate ($NaPP_i$), and concentrations of divalent cations were varied as indicated. Symbols: ● = Mg^{2+}; ■ = Zn^{2+}; ▲ = Co^{2+}; and ◆ = Mn^{2+}.

Table 1. Substrate specificity of the *Pyroccocus horikoshii* pyrophosphatase (*Ph*PPase). Activities were determined as described in Materials and methods. Each substrate was tested at a concentration of 1 mM in the presence of 5 mM Mg^{2+}.

Substrate	Relative activity (%)
PP_i	100
PPP_i	2.7
ATP	5.9
ADP	2.9
AMP	0
PEP	0
pNP	0

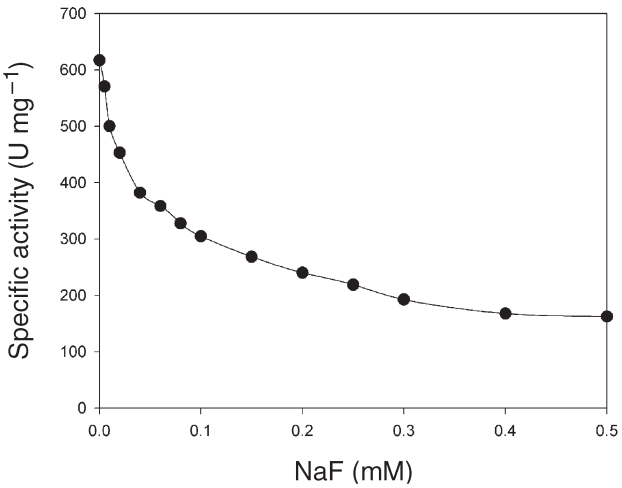

Figure 4. Inhibition of *Ph*PPase activity by NaF. The specific activity was determined at the indicated NaF concentrations, as described in Materials and methods.

Therefore, kinetic data were analyzed according to the procedure of Hill by the EZ-Fit program (Perrella 1988). At pH 7.5 and 60 °C, the values of K_m and maximum velocity (V_{max}) for PP_i were determined to be 113 ± 12 μM and 930 ± 40 U mg^{-1}, respectively, which corresponds to a k_{cat} of 744 s^{-1}. An apparent K_m for Mg^{2+} was determined to be 303 ± 9 μM.

Effect of fluoride

The influence of fluoride, a potent inhibitor of PPases, was examined. A plot of activity for NaF concentration (Figure 4) indicated that 0.5 mM NaF inhibits PPase activity by 75%. The I_{50} (concentration for 50% inhibition) was 0.1 mM. The fluoride sensitivity of *Ph*PPase is higher than that of bacterial and archaeal PPases: *E. coli* (91%, 1 mM) (Josse 1966), *Thiobacillus thiooxidans* (81%, 1.25 mM) (Tominaga and Mori 1977), *Rhodopseudomonas palustris* (50%, 5 mM) (Schwarm et al. 1986) and *Methanobacterium thermoautotrophicum* (50%, 0.9 mM) (van Alebeek et al. 1994).

In conclusion, we have identified a gene that codes for PPase protein from the *P. horikoshii* genome and also compared the properties of recombinant *Ph*PPase with those of other PPases. The *Ph*PPase is more similar to bacterial and archaeal enzymes than to eukaryotic enzymes, based on the molecular mass of a single subunit. Cytoplasmic PPase has no function in energy conservation. Its function seems to keep the cellular PP_i concentration low, through the hydrolysis of PP_i. Whether *Ph*PPase is located in the cytoplasm has not been reported. This work increased our understanding of the metabolism of a hyperthermophilic archaeon. The hyperthermostability of *Ph*PPase suggests that it can be used to suppress pyrophosphorolysis in sequencing reactions using thermostable DNA polymerases (Tabor and Richardson 1990, Vander Horn et al. 1997).

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