**A Simple Assay for Determining Activities of Phosphopentomutase
from a Hyperthermophilic Bacterium *Thermotoga maritima***

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Abstract

Phosphopentomutase (PPM) catalyzes the inter-conversion of α -D-(deoxy)-ribose 1-phosphate and α -D-(deoxy)-ribose 5-phosphate. We developed a coupled or uncoupled enzymatic assay with an enzyme nucleoside phosphorylase for determining phosphopentomutase (PPM) activities on D-ribose 5-phosphate at a broad temperature range from 30 to 90°C. This assay is not only simple and highly sensitive, but also does not require any costly special instrument. Via this technology, an open reading frame TM0167 from a thermophilic bacterium *Thermotoga maritima* putatively encoding PPM was cloned. The recombinant PPM was over-expressed in *Escherichia coli* Rosetta. This enzyme has the highest activity at 90 °C. MnCl_2 (0.1 mM) and 50 μM α -D-glucose 1, 6 bisphosphate are cofactors. The kinetic parameters of K_m and k_{cat} are 1.2 mM and 185 s^{-1} at 90 °C, respectively. The enzyme has a half-life time of up to 156 min at 90 °C. This enzyme is the most active and thermostable PPM reported until now.

Keywords: Biocatalysis, Enzymatic Assay, Phosphopentomutase, Thermophilic Enzyme, *Thermotoga maritima*

List of abbreviations

PPM: phosphopentomutase
DERA: deoxyribose 5-phosphate aldolase
dR5P: deoxyribose 5-phosphate
Pu-NPase: purine nucleoside phosphorylase
POE-PCR: prolonged overlap extension PCR
IPTG: isopropyl- β -D-thiogalactoside
DTT: dithiothreitol

1. Introduction

Phosphopentomutase (PPM) with a systematic name of α -D-ribose 1, 5-phosphomutase (EC 5.4.2.7) catalyzes the inter-conversion of α -D-ribose 1-phosphate and α -D-ribose 5-phosphate. This enzyme can utilize both D-ribose 5-phosphate and deoxyribose 5-phosphate [1-3]. Its major physiological function is a bridge between the carbohydrate and purine metabolism by converting D-ribose 5-phosphate from the pentose phosphate pathway to D-ribose 1-phosphate (Equation 1). D-ribose 1-phosphate is reacted with adenine catalyzed by adenosine phosphorylase, yielding adenosine (a purine nucleoside) (Equation 2) [4]. Also, this enzyme can be coupled with deoxyribose phosphate aldolase (DERA) and convert deoxyribose 1-phosphate obtained from the degradation of pyrimidine deoxyribonucleosides to deoxyribose 5-phosphate, which is further cleaved to acetaldehyde and glyceraldehyde 3-phosphate (**Fig. 1A**). Therefore, PPM is of importance in the salvage pathway for synthesis of nucleotides and use of the nucleosides as an energy source in a variety of microorganisms and eukaryotic cells [5].

Several methods have been developed to measure PPM activity on a substrate of either deoxyribose 5-phosphate or ribose 5-phosphate [4]. Starting from deoxyribose 1- ^{32}P , one of the oldest methods based on the conversion of acid-labile radio-labelled deoxyribose 1- ^{32}P to acid stable ^{32}P , where the release of ^{32}P was measured with a scintillation counter [6]. To avoid using a scintillation counter, PPM activity has been measured spectrophotometrically by coupling with deoxyribose 5-phosphate aldolase (DERA) and alcohol dehydrogenase (**Fig.**

1A). In this assay the enzyme activity was measured by conversion of acetaldehyde, one of the products formed by cleavage of deoxyribose 5-phosphate (dR5P), to ethanol accompanied with the oxidation of NADH catalyzed by alcohol dehydrogenase [5, 7]. An alternative way is the formation of a hydrazone from deoxyribose 5-phosphate and phenyl hydrazine (**Fig. 1B**) [8]. Starting from D-ribose 5-phosphate, three methods have been reported. The first method was carried out in three steps (**Fig. 1C**). The initial two steps were carried out by coupling PPM with adenosine phosphorylase, yielding adenosine, which was converted to inosine catalyzed by adenosine deaminase. The decrease of absorbance at 265 nm was monitored to measure PPM activity. This assay requires two additional enzymes and a quartz cuvette and also suffers from high background of adenosine absorbance. To overcome the drawbacks of this assay, another assay was carried out in two steps (**Fig. 1D**). PPM coupled with adenosine phosphorylase, yielding adenosine. The formation of adenosine was measured by using HPLC but this modified method requires HPLC equipped with a special column and is not suitable for measuring a large number of samples within a short time. Similarly, another modified assay has been developed to measure PPM by monitoring the formation of didanosine, a nucleoside analog (**Fig. 1E**) [9]. This assay was based on the PPM conversion of 2,3-dideoxyribose 5-phosphate to 2,3-dideoxyribose 1-phosphate and the addition of hypoxanthine and purine nucleoside phosphorylase resulted in the formation of didanosine, where hypoxanthine consumption was measured by xanthine oxidase at endpoints using a typical hypoxanthine detection assay based on the absorbance of the colored formazan at 546 nm [9].

Enzyme-based biocatalysis and *in vitro* synthetic enzymatic biosystems have become an attractive alternative to chemical catalysis and microbial fermentation because of its higher reaction selectivity and more modest reaction conditions [10, 11], but most enzymes are not

suitable for industrial applications due to their relatively poor stability. Discovery and utilization of thermostable enzymes from hyperthermophilic microorganisms and rapidly-growing mega-genome database are of great interest for numerous industrial applications [11]. *Thermotoga maritima* is an anaerobic, rod-shaped eubacterium, which was isolated from geothermally heated marine sediment from a volcano in Italy. It has an optimum growth temperature of ~85°C [12]. *T. maritima* is regarded as an invaluable source of intrinsically thermostable enzymes [13, 14]. The open reading frame (ORF) encoding the *ppm* gene was not designated in the genomic DNA sequence of *T. maritima* [15], but recently KEGG designated ORF TM0167 encoding the *ppm* gene. However, its biochemical function has not yet been confirmed.

In this study, we developed a simple assay to measure the activities of a thermophilic PPM requiring the addition of one enzyme nucleoside phosphorylase (Pu-NPase) (**Fig. 1F**). In the first step, PPM converts D-ribose 5-phosphate to D-ribose 1-phosphate at various temperatures. The reaction is stopped by the addition of HClO₄ followed by KOH neutralization. D-Ribose 1-phosphate is reacted with adenine catalyzed by Pu-NPase, yielding phosphate and adenosine. Inorganic phosphate is measured by a mild pH phosphate assay [16, 17]. This simple PPM assay has something in common with the previous method [9], for example, the use of Pu-NPase, stopping the reaction by using an acid followed by alkali neutralization (**Fig. 1E**). However, this method has some advantages: only one enzyme (Pu-NPase) is required, that is, no xanthine oxidase needed; the mild-pH phosphate assay is simpler and less costly than the hypoxanthine detection assay.

2. Materials and Methods

2.1. Chemical and strains

All chemicals were reagent grade, purchased from Sigma- Aldrich (St. Louis, MO, USA) and Fischer Scientific (Pittsburg, PA, USA), unless otherwise noted. The DNA polymerase used was Phusion high-fidelity DNA polymerase from New England Biolabs (Ipswich, MA, USA). The protein marker (2–212 kDa) was purchased from New England Biolabs (Ipswich, MA, USA). Primers were purchased from IDT (Coralville, IA, USA). The PCR thermocycler was Eppendorf temperature gradient master cycler (Hauppauge, NY, USA). *T. maritima* MSB8 (ATCC 43589) genomic DNA was purchased from the American Type Culture Collection (Manassas, VA, USA). *E. coli* Top 10 was used for DNA manipulation; *E. coli* Rosetta was used for protein expression. Plasmid pET20b (+) (Novagen, Germany) was used for gene expression. *E. coli* strains were cultivated in the Luria–Bertani (LB) medium supplemented with ampicillin at 100 mg/mL. Purine nucleoside phosphorylase (Pu-NPase) was purchased from Sigma Aldrich with catalog number N8264.

2.2. Plasmid construction

The DNA fragment containing ORF TM0167 was amplified by PCR with a pair of primers; forward primer IF (5′GTTTA ACTTTA AGAAG GAGAT ATACAT ATGAG GGTGG TACTGA TAGTCC T′3) and reverse primer IR (5′CAGTGG TGGTG GTGGT GGTGC TCGAG CTCCC ATATG AGGTT TTTGA AG′3) based on genomic DNA of *T. maritima* MSB8 (ATCC 43589). A linear vector backbone was amplified based on pET-20b (+) with a pair of primers; forward primer VF (5′CTTCA AAAAC CTCAT ATGGG AGCTC GAG CACCACCACCACCACCACTG′3) and the reverse primer VR (5′AGGAC TATCA GTACC ACCCT CATAT GTATA TCTCC TTCTT AAAGT TAAAC′3). VF and VR contain the last 23 bp of the 3′ terminus of the insertion sequence (underlined) and the first 25 bp of the 5′ terminus of the vector sequence (underlined). IF and IR have reverse

complementary sequences of VR and VF, respectively. The PCR products of both the insert and vector were assembled by prolonged overlap extension PCR (POE-PCR). POR-PCR conditions were as followings: initial denaturation (30 s at 98 °C), 30 cycles of denaturation (10 s at 98 °C, annealing 10 s at 60 °C, and elongation 72 °C at a rate of 2 kb/min), and a final extension step (10 min at 72 °C). The POE-PCR product was transformed to *E. coli* Top 10 [18], yielding plasmid pET20b-TmPPM. These restriction enzymes-and ligase-free subcloning method was called Simple Cloning [19].

2.3. Protein expression and purification

The recombinant plasmid pET20b-TmPPM was transformed into *E. coli* Rosetta yielding *E. coli* Rosetta (pET20b-TmPPM) and recombinant protein expression was conducted in the LB medium. Recombinant *E. coli* cells were grown at 37 °C in 1.0 L Erlenmeyer flasks containing 250 ml of the LB medium supplemented with 100 mg/mL of ampicillin. Induction of protein expression was carried out at 18 °C for 18 h by addition of 1 mM isopropyl- β -D-thiogalactoside (IPTG) when the absorbance at 600 nm reached 0.6-0.8. The cells were harvested by centrifugation at 4 °C and washed twice with 50 mM of Tris-HCl buffer (pH 7.5) containing 50 mM NaCl. The cell pellets were suspended in the same buffer and lysed by ultra-sonication in an ice bath (Fisher Scientific sonic dismembrator Model 500; 3 s pulses on and 8 s off, total 180 s at 50 % amplitude). After centrifugation, the soluble His-tagged PPM in the supernatant (i.e., 30 mL with a protein concentration of 1.8 mg/mL) was purified at room temperature by using a packed Ni-charged resin column (Bio-Rad, Profinity IMAC Ni-Charged Resin) with a bed volume of 2 mL and an inner diameter of 5 mm. The *E. coli* cellular proteins were washed away with 20 mL of the binding buffer (100 mM HEPES buffer (pH 7.5) containing 50 mM NaCl and 10 mM imidazole). The recombinant PPM was eluted with 10 mL of the 100 mM HEPES (pH 7.5) buffer containing 50 mM NaCl and 150 mM imidazole. The purity of PPM was analyzing using 12% sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 10 μ g of both total cell lysate and soluble proteins in the supernatant and 5 μ g of both pellet protein and purified PPM were loaded in each lane. The pellet proteins were solubilized by using the SDS-PAGE protein denaturing buffer. The protein bands were visualized by using the Bio-Rad Coomassie Blue 250 staining kit. The protein concentration was measured with the Bio-Rad Bradford protein kit with bovine serum albumin as a reference.

2.4. PPM assay

Phosphopentomutase activities at 60 °C or a lower temperature were measured by using the coupled enzyme assay described previously [20] with some modifications. Instead of the measurement of the synthesis of adenosine from D-ribose 5-phosphate and adenine in presence of 4 units of Pu-NPase, PPM activity was measured based on the release of inorganic phosphate. The standard mixture (1 mL) used contained 100 mM MOPS buffer (pH 7.5), 3 mM D-ribose 5-phosphate, 4 mM adenine-HCl, 0.1 mM MnCl_2 , 0.5 mM β -mercaptoethanol, 50 μ M D-glucose 1,6 bisphosphate, and 4 units Pu-NPase. The reaction was started by the addition of 0.003 g/L PPM (final concentration). The reaction was stopped by dipping the tubes containing the reaction mixture in an ice bath. Inorganic phosphate (P_i) concentration was measured by the Sahiki method, which was conducted at mild pH to avoid the degradation of sugar phosphates [16]. Twenty microliters of the reaction solution was added to 200 μ L of the molybdate reagent followed by addition of 50 μ L of 10% ascorbic acid. After mixing well, the reaction solution components were maintained at 30 °C for 20 min. Absorbance at 850 nm was measured with reference of the standard phosphate curve. One unit of enzyme activity is defined as the release of 1 μ mole of P_i per min. To measure the PPM activity at more than 60 °C, the same reaction mixture was prepared without the addition of Pu-NPase. The reaction was carried out in two steps. First, the reaction was started by addition of PPM. The reaction (1 mL of the solution) was stopped by adding 250 μ L of 4 M

HClO₄ followed by neutralization with 120 µL of 10 M KOH. After centrifugation at 12,000 x g for 5 min [21], the supernatant containing ribose 1-phosphate and adenine was used for the second-step assay. The second enzymatic reaction was started by addition of 4 units of PuNPase followed by incubation at 60 °C for 5 min. The phosphate concentration was measured as mentioned above. In all reactions regardless of temperature, the reaction mixtures without addition of PPM were used as negative controls.

2.5. Enzyme characterization

The optimum pH of PPM was examined in a series of buffers: 100 mM sodium citrate buffers (pH 5 and pH 6), 100 mM MOPS buffers (pH 6.5, 7, 7.5 and 8), and Tris-HCl buffers (pH 6.5, 7, 7.5 and 8). The reaction was carried out at 90 °C for 5 min. The effects of reaction temperature from 30 to 100°C were tested in 100 mM MOPS buffer (pH 7.5). The activation energy of PPM enzyme was measured based on the Arrhenius equation [22]. The effect of 0.1 mM di-valiant metal ions was investigated in 100 mM MOPS buffer (pH 7.5) at 90 °C. The effects of α-D-glucose 1,6-bisphosphate as an activator and reducing reagents (e.g., 0.5 mM β-mercaptoethanol and 0.5 mM dithiothreitol (DTT)) on PPM were investigated compared to positive controls containing 50 µM α-D-glucose 1,6-bisphosphate and 0.5 mM β-mercaptoethanol. All experiments were carried out in duplicates.

2.6. Kinetic study

Kinetic parameters of PPM were measured based on initial reaction rates (3-min reaction at 60 °C and 1.5-min of reaction at 90 °C) at a PPM concentration of 0.003 g/L. The substrate D-ribose 5-phosphate concentrations were 0.174, 0.435, 0.87, 1.74 and 4.35 mM in 100 mM MOPS buffer (pH 7.5) contained 4 mM adenine, 0.1 mM MnCl₂, 50 µM of α-D-glucose 1, 6-bisphosphate, and 0.5 mM of DTT. Data analysis was performed by Graph Prism software (Graph Pad software, San Diego, California, USA). All results are an average of three replicates.

2.7. Thermal stability

The thermal stability of PPM 0.001 and 0.01 g/L in 100 mM MOPS buffer (pH 7.5) was examined at 90 °C. The residual PPM activity was measured at 90 °C as described above. All reactions were carried out at least two times.

3. Results

3.1. Cloning, protein expression and purification

The ORF TM0167 putatively encoding for PPM was subcloned into plasmid pET20b by using sequence-independent and restriction enzyme- and ligase-free Simple Cloning, yielding 4.7-kb plasmid pET20b-TmPPM. This plasmid was transformed into *E. coli* BL21(DE3) and up to 1 mM IPTG was added to induce PPM expression. However, no clear protein band responsible for the targeted PPM was observed in SDS-PAGE analysis (data not shown), possibly due to the codon usage of ORF TM0167, whose rare codon adaptation index expressed in *E. coli* is 0.60 only, calculated by the GenScript Rare Codon Analysis Tool (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). Therefore, *E. coli* Rosetta was used for recombinant PPM expression. *E. coli* cells harboring the plasmid were cultured in the LB medium and recombinant PPM was induced at 18 °C with 1 mM IPTG. SDS-PAGE of the whole cell lysate is shown in **Fig 2**, lane T. A majority of the recombinant enzyme was present in the soluble fraction of the *E. coli* cell lysate (**Fig 2**, lane SN). Almost no protein representing PPM is present in the insoluble fraction of the cell lysate (**Fig 2**, lane pellet). PPM with a C-terminal His-tag was purified to homogeneity by using nickel charged resins (**Fig 2**, lane P). A single band representing the purified PPM had an estimated molecular weight of approximately 42,000 (Fig 2, lane P), in agreement with the molecular weight of 43,500 based on its deduced amino acid sequence. The PPM recovery and purification fold

were 90.1 % and 4.1, respectively (**Table 1**). The specific activity of the purified PPM was 57.8 U/mg at 90 °C.

3.2. Basic Characterization

Different buffers: 100 mM sodium citrate buffers (pH 5 and pH 6), 100 mM MOPS buffers (pH 6.5, 7, 7.5 and 8), and Tris-HCl buffers (pH 6.5, 7, 7.5 and 8) were used to examine the effect of pH on PPM activity at 37 °C and 90 °C. It was found that at 37 °C PPM displayed a higher activity in the Tris-HCl buffers than in MOPS buffers at a pH range of 6.5, 7, 7.5 and 8 (data not shown). In contrast, PPM exhibited lower activities in Tris-HCl buffers at 90 °C, compared to those in MOPS buffers. This difference may be due to the decrease in the pH level of Tris-HCl buffer at higher temperatures (Δ pka per 10 °C is 0.3). Therefore, PPM activities at high temperature were measured in MOPS buffers. This PPM displayed a maximum activity in 100 mM MOPS buffer (pH 7.5) (**Fig. 3A**). Also, the negative controls (the substrate D-ribose 5-phosphate and the product D-ribose 1-phosphate in the MOPS buffer) did not released significant amounts of inorganic phosphate even at 90 °C (data not shown), suggesting that sugar phosphates are stable under these conditions.

Effects of temperature on PPM activities from 30°C to 100 °C were carried out in 100 mM MOPS buffer (pH 7.5). The optimum temperature of this PPM was 90 °C (**Fig. 3B**), in good agreement with the optimum growth temperature of *T. maritima* [12]. The activation energy of PPM from 30 to 90 °C is 67.4 kJ per mole based on the Arrhenius plot.

Because PPM belonged to the alkaline phosphatase super family, a family of di-metalloenzymes [23], effects of divalent metal ions on the enzyme activity were measured (**Fig. 3C**). No metal ions added resulted in a very low enzyme activity, suggesting that this

enzyme is a typical metalloenzyme. The addition of 0.1 mM MnCl_2 had the highest activity. The presence of Ni^{+2} , Co^{+2} , Zn^{+2} and Cu^{+2} enhanced PPM activities, but not as much as Mn^{2+} (**Fig. 3C**). This enzyme required Mn^{2+} as a cofactor, in agreement with the reported bacterial PPM enzyme [4].

More possible activators were investigated. α -D-glucose 1,6-bisphosphate was reported to be a key activator previously [4, 23]. It was found that the highest activity in the present work was obtained in the presence of 50 μM of glucose 1, 6-bisphosphate. Without it, the enzyme exhibited only 7.5% activity of that in the presence of glucose 1, 6-bisphosphate (**Fig. 3D**). Also, the addition of 0.5 mM of DTT was found to stimulate the enzyme activity. Without DTT, this PPM had a 58% activity decrease (**Fig. 3D**). The addition of 0.5 mM DTT gave somewhat higher activities than obtained by addition of 0.5 mM β -mercaptoethanol (**Fig. 3D**).

This PPM displayed a typical Michaelis–Menten kinetics (**Fig. 4**). The kinetic parameters were determined by changing the concentration of D-ribose 5-phosphate from 0.174 to 4.35 mM at 60 °C and 90°C. The K_m , k_{cat} and k_{cat}/K_m values are 0.75 ± 0.1 mM, 55.6 ± 0.4 s⁻¹ and 74.1 ± 9.9 mM s⁻¹ at 60 °C. At its optimum temperature of 90 °C, the K_m , k_{cat} and k_{cat}/K_m are 1.2 ± 0.1 mM, 185 ± 6 s⁻¹ and 154 ± 13 mM s⁻¹. High reaction temperatures lead to decreased substrate binding capacity and increased catalytic rate, resulting 2.1-fold higher catalytic coefficient (**Table 2**).

This PPM is very stable in 100 mM MOPS buffer (pH 7.5) at 90 °C at both low and high concentrations (0.001 and 0.01 g/L). High concentration of PPM showed slightly higher stability than low concentration PPM (**Fig. 5**). The degradation constants and the half-life times of PPM are -0.0066 min⁻¹ and -0.0041 min⁻¹, and 96 min and 156 min for 0.001 and

0.01 g PPM/L, respectively. Therefore, total turn-over numbers (TTN) of this enzyme at 90 °C are 1.68×10^6 and 2.71×10^6 mole of product per mole of enzyme at 0.001 and 0.01 g/L PPM, respectively, according to the equation ($TTN = k_{cat}/k_d$).

4. Discussion

In this study we developed a modified simple assay to determine thermophilic PPM activities at a broad temperature range. At a low temperature below 60°C, PPM was coupled to Pu-NPase and the final product was determined by a mild pH phosphate assay that does not degrade sugar phosphates (D-ribose 5-phosphate and D-ribose 1-phosphate). At a high temperature above 60°C, PPM was added first followed by the reaction termination by using ice-cold HClO₄. The addition of KOH neutralized HClO₄; generating KClO₄ precipitates and removing deactivated PPM. Pu-NPase was added in the pH neutral supernatant to release phosphate from D-ribose 1-phosphate. The negative controls showed no release of inorganic phosphate even at 90 °C. This assay has several advantages. First, it can be applied to both mesophilic and thermophilic enzymes. Second, it requires only one enzyme addition rather than the two-enzyme assay used previously [4] (**Fig. 1C**). Third, it does not require costly HPLC. Fourth, as compared to other UV-based spectrophotometric assay [4], the phosphate assay can be conducted in a visible light spectrophotometer. Also, the phosphate assay has a very high sensitivity so that the overall assay can be used to determine very low PPM activities.

The PPM from *T. maritima* was expressed, purified, characterized for the first time and its biochemical function was confirmed. This PPM exhibited maximum activity in 100 mM MOPS buffer (pH 7.5) contains 0.1 mM MnCl₂, 50 µM of α-D-glucose 1, 6-bisphosphate and 0.5 mM of DTT. As shown in **Table 2**, the *T. maritima* PPM has the highest K_{cat} and catalytic

efficiency at 90°C compared to other bacterial and mailman PPMs. Compared to another hyperthermophilic PPM from *T. kodakaraensis* [5], *T. maritima* enzyme has a 2.3 fold better catalytic coefficient. Moreover, the calculated TTN was more than 1×10^6 mole of product per mole of enzyme at 90°C, suggesting that the discovery of thermostable enzyme building blocks from a genomic DNA database is efficient to obtain more stable enzymes suitable for future industrial biocatalysis.

In conclusion, we develop a simple PPM activity assay that can be used to measure PPM at a wide temperature range. Also, we confirmed the biochemical function of PPM from *T. maritima*.

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TABLES

Table 1.

PPM purification from 250 mL of the *E. coli* cell culture growing on the LB medium.

Protein Fraction	Vol. (mL)	Protein Conc. (mg/mL)	Total protein (mg)	Sp. Act. * (U/mg)	Total Act. (U)	Yield (%)	Fold purification
Total cell lysate	30	1.89	56.7	14.0	796	100	1
Purified	10	1.24	12.4	57.8	717	90.1	4.1

* Specific activity was measured at 90 °C in 100 mM MOPS buffer pH 7.5 contains 4 mM adenine, 0.1 mM MnCl₂, 50 μM of α-D-glucose 1, 6-bisphosphate, 0.5 mM of DTT, and 3 mM D-ribose 5-phosphate.

Table 2.

Comparison of kinetic characteristics of PPM from different organisms.

Organism name	Temp (°C)	pH	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM.s ⁻¹)	Ref.
Bacterial PPM						
<i>T. maritima</i> MSB8 ⁽¹⁾	60	7.5	0.75 ± 0.1	55.6 ± 0.4	74.1 ± 9.9	This study
	90	7.5	1.2 ± 0.1	185 ± 6	154 ± 13	
<i>T. kodakaraensis</i> ⁽²⁾	90	7.5	2.5 ± 0.2	173 ± 9	69.2 ± 6.6	[5]
<i>B. stearothermophilus</i> TH6-2 ⁽¹⁾	65	7.5	NA*	NA	NA	[20]
<i>Bacillus cereus</i> DSM 31 ⁽³⁾	22	8.0	NA	NA	NA	[23]
<i>Bacillus cereus</i> ATCC 14579 ⁽¹⁾	23	8.0	0.26 ± 0.03	10.2 ± 0.6	39 ± 5	[3]
<i>E. coli</i> W ⁽¹⁾	37	8.5	0.87	NA	NA	[4]
Mammalian PPM						
<i>Rattus norvegicus</i>	50	7.5	0.083 ⁽²⁾	NA	NA	[24]
		8.5	0.130 ⁽³⁾			

(1) Characterization of PPM utilizing D-ribose 5-phosphate as a substrate.

(2) Characterization of PPM utilizing 2-deoxyribose 1-phosphate as a substrate.

(3) Characterization of PPM utilizing D-ribose 1-phosphate as a substrate.

* NA: not available

Figure Legends

Figure 1. Scheme of phosphopentomutase (PPM) activity assays, where the products to be measured are shown in the box. A, the coupled PPM assay with deoxyribose 5-phosphate aldolase (DERA) and alcohol dehydrogenase, where NADH consumption was monitored; B, Enzymatic assay in which enzyme activity is measured based on formation of a hydrazone of deoxyribose 5-phosphate with phenyl hydrazine; C, Enzymatic assay in which activity is measured based on consumption of adenosine by adenosine deaminase and production of inosine. D, the coupled enzyme assay supplemented with Pu-NPase where adenosine is measured; E) the coupled enzyme assay supplemented with Pu-NPase where didanosine was measured, and F) the suggested modified coupled (below 60°C) or uncoupled (above 60°C) PPM assay, where phosphate is measured by a visible light photospectrometer.

Figure 2. 12% SDS-PAGE analysis of protein expression and purification of *T. maritima* PPM expressed in *E. coli*. PM, protein marker; T, total cell lysate; S, soluble proteins of cell lysate; Pellet, insoluble proteins of cell lysate; and P, purified protein.

Figure 3: Biochemical characteristics of *T. maritima* PPM. A, pH profile in 100 mM MOPS buffer at 90 °C; B, temperature profile in 100 mM MOPS buffer (pH 7.5); C, effect of different divalent metal ions at 0.1 mM final concentration on enzyme activity in 100 mM MOPS buffer (pH 7.5) at 90 °C; and D, effects of the presence or absence of either activator (α -D- glucose 1,6-bisphosphate) or reducing agents (β -mercaptoethanol or DTT). Error bars represent standard deviations.

Figure 4. Kinetic study of the *T. maritima* PPM at 60 °C and 90 °C in 100 mM MOPS buffer (pH 7.5) containing 4 mM adenine, 0.1 mM MnCl₂, 50 μ M of α -D-glucose 1, 6-bisphosphate and 0.5 mM of DTT, The MM-curves were fitted by using Graph Prism software. Error bars represent standard deviations of the three samples measured.

433 **Figure 5.** Thermo-inactivation of the *T. maritima* PPM in 100 mM MOPS buffer pH 7.5 at 90 °C at
434 enzyme loading of 0.001 and 0.01 g/L. Error bars represent standard deviations.

Figure 2 SDS-PAGE

Figure 3 Characterization

A

B

C

D

Figure 4 kinetics

Figure 5