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Thermophilic *Thermotoga maritima* ribose-5-phosphate isomerase RpiB: Optimized heat treatment purification and basic characterization

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ABSTRACT

The open reading frame TM1080 from *Thermotoga maritima* encoding ribose-5-phosphate isomerase type B (RpiB) was cloned and over-expressed in *Escherichia coli* BL21 (DE3). After optimization of cell culture conditions, more than 30% of intracellular proteins were soluble recombinant RpiB. High-purity RpiB was obtained by heat pretreatment through its optimization in buffer choice, buffer pH, as well as temperature and duration of pretreatment. This enzyme had the maximum activity at 70 °C and pH 6.5–8.0. Under its suboptimal conditions (60 °C and pH 7.0), k_{cat} and K_m values were 540 s^{-1} and 7.6 mM, respectively; it had a half lifetime of 71 h, resulting in its turn-over number of more than 2×10^8 mol of product per mol of enzyme. This study suggests that it is highly feasible to discover thermostable enzymes from exploding genomic DNA database of extremophiles with the desired stability suitable for in vitro synthetic biology projects and produce high-purity thermoenzymes at very low costs.

Introduction

The production of biofuels and biochemicals from renewable carbohydrates would decrease net greenhouse gas emissions, create new biomanufacturing jobs, and enhance national energy security [1]. Numerous biofuels have been produced based on different key metabolites through natural or non-natural biochemical pathways, for example, ethanol production from pyruvate [2], non-fermentative alcohols from keto acids [3], fatty acid-derived hydrocarbons from malonyl-CoA [4], isoprenoid-derived hydrocarbons from isopentenyl pyrophosphate [5]. On a basis of the pentose phosphate pathway, theoretical yield hydrogen (i.e., 12 mol of dihydrogen per mol of glucose and water) was accomplished by in vitro enzyme cocktails [6,7]. Hydrogen produced from renewable sugar is believed to be the best future biofuel based on its production efficiency [8] and energy utilization efficiency [1]. However, its large-scale production requires low-cost production of stable enzymes [9,10].

In vitro synthetic pathway biotransformation, a new direction of synthetic biology, is an emerging low-cost biomanufacturing platform due to its unique advantages, such as high product yield, fast reaction rate, easy access and control, broad reaction condition [9–12]. To produce low-value biofuels by cascade enzyme factories, one of the perquisites is highly-stable enzyme building blocks with total turn-over number (TTN)[1] values of more than 10^7–10^8 mol of product per mol of enzyme [9,10,13]. Therefore, the discovery of stable enzymes from extremophiles, enzyme engineering, and enzyme immobilization are among top priorities for the preparation of build blocks for in vitro synthetic biology projects [9,10].

Ribose-5-phosphate isomerase (Rpi, EC 5.3.1.6) is responsible for catalyzing the reversible conversion between an aldose phosphate – ribose-5-phosphate (R5P) and a ketose phosphate – ribulose-5-phosphate (Ru5P) in the non-oxidative pentose phosphate pathway, the Kevin cycle, and in the process of photosynthesis [14]. This enzyme was first described from yeast [15] and then further studied in *Escherichia coli* [16]. Mutant studies in *E. coli* have shown that Rpi is important to bacterial growth [17]. Another medical research has revealed that a deficiency in human Rpi can cause the destruction of myelin sheaths, which in turn leads to brain abnormalities [18,19]. Two completely unrelated RpiA and RpiB have been identified. RpiA is the most common, occurring in all three kingdoms of life, including most eukaryotic organisms, fungi and some bacteria; while RpiB has so far only been found in the genomes of some bacterial and protozoa [20]. RpiA is a highly conserved enzyme with no primary sequence similarities to other

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[1] *Abbreviations used:* TTN, total turn-over number; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; ORF, open reading frame; RpiB, ribose-5-phosphate isomerase B; IPTG, β-D-1-thiogalactopyranoside.

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known protein families [16]. RpiB belongs to the RpiB–LacAB family of enzymes, including RpiB as well as both LacA and LacB subunits of the galactose 6-phosphate isomerase [17].

In addition to high-level expression of recombinant enzymes in *E. coli*, it is vital to purify recombinant enzymes at low costs. Although affinity chromatographic techniques are the most popular protein purification technology in academic laboratories, they are too costly for the production of industrial bulk enzymes with selling prices of tens of dollars per kg of dry weight enzyme. Several low-cost scalable protein purification technologies have been developed, such as heat treatment, ammonia sulfate precipitation, self-cleaving aggregation [21], one-step adsorption and immobilization [22]. Among them, heat treatment may be the most promising because it does not involve any chemical reagent, protein tag, and adsorbent. Its application, however, was limited to thermostable enzyme production in mesophilic hosts like *E. coli*. During the process, crude cell extract containing the heat-resistant recombinant protein was heated. The denatured *E. coli* cellular proteins can be removed by centrifugation. For example, a recombinant *Thermus aquaticus* DNA polymerase (*Taq* polymerase) produced in *E. coli* was often purified by simple heat treatment. However, there lack detailed studies of heat treatment purification for thermostable proteins.

Thermotoga maritima is an anaerobic, rod-shaped bacterium, originally isolated from geothermally heated marine sediment. It has an optimum growth temperature of ~80 °C [23]. *T. maritima* is regarded as an invaluable source of intrinsically thermostable enzymes [24]. The open reading frame (ORF) TM1080 was designated to be a ribose-5-phosphate isomerase B (RpiB) and its crystalline structure has been determined [25]. However, its basic biochemical properties were not characterized yet. In this study, the ORF TM1080 encoding RpiB was cloned and over-expressed in *E. coli*. High-purity RpiB was obtained through optimized heat treatment. Its basic properties were characterized for the first time.

Materials and methods

Chemicals and strains

All chemicals were regent grade, purchased from Sigma–Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. Ribose-5-phosphate and ribulose-5-phosphate were purchased from Sigma–Aldrich. Restriction enzymes, Taq DNA polymerase, T4 ligase, and a broad range protein marker (2–212 kDa) were purchased from New England Biolabs (Ipswich, MA, USA). The *T. maritima* MSB8 genomic DNA was purchased from the American Type Culture Collection (Manassas, VA, USA). *E. coli* DH5α was used as a host for recombinant DNA manipulation; *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA, USA) was used as a host strain for recombinant protein expression. The Luria–Bertani (LB) medium was used for *E. coli* cell growth and recombinant protein expression. Ampicilin (100 μg/mL) was added in the *E. coli* media. The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA, USA).

Protein expression plasmid construction

The DNA fragment containing the ORF TM1080 (GenBank Accession No AE0005120) was amplified by PCR from genomic DNA of *T. maritima* MSB8 by using a forward primer (5′-GCA TAC CAT ATG ATG AAG ATC GCT ATT GCA TCG-3′, NdeI site underlined) and a reverse primer (5′-CCT CAG CTC GAG TTA AAC CTC ATC GAT CTT TCT G-3′, XhoI site underlined). PCR conditions were as the following: initial denaturation (5 min at 94 °C), 30 cycles of denaturation (30 s at 94 °C, annealing 30 s at 55 °C, and elongation 60 s at 72 °C), and a final extension step (10 min at 72 °C). After *Nde*I and *Xho*I digestion of the PCR product and plasmid pET20b, the ligated product was transformed to *E. coli* DH5α, yielding plasmid pET20b–rpiB. The DNA sequence of pET20b–rpiB was validated by sequencing by Virginia Bioinformatics Institute (Blacksburg, VA).

Expression and purification of recombinant protein

The protein expression plasmid pET20b–rpiB was transformed into the strain *E. coli* BL21 (DE3) for yielding *E. coli* BL21 (pET20b–rpiB). The freshly-prepared strain *E. coli* BL21 (pET20b–rpiB) was cultivated in 250 mL of the LB medium supplemented with 100 μg/mL ampicillin in 1-L Erlenmeyer flasks at a rotary shaking rate of 220 rpm at 37 °C. The recombinant protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM, final) when A_{600} reached 0.6 ~ 0.8. The cell cultures were incubated at 18 °C for 16 h. The cells were harvested by centrifugation at 4 °C, washed once in a 50 mM Tris–HCl buffer (pH 7.0), re-suspended by ~30 mL of 50 mM Tris–HCl buffer (pH 7.0). The cell pellets were lysed in an iced bath by ultra-sonication by Fisher Scientific Sonic Dismembrator Model 500 (3-s pulse, total 90 s, at 50% amplitude). After centrifugation at 10,000 rpm for 20 min, the supernatant of the cell lysate was used for protein purification by heat treatment. The pellets from 1 mL of the cell lysate were washed with 1 mL of 50 mM Tris–HCl buffer (pH 7.0). After centrifugation and re-suspension in 0.1 mL of the SDS–PAGE protein loading buffer followed by 5-min water boiling, 10 μL of the boiled sample was analyzed by SDS–PAGE. RpiB was purified by heating the supernatant which was recovered after the centrifugation of the cell culture. The samples were heated 40 min at 80 °C and then centrifuged at 14,000 rpm for 8 min. The supernatant containing high-purity RpiB was analyzed by SDS–PAGE.

SDS–PAGE and determination of protein concentration

The protein samples were analyzed by SDS–PAGE with 15% separate gel and 5% stacking gel. The gel was stained by the Bio-Rad Coomassie Blue 250 kit. Protein concentration was measured by the Bio-Rad Bradford protein kit with bovine serum albumin as a reference protein.

Enzyme activity assay

One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of ribulose 5-phosphate per min from ribose-5-phosphate. Ribose phosphate isomerase activity was assayed by a modified Dische's cysteine–carbazole method [16,26]. The enzyme (20 μL, the final enzyme concentration in the reaction mixtures ranged between 2.5 and 8 ng/μL) was mixed with 180 μL of a pre-warmed substrate solution containing 5 mM ribose 5-phosphate in 50 mM Tris–HCl buffer (pH 7.0). After 5 min incubation at 50 °C, 20 μL of 2 M HCl was added (final concentration is 0.2 M) to stop the reaction and then the mixture was put in an iced water bath. One mL of 66% (vol/vol) H_2SO_4 containing 35 μL of 0.12% ethanol-dissolved carbazole and 35 μL of 1.5% (wt/vol) cysteine chloride was added into each tube and mixed by vortexing. After incubation at 37 °C for 30 min, the absorbency was measured as 540 nm.

The optimum pH of RpiB was determined in a series of buffers at 65 °C for 5 min. The buffers were 100 mM sodium dihydrogen phosphate–citric acid (pH 4.6, 4.8, 5.0, 5.2, 6.4, 7.0, 7.6, and 8.0), Tris–HCl (pH 7.5, 8.0 8.5 and 9.0), as well as glycine–NaOH (pH 8.8, 9.6 and 10.6). The optimum temperature was tested at different temperatures (30–99 °C) in 50 mM Tris–HCl buffer (pH 7.0) for 5 min. The kinetic parameters of RpiB were determined based on

initial reaction rate at the first 2 min. The reactions were conducted in a 50 mM Tris–HCl buffer (pH 7.0).

Thermostability

Thermostability of the purified RpiB was studied by incubating the different concentration enzyme (0.1, 0.01 and 0.001 mg/mL) in 50 mM Tris–HCl (pH 7.0) at 60, 70 and 80 °C. After heat incubation, the residual RpiB activity was measured according to the RpiB activity assay as described above. Each result was an average of at least three repetitions.

Results

Overexpression of RpiB

The 456-bp ORF TM1080 from *T. maritima* encoding a putative RpiB was amplified by PCR. After digestion with *Nde*I and *Xho*I, the PCR product was inserted into pET20b vector, yielding a recombinant protein expression vector pET20b–rpiB. The sequence of the cloned RpiB gene was confirmed by DNA sequencing.

Expression conditions of RpiB in *E. coli* BL21 (DE3) were optimized by varying several factors: cultivation temperature (18 and 37 °C), IPTG concentration (0.01, 0.1, and 1 M), and different induction time. It was found that the optimal protein expression conditions were 0.1 M IPTG, induction at A_{600} = *ca.*0.6–0.8, and 18 °C for cultivation for 16 h. Under this condition, the target recombinant protein was highly soluble (Fig. 1A, lane 1), accounting for approximately 30% of the overall soluble cellular protein. No obvious protein bands were observed for the pellets of the *E. coli* cell lysate (Fig. 1A, lane 2), suggesting that no RpiB inclusion body was formed.

Optimized purification of RpiB

In our initial attempts, we planned to purify this protein based on its His-tag. Due to its high expression levels and thermostability, we investigated the feasibility of simple protein purification through heat treatment. Heat treatment has been widely-used as the first step for purifying thermostable enzymes produced in mesophilic hosts because most mesophilic enzymes denatured and aggregated during the heat treatment. To our limited knowledge, no one mentioned its optimization by testing different buffers and at different pH values. First, it was found that the buffer used had large influences on the purity of the target protein. As shown in Fig. 1A, heat treatment was conducted in 50 mM Tris–HCl buffer (pH 7.0) at 80 °C for 40 min, yielding high-purity RpiB (Fig. 1A, Lane 3), much better than that in 50 mM HEPES buffer at the same pH (Fig. 1A, Lane 4). Second, it was found that pH values of the Tris–HCl buffer from 6.5, 7.0, 7.5 to 8.5 greatly influenced the purity of the targeted protein (Fig. 1B, Lanes 5–8). When pH was 8.5, the supernatant protein contained numerous impurities (Fig. 1B, Lane 8). The optimal pH of the Tris–HCl buffer was 7.0. Also, heat treatment temperatures from 60 to 95 °C and treatment duration were optimized (data not shown). In general, longer heat treatment time and higher pretreatment temperature resulted in higher purity of the targeted protein, examined by SDS–PAGE. However, too long treatment time and too high temperature also deactivated RpiB, resulting in lower specific activities of RpiB (data not shown). The optimal purification conditions were the preparation of the cell lysate in 50 mM Tris–HCl buffer (pH 7.0) followed by heat treatment at 80 °C for 40 min. After centrifugation, the purity of the purified RpiB in the supernatant was more than 95%, examined by SDS–PAGE (Fig. 1A, Lane 3). Molecular mass of RpiB without any tag was 15,867 based on its deduced amino acid sequence, close to the estimated value from the SDS–PAGE analysis. Approximately 11.2 mg of RpiB was purified from 250 mL of the cell culture with a purification yield of 31% (Table 1). This enzyme had a specific activity of 290 U/mg on ribose-5-phosphate at pH 7.0 and 50 °C.

Basic properties

Previous research suggested that RpiB was metal-independent aldose–ketose isomerase [27]. Therefore, the effect of metal ions on its activity was not investigated. The pH profile of RpiB activity at 50 °C is shown in Fig. 2A. It had the maximum activities in the sodium dihydrogen phosphate–citric acid buffer at pH 6.5–8.0 and in 50 mM Tris–HCl buffer pH 7.0–9.0. Because pH value of the Tris–HCl buffer decreases with increasing temperature (i.e., ~0.03 per °C), this enzyme had optimal pH values ranging from 6.5 to 8.0. This enzyme remained ~65% activity from pH 8.8–11.0 in the glycine–NaOH buffer. At low pH values, it exhibited very low activities.

The temperature effect on RpiB activity from 30 to 99 °C was measured in a 50 mM Tris–HCl buffer (pH 7.0). Its activity increased significantly when the temperature increased until 80 °C (Fig. 2B). The optimum temperature was 80 °C. The activity at 30 °C was 25% of that at 80 °C. The enzyme activity decreased after 80 °C. It was still highly active when the temperature reached 99 °C, 90% activity of that of 80 °C. This enzyme showed relatively high activity (above 50% of its maximum activity) over a large temperature range of 40–99 °C.

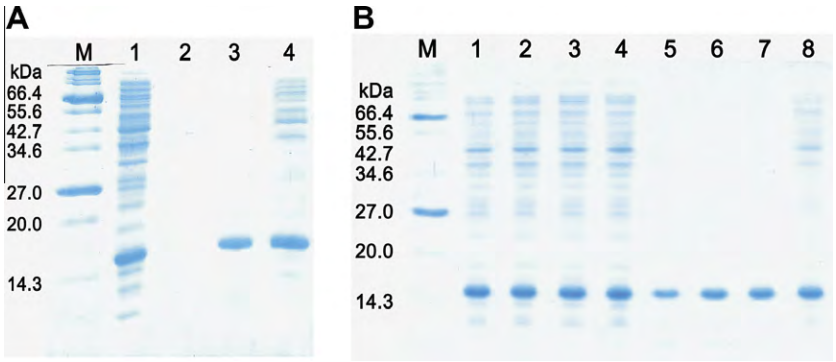

Fig. 1. SDS–PAGE analysis of RpiB purification in 50 mM Tris–HCl buffer (pH 7.0) and 50 mM HEPES buffer (pH 7.0) by heat treatment at 80 °C for 40 min. M, marker; lane 1, the supernatant of the soluble cell lysate containing RpiB; lane 2, the pellets of the cell lysate; lane 3, the supernatant of heat-treated cell lysate in 50 mM Tris–HCl buffer (pH 7.0); lane 4, the supernatant of heat treated cell lysate in 50 mM HEPES buffer (pH 7.0). (A) SDS–PAGE analysis of RpiB purification in 50 mM Tris–HCl buffer with different pH. M, marker; lane 1–4, the cell lysate containing RpiB at pH 6.5, 7.0, 7.5 and 8.5; lane 5–8, the supernatant containing RpiB at pH 6.5, 7.0, 7.5 and 8.5 (B).

Table 1
The RpiB purification from 250 mL of the *E. coli* cell culture.

Fraction (mg)	Vol. (mL)	Protein (mg/mL)	Total protein (mg)	Sp. act. (U/mg)	Total act. (U)	Yield (%)
Cell lysate	35	1.57	55	187	10296	100
Purified RpiB	34	0.33	11.2	290	3254	31.7

Sp. act., specific activity was measured in 50 mM Tris–HCl (pH 7.0) at 50 °C.

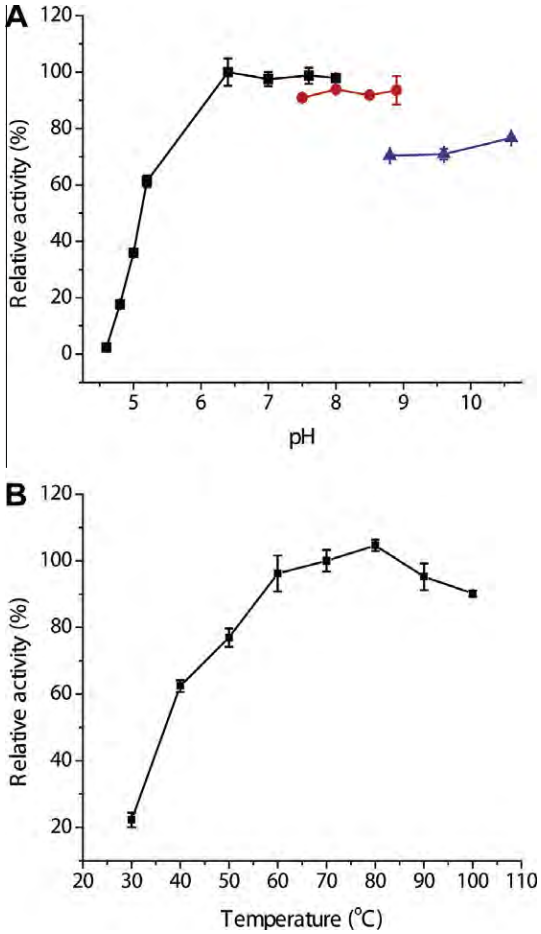

Fig. 2. The profiles of pH (A) and temperature (B) of the purified RpiB. Black square symbols represent activities measured in the sodium dihydrogen phosphate–citric acid at pH 4.6, 4.8, 5.0, 5.2, 6.4, 7.0, 7.6 and 8.0; red cycle symbols represent activities in the Tris–HCl buffer at pH 7.5, 8.0, 8.5 and 9.0; and blue triangle symbols represent activities in the glycine–NaOH buffer at pH 8.8, 9.6 and 10.6.

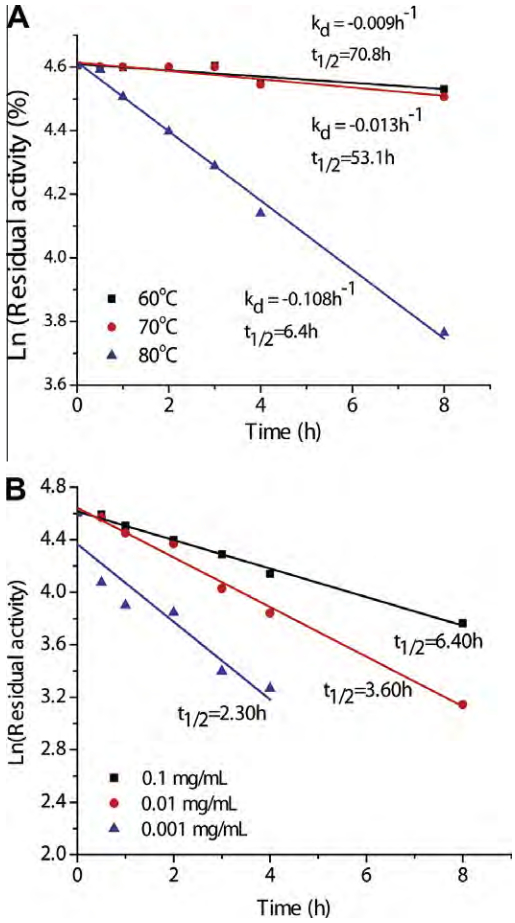

Fig. 3. Thermo-inactivation of the purified RpiB (0.1 mg/mL) at 60, 70 and 80 °C in 50 mM Tris–HCl buffer (pH 7.0) (A), and different concentration RpiB from 0.001 to 0.1 mg/mL at 80 °C in 50 mM Tris–HCl buffer (pH 7.0) (B). k_d, degradation of constant; $T_{1/2}$, half lifetime.

The kinetic parameters of RpiB were examined at 60, 70 and 80 °C (Table 2). The k_{cat} value increased from 540 ± 6 to 1192 ± 88 s^{-1} when the temperatures increased from 60 to 80 °C. The K_m value ranged around 7.6–12.1 mM (Table 2).

Thermostability

Thermostability of RpiB in 50 mM of Tris–HCl buffer (pH 7.0) strongly depended on temperature (Fig. 3A). RpiB at 0.10 mg/mL was very stable at 60 and 70 °C but it deactivated at 80 °C. The degradation constants were −0.009, −0.013 and −0.108 h^{-1}, at 60, 70 and 80 °C, respectively. That is, it had half lifetimes of 70.8, 53.1 and 6.4 h at 60, 70 and 80 °C, respectively.

Also, the half lifetime of RpiB at 80 °C greatly depended on its mass concentration from 0.10, 0.010 to 0.0010 mg/mL (Figure 3B). The half-life time of thermo-inactivation was about 6.4 h at 0.10 mg/mL but decreased to only 2.3 h at a low concentration of 0.0010 mg/mL. Under its suboptimal condition (pH 7.0, 60 °C, and 0.10 mg/mL), this enzyme had the turn-over number (TTN) of 2.2×10^8 mol of product per mol of enzyme, where TTN value can be calculated as TTN = k_{cat}/k_d [22,28].

Discussion

Heat treatment could be widely-used as the first step for protein purification. However, our study demonstrated to obtain high-purity RpiB through the optimization of heat treatment conditions – choice of buffer, buffer pH, treatment temperature, and treatment duration (Fig. 1). Therefore, it was feasible to obtain high-purity thermostable enzymes through temperature treatment only at very low costs.

The development in basic enzyme building blocks was urgently needed for the commercialization of in vitro synthetic biology projects [9–12]. Economic analysis suggested that enzyme costs in

Table 2
The kinetic characteristics of RpiB based on ribose-5-phosphate as the substrate.

Temp. (°C)	Conc. (mg/mL)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_d (h^{-1})	$T_{1/2}$ (h)	TTN (mol/mol)
60	0.10	540 ± 6	7.6 ± 0.2	71.1	0.009	71	2.2×10^8
70	0.10	767 ± 35	10.3 ± 1.0	74.5	0.013	53	2.1×10^8
	0.010				0.110	6.3	2.5×10^7
80	0.10	1192 ± 88	12.1 ± 1.9	98.5	0.108	6.4	3.9×10^7
	0.010				0.189	3.6	2.3×10^7

Table 3
Comparison of ribose-5-phosphate isomerases from different sources in the literature.

Organism	T_{opt} (°C)	Sp. act. (U/mg) (Condition)	Half lifetime (Condition)	Ref.
Arabidopsis thaliana	37	NA	1 h (50 °C)	[29]
Bacillus caldolyticus	NA	52.7 (65 °C, pH 6.5)	1 h (91 °C)	[30]
Clostridium thermocellum	65	25690 (65 °C, pH 7.5)	96 h (50 °C) 4.7 h (65 °C)	[31]
Escherichia coli	37	NA	2.2 min (60 °C)	[32]
Mycobacterium tuberculosis	37	NA	NA	[33]
Pichia jadinii	50	356 (50 °C, pH 7.5)	NA	[34]
Thermotoga maritima	80	290 (50 °C, pH 7.0)	70.8 (60 °C) 53.1 h (70 °C) 6.4 h (80 °C) 1.2 h (90 °C)	This study
Thiobacillus thioparus	NA	544 (40 °C, pH 6.5)	1 h (57 °C)	[30]

T_{opt}, optimal temperature of the enzyme; Sp. act., specific activity; NA, not available.

cascade enzyme factories could be minimal when all of the enzymes have TTN values of more than 10^7–10^8 mol of product per mol of enzyme [8,9,13]. In this study, another enzyme suitable for in vitro synthetic biology projects was obtained. As compared to other reported Rpi enzymes (Table 3), the *T. maritima* RpiB seemed to be a good building block, which had high activities at a broad temperature range and nearly neutral pH values as well as had great stability even without enzyme immobilization.

In summary, high-purity *T. maritima* RpiB was simply purified by optimized heat treatment and it exhibited high TTN values under its suboptimal conditions.

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References

[1] Y.-H.P. Zhang, What is vital (and not vital) to advance economically-competitive biofuels production, Proc. Biochem. 46 (2011) 2091–2110.
[2] A.J. Shaw, K.K. Podkaminer, S.G. Desai, J.S. Bardsley, S.R. Rogers, P.G. Thorne, D.A. Hogsett, L.R. Lynd, Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield, Proc. Natl. Acad. Sci. USA 105 (2008) 13769–13774.
[3] S. Atsumi, T. Hanai, J.C. Liao, Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels, Nature 451 (2008) 86–89.
[4] T. Liu, H. Vora, C. Khosla, Quantitative analysis and engineering of fatty acid biosynthesis in *E. coli*, Metab. Eng. 12 (2010) 378–386.
[5] D. Klein-Marcuschamer, P.K. Ajikumar, G. Stephanopoulos, Engineering microbial cell factories for biosynthesis of isoprenoid molecules: beyond lycopene, Trends Biotechnol. 25 (2007) 417–424.
[6] X. Ye, Y. Wang, R.C. Hopkins, M.W.W. Adams, B.R. Evans, J.R. Mielenz, Y.-H.P. Zhang, Spontaneous high-yield production of hydrogen from cellulosic materials and water catalyzed by enzyme cocktails, Chem. Sus. Chem. 2 (2009) 149–152.
[7] Y.-H.P. Zhang, B.R. Evans, J.R. Mielenz, R.C. Hopkins, M.W.W. Adams, High-yield hydrogen production from starch and water by a synthetic enzymatic pathway, PLoS One 2 (2007) e456.
[8] W.D. Huang, Y.-H.P. Zhang, Analysis of biofuels production from sugar based on three criteria: thermodynamics, bioenergetics, and product separation energy, Environ. Sci. 4 (2011) 784–792.
[9] Y.-H.P. Zhang, Simpler is better: high-yield and potential low-cost biofuels production through cell-free synthetic pathway biotransformation (SyPaB), ACS Catal. 1 (2011) 998–1009.
[10] Y.-H.P. Zhang, Production of biocommodities and bioelectricity by cell-free synthetic enzymatic pathway biotransformations: challenges and opportunities, Biotechnol. Bioeng. 105 (2010) 663–677.
[11] J.R. Swartz, Transforming biochemical engineering with cell-free biology, AIChE J. 58 (2011) 5–13.
[12] C. Eric Hodgman, M.C. Jewett, Cell-free synthetic biology: thinking outside the cell, Metab. Eng. (2012), doi:10.1016/j.ymben.2011.1009.1002.
[13] Y. Wang, W. Huang, N. Sathitsuksanoh, Z. Zhu, Y.-H.P. Zhang, Biohydrogenation from biomass sugar mediated by *in vitro* synthetic enzymatic pathways, Chem. Biol. 18 (2011) 372–380.
[14] K. Ishikawa, I. Matsui, F. Payan, C. Cambillau, H. Ishida, Y. Kawarabayasi, H. Kikuchi, A. Roussel, A hyperthermostable D-ribose-5-phosphate isomerase from *Pyrococcus horikoshii* characterization and three-dimensional structure, Structure 10 (2002) 877–886.
[15] B.L. Horecker, P.Z. Smyrniotis, J.E. Seegmiller, The enzymatic conversion of 6-phosphogluconate to ribulose-5-phosphate and ribose-5-phosphate, J. Biol. Chem. 193 (1951) 383–396.
[16] B. Hove-Jensen, M. Maigaard, *Escherichia coli* rpiA gene encoding ribose phosphate isomerase A, J. Bacteriol. 175 (1993) 5628–5635.
[17] K.I. Sorensen, B. Hove-Jensen, Ribose catabolism of *Escherichia coli*: characterization of the rpiB gene encoding ribose phosphate isomerase B and of the rpiR gene, which is involved in regulation of rpiB expression, J. Bacteriol. 178 (1996) 1003–1011.
[18] J.H. Huck, N.M. Verhoeven, E.A. Struys, G.S. Salomons, C. Jakobs, M.S. van der Knaap, Ribose-5-phosphate isomerase deficiency: new inborn error in the pentose phosphate pathway associated with a slowly progressive leukoencephalopathy, Am. J. Human Genet. 74 (2004) 745–751.
[19] A.K. Roos, E. Burgos, D.J. Ericsson, L. Salmon, S.L. Mowbray, Competitive inhibitors of *Mycobacterium tuberculosis* ribose-5-phosphate isomerase B reveal new information about the reaction mechanism, J. Biol. Chem. 280 (2005) 6416–6422.
[20] R.G. Zhang, C.E. Andersson, T. Skarina, E. Evdokimova, A.M. Edwards, A. Joachimiak, A. Savchenko, S.L. Mowbray, The 2.2 A resolution structure of RpiB/AlsB from *Escherichia coli* illustrates a new approach to the ribose-5-phosphate isomerase reaction, J. Mol. Biol. 332 (2003) 1083–1094.
[21] M.R. Banki, L. Feng, D.W. Wood, Simple bioseparations using self-cleaving elastin-like polypeptide tags, Nat. Methods 2 (2005) 659–662.
[22] S. Myung, X.-Z. Zhang, Y.-H.P. Zhang, Ultra-stable phosphoglucose isomerase through immobilization of cellulose-binding module-tagged thermophilic enzyme on low-cost high-capacity cellulosic adsorbent, Biotechnol. Prog. 27 (2011) 969–975.
[23] R. Huber, T.A. Langworthy, H. Konig, M. Thomm, C.R. Woese, U.B. Sleytr, K.O. Stetter, Thermotoga maritima sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90 °C, Arch. Microbiol. 144 (1986) 324–333.
[24] Y. Wang, Y.-H.P. Zhang, Overexpression and simple purification of the *Thermotoga maritima* 6-phosphogluconate dehydrogenase in *Escherichia coli* and its application for NADPH regeneration, Microb. Cell Fact. 8 (2009) 30.
[25] Q. Xu, R. Schwarzenbacher, D. McMullan, F. von Delft, L.S. Brinen, J.M. Canaves, X. Dai, A.M. Deacon, M.-A. Elsliger, S. Eshagi, R. Floyd, A. Godzik, C. Grittini, S.K. Grzechnik, L. Jaroszewski, C. Karlak, H.E. Klock, E. Koesema, J.S. Kovarik, A. Kreusch, P. Kuhn, S.A. Lesley, I. Levin, T.M. McPhillips, M.D. Miller, A. Morse, K. Moy, J. Ouyang, R. Page, K. Quijano, A. Robb, G. Spraggon, R.C. Stevens, H. van den Bedem, J. Velasquez, J. Vincent, X. Wang, B. West, G. Wolf, K.O. Hodgson, J. Wooley, I.A. Wilson, Crystal structure of a ribose-5-phosphate isomerase RpiB (TM1080) from *Thermotoga maritima* at 1.90 Å resolution, Proteins 56 (2004) 171–175.
[26] Z. Dische, E. Borenfreund, A new spectrophotometric method for the detection and determination of keto sugars and trioses, J. Biol. Chem. 192 (1951) 583–587.
[27] A.L. Stern, E. Burgos, L. Salmon, J.J. Cazzulo, Ribose 5-phosphate isomerase type B from *Trypanosoma cruzi*: kinetic properties and site-directed mutagenesis reveal information about the reaction mechanism, Biochem. J. (2007) 279–285.
[28] T.A. Rogers, A.S. Bommarius, Utilizing simple biochemical measurements to predict lifetime output of biocatalysts in continuous isothermal processes, Chem. Eng. Sci. 65 (2010) 2118–2124.

[29] Y. Xiong, C. DeFraia, D. Williams, X. Zhang, Z. Mou, Deficiency in a cytosolic ribose-5-phosphate isomerase causes chloroplast dysfunction, late flowering and premature cell death in *Arabidopsis*, Physiol. Plant 137 (2009) 249–263.
[30] C.R. Middaugh, R.D. MacElroy, The effect of temperature on ribose-5-phosphate isomerase from a mesophile, *Thiobacillus thioparus*, and a thermophile, *Bacillus caldolyticus*, J. Biochem. 79 (1976) 1331–1344.
[31] R.Y. Yoon, S.J. Yeom, H.J. Kim, D.K. Oh, Novel substrates of a ribose-5-phosphate isomerase from *Clostridium thermocellum*, J. Biotechnol. 139 (2009) 26–32.
[32] M.K. Essenberg, R.A. Cooper, Two ribose-5-phosphate isomerases from *Escherichia coli* K12: partial characterisation of the enzymes and consideration of their possible physiological roles, FEBS J. 55 (1975) 323–332.
[33] S. Mariano, A.K. Roos, S.L. Mowbray, L. Salmon, Competitive inhibitors of type B ribose 5-phosphate isomerases: design, synthesis and kinetic evaluation of new D-allose and D-allulose 6-phosphate derivatives, Carbohydr. Res. 344 (2009) 869–880.
[34] G.F. Domagk, K.M. Doering, R. Chilla, Purification and properties of ribose-phosphate isomerase from *Candida utilis*, FEBS J. 38 (1973) 259–264.