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Fructose-1,6-bisphosphatase from a hyper-thermophilic bacterium *Thermotoga maritima*: Characterization, metabolite stability, and its implications

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ABSTRACT

Fructose-1,6-bisphosphatase gene from a hyperthermophilic bacterium *Thermotoga maritima* was cloned, and the recombinant protein was produced in *E. coli*, purified, and characterized. The fructose-1,6-bisphosphatase (FBPase) with a molecular mass of *ca.* 28 kDa was purified from the fusion protein cellulose-binding module (CBM)-intein-FBPase by affinity adsorption on regenerated amorphous cellulose followed by intein self-cleavage. The substrate fructose 1,6-bisphosphate was not stable at high temperatures, especially at high pHs. The degradation constants of fructose 1,6-bisphosphate, glucose-6-phosphate, and fructose-6-phosphate were determined at different temperatures (37, 60, and 80 °C) and pH 7.5 or 9.0. The k_{cat} and K_m values of FBPase were 8.57 s^{-1} and 0.04 mM at 60 °C, as well as 58.7 s^{-1} and 0.12 mM at 80 °C. This enzyme was very stable at its suboptimal temperatures, with half-life times of *ca.* 1330 and 55.6 h at 60 and 80 °C, respectively. At 60 °C, this enzyme had an estimated total turn-over number of 20,500,000 (mol product/mol enzyme) and weight-based total turn-over umber of 192,000 (kg product/kg enzyme), respectively. These results indicated that this enzyme would be a stable building block for cell-free synthetic pathway biotransformation (SyPaB) that can implement complicated biochemical reactions. In order to obtain high-yield desired products, we suggest that over-addition or over-expression of the enzymes responsible for converting easily degraded metabolites should be important to prevent unnecessary metabolite loss for *in vitro* or *in vivo* synthetic pathway design.

1. Introduction

Fructose-1,6-bisphosphatase (EC 3.1.3.11, FBPase), a key enzyme of gluconeogenesis, catalyzes the hydrolysis of fructose-1,6-bisphosphate (F16P) to fructose-6-phosphate (F6P) and inorganic phosphate (P_i) [1–4]. By contrast, phosphofructokinase (EC 2.7.1.11) is responsible for catalyzing the reverse reaction—phosphorylation of F6P to F16P at a cost of ATP in the glycolysis pathway. Both unidirectional enzymes work closely to regulate sugar metabolisms. Recently, cell-free synthetic enzymatic pathway transformations (SyPaB) have been designed and demonstrated to produce nearly 12 moles of hydrogen from per mole of glucose equivalent of polysaccharides and water [5,6]. In these pathways, FBPase has an important role in regenerating glucose-6-phosphate in the pentose phosphate pathway. In order to prolong lifetime of enzymes and increase reaction rates at elevated temperatures, the developments in thermostable enzymes as building blocks are in high demands [7–9].

Hyperthermophiles and thermophiles are great resources for isolation and discovery of thermostable enzymes for potential applications in molecular biology and industrial biocatalysis [8,10,11]. *Thermotoga maritima* is a rod-shaped bacterium, originally isolated from geothermal heated marine sediments. This thermophilic organism has an optimum growth temperature of ~80 °C and can utilize many simple and complex carbohydrates, including glucose, sucrose, starch, and xylan [12]. *T. maritima* is regarded as an invaluable source of intrinsically thermostable enzymes [12–14]. In 1999, most open reading frames (ORF) in the *T. maritima* genome were annotated but FBPase was not identified [15]. TM1415 encoding inositol monophosphate (IMPase, EC 3.1.3.25) for *myo*-inositol biosynthesis has been characterized [16]. Later, Stec et al. found out that several thermophilic inositol monophosphatases (IMP) have FBPase activities, including *T. maritima* IMPase [17]. But the kinetics parameters of the *T. maritima* FBPase are not available. Thermostable FBPase would play an important role in producing low-cost hydrogen from renewable sugars mediated by cell-free synthetic pathway biotransformation [5–7]. Great opportunities are driving us to discover, produce,

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and characterize thermophilic enzymes in the synthetic pathways [9,18].

Little attention has been paid pertaining to stability of metabolites in hyperthermophiles, although intensive efforts have been made to study the stability mechanisms of thermoenzymes at elevated temperature [11,19–22]. Studies of degradation kinetics of labile metabolites would be important to understand how hyperthermophiles prevent degradation of labile metabolites and minimize degradation of metabolites *in vivo* [7,23], and to re-construct cell-free synthetic enzymatic pathways at high temperatures [7,8].

In this study, the FBPase gene from *T. maritima* was cloned and over-expressed in *E. coli* and the recombinant protein was purified through affinity adsorption on a cellulosic adsorbent, followed by intein cleavage. It was found that the substrate fructose 1,6-bisphosphate degraded quickly at high temperatures, especially at high pH. Basic biochemical properties of the purified FBPase were characterized. Our results suggested that some basic properties of the published thermophilic FBPase may be re-checked.

2. Materials and methods

2.1. Chemicals and strains

All chemicals were regent grade, purchased from Sigma–Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Avicel PH105, microcrystalline cellulose, was purchased from FMC (Philadelphia, PA). Regenerated amorphous cellulose (RAC) with a high adsorption capacity was made from Avicel through cellulose slurrying in water, cellulose dissolution in concentrated phosphoric acid, and cellulose regeneration in water [24]. The *T. maritima* genomic DNA was purchased from the American Type Culture Collection (Manassas, VA). *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA, USA) containing a protein expression plasmid was used for producing the recombinant protein. The Luria-Bertani (LB) medium was used for *E. coli* cell growth and recombinant protein expression. Ampicillin (100 μg/mL) was added in the *E. coli* media. The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA).

2.2. Protein expression plasmid construction

The pCIF plasmid encoding the CBM-intein-FBPase (CIF) fusion protein was prepared based on the pCIG plasmid which consists of CBM-intein-GFP fusion protein [25]. The DNA fragment containing the ORF TM1415 was amplified by PCR with a pair of primers 5′-AGG ACT CCT CGA GAT GGA CAG ACT GGA CTT TTC-3′ (XhoI site underlined) and 5′-GTT GCA CCT GCA GTC ACT TTC CTC CTA TTT CTT CTA CC-3′ (PstI site underlined). After XhoI and PstI digestion of the PCR product and plasmid pCIG, the ligated product was transformed to *E. coli* JM109, yielding plasmid pCIF. The DNA sequence of pCIF was validated by sequencing by Virginia Bioinformatics Institute (Blacksburg, VA).

2.3. Recombinant protein production and purification

Two hundred milliliters of the LB culture in 1-L Erlenmeyer flasks were incubated at 18 or 37 °C with a rotary shaking rate of 250 rpm. The recombinant protein expression was induced by adding IPTG (0.01 mM, final concentration) when the absorbance (A_{600}) reached *ca*. 0.6. The culture was incubated at 37 °C for 4 h or 18 °C for 23 h. The cells were harvested by centrifugation at 4 °C, washed once with 50 mM of Tris–HCl buffer (pH 7.5), and re-suspended with a 10 mL of 30 mM Tris–HCl buffer (pH 8.5) containing 0.5 M of NaCl and 1 mM of EDTA. The cell pellets were lysed by ultra-sonication by Fisher Scientific Sonic Dismembrator Model 500 (5-s pulse on and off, total 180 s, at 20% amplitude). After centrifugation, the supernatant of cell lysate containing the fusion protein CBM-intein-FBPase was purified through affinity adsorption on a large surface area RAC [26], followed by intein cleavage [25]. Two mL of RAC (4 mg/mL) was mixed with the 15 mL of cell lysate at room temperature for 10 min. After adsorption, the mixture was centrifuged by 6000 rpm at 4 °C for 5 min. The pellet was suspended with 20 mL of 50 mM of Tris–HCl buffer (pH 8.5) to remove other proteins. After centrifugation, the pellets were washed with 20 mL of 50 mM HEPES buffer (pH 6.5) containing 0.5 M NaCl. After centrifugation, the RAC slurry was suspended with 5 mL of 50 mM HEPES buffer (pH 6.5) containing 0.5 M NaCl at 40 °C for 9 h. After centrifugation, the cleaved FBPase was obtained in the supernatant.

2.4. FBPase assays

The enzyme activity was measured in 50 mM HEPES buffer containing 10 mM $MgCl_2$ and 20 mM F16P at 60 °C. The hydrolytic product inorganic phosphate (P_i) was measured by using the Sahiki method assayed at mild pH [27]. Ten μL of the enzyme solution was mixed with 100 μL of the molybdate reagent, followed by 25 μL of the 10% ascorbic acid. After the reaction at 30 °C for 20 min, the absorbance was read at 850 nm. The temperature effects on FBPase activities (1.8 mg/mL) were studied at pH 7.5 and 9.5 for 10 min. The kinetics of the purified protein was determined in 50 mM HEPES (pH 7.5) containing 10 mM $MgCl_2$ and different F16P concentrations at different temperatures. The effects of the substrate degradation were subtracted from product formation for the kinetics calculation.

Determination of half-life time of FBPase. Thermostability of the purified FBPase (0.225 mg/mL) was studied in 100 mM of HEPES buffer (pH 7.5) containing 150 mM of NaCl and 10 mM of $MgCl_2$ at 60 and 80 °C, respectively. The residual FBPase activity was measured as described above.

2.5. Degradation of hexose phosphate

Ten mM of the substrate (F16P, glucose-6-phosphate (G6P), or F6P) was used in presence of 10 mM Mg^{2+} in 50 mM HEPES buffer (pH 7.5) or sodium carbonate buffer (pH 9.5) at 37, 60, and 80 °C. The degradation product phosphate was measured by the mild pH phosphate assay method as described above.

3. Results

3.1. Expression and purification

The fusion protein CBM-intein-FBPase was over-expressed in *E. coli* BL21(DE3)/pCIF under different experimental conditions, such as protein expression temperature of 18 *vs* 37 °C, IPTG concentration of 0.01, 0.02, 0.1 or 1 mM, and different induction time. It was found that the inclusion body formation was drastically reduced at 18 °C as compared to 37 °C (data not shown). Increasing IPTG concentration did not significantly improve the expression of soluble CIF (data not shown). Fig. 1 shows SDS-PAGE analysis of the recombinant protein expression at 18 °C with 0.01 mM IPTG, as well as of protein purification. The cell lysate containing the fusion protein CBM-intein-FPBase was mixed with an ultra-high adsorption capacity regenerated amorphous cellulose. After centrifugation, washing, and intein self-cleavage at a high salt buffer (pH 6.5), the cleaved FBPase in the aqueous phase appeared homogeneous (Fig. 1, lane P). Approximately 30 mg of the purified FBPase

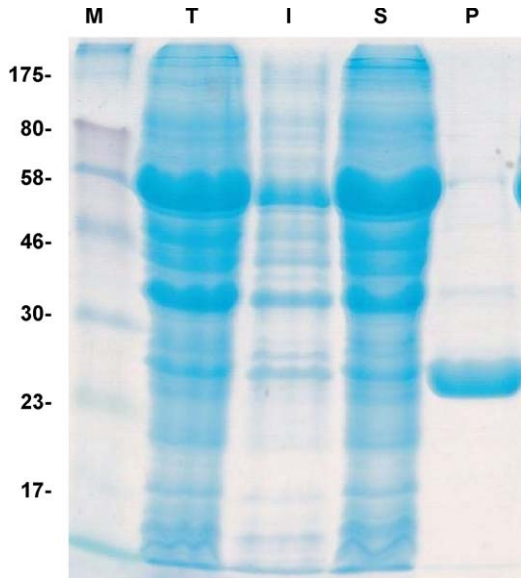

Fig. 1. 5% SDS-PAGE analysis of the FPBase purification. M, protein markers; T, total fraction of cell lysate; I, insoluble protein fraction; S, soluble fraction of the cell lysate; and P, the purified FBPase in the supernatant after its self-cleavage. The molecular weights of the CBM-intein-FBPase and cleaved FBPase are 65 and 28 kDa, respectively. ~5 μg of the protein was loaded per well and the protein in the gel was stained by the Bio-safe Coomassie blue. The insoluble cell pellets were re-suspended in a 10 mL of 30 mM Tris–HCl buffer (pH 8.5) containing 0.5 M of NaCl and 1 mM of EDTA, and denatured by boiling with the SDS-PAGE buffer.

Table 1
The FBPase purification from 200 mL of the *E. coli* cell culture.

Fraction	Vol. (mL)	Protein (mg/mL)	Sp. Act. (U/mg)	Activity (U/mL)	Total act. (U)	Recovery (%)	Purif. fold
Cell lysate	11	6.82	2.16	14.73	162.0	100	1
Purified protein	5	1.27	12.13	15.41	77.1	47.6	5.62

Sp. Act., specific activity.

were obtained per liter of the culture (Table 1). The FBPase recovery and purification fold were 47.6% and 5.62, respectively.

3.2. Degradation of hexose phosphates

It was found that a significant amount of inorganic phosphate was spontaneously generated from F16P in the absence of FBPase, especially at high temperatures and high pH. In order to eliminate the influence of the substrate degradation on FBPase activity assays, the kinetics of F16P degradation was measured. Fig. 2 presented the first-order degradation kinetics of F16P at 37, 60 and 80 °C at pH 7.5 (A) and pH 9.0 (B). At pH 7.5, the degradation constant increased greatly from −0.00038 to −0.0476 h^{-1} by 125 fold when temperature increased from 37 to 80 °C. The similar trend occurred with the cases of pH 9.0. Fructose 1,6-biphophate degraded faster at pH 9.0 than at pH 7.5. Table 2 presents the degradation constants of two other hexose phosphates (G6P and F6P). Obviously, F6P and G6P were more stable than F16P under the tested conditions.

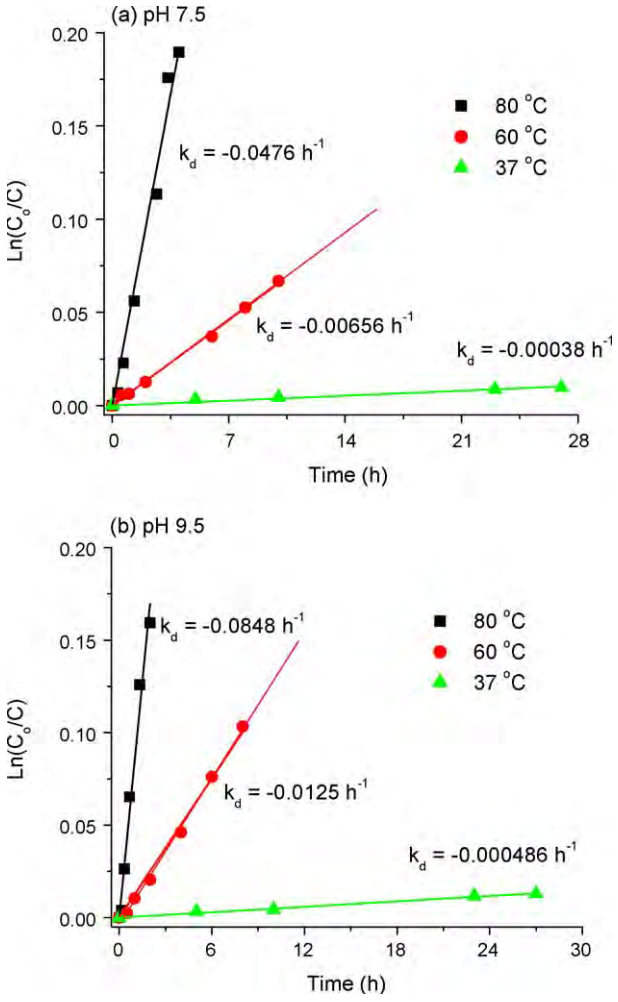

Fig. 2. Degradation kinetics of fructose 1,6-bisphosphate at pH 7.5 (a) and pH 9.0 (b). k_d, first-order degradation constant of F16P = ln(C/C_0)/time.

3.3. FBPase properties

Generally, a divalent metal ion, such as $Mg^{2+}$and Mn^{2+}, is required for activities of thermophilic FBPase [1,2] and IMPase [16,28]. Ten mM Mg^{2+} was added for all FBPase activity assays. Fig. 3 shows the pH profile of FBPase activity at 60 °C. FBPase had the maximum activities at pH 9.0–9.5, and was relatively active at pH 7.5–8.0. When pHs were higher than 9.5, large differences between the specific activities of FBPase and total phosphate release were observed due to F16P degradation at high pHs.

Fig. 4 shows the temperature effects on FBPase activities at pH 7.5 and 9.5. When temperature increased, free phosphate ions increased rapidly due to F16P degradation, especially at pH 9.5. At pH 7.5, FBPase showed the maximum specific activity of 26.6 U/mg at 90 °C. FPBase had higher specific activities at pH 9.5 than at pH 7.0. FBPase had a highest specific activity of 29.8 U/mg at pH 9.5, although it appeared not to be stable at a high pH and high temperature.

Table 3 presents the kinetic constants of the FBPase at different temperatures (37, 60, 80, and 95 °C) at pH 7.5. The FBPase exhibited increased k_{cat} values from 1.35 to 62.9.1 s^{-1} when the temperatures increased from 37 to 95 °C. At 95 °C, the specific activity of this FBPase was 62.9 s^{-1}, only *ca.* one quarter of the value that was previously published [17]. This large difference could be attributed to whether the influence of the labile substrate degradation was included. This enzyme's activity at 37 °C was only 2.2% of its maximum activity at 95 °C.

3.4. Thermostability

The FBPase is highly thermostable in 100 mM of HEPES buffer (pH 7.5) containing 150 mM NaCl and 10 mM $MgCl_2$ (Fig. 5). The

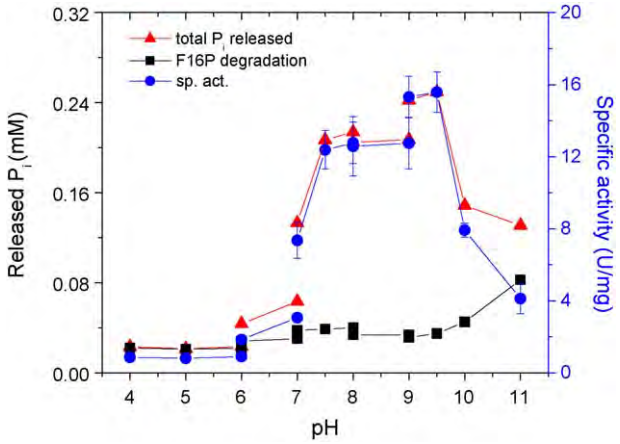

Fig. 3. Effect of pH on the FBPase activity. The specific activity of FBPase was measured by 20 mM F16P in the presence of 10 mM $MgCl_2$ at 60 °C for 10 min. The buffers were citric buffer (pH 4–6), Tris–HCl buffer (pH 6–7), HEPES buffer (pH 7–8), TAPS buffer (pH 8–9), and sodium carbonate buffer (pH 9–11). Error bar means the standard deviation of the duplicate measurements.

Table 2
Substrate degradation constants at different pHs and temperatures.

Substrate	pH	Temp. (°C)	k_d (h^{-1})	R^2
Fructose 1,6-bisphosphate (F16P)	pH 9.5	80	-8.48×10^{-2}	0.990
		60	-1.25×10^{-2}	0.998
		37	-4.86×10^{-4}	0.995
	pH 7.5	80	-4.76×10^{-2}	0.993
		60	-6.56×10^{-3}	0.999
		37	-3.80×10^{-4}	0.989
Fructose 6-phosphate (F6P)	pH 9.5	80	-2.52×10^{-2}	0.984
		60	-7.10×10^{-3}	0.998
		37	-4.30×10^{-4}	0.988
	pH 7.5	80	-1.43×10^{-2}	0.999
		60	-1.35×10^{-3}	0.997
		37	-2.09×10^{-4}	0.916
Glucose 6-phosphate (G6P)	pH 9.5	80	-3.89×10^{-3}	0.989
		60	-8.29×10^{-4}	0.727
		37	ND	–
	pH 7.5	80	-2.20×10^{-3}	0.999
		60	-2.99×10^{-4}	0.966
		37	ND	–

k_d, first-order degradation constant; R^2, regression-squared value; and ND, not determined.

degradation constants were −0.00052 and −0.0125 h^{-1}, at 60 and 80 °C, respectively. That is, it had half lifetimes of 1330 and 55.5 h at 60 and 80 °C. Although FBPase at 60 °C was not as active as that at 80 °C, it had much longer lifetime. In potential practical operations at its half maximum velocity, total turn-over number and weight-based total turn-over number of FBPase were estimated be 8.57 s^{-1}/2 × 3600 s/h × 1330 h = 20,500,000 mol product per mol enzyme and 19,600,000 × 262/28,000 = 192,000 kg product per kg enzyme, respectively.

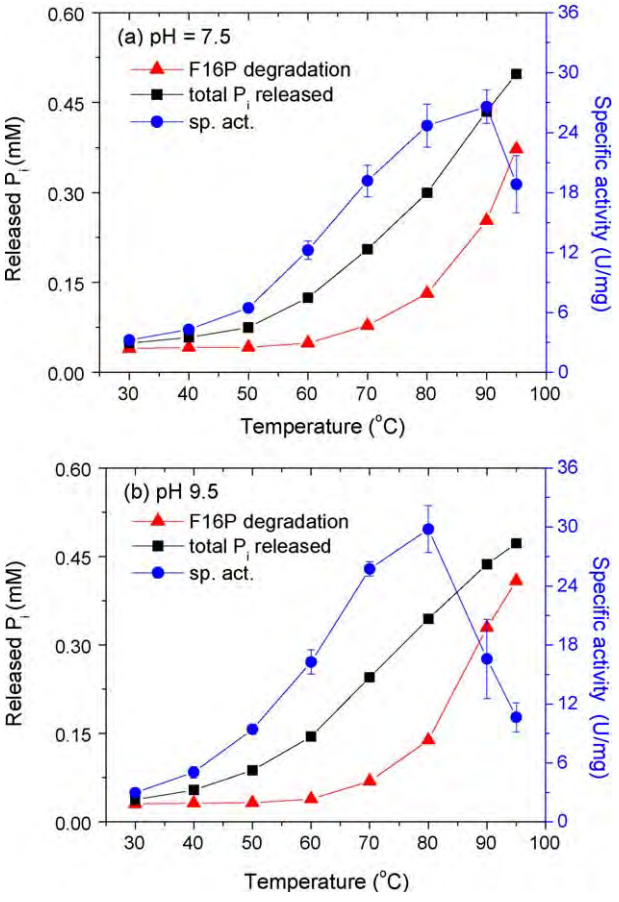

Fig. 4. The temperature profile of the FBPase at pH 7.5 (A) and pH 9.5 (B). Error bar means the standard deviation of the duplicated experiments.

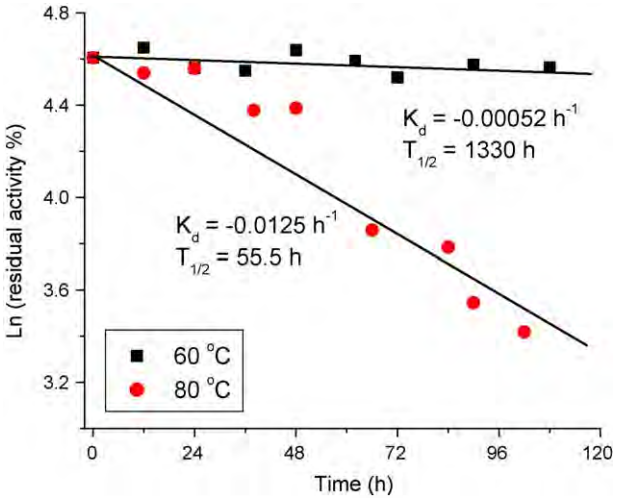

Fig. 5. Thermal stability of the FBPase at 60 (●) and 80 °C (■). k_d, degradation constant of FBPase; and $T_{1/2}$, a half lifetime of FBPase.

4. Discussion

Thermostable enzymes from thermophilic microorganisms are playing important roles in molecular biology and industrial applications [8,10]. The production of recombinant proteins from thermophilic organisms in mesophilic hosts (e.g. *Escherichia coli*)

Table 3
The kinetic characteristics of FBPase at different temperatures (pH 7.5).

Temp. (°C)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
95[a]	0.07 ± 0.004	62.9 ± 0.7	899
80[b]	0.12 ± 0.01	58.7 ± 3.3	489
60[c]	0.038 ± 0.0015	8.5 ± 1.1	226
37[d]	0.29 ± 0.02	1.36 ± 0.21	4.7

K_m, Michaelis–Menten constant; k_{cat}, reaction rate constant.
[a] Enzyme concentration = 0.113 mg/L, time = 5 min.
[b] Enzyme concentration = 0.225 mg/L, time = 5 min.
[c] Enzyme concentration = 0.90 mg/L, time = 5 min.
[d] Enzyme concentration = 4.5 mg/L, time = 10 min.

Table 4
Kinetic characteristics of the FBPases from different organisms.

Organism	Temp. (°C)	pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	Reference
Thermotoga maritima	95	8.0	–	268	–	[17]
	95	7.5	0.07	62.9	899	This study
	60	7.5	0.04	8.5	213	This study
Pyrococcus furiosus	85	8.0	0.32	5.7	17.7	[1]
	50	8.0	0.31	0.35	1.12	[1]
Thermococcus kodakaraensis	95	8.0	0.1	17	170	[2]
Corynebacterium glutamicum	30	7.5	0.014	3.2	236	[3]
Escherichia coli	30	7.8	0.015	14.6	973	[4]

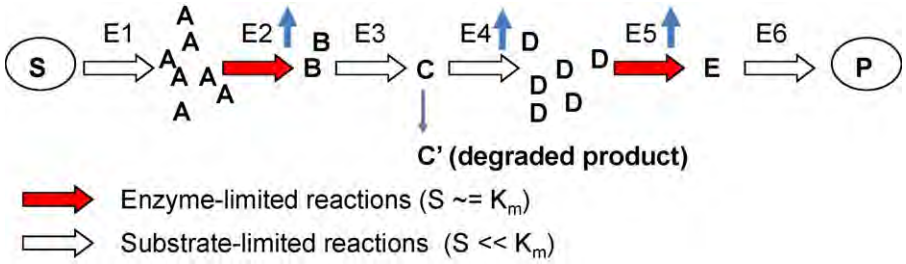

Fig. 6. Scheme of enzymatic pathway containing enzyme-limited reactions and substrate-limited reactions. S, an initial substrate; P, a final product; E1–E6, enzymes; A–E, each metabolites.

can take the advantage of easy fermentation, over-expression and simplified purification [18]. The economic analysis suggests that cell-free SyPaB requires each enzyme building block with a weight-based total turn-over number of more than 20,000 so that it might be cost-competitive with microbial fermentation [8]. The hyperthermostable FBPase from *T. maritima* showed long half-life times of 1330 and 55.5 h under its suboptimal conditions of 60 and 80 °C, respectively. At 60 °C, one kg of FBPase is estimated to produce ~195,000 kg product before it loses its half activity, suggesting that this enzyme is stable enough for the production of low-value biofuels (such as, hydrogen and alcohols) through cell-free SyPaB [8]. As shown in Table 4, the *T. maritima* FBPase was among the active enzymes. As compared to other thermostable enzymes [1,2], the *T. maritima* FBPase was the most active, suggesting its great potential for the applications at high reaction temperatures [7].

The control steps of metabolic flux are distributed among several enzymes in most pathways (Fig. 6). For example, E2 and E5 are responsible for enzyme-limited reactions, resulting in accumulation of metabolites A and D, respectively. Based on traditional metabolic engineering principles, increasing enzyme E2&E5 concentrations through over-expression would increase the overall metabolic flux. Here we suggested the supplementary rule for enhancing high-product yields that improvement of the enzymes capabilities or performances responsible for converting a labile metabolite C should be important to increase the yield of desired products when the unstable metabolite C can be degraded to a by-product C′. In nature, it was speculated that living biological entities could over-express certain enzymes to catalyze labile metabolites and avoid metabolite loss. For example, since dihydroxyacetone phosphate (DHAP) is famous for its rapid degradation, the respective enzyme triphosphate isomerase (TIM) has evolved to a kinetically perfect enzyme. A combination of high-catalytic efficiency TIM, its over-expression or its high local concentrations (micro-compartmentation effect) results in a very low level of DHAP intracellularly, i.e., fast turn-over rates [29]. This theory may be applied to explain why hyperthermophilic microorganisms need stabilize heat-sensitive metabolites, such as F16P. It has been suggested that labile NADPH [30,31] must be recycled efficiently for an extreme thermoacidophile *Picrophilus torridus* due to its short halftime of 1.7 min at pH 4.5 and 65 °C [32].

For cell-free SyPaB that can produce biocommodities with theoretically high yields [8,33], we suggested another supplementary design principle that the enzymes that are responsible for converting labile metabolites to more stable metabolites should be over-loaded although these enzymes are not responsible for the enzyme-limited reactions (Fig. 6). In addition to over-addition of these enzymes, the construction of cascade enzyme complex that allows metabolite channeling that one product of this first enzyme is transferred to the second enzyme without complete equilibrium with the bulk phase may be another efficient solution. In nature, metabolite channeling may be another important mechanism for preventing degradation of labile metabolites, such as tryptophan synthetase [34], carbomoyl-phosphate synthase [35], fatty acid synthase [36], and so on.

In a word, another thermostable building block for cell-free SyPaB projects—*T. maritima* fructose 1,6-bisphosphatase was obtained. Along with the previously obtained stable enzyme *T. maritima* 6-phosphogluconate dehydrogenase [18] and *Clostridium thermocellum* phosphoglucomutase [9], it suggested that discovery of thermostable building blocks from (hyper)thermophiles are very operative. Also, we suggest that the degradation of labile metabolites in synthetic pathways can be addressed either by over-addition of the respective enzymes or construction of the enzyme complex.

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