Appl Microbiol Biotechnol
DOI 10.1007/s00253-011-3458-1

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

One-step purification and immobilization of thermophilic polyphosphate glucokinase from *Thermobifida fusca* YX: glucose-6-phosphate generation without ATP

Hehuan Liao · Suwan Myung · Y.-H. Percival Zhang

Received: 25 May 2011 / Revised: 17 June 2011 / Accepted: 19 June 2011

Abstract The discovery of stable and active polyphosphate glucokinase (PPGK, EC 2.7.1.63) would be vital to cascade enzyme biocatalysis that does not require a costly ATP input. An open reading frame Tfu_1811 from *Thermobifida fusca* YX encoding a putative PPGK was cloned and the recombinant protein fused with a family 3 cellulose-binding module (CBM-PPGK) was overexpressed in *Escherichia coli*. Mg^{2+} was an indispensible activator. This enzyme exhibited the highest activity in the presence of 4 mM Mg^{2+} at 55°C and pH 9.0. Under its suboptimal conditions (pH 7.5), the k_{cat} and K_m values of CBM-PPGK on glucose were 96.9 and 39.7 s^{-1} as well as 0.77 and 0.45 mM at 37°C and 50°C respectively. The thermoinactivation of CBM-PPGK was independent of its mass concentration. Through one-step enzyme purification and immobilization on a high-capacity regenerated amorphous cellulose, immobilized CBM-PPGK had an approximately eightfold half lifetime enhancement (i.e., $t_{1/2}$=120 min) as compared to free enzyme at 50°C. To our limited knowledge, this enzyme was the first thermostable PPGK reported. Free PPGK and immobilized CBM-PPGK had total turnover number values of 126,000 and 961,000 mol product per mol enzyme, respectively, suggesting their great potential in glucose-6-phosphate generation based on low-cost polyphosphate.

Keywords Enzymatic building block · Cascade enzyme reaction · One-step purification and immobilization · Polyphosphate glucokinase · Synthetic pathway biotransformation (SyPaB) · *Thermobifida fusca*

H. Liao · S. Myung · Y.-H. P. Zhang (✉)
Biological Systems Engineering Department,
Virginia Polytechnic Institute and State University (Virginia Tech),
210-A Seitz Hall,
Blacksburg, VA 24061, USA
e-mail: ypzhang@vt.edu

S. Myung · Y.-H. P. Zhang
Institute for Critical Technology and Applied Science (ICTAS),
Virginia Polytechnic Institute and State University,
Blacksburg, VA 24061, USA

Y.-H. P. Zhang
DOE BioEnergy Science Center (BESC),
Oak Ridge, TN 37831, USA

Y.-H. P. Zhang
Gate Fuels Inc.,
3107 Alice Drive,
Blacksburg, VA 24060, USA

Introduction

Polyphosphate glucokinase (PPGK) or polyphosphate–glucose phosphotransferase (E.C.2.7.1.63) is responsible for transferring a terminal phosphate group from polyphosphate [poly(P_i)] to glucose for yielding glucose 6-phosphate (Hsieh et al. 1996a),

$$\text{glucose} + \text{poly}(P_i)(DP = n) \xrightarrow{\text{PPGK}} \text{glucose} - 6 - \text{phosphate} + \text{poly}(P_i)(DP = n - 1)$$

where DP is the degree of polymerization of $\text{poly}(P_i)_n$, ranging from tens or up to thousands. Because $\text{poly}(P_i)_n$ can be found in abundance in volcanic condensates and deep oceanic steam vents, ancient organisms may have utilized $\text{poly}(P_i)_n$ instead of adenosine triphosphate (ATP) in their metabolisms without the participation of the ATP–ADP system (Hsieh et al. 1996a; Mukai et al. 2003).

$\text{Poly}(P_i)_n$ can be produced by dehydration of phosphoric acid at an elevated temperature or through biological linkage by polyphosphate-accumulating microorganisms in

Published online: 16 July 2011

wastewater treatment processes (Kuroda et al. 2001). Because it is less costly and highly stable as compared to other phosphoryl donor compounds, such as creatine phosphate, phosphoenolpyruvate (PEP), and acetate phosphate (Wang and Zhang 2009a), polyphosphate is an attractive phosphoryl donor for ATP generation in preparative-scale organic synthesis and biocatalysis (Hoffman et al. 1988; Iwanoto et al. 2007; Resnick and Zehander 2000; Zhang et al. 2011).

The PPGK was firstly discovered in *Mycobacterium phlei* in 1957 (Szymona 1957). Later these enzymes were found in a variety of microorganisms (Phillips et al. 1999) such as mycobacterial species (Szymona 1978), *Nocardia minima* (Szymona 1979), *Corynebacterium diphtheria* (Szymona 1961), *Propionibacterium shermanii* (Pepin and Wood 1986), *Mycococcus coralloudes* (Gonzales et al. 1990), *Microlunatus phosphovorus* (Tanaka et al. 2003), and *Arthrobacter* sp. (Mukai et al. 2003). All reported PPGKs are isolated from mesophilic sources and they deactivate so fast that they cannot be used for potential enzyme-mediated biocatalysis. For example, PPGK from *Arthrobacter* sp. loses ~50% of its activity in 5 min at 40°C (Mukai et al. 2003).

Thermophiles are great resources for isolation and discovery of thermostable enzymes for potential industrial applications (Myung et al. 2010; Vieille and Zeikus 2001; Wang and Zhang 2009b). *Thermobifida fusca* YX is a rod shaped, aerobic, moderate thermophilic, filamentous, and gram-positive soil bacterium. It has an optimal temperature of 55°C and can grow at a broad pH range of 4–10 (McGrath and Wilson 2006; Wilson 2004). This microbe can degrade plant cell walls in heated organic materials, such as compost heaps, rotting hay, and manure piles, by secreting numerous extracellular cellulases and hemicellulases. Most of its cellulases and hemicellulases have been extensively studied (Li et al. 2010; McGrath and Wilson 2006; Vuong and Wilson 2009; Wilson 2004). The genomic sequence of *T. fusca* YX has been sequenced by the Joint Genome Institute of the Department of Energy and most open reading frames (ORFs) have been annotated (Lykidis et al. 2007). It has a single circular chromosome of 3,642,249 bp predicted to encode 3,110 proteins and 65 RNA species with a coding density of 85% (Lykidis et al. 2007). Among them, an ORF Tfu_1811was annotated to encode a putative PPGK but its function has not been determined or characterized yet. It is speculated that the *T. fusca* YX PPGK would have high stability suitable for biocatalysis.

In this study, the ORF Tfu_1811 encoding a putative PPGK was cloned and the recombinant protein was expressed in *E. coli* BL21 Star (DE3), purified, and characterized. To facilitate its easy purification and immobilization, a family 3 cellulose-binding module (CBM) was added on its N-terminal. As a result, the recombinant protein was purified as a fusion protein through one-step purification and immobilization on regenerated amorphous cellulose (RAC). The immobilized CBM-PPGK exhibited enhanced half lifetime in thermoinactivation, as compared to free enzyme. This CBM-PPGK would have great potential in cell-free biocatalysis without costly ATP.

Materials and Methods

Chemicals and strains All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Sodium hexametaphosphate with an averaged degree of polymerization of 17 was 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). Microcrystalline cellulose—Avicel PH105—was purchased from FMC (Philadelphia, PA). RAC with a high external binding capacity was prepared through sequential steps: water slurrying, cellulose dissolution in H_3PO_4, and regeneration in water as described elsewhere (Zhang et al. 2006). *T. fusca* YX genomic DNA was a gift from Dr. David Wilson at Cornell University (Ithaca, NY). *E. coli* DH5α was used as a host cell for DNA manipulation; *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) was used as a host strain for recombinant protein expression. The Luria–Bertani (LB) medium with 100 μg/mL ampicillin was used for *E. coli* cell growth and recombinant protein expression.

Plasmids construction Plasmids pC-ppgk and pCI-ppgk expressing the fusion proteins CBM-PPGK and CBM-intein-PPGK were constructed by replacing the *gfp* gene fragment with the *T. fusca ppgk* gene (ORF Tfu1811) based on plasmids pCG (Hong et al. 2008b) and pCIG (Hong et al. 2008a), respectively (Fig. 1). The *T. fusca ppgk* DNA fragment was amplified by polymerase chain reaction (PCR) using a forward primer (5′-GCATC CTCGAG ATGGCATCTC GGGGACGGGT-3′, XhoI site underlined) and a reverse primer (5′-GCTAT GGATCC TCAGGCA GAG ACCCGGTCAC, BamHI site underlined) based on the *T. fusca* genomic DNA. The PCR products after double digestion of XhoI/BamHI were ligated with the XhoI/BamHI-digested pCG and pCIG vectors for yielding plasmids pC-ppgk and pCI-ppgk, respectively. The plasmid sequences were validated by DNA sequencing.

Recombinant protein expression and purification The plasmids pC-ppgk and pCI-ppgk were transformed into *E. coli* BL21 (DE3) for yielding *E. coli* BL21 (pC-ppgk) and *E. coli* BL21 (pCI-ppgk), respectively. The strains *E. coli* BL21 (pC-ppgk) and *E. coli* BL21 (pCI-ppgk) were

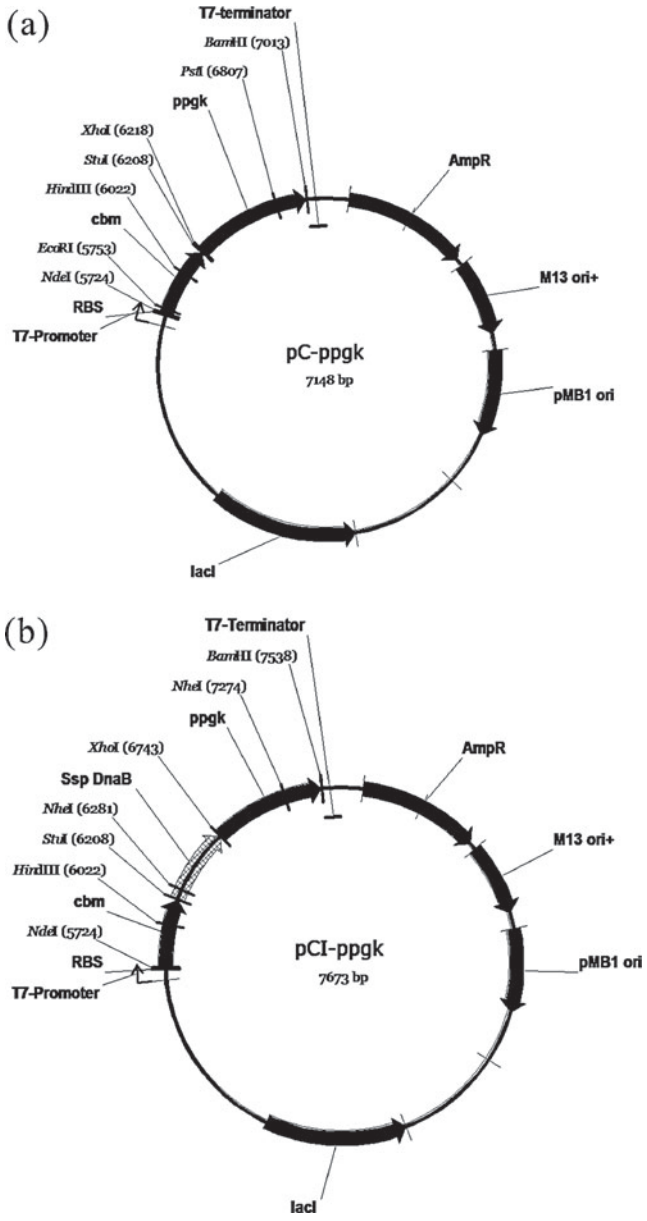

Fig. 1 Plasmid maps of plasmid pC-ppgk expressing CBM-PPGK (**a**) and plasmid pCI-ppgk expressing CBM-intein-PPGK (**b**)

cultivated in 250 mL of LB medium supplemented with 100 μg/mL ampicillin at 37°C, with a rotary shaking rate of 250 rpm. Isopropyl-beta-D-thiogalactopyranoside (100 μM) was added to the culture when A_{600} reached ~0.8, and then cultivation temperature was decreased to 18°C for 16 h. For the purification of CBM-PPGK, the cells were harvested by centrifugation at 4°C, washed twice in 50 mM Tris–HCl buffer (pH 7.5), and re-suspended in 20 mL of 50 mM Tris–HCl buffer (pH 8.5). The cell pellets were lysed by Fisher Scientific Sonic Dismembrator Model 500 (3-s pulse, total 300 s, at 50% amplitude) in an ice bath. After centrifugation, the supernatant was used for purification through affinity adsorption on RAC followed by ethylene glycol (EG) elution (Hong et al. 2008b). Five milliliters of 10 g/L RAC was mixed with 20 mL of the cell lysate at room temperature for 15 min followed by centrifugation. The RAC pellet was suspended in 20 mL of 50 mM Tris–HCl buffer (pH 8.5) to remove impure proteins in RAC matrix. After centrifugation, the RAC pellets containing the adsorbed CBM-PPGK were suspended in 4 RAC pellet volumes of 100% EG (i.e., the final EG concentration was ~80% *v*/*v*). After centrifugation, the purified CBM-PPGK was obtained from the supernatant.

For the purification of CBM-intein-PPGK, the cells were harvested by centrifugation at 4°C, washed twice in 50 mM Tris–HCl buffer (pH 7.5), and re-suspended in 20 mL of 50 mM Tris–HCl buffer (pH 8.5). The cells were lysed by sonication. After centrifugation, the supernatant was used for purification through affinity adsorption on RAC followed by intein self-cleavage (Hong et al. 2008a). After centrifugation, the cleaved CBM-free PPGK was collected from the supernatant.

One-step PPGK purification and immobilization For the preparation of immobilized CBM-PPGK (iCBM-PPGK), the cell lysate containing CBM-PPGK was mixed with RAC at a mass ratio of ~300 mg of CBM-PPGK per gram of RAC, followed by 15-min incubation at room temperature (Myung et al. 2011). The pellets were washed twice in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5). The washed pellets were suspended in 50 mM HEPES buffer (pH 7.5) containing 4 mM Mg^{2+}, being the iCBM-PPGK slurry. The mass protein concentration for iCBM-PPGK was determined by the modified ninhydrin assay (Zhu et al. 2009).

Optimization of CBM-PPGK conditions The effects of magnesium ion on CBM-PPGK activity were optimized in 50 mM HEPES buffer (pH 7.5) supplemented with a different magnesium sulfate concentration of 1, 2, 3, 4, 5, 6, 8, and 10 mM. The pH effects were tested in 50 mM citric acid/sodium citrate buffer (pH 4.0, 5.0, and 6.0), HEPES buffer (pH 7.0, 7.5, and 8.0), as well as glycine–NaOH (pH 8.5, 9.0, 9.5, 10.0, 10.5, and 11) at 37°C. The temperature effects were tested from 20°C to 80°C in 50 mM glycine–NaOH (pH 9.0) containing 4 mM Mg^{2+}.

CBM-PPGK activity assay The CBM-PPGK activity was measured by using a discontinuous approach. CBM-PPGK activity was measured based on the generation of G6P from polyphosphate and glucose in a 50-mM HEPES buffer (pH 7.5) containing 4 mM Mg^{2+}, 5 mM D-glucose, and 1 mM polyphosphate (17 mM phosphate units) or 5 mM ATP at 50°C for 5 min. The formation of G6P was measured in 50 mM HEPES buffer, containing 1 mM NAD^{+}, and 0.5 U/mL glucose-6-phosphate-dehydrogenase

at room temperature for 15 min. An increase of absorbance at 340 nm due to the formation of NADH (ε 340= 6220 M^{-1} cm^{-1}) was measured by a UV spectrophotometer. One unit of CBM-PPGK activity was defined as 1 μmol of G6P generated per minute. The kinetics of CBM-PPGK was determined based on initial reaction rates, where reaction time was less than 5 min. The reactions were conducted in a 50-mM HEPES buffer (pH 7.5) or 50 mM glycine–NaOH (pH 9.0) containing 4 mM Mg^{2+}, 0.5 mM polyphosphate, and D-glucose (0.2–5 mM) at 30°C or 50°C. The enzyme concentration for the kinetic assay of was 0.191 μg/mL. All the reactions were conducted in duplicate in 5-mL glass tubes (12×75 mm, Fisher Scientific).

Other assays The remaining activities of the purified CBM-PPGK and iCBM-PPGK) on RAC were examined in 50 mM HEPES buffer (pH 7.5) containing 4 mM Mg^{2+} and 1 mg/mL bovine serum albumin (BSA) at 50°C. The residual CBM-PPGK activity was measured according to the CBM-PPGK activity assay as described above. The mass concentration of soluble protein was measured by the Bio-Rad-modified Bradford protein kit with BSA as a standard protein, as described elsewhere (Zhu et al. 2009). The purity of the enzymes was examined by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Tris–glycine buffer.

Results

Identification of a putative PPGK by using sequence analysis

The 789-nt ORF Tfu_1811 from *T. fusca* YX was designated to encode a putative PPGK (Lykidis et al. 2007). The deduced 262 amino acid PPGK has a molecular mass of 27,392. Homology analysis of protein sequence of this putative PPGK was performed by using BL2SEQ tool in Biology Workbench (http://workbench.sdsc.edu/). The amino acid sequence was found to be homologous with PPGK from *M. phosphovorus* (Tanaka et al. 2003) (61% identity over 255 amino acids), *Arthrobacter* sp. KM (Mukai et al. 2003, 2004) (59% identity over 244 amino acids), *P. shermanii* (Pepin and Wood 1986) (58% identity over 250 amino acids), and *Mycobacterium tuberculosis* H37Rv (Hsieh et al. 1996a, b, 1993) (55% identity over 249 amino acids).

Multiple sequence alignment of this putative *T. fusca* YX PPGK and its homologs clearly suggests that the key amino acid sequences responsible for substrate binding and catalysis are highly conserved (Fig. 2). 'Glucose' is suggested to be responsible for the binding of glucose. Five regions are suggested to be common motifs interacting within the vicinity of the ATP molecule: 'phosphate-1' and 'phosphate-2' motifs contacting the β-phosphates and γ-phosphates of ATP, the 'connect-1' and 'connect-2' motifs that are the two hinge regions at the interface between the subdomains, and an 'adenosine' motif that contacts the adenine ring of ATP. 'Phosphate-3' region is suggested to be responsible for the binding of polyphosphate. In the phosphate-3 region of the primary structure of *M. tuberculosis* PPGK, Trp 193, Trp 198, and several charged groups around this region (Lys 188, Glu 189, Lys 190, Asp 192, Lys 197, and Lys 200) have been reported to be vital for the binding of polyphosphate (Mukai et al. 2003; Phillips et al. 1999). For example, the site-directed mutagenesis of Trp 198 in the *M. tuberculosis* PPGK resulted in a complete loss of catalytic activity (Phillips et al. 1999). It was found that the phosphate-3 region of the *T. fusca* PPGK was highly similar with other homologous PPGK proteins.

Expression and purification of CBM-PPGK and PPGK

Two fusion proteins CBM-PPGK and CBM-intein-PPGK were overexpressed in *E. coli* BL21 (DE3) at 18°C. The expression level of CBM-intein-PPGK was much lower than that of CBM-PPGK (data not shown), suggesting that an insertion of intein had a negative effect of recombinant protein expression. This phenomenon was not reported before (Hong et al. 2008a; Wang and Zhang 2010). This result along with previous reports (Hong et al. 2008a; Wang and Zhang 2010) suggests whether intein influences fusion protein expression or not is case-by-case. The crude cell lysate containing CBM-PPGK and its supernatant were examined by SDS-PAGE (lanes 1 and 2, Fig. 3). Lanes 1 and 2 presented similar patterns, suggesting that most recombinant CBM-PPGK was soluble. Without the addition of CBM, the recombinant PPGK expressed in *E. coli* formed a significant amount of inclusion body (data not shown), suggesting that CBM was a useful protein-folding helper (Hong et al. 2008b; Murashima et al. 2005). Since the CBM-PPGK can be specifically bound on a high binding capacity cellulosic adsorbent—RAC, the other proteins without CBM remained in the supernatant after adsorption (lane 3, Fig. 3). After EG elution, the purified CBM-PPGK appeared to be a single band (Fig. 3, lane 4). Molecular mass of the CBM-PPGK is 45,300 based on the deduced amino acid sequence, close to the estimated value from 10% SDS-PAGE (Fig. 3). Approximately 22.8 mg CBM-PPGK was purified from 1 L of the cell culture with a purification yield of 24.9% (Table 1). This enzyme had a specific activity of 64.6 U/mg on polyphosphate at pH 7.5 and 50°C. This enzyme had a much lower activity of ~3.2 U/mg when 5 mM ATP was a substrate instead of

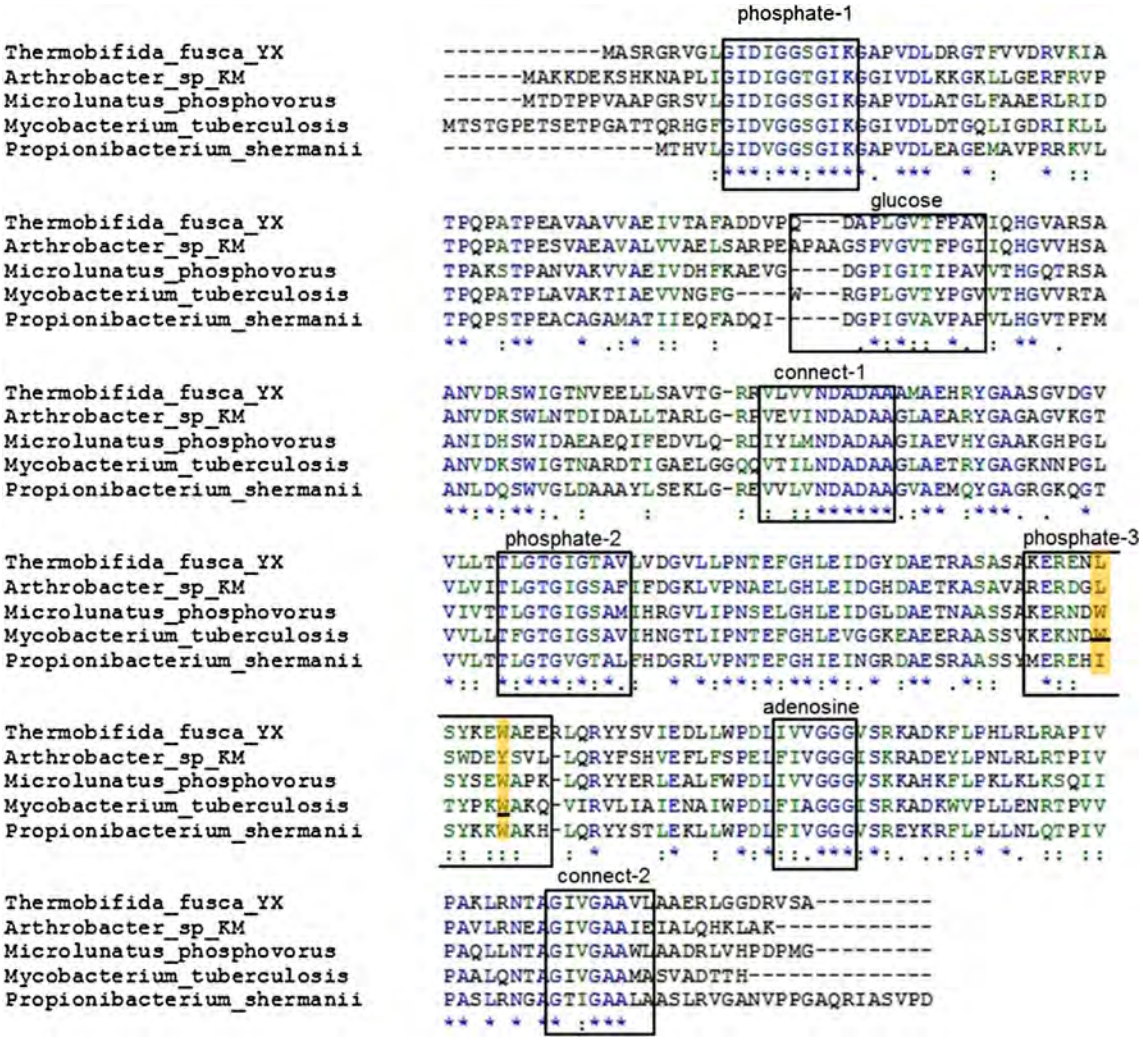

Fig. 2 Multiple sequence alignment of the primary structure of PPGK from *T. fusca* (YX) versus PPGKs from different organisms using a Clustalw (1.81) tool in Biology WorkBench. Seven conserved regions, the phosphate-1, phosphate-2, phosphate-3, connect-1, connect-2, glucose, and adenosine regions, are presented in boxes. The '*asterisk*' symbol indicates single, fully conserved residue; '*colon*' indicates conservation of strong groups; '*period*' indicates conservation of weak groups; and '*blank space*' indicates no consensus. Trp 193 and Trp 198 in the phosphate-3 region of PPGK from *M. tuberculosis* H37Rv are *underlined*. The amino acid residues aligned with Trp 193 and Trp 198 are *shaded*

polyphosphate. These results suggest that this enzyme has some promiscuous activities on ATP but prefers polyphosphate. Since most times the addition of CBM did not influence enzyme activity on soluble substrates (i.e., CBM-free PPGK had similar specific activities of CBM-PPGK, data not shown), we investigated the basic characteristics of CBM-PPGK in the following experiments.

Basic biochemical characterization

Because Mg^{2+} is an indispensible cofactor for known PPGKs, the effects of Mg^{2+} on CBM-PPGK activities were examined (Fig. 4a). CBM-PPGK enzyme activity increased sharply with an increase in Mg^{2+} and exhibited a maximum activity when the Mg^{2+} concentration was 4 mM. Higher Mg^{2+} levels decreased CBM-PPGK activity. These biphasic effects of Mg^{2+} on CBM-PPGK activities may be explained by the hypothesis that a proper concentration of Mg^{2+} was necessary for PPGK functions, while an excess of Mg^{2+} might be toxic, like most kinases (e.g., hexokinase). The pH profile of CBM-PPGK activity was examined at 37°C (Fig. 4b). This enzyme had the maximum activity at pH 8.5–9.0 and remained relatively active at pH 7.0–7.5 (~60% activity), whose pH range is compatible with most enzymes in cascade enzyme cocktails (Wang et al. 2011; Ye et al. 2009; Zhang et al. 2007). The temperature effect on CBM-PPGK activity was examined at pH 9.0 over a range of 20–80°C (Fig. 4c). The optimum temperature of CBM-PPGK was around 55°C, much higher than other PPGKs reported.

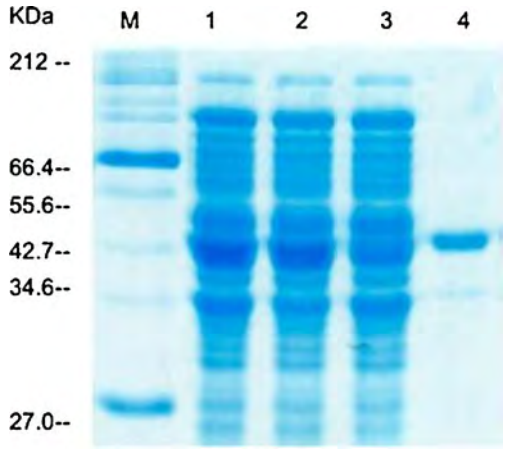

Fig. 3 10% SDS-PAGE analysis of CBM-PPGK purification. *Lane M*, protein marker; *1* total *E. coli* cell lysate containing CBM-PPGK; *2* the supernatant of the total *E. coli* cell lysate containing CBM-PPGK; *3* the supernatant of the total *E. coli* cell lysate after RAC adsorption; and *4* 1 μg of purified CBM-PPGK by ethylene glycol elution

The k_{cat} and K_m values of CBM-PPGK on glucose were 129±2 s^{-1} and 0.85±0.03 mM at 50°C and pH 9.0, respectively (Table 2). When pH was 7.5, k_{cat} decreased to 97±6 s^{-1} at 50°C. When its reaction temperature decreased to 30°C, k_{cat} was 39.7±0.1 s^{-1}, about ~40% of its activity at 50°C and pH 7.5 and ~31% of its maximum activity at 50°C and pH 9.0.

Free CBM-PPGK versus iCBM-PPGK

Most recombinant proteins produced in *E. coli* are usually purified based on their purification tags and then are immobilized on a solid support such as silica gel, alginate beads, or matrix. As compared to costly synthetic solid supports and relatively complicated chemical reactions for covalent immobilization, one-step purification and immobilization of a family 3 CBM-tagged enzyme on low cost, biodegradable, high capacity regenerated amorphous cellulose was developed previously (Myung et al. 2011). Without EG elution, the adsorbed CBM-PPGK on a large surface regenerated amorphous cellulose was iCBM-PPGK. CBM-oriented adsorption and immobilization can avoid random enzyme adsorption on the support, while such random adsorption may greatly decrease the activity of the

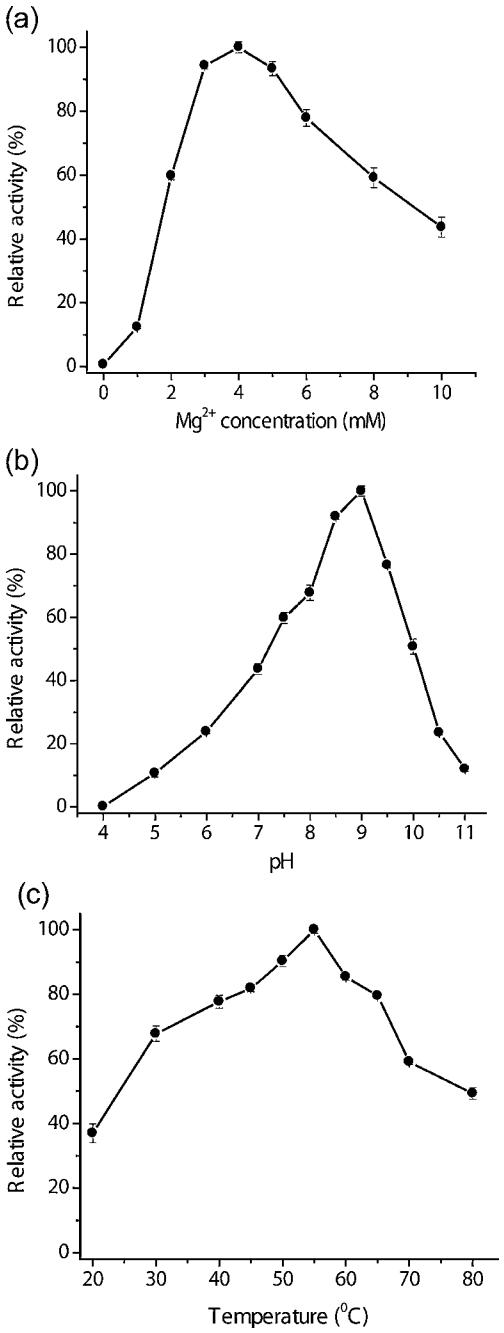

Fig. 4 Effects of Mg^{2+} concentration on the CBM-PPGK activity (**a**), of pH on the CBM-PPGK activity (**b**), and of temperature on the CBM-PPGK activity (**c**)

Table 1 The CBM-PPGK purification from 250 mL of the *E. coli* cell culture

Fraction	Vol. (mL)	Protein (mg/mL)	Total (mg)	Sp. act. (U/mg)	Total act. (U)	Yield (%)	Purif. fold
Cell lysate	23	3.2	73.6	20.1	1,479	100	1
Purified protein	5	1.14	5.7	64.6	368	24.9	3.2

Specific activity was measured in 50 mM HEPES (pH 7.5) containing 4 mM Mg^{2+} at 50°C

Vol. volume, *Sp. act.* specific activity, *total act.* total activity, *Purif. fold* purification fold

Table 2 The kinetic characteristics of CBM-PPGK and iCBM-PPGK

Enzyme	pH	Temp. (°C)	Glucose		
			K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (10^3 M^{-1} s^{-1})
CBM-PPGK	9.0	50	0.85±0.03	129±2	151
	7.5	50	0.77±0.03	96.9±6.1	126
	7.5	30	0.45±0.01	39.7±0.1	88.5
iCBM-PPGK	9.0	50	0.94±0.1	136±4	145
	7.5	50	0.80±0.02	92.6±1.0	116
	7.5	30	0.52±0.01	33.6±1.2	64.4

Temp. temperature, CBM cellulose-binding module, *PPGK* polyphosphate glucokinase, iCBM-PPGK immobilized CBM-PPGK

immobilized enzyme because the active site of the enzyme may be blocked by the support (Myung et al. 2011).

iCBM-PPGK had similar kinetic characteristics of free CBM-PPGK (Table 2). When temperature increased from 30°C to 50°C at pH 7.5, both of them exhibited approximately threefold increases in k_{cat}. Both CBM-PPGK and iCBM-PPGK showed an increase in k_{cat} values with a swift from pH 7.5 to 9.0. The above results suggested that the CBM-PPGK immobilization on RAC through simple affinity adsorption did not significantly change the CBM-PPGK performance.

Two concentrations of CBM-PPGK (0.01 and 0.1 g/L) had nearly similar residual activities (Fig. 5), suggesting that CBM-PPGK thermoinactivation was independent of its mass concentration, different from most enzymes (Liu et al. 2010; Myung et al. 2011; Wang and Zhang 2010). CBM-PPGK lost 50% of its activity after 15-min incubation at 50°C. Immobilized CBM-PPGK had approximately 70% activity of free counterpart, but it lost its activities much more slowly. Its half lifetime was prolonged to ca. 2 h for both 0.01 and 0.1 g/L iCBM-PPGK. Since the degradation

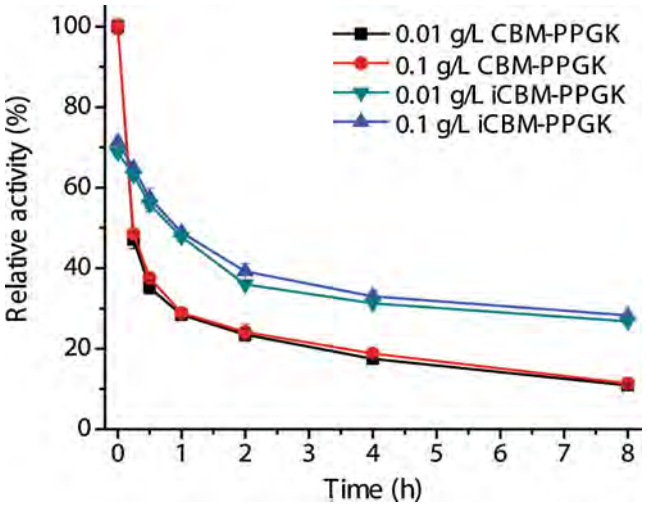

Fig. 5 Residual activities of CBM-PPGK and iCBM-PPGK at two different protein concentrations. Enzymes were incubated at 50 mM HEPES buffer (pH 7.5) containing 4 mM Mg^{2+} and 1 mg/mL BSA solution at 50°C

constant (k_d) can be calculated by $k_d = \ln 2/t_{1/2}$, k_d values for CBM-PPGK and iCBM-PPGK were estimated to be 2.77 and 0.347 h^{-1}, respectively, at pH 7.5 and 50°C. Therefore, total turnover number (TTN) values for CBM-PPGK and iCBM-PPGK were estimated to be 126,000 and 961,000 mol product per mol enzyme at pH 7.5 and 50°C, respectively, according to TTN = k_{cat}/k_d.

Discussion

An ORF Tfu_1811 from the thermophilic bacterium *T. fusca* was cloned. Two forms of recombinant proteins (CBM-PPGK and PPGK) were expressed in *E. coli* BL21 (DE3) and purified through affinity adsorption on a high binding capacity RAC. CBM-PPGK had a high specific activity of 64.6 U/mg on polyphosphate, while exhibited only a weak activity on ATP, suggesting that it is a polyphosphate-preferred enzyme. This CBM-PPGK is the first thermostable PPGK reported. This enzyme had higher optimal temperature and better stability than other PPGKs (Table 3). Free CBM-PPGK had a half lifetime of 15 min at 50°C, while its half lifetime was prolonged to 2 h after simple adsorption on

Table 3 A comparison of basic properties of different PPGKs

Organism	$T_{opt.}$ (°C)	Sp. act. (U/mg) (conditions)[a]	Half lifetime (conditions)	References
Arthrobacter sp.	45	220 (pH 7.0, 30°C)	5 min (pH 7.5, 40°C)	(Mukai et al. 2003)
M. phlei	N. A.	N. A.	24 h (pH 3.5, 3°C)	(Szymona and Ostrowski 1964)
M. phosphovorus	30	N. A.	<5 min (pH 7.5, 25°C)	(Tanaka et al. 2003) This study
M. tuberculosis	N. A.	203 (pH 7.5, 30°C)	N. A.	(Hsieh et al. 1996b)
P. shermanii	N. A.	15.3 (pH 7.5, 30°C)	N. A.	(Pepin and Wood 1986)
T. fusca	55	64.6, free 48.1, immobilized (pH 7.5, 50°C)	0.25 h, free 2 h, immobilized (pH 7.5, 50°C)	This study

[a] One unit of enzyme activity was defined as the production of 1 μmol of product per min

Sp. act. specific activity, *N. A.* not available

RAC. Free CBM-PPGK and immobilized CBM-PPGK had TTN values of 126,000 and 961,000 mol product per mol enzyme, respectively, indicating its great potentials in multienzyme-mediated biocatalysis.

Discovery and utilization of thermostable enzyme are playing an increasing important role in life science studies, industrial biocatalysis, and cell-free cascade enzyme-mediated biocatalysis. A lack of stable PPGK may restrict its potential in vitro biocatalysis that requires ATP supplies. Prior to cloning and expression of the *T. fusca* PPGK, we attempted to expressed the *M. phosphovorus* PPGK (MpPPGK), a strictly PPGK enzyme that cannot utilize ATP (Tanaka et al. 2003). The synthetic gene (accession number AB075018) made by DNA 2.0 was inserted into several T7 promoter plasmids with different protein folding tags, such as cellulose-binding module, thioredoxin, as used before (Hong et al. 2007; Wang and Zhang 2009b, 2010). Later, it was found that the MpPPGK lost its activity very fast at ambient temperature even in the presence of added salts, surfactants, and BSA (Wang and Zhang 2010) (data not shown).

Thermo-stable PPGK would have numerous applications in enzyme-mediated biotransformations from the production of biocommodities (e.g., hydrogen) to high-value products. For example, Woodward and his coworkers demonstrated the production of nearly 12 mol of hydrogen from per mole of glucose-6-phopshate and water by using an enzyme cocktail (Woodward et al. 2000) but this process cannot be scaled up due to high costs of G6P or ATP consumption for G6P generation (Zhang 2010; Zhang et al. 2010). Although 12 mol of hydrogen has been produced from per mol of glucose unit from starch or cellulosic materials without ATP input (Ye et al. 2009; Zhang et al. 2007), one glucose unit per polysaccharide cannot be utilized. The utilization of less costly polyphosphate rather than ATP made it possible to produce high-yield hydrogen at low costs without ATP. Another potential application may be cell-free protein synthesis, which required a large amount of ATP input. By integration of PPGK that can produce G-6-P from low-cost polyphosphate with enzymes in the glycolysis pathway in the *E. coli* cell lysate (Calhoun and Swartz 2005; Wang and Zhang 2009a), it could be possible to synthesize proteins from low-cost substrates rather than costly substrates such as creatine phosphate, PEP, and acetate phosphate.

One-step CBM-PPGK purification and immobilization was conducted by affinity adsorption of a CBM-fused protein on an ultra-large capacity adsorbent RAC. The advantages of this method included (a) improved expression of soluble CBM-PPGK, (b) integrated protein purification and immobilization, (c) the use of low-cost renewable biodegradable enzyme immobilization support—high binding capacity adsorbent RAC, and (d) easy scale-up protein purification by using simple solid/liquid unit operations (e.g., centrifugation).

Acknowledgments This work was not possible without support from the Biological Systems Engineering Department of Virginia Tech, the Air Force Office of Scientific Research (FA9550-08-1-0145), the USDA Biodesign and Bioprocess Center, and DOE BESC to YPZ. HL was partially supported by the China Scholarship Council. SM was partially supported by ICTAS through ICTAS Scholarship. We were grateful for the genomic DNA sample provided by Dr. David Wilson at Cornell University.

References

Calhoun KA, Swartz JR (2005) Energizing cell-free protein synthesis with glucose metabolism. Biotechnol Bioeng 90(5):606–613

Gonzales F, Fernandes-Vivas A, Arias JM, Montoya E (1990) Polyphosphate glucokinase and ATP glucokinase activities in *Mycococcus coralloides* D. Microbiology 154:438–442

Hoffman RJ, Wyman P, Smith L, Nolt C, Conley J, Hevel J, Warren J, Reiner G, Moe OJ (1988) Immobilized polyphosphate kinase: preparation, properties, and potential for use in adenosine 5′-triphosphate regeneration. Biotechnol Appl Biochem 10(2):107–117

Hong J, Ye X, Zhang Y-HP (2007) Quantitative determination of cellulose accessibility to cellulase based on adsorption of a nonhydrolytic fusion protein containing CBM and GFP with its applications. Langmuir 23(25):12535–12540

Hong J, Wang Y, Ye X, Zhang Y-HP (2008a) Simple protein purification through affinity adsorption on regenerated amorphous cellulose followed by intein self-cleavage. J Chromatogr A 1194(2):150–154

Hong J, Ye X, Wang Y, Zhang Y-HP (2008b) Bioseparation of recombinant cellulose binding module-protein by affinity adsorption on an ultra-high-capacity cellulosic adsorbent. Anal Chim Acta 621:193–199

Hsieh P-C, Shenoy BC, Jentofe JE, Phillips NFB (1993) Purification of polyphosphate and ATP glucose phosphotransferase from *Mycobacterium tuberculosis* H37Ra: evidence that poly(P) and ATP glucokinase activities are catalyzed by the same enzyme. Prot Expr Purif 4:76–84

Hsieh P-C, Kowalczyk TH, Phillips NFB (1996a) Kinetic mechanisms of polyphosphate glucokinase from *Mycobacterium tuberculosis*. Biochemistry 35:9772–9781

Hsieh P-C, Shenoy BC, Samols D, Phillips NFB (1996b) Cloning, expression, and characterization of polyphosphate glucokinase from *Mycobacterium tuberculosis*. J Biol Chem 271(9):4909–4915

Iwanoto S, Motomura K, Shinoda Y, Urata M, Kato J, Takiguchi N, Ohtake H, Hirota R, Kuroda A (2007) Use of an *Escherichia coli* recombinant producing thermostable polyphosphate kinase as an ATP regenerator to produce fructose 1,6-diphosphate. Appl Environ Microbiol 73(17):5676–5678

Kuroda A, Nomura K, Ohtomo R, Kato J, Ikeda T, Takiguchi N, Ohtake H, Kornberg A (2001) Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. Science 293(5530):705–708

Li Y, Irwin DC, Wilson DB (2010) Increased crystalline cellulose activity via combinations of amino acid changes in the family 9 catalytic domain and family 3c cellulose binding module of *Thermobifida fusca* Cel9A. Appl Environ Microbiol 76(8):2582–2588

Liu W, Zhang X-Z, Zhang Z-M, Zhang Y-HP (2010) Engineering of *Clostridium phytofermentans* endoglucanase Cel5A for improved thermostability. Appl Environ Microbiol 76(14):4914–4917

Lykidis A, Mavromatis K, Ivanova N, Anderson I, Land M, DiBartolo G, Martinez M, Lapidus A, Lucas S, Copeland A et al (2007)

Genome sequence and analysis of the soil cellulolytic actinomycete *Thermobifida fusca* YX. J Bacteriol 189(6):2477–2486

McGrath CE, Wilson DB (2006) Characterization of a *Thermobifida fusca* beta-1,3-glucanase (Lam81A) with a potential role in plant biomass degradation. Biochemistry 45:14094–14100

Mukai T, Kawai S, Matsukawa H, Matuo Y, Murata K (2003) Characterization and molecular cloning of a novel enzyme: inorganic polyphosphate/ATP-glucomannokinase, of *Arthrobacter* sp. strain KM. Appl Environ Microbiol 69(7):3849–3857

Mukai T, Kawai S, Mori S, Mikami B, Murata K (2004) Crystal structure of bacterial inorganic polyphosphate/ATP-glucomannokinase. J Biol Chem 279(48):50591–50600

Murashima K, Kosugi A, Doi RH (2005) Site-directed mutagenesis and expression of the soluble form of the family IIIa cellulose binding domain from the cellulosomal scaffolding protein of *Clostridium cellulovorans*. J Bacteriol 187(20):7146–7149

Myung S, Wang YR, Zhang Y-HP (2010) Fructose-1,6-bisphosphatase from a hyper-thermophilic bacterium *Thermotoga maritima*: characterization, metabolite stability and its implications. Process Biochem 45:1882–1887

Myung S, Zhang X-Z, Zhang Y-HP (2011) Ultra-stable phosphoglucose isomerase through immobilization of cellulose-binding module-tagged thermophilic enzyme on low-cost high-capacity cellulosic adsorbent. Biotechnol Prog. doi:10.1002/btpr.606

Pepin CA, Wood HG (1986) Polyphosphate glucokinase from *Propionibacterium shermanii*. J Bio Chem 261(10):4476–4480

Phillips NFB, Hsieh P-C, Kowalczyk TH (1999) Polyphosphate glucokinase. Prog Mol Subcell Biol 23:101–125

Resnick SM, Zehander AJB (2000) In vitro ATP regeneration from polyphosphate and AMP by polyphosphate: AMP phosphotransferase and adenylate kinase from *Acinetobacter johnsonii* 210A. Appl Environ Microbiol 66(5):2045–2051

Szymona M (1957) Utilization of inorganic polyphosphates for phosphprylation of glucose in *Mycobacterium phlei*. Bull Acad Pol Sci Ser Sci Biol 5:379–381

Szymona M (1961) Participation of volutin in the hexokinase reaction of Corynebacterium diphtheriae. Bull Acad Pol Sci Ser Sci Biol 9:371–372

Szymona M (1978) Multiple forms of polyphosphate-glucose phosphotransferase in various *Mycobacterium* strains. Acta Microbiol Pol 27:73–74

Szymona O (1979) Polyphosphate-and ATP-glucose phosphotransferase activities of *Nocardia minima*. Acta Microbiolog Pol 28(2):153–160

Szymona M, Ostrowski W (1964) Inorganic polyphosphate glucokinase of *Mycobacterium phlei*. Biochem Biophys Acta 85:283–295

Tanaka S, Lee S-O, Hamaoka K, Kato J, Takiguchi N, Nakamura K, Ohtake H, Kuroda A (2003) Strictly polyphosphate-dependent glucokinase in a polyphosphate-accumulating bacterium, Microlunatus phosphovorus. J Bacteriol 185(18):5654–5656

Vieille C, Zeikus GJ (2001) Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol Mol Biol Rev 65(1):1–43

Vuong TV, Wilson DB (2009) Processivity, synergism and substrate specificity of *Thermobifida fusca* Cel6B. Appl Environ Microbiol 71:6655–6661

Wang Y, Zhang Y-HP (2009a) Cell-free protein synthesis energized by slowly-metabolized maltodextrin. BMC Biotechnol 9:58

Wang Y, Zhang Y-HP (2009b) Overexpression and simple purification of the *Thermotoga maritima* 6-phosphogluconate dehydrogenase in *Escherichia coli* and its application for NADPH regeneration. Microb Cell Fact 8:30

Wang Y, Zhang Y-HP (2010) A highly active phosphoglucomutase from *Clostridium thermocellum*: cloning, purification, characterization, and enhanced thermostability. J Appl Microbiol 108:39–46

Wang Y, Huang W, Sathitsuksanoh N, Zhu Z, Zhang Y-HP (2011) Biohydrogenation from biomass sugar mediated by in vitro synthetic enzymatic pathways. Chem Biol 18:372–380

Wilson DB (2004) Studies of *Thermobifida fusca* plant cell wall degrading enzymes. Chem Rec 4:72–82

Woodward J, Orr M, Cordray K, Greenbaum E (2000) Enzymatic production of biohydrogen. Nature 405:1014–1015

Ye X, Wang Y, Hopkins RC, Adams MWW, Evans BR, Mielenz JR, Zhang Y-HP (2009) Spontaneous high-yield production of hydrogen from cellulosic materials and water catalyzed by enzyme cocktails. ChemSusChem 2(2):149–152

Zhang Y-HP (2010) Production of biocommodities and bioelectricity by cell-free synthetic enzymatic pathway biotransformations: challenges and opportunities. Biotechnol Bioeng 105:663–677

Zhang Y-HP, Cui J, Lynd LR, Kuang LR (2006) A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: evidence from enzymatic hydrolysis and supramolecular structure. Biomacromolecules 7(2):644–648

Zhang Y-HP, Evans BR, Mielenz JR, Hopkins RC, Adams MWW (2007) High-yield hydrogen production from starch and water by a synthetic enzymatic pathway. PLoS One 2(5):e456

Zhang Y-HP, Sun J-B, Zhong J-J (2010) Biofuel production by in vitro synthetic pathway transformation. Curr Opin Biotechnol 21:663–669

Zhang Y-HP, Myung S, You C, Zhu ZG, Rollin J (2011) Toward low-cost biomanufacturing through cell-free synthetic biology: bottom-up design. J Mater Chem. doi:10.1039/C1JM12078F

Zhu Z, Sathitsuksanoh N, Zhang Y-HP (2009) Direct quantitative determination of adsorbed cellulase on lignocellulosic biomass with its application to study cellulase desorption for potential recycling. Analyst 134:2267–2272