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Ultra-stable phosphoglucose isomerase through immobilization of cellulose-binding module-tagged thermophilic enzyme on low-cost high-capacity cellulosic adsorbent

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Running title: One-step protein purification and immobilization

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Abstract

One-step enzyme purification and immobilization was developed based on simple adsorption of a family 3 cellulose-binding module (CBM)-tagged protein on the external surface of high-capacity regenerated amorphous cellulose (RAC). An ORF Cthe0217 encoding a putative phosphoglucose isomerase (PGI, EC 5.3.1.9) from a thermophilic bacterium *Clostridium thermocellum* was cloned and the recombinant proteins with or without cellulose-binding module (CBM) were over-expressed in *E. coli*. The k_{cat} and K_m of CBM-free PGI at 60 ^{o}C were 2765 s^{-1} and 2.89 mM, respectively. PGI was stable at a high protein concentration of 0.1 g/L but deactivated rapidly at low concentrations. Immobilized CBM-PGI on RAC (iCBM-PGI) was extremely stable at ~60^{o}C, nearly independent of its mass concentration in bulk solution, because its local concentration on the solid support was constant. iCBM-PGI at a low concentration of 0.001 g/L had a half-life time of 190 h, approximately 80-fold of that of free PGI. Total turn-over number of iCBM-PGI was as high as 1.1×10^9 mole of product per mole of enzyme at 60 ^{o}C. These results suggest that a combination of low-cost enzyme immobilization and thermoenzyme led to an ultra-stable enzyme building block suitable for cell-free synthetic pathway biotransformation (SyPaB) that can implement complicated biochemical reactions *in vitro*.

Keywords: cell-free synthetic biology, cellulose-binding module, enzyme immobilization, phosphoglucose isomerase, protein purification, synthetic pathway biotransformation (SyPaB), thermoenzyme

Introduction

Biocatalysis mediated by enzymes is becoming more and more acceptable because of the high selectivity and specificity, modest reaction conditions, low energy consumption, and environmentally friendly conditions [1]. But a single enzyme cannot catalyze complicated biochemical reactions, such as complete oxidation of glucose, complete hydrolysis of lignocellulosic biomass, protein synthesis, and so on. Multiple enzymes in one pot and cell-free biotransformation/ biosynthesis mediated by numerous enzymes are gaining more attention [2-5]. Since enzymes cannot duplicate themselves, stabilization of enzymes is vital for economically viable industrial bioprocesses. Enzyme immobilization prolongs life-time of the enzyme and facilitates enzyme/product separation [6, 7].

Synthetic pathway biotransformation (SyPaB) is the implementation of complicated biochemical reactions by employing the *in vitro* assembly of a large number of enzymes and coenzymes [5, 8]. For example, nearly 12 moles of dihydrogen have been produced from per glucose equivalent of polysaccharides and water by the non-natural synthetic pathways containing 13-14 enzymes [9, 10], breaking the Thauer limit for anaerobic hydrogen-producing microorganisms [11]. SyPaB would be economically competitive with microbial fermentation for the production of biocommodities only when (i) all of the enzymes in the SyPaB systems have total turn-over number (TTN) values of more than 10^7-10^8 mole of product per mole of enzyme, and (ii) low-cost bulk enzyme production and purification are available [5, 8, 12]. SyPaB has numerous potential applications, such as the production of hydrogen, alcohols, and polyols from biomass sugars [8, 12], CO_2 utilization [13, 14], sugar battery [5], and so on. Therefore, it is vital to develop low-cost enzyme purification and immobilization technologies so that stabilized bulk enzymes would be Lego-like building blocks for SyPaB projects.

Cellulose-binding module (CBM), a key component of cellulase, is responsible for specifically binding to cellulose and increasing the reaction rates of enzymatic cellulose hydrolysis [15-17]. Several CBMs have been exploited as an affinity tag for the purification and immobilization of heterologous fusion proteins on cellulosic supports [16, 18-22]. Among more than 60 families of carbohydrate-binding modules, a family 3 CBM from the *Clostridium thermocellum* scaffoldin was chosen as a tag because it can tightly bind to both crystalline and amorphous cellulose [19, 23]. Cellulose is an ideal low-cost support for enzyme purification and immobilization because it is cheap, inert, stable, biodegradable, and readily available in different forms. Commercial microcrystalline cellulose (Avicel, cellulose nanocrystal) has a low binding capacity and more than 80% of its binding surface is internal [23]. In contrast, regenerated amorphous cellulose (RAC) is made from microcrystalline cellulose through cellulose dissolution in a cellulose solvent followed by cellulose regeneration in water [24]. RAC has a much higher binding capacity than cellulose nanocrystal, nano-cellulose fibers (e.g., bacterial microcrystalline cellulose), and cotton fibers [25]. In addition, all the binding capacity of RAC is external. The use of RAC for enzyme immobilization would bring three potential benefits as compared to Avicel: less cellulose use, higher volumetric enzyme concentration, and better substrate/product mass transfer.

Phosphoglucose isomerase (PGI, EC 5.3.1.9), a key enzyme in the glycolysis and glycogenesis pathways, can reversibly convert glucose-6-phosphate (G6P, an aldose phosphate) to fructose-6-phosphate (F6P, a ketose phosphate) [26]. For hydrogenesis mediated by SyPaB, PGI is responsible for regenerating glucose-6-phosphate from frutose-6-phosphate. Utilization of a rapidly expanding library of thermostable enzymes isolated from extremophiles is increasingly more important for molecular biology R&D and industrial

biocatalysis [5, 8, 27]. In this study, we cloned, expressed, and characterized the putative PGI from a thermophilic bacterium *Clostridium thermocellum*. To prolong the life-time of the *C. thermocellum* PGI and decrease enzyme purification and immobilization costs, one-step purification and immobilization was developed. A combination of thermoenzyme and simple immobilization led to an ultra-stable enzyme that can work at elevated temperatures.

Materials and Methods

Chemicals and microorganisms. 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 a series of steps – cellulose slurrying in water, cellulose dissolution in concentrated phosphoric acid, and cellulose regeneration in water [24]. The *C. thermocellum* ATCC 27405 genomic DNA was purchased from ATCC [28]. *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) 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 supplemented with 100 μg/mL ampicillin. The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). The liquid glucose reagent containing hexokinase/glucose-6-phosphate dehydrogenase was purchased from Pointe Scientific Inc. (Canton, MI).

Protein expression plasmid construction. Plasmid pCIP encoding the CBM-intein-PGI (CIP) and plasmid pCP encoding the CBM-PGI (CP) fusion protein were prepared based on plasmids pCIG [19] and the pCG [25], respectively. The ORF Cthe0217 encoding a putative PGI was amplified by PCR with a pair of primer 5'- CGG CCT CGA GAT GGA AAG AAT AAA ATT TGA C - 3` (XhoI site underlined) and 5'- CTA GCT GCA GTT ACA AAC GCT CTT C

-3' (PstI site underlined). After XhoI and PstI digestion of the PCR product and plasmid pCIG and pCG, the ligated products were transformed to *E. coli* JM109, yielding plasmids pCIP and pCP. The DNA sequences of pCIP and pCP were validated by sequencing service from the Virginia Bioinformatics Institute (Blacksburg, VA).

Recombinant protein expression and purification. Two hundred milliliters of the LB culture in 1-L Erlenmeyer flasks were incubated with a rotary shaking rate of 250 rpm at 37 ^{o}C. After the absorbance (A_{600}) reached *ca.* 0.6, the recombinant protein expression was induced by adding IPTG (0.1 mM, final concentration). The culture was incubated at 18 ^{o}C for 20 h. The cells were harvested by centrifugation at 4 ^{o}C, washed twice by 50 mM of Tris-HCl buffer (pH 7.5), and 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. The cell pellets were lysed by Fisher Scientific Sonic Dismembrator Model 500 (5-s pulse on and off, total 360 s, at 20% amplitude) in an ice bath. After centrifugation, the fusion protein CBM-intein-PGI or CBM-PGI was purified through affinity adsorption on RAC, followed by intein cleavage [19] or ethyl glycol elusion method [25]. Two milliliters of 4 g RAC/L was mixed with the 15 mL of the cell lysate at room temperature for 10 min. After adsorption, the mixture was centrifuged by 6,000 rpm at 4 ^{o}C for 5 min. The pellets were suspended with 20 mL of 50 mM Tris-HCl buffer (pH 8.5) to remove impure proteins, followed by centrifugation. For purification of PGI from CBM-intein-PGI, the pellet was 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 ^{o}C for 9 h. After centrifugation, the cleaved CBM-free PGI was obtained in the supernatant. For purification of CBM-PGI, the RAC pellets containing the adsorbed CBM-target protein were suspended in four RAC pellet volumes of 100% ethyl glycol (EG), i.e., the final EG concentration was ~80% (v/v). After by 10 min incubation at room temperature followed by

centrifugation, the purified CBM-PGI was obtained in the supernatant. Ethylene glycol was removed by dialysis against 50 mM tris-HCl buffer (pH 7.5) including 100 mM NaCl in an ice bath. The diluted purified protein was re-concentrated by using the Pall ultra-filtration centrifugal tubes.

PGI activity assay. Thermophilic PGI activity was measured by using a discontinuous approach since thermophilic glucose-6-phosphate dehydrogenase was not available. The initial reaction rates of PGI were measured based on the generation of the product G6P from the substrate F6P (Eq. 1). G6P was measured by using glucose-6-phosphate dehydrogenase (G6PDH) in the presence of NAD^+ (Eq. 2). For simplicity, the amount of G6P was measured by using the Pointe Scientific glucose assay kit (Canton, MI) and the absorbance of NADH was measured at 340 nm.

$$F6P \overset{PGI}{\leftrightarrow} G6P \quad [1]$$

$$G6P + NAD^+ \overset{G6PDH}{\leftrightarrow} 6-phosphogluconate + NADH \quad [2]$$

The PGI activity was measured in a 100 mM HEPES buffer containing 10 mM Mg^{2+}, 0.5 mM Mn^{2+}, and 5 mM F6P at 60 or 37 ^{o}C. The pre-warmed substrate solution and the PGI enzyme solution were mixed well and incubated for 5 min at the tested temperature. The reactions were stopped by boiling for 3 min. One mL of the reaction solution was mixed with 1.0 mL of the liquid glucose reagent at 37 °C for 3 min. The absorbance was read at 340 nm with a reference of the blank enzyme solution.

Adsorption and one-step purification and immobilization. The crude cell lysate containing CBM-PGI and other *E. coli* cellular proteins was mixed with RAC and Avicel at room temperature for 10 min. After centrifugation at 12,000 rpm for 3 min, the residual PGI activity in the supernatant was measured as described above. The maximum adsorption

capacity of RAC or Avicel (A_{max}) was calculated following the Langmuir isotherm as described elsewhere [23, 25]. For preparation of iCBM-PGI, the cell lysate was mixed with RAC at a ratio of ~300 mg of CBM-PGI per gram of RAC added. The pellets were washed twice in 100 mM of HEPES buffer (pH 7.5) followed by centrifugation. The washed pellets were suspended in the HEPES buffer containing 10 mM Mg^{2+} and 0.5 mM Mn^{2+}, being the iCBM-PGI slurry. The iCBM-PGI activity was measured as described above and the protein mass concentration was measured by the ninhydrin assay [29]. The leakage of iCBM-PGI was investigated following the below protocol. The iCBM-PGI slurry was suspended in 100 mM HEPES buffer (pH 7.5) containing 10 mM Mg^{2+} and 0.5 mM Mn^{2+} at 60 ^{o}C, followed by centrifugation at 12,000 rpm for 3 min. After the supernatant was decanted, the pellets were suspended in the HEPES buffer again. These washing steps were repeated 50 times. Small amounts of the suspended pellets were withdrawn for the PGI activity assay.

Thermostability of PGI and iCBM-PGI. The thermostability of the purified CBM-PGI and immobilized CBM-PGI were measured at different concentrations of enzyme (0.1, 0.01. 0.001 g/L) in 100 mM of HEPES buffer (pH 7.5) containing 150 mM of NaCl and 10 mM of $MgCl_2$ at 60 ^{o}C. The residual PGI activity was immediately measured according to the PGI activity assay as described above.

Other assays. Mass concentration of soluble protein was measured by the Bio-Rad modified Bradford protein kit with bovine serum albumin as a standard protein. 10% SDS-PAGE was performed in the Tris–glycine buffer as described elsewhere [13, 28].

Results

One-step purification and immobilization

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.

One-step purification, covalent immobilization, and additional stabilization of a poly-His-tagged-galactosidase by using heterofunctional chelate-epoxy sepabeads has been developed [30]. As compared to costly synthetic solid supports and relatively complicated chemical reactions for covalent immobilization, we proposed one-step purification and immobilization of a family 3 cellulose-binding module (CBM)-tagged enzyme on low-cost, biodegradable, high-capacity regenerated amorphous cellulose (Fig. 1). After recombinant protein expression and cell lysis, the CBM-tagged target protein can be specifically adsorbed onto RAC. After centrifugation followed by washing, other *E. coli* soluble proteins were removed. Finally, the pellets contained the immobilized purified protein on the external surface of RAC. This immobilization was conducted based on simple adsorption without chemical covalent bonds because later we found out that the relatively high ionic strength HEPES buffer did not wash the adsorbed enzyme from the surface of RAC. CBM-oriented adsorption and immobilzation can avoid random enzyme adsorption on the support. Such random adsorption may greatly decrease the activity of the immobilized enzyme because the active site of the enzyme may be blocked by the support.

Conversion of commercial Avicel by a cellulose solvent to RAC can greatly increase binding capacity by at least 20-fold, i.e., far more enzymes can be immobilized on the same weight cellulosic support. The adsorption profiles of the CBM-tagged PGI in the whole cell lysate were examined on RAC and Avicel. Clearly, their adsorptions obeyed the Langmuir isotherm (Fig. 2). The maximum binding capacity on RAC was as high as 483 mg protein per gram of RAC, nearly 54 times of that on Avicel (i.e., 8.88 mg per gram of Avicel). High enzyme immobilization capacity on RAC meant high volumetric enzyme loading. In addition, the externally-bound enzyme on the surface of RAC may lower mass diffusional hindrance.

Basic characteristics of PGI enzymes

Four different purified forms of PGI enzymes -- free CBM-free PGI, free CBM-PGI, and immobilized CBM-PGI (iCBM-PGI) on RAC and Avicel -- were obtained in this study. CBM-free PGI was produced by *E. coli* BL21(DE3)/pCIP ; CBM-PGI and immobilized CBM-PGI were produced by *E. coli* BL21(DE3)/pCP. It was noted that the adsorption of CBM-tagged enzyme on cellulosic materials is reversible and a fraction of the adsorbed enzyme can be washed by using ethylene glycol (EG) or low ionic strength buffers [25]. Therefore, free CBM-PGI was purified from immobilized CBM-PGI on RAC by EG washing. The purified CBM-free PGI appeared to be homogenous (Fig. 3, Lane 2). Without EG elution, immobilized CBM-PGI was obtained after RAC adsorption and washing. The CBM-free PGI was prepared from a fusion protein CBM-intein-PGI (Fig.3, Lane 3). Through simple adsorption and self-cleavage of intein [19], the purified CBM-free PGI in the supernatant appeared to be a single band (Fig. 3, Lane 4). The molecular weights of the CBM-PGI and CBM-free PGI were 67 and 49 kDa, respectively. Approximately 56 mg of the purified PGI and 70 mg of the purified CBM-PGI were obtained per liter of the culture with the purification yields of 37.5% and 18.2 %, respectively. It was estimated that approximately 350 mg of iCBM-PGI per liter the culture was obtained through one-step purification and adsorption.

PGI and CBM-PGI activities had the same optimum temperature (60 ^{o}C) at pH 7.5. Each enzyme retained approximately 90 % of its maximum activity at 50 and 70 ^{o}C. But only approximately 40% of each enzyme's optimum activity was exhibited at 30 ^{o}C. The influence of pH on both PGI and CBM-PGI activity was examined in the following buffers: citric acid (pH 4, 5 and 6), Tris-HCl (pH 6 and 7), HEPES (pH 7 and 8), TAPS (pH 8 and 9),

and sodium carbonate (pH 9, 10 and 11) at 60°C. Both enzymes exhibited the same pH profiles. PGI had the maximum activity at pH 8.0 in the buffers of HEPES and TAPS. They had approximately 75% and 40% of their maximum activity at pH 7.0 and pH 9.0, respectively. PGI in Tris-HCl buffer had 30% lower observed activity than that in the HEPE buffer at pH 7.0.

The kinetic constants of PGI, CBM-PGI, and iCBM-PGI were examined at 37 and 60 °C (Table 1). The CBM-PGI showed slightly low k_{cat} values than PGI and both exhibited approximately 3-fold k_{cat} values when the temperature increased from 37 to 60 °C (Table 1). CBM-PGI had somewhat lower K_m value (1.86 ± 0.08 mM) than that of PGI (2.89 ± 0.42 mM) at 60 °C. The above results suggested that the fusion of CBM-tag on PGI and immobilization on RAC or Avicel did not change PGI properties greatly.

Prolonged stability of iCBM-PGI

The half-life time of free PGI at 60°C greatly depended on its mass concentration from 0.1, 0.01 and 0.001 g/L (Fig. 4a). The half-life time of thermo-inactivation was *ca.* 180 h at 0.1 g/L but decreased to only 2.4 h at a low concentration (0.001 g/L). A relatively low mass concentration of PGI from 0.001-0.01 g/L lose its activity so fast that TTN of free PGI was far below the threshold value of $10^{7\text{-}8}$ mole of product per mole of enzyme. The thermostability of CBM-PGI was almost same as purified PGI (data not shown), suggesting that CBM tag only did not enhance the thermostablity of PGI.

iCBM-PGI exhibited drastically prolonged half-life times of 310 and 190 h at 0.01 g/L and 0.001 g/L as compared to those of free PGI by 6.3- and 80-fold, respectively (Fig. 4b). At a high concentration of 0.1 g/L, iCBM-PGI only doubled the half-life time to 360 h as

compared to free PGI. The half-life times of iCBM-PGI at different protein concentration were nearly constant because CBM-PGI concentration on the solid support was nearly constant. The kinetic constants of iCBM-PGI were examined at 37 and 60 ^{o}C (Table 1). The k_{cat} and K_m values of iCBM-PGI on RAC were 2198 ± 43 s^{-1} and 2.43 ± 0.15 mM, respectively, small changes as compared to free CBM-PGI and iCBM-PGI on Avicel. The above results suggested that simple adsorption of CBM-tagged PGI greatly prolonged the enzyme's life-time but retained most of its activity. Since the TTN value can be simply calculated without running a continuous biocatalytic process according to the equation of TTN = k_{cat}/k_d [31], iCBM-PGI at 0.001 g/L had a TTN value of 1.1 × 10^9 mole of product per mole of enzyme, where the observed reaction rate was a half of its maximum reaction rate, i.e., [S] = 1.2 mM. Such high TTN values for iCBM-PGI are among the most stable enzymes in the literature, far higher than the critical values of 10^{7-8} for SyPaB [8].

It is well-known that adsorbed CBM-tagged protein on RAC may be washed out reversibly, depending on buffer type and ionic strength. For example, high concentration glycerol or ethyl glycol, low ionic strength buffer or water can partially wash the adsorbed CBM-tagged proteins [25]. Therefore, the leaking behavior of iCBM-PGI was investigated under its working condition [100 mM HEPES buffer (pH 7.5) containing 10 mM Mg^{2+} and 0.5 mM Mn^{2+} at 60 ^{o}C] (Fig. 5). It was found that no significant PGI activity was lost after 50 washings, indicating that iCBM-PGI was stable under this relatively high ionic strength working buffer for a long time. Therefore, it was not necessary to conduct covalent linking between RAC and CBM-PGI.

Discussion

A simple protein purification and immobilization protocol was developed based on high

affinity of CBM-tagged protein on a large binding capacity cellulosic material - RAC. As compared to other enzyme immobilization supports, cellulosic materials are very attractive because they are cheap, biodegradable, and abundant [18-20]. The use of RAC rather than Avicel can increase enzyme binding capacity by 54 fold. As compared to other cellulosic materials (such as bacterial cellulose), RAC also presents higher accessibility and is much less costly [23, 25]. The use of RAC for enzyme immobilization would bring more advantages: (i) a reduction in the mass use of cellulosic support, (ii) the potential for increasing enzyme loading for high-speed biocatalysis, like carrier-free CLEA vs routine carrier-based enzyme immobilization [32], and (iii) a potential reduction in mass diffusion hindrance because all enzymes are immobilized on the external surface of RAC. Given 300 mg of CBM-PGI adsorbed by per gram of RAC, one kg of enzyme requires 3.3 kg of RAC for immobilization, resulting in 4.33 kg of iCBM-PGI. Assuming that the crude recombinant PGI production costs was \$30/kg enzyme and RAC cost was \$2/kg, the production costs of iCBM-PGI can be estimated to be \$36.6/kg of CBM-PGI or \$8.45/kg of iCBM-PGI.

Simple enzyme adsorption on the support may have no or some damage to enzyme activity, depending on the orientation between the active sites of the enzyme and support [6]. To minimize steric hindrance and environmental effects imposed by the surface properties of the matrices, a proper distance between the surface of the support and the enzyme is preferred for efficient immobilization of biocatalytic molecules on solid support materials [33, 34]. Sometimes, enzyme adsorbed on an adsorbent was easily leached out from the support because of weak interaction between enzyme molecule and surface of supports [35] and extra covalent bonding may be conducted [30]. In this study, CBM can orient the enzyme adsorption on the surface of RAC (Fig. 1) so that the active sites of the enzymes had enough distance from RAC and iCBM-PGI had comparable activity to free enzyme (Table 1). It

was noted that the high ionic strength working buffer allowed such simple immobilization to be stable enough even after 50 washings at 60 ^{o}C. Therefore, covalent attachment does not appear to be necessary.

The thermostability of free PGI was strongly associated with its concentration (Fig. 4a), implying that this enzyme is a multimeric protein. The cloned *C. thermocellum* PGI has a similarity of 85% as compared to *Bacillus stearothermophilus* PGI [36, 37], which is a dimeric enzyme. In support of it, the binding capacity ratio of iCBM-PGI on RAC/Avicel was 54, approximately two times than the value of a monomeric protein of thioredoxin-green fluorescence protein-CBM [23]. After enzymes were immobilized on the surface of RAC, their stability depended on their local concentration on the solid support rather than their bulk concentration. When immobilized enzymes were diluted to very low concentrations in a liquid solution, their local concentration was not changed so that they retained their activity and stablity, nearly independent of its bulk concentration (Fig. 4b).

To enhance the overall reaction rate in a complicated biological reaction network, it is often expected that there is no rate-limiting step controlled by one of several enzymes [38, 39]. However, the k_{cat} values of several enzymes often differ by three or four orders of magnitude [9, 10]. When evenly distribution of the rate-controlling theory for each enzyme was implemented, it meant that it was necessary for these enzymes to have the respective roughly three or four orders of magnitude difference in mass concentration. For high-activity enzymes, it was important to increase their half-time, especially at low enzyme concentration at the same level as those of low-activity enzymes at high mass concentration. Enzyme immobilization was a good solution to address such challenge because immobilized enzymes had nearly a constant life time, independent of its mass concentration in the bulk phase.

In conclusion, one-step simple protein purification and immobilization method was developed by adsorbing CBM-tagged proteins by using the low-cost ultra-high capacity adsorbent RAC. As compared to Avicel as a support [16, 22], iCBM-PGI on RAC decreased the cellulosic support use based on weight by 54-fold so that high enzyme loadings were possible, especially important in SyPaB where more than 10 enzymes were used together. As compared to carrier-free cross-linked enzyme aggregate [32], iCBM-PGI avoided random cross linking, which may hurt enzyme activity, and may have better mass transfer because of mono-layer adsorption of the enzyme on the large surface solid support -- RAC. Different from the previous thermoenzymes [28, 39, 40], the free thermophilic PGI was not stable enough in a free form for SyPaB but simple immobilization enabled it to be far more stable than what we required. This simple and low-cost protein purification and purification technology would have broad applications in enzyme-mediated biomanufacturing.

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Figure Legends

Figure 1. Scheme of one-step purification and immobilization of a CBM-tagged protein on ultra-large capacity RAC.

Figure 2. The profile of CBM-PGI adsorption on RAC and Avicel at room temperature. The curves were fitted by the Langmuir equations.

Figure 3. SDS-PAGE analysis of CBM-PGI and PGI purifications. M, marker; lane 1, soluble CBM-PGI; lane 2, purified CBM-PGI; lane 3, soluble CBM-intein-PGI; lane 4, purified PGI in the supernatant after its self-cleavage.

Figure 4. Thermo-inactivation profile of different concentration free PGI (a) and immobilized CBM-PGI (b) in a100 mM of HEPES buffer (pH 7.5) containing 150 mM NaCl and 10 mM $MgCl_2$ at 60 ^{o}C.

Figure 5. Leakage of the immobilized CBM-PGI washing in a100 mM of HEPES buffer (pH 7.5) containing 10 mM Mg^{2+} and 0.5 mM Mn^{2+}.

Table 1. The kinetic parameters of PGI and immobilized PGI at different temperatures (pH 7.5).

		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (10^5 $s^{-1}M^{-1}$)
PGI	37 °C	1.90 ± 0.24	929 ± 25	4.89
	60 °C	2.89 ± 0.42	2765 ± 335	9.57
CBM-PGI	37 °C	1.02 ± 0.06	729 ± 53	7.15
	60 °C	1.86 ± 0.08	2433 ± 79	13.08
iCBM-PGI	37 °C	2.11 ± 0.14	1091 ± 35	5.17
	60 °C	2.43 ± 0.15	2198 ± 43	9.06
Avicel-CBM-PGI	37 °C	1.53 ± 0.14	946 ± 27	6.18
	60 °C	1.65 ± 0.02	2009 ± 16	12.18

K_m, Michaelis-Menten constant; k_{cat}, rate constant

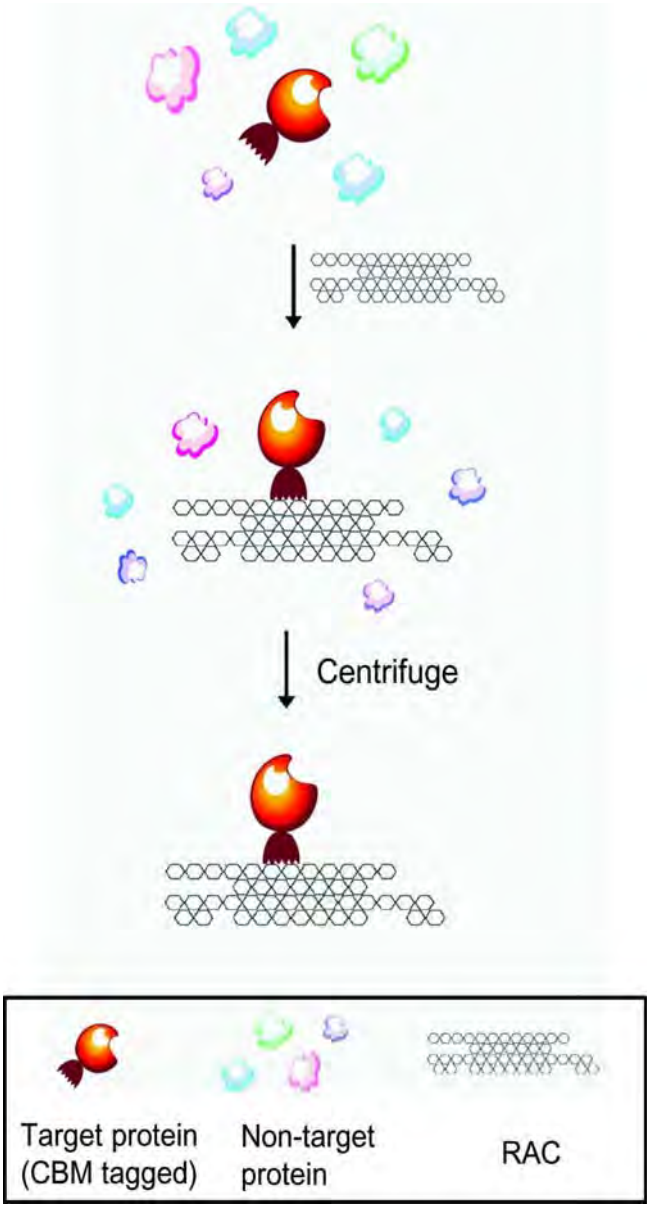

Figure 1
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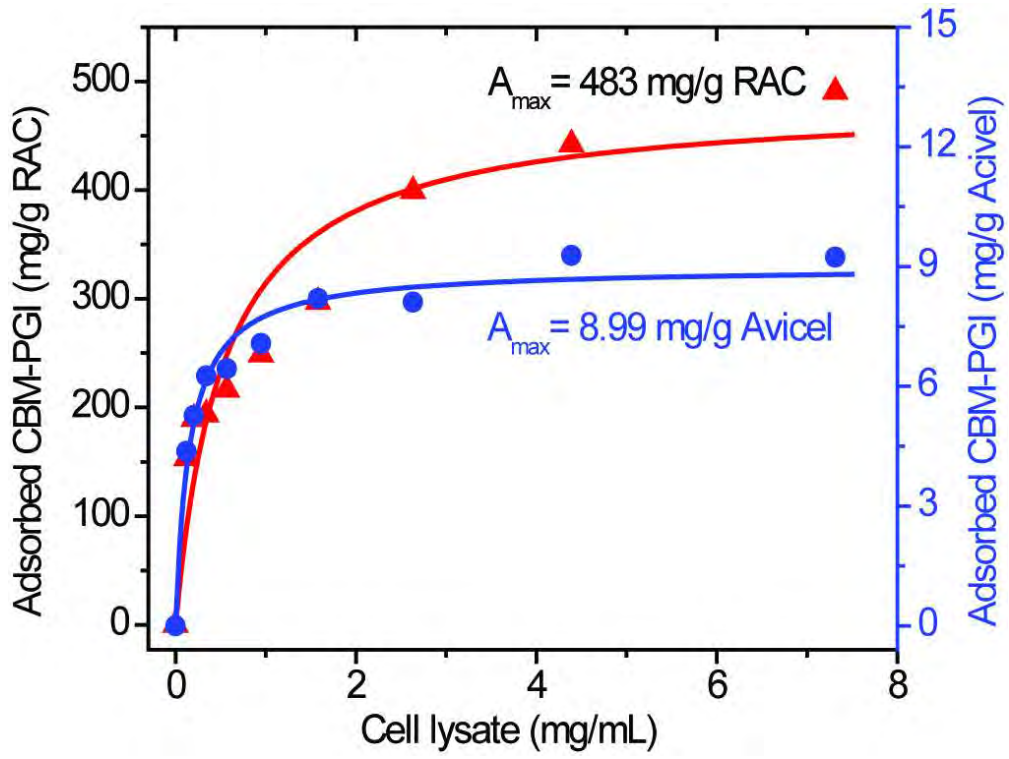

Figure 2
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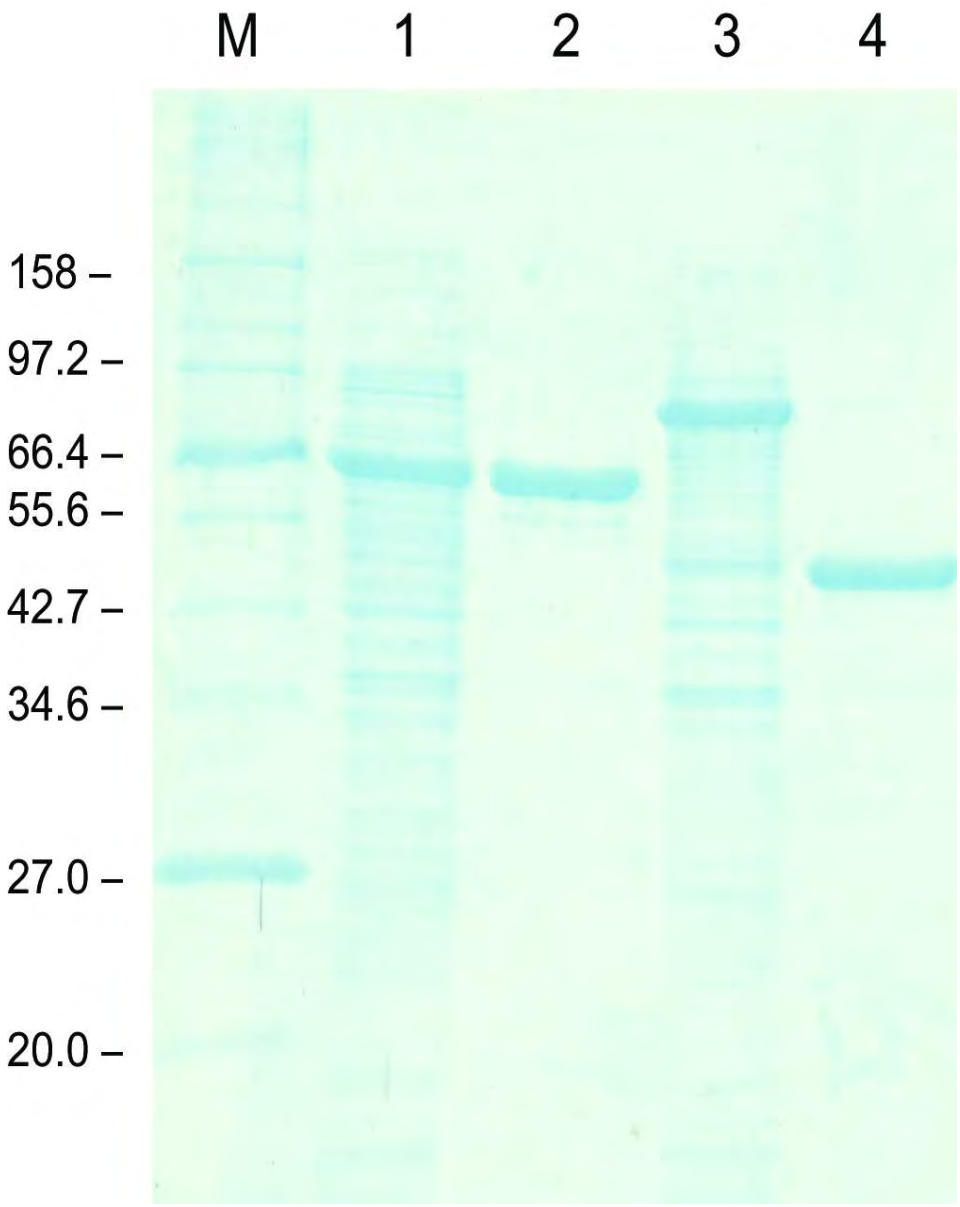

Figure 3
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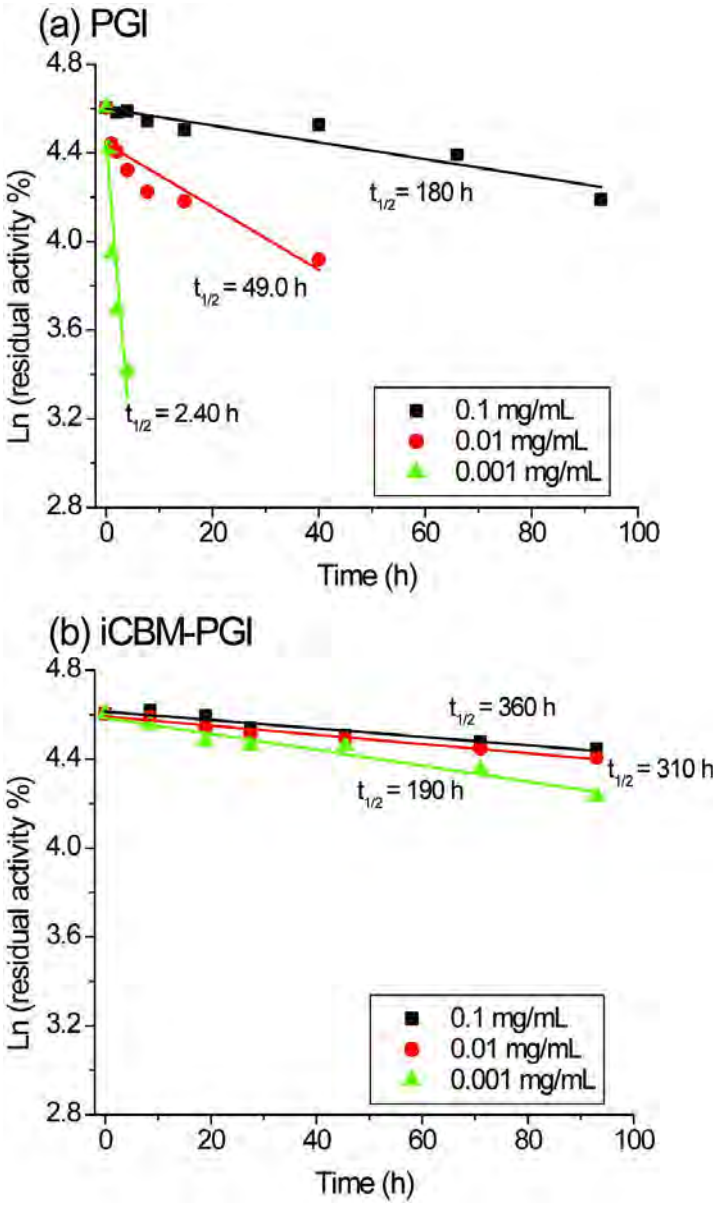

Figure 4
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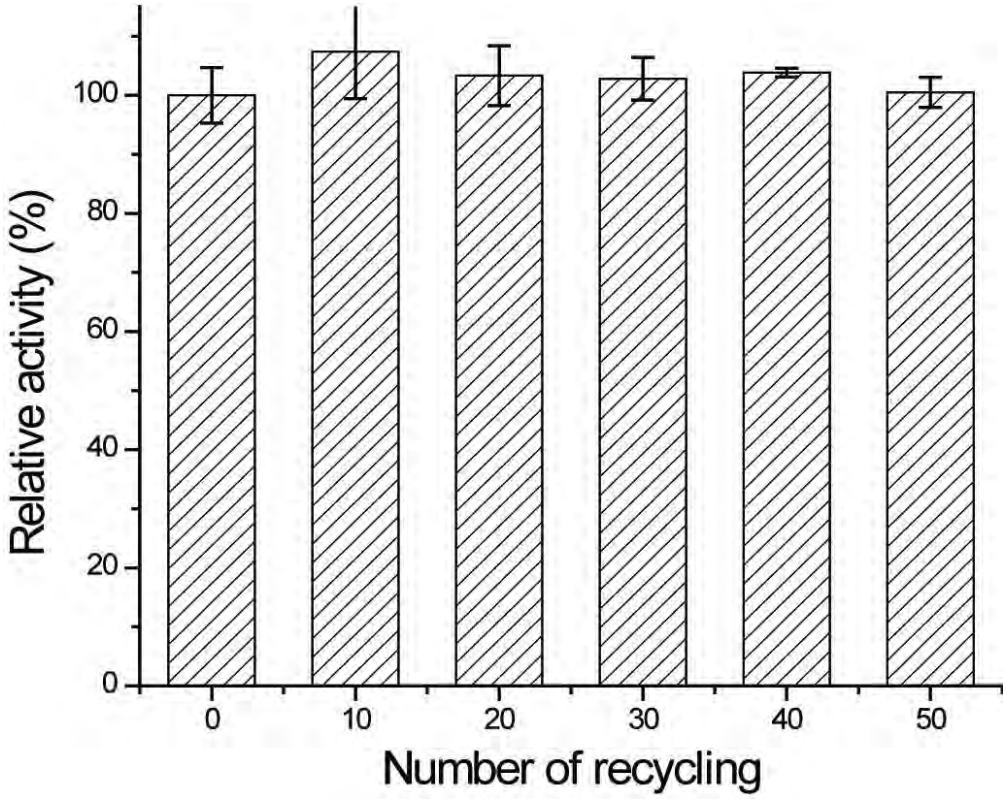

Figure 5
87x69mm (300 x 300 DPI)