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Feasibility of thermophilic adenosine triphosphate-regeneration system using *Thermus thermophilus* polyphosphate kinase

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ABSTRACT

The gene encoding polyphosphate kinase from *Thermus thermophilus* (TtPPK) was expressed in *Escherichia coli* Rosetta2 (DE3) pLysS. The *E. coli* recombinant cells were heated at 70 °C to inactivate indigenous enzymes and used for regenerating adenosine triphosphate (ATP) from exogenous polyphosphate (polyP) and adenosine diphosphate (ADP). The heat-treated cells having TtPPK were able to regenerate ATP at rates similar to those detected in cell-free extracts, suggesting that exogenous polyP and ADP could freely access TtPPK through the heat-damaged cell envelope. More than 80% of TtPPK activity was retained in the heated cells after incubation for at least 40 min at 70 °C. TtPPK in the heated cells could be easily recovered from the reaction mixture by centrifugation at 12,000 × *g* for 10 min. The gene encoding thermophilic ATP-dependent glycerol kinase from *Thermococcus kodakaraensis* KOD1 (TkGK) was expressed in *E. coli* Rosetta2 (DE3) pLysS. Using the mixture of *E. coli* recombinants expressing TkGK and TtPPK, the production of glycerol 3-phosphate (G3P) from glycerol was examined at 70 °C as a model reaction. When polyP was added to the reaction mixture in a fed-batch mode, 100 mM glycerol was stoichiometrically converted to 80 mM G3P (a molar yield of 80%).

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1. Introduction

Adenosine triphosphate (ATP) is a multifunctional nucleotide that plays an important role in cell biology [1]. The enzymatic system for ATP regeneration is of great importance in the use of ATP-dependent enzymes for industrial purposes. Direct use of ATP is cost-ineffective and often problematic because of the inhibitory effects due to the formed adenosine diphosphate (ADP) and adenosine monophosphate (AMP) [2]. Several ATP regeneration systems have been developed by employing biological agents including whole cells, organelles, and enzymes [3]. Kimura et al. [4] have applied immobilized yeast cells capable of regenerating ATP to the production of cytidine diphosphate choline (CDP choline). Mori et al. [5] have used metabolically active *Corynebacterium ammoniagenes* cells as the ATP regeneration system for the production of inosine 5′-monophosphate (5′-IMP). The use of living cells could eliminate laborious and time-consuming procedures for enzyme purification. However, an ATP regeneration system is strongly dependent on the respiratory activity of living cells. To maintain respiratory activity, culture conditions need to be optimized by controlling the rates of aeration and agitation. Poor membrane permeability of substrates has also impeded the expanded use of living cells for ATP regeneration.

Polyphosphate (polyP) is an inexpensive polymer consisting of inorganic phosphate residues. ATP could be generated from AMP and polyP using polyP-AMP phosphotransferases (PAPs) from *Acinetobacter johnsonii* [6,7] and *Myxococcus xanthus* [8]. The formation of ATP from polyP and ADP has also been demonstrated using polyphosphate kinase (PPK) from *Escherichia coli* [9]. This ATP regeneration system has been applied to the synthesis of an oligosaccharide, *N*-acetyllactosamine [10]. Sato et al. [11] have developed a thermophilic ATP regeneration system using PPK from *Thermosynechococcus elongates* BP-1 (TePPK). The TePPK-mediated ATP regeneration enables the production of D-alanyl-D-alanine from D-alanine with a molar yield of nearly 80%. Another thermophilic ATP regeneration system using PPK from *Thermus thermophilus* (TtPPK), which is more thermostable than TePPK has been proposed [12].

Thermophilic enzymes, including TtPPK and TePPK, have enormous potential for industrial applications [13–16]. They can be expressed in mesophilic hosts such as *E. coli* and *Bacillus subtilis*. By heating the recombinant cells at a temperature of 70 °C or higher, all indigenous enzymes can be inactivated to minimize unwanted side reactions. Additionally, the cell membrane barrier of mesophilic cells is disrupted by the heat treatment. This improves the accessibility of substrates to target enzymes in the heat-damaged cells.

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Honda et al. [17] have demonstrated the combined use of multiple thermophilic enzymes to construct an artificial biosynthetic pathway for production of 2-deoxyribose 5-phosphate from fructose. Iwamoto et al. [12] have also demonstrated the production of fructose 1,6-diphosphate from fructose with a molar yield of nearly 100% using *E. coli* recombinants producing thermophilic fructose kinase, thermophilic phosphofructo kinase, and TtPPK.

Although previous works have demonstrated the potential of a thermophilic ATP regeneration system for the production of value-added chemicals [11,12,17], the feasibility has not been well examined particularly on the biotechnological aspects, including (i) the optimization of reaction conditions; (ii) the use of high substrate concentrations; (iii) the stability and reusability of thermophilic enzymes; and (iv) the applicability to different reaction scales. In the present study, we examined the feasibility of a thermophilic ATP regeneration system with TtPPK and polyP, by employing the production of glycerol 3-phosphate (G3P) from glycerol as a model conversion. To produce G3P from glycerol, the thermophilic ATP regeneration system was coupled with glycerol kinase from *Thermococcus kodakaraensis* KOD1 (TkGK).

2. Materials and methods

2.1. Bacterial strains and culture conditions

The expression vector for TtPPK, pET-TtPPK, was described previously [12]. *ppk* encoding *E. coli* PPK (EcPPK) was amplified by polymerase chain reaction (PCR) using the following oligonucleotides; 5′-TA*CATATG*GTCAGGAAAAGCTATA-3′ (the *Nde*I restriction site is italicised) and 5′-AT*GAATTC*TTATTCAGGTTGTTCGAGTGA-3′ (the *Eco*RI restriction site is italicised). After digestion with *Nde*I and *Eco*RI, a DNA fragment containing *ppk* was cloned into pET-21a (Novagen, Madison, WI, USA), sequenced to verify the integrity, and introduced into *E. coli* Rosetta 2 (DE3) pLysS (Novagen). The vectors for TkGK [18,19] and glycerol kinase from *E. coli* (EcGK), which were designed as pET-TkGK and pET-EcGK, respectively, were kind gifts from Dr. Y. Koga, Osaka University [18]. They were introduced into *E. coli* Rossetta 2 (DE3) pLysS by transformation. *E. coli* cells were aerobically grown at 37 °C in Luria-Bertani broth supplemented with 100 mg/l ampicillin and 34 mg/l choramphenicol. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.2 mM in the late-log phase. Cells were harvested by centrifugation and suspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH buffer (pH 7.0). The cell concentrations of *E. coli* recombinants harboring pET-TtPPK and pET-TkGK were adjust to 400 and 50 mg wet cells/ml, respectively. The cell suspensions were incubated at 70 °C for 20 min before their use for G3P production.

2.2. Enzyme assay

The standard reaction mixture for PPK assay contained 50 mM HEPES-KOH (pH 7.0), 40 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2{\cdot}6H_2O$, 1 mM sodium phosphate glass (Type 65 polyP, $Na_{n+2}P_nO_{3n+1}$; $n = 65 \pm 5$, Sigma), 1 mM ADP, and 100 mg/ml *E. coli* cells having PPK. The reaction was carried out for 60 min at either 70 °C for TtPPK or 37 °C for EcPPK. ATP concentration was quantified using an ATP Bioluminescence Assay Kit (CLS II, Rosche) in accordance with the manufacturer's instruction. GK assay was performed in a 0.1-ml mixture consisting of 50 mM HEPES-KOH (pH 7.0), 40 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2{\cdot}6H_2O$, 25 mM glycerol, 25 mM ATP, and 1.25 mg/ml *E. coli* cells having GK. The reaction mixture was incubated for 5 min at 70 °C for TkGK or 37 °C for EcGK. G3P was quantified as described previously [20]. The detection solution consisted of 0.3 mg/ml phenol, 0.1 mg/ml 4-aminoantipyrine, 8 U/ml peroxidase (Toyobo, Kyoto, Japan), 20 U/ml G3P oxidase (Toyobo), and 100 mM HEPES-KOH (pH 7.9). After 20-min incubation at 37 °C, the absorbance at 500 nm was measured. For enzyme assay using cell-free extracts, cells were suspended in an appropriate volume of 50 mM HEPES-KOH (pH 7.0) and disrupted by UD-201 ultrasonicator (Kubota, Osaka, Japan) at 80 W for 3 min. The lysate of *E. coli* cells producing either TtPPK or TkGK was heated for 20 min at 70 °C. After the heat treatment, cell debris was removed by centrifugation at 12,000 × *g* for 10 min. Cell-free extract assays were performed using the supernatant prepared from the same quantity of *E. coli* cells as those used in the whole-cell assays.

2.3. Coupling reaction of PPK and GK

The standard reaction mixture was composed of 25 mM glycerol, 1 mM ADP (Oriental Yeast, Osaka Japan), 1 mM polyP, 10 mM $MgCl_2{\cdot}6H_2O$, 40 mM $(NH_4)_2SO_4$, 50 mM HEPES-KOH (pH 7.0), 1.25 mg/ml *E. coli* cells having GK, and 98.8 mg/ml *E. coli* cells having PPK. The 0.1-ml reaction mixture was placed in a 1.5-ml microtube and incubated for 20 min either at 70 °C for the coupling reaction with TtPPK/TkGK or at 37 °C for that with EcPPK/EcGK.

2.4. Leakage of TtPPK and TkGK following heat treatment

One hundred micrograms of *E. coli* wet cells having either TtPPK or TkGK was suspended in 10 ml of 50 mM HEPES-KOH (pH 7.0) and heated at 70 °C. After 20 and 40 min of incubation, the cell suspension was centrifuged at 12,000 × *g* for 10 min at 4 °C to collect the supernatant for enzyme assays. The cell pellet was washed with 50 mM HEPES-KOH, resuspended in 10 ml of the buffer, and subjected to enzyme assays.

2.5. Analytical methods

For glycerol quantification, the reaction mixture was mixed with an equal volume of acetone containing 1 mg/ml 1,2,4-butanetriol as an internal standard and centrifuged at 12,000 × *g* for 10 min. The resulting mixture was analyzed using a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a DB-17 capillary column (J&W Scientific, 0.25 mm × 30 m). Nitrogen was supplied as the carrier gas at a flow rate of 72.3 ml/min. The oven program was set at 80 °C for 1 min and increased to 180 °C at a rate of 15 °C/min, to 230 °C at 7 °C/min, and to 300 °C at 10 °C/min.

Polyacrylamide gel electrophoresis (PAGE) was performed to assess the chain length of polyP during the reaction. An 18-μl sample was mixed with 2 μl of a 10× DNA loading buffer (Takara Bio, Shiga, Japan) and loaded onto a 20% polyacrylamide slab gel. Gel preparation and electrophoresis were carried out as described elsewhere [21]. The gel was stained with 0.05% toluidine blue in 5% glycerol and 25% methanol.

3. Results and discussion

3.1. Optimization of PPK/GK coupling reaction

To investigate the feasibility of the TtPPK-mediated ATP regeneration system, the phosphorylation of glycerol to G3P catalyzed by GK was employed as a model reaction. Glycerol is currently considered as a potential low-cost substrate, which can be abundantly produced as a byproduct of biofuel production [22]. G3P is an important intermediate precursor for some cosmetics and other value-added chemicals [23,24].

E. coli pET-TtPPK exhibited the highest specific ATP production rate of 48 pmol/min/mg-wet cells at 70 °C and pH 7.0 (Fig. 1A and B). Although the optimum conditions for TkGK were 90 °C and pH 7.9 or higher, the specific rate of G3P production by TkGK-producing cells was much higher even at 70 °C and pH 7.0 than that of ATP production by TtPPK-producing cells. The TtPPK/TkGK coupling reaction was, therefore, performed at 70 °C and pH 7.0. The optimum mass ratio of *E. coli* cells (TtPPK:TkGK) was experimentally determined to be 98.8 mg/ml: 1.2 mg/ml (or 80:1) by performing the coupling reactions using various mass ratios of the cells at a total concentration of 100 mg/ml (data not shown). The optimum temperature and pH for the TtPPK/TkGK coupling reaction at this mass ratio was essentially identical to those detected using only TtPPK (Fig. 1C and D).

The G3P production by the coupling reaction was then assessed at different concentrations of polyP and ADP. PolyP concentrations higher than 1 mM were inhibitory for the coupling reaction (Fig. 1E). TtPPK and TkGK used individually were also inhibited by polyP at 2 mM or higher. The ATP production rate of TtPPK with 2 mM polyP was 58% of that obtained with 1 mM polyP. TkGK activities in the presence of 1 and 2 mM polyP were 46% and 27% relative to that in the absence of polyP, respectively. The production of G3P increased with increasing initial concentration of ADP up to 1 mM. However, further increase in ADP concentration showed no significant effect on G3P production for coupling reaction (Fig. 1F).

Similarly, the optimum temperature and pH for EcPPK and EcGK were determined using cell-free extracts (data not shown). The optimum conditions for EcPPK were 37 °C and pH 7.0. The specific rate of ATP production reached 4.5 pmol/min/mg protein under these conditions. On the other hand, the specific rate of G3P production by EcGK at 37 °C and pH 7.0 was 16.3 μmol/min/mg protein, which was much higher than that of ATP production by EcPPK. Thus, EcPPK/EcGK coupling reaction was performed at 37 °C and pH 7.0.

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