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1. Introduction

1.1. Impacts of metabolic engineering and its limitation

Production of value-added chemicals from renewable feedstocks, namely biorefinery, has been one of the most important issues to reduce the negative environmental impacts of fossil-fuel based industry. During the last decades, due to the periodic crises in some oil exporting countries, bioethanol becomes a viable and realistic alternative in the energy market (Cardona and Sánchez, 2007). Many industries in Europe have produced bioethanol in commercial plants. For example, in 2006, Abengoa Bioenergy in Spain produced approximately 510,000,000 liters per year of bioethanol from wheat (www.abengoabioenergy.com). The efficiency of biorefinery processes is often dependent on the metabolic ability of microbial cells, so that the development of metabolically engineered microorganisms is highly required. The concept of metabolically engineered microorganisms is highly required. The concept of metabolic engineering for providing the microorganisms with desirable properties have been established since few decades ago (Bailey, 1991; Stephanopoulos and Valino, 1991). The metabolic engineering is defined as the directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new

one(s) with the use of recombinant DNA technology (Stephanopoulos et al., 1998). Metabolic engineering technologies have directed to the construction of biocatalysts with bespoke activity and selectivity (Villeneuve et al., 2000; Zaks, 2001). It has contributed significantly to the enhanced production of various value-added chemicals and materials from renewable resources in past few decades. Nowadays, number of biotransformation processes, in which metabolically engineered microbes play crucial roles, has been running on the commercial scale (Straathof et al., 2002; Lee et al., 2011).

A metabolically engineered *Escherichia coli* for 1,3-propanediol synthesis from corn sugar has been co-developed by Dupont and Genencor (Nakamura and Whited, 2003). This process can reduce approximately 40% of energy consumption and 20% of greenhouse gas emission compared to the conventional process (Muska and Alles, 2005). Cargill Dow Polymers has built the fermentation plant, which can produce 140,000 ton/y of lactic acid from hydrolyzed corn starch, for the production of polylactide-based biodegradable plastic. Hoffman La Roche has successfully replaced the 6-step chemical process for riboflavin production (vitamin B2) with a single step bioconversion using a metabolically engineered *Bacillus subtilis* (Rogers et al., 2005). The evolutionary of metabolic engineering which involves a screening technique for the desired phenotypes from variant cell libraries (Lee et al., 2011) could produce 114 g/L of L-alanine with a selectivity of 95% and a purity greater than 99.5% (Zhang et al. 2007).

Although metabolic engineering is a very powerful approach to improve the industrial fermentation processes, it is not always easy to improve the microbial cells as we expected. This is due to the complexity and unidentified regulatory mechanisms in the cellular metabolisms of living organisms. The biocatalytic processes using living organisms need tight operational control. In addition, unlike *in vitro* enzymatic synthesis, degradation the product and byproduct formation by the co-existing enzymes often take place in living whole-cell systems (Ruffing and Chen, 2006).

1.2. Synthetic metabolic engineering

Recently, much attention has been paid on utilization of thermophilic enzymes as potential catalysts for industrial bioprocesses (Niehaus et al., 1999; Eichler, 2001; Taylor et al., 2004; Turner et al., 2007). High-reaction temperatures offer advantages, such as ease of mixing, better substrate solubility, high mass transfer rate, and low risk of contamination (Tunner et al., 2007). The cloning and expression of the gene encoding a thermophilic enzyme in an appropriate mesophilic host allows us a quick and simple purification of the enzyme by heat treatment (Patchett et al., 1989; Engelke et al., 1990). Motoshima et al. (1990) have successfully expressed the gene encoding aminopeptidase T of *Thermus aquaticus* YT-1 in *E. coli* and found that the thermostable enzyme can be easily purified by heating the cell extract at 80°C for 30 min. Although *E. coli* proteins were proficiently removed at 80°C or

higher, heat treatment at 70°C was also capable of removing cell debris, small particles, and the majority of proteins (Kirk and Cowan, 1995). Coolbear et al. (1992) demonstrated that incubation of recombinant mesophilic microorganisms producing thermophilic enzymes at high temperatures (65-90°C for 15-30 min) results in the irreversible denaturation and inactivation of the relatively instable indigenous enzymes. Consequently, whole-cell catalysts without undesired side activities can be easily obtained. The cell membrane barrier of the mesophilic host is partially disrupted by the heat treatment and a better accessibility between the enzyme and substrates can be achieved (Tsuchido et al., 1985; Giuliano et al., 2004; Ren et al., 2007a,b). Moreover, Giuliano et al. (2004) cloned a heat-stable α -glucosidase from *Sulfolobus solfataricus* (SS α -glu) into a mesophile of *Lactococcus lactis*. They found that the activity of SS α -glu can be highly recovered during heat treatment. The whole-cell lysate, which was obtained from glass bead disruption method, can only retained approximately 74% of residual activity of SS α -glu compared to permeabilized cells after thermal treatment, possibly due to denaturation of SS α -glu following mechanical stress.

Note that this approach is, in principle, applicable to all thermophilic enzymes as long as they can be functionally produced in an appropriate mesophilic host strain. Combination of multiple thermophilic whole-cell catalysts, which were prepared using this approach, would allow us to construct an *in vitro* metabolic pathway for producing value-added chemicals. Iwamoto et al. (2007) demonstrated the combined use of heat treated *E. coli* recombinants

producing TtPPK, fructokinase (FK), and phosphofructokinase (PFK) to synthesize nearly 100% yield of fructose-1,6-diphosphate (FDP) from fructose after 6-h reaction at 70°C. Honda et al. (2010) successfully constructed an *in vitro* pathway for the production of 2-deoxyribose-5-phosphate (DR5P) from fructose using *E. coli* recombinants producing six thermophilic enzymes. They designated this simple and universal strategy as “synthetic metabolic engineering (SME)”. In a SME-based bioproduction, a substrate can be straightforwardly converted to the desired product without being converted to undesired byproduct. This stoichiometric conversion allows us the thermodynamical prediction of the product yield.

1.3. Heat-induced leakage of enzymes

E. coli have been considered as potential cell factories for the production of recombinant proteins. Owing to the outer membrane of gram-negative bacteria, *E. coli* typically has low substrate permeability (Nikaido, 1976). Heat-induced disruption of this permeability barrier has been reported in literature (Grau, 1978; Tsuchido et al. 1985; Giuliano et al., 2004). The heat-induced disruption of cell membrane barrier causes the release of soluble proteins out of the cells (Kucharczyk et al., 1991; Umakoshi et al., 1998; Ferkade et al., 2005).

Heat treatment is thereafter used to release and recover the thermophilic proteins, which are cloned and expressed in *E. coli* and other mesophilic hosts (Takesawa et al., 1990; Ferkade et al., 2005; Ren et al.,

2007a,b; Balasudaram et al., 2009). Ferkade et al. (2005) have found that the combination of heat treatment and ultrasonication can reduce the energy required for disrupting the cells. In order to obtain maximum amount of the target enzyme activity, cell suspension should be heated at a temperature of 45°C for 8-10 min and followed by sonication for 10 min (Farkade et al., 2005). Ren et al. (2007b) claimed that thermolysis gave the selective release of thermophilic enzyme which is expressed in mesophilic recombinants. They successfully recovered 91% of thermophilic esterase from *E. coli* with a 12.1 fold of purification factor after heating at 80°C.

The heat-induced leakage, however, impedes the repetitive use of whole cell catalyst, so that the prevention of enzyme leakage is needed in SME-based bioproduction. One of the possible approaches is the chemical immobilization of enzymes. However, it requires a great amount of enzyme and frequently reduces the reaction rates and product yield (Tischer and Kasche, 1999). Those disadvantages are primarily owing to the multisite attachment, multiple orientations, and steric hindrance (Shao et al., 2000).

During the course of this work, the author found that a membrane-associated thermophilic enzyme was retained on the cell debris after the heat lysis. On the basis of this finding, integration of membrane-anchoring domain to a soluble thermophilic enzyme was attempted to prevent the heat-induced enzyme leakage.

1.4. Objective and outline of thesis

The basic strategy of SME is illustrated in Fig 1.1. An expression vector for thermophilic enzyme was transformed into a mesophilic cell. The recombinant mesophiles were cultivated at 37°C and the thermophilic enzymes were overproduced. The cells were subjected to heat treatment at 70°C for 20 min to denature indigenous proteins and partially disrupt the cell membrane barrier of *E. coli*. The heated *E. coli* recombinants can be directly used as biocatalyst at the optimum temperature for the thermophilic enzymes.

This thesis addresses a feasibility study of the SME-based bioproduction. Particularly, the author focused on an ATP-regenerating reaction catalyzed by *Thermus thermophilus* HB27 polyphosphate kinase (TtPPK). Since no living microorganism is used in SME, the addition of stoichiometric amounts of energy and redox cofactors is required to accomplish the pathway involving cofactor-dependent enzymes. However, the direct addition of cofactor causes some problems, such as an expensive process, alteration of reaction equilibrium, accumulation of inhibitory cofactor byproducts, and complex recovery of end products (Whitesides et al., 1995). Integration of a thermophilic cofactor regeneration system is an important issue for constructing a variety of *in vitro* pathway. In this study, the production of glycerol-3-phosphate (G3P) from glycerol was used as a model reaction (Fig. 1.2). The phosphorylation of glycerol to G3P, catalyzed by an ATP-dependent enzyme of *Thermococcus kodakaraensis* KOD1 glycerol kinase (TkGK), was coupled with the ATP

regeneration system using TtPPK. In an ATP-regenerating system, an adenosine scaffold is recycled and large amount of ATP or ADP is not required, but polyP is consumed as a phosphate donor (Sato et al., 2007). The objective of this work is to assess the catalytic performance of metabolically inactive *E. coli* cells producing TtPPK and TkGK by focusing on the biotechnological aspects such as the optimization of reaction conditions, the use of high substrate concentration, the applicability to the different reaction scales, the thermostability, and repetitive use of the biocatalyst.

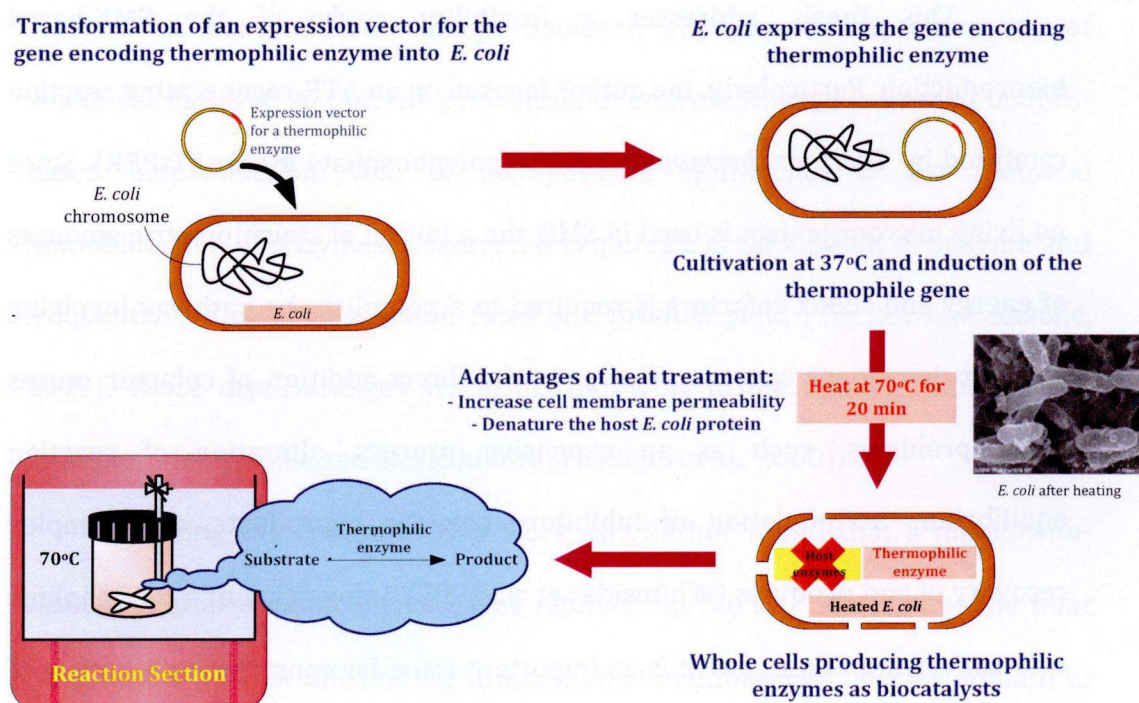


Fig 1.1. Basic strategy of SME

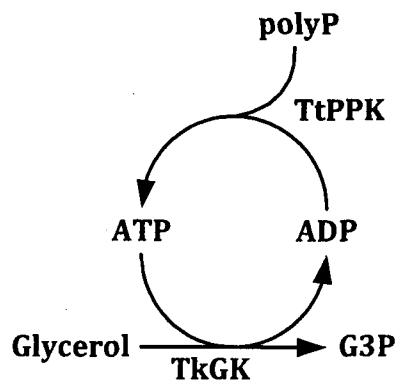


Fig. 1.2. Coupling reaction scheme of G3P production from glycerol and polyP using TkGK and TtPPK.

Overview of this thesis can be described as follows: the chapter 2 deals with the feasibility study of ATP-regenerating system of TtPPK in G3P production from glycerol. The heat-treated *E. coli* recombinants having TtPPK were able to regenerate ATP at rates similar to those detected in cell-free extracts, suggesting the exogenous polyP and ADP could freely access TtPPK through the heat-damage cell envelope. More than 80% of TtPPK activity was retained in the heated cells after incubation at least 40 min at 70°C, whereas TkGK was readily released out of the cells. This result indicated that TtPPK was associated to the membrane fraction and could be easily recovered and repetitively used as catalyst. Using the mixture of *E. coli* recombinants expressing TkGK and TtPPK, the production of G3P from glycerol was examined. 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%).

The chapter 3 describes the construction of membrane-anchoring fusion proteins of TkGK and their application to repetitive batchwise reactions. In this study, TkGK was fused with either TtPPK or an *E. coli* membrane-intrinsic protein, YedZ, to minimize the heat-induced leakage of TkGK. When the *E. coli* recombinants having these fusion proteins were incubated at 70°C for 2 h, more than 80% of TkGK activity was retained in the heated *E. coli* cells. However, the yield of G3P production by *E. coli* having the fusion proteins of TtPPK and TkGK was only less than 35%, probably owing to the inhibitory effect of polyphosphate on TkGK activity. The mixture of *E. coli* cells having TtPPK and those having YedZ::TkGK converted 80% of glycerol into G3P. These recombinant cells could be easily recovered from the reaction mixture by centrifugation and repeatedly used without a significant loss of enzyme activities.

The results shown in the chapters 2 and 3 are summarized at the end of those chapters. The conclusions and prospective of future research are given in the chapter 4.

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

2.1. Introduction

Adenosine triphosphate (ATP) is a multifunctional nucleotide that plays an important role in cell biology (Knowles, 1980). 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) (Whitesides et al., 1995). Several ATP regeneration systems have been developed by employing biological agents including whole cells, organelles, and enzymes (Langer et al., 1976). Kimura et al. (1978) have applied immobilized yeast cells capable of regenerating ATP to the production of cytidine diphosphate choline (CDP choline). Mori et al. (1997) 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* (Resnick and Zehnder, 2000; Tanaka et al., 2001) and *Myxococcus xanthus* (Kameda et al., 2001). The formation of ATP from polyP and ADP has also been demonstrated using polyphosphate kinase (PPK) from *E. coli* (Kornberg, 1995). This ATP regeneration system has been applied to the synthesis of an oligosaccharide, *N*-acetyllactosamine (Noguchi and Shiba, 1998). Sato et al. (2007) 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 (Iwamoto et al., 2007).

Thermophilic enzymes, including TtPPK and TePPK, have enormous potential for industrial applications (Coolbear et al., 1992; Persidis, 1998; Niehaus et al., 1999; Eichler, 2001). 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. Honda et al. (2010) have demonstrated the combined use of multiple thermophilic enzymes to construct an artificial biosynthetic pathway for production of DR5P from fructose. Iwamoto et al. (2007) have also successfully demonstrated the production of FDP from fructose using *E. coli* recombinants having 3 thermophilic enzymes.

Although previous works have demonstrated the potential of a thermophilic ATP regeneration system for the production of value-added chemicals (Sato et al., 2007; Iwamoto et al., 2007; Honda et al., 2010), 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 this study, the feasibility of a thermophilic ATP regeneration system with TtPPK and polyP was examined by employing the production of G3P from glycerol (Fig. 1.2) as a model conversion. To produce G3P from glycerol, the thermophilic ATP regeneration system was coupled with glycerol kinase from *Thermococcus kodakaraensis* KOD1 (TkGK).

2.2. Materials and methods

2.2.1. Bacterial strains and culture conditions

The expression vector for TtPPK, pET-TtPPK, was described previously (Iwamoto et al., 2007). *ppk* encoding *E. coli* PPK (EcPPK) was amplified by polymerase chain reaction (PCR) using the following oligonucleotides; 5'-TACATATGGTCAG-GAAAAGCTATA-3' (the *Nde*I restriction site is underlined) and 5'-ATGAATTC-TTATTCAGGTTGTTTCGAGTGA-3' (the *Eco*RI restriction site is underlined). 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* Rosetta2(DE3)pLysS (Novagen). The vectors for TkGK (Koga et al., 2001; Fukui et al., 2009) 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 (2001). They were introduced into *E. coli* Rosetta2(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 chloramphenicol. 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 adjusted 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.

2.2.2. Enzyme assay

The standard reaction mixture for PPK assay contained 50 mM HEPES-KOH (pH 7.0), 40 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM sodium phosphate glass (Type 65 polyP, $\text{Na}_{n+2}\text{P}_n\text{O}_{3n+1}$; $n = 65 \pm 5$, Sigma), 1 mM ADP (Oriental Yeast, Osaka Japan), 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 $(\text{NH}_4)_2\text{SO}_4$, 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 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 (Koga et al. 1998). 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 $12,000 \times g$ 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.

2.2.3. Coupling reaction of PPK and GK

The standard reaction mixture was composed of 25 mM glycerol, 1 mM ADP, 1 mM polyP, 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 40 mM $(\text{NH}_4)_2\text{SO}_4$, 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.

2.2.4. 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 $12,000 \times g$ 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.

2.2.5. 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 $12,000 \times g$ 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 \times 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- μ l sample was mixed with 2 μ l of a 10 \times 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 (Clark and Wood, 1987). The gel was stained with 0.05% toluidine blue in 5% glycerol and 25% methanol.

2.3. Results and discussion

2.3.1. 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 (da Silva et al. 2009). G3P is an important intermediate precursor for medicine, cosmetics and other value-added chemicals (Nguyen, 2004; Chanda et al., 2008; Vigeolas and Geigenberger, 2004).

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 (Figs. 2.1A 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). Obviously, ATP regeneration by TtPPK was likely to be the rate limiting step of coupling reaction (Table 2.1). It should be noted that the predicted ATP-production rate, which was calculated on the basis of specific activity of *E. coli* having TtPPK under the standard assay conditions, was considerably lower than the actual G3P production rate obtained from coupling reaction between TtPPK and TkGK (Table 2.1). It was suggested that ATP-production rate by TtPPK under the standard assay conditions was underestimated compared with that by TtPPK/TkGK coupling reaction. Under the TtPPK assay conditions, ADP was time-dependently phosphorylated to ATP and the decrease of ADP

concentration would result in the decrease of reaction rate. However, in coupling reaction of TtPPK/TkGK, ATP regenerated from polyP was continuously consumed by phosphorylation of glycerol to produce G3P and ADP (Fig. 1.2). On the other hand, G3P-production rate by TkGK under the standard assay condition was likely to be overestimated compared with that by TtPPK/TkGK coupling reaction, due to the high initial concentration of ATP (25 mM).

When the cell concentration of *E. coli* pET-TtPPK was decreased to 50 and 25 mg/ml, production rate of G3P by the TtPPK/TkGK coupling reaction also decreased (Table 2.1). On the other hand, increase in the cell concentration of *E. coli* pET-TkGK did not significantly affect the G3P-production rate. These facts confirmed that the ATP-regenerating reaction by TtPPK was a reaction-limiting step of the coupling reaction.

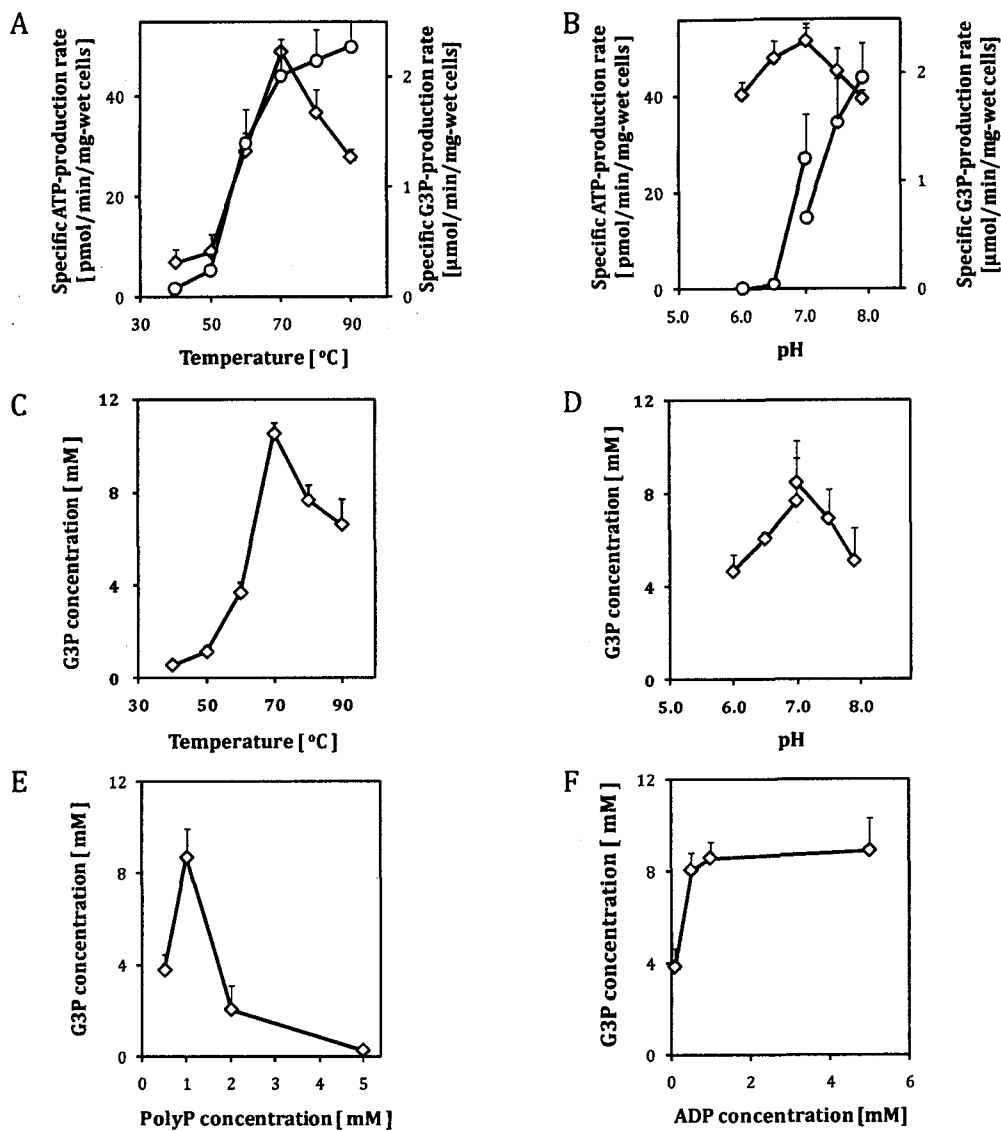


Fig. 2.1 Optimization of reaction conditions. The effects of temperature (A) and pH (B) on the activities of TtPPK (diamonds) and TkGK (circles) were assessed. The coupling reaction of TtPPK and TkGK was performed at various temperatures (C), pHs (D), polyP concentrations (E), and ADP concentrations (F). The pH of a reaction mixture was adjusted to 6.0–7.0 by adding 50 mM MOPS-KOH and to 7.0–7.9 by adding 50 mM HEPES-KOH. The data are shown as average \pm standard deviation ($n=3$).

Table 2.1. Comparison between the predicted production rate of ATP and G3P and the actual G3P production rate by the coupling reaction of TtPPK/TkGK.

<i>E. coli</i> pET-TtPPK (mg/ml)	<i>E. coli</i> pET-TkGK (mg/ml)	Predicted ATP production rate ($\times 10^{-3}$ mM/min)	Predicted G3P production rate (mM/min)	G3P production by the coupling reaction TtPPK/TkGK (mM/min)
98.8	1.2	4.7	2.4	0.53
50	1.2	2.4	2.4	0.37
25	1.2	1.2	2.4	0.21
50	50	2.4	100	0.39

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. 2.1E). 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. 2.1F).

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 μ mol/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. The mass ratio of *E. coli* cells having EcPPK to those having EcGK was set at 80:1 as used for TtPPK/TkGK. The production of G3P by EcPPK/EcGK reached a maximum at 1 mM polyP.

2.3.2. Elimination of cell surface barrier and unwanted side reactions

The surface of a microbial cell provides a semipermeable barrier to chemical compounds (Chen, 2007). This often inhibits the conversion of chemical compounds by whole-cell catalysis. Fig 2.2A shows a comparison of the time courses of G3P production between the cell-free extract and the whole cells of *E. coli* recombinants having EcPPK and EcGK at 37°C. No significant production of G3P was detected in the whole cells, whereas the cell-free extract produced approximately 12 mM G3P at 90 min. By contrast, in the TtPPK/TkGK coupling reaction at 70°C, no significant difference was observed between the cell-free extract and the whole cells of *E. coli* recombinants producing TtPPK and TkGK (Fig. 2.2B). This finding suggests that heating *E. coli* cells at 70°C disrupted the cell surface barrier and improved the accessibility of substrates (ADP, polyP, and glycerol) to enzymes (TtPPK and TkGK). EcGK assays showed no significant

difference in enzymatic activity between the cell-free extract and whole cells (Fig. 2.3A). This indicates that the accessibility of substrates to GK was not the rate-limiting factor for the coupling reaction at 37°C. On the other hand, the specific rate of ATP production by the cell-free extract was approximately 60% higher than that by whole cells (Fig. 2.3B). The poor membrane permeability of polyP likely impedes the ATP regeneration by whole cells producing EcPPK.

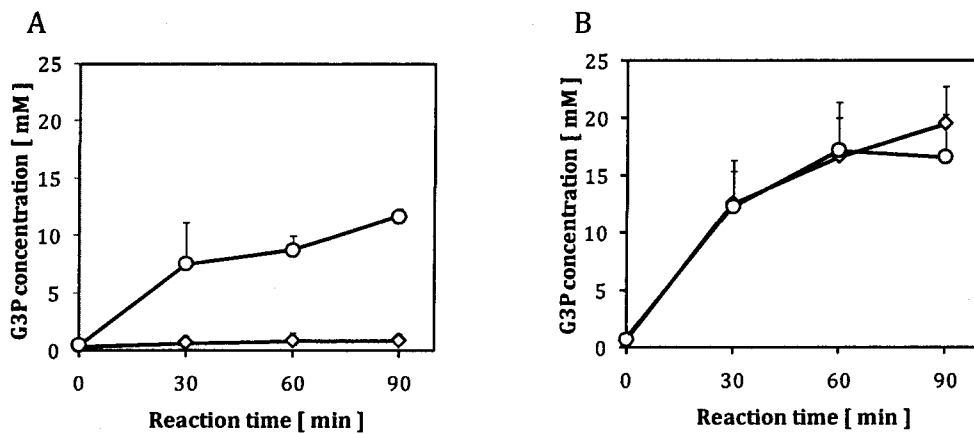


Fig. 2.2 G3P production by coupling reactions of EcPPK/EcGK (A) and TtPPK/TkGK (B) using whole cells (diamonds) and cell-free extracts (circles) as catalysts. The data are shown as average \pm standard deviation (n=3).

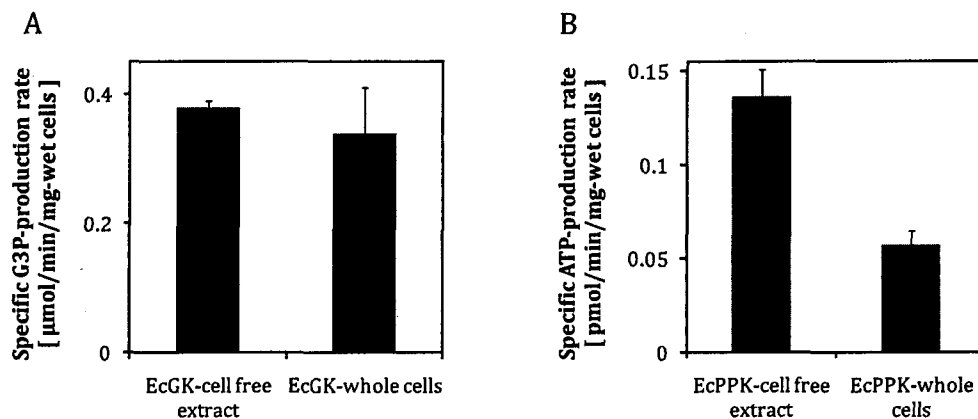


Fig. 2.3 EcGK (A) and EcPPK (B) assays using cell-free extracts and whole cells as catalysts. The data are shown as average \pm standard deviation (n=3).

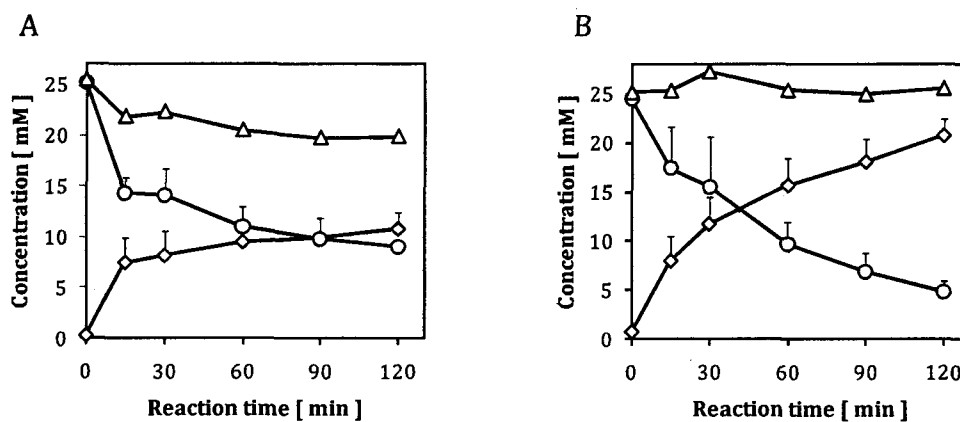


Fig. 2.4 Mass-balance analysis of G3P production by coupling reactions of EcPPK/EcGK (A) and TtPPK/TkGK. Total concentrations of glycerol and G3P (triangles) were calculated from the respective concentrations of glycerol (circles) and G3P (diamonds). The data are shown as average \pm standard deviation (n=3).

In the EcPPK/EcGK coupling reaction at 37°C, the cell-free extract consumed glycerol at a rate higher than that for the production of G3P (Fig. 2.4A). The rate of glycerol consumption is higher than that of the G3P production. Although 65% of glycerol was consumed after 2 h, the production yield of G3P was only 44%. The productivity of G3P over glycerol consumption was 0.68 mol/mol, which gave inconsistency of molar balance. On the basis of this finding, approximately 32% of glycerol was converted to some products other than G3P. By contrast, the total amount of glycerol and G3P was nearly constant during the TtPPK/TkGK coupling reaction for 120 min at 70°C (Fig. 2.4B). The productivity of G3P over glycerol consumption was 1 mol/mol, suggesting that unwanted side reactions were eliminated by heating at 70°C.

2.3.3. Heat-induced leakage of TtPPK and TkGK

Heat-induced lysis of microbial cells has been reported by many researchers. Tsuchido et al. have reported the disruption of the outer membrane barrier of *E. coli* following heat treatment (Tsuchido et al., 1985). Ren et al. (2007b) have demonstrated that the leakage of hyperthermophilic esterase from *E. coli* recombinants was induced by heat treatment at 60°C or higher. Giuliano et al. (2004) have also reported that approximately 40% of *Sulfolobus solfataricus* α -glucosidase expressed in *Lactococcus lactis* was detected in supernatant after incubation at 75°C.

As shown in Fig. 2.5A, approximately 50% of TkGK activity was detected in the supernatant of *E. coli* cell suspension after heat treatment at 70°C for 20 min. Further incubation at 70°C slightly increased the enzyme activity in the supernatant. On the other hand, approximately 80% of TtPPK activity was retained in the cells even after 40-min incubation at 70°C (Fig. 2.5B). This finding implied that the accessibility of polyP to TtPPK was improved by the increased membrane permeability of polyP.

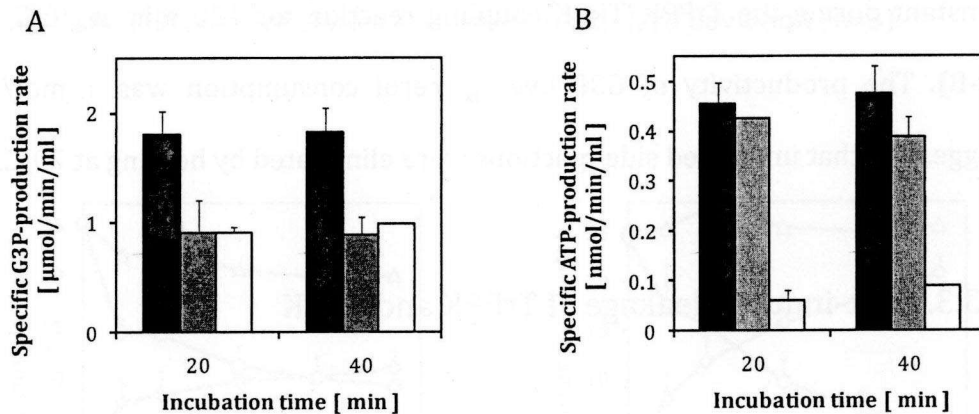


Fig. 2.5 TkGK (A) and TtPPK (B) activities in whole-cell suspension (black), remaining cell suspension (grey), and supernatant (white) after incubation at 70°C for 20 and 40 min. The data are shown as average \pm standard deviation (n=3).

Prokaryotic PPKs are classified into the PPK1 and PPK2 families (Brown and Kornberg, 2008). TtPPK belongs to the PPK1 family. Although the intracellular localization of TtPPK is not known, PPKs of the PPK1 family have

been found in the outer membrane fraction of cell lysates and solubilized by ultrasonication and the addition of high concentration of salts (Ahn and Kornberg, 1990). It seems possible that TtPPK is weakly associated with the outer membrane of *E. coli* recombinants. TtPPK assays using the cell-free extract and whole cells of *E. coli* pET-TtPPK revealed that approximately 85% of the enzyme activity was recovered in the cell-free extract, which was prepared by disrupting the cells using ultrasonication. On the other hand, TtPPK did not liberate from the membrane fraction by heat treatment and could be easily recovered from the reaction mixture by centrifugation of the cells.

2.3.4. Effect of glycerol concentration on G3P production

The effect of glycerol concentration on the production of G3P was investigated using *E. coli* cells having TtPPK and TkGK at 70°C (Fig. 2.6A). When the initial concentration of glycerol was 25 mM or lower, approximately 80% of glycerol was converted to G3P by 60 min. PAGE analysis of the reaction mixture with 25 mM glycerol demonstrated that the apparent chain length of residual polyP was similar to that of a standard polyP with an average chain length of 45 (Fig. 2.6B). This observation implies that phosphate residues of polyP were stoichiometrically transferred to ADP by TtPPK and then to glycerol by TkGK. No further conversion was observed mainly because of the chemical equilibrium between glycerol and G3P.

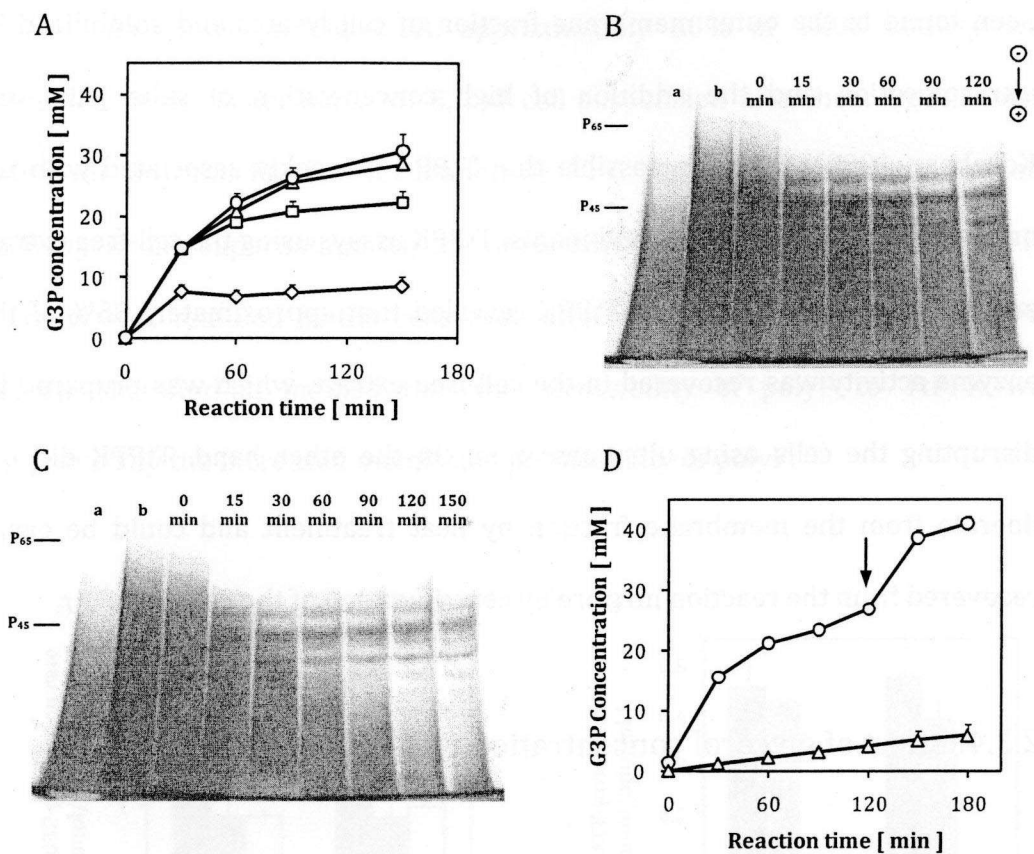


Fig. 2.6 Effects of initial concentrations of glycerol. (A) TtPPK/TkGK reaction was carried out at initial glycerol concentrations of 10 (diamonds), 25 (squares), 50 (triangles), and 100 mM (circles) in the presence of 1 mM polyP. (B and C) The supernatant of the reaction mixture with initial glycerol concentrations of 25 mM (B) and 50 mM (C) was subjected to 20% PAGE analysis after the reaction for the indicated time. PolyPs with average chain length of 45 and 65 were loaded on lanes a and b, respectively. PolyP appears as smear bands. (D) Reaction was carried out using 1 mM polyP and 50 mM glycerol (circles). After 120 min, polyP was added again at a concentration of 1 mM (indicated by arrow). G3P productions at an initial polyP concentration of 2 mM are indicated by triangles. The data are shown as average \pm standard deviation (n=3).

If all phosphate residues could be stoichiometrically transferred to glycerol, more than 50 mM of G3P should be produced. However, the final G3P concentration did not exceed 30 mM when the initial concentration of glycerol was 50 mM or higher. This is probably due to the fact that TtPPK prefers to use long-chain polyPs as a phosphate donor (Iwamoto et al., 2007). In fact, PAGE analysis of reaction mixture with 50 mM glycerol revealed that short-chain polyP remained in mixture without serving as a substrate of TtPPK (Fig. 2.6C).

G3P was hardly produced by the coupling reaction in the presence of 2 mM polyP (Fig. 2.6D), since both TtPPK and TkGK were inhibited by high concentrations of polyP (data not shown). On the basis of this finding, polyP was added to the reaction mixture in a fed-batch mode. As shown in Fig. 2.6D, 50 mM glycerol was converted to G3P with a molar yield of 80% by adding 1 mM polyP approximately 120 min after the start of incubation.

2.3.5. G3P production at different reaction scales

G3P can be produced by several methods which are, however, laborious, expensive (Nguyen, 2004) and low productivity. The pure G3P can be generated by chemical synthesis starting from D-acetone glycerol, but this process requires many steps for protection and deprotection of hydroxyl groups (Baer and Fisher, 1939). Glycerol can also be enzymatically phosphorylated by glycerol kinase to produce G3P. In this study, the production of G3P using *E. coli* recombinants having EcGK and EcPPK as ATP regeneration system was

performed in 0.1- μ l reaction scale at 37°C. The G3P was insignificantly produced due to the impermeability of polyP into the cells. The cell-free extract of *E. coli* recombinants having EcGK and EcPPK was also performed. The productivity of G3P was 0.68 mol for each mol glycerol consumed. Besides the byproduct was obtained in this reaction, the stability of the cell-free extract could be low for the long reaction time.

Nguyen (2004) investigated G3P production from glucose using engineered *Saccharomyces cerevisiae* with deletions in both genes encoding specific G3Pase (GPP1 and GPP2) and multicopy overexpression of G3P dehydrogenase (GDP1). Under oxygen limited, this fermentation could produce 26 mg/L of G3P from 15.5 g/L of glucose consumed. The yield of G3P could be improved by optimizing the production of biomass under aerobic condition and then shifting to anaerobic conditions for G3P production with high cell density (Popp et al., 2008). In 100-ml batch cultivation, total G3P (intra and extracellular) obtained after 55-h fermentation was approximately 80 mg/L (\approx 465 μ M) from 12 g/L of glucose consumed. Up-scaling and optimizing the process employed fed-batch fermentation with repeated glucose feeding were able to produce a final product titer of about 325 mg total G3P per liter (\approx 1.89 mM) of fermentation broth from about 210 g/L glucose consumed. Using SME-based process, approximately 21 mM G3P (\approx 3.6 g/L) can be produced from 21 mM glycerol consumed (\approx 1.9 g/L) in 0.1 ml reaction mixture.

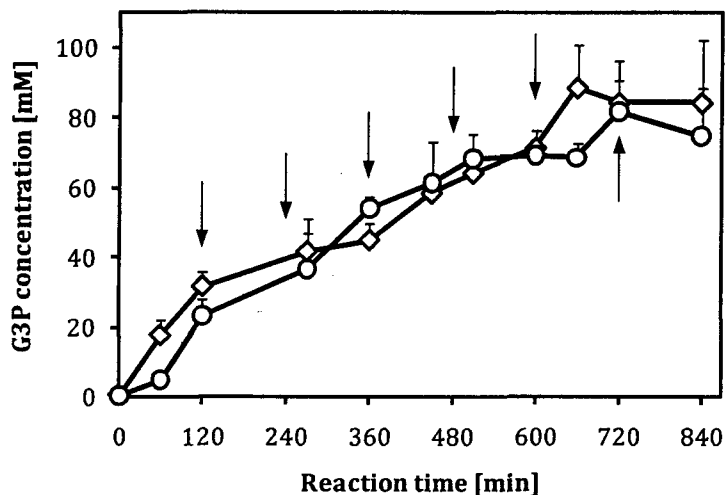


Fig. 2.7 Effect of reaction scale on G3P production. TtPPK/TkGK coupling reaction was performed in 0.1 ml (diamonds) and 100 ml (circles) of reaction mixtures. Arrows indicate the time of polyP addition. The data are shown as average \pm standard deviation (n=3).

Up scaling of G3P production was examined in a screw-capped cylinder vessel (ϕ 40 mm) containing 100 ml of the reaction mixture. The initial concentration of glycerol was 100 mM. The reaction mixture was gently agitated with a magnetic stirrer throughout the reaction. To prevent the inhibitory effect of a high concentration of polyP on enzymes, polyP was added in a fed-batch mode to the reaction mixture. A 1/50 volume of 50 mM polyP solution was added to the mixture at time intervals of 2 h. As shown in Fig. 2.7, the rate of G3P production in the vessel was essentially identical to that observed in a 1.5-ml microtube containing a 0.1-ml reaction mixture. After 720-min incubation, the concentration of G3P increased up to 81 mM (\approx 13.9 g/L). This value is much

higher than ones obtained from the process using living cells. We also assessed the thermo-stability of G3P by incubating a commercially available G3P (Biochemika, USA) at 70°C and confirmed that the decrease in G3P concentration after 840-min incubation was less than 2% of the initial concentration (80 mM). The thermophilic ATP regeneration system could be kept active when the reaction temperature was controlled at 70°C.

2.4. Summary

In this chapter, the feasibility of TtPPK as a thermophilic ATP regenerator was investigated. The ATP-regenerating reaction was coupled with the phosphorylation of glycerol to G3P catalyzed by TkGK. The potential of ATP regeneration system using thermophilic PPKs has been demonstrated in production of value-added chemicals (Sato et al., 2007; Iwamoto et al., 2007; Honda et al., 2010), but their feasibility has not been well examined. The author addressed on the demonstration of the superiority of *E. coli* recombinants producing thermophilic enzymes compared to those producing mesophilic enzymes. It was revealed that the membrane permeability of polyP was the rate limiting step of the reaction catalyzed by *E. coli* recombinants having EcPPK at 37°C. At 70°C, the membrane permeability of polyP markedly increased and no significant different was observed between the reaction rates using the cell-free extract and the whole cells of *E. coli* recombinants producing TtPPK and TkGK. *E. coli* having TtPPK, which is associated with the membrane fraction of *E. coli*

recombinants, could retain more than 80% of the TtPPK activity after the heat treatment. This finding implies that the enhanced accessibility between polyP and TtPPK at 70°C was mainly attributed to the increased membrane permeability of polyP, but not to leakage of TtPPK out of the cells. Furthermore, this result indicated TtPPK could be easily recovered from reaction mixture. On the other hands, significant amount of TkGK, which is produced as a soluble protein in the *E. coli* recombinant, was leaked out of the cells after the heat treatment. Some possible ways to prevent this heat-induced leakage of TkGK will be dealt with chapter 3. The mass balance analysis of the coupling reaction of GK/PPK indicated that unwanted side reactions were eliminated by heating at 70°C, and the stoichiometric conversion of glycerol to G3P could be achieved. Due to the inhibitory effect of high concentration of polyP, the fed-batch operation was employed for the addition of polyP in the reaction with a high concentration of glycerol. Finally, the effect of reaction scales on the production rate was investigated in 0.1-ml and 100-ml reaction mixture. No significant difference was observed in the conversion rate and the yield of product at both reaction scales. Approximately 80 mM G3P was obtained from 100 mM of initial concentration of glycerol by applying the fed-batch mode of polyP addition. This concentration, to the best of the author's knowledge, is much higher than the G3P concentration obtained from living microorganisms.

3. Construction of membrane-anchoring fusion protein of *Thermococcus kodakaraensis* glycerol kinase and its application to repetitive batchwise reactions

3.1. Introduction

In chapter 2, it was demonstrated that approximately 80% of the total TtPPK activity could be retained in *E. coli* cells after a 40-min incubation at 70°C. Although the intracellular location of TtPPK has not yet been fully investigated, the enzyme is likely associated with the cell membrane. TtPPK could be easily recovered from the reaction mixture by centrifugation.

On the other hand, thermophilic enzymes that are produced as soluble proteins in heterologous mesophilic hosts are readily released from the cells by heat treatment (Tsuchido et al., 1985; Giuliano et al., 2004; Ren et al., 2007a,b). As shown in chapter 2, an ATP-dependent glycerol kinase of *Thermococcus kodakaraensis* KOD1 (TkGK), which catalyzes the production of G3P from glycerol, was also easily leaked out of the *E. coli* recombinants. Approximately 50% of the total TkGK activity was detected in the supernatant of the cell suspension after heating at 70°C for 20 min. The heat-induced leakage of

enzymes impedes the repeated use of thermophilic whole-cell catalysts. One possible approach to overcome this drawback is the introduction of a membrane-anchoring domain to the thermophilic enzymes and their integration to the cell membrane fraction. In this study, the author examined the effect of the membrane association of TkGK on the heat-induced leakage from the cells. To perform this, TkGK was fused with either TtPPK or YedZ, which is a membrane-binding protein of *E. coli*. The heat-induced leakage of TkGK was decreased by fusing with TtPPK or YedZ. As a result, it was possible to use *E. coli* recombinants for the production of G3P from glycerol in a repeated batchwise manner.

3.2. Materials and methods

3.2.1. Bacterial strains and culture conditions

E. coli Rosetta2(DE3)pLysS (Novagen, Madison, WI) was used as the host cell for gene expression. The *E. coli* recombinants were cultured in a 500-ml Erlenmeyer flask containing 200 ml of Luria-Bertani broth supplemented with 100 mg/l ampicillin and 34 mg/l chloramphenicol. Cultivation was carried out in a rotary shaker at 175 rpm and 37°C. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.2 mM in 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 suspensions were incubated at 70°C for 20 min before use.

3.2.2. Plasmid construction

The plasmids and oligonucleotide primers used in this study are listed in Table

3.1. PCR-amplifications of *tppk* and *tkgk* were performed using pET-TtPPK

(Iwamoto et al., 2007) and pET-TkGK (Koga et al, 1998) as template DNAs,

respectively. The genomic DNA of *E. coli* W3110 was used as the template for

the amplification of *yedZ*. Four plasmid vectors were constructed for the

expression of the variant fusion proteins of TtPPK and TkGK, namely,

TtPPK::TkGK, TkGK::TtPPK, TtPPK::(-gly)₅::TkGK, and TkGK::(-gly)₅::TtPPK

(Table 3.1). To construct pET-TtPPK::TkGK and pET-TtPPK::(-gly)₅::TkGK, two

PCRs were conducted to amplify the gene encoding TkGK using primer sets of

TkGK-CF/TkGK-CR and Lin-gk-CF/TkGK-CR, respectively. These amplicons

were gel-purified, digested with *EcoRI* and *Sall*, and then introduced into the

corresponding restriction sites of pET-21a (Novagen). The resulting plasmids

were digested with *NdeI* and *EcoRI*, and ligated with *tppk*, which was amplified

by PCR using a primer set of TtPPK-NF/TtPPK-NR and then digested with *NdeI*

and *EcoRI*. Similarly, to construct pET-TkGK::TtPPK and pET-TkGK::(-gly-

)₅::TtPPK, the gene encoding TtPPK was amplified by two PCRs using primer

sets of TtPPK-CF/TtPPK-CR and Lin-ppk-CF/TtPPK-CR, respectively. They were

digested with *EcoRI* and *Sall*, and then introduced into the corresponding sites

of pET-21a. The gene encoding TkGK was amplified using a primer set of TkGK-NF/TkGK-NR, digested with *NdeI* and *EcoRI*, and then introduced into the corresponding sites of those plasmids.

The gene coding for the fusion protein of YedZ and TkGK was constructed by two-step PCR. The first PCR was conducted to amplify *yedZ* and *tkgk* using primer sets of *yedZ-F/yedZ-R* and *Fus-gk-F/TkGK-CR*, respectively. The amplicons were then gel-purified. An equivalent mixture of the purified amplicons was used as the template of the second PCR. The second PCR was performed using *yedZ-F* and *TkGK-CR* as primers. The PCR product was digested with *NdeI* and *Sall*, and then introduced into the corresponding sites of pET-21a. The resulting plasmid was designated as pET-YedZ::TkGK.

3.2.3. Enzyme assay

The standard reaction mixture for TtPPK assay contained 50 mM HEPES-KOH (pH 7.0), 40 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM sodium phosphate glass, 1 mM ADP, and an appropriate concentration of *E. coli* recombinants. The reaction was carried out for 60 min at 70°C. ATP was quantified using an ATP Bioluminescence Assay Kit (CLS II, Rosche) in accordance with the manufacturer's instruction. TkGK assay was performed in a reaction mixture consisting of 50 mM HEPES-KOH (pH 7.0), 40 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 25 mM glycerol, 25 mM ATP, and an appropriate concentration of

E. coli recombinants. The reaction mixture was incubated for 5 min at 70°C. G3P was quantified as described previously (Koga et al., 1998).

3.2.4. Coupling reaction of TtPPK and TkGK

The standard reaction mixture for the coupling reaction between TtPPK and TkGK was described in chapter 2. For enzyme assays with TtPPK::TkGK, TkGK::TtPPK, TtPPK::(-gly-)₅::TkGK, or TkGK::(-gly-)₅::TtPPK, the reaction mixture was composed of 25 mM glycerol, 1 mM ADP, 1 mM polyP, 10 mM MgCl₂·6H₂O, 40 mM (NH₄)₂SO₄, 50 mM HEPES-KOH (pH 7.0), and 100 mg/ml *E. coli* recombinants having the fusion protein. The coupling reaction between TtPPK and YedZ::TkGK was performed in the same manner, except that 50 mg/ml *E. coli* pET-TtPPK and 50 mg/ml *E. coli* pET-YedZ::TkGK were used as catalysts.

Table 3.1. Plasmids and oligonucleotide primers used in this study

Plasmid and primer	Description and sequence (5'-3')	Source or reference
Plasmid		
pET-TtPPK	Expression vector for TtPPK	11
pET-TkGK	Expression vector for TkGK	12
pET-TtPPK::TkGK	Expression vector for TkGK fused to the COOH-terminal of TtPPK	this work
pET-TkGK::TtPPK	Expression vector for TkGK fused to the NH ₂ -terminal of TtPPK	this work
pET-TtPPK::(-gly-) ₅ ::TkGK	Expression vector for TkGK fused to the COOH-terminal of TtPPK via a penta-glycine linker	this work
pET-TkGK::(-gly-) ₅ ::TtPPK	Expression vector for TkGK fused to the NH ₂ -terminal of TtPPK via a penta-glycine linker	this work

Table 3.1. (continue)

pET-YedZ::TkGK

Expression vector for TkGK fused to the COOH-terminal of YedZ this work

Primer

TkGK-NF	5'- <u>CATATG</u> ^a GAAAAGTTCGTTCTTTCTCTT-3'
TkGK-NR	5'- <u>GAATTC</u> ^b ATTTGATTTTGCACTATCAACGAC-3'
TkGK-CF	5'- <u>GAATTC</u> ^b ATGGAAAAGTTCGTTCTTTCTCTT-3'
TkGK-CR	5'- <u>GTCGAC</u> ^c TCAATTTGATTTTGCACTATCAAC-3'
TtPPK-NF	5'- <u>CATATG</u> ^a CACCTCCTTCCCGAAGCA-3'
TtPPK-NR	5'- <u>GAATTC</u> ^b TAGCTCCAGGCGCTGGGC-3'
TtPPK-CF	5'- <u>GAATTC</u> ^b ATGCACCTCCTTCCCGAA-3'
TtPPK-CR	5'- <u>GTCGAC</u> ^c TTATAGCTCCAGGCGCTGG-3'
Lin-gk-CF	5'- <u>GAATTC</u> ^b GGCGGTGGTGGTGGCATGAAAAGTTCGTTCTTTC-3'
Lin-ppk-CF	5'- <u>GAATTC</u> ^b GGCGGTGGTGGTGGCATGCACCTCCTTCCCGAAGC-3'

Table 3.1. (continue)

yedZ-F	5'- <u>CATATG</u> ^a CGTCTGACAGCAAAACAG-3'
yedZ-R	5'-CGAACTTTTCTACAGATAATTTGTTGTGAA-3'
Fus-gk-F	5'-ATTATCTGTAGAAAAGTTCGTTCTTTCTCTTG-3'

The italic sequences represent additional (-gly-)₅ coding sequences.

^a*Nde*I restriction sites are underlined.

^b*Eco*RI restriction sites are underlined.

^c*Sal*I restriction sites are underlined.

3.2.5. Leakage of enzyme by heat treatment

One hundred micrograms of *E. coli* wet cells was suspended in 10 ml of 50 mM HEPES-KOH (pH 7.0). After incubation at 70°C, the cell suspension was centrifuged at $5,000 \times g$ for 10 min at 4°C to obtain the cell supernatant. The supernatant was directly subjected to TkGK assay. The cell pellets were washed with 50 mM HEPES-KOH and then subjected to the assay. The percentage of enzyme leakage was estimated using Eq. (3.1):

$$Leakage(\%) = 100 \times A_{sup} / (A_{sup} + A_{cell}) \quad (3.1)$$

where A_{sup} and A_{cell} are the TkGK activities in the supernatant and remaining cells, respectively.

3.2.6. Reusability test

The coupling reactions with TtPPK/TkGK or TtPPK/YedZ::TkGK were repeatedly performed to assess the reusability of *E. coli* recombinants having these enzymes. After each cycle of reaction, the cells were harvested by centrifugation at $12,000 \times g$ and 4°C for 5 min. The cells were washed with 50 mM HEPES-KOH (pH 7.0) and resuspended in a fresh reaction mixture.

3.3. Results and discussion

3.3.1. Fusion proteins of TtPPK and TkGK

TkGK was fused with TtPPK to minimize the heat-induced leakage of the enzyme. Prokaryotic PPKs are classified into the PPK1 and PPK2 families (Brown and Kornberg, 2008). TtPPK belongs to the PPK1 family. Although the intracellular localization of TtPPK is not known, PPKs of the PPK1 family have been found in the outer membrane fraction of cell lysates and solubilized by ultrasonication and the addition of salts at high concentration (Ahn and Kornberg, 1990). The heat-induced leakage of the fusion proteins was estimated by determining TkGK activity in the cell supernatant. As shown in Table 3.2, the heat-induced leakage of fusion proteins was similar to that of TtPPK. The GK and PPK activities of the *E. coli* recombinants having TtPPK::TkGK or TkGK::TtPPK were examined (Table 3.2). No significant change in the specific enzyme activity was detected with the enzyme, which was located on the COOH-terminal side of the fusion protein. By contrast, the specific activity of the enzyme was considerably decreased when it was located on the NH₂-terminal side. This indicates that both TkGK and TtPPK have their functionally important domains on the COOH-terminal side. To avoid the conformational defects, we constructed fusion proteins having a penta-glycine peptide linker between TtPPK and TkGK. Unfortunately, however, no significant improvement was observed in the TtPPK and TkGK activities of these fusion enzymes and in

decreasing the heat-induced leakage of fusion protein (Table 3.2). To the best of the author's knowledge, the crystal structure of TtPPK has not been known yet. On the other hand, the crystal structure of TkGK was previously reported by Koga et al. (2008). The glycerol binds to the active side of TkGK and EcGK in similar manner. This site is located at the bottom of interdomain cleft. TkGK exists as a dimer in solution at a wide range of protein concentrations. The dimer structure is interfaced by both COOH-terminal sides of 2 monomers of TkGK (Koga et al., 2008). TtPPK, which weakly attached to cell membrane (Ahn and Kornberg, 1990), is expectedly easy to translocate. Due to these circumstances, if TkGK is located in COOH-terminal side of chimeric protein, it is possible to form a dimer structure after translation phase. On the contrary, if TkGK was put to NH₂-terminal side of chimeric protein, it is difficult to perform the dimerization.

E. coli recombinants having the fusion proteins were used as catalysts for the coupling reaction between TtPPK and TkGK. In the previous chapter, the author examined the effect of the mass ratio of *E. coli* cells having TtPPK to those having TkGK on G3P production. It was found that the optimum mass ratio was 98.8 mg/ml:1.2 mg/ml (or 80:1). At this optimum mass ratio, the total activities of TtPPK and TkGK in the reaction mixture (100 μ l) are 4.7 nmol/min/ml and 2.4 μ mol/min/ml, respectively. When wet cells of *E. coli* having TkGK::TtPPK or TkGK::(-gly-)₅::TtPPK were used at 100 mg/ml in 100- μ l reaction mixture, the TkGK and TtPPK activities were respectively higher than and similar to those detected in the previous work. Nevertheless, the G3P yield with fusion proteins

was considerably low compared to that with the mixture of individual TkGK and TtPPK. TkGK requires the magnesium ion for its activity (Koga et al., 2001). On the other hand, polyP is a strong chelator of metal ions owing to its polyanionic feature (Kornberg, 1995), suggesting that polyP has an inhibitory effect on TkGK activity. In fact, TkGK activities in the presence of 1 and 2 mM polyP decreased to 46% and 27%, respectively, compared with those detected in the absence of polyP (data not shown). Although the G3P yields by TtPPK::(-gly)₅::TkGK and TkGK::(-gly)₅::TtPPK could be improved by the addition of higher concentration of MgCl₂, the levels of improvement was modest. In the presence of 30-60 mM of MgCl₂, the final G3P yields increased to 40-50% (data not shown). Further addition of MgCl₂ resulted in lower G3P yields, probably due to the formation of the insoluble salt of polyP and Mg²⁺.

The crystal structure of *E. coli* PPK revealed that the active site of the enzyme is located in a tunnel, which contains a unique ATP-binding site and may accommodate a polyP chain. Amino acid residues located in the tunnel are highly conserved among the PPK1 family enzymes. These positively charged residues may interact with a polyP chain (Zhu et al., 2005). The insufficient space between TtPPK and TkGK may enhance the inhibitory effect of polyP on the TkGK activity of the fusion protein. This is supported by the observation that fusion proteins having a penta-glycine linker between TtPPK and TkGK gave better G3P yields than those without the linker peptide (Table 3.2).

Table 3.2. Heat-induced leakage and enzyme activity of TtPPK, TkGK, and fusion enzymes

Enzyme	Leakage ^a	Specific TkGK activity	Specific TtPPK activity	G3P yield ^b
	(%)	($\mu\text{mol}/\text{min}/\text{mg-wet cells}$)	($\text{pmol}/\text{min}/\text{mg-wet cells}$)	(%)
TtPPK	13 \pm 1	N/A	48 \pm 7.3	
TkGK	68 \pm 2.7	2 \pm 0.3	N/A	83 \pm 5 ^c
TtPPK::TkGK	9.0 \pm 2.0	1.7 \pm 0.6	21 \pm 2.8	8 \pm 2.5 ^d
TkGK::TtPPK	14 \pm 0.5	0.1 \pm 0.1	43 \pm 2.6	11 \pm 4.0 ^d
TtPPK::(-gly-) ₅ ::TkGK	12 \pm 0.3	1.6 \pm 0.1	23 \pm 7.4	34 \pm 0.5 ^d
TkGK::(-gly-) ₅ ::TtPPK	16 \pm 0.1	0.2 \pm 0.2	42 \pm 7.0	31 \pm 1.0 ^d
YedZ::TkGK	16 \pm 0.6	0.1 \pm 0.2	N/A	83 \pm 3 ^e

N/A = not available

^aLeakage after incubation of *E. coli* recombinants at 70°C for 120 min.

^bCoupling reaction was performed at 70°C for 120 min.

^cG3P yield of coupling reaction using wet cells of recombinant *E. coli* having TtPPK and those having TkGK at concentrations of 98.8 and 1.25 mg/ml, respectively.

^dG3P yield of coupling reaction using wet cells of recombinant *E. coli* having fusion proteins at concentration of 100 mg/ml.

^eG3P yield of coupling reaction using wet cells of recombinant *E. coli* having TtPPK and those having fusion protein of YedZ::TkGK at concentration of 50 and 50 mg/ml, respectively.

3.3.2. Fusion protein of TkGK and *E. coli* membrane protein YedZ

The low G3P yield with the fusion proteins of TtPPK and TkGK motivated the author to look for other candidates for constructing a fusion protein with TkGK. After a preliminary search for such candidate proteins, the author employed YedZ, which is an inner-membrane protein of *E. coli*, as the membrane-anchor for TkGK. YedZ has a molecular mass of 24.1 kDa and serves as a membrane-anchor for YedY, which is a soluble periplasmic oxidoreductase in *E. coli* (Drew et al., 2002; Wagner et al., 2007; Brox et al., 2005). A protocol for the overexpression of a fusion protein of YedZ with the green fluorescent protein (GFP) in *E. coli* has been established by Drew et al. (2006).

On the basis of the finding that TtPPK integration at the COOH terminal of TkGK adversely affects the specific TkGK activity, YedZ was assigned at the NH₂-terminal of TkGK. However, *E. coli* recombinants harboring pET-YedZ::TkGK showed a specific TkGK activity of 0.1 $\mu\text{mol}/\text{min}/\text{mg-wet cells}$, which was only 6% of that detected with *E. coli* recombinants having TtPPK::TkGK or TtPPK::(-gly)₅::TkGK (Table 3.2). The crystal structural analysis of TkGK has revealed that a marked conformational change is associated with the binding of a substrate to the enzyme (Koga Y., personal communication). TkGK forms dimer in the solution with a wide range of concentrations (Koga et al., 2001). It is not like TtPPK which is weakly attached to membrane cell, YedZ is a membrane-intrinsic enzyme with six putative transmembrane helices. A tight integration of TkGK to the *E. coli* membrane may

prevent this conformational change, resulting in a decreased specific activity. On the other hand, the thermostability of TkGK was not significantly affected by membrane anchoring (Fig. 3.1). When the cell suspension of *E. coli* having YedZ::TkGK was incubated at 70°C for 150 min, 84% of the total TkGK activity was retained in the remaining cells (Fig. 3.2A). Furthermore, more than 80% activity was retained in the cells even after 48-h incubation (Fig. 3.2B).

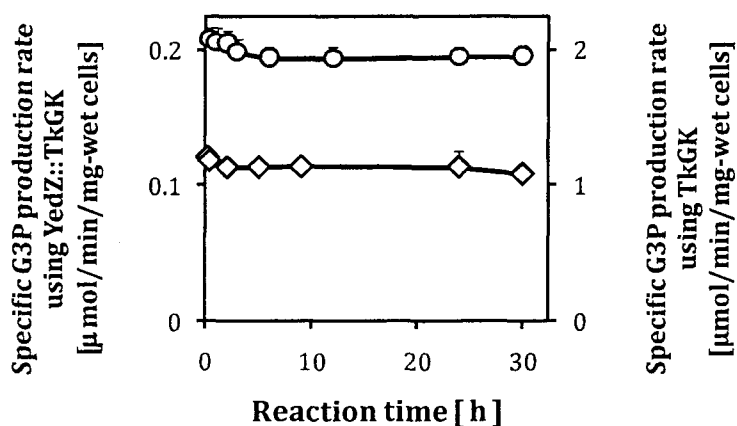


Fig 3.1 Thermostabilities of YedZ::TkGK (diamonds) and TkGK (circles). *E. coli* recombinants were incubated at 70°C for the period of time and then subjected to TkGK assay. The data are shown as average \pm standard deviation (n=3).

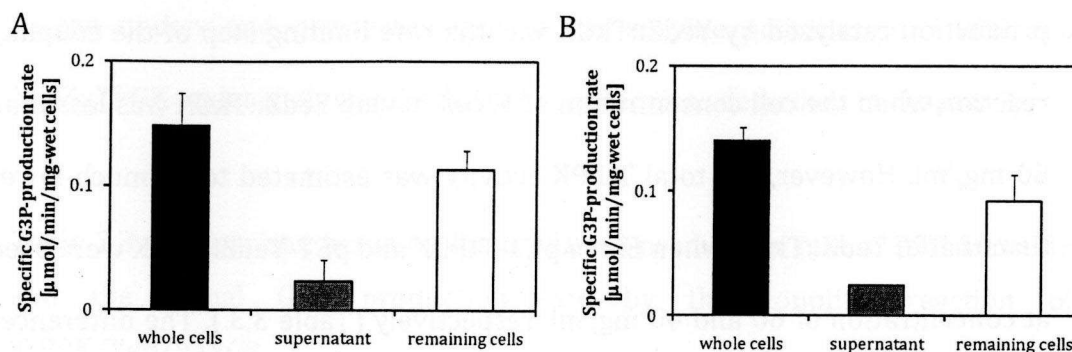


Fig 3.2 Leakage of YedZ::TkGK from *E. coli* recombinants after incubation at 70°C for 2.5 (A) and 48 h (B).

3.3.3. Coupling reaction with TtPPK and YedZ::TkGK

The mass ratio of *E. coli* recombinants is a critical parameter for the efficiency of the TtPPK/TkGK coupling reaction. In the previous chapter, the optimum mass ratio of *E. coli* cells having TtPPK to those having TkGK was experimentally determined to be 80:1. However, when *E. coli* cells having TtPPK and those having YedZ::TkGK were used at this mass ratio, the coupling reaction rate was very low, probably owing to the low specific activity of YedZ::TkGK (Fig. 3.3). Therefore, the author reexamined the optimum mass ratio using *E. coli* cells having TtPPK and those having YedZ::TkGK. Although the G3P production rates among mass ratio 60:40, 50:50, and 40:60 were similar, the highest G3P production rate was achieved at a mass ratio of 50:50 when the total cell concentration was kept at 100 mg/ml (Table 3.3). This fact implied that the G3P

production catalyzed by YedZ::TkGK was the rate limiting step of the coupling reaction when the cell concentration of *E. coli* having YedZ::TkGK was less than 50 mg/ml. However, the total TtPPK activity was estimated to be much lower than that of YedZ::TkGK when *E. coli* pET-TtPPK and pET-YedZ::TkGK were used at concentration of 60 and 40 mg/ml, respectively (Table 3.3.). The differences between the predicted ATP- and G3P-production rates and the actual G3P-production rate by the TtPPK/YedZ::TkGK coupling reaction were probably due

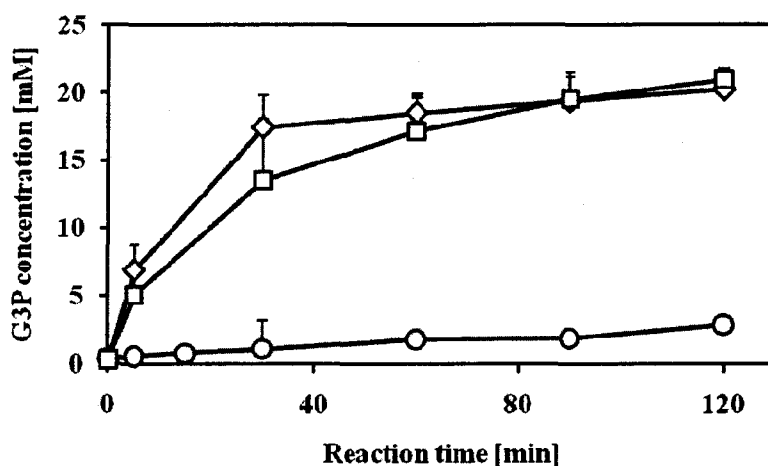


Fig 3.3 Effects of mass ratio of *E. coli* recombinants producing TtPPK to those producing YedZ::TkGK on G3P production. The coupling reactions were performed using recombinant cells having TtPPK and those having YedZ::TkGK at cell concentrations (mg/ml: mg/ml) of 98.8:1.2 (open circles) and 50:50 (open squares). The coupling reaction was also conducted using a mixture of TtPPK- and TkGK-producing cells at a mass ratio of 98.8:1.2 (open diamonds). The data are shown as average \pm standard deviation (n=3).

to the under- and over-estimation of the enzyme activities of TtPPK and YedZ::TkGK under their standard assay conditions as described in chapter 2.

Table 3.3. Comparison between the predicted production rate of ATP and G3P and the actual G3P production rate by the coupling reaction of TtPPK/YedZ::TkGK.

<i>E. coli</i> pET-TtPPK (mg/ml)	<i>E. coli</i> pET- YedZ::TkGK (mg/ml)	Predicted ATP production rate ($\times 10^{-3}$ mM/min)	Predicted G3P production rate (mM/min)	G3P production by the coupling reaction of TtPPK/YedZ::TkGK, (mM/min)
98.8	1.2	4.7	0.12	0.1
60	40	2.9	4	0.32
50	50	2.4	5	0.39
40	60	1.9	6	0.23

3.3.4. Reusability of recombinant *E. coli* cells as biocatalyst

The coupling reaction between TtPPK and TkGK was repeatedly performed using the mixture of *E. coli* cells having TtPPK and those having either TkGK or YedZ::TkGK. Although the coupling reaction with TtPPK/TkGK at a mass ratio of 80:1 exhibited a high reaction rate and G3P yield in the first reaction, the reaction rate markedly decreased in the second reaction (Fig. 3.4A and Table 3.4). When the reaction was performed at a mass ratio of 50:50, the rate of the first reaction was lower but those of the second and third reactions were

improved, compared with those detected at a mass ratio of 80:1. However, no increase in the final yield of G3P was observed with the change in mass ratio. Evidently, the loss of TkGK with the heat-induced leakage was attributed to the low reaction efficiency. On the other hand, the reaction rate and G3P yield of the coupling reaction with TtPPK/YedZ::TkGK were kept nearly constant when the reaction was repeated three times (Fig. 3.4B and Table 3.4). The *E. coli* recombinants could be recovered from the reaction mixture by centrifugation at $12,000 \times g$ for 5 min.

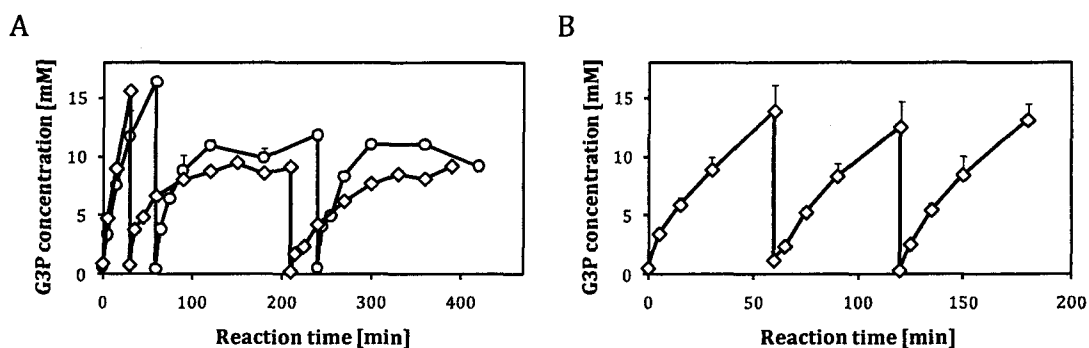


Fig 3.4 Repeated batchwise reactions using TtPPK- and TkGK-producing cells (A), and those using TtPPK- and YedZ::TkGK-producing cells (B). Coupling reactions with TtPPK/TkGK were conducted using cell concentration ratios (mg/ml: mg/ml) of 98.8:1.2 (diamonds) and 50:50 (circles). Coupling reaction with TtPPK/YedZ::TkGK was performed at a cell concentration ratio of 50:50. The data are shown as average \pm standard deviation ($n=3$).

Table 3.4. Initial G3P yield of repeated reaction of TtPPK/TkGK and TtPPK/YedZ::TkGK

Coupling reaction	Mass ratio of TtPPK:TkGK or TtPPK:YedZ::TkGK	Initial G3P production rate (mM/min)		
		1 st cycle of reaction	2 nd cycle of reaction	3 rd cycle of reaction
TtPPK/TkGK	98.8:1.2	0.53	0.32	0.16
TtPPK/TkGK	50:50	0.39	0.29	0.27
TtPPK/YedZ::TkGK	50:50	0.39	0.36	0.37

3.4. Summary

In chapter 2, the author demonstrated the stoichiometric conversion of glycerol to G3P using *E. coli* recombinants producing the ATP-dependent GK of the hyperthermophile *T. kodakaraensis* KOD1 (TkGK) and the PPK of *T. thermophilus* HB27 (TtPPK). TtPPK was associated with the membrane fraction of *E. coli* recombinants, whereas TkGK was released from the cells during the reaction at 70°C. In this chapter, membrane-anchoring fusion proteins of TkGK were constructed to minimize the heat-induced leakage of the enzyme. TkGK was fused with either TtPPK or an *E. coli* membrane-intrinsic protein, YedZ. When the *E. coli* recombinants having the fusion proteins of TkGK and TtPPK were incubated at 70°C for 2 h, more than 80% of TkGK activity was retained in the heated *E. coli* cells. However, the yield of G3P production by these *E. coli* recombinants was much lower than of that by the mixture of *E. coli* recombinants having TkGK and those having TtPPK, probably owing to the

inhibitory effect of polyphosphate on TkGK activity. Insertion of a pentaglycine peptide linker between TtPPK and TkGK resulted in a slight increase in G3P yield. This finding supported the observation that inhibitory effect of polyP is enhanced by insufficient space between TtPPK and TkGK.

YedZ, an inner-membrane protein of *E. coli*, was also tested as the membrane-anchor for TkGK. Due to the low specific activity of YedZ::TkGK, the optimum mass ratio using *E. coli* recombinants having TtPPK to those having YedZ::TkGK was examined. The heat-induced leakage of YedZ::TkGK was less than 20% after 48-h incubation at 70°C. The application of YedZ::TkGK to repetitive batchwise reaction with TtPPK was investigated and compared to the coupling reaction of TtPPK/TkGK. The recovered *E. coli* recombinant having YedZ::TkGK exhibited better performance than that having TkGK. The repetitive coupling reaction TtPPK/YedZ::TkGK can be done at least 3 times without a significant loss of enzyme activities.

4. Conclusions and Prospective

4.1. Conclusions

The basic advantages of “synthetic metabolic engineering”, *i.e.* an enhanced membrane permeability, the elimination of undesired side reaction, and the simplification of process control have been successfully demonstrated using *E. coli* recombinants having thermophilic enzymes (TtPPK and TkGK). The use of *E. coli* recombinants producing the thermophilic enzymes at 70°C remarkably increased the membrane permeability of polyP. After the optimization of reaction conditions, including temperature, pH, and mass ratio of *E. coli* cells, 100 mM glycerol was converted into G3P with a molar yield of approximately 80% using a periodic fed-batch operation for polyP addition. The conversion rates were almost identical at both 0.1-ml and 100-ml scales without using a complex process control. The stoichiometric conversion of glycerol to G3P could be achieved, suggesting that denaturation of indigenous enzymes of *E. coli* at 70°C could eliminate undesired side reactions.

Fusion enzymes of TkGK to TtPPK, a membrane bound protein, and also to YedZ, an *E. coli* membrane protein, can suppress the heat-induced leakage. Due to insufficient space between TtPPK and TkGK, the chelator of polyP gave

strongly inhibitory effect to TkGK activity which required Mg ion. As a result of the inhibitory effect, the coupling reaction using fusion proteins of TkGK and TtPPK gave low productivity of G3P. Although *E. coli* cells having YedZ::TkGK exhibited a lower specific enzyme activity, the coupling reaction between TtPPK and fusion protein of YedZ::TkGK can give approximately 80%-molar yield of G3P after adjusting cell mass ratio between TtPPK and YedZ::TkGK. YedZ::TkGK-producing cells could be used as whole-cell catalysts in the repeated batch reaction without losing enzyme activity. This approach is potentially applicable to a wide variety of thermophilic enzymes, allowing the full use of their excellent thermal stability.

4.2. Prospective

The basic advantages of synthetic metabolic engineering have been successfully demonstrated by simple coupling reaction using 2 thermophilic enzymes which were expressed separately in *E. coli*. The SME strategy can be applied to other complicated pathway which uses more than 2 enzymes. Iwamoto et al. (2007) and Honda et al. (2010) have run the synthetic pathway using more than 2 thermophilic enzymes expressed in *E. coli*.

In this study, the ATP-production rate using the regenerating system of TtPPK is very slow compared to the G3P-production rate of TkGK. Due to the unbalanced fluxes, recombinant cells having TtPPK was added much more than *E. coli* pET-TkGK with a mass ratio of 80:1. By using this mass ratio, the coupling

reaction proceeds stoichiometrically due to the absence of side reaction. The improvement of G3P production can be prospectively done by employing enzymes with better performance. For instance, use of mutant enzymes of TtPPK and TkGK, which can be potentially generated by protein-engineering techniques, with lower K_m for ATP at the optimum conditions of coupling reaction would allow us to use lower concentration of ATP for G3P production.

Furthermore, although the direct use of whole cells of *E. coli* recombinants having thermophilic enzymes at a high temperature is a simple and universal approach in the biocatalytic production, the immobilization of the soluble enzymes is required to enable the recovery of biocatalysts. The immobilization of the enzyme to the cell membrane could successfully prevent the heat-induced leakage of the enzyme. This thermophilic whole cell catalyst is potentially applicable to bioconversions in a series of continuous bio-reactors, such as repetitive batch reactors, continuous reactors with membrane-separation systems, and the fixed-bed reactors.

The combination of multiple enzymes is required for the construction of a synthetic pathway. Co-expression of multiple thermophilic enzymes in a mesophilic cell would be one of promising approaches to increase the biocatalytic performance of the synthetic pathway. Co-expression of multiple enzymes would allow a physical proximity between the catalytic sites of individual enzymes. This method can reduce the diffusional distance of intermediates and decrease their transit time. Iwamoto et al. (2007) constructed *E. coli* recombinant co-expressing enzymes (FK, PFK, and PPK) from

T. thermophilus to produce FDP from fructose. By using this *E. coli* recombinant, the production rate of FDP increased approximately twofold compared to that of the mixture of cells separately producing those enzymes. Duebert et al. (2009) successfully demonstrated that an introduction of a protein scaffold for synthetic enzymes might be a complement conventional strategy for balancing the level of co-expressed enzymes. These techniques would be applicable to improve the performance of SME-base production.

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RELATED PUBLICATIONS

Restiawaty, E., Iwasa, Y., Maya, S., Honda, K., Omasa, T., Hirota, R., Kuroda, A., and Ohtake, H.: Feasibility of thermophilic adenosine triphosphate-regeneration system using *Thermus thermophilus* polyphosphate kinase. *Process Biochem.*, **46**, 1747-1752 (2011).

Restiawaty, E., Honda, K., Okano, K., Omasa, T., Hirota, R., Kuroda, A., and Ohtake, H.: Construction of membrane-anchoring fusion protein of *Thermococcus kodakaraensis* KOD1 glycerol kinase and its application to repetitive batchwise reactions. *J. Biosci. Bioeng.*, doi: 10.1016/j.jbiosc.2011.11.016 (2011).

PRESENTATIONS OF INTERNATIONAL CONFERENCES

Restiawaty, E., Honda, K., Omasa, T., Kuroda, A., and Ohtake, H.: Feasibility of a thermo-tolerant ATP regeneration system using *Thermus thermophilus* polyphosphate kinase. Asian Congress on Biotechnology, 2011, May 11-15, Shanghai, China (Poster presentation: Outstanding award of young scientist and student for poster presentation).

Restiawaty, E., Honda, K., Okano, K., Kuroda, A., and Ohtake, H.: Production of Glycerol-3-phosphate Using *Thermococcus kodakaraensis* KOD1 Glycerol Kinase and In Vitro ATP-Regenerating System of *Thermus thermophilus* HB27 Polyphosphate Kinase. Soehadi Reksowardojo 2011 - International Seminar on Chemical Engineering, 2011, October 5-7, Bandung, Indonesia (Oral presentation).

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