Efficient Production of 2-Deoxyribose 5-Phosphate from Glucose and Acetaldehyde by Coupling of the Alcoholic Fermentation System of Baker’s Yeast and Deoxyriboalolase-Expressing Escherichia coli

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2-Deoxyribose 5-phosphate production through coupling of the alcoholic fermentation system of baker’s yeast and deoxyriboaldolase-expressing Escherichia coli was investigated. In this process, baker’s yeast generates fructose 1,6-diphosphate from glucose and inorganic phosphate, and then the E. coli convert the fructose 1,6-diphosphate into 2-deoxyribose 5-phosphate via D-glyceraldehyde 3-phosphate. Under the optimized conditions with toluene-treated yeast cells, 356 mM (121 g/l) fructose 1,6-diphosphate was produced from 1,111 mM glucose and 750 mM potassium phosphate buffer (pH 6.4) with a catalytic amount of AMP, and the reaction supernatant containing the fructose 1,6-diphosphate was used directly as substrate for 2-deoxyribose 5-phosphate production with the E. coli cells. With 178 mM enzymatically prepared fructose 1,6-diphosphate and 400 mM acetaldehyde as substrates, 246 mM (52.6 g/l) 2-deoxyribose 5-phosphate was produced. The molar yield of 2-deoxyribose 5-phosphate as to glucose through the total two step reaction was 22.1%. The 2-deoxyribose 5-phosphate produced was converted to 2-deoxyribose with a molar yield of 85% through endogenous or exogenous phosphatase activity.

Key words: 2-deoxyribose 5-phosphate; 2-deoxyribose; deoxyriboaldolase; alcoholic fermentation; 2'-deoxyribonucleoside

The synthesis of antiviral 2'-deoxyribonucleosides such as AZT requires a deoxyribose component as a starting material, but chemical synthesis of the deoxyribose frame is a tedious process with many protection and deprotection steps.\(^1\)\(^–\)\(^4\)

We performed 2-deoxyribose 5-phosphate (DR5P) production by means of deoxyriboaldolase 5–7) (DERA) of Klebsiella pneumoniae B-4-4 with triosephosphates such as D-glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) and acetaldehyde as starting materials.\(^8\) Triosephosphates are the intermediates of glycolysis, so if triosephosphates are supplied through glycolysis from cheap sugars, the process should become more practical. In a previous study,\(^9\) we investigated the glycolytic function of DERA-expressing E. coli with several intermediates of glycolysis, such as glucose, fructose, fructose 6-phosphate, and fructose 1,6-diphosphate (FDP), and found that the phosphorylated intermediates, especially FDP, served as suitable precursors for G3P.

Processes for the production of various substances through coupling with the powerful fermentative ability of baker’s yeast as the energy source were established by Tochikura and colleagues.\(^10\)\(^–\)\(^12\) The sugar-fermentative system of baker’s yeast was used as the ATP donor in these processes. Temporary accumulation of FDP in the course of ATP regeneration has been reported.\(^13\)\(^–\)\(^15\)

Based on these reports, we designed a novel metabolic and enzymatic DR5P production system consisting of glucose fermentation and DERA-catalyzed aldol condensation with glucose and acetaldehyde as starting materials. In this study, we tried to optimize FDP production from glucose and inorganic phosphate with toluene-treated baker’s yeast (toluene-treated yeast), and DR5P production from enzymatically prepared FDP and acetaldehyde by DERA-expressing E. coli (Fig. 1). Further convenient conversion of DR5P to 2-deoxyribose (DR) was also examined.

Materials and Methods

Preparation of a toluene-treated yeast. Pressed

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Abbreviations: DR5P, 2-deoxyribose 5-phosphate; DERA, deoxyriboaldolase; FDP, fructose 1,6-diphosphate; G3P, D-glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; LB, Luria-Bertani; DR, 2-deoxyribose
baker’s yeast (Oriental Yeast, Tokyo) was incubated with 16.6% (v/v) toluene in 176 mM potassium phosphate buffer (pH 7.0) at 37°C for 1 h with standing. The suspension was centrifuged (3,500 x g, 20 min), and the resulting pellet was used as the source of glycolytic enzymes (toluene-treated yeast).

Culture conditions for DERA-expressing E. coli. DERA-expressing E. coli 10B5/pTS8 was cultivated at 37°C in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.2) for 12 h. Cells were harvested by centrifugation (8,000 x g, 10 min). After washing with a 0.85% NaCl solution, the cells were used for DR5P synthesis.

Reaction conditions. FDP production: The standard reaction mixture comprised, in 10 ml, 1,111 mM glucose, 500 mM K2HPO4, 30 mM MgSO4·7H2O, 15 mM AMP·2Na and 60% (w/v) toluene-treated yeast. The reactions were carried out at 37°C for 3–7 h with standing. The supernatants obtained on centrifugation (15,000 x g, 15 min) were subjected to FDP analysis and then used for DR5P production as an enzymatically prepared FDP solution. The averages of three separate experiments, which were reproducible within ±10%, are presented in the text and figures.

DR5P production: The standard reaction mixture comprised, in 0.5–60 ml, 50% (v/v) enzymatically prepared FDP solution, 200 mM acetaldehyde, 200 mM potassium phosphate buffer (pH 7.0), 15 mM MgSO4·7H2O, 0.4% (v/v) polyoxyethyleneaurylamine, 1.0% (v/v) xylene, and 12.5% (w/v) wet cells of E. coli 10B5/pTS8. The reactions were carried out at 28°C for 2–4 h with shaking (120 rpm), followed by centrifugation (15,000 x g, 15 min). The amounts of DR5P in the resulting supernatants were measured as described below after checking the accumulation of DR by TLC. The averages of three separate experiments, which were reproducible within ±10%, are presented in the text.

Analytical methods. FDP analysis: FDP was measured enzymatically by monitoring a decrease in absorbance at 340 nm of NADH through coupled reactions catalyzed by FDP aldolase, triosephosphate isomerase (TPI), and a-glycerophosphate dehydrogenase (GDH). Twenty μl of FDP-containing sample solution was added to 140 μl of a reaction mixture comprising 150 mM Tris/HCl (pH 7.4) and 0.5 mM NADH. Then 20 μl of a TPI/GDH solution (Sigma, St. Louis, MO) containing 50 U of TPI and 5 U of GDH was added to decompose contaminating triosephosphates. After 10 min incubation at 30°C, 20 μl of FDP aldolase solution (Sigma) containing 10 U of FDP aldolase from rabbit muscle was added, and then...
the decrease in NADH was monitored at a wavelength of 340 nm with a Spectra Max 190 (Molecular Devices, Sunnyvale, CA) after 20 min incubation at 30 °C. The FDP concentration was calculated based on the decrease in NADH using a calibration curve obtained with authentic FDP solutions of known concentrations.

DR5P analysis: Qualitative analysis of DR5P and DR was performed by TLC with Kieselgel 60 F254 (Merck, Darmstadt, Germany). The developing system consisted of n-butanol, 2-propanol, and H2O in a ratio of 3:12:4 (v/v/v). DR5P and DR were detected with 1% (v/v) anisaldehyde and 2% (v/v) H2SO4 in acetic acid as purple spots. Quantitative analysis of DR5P was performed with cysteine-sulfate as described previously, after checking for the absence of DR by TLC.

DR analysis: DR was measured by HPLC with a refractive index detector (Shimadzu RID-6A, Kyoto, Japan) (column, Shodex Sugar KS-801 [8.0 x 300 mm], Showa Denko, Tokyo; eluent, H2O; flow rate, 0.5 ml/min; temperature, 55 °C).

Glucose analysis: The amounts of glucose in the reaction mixtures were determined by glucose oxidase (Glucose C2 Test WAKO; Wako Pure Chemical Industries, Osaka, Japan).

Inorganic phosphate analysis: The amounts of inorganic phosphate in the reaction mixtures were determined with a Phospha-C Test WAKO (Wako Pure Chemical Industries).

Result

Optimization of FDP production with baker’s yeast

Reaction pH and temperature: The effect of the reaction pH (in a range of 6.0–9.0) and temperature (in a range of 18–60 °C) were examined. The FDP production proceeded well at pH 6.4 (500 mM potassium phosphate buffer) and 37 °C (data not shown).

Inorganic phosphate concentration: The highest FDP production was attained with 1,000 mM inorganic phosphate in 5 h (Fig. 2A). The enzymatically prepared FDP solutions obtained with various phosphate concentrations and different reaction times were centrifuged, and the resulting supernatants were used directly for DR5P production with E. coli 10B5/pTS8. When the enzymatically prepared FDP solution obtained with 750 mM phosphate (initial concentration) was used as the substrate, the highest production of DR5P was achieved (Fig. 2B). As a result, the optimum initial phosphate concentration for FDP production was determined to be 750 mM in relation to DR5P production.

Energy carrier: The addition of an energy carrier was effective, although approximately 130 mM of FDP was produced even without the addition of an endogenous energy carrier. Among the energy carriers tested (ATP, ADP, AMP, adenosine, and adenine, each 15 mM), ATP, ADP, and AMP enhanced FDP production (Fig. 3). AMP was selected for further optimization.

Sugar type and concentration: Monosaccharides (glu-
cose, fructose, galactose, rhamnose, mannose, and sorbitol) and disaccharides (sucrose, maltose, and lactose) were examined. Glucose, fructose, mannose, sucrose, and maltose served as substrates and energy sources for FDP production, while galactose, sorbitol, and lactose did not (Fig. 4). Glucose was selected for further investigation, and the effect of its concentrations was examined. FDP production increased with increasing initial concentration of glucose up to 1,111 mM (data not shown). The initial glucose concentrations did not have any effect on DR5P productivity.

**Mg$^{2+}$ concentration:** The effect of the Mg$^{2+}$ concentration on ATP generation was examined. FDP production increased with increasing Mg$^{2+}$ concentrations up to 30 mM, but decreased with higher concentrations of Mg$^{2+}$ (data not shown).

**Acetaldehyde:** The addition of acetaldehyde to the reaction mixture enhanced FDP production. FDP production increased with increasing concentrations of acetaldehyde up to 250 mM, but decreased with higher concentrations of acetaldehyde (data not shown).

Based on these results, the optimum reaction con-
Conditions for FDP production with toluene-treated yeast were determined to be as follows: The reaction mixture comprised 1,111 mM glucose, 750 mM potassium phosphate buffer (pH 6.4), 15 mM AMP·2Na, 30 mM MgSO₄·7H₂O, 250 mM acetaldehyde, and 60% (w/v) toluene-treated yeast. Two hundred ml of the reaction mixture was poured into a 200-ml Erlenmeyer flask and then incubated at 37°C with standing. Shaking was less effective, because soluble oxygen inhibited anaerobic alcoholic fermentation with toluene-treated yeast. As shown in Fig. 5, 356 mM FDP (121 g/l) accumulated in 5 h, and the inorganic phosphate added was almost completely consumed (the yield of inorganic phosphate was 95.9%).

Optimization of DR5P production from enzymatically prepared FDP and acetaldehyde with E. coli 10B5/pTS8

The FDP-containing reaction mixture prepared above was centrifuged, and the resulting supernatant was used directly as the FDP source for DR5P production with DERA-expressing E. coli 10B5/pTS8. The reaction proceeded well under both aerobic (shaking) and anaerobic (standing) conditions. To mix the reaction mixtures well, the reactions were carried out with shaking (120 rpm).

Reaction pH and temperature: The effect of the reaction pH (in a range of 5.0–6.3 with 200 mM potassium phosphate buffer) on DR5P production was examined. The reaction proceeded well at pH 6.0 (data not shown). The effect of potassium phosphate buffer (pH 6.0) concentration was examined. The reaction was inhibited with increasing concentrations of potassium phosphate buffer and proceeded well without the buffer. Furthermore, the pH of the reaction mixture remained at about 6.0 without the addition of any buffer. Hence the reactions were carried out without any buffer. The effect of reaction temperature (in a range of 18–57°C) on DR5P was examined. At higher than 28°C, dephosphorylation of DR5P to DR was observed, which resulted in low DR5P accumulation (Fig. 6). Hence the reaction temperature was kept at 28°C.

Substrate concentration: DR5P production increased with increasing concentrations of acetaldehyde up to 400 mM (data not shown). With 400 mM acetaldehyde, the best amount of the enzymatically prepared FDP solution was equivalent to half the volume (50%, v/v) of the reaction mixture (FDP concentration, 178 mM). A
higher amount of FDP solution was less effective for DR5P production (Fig. 7).

Based on these results, the optimum conditions for DR5P production from enzymatically prepared FDP and acetaldehyde with E. coli 10B5/pTS8 were determined to be as follows: The reaction mixture comprised 50% (v/v) enzymatically prepared FDP solution (178 mM FDP), 400 mM acetaldehyde, 15 mM MgSO$_4$·7H$_2$O, 0.4% (v/v) polyoxyethylene laurylamine, 1.0% (v/v) xylene, and 12.5% (w/v) wet cells of E. coli 10B5/pTS8. Improvement of the FDP-permeability of the catalyst E. coli cells by detergent treatment was effective. Xylene and polyoxyethylene laurylamine were used for this purpose. Sixty ml of the reaction mixture was poured into a 200-ml Erlenmeyer flask, followed by incubation at 28 °C for 2 h with agitation by magnetic stirring. Under these conditions, 246 mM DR5P (52.6 g/l) accumulated in 2 h (Fig. 8). Theoretically, 2 mol of DR5P is produced from 1 mol of FDP, so the yield of DR5P to FDP in this reaction was calculated to be 69.1%.

Transformation of DR5P into DR
Dephosphorylation of DR5P into DR was carried out by incubating the intact DR5P-containing reaction mixture prepared as above at 47 °C to trigger endogenous phosphatase activity (Fig. 6). After 10 h incubation, about 50% of the DR5P was dephosphorylated to DR, and after 22 h, 210 mM (28.1 g/l) DR was obtained, with a molar yield of DR5P of 85%. Almost the same amount of DR (approximately 210 mM) was obtained on 4 h incubation of the DR5P-containing reaction supernatant obtained on centrifugation with the addition of exogenous phosphatase (Sumizyme PM-L, 2% w/v) at 37 °C.

Discussion
In this study, we optimized FDP production using toluene-treated yeast, and also further transformation to DR5P and DR with DERA-expressing E. coli.

In this two-step reaction system, it is important that almost all the inorganic phosphate is consumed in the
first step (FDP production) because the second step (DERA reaction) is inhibited by inorganic phosphate. When FDP production was carried out with 750 mM inorganic phosphate, almost all the inorganic phosphate was converted into FDP, so the subsequent DR5P production proceeded well. The addition of acetaldehyde was effective for FDP production. In the presence of a downstream intermediate of alcoholic fermentation, acetaldehyde, FDP degradation might be depressed, resulting in an increase in FDP accumulation. A standing reaction with an almost fully filled Erlenmeyer flask, to maintain a low soluble oxygen concentration, was effective. Under the preparative reaction conditions, 356 mM FDP (121 g/l) accumulated in 5 h. The molar yields of FDP to glucose and inorganic phosphate were 32% and 95.9% respectively, and the apparent AMP turnover was 47.5.

For optimization of DR5P production from the enzymatically prepared FDP solution and acetaldehyde with DERA-expressing E. coli 10B5/pTS8, surfactants, polyoxyethylene laurylamine and xylene, were added for improvement of the permeation of phosphorylated compounds. For efficient accumulation of DR5P, incubation of the reaction mixture at 28 °C was better than at higher temperatures. If the reaction temperature is higher, phosphatases of the E. coli host might become active, and in this case DR5P will be dephosphorylated into DR. This is a disadvantage for DR5P production, but is favorable for DR production. Under the preparative reaction conditions, 246 mM DR5P (52.6 g/l) was produced from the enzymatically prepared FDP solution and acetaldehyde in 2 h. The molar yields of DR5P to FDP and acetaldehyde were 69.1% and 61.5% respectively. The molar yield to glucose through the total two-step reaction was 22.1%. If the further transformation of DR5P to 2'-deoxyribonucleosides proceeded with almost 100% yield, the yield of DR5P to glucose obtained here (22.1%) might be comparable the yield of ribonucleosides.

In a previous paper, we reported DR5P production from glucose and acetaldehyde with a great deal of ATP as the energy carrier for acceleration of glycolysis by E. coli itself. One hundred mM ATP was required for the production of 100 mM DR5P. But from the viewpoint of industrial production, ATP is an expensive compound. It is noteworthy that coupling of the glycolytic pathway of baker’s yeast to DR5P synthesis greatly reduced the amount of endogenous energy required. The same coupling of the alcoholic fermentation system of baker’s yeast and aldolase-expressing E. coli might be promising for other aldolases and transketolases that use G3P or DHAP as a substrate, such as FDP aldolase, tagatose 1,6-diphosphate aldolase, L-fuculose 1-phosphate aldolase, rhamnulose 1-phosphate aldolase, and 1-deoxy-d-xylulose 5-phosphate synthase etc. Using the method presented here, glucose can be used as a starting material for these aldolase-catalyzing reactions instead of expensive phosphorylated compounds as substrates.

The DR5P produced was easily converted to DR through phosphatase-catalyzing reactions. Furthermore, the DR5P was enzymatically converted into 2'-deoxyribonucleoside derivatives. Investigation of the latter enzymatic process will be reported elsewhere.

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