Enzymatic Production of Fructose 1,6-Diphosphate Using Crude Cell Extract of Bacillus stearothermophilus

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The enzymatic production of fructose 1,6-diphosphate (FDP) from glucose was performed in a batch reactor and a semibatch reactor using the crude cell extract of Bacillus stearothermophilus which contains all four enzymes required for the synthesis. The experimental results of the yield and the time courses of FDP production obtained using various enzyme concentrations were in good agreement with the theoretical predictions calculated based on the differential equations including the rate equations of the four enzymes, which were determined using the purified enzymes of B. stearothermophilus.

[Key words: acetyl phosphate, glucose, glucose 6-phosphate, fructose 6-phosphate, FDP, batch reactor, semibatch reactor]

In a previous paper, we synthesized fructose 1,6-diphosphate (FDP) from glucose using purified enzymes of Bacillus stearothermophilus in a batch reactor and compared the FDP yield and the space-time yield with the theoretical predictions (1). The process consisted of three synthetic reactions catalyzed by glucokinase (GK), phosphoglucone isomerase (PGI) and phosphofructokinase (PFK), and the ATP regeneration reaction catalyzed by acetate kinase (AK) (2). The experimental results of the yield obtained using various enzyme and substrate concentrations were in good agreement with the theoretical predictions calculated based on the differential equations derived in our previous paper (1) and the rate equations determined for the reactions catalyzed by the purified enzymes of B. stearothermophilus (3-5, and Shirosima, M., M. E. thesis, Osaka Prefecture University, Osaka, 1994). These results proved the validity of the rate equations and the kinetic parameters used in the analysis. For industrial application of this enzymatic FDP synthesis, the use of crude enzymes is economically advantageous because they are much less expensive than purified enzymes. The crude cell extract of B. stearothermophilus contains all four enzymes required for the synthesis. Therefore, in the present paper the experiments using the crude cell extract were performed at pH 8.0 and 30°C using various enzyme and substrate concentrations. The yield of FDP and the space-time yield were compared with the theoretical predictions.

The crude cell extract of B. stearothermophilus was prepared as follows: the strain NCA 1503 of B. stearothermophilus was grown in a 2-1 jar fermentor. One liter of medium contained 5 g Polypepton, 5 g yeast extract, 10 g sucrose, 6.44 g Na2HPO4·12H2O, 7 mg FeCl3·6H2O, 0.32 g MgSO4·7H2O, 1.3 g K2SO4, 15 mg MnCl2·4H2O, and 0.27 g citric acid. The pH of the medium was adjusted to 7.1 using a 10 N KOH solution. The medium was sterilized for 30 min at 120°C and then cooled to 60°C. A 5% (v/v) inoculum, which was prepared by growing cells for 2 h at 60°C on a shaker, was added to the medium and the cells were grown with vigorous aeration until the late phase of exponential growth for 2-3 h at 60°C. The cells were harvested and washed with 0.1 M Tris-HCl buffer (pH 7.6) containing 0.1 M KCl and 10 mM MgCl2. The cell paste was stored at -20°C. Approximately 5.6 g of cells (wet mass) was obtained from a 1.5-l culture. The cells were disrupted by the freez ing and thawing method. The mixture containing the disrupted cells was centrifuged at 5000 x g for 5 min and the supernatant was recovered as the crude cell extract.

The concentration of each enzyme in the crude cell extract was determined by the following procedure: the crude cell extract prepared above was diluted 20 times using 0.1 M TEA buffer (pH 8.0) prior to the enzyme assay. The GK concentration was measured by a coupled assay as we previously reported (3) using glucose 6-phosphate dehydrogenase (G6PDH) as the coupling enzyme. The assay was carried out at 30°C in a total volume of 3 ml of 0.1 M-TEA buffer (pH 8.0) containing 1 mM glucose, 0.4 mM ATP, 1 mM NADP+, 10 mM MgCl2, 5 kU·l⁻¹ G6PDH, and 0.2 ml of the 20-fold dilution of the crude cell extract prepared above. The GK concentration was determined from the change in absorbance at 340 nm due to the production of NADPH (εmax=6200 M⁻¹·cm⁻¹) using a Shimadzu UV-2100 spectrophotometer (Shimadzu Co. Ltd., Kyoto). In this assay, the concentration of G6PDH was so determined that the effect of the reaction catalyzed by PGI became negligible because there are two reactions which consume G6P, i.e. the reaction catalyzed by G6PDH and that by PGI.

The GK concentration was determined by measuring the amount of G6P produced according to the procedure reported in our previous paper (4). The experiments were carried out at 30°C in a total volume of 3.0 ml of 0.1 M-TEA buffer (pH 8.0) containing 4.0 mM F6P, 1.0 mM NADP+, 1.0 kU·l⁻¹ G6PDH, and 0.05 ml of the 20-fold dilution of the crude cell extract. In this assay, the G6PDH concentration was so determined to ensure that the reaction catalyzed by PGI would become
the rate-controlling step.

To measure the PFK concentration, the F6P, formed by the reverse reaction catalyzed by PFK, is converted to G6P. Applying the coupled assay used above to determine the GK concentration with G6PDH as the coupling enzyme, the G6P concentration was monitored by measuring the absorbance change due to the production of NADPH at 340 nm using a spectrophotometer. The experiments were carried out at 30°C in a total volume of 3 ml of 0.1 M-TEA buffer (pH 8.0) containing 4 mM FDP, 2 mM ADP, 1 mM NADP⁺, 5 mM MgCl₂, 12 kU l⁻¹ G6PDH, and 0.2 ml of the 20-fold dilution of the crude cell extract. As in the assays of the enzymes GK and PGI, the rate of the reaction catalyzed by G6PDH must be the highest among the reactions involved. Furthermore, the reaction catalyzed by PFK must be the rate-controlling step of all the reactions. Therefore, even though the crude cell extract contained PGI, purified PGI was added to the assay mixture so that the rate of reaction catalyzed by PGI was much higher than that of PFK.

The AK concentration was determined using the method reported previously (5). The reactions were carried out at 30°C in a total volume of 3 ml of 0.1 M TEA buffer (pH 8.0) containing 8 mM ATP, 400 mM acetic acid, 4 mM PEP, 0.2 mM NADH, 10 mM MgCl₂, 10 kU l⁻¹ PK, 10 kU l⁻¹ LDH, and 0.05 ml of the 20-fold dilution of the crude cell extract.

To determine the concentration of each enzyme in g⁻¹, the activity values in U l⁻¹ measured according to the above procedures were divided by the specific activity (kU g⁻¹) of each enzyme. For the specific activities, the values reported by Unitika (data attached to the vial of the enzymes) were used. An additional procedure was required in the case of PFK, since the specific activity provided by Unitika was based on the forward reaction of this enzyme (the reaction which forms FDP from F6P), while the procedure described above was based on the reverse reaction catalyzed by PFK (reaction which forms F6P from FDP). Therefore, to obtain the specific activity of purified PFK based on the reverse reaction, the ratio of the activity based on the forward reaction to that based on the reverse reaction was measured. Ten microliters of 50% glycerol solution containing purified PFK (commercially obtainable PFK solution) was used to measure the activity based on the forward reaction following the procedure described by Unitika (Enzymes, a brochure published by Unitika). The activity based on the reverse reaction was measured by a procedure similar to that described above for measuring the PFK concentration, except that 8 mM FDP and 10 μl of the PFK solution were used. The ratio of the activity based on the forward reaction to that based on the reverse reaction was determined to be 14.0. The molar concentrations of the enzymes were calculated based on the molecular masses of the enzymes (1). The values thus determined for each enzyme in the crude cell extract prepared above are listed in Table 1.

If glucose, G6P, F6P, ATP and/or aldolase are present in the crude cell extract, the above procedure will introduce considerable error to the determination of enzyme concentrations. Therefore, their presence was investigated. The presence of glucose was tested by a procedure similar to that used to determine the GK concentration, but without the addition of glucose to the reaction mixture. The presence of G6P or F6P was tested by the following procedure: the crude cell extract was added to a mixture containing 1 mM NADP⁺ and 0.2 kU l⁻¹ G6PDH and the change in the absorbance due to the production of NADPH at 340 nm was observed using a spectrophotometer. The presence of ATP was tested by a procedure similar to that used to determine the GK concentration, but without the addition of ATP to the reaction mixture. The presence of aldolase, which is known to be involved in the glycolytic pathway and converts FDP to two triose phosphates, was also investigated. The experiment was performed using the usual procedure to determine the FDP concentration as described in our previous paper (1) but without the addition of aldolase. Results from the tests showed no detectable amount of the above compounds (data not shown), so that the enzyme concentrations determined above were believed to be valid.

An experiment investigating FDP synthesis using the crude cell extract of B. stearothermophilus was performed in a 100-ml-volume batch reactor, the same reactor used for the experiments using purified enzymes (1). The experimental procedure was almost the same as that for the experiments when the purified enzymes were used. The only difference was that in the present experiment, the crude cell extract containing all four enzymes was added to the reaction mixture to initiate the reaction. The reaction mixture consisted of 0.1 M TEA buffer (pH 8.0) containing 10 mM glucose, 20 mM acetyl phosphate, 0.01 mM ATP, 1 mM MgCl₂, 3.75 × 10⁻⁶ M GK, 3.49 × 10⁻⁸ M PGI, 5.18 × 10⁻⁸ M PFK and 9.12 × 10⁻³ M AK. Figure 1 shows the experimental result as a plot of the yield of FDP vs. time. For comparison, the experimental results obtained using the purified enzymes using the same concentrations of glucose and acetyl phosphate (1) are also shown in the figure. The concentrations of each enzyme and their ratios to the GK concentration for both experiments are given in Table 2. The final yield was 0.89, which was almost the same as that obtained using the purified enzymes (1). However, the figure shows that the time required to attain the final FDP yield using the crude cell extract was 50 mlm longer than that using the purified enzymes. This resulted in a productivity of 1.23 mol m⁻³ h⁻¹ for the crude cell extract, which was 30% lower than that

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (kU l⁻¹)</th>
<th>Mass concentration (g l⁻¹)</th>
<th>Molecular concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucokinase (GK)</td>
<td>25.6</td>
<td>5.69 × 10⁻²</td>
<td>8.49 × 10⁻⁷</td>
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<tr>
<td>Phosphoglucose isomerase (PGI)</td>
<td>68.6</td>
<td>1.72 × 10⁻¹</td>
<td>9.10 × 10⁻⁷</td>
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<tr>
<td>Phosphofructokinase (PFK)</td>
<td>3.0</td>
<td>1.75 × 10⁻¹</td>
<td>3.15 × 10⁻⁴</td>
</tr>
<tr>
<td>Aldolase (AK)</td>
<td>63.0</td>
<td>4.04 × 10⁻²</td>
<td>2.38 × 10⁻⁷</td>
</tr>
</tbody>
</table>

* Based on the specific activities of 450 kU g⁻¹, 400 kU g⁻¹, 240 kU g⁻¹ and 1560 kU g⁻¹ for GK, PGI, PFK and AK, respectively (Enzymes, a brochure published by Unitika).

* Based on the value of 14, which is the ratio of the activity based on the forward reaction to that based on the reverse reaction measured in the present work.

* Based on the molecular masses of 67 kDa, 189 kDa, 130 kDa and 170 kDa for GK, PGI, PFK and AK, respectively, as mentioned in our previous paper (1).
obtained using the purified enzymes. This is because the composition of the enzymes in the crude cell extract was not necessarily optimal for the FDP synthesis as shown in Table 2. The concentration of the crude cell extract was prepared such that the GK concentrations in the two series of experiments were consistent. However, the concentrations of PGI and AK in the crude cell extract were considerably different from those used in the experiments using purified enzymes (1). The lower AK concentration in the crude cell extract was responsible for the lower productivity. The solid line in Fig. 1 represents the theoretical prediction calculated based on the differential equations described in our previous paper (1) and the rate equations determined for the reactions catalyzed by the purified enzymes of B. stearothermophilus (3-5, and Shiroshima, M., M. E. thesis, Osaka Prefecture University, Osaka, 1994). The dotted line represents the theoretical prediction using the purified enzymes (1). The agreement between the theoretical line and the experimental result is satisfactory.

Experiments were also performed using 200 mM glucose using various enzyme concentrations. In these experiments, a semibatch reactor with a volume of 100 ml was used into which 200 mM acetyl phosphate was fed continuously at a liquid flow rate of 18 ml·h⁻¹ in order to avoid the thermal decomposition of acetyl phosphate until a predetermined amount of acetyl phosphate was fed, which was equivalent to twice the initial molar amount of glucose. The reactions were carried out at 30°C in a 30-ml initial volume of 0.1 M TEA buffer (pH 8.0) containing 200 mM glucose, 0.2 mM ATP and 20 mM MgCl₂. The concentrations of the enzymes in the reaction mixtures were (3.25-13.12) x 10⁻⁸ M GK, (3.49-13.96) x 10⁻⁸ M PGI, (5.18-20.72) x 10⁻⁸ M PFK and (9.12-36.48) x 10⁻⁹ M AK. Figure 2 shows the experimental results obtained using three levels of enzyme concentration. In this figure, the molar amount of FDP produced is shown because the volume of the reaction mixture changed with time. When the syntheses were performed using higher concentrations of the crude cell extract, larger molar amounts of FDP were produced and higher FDP production rates were attained, resulting in higher yields of FDP (the yields were 0.67, 0.90 and 0.98 for the data represented by ○, Δ and △, respectively). Furthermore, since the time required to complete the reaction was reduced, the productivity was also effectively improved by using higher enzyme concentrations (the productivities were 3.80, 7.10 and 11.56 mol·m⁻³·h⁻¹ for the data represented by ○, Δ and △, respectively). The figure shows the good agreement between the experimental results and the theoretical predictions as indicated by the solid lines.

The crude cell extract of B. stearothermophilus which contains all the four enzymes required for FDP synthesis was examined as the catalyst with good results. Similar to the results obtained using the purified enzymes, the agreement between the experimental data and the theoretical predictions was satisfactory. This indicates that the crude cell extract of B. stearothermophilus does not contain detectable amounts of inhibitors, activators or aldolase, and that the rate equations derived using the purified enzymes can be used for the analysis of the experimental results obtained using the crude enzyme. These results will in turn be very important in developing an efficient method for FDP synthesis on a larger scale using the crude cell extract of B. stearothermophilus.

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