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Enzymatic recycling of NADPH at high temperature utilizing a thermostable glucose-6-phosphate dehydrogenase from *Bacillus stearothermophilus*

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Abstract

A high temperature NADPH recycling system was designed utilizing a thermostable glucose-6-phosphate dehydrogenase (tG6PDH) from *Bacillus stearothermophilus* with glucose-6-sulfate (G6S) as the substrate. The thermostable alcohol dehydrogenase (tADH) from *Thermoanaerobacter* (formerly *Thermoanaerobium*) *brockii* was employed to catalyze the reduction of 2-butanone to 2-butanol at 55 °C. It was established that the *B. stearothermophilus* G6PDH is capable of utilizing G6S as the substrate (K_M = 50 mM at 55 °C). NADPH recycling based on the G6S/tG6PDH system performed optimally with a 3:1 ratio of tG6PDH:tADH, and with 1 mM NADP. The system produced 75 mM 2-butanol and retained 60% of NADPH after 5 h at 55 °C. Under the same conditions, the system using glucose-6-phosphate (G6P), the natural substrate for tG6PDH, produced only 25 mM 2-butanol and retained negligible amount of the co-factor.

Keywords: Alcohol dehydrogenase; Biotransformation; Glucose-6-phosphate dehydrogenase; NADPH recycling; Thermostable

1. Introduction

Enzymes isolated from thermophilic microorganisms exhibit remarkable temperature tolerance and are generally resistant to chemical denaturation, proteolytic degradation, and organic solvents [1]. The use of high temperature in biotransformations increases the solubility of substrates and improves mixing by decreasing the viscosity of the reaction mixture, resulting in an overall increase in the reaction rate. It has also been observed that the enantiomeric excess of the product depends on the reaction temperature. For example, in the reduction of 2-butanone by a secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*, an increase in temperature favors the formation of (*R*)-2-butanol [2]. Furthermore, the use of high temperature is advantageous for endothermic reactions [3]. Hence, thermostable enzymes present significant advantages over enzymes isolated from mesophilic microorganisms, because they expand the range of conditions under which catalysis can be carried out.

Dehydrogenases are used in biotransformations for the stereoselective reduction of prochiral ketones into chiral alcohols for the synthesis of fine chemicals and pharmaceutical products [4–6]. Thermostable dehydrogenases offer the possibility of increasing the temperature range of the catalytic asymmetric reduction. Thermostable alcohol dehydrogenase (tADH) from *Thermoanaerobacter* (formerly *Thermoanaerobium*) *brockii* [7–9] has been successfully employed in a number of organic reactions [10–12]. However, a limitation in biotransformations employing dehydrogenases is that they require stoichiometric amounts of the reduced form of the co-factors β-nicotinamide adenine dinucleotide (NADH) or β-nicotinamide adenine dinucleotide phosphate (NADPH) relative to the substrate. In addition to the cost constraints, the oxidized and reduced forms of both nicotinamide co-factors are unstable in solution. NADP(H), because of the 2′-phosphate group that acts as an intramolecular acid catalyst, degrades more rapidly than NAD(H) [13,14]. The degradation of co-factors is accelerated at higher temperatures and has been studied extensively

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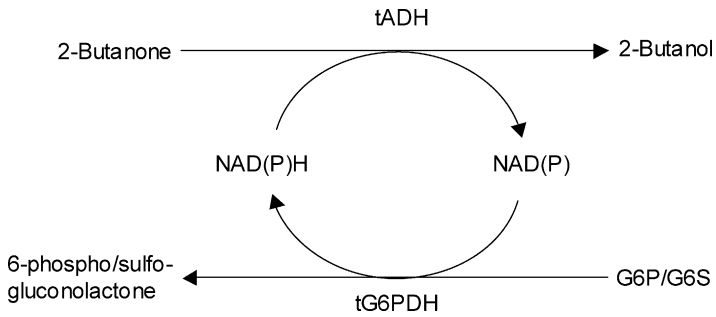

Fig. 1. NADPH recycling using a coupled-enzyme system with a thermostable glucose-6-phosphate dehydrogenase (tG6PDH) from *Bacillus stearothermophilus* and a thermostable alcohol dehydrogenase (tADH) from *Thermoanaerobacter brockii*.

[14]. Therefore, the major challenge in these systems is efficient co-factor regeneration.

Whole-cell biocatalysis in which the microorganism itself is used for the catalytic reaction, and the recycling of NAD(P)H have been described [15,16]. There have also been several electrochemical methods for regenerating nicotinamide co-factors [17–21]. Electrochemical methods generally succeed better in the oxidative direction (NADPH to NADP). The stringent demand for regioselectivity in reductive regeneration (NADP to NADPH) makes enzymatic methods more suitable. Most of the regeneration systems described are either not suitable for high temperature catalysis or are specific to a particular system. For example in tADH-catalyzed reactions, at high temperatures, the co-factor has been recycled using the same enzyme (i.e. tADH was used for both the forward and the recycling reactions) and isopropanol by a coupled-substrate approach [22]. With a growing interest in thermostable enzymes and their utility in biotransformations [3,23], there is a need for a universal high-temperature NAD(P)H recycling system that can be used with any dehydrogenase. Herein, we report the regeneration of NADPH at high temperature using a thermostable glucose-6-phosphate dehydrogenase (tG6PDH) from *Bacillus stearothermophilus*. The reduction of 2-butanone to 2-butanol by the NADP-dependent tADH was chosen as a model reaction, and the NADPH was recycled by tG6PDH as indicated in Fig. 1.

2. Experimental

2.1. Materials

Bacillus stearothermophilus G6PDH (EC 1.1.1.49) was kindly provided by Dr. Yukari Kaji (Unitika Ltd., Japan). Thermostable alcohol dehydrogenase (EC 1.1.1.2) from *T. brockii*, glucose-6-phosphate (G6P), Glucose-6-sulfate (G6S), NADP, NADPH, and methyl *tert*-butyl ether (MTBE) were obtained from Sigma (St. Louis, MO, USA), while tris(hydroxymethyl)aminomethane (Tris) was from Research Organics (Cleveland, OH, USA). Centricon (30,000 MWCO) filters were purchased from Millipore (Bedford, MA). Deionized water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). All chemicals used were of analytical grade.

2.2. Determination of K_M for G6S

G6PDH activity was measured at 55 °C by following the increase in absorbance of NADPH formed at 340 nm on a HP 8453 diode array spectrophotometer, equipped with a thermostated cuvette holder. The reaction mixture containing tG6PDH and variable amounts of G6S in 100 mM Tris–HCl, 0.5 M NaCl, pH 8.2, was allowed to reach the desired temperature and the reaction was initiated by injecting NADP. The concentration of NADP was always kept saturating. Data from these measurements were plotted as Eadie-Hofstee plots.

2.3. Recycling reaction and product analysis

The recycling reactions were performed at 55 °C in a 50-ml round-bottom flask equipped with a water condenser in 10 ml of 100 mM Tris–HCl, 0.5 M NaCl, pH 8.2. The recycling reaction mixture contained 0.5 U/ml (based on 2-butanone and NADPH as substrates at 55 °C) of tADH, 100 mM 2-butanone, 100 mM G6S or G6P, and varying concentrations of NADP and tG6PDH. Since NADP is less expensive and more stable than NADPH in solution at 55 °C, initially (at $t = 0$ min) all the co-factor was in the form of NADP. The reaction progress was analyzed by extracting 500 μl of sample with 100 μl of methyl *tert*-butyl ether at regular time intervals. The organic phase was analyzed on a GC-17A gas chromatograph from Shimadzu using a DB 210 capillary column (30 m × 0.326 mm) and a flame ionization detector. The column, injection, and detection temperatures were 40, 120, and 200 °C, respectively, with helium as the carrier gas. The percent conversion of 2-butanone to 2-butanol was calculated from the corresponding GC peaks. The two systems, G6P/tG6PDH and G6S/tG6PDH, were individually optimized to achieve maximum 2-butanol concentration that was measured after 30 min. The stability of NADPH was determined in stoppered cuvettes by measuring the decrease in absorbance at 340 nm. The half-life was calculated from semi-log plots of the data obtained.

3. Results and discussion

As mentioned earlier, there are advantages in performing biocatalysis at higher temperatures. This requires the availability of enzymes and co-factors that are thermostable. This report describes a NADPH recycling system capable of operating at high temperatures using a tG6PDH from *B. stearothermophilus*. G6PDH catalyzes the oxidation of G6P to 6-phosphoglucono-δ-lactone yielding reducing equivalents, in the form of NADH/NADPH, for reductive biosynthesis. At low temperatures, G6PDH from *Leuconostoc mesenteroides* has been used for NADPH recycling [24]. Glucose-6-sulfate was used as an alternate substrate because it was observed that NADPH was unstable in the presence of glucose-6-phosphate [25], the natural substrate for G6PDH. The decomposition of NADPH in the presence of G6P was attributed to the phosphate group of G6P that acts as an acid catalyst [25]. We found that this degradation is more rapid at 55 °C compared to room temperature (Table 1). On the contrary, the half-life of NADPH is not altered when G6S was used instead of G6P in the buffer solution. Therefore, in order to efficiently regenerate NADPH, and at the same time significantly reduce the degradation of the co-factor at high temperatures, G6S was evaluated as an alternate substrate for tG6PDH at 55 °C. G6S is commercially available or can be chemically synthesized in the laboratory by reacting glucose with pyridine–sulfur trioxide in DMF [24]. We established that, like the *L. mesenteroides* enzyme, the *B. stearothermophilus* G6PDH is able to utilize G6S as an alternate substrate. The enzyme showed a K_M of 50 ± 7 mM for G6S and 280 ± 35 μM for G6P, with NADP as the co-factor at 55 °C. The G6S/tG6PDH reaction showed a fourfold lower specific activity than the G6P/tG6PDH reaction.

3.1. Effect of enzyme ratio

The amount of tG6PDH required to achieve the highest product conversion rate was individually optimized for the two systems. The activity of tADH was kept constant (0.5 U/ml) (Fig. 2). It was found that the G6S/tG6PDH system needed approximately threefold excess of tG6PDH relative to tADH to operate optimally, whereas the G6P/tG6PDH system required a corresponding ∼10-fold excess. This difference in the amount of tG6PDH needed for the G6P/tG6PDH could be explained because of the rapid degradation of NADPH in the presence of G6P at 55 °C, failing to saturate the tADH during the entire 30 min period. Indeed as shown in Table 1, the NADPH concentration halves every 10 min in the presence of 100 mM G6P, whereas $t_{1/2} = 16$ h in the presence of G6S.

Table 1
Observed half-life of 1 mM NADPH in a buffer containing G6P/G6S at 55 °C

Buffers	$t_{1/2}$ at 55 °C
100 mM Tris–HCl, 0.5 M NaCl, 0.1 M G6P, pH 8.2	10 min
100 mM Tris–HCl, 0.5 M NaCl, 0.5 M G6P, pH 8.2	2 min
100 mM Tris–HCl, 0.5 M NaCl, 0.1 M G6S, pH 8.2	16 h
100 mM Tris–HCl, 0.5 M NaCl, 0.5 M G6S, pH 8.2	16 h
100 mM Tris–HCl, 0.5 M NaCl, pH 8.2	16 h

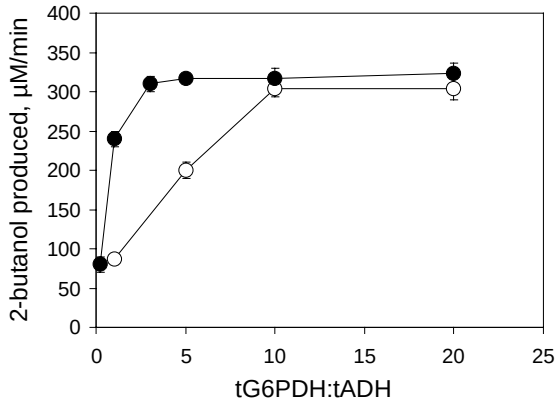

Fig. 2. Effect of tG6PDH:tADH ratio on the rate of 2-butanol production. G6S/tG6PDH (closed circle) and G6P/tG6PDH (open circle). The reaction was performed at 55 °C in 10 ml of 100 mM Tris–HCl, 0.5 M NaCl, pH 8.2, 100 mM G6S or G6P, 2 mM NADP, and 5 U of tADH. Error bars denote standard deviations ($n = 3$).

3.2. Effect of NADP concentration

In order to minimize the amount of co-factor required for the recycling, the G6S/G6P systems were optimized by varying the NADP concentration from 0.1 to 10 mM (Fig. 3). The rate of 2-butanol production was highest with 1 and 2 mM NADP for the G6S/tG6PDH and G6P/tG6PDH systems, respectively. The rate was lowered at high concentrations of the co-factor. It has been observed that tADH is inhibited by NADP ($K_{i,NADP} = 50$ μM) [26]. Although the $K_{i,NADPH}$ of the *B. stearothermophilus* G6PDH has not been reported, it is known that G6PDHs in general are inhibited by NADPH

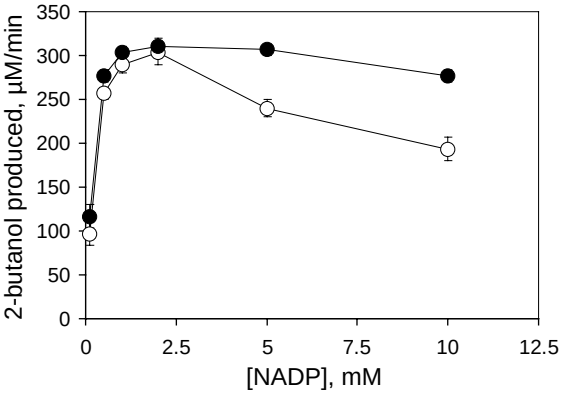

Fig. 3. Effect of NADP concentration on the rate of 2-butanol production. G6S/tG6PDH (3:1 tG6PDH:tADH, closed circle) and G6P/tG6PDH (10:1 tG6PDH:tADH, open circle) systems. The reaction was performed at 55 °C in 10 ml of 100 mM Tris–HCl, 0.5 M NaCl, pH 8.2, 100 mM G6S or G6P, and 5 U of tADH. Error bars denote standard deviations ($n = 3$).

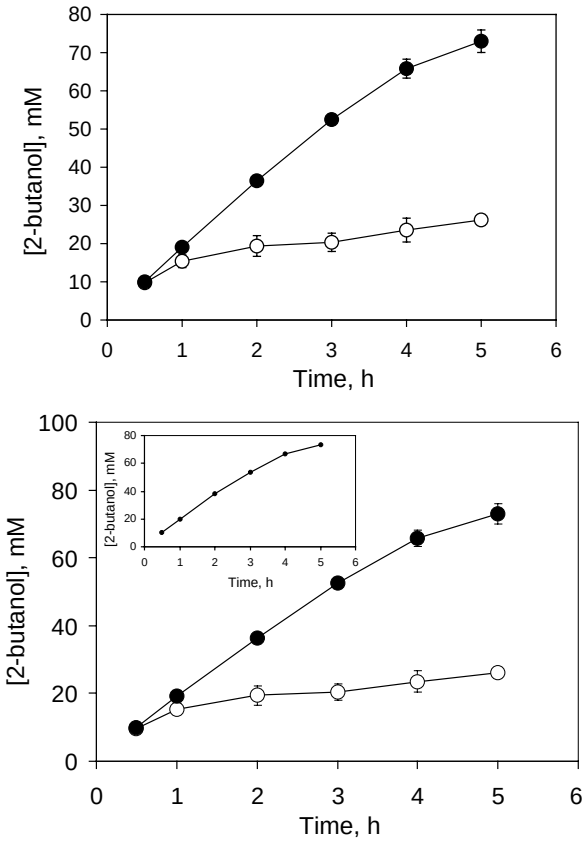

Fig. 4. Influence of substrate on reaction progress. The tG6PDH:tADH enzyme ratios were 3:1 (G6S, closed circle) and 10:1 (G6P, open circle). The reaction was performed at 55 °C in 10 ml of 100 mM Tris–HCl, 0.5 M NaCl, pH 8.2, 100 mM G6S or G6P, 2 mM NADP, and 5 U of tADH. Error bars denote standard deviations ($n = 3$). Inset depicts the progress of the reaction at 500 mM G6S.

[27]. It can be assumed that the lowered efficiency of recycling at increased co-factor concentration is due to the combined inhibition of the two enzymes by both forms of the co-factor.

3.3. Effect of G6S/G6P substrate

The effect of substrate (100 mM G6S or G6P) on the progress of the reaction was investigated with a co-factor concentration of 2 mM for the two systems (Fig. 4). The reaction progress was linear for the G6S/tG6PDH system for ~3 h, exhibiting maximum efficiency during that period. The tADH reaction is reversible and, in fact, the reverse reaction (2-butanol to 2-butanone) is more favorable [7]. As the concentration of 2-butanol increases, the reverse reaction competes, and thus, slows down the overall biotransformation reaction progress. After 5 h, the concentration of 2-butanol reached 75 mM. The progress of the reaction followed the same profile with 500 mM G6S (Fig. 4, inset), indicating that the concentration of G6S was not the limiting factor. On the contrary, the G6P/tG6PDH system did not show any linearity and the reaction progress profile is consistent with the degradation of NADPH with time. The product concentration reached 25 mM after 4 h (19 mM 2-butanol is formed in 2 h). The product concentration at 5 h was statistically indistinguishable from that at 4 h. In the presence of 500 mM G6P, only 5 mM 2-butanol was formed after 30 min. It should be noted that the half-life of NADPH at 55 °C in 500 mM G6P was determined to be 2 min (Table 1).

In a separate experiment, the residual concentration of NADPH was analyzed after separating the enzymes by using Centricon (30,000 MWCO) centrifugal filters and converting all the NADP to NADPH by glucose/glucose dehydrogenase. The G6S/tG6PDH system retained 60% of NADPH after 5 h at 55 °C, whereas the co-factor concentration was negligible in the G6P/tG6PDH system under the same conditions.

4. Conclusion

In summary, a high temperature NADPH recycling system was developed utilizing a thermostable G6PDH from *B. stearothermophilus* with G6S as the substrate. The G6S/tG6PDH system showed excellent recycling (75 mM 2-butanol is formed after 5 h at 55 °C) and enhanced stability over the corresponding G6P-based system. The *B. stearothermophilus* G6PDH exhibits dual co-enzyme specificity, in that it utilizes both NAD and NADP as the co-factor [28]. Thus, the recycling system reported here should be applicable to any dehydrogenase that uses NADPH or NADH as the co-factor, thereby enabling a wide spectrum of applications at high temperatures.

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