Note

NADPH-dependent Reduction of Ethyl Acetoacetate Coupled with Ethanol Oxidation in Kloekera magna

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We evaluated the catalytic ability of 29 yeast strains to reduce ethyl acetoacetate (EA) in the presence of ethanol or glucose. In 18 yeast strains, the reduction in the presence of ethanol proceeded as well as in the presence of glucose. Among them, Kloekera magna (AKU 4704) effectively catalyzed the NADPH-dependent reduction of EA in the presence of ethanol. In this reduction, 1 mol of EA was reduced by consuming 1 mol of ethanol. We found that the NADPH regeneration system responsible for EA reduction in K. magna was coupled with the oxidation of acetaldehyde to acetic acid catalyzed by an NADPH-dependent aldehyde dehydrogenase.

Key words: Kloekera magna, asymmetric reduction; NADPH regeneration; ethanol, aldehyde dehydrogenase

We have described the large-scale production of optically active alcohols through the asymmetric reduction of ketones catalyzed by NADPH-dependent carbonyl reductases in baker’s yeast, Saccharomyces cerevisiae.1-2 In yeast-mediated reduction, the endogenous regeneration to NADPH from NADP⁺ formed on the reduction of ketone is important to proceed with successive reduction and to produce chiral alcohols on a preparative scale. The NADPH regeneration usually proceeds through sugar consumption.3,4 Recently, we established an efficient method based on ethanol oxidation, which is applicable to the large-scale production of a chiral alcohol, (S)-ethyl 3-hydroxybutanoate (E3-HB).5-7 In baker’s yeast cells, NADPH was regenerated from NADP⁺ through the oxidative pathway of ethanol to carbon dioxide. The method is clean and more efficient than the conventional procedure.8 This paper describes the reduction ability of other yeast strains in the presence of ethanol. The reduction of ethyl acetoacetate (EA) to E3-HB, an important chiral starting material for many biologically active compounds,9 was examined in the presence of ethanol or glucose. We found that Kloekera magna (AKU 4704) as well as baker’s yeast effectively catalyzed the reduction and that the enzyme system responsible for NADPH regeneration coupled with reduction of EA in K. magna was acetaldehyde oxidation to acetate by aldehyde dehydrogenase (ALDH). This regeneration system is the third one for microbial reduction of carbonyl compounds, as two other systems, the hexose monophosphate pathway and the acetate oxidation to carbon dioxide, have already been reported.10,11

Yeast strains were obtained from the culture collection of the Faculty of Agriculture, Kyoto University (AKU), the Faculty of Engineering, Hiroshima University (HUT), and the Institute for Fermentation, Osaka (IFO). Compressed baker’s yeast was a product of the Oriental Yeast Co. (Tokyo) and was used directly. Other yeast cells were cultured in 500 ml of the medium composed of 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and 1% glucose (pH 5.5) at 30°C for 48 h with shaking. The cells were harvested by vacuum filtration, washed with 0.9% saline, and then used. A suspension of 1.0 g of yeast cells as wet weight in 20 ml of 0.1 M phosphate buffer (pH 7.0) containing 77 mm EA (10 g/liter) and 200 mm ethanol (or 300 mm glucose) was placed in a 100-ml Erlenmeyer flask and agitated at 80 rpm on a rotary shaker at 30°C. The control contained neither ethanol nor glucose. The concentrations of EA, E3-HB, ethanol, and acetic acid were measured by gas-liquid chromatography as described.9 The specific reduction rate by each yeast strain was calculated from the concentration of E3-HB after 8 h per unit weight of dry cells. The actual specific reduction rate in the presence of ethanol or glucose was obtained by deducting the specific reduction rate of the control without an energy source from the corresponding specific reduction rate and is represented as (E-N) or (G-N) [mmol/h/g-dry-cell], respectively.

Alcohol dehydrogenase (ADH) and ALDH activities in the presence of NAD⁺ or NADP⁺ as co-enzymes were measured as follows. After grinding 1 g of the cells with 2 g of aluminum oxide at 4°C, the resultant mass was mixed with 8 ml of potassium phosphate buffer (pH 7.5) and centrifuged at 4500 × g for 15 min. A portion of the supernatant, containing 5-10 mg/ml of protein measured by the Lowry method (bovine serum albumin as the protein standard), was used for the measurement. Oxidation of ethanol or acetaldehyde in the presence of NAD⁺ or NADP⁺ was monitored by absorption at 340 nm.12 One unit was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol of substrate per min at 25°C.

The reduction abilities of 29 yeast strains were evaluated by measuring the specific reduction rate of EA. Figure 1 shows a comparison between the (E-N) and (G-N) of 25 yeast strains. The results of the remaining 4 yeast strains were omitted, as they assimilated EA and E3-HB during the reaction. In more than half of the yeast strains tested, reduction in the absence of ethanol proceeded as well as in the presence of glucose, although the rates in the presence of glucose were faster. For Endomyces variabilis and Lipomyces starkeyi, the reduction hardly proceeded in the presence of ethanol. The reduction by Rhodotorula spp. (2 strains) and Sporabolomyces spp. (3 strains) proceeded quickly without an energy source. These results indicate that most of yeast strains had the reduction ability in the presence of ethanol as well as in the presence of glucose.

Figure 1 also indicates that K. magna (AKU 4704) and commercial baker’s yeast effectively catalyzed the reduction of EA in the presence of ethanol, as well as glucose. In the baker’s yeast-mediated reduction of EA in the presence of ethanol, NADPH was continuously regenerated from NADP⁺ through acetate oxidation into carbon dioxide.11 In the reaction with K. magna, however, ethanol was oxidized to acetate and the acetate

[Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; EA, ethyl acetoacetate; E3-HB, ethyl 3-hydroxybutanoate.]

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accumulated in the reaction mixture. As we confirmed that the reductases for EA in *K. magna* were also NADPH-dependent by the standard procedure, NADPH should be regenerated through ethanol oxidation to acetate.

Table shows ADH and ALDH activities in the presence of NAD⁺ or NADP⁺. The NAD⁺-dependent ADH and NADP⁺-dependent ALDH activities were significantly higher than the NADP⁺-dependent ADH and NAD⁺-dependent ALDH activities. This result suggests that the NADPH regeneration system responsible for EA reduction in *K. magna* was coupled with oxidation of acetaldehyde to acetate catalyzed by NADP⁺-dependent ALDH. Thus, the scheme for EA reduction through ethanol oxidation to acetate by *K. magna* is presented in Fig. 2.

This scheme was confirmed by the following experiments. Figure 3 shows changes in the EA reduction by *K. magna* in the presence of ethanol under aerobic (A) and anaerobic (B) conditions. Under aerobic conditions, the specific rates of EA reduction to E3-HB and ethanol oxidation to acetate were almost identical. These results suggest that the reduction of EA was coupled with the oxidative pathway of ethanol to acetate and 1 mol of EA was reduced consuming 1 mol of ethanol. On the other hand, EA reduction and ethanol oxidation did not proceed satisfactorily under anaerobic conditions. The reason may be that NADH, which was formed from NAD⁺ through oxidation of ethanol to acetaldehyde, was not oxidized to NAD⁺ through the respiratory chain under these conditions as in the baker’s yeast cells.

The NADH and NADPH regeneration shown in Fig. 2 were confirmed by examining the relationship between E3-HB production and oxygen consumption in a closed reaction system connecting a gas burette under several reaction conditions on aeration as reported. The plots of E3-HB production against oxygen consumption at several agitation speeds gave nearly straight lines with slopes ranging from 1.1 to 1.8 [E3-HB mmol/O₂ mmol] (Fig. 4). As the slope is theoretically 2.0, oxygen was effectively used in the scheme for EA reduction at an agitation speed of 40 rpm (90% efficiency). At higher agitation speeds, oxygen was wastefully used in the cells, though the specific rate of EA reduction increased (data not shown).

![Fig. 2. Possible Scheme for Reduction of EA Coupled with Ethanol Oxidation in *K. magna.*](image)

![Fig. 3. Changes in EA Reduction with *K. magna* in the Presence of Ethanol under Aerobic (A) and Anaerobic Conditions (B).](image)

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**Table ADH and ALDH Activities of *K. magna***

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>NAD⁺-ADH</th>
<th>NAD⁺-ALDH</th>
<th>NADP⁺-ADH</th>
<th>NADP⁺-ALDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity [U/mg protein]</td>
<td>1.79</td>
<td>0.06</td>
<td>0.06</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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*Fig. 1. Comparison of the Actual Specific Reduction Rate in the Presence of Ethanol and Glucose among 25 Yeast Strains.*

A suspension of 1.0 g of yeast in 20 ml of 0.1 M phosphate buffer (pH 7.0) containing 77 mM EA and energy source (or none) was shaken at 80 rpm at 30°C under anaerobic conditions. E. G. and N mean specific reduction rate of EA in the presence of ethanol, glucose, or none, respectively.

- *Saccharomyces* spp.: ▲
- *Candida* spp.: ○
- *Hansenula* spp.: △
- Others: □

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*Fig. 3. Changes in EA Reduction with *K. magna* in the Presence of Ethanol under Aerobic (A) and Anaerobic Conditions (B).*

A suspension of 1.0 g of *K. magna* in 20 ml of 0.1 M phosphate buffer (pH 7.0) containing 77 mM EA and 200 mM ethanol was shaken at 80 rpm at 30°C under aerobic or anaerobic conditions.

- *E3-HB*: ▲
- Ethanol: □
- Acetate: △
The reaction product, E3-HB, with *K. magna* was the S-configuration, as with baker’s yeast-mediated reduction and its enantiomeric excess was 76% by the same procedure as reported.\(^8\)

In baker’s yeast and other fungal strains, two NADPH regeneration systems coupled with reduction of carbonyl compounds have been reported.\(^10,11\) One was in the hexose monophosphate pathway for glucose oxidation and the other in acetate oxidation to carbon dioxide. In the two systems, the latter was thought to be more efficient. However, in the baker’s yeast-mediated reduction using the latter system, only 1 mol of EA was reduced by consuming about 2 mol of ethanol,\(^14\) while 1 mol of EA was reduced by consuming 1 mol of ethanol in *K. magna* using the regeneration system shown in Fig. 2, which was very efficient compared with that by baker’s yeast. In this study, we found a new and efficient NADPH regeneration system coupled with asymmetric reduction of ketone in yeast cells.

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**References**