Highly Efficient and Convenient Regeneration of NADPH for Asymmetric Reduction Using Bakers' Yeast Cell Free Extract

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An asymmetric reduction of ethyl pyruvate was carried out using bakers' yeast cell free extract with addition of NADPH in catalytic amounts and glucose as a hydride source. The total turnover number of NADPH reached 7680. The efficiency of the cell free extract is discussed.

The asymmetric reduction of ketones by use of isolated dehydrogenases has been well documented as a useful method in organic synthesis.[1] High enantioselectivities and high yields have been achieved by the method but the high cost of nicotinamide cofactor, NAD(P)H, has necessitated its efficient regeneration *in situ*. Thus many enzymatic methods have been developed for the NAD(P)H regeneration using various substrate/enzyme systems such as glucose/glucose dehydrogenase, glucose-6-phospate/glucose-6-phosphate dehydrogenase, alcohols/alcohol dehydrogenase, and so on.

In the previous papers,[2] we have demonstrated that asymmetric reductions by use of bakers' yeast cell free extract, compared with bakers' yeast whole cells, can be carried out with higher chemical and optical yields with additives to control the activity of enzymes contained. Herein we wish to report another striking feature of the cell free extract that it can regenerate NADPH from $NADP^+$ efficiently to give a large total turnover number of 7680 requiring only glucose as a hydride source without addition of any dehydrogenase for the cofactor regeneration.

Figure 1 shows the time course of the reduction of ethyl pyruvate (1.00 mmol) to ethyl lactate by use of bakers' yeast cell free extract (20 mL from 10 g of pressed bakers' yeast) with glucose (3.0 mmol) and NADPH (1.00, 0.50, 0.25, and 0.125 μmol) at 30 °C.[3] The reduction of ethyl pyruvate proceeded to completion after 70–90 min when 1.00 μmol of NADPH was added, with a total turnover number of 1000 (mol product formed/mol NADPH added). The reduction was slowed down by decreasing the amount of NADPH added, but the maximum conversion of 96% was attained with 0.125 μmol of NADPH after 240 min[4] to give a total turnover number of 7680.

In order to clarify the factors giving such a high total turnover number, kinetic measurements were carried out as shown in

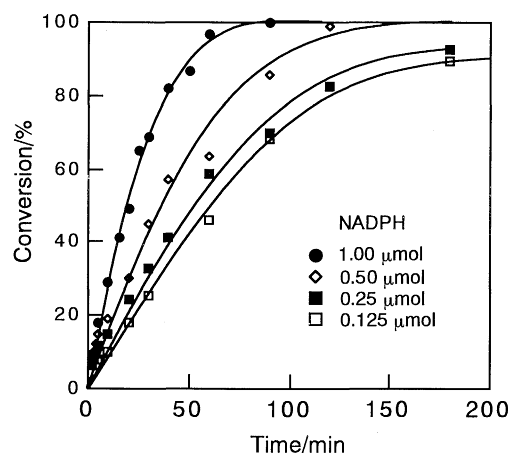

Figure 1. The time course of asymmetric reduction of ethyl pyruvate to ethyl lactate using bakers' yeast cell free extract with NADPH in catalytic amounts. Ethyl pyruvate 1.00 mmol, cell free extract 20 mL (0.1 M MES buffer/NaOH, pH 6, pressed bakers' yeast 10 g), glucose 3 mmol, 30 °C.

Table 1. Effect of NADPH concentrations on reduction rates and NADPH turnover numbers

Reduction[a]	NADPH /μmol	NADPH (/μM)	Initial rate /mmol min^{-1}	NADPH Turnover number /min^{-1}	NADPH Total turnover number / $\frac{\text{mol product}}{\text{mol NADPH}}$	K_m /mM
O, CO_2Et → OH, CO_2Et	1.00	(50.0)	0.036	36	1000	0.01
	0.50	(25.0)	0.029	58	2000	
	0.25	(12.5)	0.023	92	>3700	
	0.125	(6.25)	0.016	130	7680	
O, OAc → OH, OAc	10	(500)	0.06	6	100	0.2
	1.0	(50.0)	0.018	18	1000	
	0.5	(25.0)	0.009	18	1540	

[a]Substrate 1 mmol (50 mM), bakers' yeast cell free extract 20 mL (0.1 M MES buffer/NaOH, pH 6, pressed bakers' yeast 10 g), glucose 3 mmol, 30 °C.

Table 1.[5] The reduction of 1-acetoxy-2-propanone was conducted for comparison.

The following three factors are worthy of remark. 1) The turnover number (min^{-1}) is high. When it is low, the NADPH is degraded in the cell free extract during a longer reaction time.[4] 2) The binding of NADPH to the reductase occurs more efficiently. As shown in Table 1, a smaller K_m value of 0.01 mM is estimated for the ethyl pyruvate-reducing enzyme but a larger one of 0.2 mM is estimated for the 1-acetoxy-2-propanone-reducing enzyme.[6] 3) The turnover number of NADPH is still increasing in the reduction of ethyl pyruvate. Fortunately and unexpectedly, the regeneration of NADPH from $NADP^+$ is fast enough. The regeneration is considered to proceed by way of the hexose monophosphate-pentose pathway. Here, one molecule of ATP is consumed in the initial conversion of glucose to its 6-phosphate catalyzed by hexokinase, and one molecule of the 6-phosphate can regenerate two molecules of NADPH in the pathway. Therefore, 1 mmol of the ketone to be reduced to completion requires at least 0.5 mmol of ATP. Thus it is obvious that the regeneration of NADPH in the present preparative scale is driven by a spontaneous supply of ATP in the bakers' yeast cell free extract.[7]

In conclusion, we have demonstrated potentialities of the bakers' yeast cell free extract in the asymmetric reduction. Further uses of the cell free extract are in progress in our laboratory.

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References and Notes

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2 K. Ishihara, T. Sakai, S. Tsuboi, and M. Utaka, *Tetrahedron Lett.*, **35**, 4569 (1994). K. Ishihara, Y. Higashi, S. Tsuboi, and M. Utaka, *Chem. Lett.*, **1995**, 253.

3 The cell free extract was prepared as follows. Pressed bakers' yeast (Oriental Yeast, 30 g) was homogenized in 0.1 M morpholinoethanesulfonate buffer (pH 6, 60 mL) containing 0.5 mM diisopropyl fluorophosphate with glass beads (0.5 mm in diameter, 60 mL) using a Vibrogen cell mill at 4 °C. The homogenate was centrifuged at 12 000 rpm for 30 min at 4 °C and the supernatant (60 mL) was used without dialysis. The cell free extract thus prepared was found to contain a trace amount of NADPH that catalyzed the reduction of 1-acetoxy-2-propanone to the extent of not more than a 2% yield. The reduction of ethyl pyruvate was followed by GLC. The isolation and identification of the product ethyl lactate were as follows. The reaction mixture catalyzed by 1 μmol of NADPH was found to contain no ethyl pyruvate after 90 min, then stirred with 4 g of Celite in an ice bath for 10 min, acidifed to pH~2 (10% HCl), and extracted with diethyl ether. The crude product obtained was found to contain ethyl lactate in 52% yield by means of GLC using an internal standard. The purified product was obtained by distillation in 50% yield, and determined to be 98% ee by GLC (CP-Cyclodextrin-β-2,3,6-M-19) with the S configuration, $[\alpha]_D$ = -12.6 (c 1.34, EtOH). When 0.125 μmol of NADPH was added, the mixture was allowed to react for 300 min, to give 96% conversion, 63% yield as determined by GLC, and 95% ee.

4 After 300 min, the conversion remained unchanged. This is attributed to degradation of NADPH in the cell free extract.

5 The initial rates were measured by GLC using the conversion of less than 10%. The K_m values for NADPH were estimated using the Lineweaver-Burk plot.

6 Recently Nakamura and co-workers have isolated and characterized four NADPH-dependent ethyl pyruvate reductases from bakers' yeast, which produced (*S*)-ethyl lactate with 93.5–98.2% ee. We have also isolated a single NADPH-dependent reductase for 1-acetoxy-2-propanone to 1-acetoxy-2-propanol with >99% ee. This reductase corresponds to one of the four reductases for the pyruvate. The presence of many reductases which can bind NADPH or reduce the substrate makes analyses of the kinetic measurements complicated. So we should take the K_m values in Table 1 as apparent or effective ones. See K. Nakamura, S.-I. Kondo, N. Nakajima, and A. Ohno, *Tetrahedron*, **51**, 687 (1995) and K. Ishihara, N. Nakajima, S. Tsuboi, and M. Utaka, *Bull. Chem. Soc. Jpn.*, **67**, 3314 (1994).

7 Yamada and Shimizu's group reported that the dialyzed bakers' yeast cell free extract itself could not regenerate NADPH from $NADP^+$ although it showed activities of hexokinase and glucose-6-phosphate dehydrogenase. They needed to add ATP to regenerate the cofactor. See M. Kataoka, Y. Nomura, S. Shimizu, and H. Yamada, *Biosci. Biotech. Biochem.*, **56**, 820 (1992). Van Steveninck detected about 1.2 μmol of ATP per gram of bakers' yeast. See J. Van Steveninck, *Arch. Biochem. Biophys.*, **130**, 244 (1969).