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Asymmetric reduction of α,β-unsaturated carbonyl compounds with reductases from *Nicotiana tabacum*

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Abstract—Two reductases, p44 and p90, isolated from *Nicotiana tabacum*, catalyzed the asymmetric reduction of α,β-unsaturated carbonyl compounds. The p44 reductase reduced α-alkylated enones to (*R*)-ketones, while the reduction using the p90 reductase gave the (*S*)-ketones. The p90 reductase reduced β-alkylated enones with excellent enantioselectivities to afford the (*S*)-ketones. On the other hand, the hydrogenation of the C–C double bond of 2-methylmaleimide with both enzymes gave (*R*)-2-methylsuccinimide.

1. Introduction

Optically active ketone derivatives are potentially useful chiral building blocks for drugs and insecticides.[1,2] Asymmetric reduction of enones with biocatalysts is very attractive for the practical preparation of α-chiral ketones because of the excellent enantioselectivities of the reductases participating in the hydrogenation of C–C double bonds.[3–12] However, little attention has been paid to the production of β-chiral ketones with these reductases, because most reductases reduce the C–C double bonds when the substrates have a hydrogen atom at the β-position to the carbonyl group.[13–21]

Recently, we isolated two reductases from *Nicotiana tabacum*; a 44 kDa reductase (p44), which is capable of reducing enones with a hydrogen atom at the β-position to the carbonyl group by the *anti*-addition of hydrogen atoms, and a 90 kDa reductase (p90), which is able to reduce β-methylated enones, catalyzing the *syn*-addition of hydrogen atoms.[10–12]

On the other hand, it has been reported that the whole cells of *N. tabacum* have the ability to catalyze the asymmetric hydrogenation of the C–C double bond of 2-methylmaleimide to give (*R*)-2-methylsuccinimide with high enantioselectivity.[20]

Herein we report the asymmetric hydrogenation of the C–C double bond of enones and 2-methylmaleimide with the reductases isolated from *N. tabacum*. This approach gives a new biocatalytic method for the enzymatic production of enantiomerically pure β-substituted ketones and 2-methylsuccinimide by the reductases.

2. Results and discussion

Two reductases, p44 and p90, were isolated from plant cell cultures of *N. tabacum* in three steps involving column chromatographies. Reductions of enones **1**–**6** and 2-methylmaleimide **7** (see Fig. 1) with the p44 reductase were performed at pH 7.7 where the enzyme activity is optimal. Enones **1** and **2**, which have an alkyl substituent at the α-position to the carbonyl group, were reduced to give the corresponding (*R*)-ketones with very high enantiomeric purities (ees of 100% and >99%), as shown in Table 1. On the contrary, no reduction occurred in the cases of β-alkylated substrates **3**–**6**. When *N*-phenyl-2-methylmaleimide **7**, which was expected to be reacted as a α,β-unsaturated carbonyl compound with either α- or β-methyl substituent, was used as the substrate, the resulting *N*-phenyl-2-methylsuccinimide **14a** had an (*R*)-configuration. The enantiomeric excess of product **14a** was 100% based on the peak analysis of methyl proton signal in the ^{1}H NMR spectrum of **14a** with Eu(hfc)$_3$. This shows that the p44 reductase

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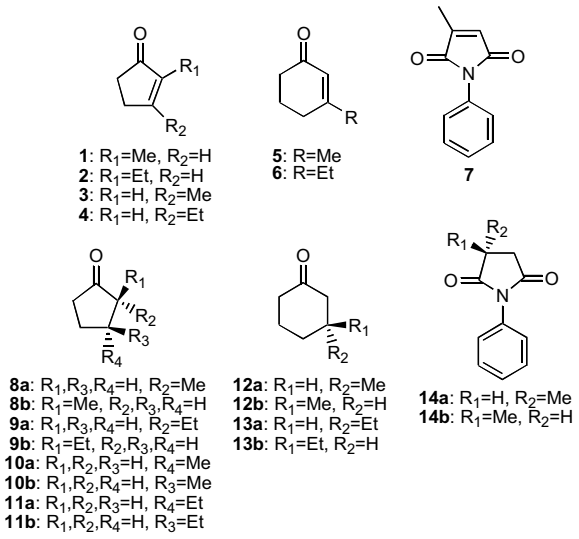

Figure 1. Structures of α,β-unsaturated carbonyl compounds and conversion products.

was able to recognize the 2-methylmaleimide derivative as a substrate and that **7** was accepted preferentially as a α-alkylated substrate by the p44 reductase. The results obtained here demonstrate that the p44 reductase from *N. tabacum* has the ability to reduce α-alkylated enones to (*R*)-ketones and catalyze the enantioface-discriminating hydrogenation of 2-methylmaleimide to give (*R*)-2-methylsuccinimide.

Substrates **1**–**7** were next subjected to the reduction with the p90 reductase at pH 7.4 (optimum pH of the enzyme). Enones **1** and **2** were reduced to give the corresponding (*S*)-ketones, and the hydrogenation at the α-position showed high enantioselectivities (ees of >99% and 98%) (Table 2). Interestingly, β-alkylated enones **3**–**6** were reduced to give the corresponding (*S*)-ketones after 12 h incubation. The enantiomeric purities of the resulting ketones **10b**, **11b**, **12b** and **13b** were ees of 100% and >99%, suggesting that the asymmetric reduction of β-alkylated enones with the p90 reductase is useful for the practical preparation of β-substituted (*S*)-ketones in their enantiomerically pure forms. The reduction of **7** gave **14a** with 89% ee, indicating that **7** was accepted preferentially as a β-alkylated substrate by the p90 reductase. These results demonstrate that the p90 reductase from *N. tabacum* is able to catalyze the asymmetric reduction of both α- and β-alkylated enones to give (*S*)-ketones and is capable of reducing 2-methylmaleimide to give (*R*)-2-methylsuccinimide.

3. Conclusions

The asymmetric hydrogenation of the C–C double bond of enones and 2-methylmaleimide has been accomplished by two reductases from *N. tabacum*. It is worth noting that the enantioselective formation of each enantiomer of the α-substituted ketones and β-substituted (*S*)-ketones has been achieved by selective use of these enzymes, which are different in substrate specificity and enantioselectivity, and that the new asymmetric hydrogenation of 2-methylmaleimide by the reductase from *N. tabacum* produced (*R*)-2-methylsuccinimide in an enantiomerically pure form.

4. Experimental

4.1. General

Chemicals such as enones, *N*-phenyl-2-methylmaleimide, (*RS*)-ketones and optically active ketones used

Table 1. Enantioselective reduction of α,β-unsaturated carbonyl compounds with the p44 reductase from *N. tabacum*

Substrates	Products	Reaction time (h)	Conversion (%)[a]	Ee (%)	Configuration
1	**8a**	24	81	100	*R*
2	**9a**	24	49	>99	*R*
3	—	24	—	—	—
4	—	24	—	—	—
5	—	24	—	—	—
6	—	24	—	—	—
7	**14a**	12	>99	100	*R*

[a] The conversions are expressed as the percentage of the product in the reaction mixture on the basis of GLC.

Table 2. Enantioselective reduction of α,β-unsaturated carbonyl compounds with the p90 reductase from *N. tabacum*

Substrates	Products	Reaction time (h)	Conversion (%)[a]	Ee (%)	Configuration
1	**8b**	24	86	>99	*S*
2	**9b**	24	44	98	*S*
3	**10b**	12	>99	100	*S*
4	**11b**	12	95	100	*S*
5	**12b**	12	99	100	*S*
6	**13b**	12	88	>99	*S*
7	**14a**	12	>99	89	*R*

[a] The conversions are expressed as the percentage of the product in the reaction mixture on the basis of GLC.

as substrates or authentic samples were purchased from Aldrich Chemical Co. The suspension cells of *N. tabacum* were cultured in 500 mL conical flasks containing 300 mL of Murashige and Skoog's (MS) medium supplemented with 3% sucrose and 10 mM 2,4-D under illumination (4000 lux) as reported previously.[22] GLC analyses were performed with FID and a capillary column of Rt-βDEX (0.25 mm × 30 m) using N_2 as a carrier gas (injector, 180 °C; detector, 180 °C; make up, 50 mL min^{-1}). Optical rotation data were obtained on a Jasco DIP-360 using a 4 mL cuvette.

4.2. Enzyme preparation

Homogenates of cultured cells of *N. tabacum* in 100 mM Na-phosphate buffer (pH 7.5) were centrifuged at 10,000*g* for 30 min to give a cell-free extract, which was then treated with ammonium sulfate (60–80% satd) to give a crude enzyme preparation. Diethylaminoethyl-Toyopearl column chromatography of the crude enzyme preparation gave a good separation of the two different reductases. Further purification by chromatography on a hydroxylapatite column and then a Red-Toyopearl column gave homogeneous reductases as judged by SDS-PAGE: p44, dimeric form composed of two identical 22 kDa subunits; p90, dimeric form composed of two identical 45 kDa subunits.[10–12]

4.3. Reduction condition

Substrates **1**–**7** (10 mg each) were administered to a mixture of the p44 reductase (ca. 30 μg) and 120 mg of NADH in 5 mL of 50 mM Na-phosphate buffer (pH 7.7) and incubated at 37 °C for 12 or 24 h. The yields of the products were determined by GLC analyses.

Reduction by the p90 reductase was performed under similar conditions except that NADPH was used as the coenzyme and that the pH of the reaction mixture was 7.4.

In order to obtain the products adequate enough for optical rotation analysis, the reaction was performed under similar conditions to the standard assay system except that the scale was 20–30 fold enlarged. Extraction from the reaction mixture with ether followed by purification using column chromatography on silica gel with pentane–ethyl acetate (95:5, v/v) gave the products.

4.4. Product identification

The products obtained by the reduction of **1** and **2** with the p44 and p90 reductases were identified as the corresponding ketones by direct comparison of GLC, GC–MS and ^{1}H NMR with those of the authentic samples. The absolute configuration and enantiomeric excesses of the resulting ketones were determined by the specific rotation of the products {**8a**: $[\alpha]_D^{25} = -112.4$ (*c* 0.51, $CHCl_3$) {lit.[23] $[\alpha]_D^{25} = -110.5$}; **8b**: $[\alpha]_D^{25} = +104.3$ (*c* 0.53, $CHCl_3$); **9a**: $[\alpha]_D^{25} = -125.7$ (*c* 0.45, $CHCl_3$); **9b**: $[\alpha]_D^{25} = +121.1$ (*c* 0.58, $CHCl_3$)} and the peak area of the corresponding enantiomers in the GLC analyses of the products on Rt-βDEX. Retention times for the ketones **8** and **9** in the GLC at the oven temperature of 100 °C were as follows: (*S*)- and (*R*)-**8**, 12.0 and 12.7 min; (*S*)- and (*R*)-**9**, 15.8 and 16.6 min.

The products obtained by the reduction of **3**–**6** with the p90 reductase were identified as the corresponding ketones by direct comparison of GLC, GC–MS and ^{1}H NMR with those of the authentic samples. The absolute configuration of the resulting ketones were determined by comparing the chiral GLC retention times with those of authentic chiral ketones and the specific rotation of the products {**10b**: CD $[\theta]_{294} = -5653$ (*c* 0.55, MeOH); $[\alpha]_D^{25} = -147.0$ (*c* 0.4, MeOH) {lit.[24] $[\alpha]_D^{25} = +154.8$ for (*R*)-enantiomer}; **11b**: CD $[\theta]_{294} = -6257$ (*c* 0.36, MeOH); $[\alpha]_D^{25} = -153.5$ (*c* 0.3, MeOH); **12b**: CD $[\theta]_{289} = -1877$ (*c* 0.59, MeOH); $[\alpha]_D^{25} = -13.2$ (*c* 0.4, MeOH) {lit.[25] $[\alpha]_D^{20} = +12.6$ for (*R*)-enantiomer}; **13b**: CD $[\theta]_{288} = -2270$ (*c* 0.50, MeOH); $[\alpha]_D^{25} = -19.9$ (*c* 0.2, MeOH)}. The enantiomeric purities of the obtained ketones were determined by the peak area of the corresponding enantiomers in the GLC analyses of the products on Rt-βDEX at the oven temperature of 80 °C. Retention times for ketones **10**–**13** in the GLC were as follows: (*R*)- and (*S*)-**10**, 15.3 and 15.9 min; (*R*)- and (*S*)-**11**, 25.2 and 26.2 min; (*R*)- and (*S*)-**12**, 25.7 and 26.6 min; (*R*)- and (*S*)-**13**, 33.8 and 34.8 min.

The products converted from **7** by the p44 and p90 reductases were identified as (*R*)-*N*-phenyl-2-methylsuccinimide **14a** {**14a** converted by the p44 reductase: $[\alpha]_D^{25} = +7.3$ (*c* 0.5, $CHCl_3$) {lit.[26] $[\alpha]_D^{22} = +8$}; **14a** reduced by the p90 reductase: $[\alpha]_D^{25} = +6.8$ (*c* 0.4, $CHCl_3$)}, respectively, by means of ^{1}H and ^{13}C NMR. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.46 (3H, d, $J = 7.1$ Hz, 2-Me), 3.04 (1H, ddq, $J = 9.3$, 4.6 and 7.3 Hz, 2-H), 2.51 (1H, dd, $J = 17.7$ and 4.5 Hz, 3-Ha), 3.10 (1H, dd, $J = 17.6$ and 9.3 Hz, 3-Hb), 7.29 (2H, d, $J = 8.3$ Hz, *o*-H), 7.39 (1H, t, $J = 7.4$ Hz, *p*-H), 7.47 (2H, t, $J = 7.7$ Hz, *m*-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 16.9 (Me), 34.9 (CH), 36.7 (CH_2), 126.4 (*o*-C in Ph), 128.6 (*p*-C in Ph), 129.1 (*m*-C in Ph), 132.0 (N–C in Ph), 175.4 (C=O), 179.5 (C=O). The intensities of the pair of methyl proton signals in the ^{1}H NMR spectra of the reduction products in the presence of Eu(hfc)$_3$ were used for the determination of the enantiomeric excess of the resulting **14a** as reported previously.[20] Methyl proton signals of **14** in the ^{1}H NMR spectrum were revealed at δ 2.56 (d, $J = 7.0$ Hz, for **14a**) and 2.64 (d, $J = 7.0$ Hz, for **14b**) in a $CDCl_3$ solution of the product and Eu(hfc)$_3$ (1:1 mol ratio). The enantiomeric purity of **14a** was confirmed by the peak area of the corresponding enantiomers in the chiral GLC analyses of the products on Rt-βDEX at the oven temperature of 80 °C. Retention times for **14** in the GLC were as follows: (*R*)- and (*S*)-**14**, 40.2 and 40.9 min.

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