Phytochemistry, 1970, Vol. 9, pp. 2319 to 2321. Pergamon Press. Printed in England.

SHORT COMMUNICATION

LIMONENE REDUCTASE SYSTEM IN THE ORANGE

V. H. POTTY* and J. H. BRUEMMER

U.S. Fruit and Vegetable Products Laboratory,† Winter Haven, Florida 33880, U.S.A.

(*Received* 14 *January* 1970, *in revised form* 20 *March* 1970)

Abstract—(+)-Limonene was reduced to Δ^1*p*-menthene by an extract from neutralized orange juice vesicles with either NADPH or NADH. This limonene reductase system was partially purified from the soluble fraction of the extract. Activity was specific for the (+)-form; (−)-limonene was inactive. The enzyme system reduced $\Delta^{8(9)}$*p*-menthene more rapidly than (+)-limonene.

INTRODUCTION

(+)-LIMONENE IS the major terpene in citrus.[1] It accumulates in peel oil of Valencia oranges during growth to reach 90% of mature oil.[2] This reservoir of limonene in citrus oil glands cannot be adequately explained as a terminus for "secondary products of metabolism", in view of the evidence reviewed by Loomis[3] of the dynamic metabolism of monoterpenes.

We have examined extracts of orange juice vesicles for ability to reduce limonene, considering reduction as a probable reaction in limonene metabolism. The reduction of limonene by an NADH/NADPH-dependent reductase in the extracts described in this report is the first instance of an enzyme-catalyzed limonene reaction in higher plants.

RESULTS AND DISCUSSION

The (+)-limonene oxidized both NADH and NADPH in the presence of neutralized extracts from orange juice vesicles. The rate of oxidation was linear and proportional to the concentration of the enzyme extract. Exposure of pH 5·0 or lower for 5 min rapidly inactivated the enzyme but activity was stable to pH values between 6 and 11. Maximal activity was obtained at pH 9.0.

Data in Table 1 show that activity for NADH oxidation was concentrated in the soluble fraction and was precipitated between 30 and 60% saturation with $(NH_4)_2SO_4$. Neither mitochondrial nor microsomal fraction showed appreciable activity.

Oxidation of NADH or NADPH by the purified enzyme preparation is specific for (+)-limonene and monoterpenes with unsaturation at $\Delta^{8(9)}$. Table 2 shows that no activity was obtained with terpenes without this functionality, such as, Δ^1*p*-menthene, $\Delta^{2,4(8)}$*p*-menthadiene, α- and γ-terpinene, *p*-cymene, β-myrcene, and α-phellandrene. Almost 2·5

* NRC-Postdoctoral Research Associate.

† One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

References to specific products of commercial manufacture are for illustration only and do not constitute endorsement by the U.S. Department of Agriculture.

[1] W. L. STANLEY, in *Flavor Research and Food Acceptance* (edited by L. B. SJOSTRON), p. 344, Reinhold, New York (1958).

[2] J. A. ATTAWAY, A. P. PIERINGER and B. S. BUSLIG, *Phytochem.* 7, 1695 (1968).

[3] W. D. LOOMIS, in *Terpenoids in Plants* (edited by J. B. PRIDHAM), p. 59, Academic Press, New York (1967).

times more activity was obtained with $\Delta^{8(9)}p$-menthene than with (+)-limonene. The results with NADH and NADPH are comparable; therefore, only NADH data is shown.

No appreciable activity was obtained with (−)-limonene and activity with the racemic mixture, dipentene, was 36% of the rate with (+)-limonene. The apparent specificity of the enzyme for C8–9 unsaturation on monoterpenes suggested that $\Delta^{1}p$-menthene was the product of limonene in the oxidation of NADH and NADPH. GLC separation of the reaction

TABLE 1. DISTRIBUTION OF LIMONENE-DEPENDENT NADH OXIDATION IN JUICE CELLS

NADH/100 g juice vesicles	Protein	Total activity NADH oxidation
	mg	nmole/min
Mitochondrial fraction	6·1	0
Microsomal fraction	1·9	40
Soluble fraction		
0–30% $(NH_4)_2SO_4$ ppt	1·1	57
30–60% $(NH_4)_2SO_4$ ppt	6·4	670
60–90% $(NH_4)_2SO_4$ ppt	4·8	24

The $(NH_4)_2SO_4$ fractions were suspended in 5 ml of 0·05 M Tris, pH 9·0, and dialyzed for 90 min before assay.

TABLE 2. SUBSTRATE SPECIFICITY FOR OXIDATION OF NADH BY PURIFIED PREP.*

Substrate†	NADH oxidation (nmole/min)	Relative activity
(+)-Limonene	22	100
(−)-Limonene	1	5
Dipentene	8	36
$\Delta^{1}p$-Menthene	0	0
$\Delta^{8(9)}p$-Menthene	52	236
$\Delta^{2,4(8)}p$-Menthadiene	0	0
γ-Terpinene	2	9
α-Terpinene	0	0
p-Cymene	2	9
β-Myrcene	2	9
α-Phellandrene	0	0

* Dowex-treated preparation, 0·2 mg protein/cuvette.
† All substrates were added as 0·02 ml of 5% solution of substrate in acetone.

products and comparison with a pure sample of $\Delta^{1}p$-menthene showed that the C8–9 double bond of limonene was reduced in the reaction.

Limonene reductase, the designation for this enzyme in orange juice vesicles that reduces $\Delta^{8(9)}$ of limonene, is the first enzyme reported for limonene metabolism in higher plants. The significance of limonene reductase in citrus is not readily apparent. We were unable to demonstrate the reversibility of the reaction using $\Delta^{1}p$-menthene as substrate with NAD or NADP and high concentrations of the enzyme preparation. In addition, the product of

limonene reduction, Δ^1*p*-menthene, has not been identified as a constituent of citrus. Purification and characterization of limonene reductase is in progress as the first step toward determining its function.

EXPERIMENTAL

Commercially immature Valencia oranges harvested from a local grove, November through February, were used as enzyme source. The juice vesicles were separated from other tissues by the freezing method described by Potty.[4] A 100 gm portion of frozen vesicles was warmed to 4° in 100 ml of 0·25 M sucrose containing enough 1 M Tris to adjust the pH of homogenate to pH 7 after blending. The suspension of thawed vesicles was blended for 3 sec and centrifuged at 500 *g* for 10 min. The residue was discarded and the supernatant was fractionated by differential centrifugation in a Beckman L2-65 Ultracentrifuge. The fraction sedimenting at 10,000 *g* after 15 min was designated "mitochondrial"; the fraction sedimenting at 100,000 *g* after 45 min was designated "microsomal" and the supernatant from the high-speed run was designated "soluble fraction". The particulate fractions were resuspended in 10 ml of 0·25 M sucrose with 0·05 M Tris, pH 9·0, for assay. All procedures were carried out at 4°.

Enzyme purification was carried out on neutralized extracts prepared from the vesicles as reported previously.[5] The 10,000 *g* supernatant from the extract was treated with $(NH_4)_2SO_4$. The protein fraction precipitating between 30 and 60% salt saturation was suspended in 0·05 M Tris, pH 9·0, dialyzed for 1·5 hr, and then treated batchwise with pre-swelled Dowex-1 (Cl^-) 50%, w/v to remove anionic pigments. The Dowex-treated filtrate was used as the purified enzyme. All procedures were carried out at 4°.

Protein was estimated by a modified Lowry procedure.[6] Enzyme preparations were stored at −96°.

The (−)-limonene, $\Delta^{8(9)}$*p*-menthene and Δ^1*p*-menthene were gifts from Glidden–Durkee Division of SCM Corporation. The other monoterpenes were isolated in this laboratory from essential oils and were better than 99% pure.[7] Each of these compounds was dissolved in acetone to give 5% solutions. NADH and NADPH were purchased from Sigma Chemical Company.

Enzyme activity was assayed spectrophotometrically by monitoring the oxidation of NADH or NADPH in the presence of enzyme and substrate at 30°. Both the reaction and reference cuvettes (1 cm light path) contained the enzyme prep. (0·1–1·0 mg protein), 0·02 ml of 0·015 M NADH or NADPH and 0·05 M Tris, pH 9·0, to total of 2·98 ml. The reaction was initiated by adding 0·02 ml of 5% solution of (+)-limonene or other monoterpenes in acetone to the reaction cuvette and 0·02 ml acetone to the reference cuvette. The rate of NADH or NADPH oxidation was measured by recording the linear decrease in absorbance at 340 nm for 5 min after acetone addition. The amount of NADH oxidized was calculated in nmole using $6{\cdot}2 \times 10^3$ as molar extinction coefficient.

To identify the product of limonene reduction, the reaction mixture was extracted with Et_2O and analyzed with a Perkin–Elmer Model 154 vapor fractometer on a 300 × 0·6 cm aluminum column packed with 6% diethylene glycol succinate-coated Chromosorb W. With column temp. of 82° and helium flow rate at 75 ml/min, limonene had a retention time of 3·8 min; Δ^1*p*-menthene, 2·8 min; and $\Delta^{8(9)}$*p*-menthene, 2·0 min.

[4] V. H. Potty, *J. Food Sci.* **34**, 231 (1969).
[5] V. H. Potty and J. H. Bruemmer, *Phytochem.* **9**, 1001 (1970).
[6] V. H. Potty, *Anal. Biochem.* **29**, 536 (1969).
[7] E. D. Lund, personal communication.