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OXIDATION OF GERANIOL BY AN ENZYME SYSTEM FROM ORANGE

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Abstract—Enzyme preparations from orange juice vesicles reversibly oxidized geraniol in the presence of NADP. NAD was ineffective for this reaction. The enzyme mediating this reaction—geraniol dehydrogenase (GeDH)—was separated from alcohol dehydrogenase by ammonium sulfate fractionation and gel filtration. GeDH also oxidized nerol, farnesol and citronellol, but at slower rates. Whereas pH9·0 was optimal for geraniol oxidation, geranial was reduced maximally at pH 6·5. Thiol-reacting compounds and metal chelators inhibited GeDH activity. Increasing the ratio of the reduced to oxidized forms of NADP progressively depressed the rate of geraniol oxidation. Thus, in juice cells the redox state of the nucleotide coenzyme may regulate the equilibrium between geraniol and geranial.

INTRODUCTION

THE CHARACTERISTIC aroma of citrus fruits is generally ascribed to mixtures of a variety of terpenoid compounds. The oxygenated terpenes, representing about 5 per cent of the citrus oils, provide the aroma typical of individual fruits.[1] Acyclic isoprenoid alcohols, such as geraniol, nerol, citronellol and farnesol, and the corresponding aldehydes, have been reported to be present also in citrus essence.[2,3] The presence of these alcohols and aldehydes in the fruit is an indication that the fruit tissue might have the necessary enzymes that could bring about their interconversions. A number of oxidoreductase enzymes use nicotinamide-adenine dinucleotide (NAD) and/or its phosphate (NADP) as cofactors. Orange and grapefruit vesicles contain sizeable levels of these cofactors both in oxidized and reduced states.[4] These observations prompted us to examine citrus fruits for the presence of enzyme(s) mediating the interconversion of acyclic alcohols and aldehydes. This paper reports the occurrence of an oxidoreductase in orange juice vesicles catalyzing the reversible oxidation of geraniol with NADP as cofactor. The enzyme was partially purified and some of its properties studied.

RESULTS

Oxidation of Geraniol to Geranial

NADP was reduced by geraniol in the presence of a crude enzyme preparation from orange juice vesicles. Geranial was identified as the product of this reaction. NAD was

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Mention of brand names does not imply endorsement.

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

[2] G. L. K. HUNTER and M. G. MOSHONAS, *Anal. Chem.* 37, 268 (1965).

[3] R. W. WOLFORD, J. A. ATTAWAY and L. J. BARABAS, *Proc. Florida State Hort. Soc.* 78, 268 (1965).

[4] J. H. BRUEMMER, *J. Agr. Food Chem.*, 17, 1312 (1969).

ineffective as a cofactor for this oxidation, but was effective in the oxidation of ethanol by the same crude enzyme preparation; NADP could not replace NAD in this latter oxidation.

Partial Purification of Geraniol Dehydrogenase

To obtain more information about the geraniol-oxidizing enzyme, purification was attempted with routine fractionation techniques. Whereas the bulk of the ethanol-oxidizing activity was concentrated in a protein fraction precipitating between 45 and 55 per cent ammonium sulfate saturation, most of the geraniol-oxidizing activity was salted out between 55 and 70 per cent ammonium sulfate saturation (Table 1). This difference in solubility indicated that separate enzymes are responsible for the oxidation of ethanol and geraniol. The terms alcohol dehydrogenase (ADH) and geraniol dehydrogenase (GeDH) are used here to refer to these two activities. GeDH was also separated from ADH by gel filtration on Sephadex G-100. GeDH was estimated to have a molecular weight of 92,000 and ADH, a molecular weight of 73,000 using yeast ADH, heart isocitrate dehydrogenase and cytochrome C as standards for calculation.

TABLE 1. SEPARATION OF GeDH AND ADH BY SALT PRECIPITATION

% $(NH_4)_2SO_4$ saturation	Activity distribution	
	% ADH	% GeDH
0–30	1·4	—
30–45	8·8	—
45–55	60·6	12·7
55–70	29·2	87·3

A six-fold purification of GeDH was achieved by dialysis, treatment with Dowex anion exchanger and Sephadex filtration (Table 2). The GeDH fraction from the Sephadex column was analyzed by disc electrophoresis on polyacrylamide gels. At least five protein bands were detected, indicating that this preparation contained other proteins besides GeDH. The enzyme was not purified beyond this stage.

TABLE 2. PURIFICATION OF GeDH FROM ORANGE JUICE VESICLES

Enzyme preparation	GeDH activity (units/mg protein)
Juice extract	Not detected
55–70% $(NH_4)_2SO_4$ ppt.	33
After 2 hr dialysis	68
After Dowex treatment	112
After Sephadex gel filtration on G-100	203

pH Optima and Stability of GeDH

The optimum pH for the oxidation of geraniol to geranial was 9·0, whereas the reverse reaction was maximal at pH 6·5. The enzyme had poor stability at acidic pH. Exposure to pH below 5 for 5 min inactivated the enzyme completely and irreversibly. In the alakaline range the enzyme was stable up to pH 11·0. Dialyzed preparations rapidly lost GeDH

activity unless stored at −19°. Undialyzed, ammonium sulfate fractionated preparations were quite active after 6 months' storage at −96°.

Substrate Specificity

Besides geraniol, nerol was also oxidized by the enzyme but at only 60 per cent of the rate with geraniol (Table 3). Citronellol and farnesol were oxidized at even slower rates, but decanol, terpineols and linalool were completely unreactive.

TABLE 3. SUBSTRATE SPECIFICITY OF GeDH

Alcohol*	Relative activity
Geraniol	100
Nerol	60·4
Farnesol	10·5
Citronellol	7·5

Alcohols were added as 5% solutions in acetone. Activities are expressed relative to rate of geraniol oxidation.

*α-Terpineol, 4-terpineol, n-decanol and linalool showed no activity.

Inhibition of GeDH

GeDH activity was inhibited by iodoacetamide. Addition of mercaptoethanol to iodoacetamide-treated enzyme preparation did not restore the activity to the original level. However, the inclusion of mercaptoethanol before adding iodoacetamide protected the enzyme from inhibition by molar equivalents of iodoacetamide. Mercaptoethanol, by itself, was stimulatory to the enzyme.

A number of well-known metal chelators inhibited GeDH activity readily (Table 4). The most potent was 8-hydroxyquinoline, which at a concentration $6{\cdot}7 \times 10^{-4}$ M caused a 50 per cent decrease in the rate of NADP reduction. Zinc ions were also found to be a strong inhibitor of GeDH activity. Oxidized glutathione at $3{\cdot}3 \times 10^{-3}$ M concentration completely

TABLE 4. INHIBITION OF GeDH ACTIVITY

Inhibitor	Concentration ($M \times 10^{-4}$)	Inhibition (%)
Sodium azide	830	50·8
8-Hydroxyquinolone	6·70	50·2
O-Phenanthroline	167	50·0
α,α′-Dipyridyl	250	50·0
EDTA	677	52·6
Zn^{++}	16·70	49·3
Glutathione, oxidized	33·33	100·0
(+)-Limonene	4·88	100·0
Linalool	11·20	56·3
α-Terpineol	5·60	57·6

Zn^{++} was added as $ZnCl_2$ solution; (+)-limonene, linalool and α-terpineol were added as acetone solutions. Test compounds were added to cuvette before geraniol.

stopped net formation of NADPH by geraniol. Some of the terpenoid compounds also were effective in reducing or stopping the rate of reduction of NADP by geraniol. (+)-Limonene at comparatively low concentration completely stopped any NADPH formation whereas linalool and α-terpineol were less effective.

Effect of Redox Ratio on GeDH

The state of redox potential existing in the reaction system profoundly influenced the oxidation of geraniol by GeDH (Table 5). When the ratio of NADPH/NADP was 1:1 the oxidation of geraniol was completely suppressed. Even when this ratio was only 0·083 there was discernible inhibition in the rate of geraniol oxidation. Thus, redox ratios, approaching unity, generally favor the formation of geraniol whereas lower ratios push the equilibrium towards the formation of geranial.

TABLE 5. EFFECT OF REDOX RATIO ON GeDH ACTIVITY

NADPH (μmoles/cuvette)	$\frac{\text{NADPH}}{\text{NADP}^*}$	GeDH activity (units/mg protein)	% Inhibition
0		72·5	0
0·125	0·083	64·5	11
0·250	0·167	55·6	23
0·500	0·333	32·3	55
1·500	1·0	0	100

*1·5 μM of NADP was present in all cases.

Michaelis Constants of GeDH and ADH

Partially purified preparation from orange juice vesicles was used to determine the K_m values of GeDH and ADH at pH 7·0 and 9·0 (Table 6). The K_m values for GeDH were lower at pH 9·0 than at pH 7·0, indicating greater affinity between the enzyme and the substrates at pH 9·0. The affinity between ethanol and ADH was higher at pH 7·0 than at pH 9·0. But ADH had a higher affinity for NAD at pH 9·0. Generally, GeDH showed a higher affinity for its substrates than ADH showed for its substrates.

TABLE 6. K_m VALUES OF GeDH AND ADH

Enzyme	K_m* at pH ($\times 10^4$) 7·0	9·0
GeDH (geraniol)	0·833	0·465
GeDH (NADP)	8·30	4·54
ADH (ethanol)	1·19	117.0
ADH (NAD)	1·05	0·567

* K_m values were determined by the method of Lineweaver and Burke[5] using dialyzed preparations from 45–55% amm. sulfate fraction for ADH and from 55–70% amm. sulfate fraction for GeDH.

[5] H. LINEWEAVER and D. BURKE, *J. Am. Chem. Soc.* **56**, 658 (1934).

DISCUSSION

The presence of an enzyme mediating the reversible oxidation of geraniol in juice vesicles is clearly established by the present studies. This enzyme converts geraniol to geranial and *vice versa* depending on the nature of the cofactor available and the pH of the reaction system. The only report of a similar enzymatic oxidation of a terpene alcohol describes the use of liver alcohol dehydrogenase for oxidation of farnesol but with NAD as the cofactor.[6] Battaile *et al.*[7] reported reduction of pulegone to methanol by cell-free extracts of peppermint shoot-tips which was dependent on NADPH.

GeDH of orange fruit vesicles differs from the conventional alcohol dehydrogenase in many respects. Whereas ADH of orange in NAD-dependent, GeDH utilizes only NADP as cofactor. Liver ADH is known to accept both NAD and NADP and this may be the reason for its broad specificity. Most of the ADH of orange precipitated between 45–55 per cent ammonium sulfate concentration, whereas GeDH was mainly concentrated in the 55–70 per cent ammonium sulfate fraction. The two enzymes also differed in their molecular weights as determined by gel filtration. In addition, ADH was observed to be more stable than GeDH during storage at $-19°$. On the basis of these differences, GeDH should be classified separately from ADH.

The redox potential of the reaction system has a great influence on the rate of geraniol oxidation. Bruemmer[4] observed that the ratio NADPH:NADP in orange juice remained relatively constant (1·3:1·5) during the period of maturation. This high redox ratio is favorable for the reverse reaction, namely reduction of geranial to geraniol. Geraniol has been implicated as a key intermediate in terpenoid biosynthesis in a number of biological systems.[8,9] If this is true in orange, terpene formation is probably regulated by the redox potential at the site of synthesis of these compounds.

Active thiol groups are probably essential for GDH activity. The enzyme was inhibited by iodoacetamide and Zn^{2+} which are known to react with thiol groups. The protective action of mercaptoethanol against iodoacetamide also points to the participation of thiol groups in the reaction. In this respect the alcohol dehydrogenase enzymes from both plants and animals show absolute requirement for —SH groups.[10] That GeDH could be a metalloprotein was indicated by the inhibition of GeDH activity by metal chelators. A number of non-flavoprotein dehydrogenases are known to contain Zn^{2+} as an integral part of their structure.[11] GeDH also might be a zinc-containing enzyme but we have no evidence on this point.

The action of oxidized glutathione was found to be due to the presence of glutathione reductase in our enzyme preparation.[12] As soon as NADPH was formed in the system, it was reoxidized by glutathione reductase, resulting in no detectable NADPH. Since the assay of GDH was based on the rate of NADPH formation, the addition of oxidized glutathione made

[6] G. Ryback, in *Terpenoids in Plants* (edited by J. B. Pridham), p. 47, Academic Press, London (1967).
[7] J. Battaile, A. J. Burbott and W. D. Loomis, *Phytochem.* 7, 1159 (1968).
[8] T. W. Goodwin, in *Biosynthetic Pathways in Higher Plants* (edited by J. B. Pridham and T. Swain), p. 57, Academic Press, London (1965).
[9] H. J. Nicholas, in *Biogenesis of Terpenes in Plants* (edited by P. Bernfeld), p. 641, Pergamon Press, Oxford (1963).
[10] H. Sund and H. Theorell, in *The Enzymes* (edited by P. D. Boyer, H. Lardy and K. Myrback), Vol. 7, p. 25, Academic Press, New York (1963).
[11] B. L. Vallee, in *The Enzymes* (edited by P. D. Boyer, H. Lardy and K. Myrback), Vol. 3, p. 225, Academic Press, New York (1960).
[12] Unpublished observations.

it appear as if geraniol oxidation was inhibited. Limonene, α terpineol and linalool also probably acted the same way as oxidized glutathione. These compounds oxidized NADH, as well as NADPH, in the presence of the enzyme preparation.[13]

The oxidation of NADPH and NADH by limonine, α-terpineol and linalool with the enzyme preparation suggests the presence in orange juice vesicles of a reductase which acts on cyclic and acyclic terpenes containing double bonds. Since geraniol, geranial, nerol and neral are also substrates for this pyridine dinucleotide oxidation, the reductase might account for the presence of citronellol and citronellal in orange juice vesicles. The relationship between these acyclic terpenoids may be summarized as shown in Fig. 1. GeDH maintains the equilibrium between the aldehyde and alcohol moieties and the terpenoid reductase saturates one of the double bonds to citronellol and citronellal. The isolation and identification of this terpenoid reductase is reported in another paper.[13]

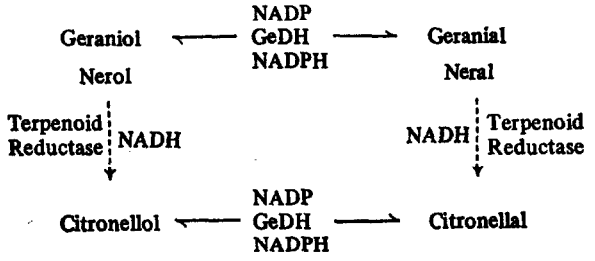

FIG. 1. RELATIONSHIP BETWEEN ACYCLIC TERPENOIDS IN ORANGE.

EXPERIMENTAL

Materials

The terpenoid compounds (purity > 99%) were provided by Dr. Eric D. Lund of this laboratory. Other chemicals were purchased from Sigma Chemical Company, Fisher Scientific Company and Matheson, Coleman and Bell.

Enzyme Source

Orange fruits with Brix/acid ratio of 6:1 and above were obtained from local groves to prepare enzyme extracts.

Enzyme Preparation

The peeled fruits were quick-frozen in liquid N_2 and juice vesicles separated from accompanying fibrous and seed materials as described earlier.[14] Neutralized juice extracts were prepared from these vesicles using insoluble polyvinyl pyrrolidone, ascorbate, and tris buffer according to the method illustrated elsewhere.[15] The 10,000 *g* supernatant was fractionated with $(NH_4)_2SO_4$. The precipitates were collected, suspended in 0·1 M tris buffer, pH 7·0, and dialyzed for 2 hr against H_2O containing 0·001 M mercaptoethanol at 4°. The dialyzed preparations were then centrifuged, the supernatants treated with 50% (w/v) pre-swelled Dowex-1-(Cl—) for 30 min and filtered through glass-wool. The filtrates were diluted to contain 1 mg protein per ml as estimated by a modified Lowry's method.[16]

Enzyme Assays

Enzyme preparations were assayed for ADH according to the spectrometric method of Racker.[17] Cuvettes with 1 cm light path were used to study the reaction. A 3 ml reaction mixture contained 270 μmoles of tris

[13] V. H. POTTY and J. H. BRUEMMER, submitted for publication in *Phytochem.*
[14] V. H. POTTY, *J. Food Sci.* **34**, 231 (1969).
[15] V. H. POTTY and J. H. BRUEMMER, *Phytochem.*, **9**, 99 (1970).
[16] V. H. POTTY, *Anal. Biochem.* **29**, 535 (1969).
[17] E. RACKER, *J. Biol. Chem.* **184**, 313 (1950).

buffer, pH 9·0, 1·5 μmoles of NAD, 0·87 mmole ethanol and about 50–100 μg enzyme protein. The reaction was initiated by adding EtOH and the increase in absorbance at 340 nm was recorded in a Beckman-DU-Gilford Spectrophotometer at 30°. NADH generated in the cuvette was calculated in mμmoles using $6{\cdot}2 \times 10^3$ as molar extinction coefficient of NADH. One unit of enzyme catalyzed the reduction of 1 mμmole NAD/min under the conditions of our experiment.

The assay system for GeDH was essentially the same as that used above except that 1·5 μmoles of NADP was substituted for NAD. Terpene alcohols could not be used as aqueous solutions because of their low solubility in water. A number of solvents were tried but acetone and methanol were found to be excellent solvents which were not inhibitory to ADH up to 1 M concentrations. The reaction was initiated by adding 20 μl of a 5% geraniol solution in acetone. The rate of NADPH generation was calculated from the time-course curve as described in ADH assay. One unit of GeDH catalyzed the formation of 1 mμmole NADPH/min under the experimental conditions.

Separation of GeDH and ADH by Gel Filtration

A Sephadex G-100 column of 430 ml bed volume was employed to separate GeDH and ADH from a 90% $(NH_4)_2SO_4$ fraction. The column was developed with 0·01 M tris buffer, pH 8·0, containing 0·001 M mercapto-ethanol. Each 5-ml elution fraction was assayed for ADH and GeDH activity ADH appeared in the 38–42 fraction and GeDH in the 40–47 fraction. In a duplicate run, cytochrome C, yeast ADH and isocitrate dehydrogenase (Sigma Chemical Company) of known molecular weights were added to the orange enzyme preparation before gel filtration. Approximate molecular weights of orange ADH and GDH were calculated from their location with respect to the reference proteins in the effluent fractions.[18]

GLC Studies

The product of GeDH oxidation of geraniol was identified by GLC on a 3 m × 6 mm aluminium column packed with diethylene glycol succinate coated (6%, w/w) chromosorb W. The reaction mixture, in a total volume of 10 ml, contained 5 mg enzyme protein (55–70% ammonium sulfate fraction), 20 μmoles NADP and 5 μl geraniol. NADP was continuously regenerated in the reaction system by the inclusion of oxidized glutathione and glutathione reductase. After 1 hr the reaction mixture was acidified and the precipitated proteins were removed. After the supernatant was extracted with Et_2O, the Et_2O was concentrated in a stream of N_2 and an aliquot of the concentrate was analyzed on the column (150°) in a Perkin–Elmer Vapor Fractometer, Model 154 (helium flow, 70 ml/min), fitted with a flame-ionization detector. The retention times for geraniol and geranial were 13·2 min and 15·7 min respectively.

[18] P. ANDREWS, *Biochem. J.* 91, 222 (1964).