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BIOSYNTHESIS OF ALLYLIC ISOPRENOID PYROPHOSPHATES BY AN ENZYME PREPARATION FROM THE FLAVEDO OF *CITRUS PARADISII*

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Key Word Index—*Citrus paradisii*; Rutaceae; grapefruit; terpene biosynthesis; sesquiterpenes; allylic pyrophosphates; mevalonic acid; isoprenoid pathway; metal stereospecificity.

Abstract—A cell-free system obtained from *Citrus paradisii* flavedo transformed mevalonic acid into mono- and sesquiterpenoids of *E*- and *Z*-conformation. The enzyme system required bivalent metal ions and the presence of sulfhydryl groups. IPP isomerase activity (EC 5.3.3.2) was independent of metal ions and almost insensitive to sulfhydryl group reagents, while prenyltransferase (EC 2.5.1.1) was inactivated by DTNB and required bivalent metals for activity. The nature of the metal ion defined the stereochemistry of the products formed by prenyltransferase. The ratio of *E*–*Z* farnesylpyrophosphates was 3:1. This *Citrus* species could, therefore, be a good starting material for the study of the stereochemistry of the enzymes forming *E* and *Z* sesquiterpenoids.

INTRODUCTION†

The biosynthesis of allylic terpenoids by enzyme systems from higher plants leads to the formation of both *E*- and *Z*-diasteromers‡ [1, 2], the latter being far less abundant that the former. The origin of the *Z*-isomers NPP and *Z*-FPP is most likely due to the activity of stereospecific prenyltransferases (EC 2.5.1.1.)§ [5, 6] and not to a direct *E*–*Z* isomerization of the pyrophosphates [5].

The flavedo of *Citrus sinensis* contains a 12-fold excess of *E*-prenyltransferases. It was not possible to dissociate completely an enzyme with *Z* stereospecificity from the enzyme forming *E* terpenoids [7], although a partial enrichment of the former activity was achieved [5]. Our aim is to pursue the study of the enzymes which form the less abundant *Z* isoprenoids. It seemed necessary, therefore, to start with a source of prenyltransferases with a more favourable *Z*–*E* ratio [5]. We report here the results obtained with enzymes from the easily available *Citrus paradisii* (grapefruit), which differ from those from other species of *Citrus* [5, 7].

RESULTS AND DISCUSSION

A cell-free extract from the flavedo of *Citrus paradisii* converted 3(*RS*)-[2-^{14}C]MVA, into different intermediates to the extent of 11.2% of the racemic substrate in 2 hr in the presence of ATP, Mn^{2+} and sulfhydryl group protectors (Table 1). Isopentenyl monophosphate was found, probably as the result of phosphatase action on IPP [8, 9]. The allylic fraction [10] was formed predominantly by C_{15} compounds, as reported for other species [11]. C_{10} and C_5 compounds were found only in small amounts. Qualitative analysis of these prenols showed that the system formed *E*-farnesyl and neryl derivatives. The absence of geranyl derivatives may be due to its utilization to form FPP; whereas neryl derivatives, which

Table 1. Utilization of [2-^{14}C]MVA by an enzyme system from *Citrus paradisii* flavedo in the presence of Mn^{2+}

Products analysed	% of initial MVA*
Pyrophosphomevalonic acid	1.9
Phosphomevalonic acid	1.6
DMAPP†‡	0.1
GPP†‡	n.d.§
NPP†‡	0.29
E-FPP†‡	3.9
Z-FPP†‡	n.d.§
IPP‡	0.37
Free lipids [10]	3.0
Total utilization of MVA	11.16

*Results are referred to 3(*RS*)-[2-^{14}C]MVA.

†Obtained from the allylic fraction [10] which was 4.29% of the initial MVA.

‡Obtained from the prenyl phosphate fraction [10] which was 4.66% of the initial MVA.

§No radioactivity could be detected in this product. The products were analysed by PC [8], TLC [5, 24, 25] or GC/RC [5].

†Abbreviations: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate (C_5); DMAPP, 3,3'-dimethylallyl pyrophosphate (C_5); GPP, geranyl pyrophosphate ($C_{10}E$); NPP, neryl pyrophosphate ($C_{10}Z$); *E*-FPP, (2*E*,6*E*)-farnesyl pyrophosphate(C_{15}); *Z*-FPP, (2*Z*,6*E*)-farnesyl pyrophosphate (C_{15}); DTNB, 5,5'-dithio-bis(nitrobenzoic acid); DTT, dithiothreitol.

‡The *E*- and *Z*-isomers will be referred to as diasteroisomers of different 'conformation' [3, 4].

§The term 'prenyltransferase' has been used in order to conform to the Enzyme Commission nomenclature. We will use the terms: 'C_{10} or C_{15}', '*E*- or *Z*'-prenyltransferases according to chain length and stereochemistry of the product formed, and not to the prenyl unit transferred.

are not substrates for prenyltransferases [5], were accumulated.

[1-^{14}C]IPP was transformed into DMAPP. The activity of IPP isomerase (EC 5.3.3.2) was 0.02 nkat/mg under initial rate conditions. In contrast with other isomerases [10, 12], the enzyme from *C. paradisii* did not require the presence of Mg^{2+} or Mn^{2+}, nor was it inhibited by EDTA at concentrations up to 10 mM. Its sensivity to sulfhydryl reagents was also less than reported for other IPP isomerases [12, 13]. It was not inhibited by 0.2 mM DTNB, but it was slowly inactivated by this reagent at 30° with a half life of more than 15 min.

Prenyltransferase activity of *C. paradisii*, corrected for phosphatase and IPP isomerase activities, had a pH optimum of 6.5 with half maxima at pH 5.0 and 8.0. The lower activity on the acid side was due to an effect on the enzyme and not to a decrease of substrate concentration produced by acid catalysed hydrolysis. Even at pH 4.0, the decrease in substrate concentration in 30 min was 0.1%. The activity of the enzyme was 0.038 nkat/mg under optimum pH and initial rate conditions, as compared with 0.028 nkat/mg for the enzyme from fresh flavedo of *C. sinensis* [5].

Prenyltransferase required the presence of sulfhydryl group protectors; it was activated by DTNB at concentrations below 0.01 mM. In the absence of substrate, the enzyme was reversibly inactivated by 0.025–0.1 mM DTNB with a second order rate constant of 26 M^{-1} sec^{-1}. The inactivation curve did not exhibit the second slower phase reported for prenyltransferase from *C. sinensis* [5, 7, 14]. The addition of 1 mM DTT caused a 90% recovery of the initial activity.

Prenyltransferase from *C. paradisii* utilized DMAPP or GPP as allylic acceptors, forming *E*-FPP and *Z*-FPP in a ratio of 3:1 in the presence of Mg^{2+} (Fig. 1). As in *Pinus radiata* or *C. sinensis*, this enzyme did not utilize NPP as prenyl acceptor [5, 15].

Table 1 and Fig. 1 suggest some degree of metal selectivity in the stereospecificity of the chain lengthening process. No *Z*-C_{15} product was formed from MVA in the presence of Mn^{2+} whereas the *Z*-C_{10} product NPP was formed. Formation of GPP from MVA may be inferred from the fact that the chain was further lengthened to (2*E*, 6*E*)-FPP, which cannot possibly occur through NPP [5].

Starting from a C_5 allylic substrate all four products, GPP, NPP, *E*- and *Z*-FPP, were formed in the presence of Mg^{2+} (Fig. 1). It is clear that the formation of *Z*-FPP is linked to the presence of Mg^{2+}, since it is not formed in the presence of Mn^{2+} neither directly from GPP plus IPP or from MVA.

C_{15} prenyltransferase requires the presence of Mg^{2+} in order to form the 2*Z* double bond, whereas C_{10} transferase does not exhibit this type of selectivity. A direct effect of the metal ion on the stereochemical mechanism of double bond formation in the condensation process catalysed by a single enzyme cannot be excluded so far [5], but it seems more plausible to assume the existence of several specific prenyltransferases [16].

As in *C. sinensis* [5], *Z*-prenyltransferase was more heat labile than the *E*-enzyme. The Mg^{2+} complexes of DMAPP, GPP or NPP protected only the *E*-enzyme from heat inactivation. Attempts to purify prenyltransferase by conventional methods have been so far unsuccessful.

The extract from *C. paradisii* contained an acid phosphatase activity (EC 3.1.3.2) with two maxima at pH 5.0 and 6.5. Metal ions or EDTA had no effect on this activity, which was 50% inhibited by 27 mM sodium fluoride. It was active in decreasing order towards pyrophosphate, *p*-nitrophenylphosphate, ATP, AMP, GPP and IPP. The activities with pyrophosphate and GPP were 25 nkat/mg and 0.46 nkat/mg, respectively, under initial rate conditions.

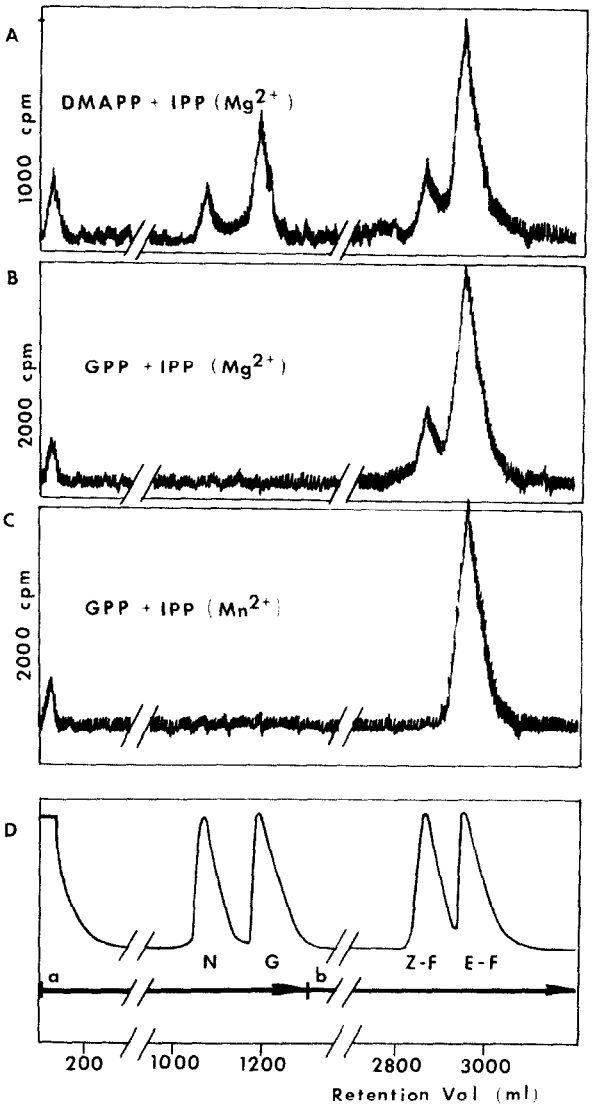

Fig. 1. Analysis of allylic pyrophosphates formed by prenyltransferases from the substrates indicated in A, B or C. D indicates carrier prenols: N, nerol, G, geraniol, Z–F, 2*Z*, 6*E*-farnesol, E–F, 2*E*,6*E*-farnesol. Column temperature: (a) isothermal at 100°, (b) isothermal at 170°. The unrearranged prenols for GC/RC analysis [5] were obtained from the phosphorylated products of prenyltransferases by enzymic hydrolysis (prenylphosphates) [10].

The enzyme system from *C. paradisii* resembles the system of *C. sinensis* [5, 7, 9, 14], but some experimentally important features have been found.

IPP isomerase differs from enzymes from other sources [10, 12] as it is less dependent on the participation of

sulfhydryl groups and independent from metal ions.

The increase in prenyltransferase activity by low concentrations of DTNB, suggests an unfolding of the enzyme upon derivatization of sulfhydryl groups, as described for other enzymes [17, 18]. Product specificity of the C_{15} prenyltransferases [5, 19] is controlled by the nature of the activating ion, suggesting the existence of several enzymes, as does the fact that the C_{10} prenyltransferase does not exhibit this specificity. These data, as well as the heat inactivation pattern, although not conclusive, point towards the existence of more than one prenyltransferase in *C. paradisii*, as is probably the case in *C. sinensis*. The higher ratio of *Z–E* products in enzymes from the former species, seems to offer better perspectives than other sources for the isolation of product specific prenyltransferases.

EXPERIMENTAL

Substrates: 3 (*RS*)-[2-^{14}C]MVA was purchased from NEN; [^{14}C]IPP was from Amersham; unlabelled IPP, GPP and NPP and [1-^{3}H]GPP were prepared as described before [20, 21].

Enzyme preparation. The flavedo (exocarpium) of ripe grapefruits, obtained from commercial sources in winter (May–Sept.) was homogenized in an Omni mixer at 3000 rpm for 3 min, with 1.5 vols. of one of the following buffer solns according to the activity to be measured: (A) 100 mM Tris–HCl, pH 7.8; 100 mM 2-mercaptoethanol and 2 mM EDTA; (B) 100 mM Tris–HCl, pH 7.0; 5 mM 2-mercaptoethanol and 1 or 100 mM EDTA; (C) 100 mM KPi, pH 7.4; 100 mM 2-mercaptoethanol and 2 mM EDTA; and (D) 100 mM sodium succinate, pH 6.0. The extract will be designated with the corresponding letter.

The homogenate thus obtained was strained through cheesecloth and centrifuged for 30 min at 17 000 *g* at 0°. The supernatant (extract) was used as the enzyme source. Proteins were determined by turbidimetry [22].

Incorporation of [2-^{14}C]*MVA.* Incubations were performed at pH 7.8, for 45 min at 37° in glass stoppered conical tubes in a final vol. of 1 ml 100 mM Tris–HCl, 20 mM 2-mercaptoethanol, 1 mM $MgCl_2$ or 2 mM $MnCl_2$, ATP–Mg^{2+} or Mn^{2+} = 2; 0.016 mM 3 (*RS*)-[2-^{14}C]MVA (sp. act. 6×10^7 dpm/μmol) and 0.7 mg proteins of extract A. The reaction was stopped by boiling the tubes for 3 min. Products were analysed as described before [10]. The hexane phases were either assayed directly for radioactivity or further analysed for products [5].

IPP isomerase. Incubations were performed at pH 6.0 for 5 min at 30° in glass stoppered conical tubes in a final vol. of 0.5 ml 150 mM sodium succinate, 20 mM 2-mercaptoethanol, 0.05 mM [1-^{14}C]IPP (sp. act. 2.4×10^7 dpm/μmol) and 0.05 mg of proteins of extract B. The reaction was quenched by cooling the tubes to 0° and allylic HCl products were assayed [23].

Prenyltransferase. Incubations were performed at pH 7.4 for 30 min in glass stoppered conical tubes in a final vol. of 0.5 ml 100 mM KPi, 6 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 0.01 mM DMAPP or GPP, 0.05 mM [1-^{14}C]IPP (sp. act. 2.4 $\times 10^6$ dpm/μmol) and 0.03 mg proteins from extract C. The reaction was stopped by cooling the tubes to 0° and prenylphosphates were analysed as described in ref. [5] and corrected for IPP isomerase [23].

Phosphatases. Incubations were performed at pH 6.0 for 10 min at 37° as described in ref. [9] using proteins from extract D.

All results are expressed in nkat, and were corrected for non-enzymic controls performed with boiled enzyme.

Radioactive prenols directly extracted with hexane from the incubation mixture or obtained from their phosphate esters by hydrolysis with added phosphatase [5] were identified by GC, after addition of 0.5–1 mg of authentic carriers [5]. The products obtained from [2-^{14}C]MVA incorporation by C_{10} and C_{15} prenyltransferase activities, were also analysed by TLC for chain length [24] and conformation [5, 25].

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