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EVIDENCE FOR ENZYMATIC FORMATION OF ISOPREPHENATE FROM ISOCHORISMATE

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Abstract: Isoprephenate, a hypothetical intermediate in the biosynthesis of *m*-carboxy aromatic amino acids, has not been demonstrated *in vitro* or *in vivo*. Evidence for The enzymatic conversion of isochorismate to isoprephenate was obtained in crude extracts derived from *Nicotiana silvestris*.

Some higher-plant species (e.g., iris) are known[1-3] to produce *m*-carboxy aromatic amino acids as secondary metabolites. The only reported work[2,3] on biosynthesis of these non-protein amino acids relates to their derivation from shikimate. Labeled shikimate was used to show that the 3-carboxy components of *m*-carboxytyrosine and *m*-carboxyphenylalanine were derived from the carboxyl group of shikimate with retention of the *pro*-6-S hydrogen atom. Chorismate and isochorismate, first established as aromatic-pathway intermediates by Gibson and coworkers,[4-6] were hypothesized by Larsen to be intermediates. The chemical structure of isochorismate differs from that of chorismic acid by the positions of the hydroxyl substituent and the double bonds (Fig. 1). The C2 hydroxyl group from isochorismate has been shown[7,8] to originate from water, rather than from molecular oxygen or via an intramolecular shift. Bentley has emphasized[9] the nature of isochorismate as a major branchpoint which leads to napthoquinones, shihunine (a plant alkaloid), siderophores, plant epoxides, 6-hydroxyanthranilic acid (an antibiotic precursor), as well as to the plant 3-carboxy-substituted aromatic amino acids.

If isochorismate is heated at 100°C at pH 7.0 for 10 min, conversion to *m*-carboxyphenylpyruvate occurs[6]. A plausible mechanism for this conversion was proposed[6], involving "isoprephenate", a hypothetical intermediate which has neither been isolated nor synthesized. Accordingly, isoprephenate was postulated[2,3] to be a likely precursor of *m*-

carboxyphenylalanine and *m*-carboxytyrosine. As discussed by Zamir et al.[8], enzymatic reactions leading to *m*-carboxyphenylalanine would involve an "isoprephenate dehydratase" followed by transamination, while those leading to *m*-carboxytyrosine would involve "isoprephenate dehydrogenase" followed by transamination.

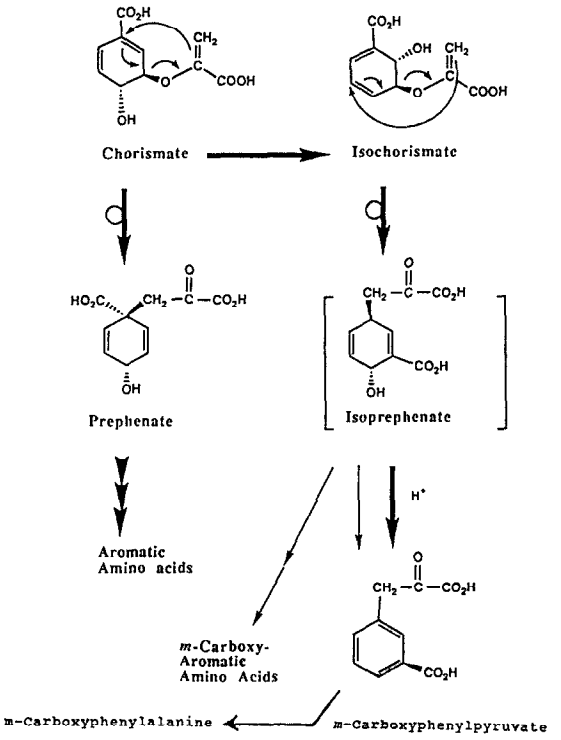

Figure 1. Chorismate-derived metabolites. The heavy arrows represent established enzymatic or non-enzymatic (H^+) steps; the looped-out arrows indicate the analagous Claisen rearrangements performed by chorismate mutase (left) and isochorismate mutase (right). Thin arrows leading from isoprephenate represent putative enzyme reactions yet to be established.

A cell culture of *Nicotiana silvestris* was used which has been characterized in detail with respect to both growth parameters[10] and expression of various enzymes of aromatic amino acid

biosynthesis[11-12]. Cells in the stationary phase of growth (8 days after subculture) were used as a source of crude extracts. Cell extracts were prepared from liquid-nitrogen ground powders[11] which were made up in 0.1 *M* K-phosphate buffer (pH 7.3) containing 20% (v/v) glycerol, 1 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride and 0.05% ß-mercaptoethanol. The protein which salted out upon addition of ammonium sulfate at 85% of saturation, was taken up in 0.1 *M* K-phosphate buffer (pH 7.3) containing 20% glycerol and dialyzed overnight with 3 changes of buffer.

Reaction mixtures in a total volume of 200 µl contained 7.5 m*M* isochorismate, 50 µl of extract protein, and 0.1 *M* K-phosphate buffer at pH 7.3. The isochorismate substrate was prepared according to ref. 8, and ^{1}H- and ^{13}C-NMR was used to confirm identity and purity. Reaction mixtures were incubated for 20 min at 37°C, and 50 µl of 1*N* HCl was added to convert any isoprephenate formed to *m*-carboxyphenylpyruvate. After 30 min at room temperature, 750 µl of 2.5 *N* NaOH was added and absorbance of the presumptive *m*-carboxyphenylpyruvate at 320 nm was recorded. Controls run to recognize possible non-enzymatic reactions were identical to the other reaction mixtures except that enzyme was added after the addition of 1 *N* HCl. Activity was found to be proportional with respect to protein concentration within a range of 20-50 µl of extract added, as shown in Table 1. Within this range proportionality with respect to elapsed time of reaction at 37°C was observed. No increase in A_{320} was obtained without the acidification step. Various reaction mixtures were frozen prior to further evaluation of the product formed.

Table 1. Formation of isoprephenate as a function of enzyme concentration.

µl of Extract[a]	A_{320}[b]
20	.065
30	.100
40	.144
50	.186
60	.177
70	.170
80	.181
90	.162
100	.151

[a] The protein content of the extract used was 7 µg per µl.

[b] Enzyme activity is expressed as the increment of absorbance at 320 nm after 20 min at 37°C, subtracting A_{320} values measured in unreacted controls.

A standard of authentic *m*-carboxyphenylpyruvate was synthesized[13] and purified[14]. A full set of reaction mixtures and the corresponding controls were worked up[15] in preparation for analysis by HPLC[16]. Five different samples were analyzed in which an increment of about 0.2 at 320 nm had been obtained enzymatically. All five samples analyzed exhibited a peak with the same retention time as the authentic *m*-carboxyphenylpyruvate standard[17], as illustrated in Fig. 2. When samples were spiked with the standard, a single enlarged peak of identical retention time was observed. Control samples contained no extraneous peaks and showed no indication of non-enzymatic conversion of isochorismate to *m*-carboxyphenylpyruvate.

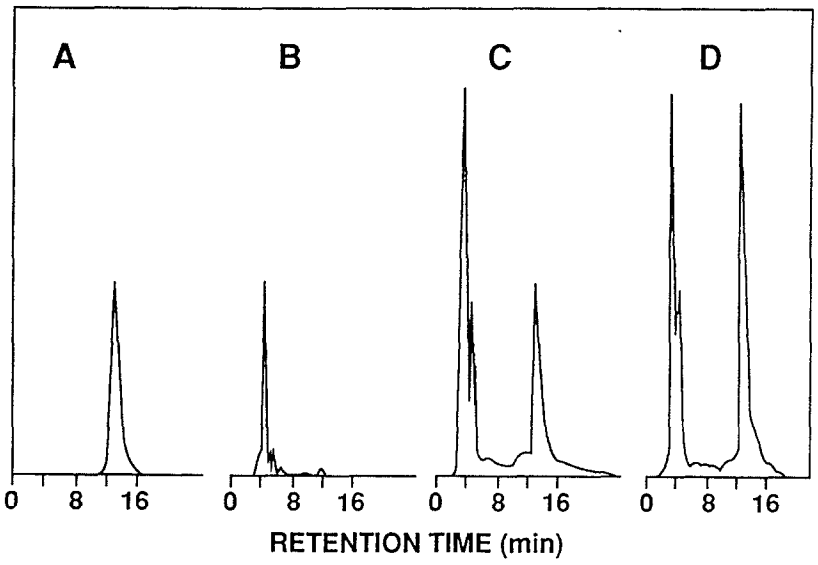

Figure 2. Identification by HPLC of *m*-carboxyphenylpyruvate following acidification of isoprephenate formed enzymatically. The following injections were made: (A) 5 μg of synthetic *m*-carboxyphenylpyruvate, (B) control reaction mixture containing denatured enzyme, (C) a half-portion of an enzyme reaction sample, and (D) the second half-portion sample spiked with 5 μg of standard.

Perspective

The conversion of isochorismate to isoprephenate is analagous to the reaction catalyzed by chorismate mutase, and hence the plant enzyme implicated in this report would appropriately be named isochorismate mutase. *N. silvestris* possesses two species of chorismate mutase[18], the plastid-localized CM-1 isoenzyme and the cytosolic CM-2 isoenzyme. It will be of interest to see whether one or both of these isoenzymes possesses sufficient breadth of substrate specificity to accommodate both reactions[19], or whether a previously unknown enzyme is dedicated to performance of the isochorismate mutase reaction. Similar considerations of substrate specificity might apply with respect to the putative isoprephenate dehydratase and isoprephenate dehydrogenase enzymes, although prephenate dehydratase and prephenate dehydrogenase enzymes have not been demonstrated in higher plants. The arogenate dehydrogenase [20] and the arogenate dehydratase[21] which exist in *N. silvestris* fail to utilize prephenate and would therefore not be expected to utilize isoprephenate. Perhaps plant species in the iris family will prove to possess broad-specificity dehydratase and dehydrogenase species which result in completion of the catalytic chain to *m*-carboxy aromatic amino acids. *N. silvestris*, like most higher plants, is not known to produce these non-protein amino acids, yet it is capable of the isochorismate to isoprephenate transformation. It may be that an additional biological role(s) for isoprephenate is yet to be elucidated.

Acknowledgements

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14. *m*-carboxyphenylpyruvate was purified by use of HPLC (Waters Delta Prep 3000) using two ODS-3 MAG·9 reversed phase semi-preparative columns in series (9.4 x 500 mm) with 5 m*M* K-phosphate buffer (pH 6.0) eluted at 3 ml min^{-1}. The fractions corresponding to a retention time of 36.0 min were lyophilized. The powder was dissolved in water, acidified to pH 2.0, and the acid was extracted with ethyl acetate. This was dried ($MgSO_4$), filtered, and evaporated. The NMR spectra corresponded to that reported in the literature[13].
15. The 1-ml samples (200 μl reaction mixture, 50 μl 1 *N* HCl, and 750 μl 2.5 *N* NaOH) were mixed with 50 ml of 0.2 *N* HCl. This mixture was saturated with NaCl and extracted with ethyl acetate (3 x 35 ml). The ethyl acetate was evaporated and the residue dissolved in 200 μl of 5 m*M* K-phosphate buffer (pH 6.0) buffer. The pH was adjusted to 6.0 with 10 μl of 2.5 *N* NaOH. All 200 μl of each control sample was injected into the HPLC system. The other samples were injected in two 100-μl portions, one being spiked with 5 μg of synthetic *m*-carboxyphenylpyruvate.
16. Samples were analyzed by HPLC using one ODS-3 analytical reversed phase column (4.6 x 250 mm) with 5 m*M* K-phosphate buffer (pH 6.0) eluted at 1 ml min^{-1} and detected by UV at a wavelength of 215 nm. As shown in Fig. 2, the retention time of the isochorismate-derived biological product after acidification was identical to that of synthetic *m*-carboxyphenylpyruvate, i.e., R_t = 15 min. The five controls all lacked *m*-carboxyphenylpyruvate, whereas *m*-carboxyphenylpyruvate was found to be present in all five reaction samples.
17. A standard curve was constructed using synthetic *m*-carboxyphenylpyruvate, and the amount of *m*-carboxyphenylpyruvate formed in each of the five samples was measured. An average of 7.8 μg of *m*-carboxyphenylpyruvate was determined within a range of 6-10 μg.
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