Volume 237, number 1,2 159–162 FEB 06305 September 1988

Geranylpyrophosphate synthase from cell cultures of *Lithospermum erythrorhizon*

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Received 6 June 1988; revised version received 26 July 1988

A prenyltransferase (EC 2.5.1.1) was isolated and partially purified from cell cultures of *Lithospermum erythrorhizon*. The product of the enzymatic reaction is geranyl pyrophosphate rather than farnesyl pyrophosphate.

Prenyltransferase; Geranylpyrophosphate synthase; Shikonin; Cell culture; (*Lithospermum erythrorhizon*, Boraginaceae)

1. INTRODUCTION

Prenyltransferase (dimethylallyl*trans*transferase; EC 2.5.1.1) catalyses the reaction of isopentenyl pyrophosphate (IPP) with an allylic substrate [1,2]. Two types of prenyltransferases have been isolated from plants: (i) farnesylpyrophosphate synthase, using dimethylallyl pyrophosphate (DMAPP) and geranyl pyrophosphate (GPP) as allylic substrates, thereby producing farnesyl pyrophosphate (FPP) for sterol biosynthesis [3–5]; (ii) geranylgeranylpyrophosphate synthase, using DMAPP, GPP and FPP as allylic substrates, forming geranylgeranyl pyrophosphate (GGPP) for carotinoid biosynthesis [3,6–9].

The existence of a third type of plant prenyltransferase, i.e. GPP synthase, is still a matter of discussion [10]. This enzyme would specifically form GPP as a precursor of secondary metabolites, e.g. monoterpenes. It was proposed [11] that such an enzyme does not exist in plants, and that GPP for monoterpene biosynthesis is formed as an intermediary product of the FPP synthase reaction. Indications for a specific GPP synthase have been found in crude extracts from *Pelargonium graveolens* [12] and *Tanacetum vulgare* [13] and a similar enzyme from *Salvia officinalis* is under investigation by Croteau [10]. An enzyme which synthezises both GPP and GGPP, but not FPP, has been found in bacteria [14].

We now report the isolation and partial purification of a GPP synthase from cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc., Boraginaceae [15]. This enzyme is likely to supply GPP for shikonin biosynthesis (fig.1) [16–18].

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2. EXPERIMENTAL

2.1. *Cell cultures*

Cell cultures of *L. erythrorhizon* M18 strain were cultured in liquid LS medium [19]. 50 ml medium in 300 ml erlenmeyer flasks were agitated at 100 rpm in a rotary shaker (25 mm stroke). Cells were harvested 7 days after inoculation.

2.2. *Enzyme assay*

In a final volume of 100 μl, the incubation mixture contained DMAPP (20 nmol), $MgCl_2$ (1 μmol), [1-^{14}C]IPP (4.5 nmol, 167 Bq), Tris-HCl, pH 7.5 (10 μmol) and purified protein (~ 0.8 μg). After incubaton for 30 min at 30°C, reaction was terminated by addition of 400 μl EtOH and 20 μl concentrated HCl. After hydrolysis for 60 min at 37°C, the mixture was extracted with 800 μl heptane. 500 μl of the organic layer was used for scintillation counting.

2.3. *Enzyme preparation*

Crude extracts [20] from 70 g cells were subjected to ammonium sulfate precipitation. The pellet of the 30–70% fraction was dissolved in 0.05 M Tris-HCl (pH 7.5) and chromatographed over 20 ml DEAE-Sephacel (gradient 0–0.3 M KCl in the same buffer). Active fractions were combined, brought to

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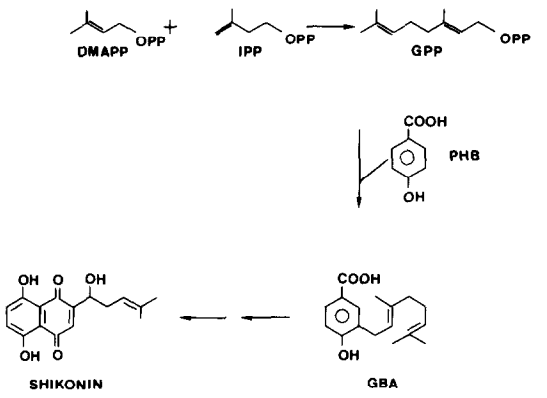

Fig. 1. Biosynthesis of shikonin.

20% saturation with ammonium sulfate, applied to a column of 20 ml phenyl-Sepharose CL-4B and eluted with a gradient (20–0% saturation of ammonium sulfate) in the same buffer. Active fractions were combined, concentrated and chromatographed over Sephadex G-150 (0.9 × 47 cm) with 0.1 M Tris-HCl, 1 mM DTT as eluent.

2.5. *Product identification*

In a final volume of 500 μl, DMAPP (100 nmol), $MgCl_2$ (5 μmol), [1-^{14}C]IPP (17 nmol; 36700 Bq), Tris-HCl, pH 7.5 (50 μmol) and purified protein (4 μg) were incubated for 70 min at 30°C.

2.5.1. Identification of geraniol

250 μl of the incubation mixture were, after addition of 20 mg (0.36 U) acid phosphatase (EC 3.1.3.2), incubated overnight at pH 4.7 and 37°C. 25 μl of 5 N NaOH, 30 μg geraniol and 60 μg farnesol were added, and the mixture was extracted with 2 × 1 ml petroleum ether. 100 μl of the organic layer was concentrated under nitrogen, and, after addition of 2 μg each of dimethylallyl alcohol, geraniol and farnesol, subjected to HPLC analysis. TLC: paraffin-impregnated cellulose; solvent: acetone-H_2O, 1:1. R_f: geraniol, 0.82; farnesol, 0.35.

2.5.2. Identification of GPP

To 50 μl of the above incubation mixture, 4 μmol GPP and 10 μmol tetrabutylammonium hydrogen sulfate were added. This mixture was used for ion-pair HPLC. TLC of GPP: SiO_2; solvent: *n*-PrOH-H_2O-NH_3, 2:1:1; R_f: GPP, 0.50; IPP and DMAPP, 0.40.

2.5.3. GC identification of geraniol

In a final volume of 2.5 ml, DMAPP (500 nmol), IPP (500 nmol), $MgCl_2$ (25 μmol), KP_i, pH 7.5 (250 μmol) and purified protein (20 μl) were incubated for 90 min at 30°C. After enzymatic hydrolysis (see above), the products were extracted with 2 × 5 ml petroleum ether. Most of the solvent was removed with N_2. The remaining solution was used for GC: DB-5 capillary column 30 m × 0.32 mm, 0.25 μm film; temperature programme 90°C, 2 min, 90–300°C, (10°C/min). Retention times: geraniol, 5.81 min; nerol, 5.48 min; farnesol, 11.42 min. GC-MS: GC as above; MS: Finnigan/Mat 1020 B, 70 eV.

3. RESULTS

In the course of our investigations of the *p*-hydroxybenzoic acid geranyltransferase in *L. erythrorhizon* cell cultures [18], indications were found for the presence of a GPP-synthase activity. The examination of this enzyme, however, was hampered by strong interference from phosphatases and DMAPP-IPP isomerase. Therefore, the enzyme was purified by ammonium sulfate fractionation and subsequent chromatography on DEAE-Sephacel, phenyl-Sepharose CL-4B and Sephadex G-150. The purified enzyme was free of phoshatases and isomerase; GPP-synthase activity was enriched 50-fold by the purification.

The prenyltransferase reaction was dependent on the presence of intact enzyme, Mg^{2+} and DMAPP (table 1). When DMAPP was replaced by GPP in the standard assay, a small amount of acid-labile products was formed. However, product formation was only 3.2% of that obtained with DMAPP. When both GPP and DMAPP were included, product formation was approximately two thirds of that with DMAPP alone.

In order to identify the reaction products, [^{14}C]IPP was incubated with DMAPP, $MgCl_2$ and purified enzyme. Products were hydrolyzed with acid phosphatase and extracted into petroleum ether. HPLC analysis of the organic layer (fig.2a) showed complete conversion of the [^{14}C]IPP to geraniol; no incorporation of radioactivity into

Table 1

Formation of acid-labile products from [^{14}P]IPP by GPP synthase

	Product formation	
	Bq	% of control
Standard assay	44.2	100
− $MgCl_2$	5.8	13.1
heat-denatured enzyme	< 0.7	< 1.6
− DMAPP	< 0.7	< 1.6
− DMAPP + GPP		
(2 mM)	1.2	2.7
(0.2 mM)	1.4	3.2
(0.02 mM)	0.9	2.0
(0.002 mM)	< 0.7	< 1.6
+ DMAPP (0.2 mM) + GPP (0.2 mM)	29.7	67.2

The standard assay contained 20 nmol (0.2 mM) DMAPP, 167 Bq [^{14}C]IPP, and other components as described in section 2

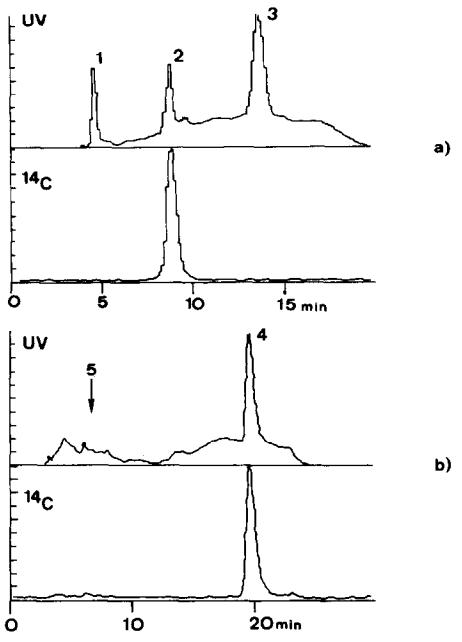

Fig. 2. HPLC identification of the products of the GPP synthase reaction. (a) After enzymatic hydrolysis of products; 1, dimethylallyl alcohol; 2, geraniol; 3, farnesol. (b) Without hydrolysis; 4, geranyl pyrophosphate; 5, isopentenyl pyrophosphate. HPLC: Lichrosorb RP 18, 7 μm, 250 × 4 mm. Solvent systems: (a) gradient MeOH-H_2O, 60–75% MeOH; (b) gradient MeOH-H_2O, containing 2.5 mM KP_i (pH 6.8) and 5 mM tetrabutylammonium hydrogen sulfate; 33–50% MeOH. Detection: absorption at 220 nm (upper track) and flow scintillation counting (lower track).

farnesol was detected under these conditions. This result was confirmed by TLC of the products on paraffin-impregnated cellulose (see section 2) followed by radioscanning. The formation of geraniol was dependent on the presence of active enzyme, DMAPP and Mg^{2+}. However, if DMAPP was replaced by GPP in the incubation mixture, a small amount of radioactivity was incorporated into farnesol rather than geraniol, as shown by HPLC analysis after enzymatic hydrolysis of the reaction products.

For direct detection of GPP, the incubation mixture, using DMAPP as substrate, was examined without enzymatic hydrolysis. Ion-pair HPLC (fig.2b) showed that [^{14}C]IPP had been completely converted to GPP. The same result was obtained by direct TLC and radioscanning of the reaction mixture (see section 2).

Table 2

Conversion of enzymatically formed [^{14}C]GPP to *m*-geranyl-*p*-hydroxybenzoic acid (GBA) by PHB geranyltransferase

	Formation of GBA	
	Bq	% of control
Complete assay	31.7	100
– PHB	< 0.7	< 1.6
– complete, but with heat-denatured PHB geranyltransferase	< 0.7	< 1.6
– complete, but with heat-denatured GPP synthase	< 0.7	< 1.6

The complete assay contained [^{14}C]IPP (367 Bq, 1.8 nmol), *p*-hydroxybenzoic acid (PHB) (100 nmol), PHB geranyltransferase, isolated by centrifugation at 100000 × *g* [18] (100 μg), and other components as described in section 2. GBA was isolated by HPLC [18]

Subsequently, unlabelled IPP was incubated with DMAPP, $MgCl_2$ and purified enzyme; products were enzymatically hydrolyzed and extracted as above without addition of carriers. When the organic layer was subjected to capillary GC, only geraniol, but not nerol or farnesol, was detected (see section 2). In GC-MS analysis, this sample showed the same retention time and fragmentation pattern as geraniol: *m/z* (relative intensity) = 154 (1%; M^+), 139 (2%), 136 (2%), 123 (8%), 111 (9%), 93 (10%), 69 (100%).

Finally, the identity of the reaction product GPP was confirmed by its enzymatic conversion to *m*-geranyl-*p*-hydroxybenzoic acid (GBA) (see fig.1). PHB geranyltransferase was prepared from *L. erythrorhizon* cell cultures [17]. Co-incubation of this enzyme with the purified GPP synthase and $MgCl_2$, DMAPP, *p*-hydroxybenzoic acid and [^{14}C]IPP (table 2) resulted in the formation of [^{14}C]GBA, which was dependent on both enzyme as well as on PHB.

4. DISCUSSION

The prenyltransferase isolated from *L. erythrorhizon* cell cultures is a GPP synthase rather than an FPP synthase (fig.2). DMAPP rather than GPP serves as allylic substrate (table 1). In shikonin biosynthesis, GPP is converted to *m*-geranyl-*p*-hydroxybenzoic acid (GBA) (fig.1). By co-

incubation of the GPP synthase with PHB geranyltransferase, this reaction sequence could be demonstrated in vitro.

This is the first report of a purified plant prenyltransferase which produces GPP rather than FPP. This finding may also be of interest for the concepts of monoterpene biosynthesis, since the existence of a specific GPP synthase for secondary metabolism in plants has been in doubt.

The properties of the GPP synthase are now under investigation in our laboratory.

Acknowledgements: I am grateful to Professor M. Tabata, Kyoto University, for the generous gift of *Lithospermum erythrorhizon* cell cultures, Dr K. Yazaki, Okayama University, for samples of IPP, DMAPP and GPP and R. Stegemann, M. Breuer and C. Lehmann for assistance in the experiments. This work has been supported by the Deutsche Forschungsgemeinschaft.

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