Purification and characterization of farnesyl pyrophosphate synthase from *Capsicum annuum*

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Farnesyl pyrophosphate synthase (FPP) displaying dimethylallyl transferase activity (EC 2.5.1.1) and geranyl transferase activity (EC 2.5.1.10) was purified from *Capsicum* fruits. This prenyltransferase has a molecular mass of 89000 ± 5000 Da resulting from the association of two apparently identical subunits having a molecular mass of 43000 ± 2000 Da. Antibodies raised against *Capsicum* FPP synthase selectively blocked the transferase activity. Analysis of the immunological relationships between FPP synthase and geranylgeranyl pyrophosphate synthase (EC 2.5.1.1, EC 2.5.1.10 and EC 2.5.1.30) revealed that these two enzymes though performing the same mechanism of catalysis and accepting identical substrates have different antigenic determinants. Thus, in connection to previous work, this immunological study suggests that *Capsicum* FPP is strictly located in the extraplastidial compartment.

Farnesyl pyrophosphate synthase; Prenyltransferase; Enzyme purification; Immunocharacterization; *Capsicum annuum*

1. INTRODUCTION

Plant prenyltransferases are involved in the biogenesis of several homogeneous or mixed isoprenoids in different cellular compartments, including plastid, mitochondria, endoplasmic reticulum and cytosol. We have purified to homogeneity GGPP synthase [1] and shown that this enzyme is exclusively plastid-localized in *Capsicum* cells [2] (and manuscript in preparation). About FPP synthase (EC 2.5.1.1, EC 2.5.1.10) much less is known. This enzyme is involved in the biogenesis of sterols, dolichols, sesquiterpenes and presumably mitochondrial ubiquinones. Furthermore it catalyzes a key step in the biogenesis of isoprenoid phytoalexins [3] and, in some cases, the presence of this enzyme has been reported in leucoplasts [4].

Although there are several reports on the purification of FPP synthase from plants [5-8], no purification to homogeneity of this enzyme has been reported, except the purification of an atypical rubber prenyltransferase from *Hevea brasiliensis* which catalyzes the synthesis of FPP and the following elongation steps [9], thus differing considerably from another recently described rubber transferase isolated from *Parthenium argentatum* [10]. As a step toward understanding how FPP synthase might fulfill its different roles, we have now purified this prenyltransferase from *Capsicum* fruits and raised polyclonal antibodies against this enzyme.

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Abbreviations: DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GC, gas chromatography; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin layer chromatography

2. MATERIALS AND METHODS

2.1. *Materials*

Green pepper fruits (*Capsicum annuum* L.) were purchased from a local market. [1-^{14}C]Isopentenyl pyrophosphate (56 mCi/mmol) was from Amersham, DMAPP, GPP and FPP were prepared as described previously [11]. Sepharcyl S-200, Phenyl Sepharose CL-4B, Q-Sepharose Fast Flow and Mono-Q HR 5/5 FPLC column were purchased from Pharmacia France. All other chemicals were of analytical grade.

2.2. *Enzyme assay*

Routine assay mixture (100 µl final volume) contained 20 mM Tris-HCl (pH 7.6), 5 mM dithiothreitol, 100 µM $MnCl_2$, 3 µM [^{14}C]IPP (620 Bq) and 10 µM DMAPP or GPP and a definite amount of enzyme. After incubation at 30°C for 15 min, the reaction was stopped by addition of 400 µl of 3 N HCl in methanol. The mixture was incubated at 37°C for 10 min prior to the extraction of prenols with 400 µl of chloroform. 300 µl of the organic layer was used for radioactivity determination by liquid scintillation counting. Protein content was determined according to the dye binding method using a Bio-Rad kit.

2.3. *Product analysis*

The incubation mixture was similar to that described above, except that final volume was 200 µl. After incubation for 30 min at 30°C, the pH was adjusted to 5.0 with CH_3COOH and 10 mg (0.2 unit) acid phosphatase was added. After hydrolysis for 2 h at 30°C, 600 µl of chloroform/methanol (2:1, v/v) were added before concentrating the organic layer under nitrogen. The products were separated by thin-layer chromatography on Kieselgel RP-18 F 254 Merck plates

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developped with MeOH/water (90:10) and autoradiography was performed using Kodak DEF5 films. Radio-gas chromatography was performed on a Varian Aerograph 1400 equipped with a flame ionization detector and a 0.25 in. × 1.8 m column of 4.5% SE30 on Gas Chrom Q60-80. Argon served as the carrier gas and the flow rate was 35 ml · min^{-1}. Radioactivity was detected by a Packard 894 gas counter and products were identified using purified standards.

2.4. *Enzyme purification*

Capsicum fruits washed with ice-cold distilled water were cut into small pieces and suspended in 50 mM Tris-HCl (pH 8.5) buffer containing 2.5% of polyvinylpyrrolidone and 10 mM 2-mercaptoethanol (1 liter per 2 kg of fruit). The suspension was then homogenized in a Waring blender for 3 × 30 s. The homogenate was filtered through cheesecloth and its pH was adjusted to 7.6 with 1 M Tris. The filtrate was then centrifuged at 30 000 × *g* for 45 min and the supernatant was used for FPP synthase purification.

Q-Sepharose equilibrated with 20 mM Tris-HCl (pH 7.6) buffer containing 10 mM 2-mercaptoethanol and 10% (v/v) glycerol (buffer A) was added to the 30 000 × *g* supernatant (1 ml gel/10 mg protein) and the suspension was stirred for 30 min at 4°C. The gel was then collected on a Buchner funnel and washed with buffer A containing 100 mM NaCl. The prenyltransferase-containing fractions were eluted with 3 bed volumes of buffer A containing 500 mM NaCl.

This fraction was applied to a hydroxylapatite column (2.5 × 40 cm) equilibrated with buffer A containing 5 mM KH_2PO_4. The column was then eluted with a linear gradient of 5 to 200 mM KH_2PO_4 in buffer A. Fractions (10 ml) were collected and active fractions were pooled, dialysed against buffer A and applied to a Q Sepharose column (1 × 15 cm) equilibrated with buffer A. The column was eluted with a linear gradient of 50 to 350 mM NaCl in buffer A and 3 ml fractions were collected and analyzed for FPP synthase activity.

Active fractions were combined, adjusted to 1 M NaCl and applied to a Phenyl Sepharose column (1 × 5 cm) equilibrated with buffer A containing 1 M NaCl. The column was washed with 25 ml of buffer A containing 500 mM NaCl before elution with 3 × 10 ml of buffer A. After dialysis against buffer A, active fractions were applied to a Mono-Q FPLC column equilibrated with buffer A. The column was eluted with a linear gradient of 50 to 300 mM NaCl in buffer A and active fractions were pooled for further purification by preparative SDS-PAGE.

2.5. *SDS-PAGE*

Analytical SDS-PAGE was performed on 12.5% acrylamide slab gels and protein bands were detected by silver staining according to a previously described procedure [12].

Preparative SDS-PAGE was carried out on slab gels (1.5 mm thickness). The band corresponding to the putative FPP synthase was located using prestained molecular weight markers (Sigma). Subsequently, the polypeptide was eluted overnight from the gel by simple diffusion in 70% CH_3COOH. After extensive dialysis the resulting solution was lyophilised.

2.6. *Gel filtration*

Degassed Sephacryl S-200 column (1 × 60 cm) was used for the determination of the native molecular weight of FPP synthase. The column was calibrated with the following gel filtration standards: alcohol dehydrogenase M_r = 150 000; phosphorylase *b* M_r = 97 000; bovine serum albumin M_r = 67 000; ovalbumin M_r = 43 000; chymotrypsinogen A M_r = 25 000. Elution was performed with buffer A containing 200 mM NaCl to reduce possible trailing artifacts.

2.7. *Preparation of polyclonal antibodies and immunoinhibition of FPP synthase*

Purified protein (500 μg) was injected into a rabbit (fauve de Bourgogne). After one month, the rabbit was immunized again four times at one week intervals. The antiserum was then tested for FPP synthase inhibition: 100 μl of partially purified FPP synthase (Q-Sepharose fraction) were incubated with 100 μl of antiserum or preimmune serum for 1 h at 25°C. After a 20 000 × *g* centrifugation for 10 min, FPP synthase activity in the supernatant was used for assay.

2.8. *Protein blot analysis*

After SDS-PAGE, the separated polypeptides were transferred electrophoretically from the gel to a nitrocellulose paper using a semi-dry blotting system (Novablot LKB). The efficiency of transfer was followed using prestained molecular weight markers (Sigma). After blocking additional binding sites with 3% of bovine serum albumin dissolved in 20 mM Tris-HCl (pH 7.6) buffer containing 500 mM NaCl (buffer B), the nitrocellulose paper was incubated with the antiserum for 3 h at 25°C. The nitrocellulose paper was then washed 3 × 10 min with buffer B containing 0.5% (v/v) of Tween-20. The protein-antibody complex was revealed using the protein A-gold kit (Bio-Rad).

3. RESULTS AND DISCUSSION

3.1. *Purification of* Capsicum *FPP synthase*

As reported previously [1], cell free extracts from *Capsicum* fruit contains IPP isomerase activity and prenyltransferase activities. The first step of the purification procedure was carried out by batch adsorption and elution of the soluble extract on Q-Sepharose using a Buchner funnel. This procedure permits a rapid elimination of the isomerase activity which elutes in the fraction containing 100 mM NaCl. FPP synthase and GGPP synthase activities elute together in the fraction containing 500 mM NaCl. Further chromatography on hydroxylapatite column eliminates the bulk of inactive proteins but allowed poor separation of FPP synthase and GGPP synthase. These two activities are finally separated on Q-Sepharose column as shown by the fraction eluting at 175 mM NaCl which contained FPP synthase activity totally devoid of GGPP synthase activity (Fig. 1). Further identification of the of FPP was done by radio-gas chromatography (Fig. 2).

An overall scheme of the purification procedure is summarized in Table I. Final specific activity of the FPP synthase is quite low compared to those previously described [6] for a partial purification of pea FPP synthase, probably because of the partial inactivation of the enzyme. Indeed, after the Mono-Q step, FPP synthase seems to be quite unstable and the rapid loss of activity prevented us to go further in the purification using column chromatography. However, we were able to correlate the FPP synthase activity with the presence of a major polypeptide having a molecular mass of 43 000 Da. As several polypeptide bands were detected after the Mono-Q step (Fig. 3A), the 43 000 Da polypeptide was isolated by preparative electrophoresis and antibodies were raised against it, to further check its involvement in the synthesis of FPP. After five injections, the antiserum directed against this polypeptide strongly inhibited the synthesis of FPP in a cell free extract of *Capsicum* fruit (Fig. 4). In contrast, no inhibition occurred in the control experiment (Fig. 4). Similar results were obtained for the partially purified enzyme. Protein blot analysis of the 30 000 × *g* supernatant revealed a single polypeptide band corresponding to a 43 000 Da protein that can now be identified as FPP synthase (Fig. 3B).

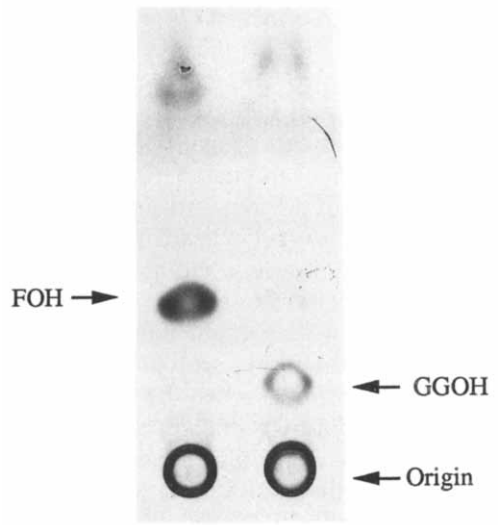

Fig. 1. Separation of FPP synthase and GGPP synthase from the crude cell free extract of *Capsicum*. The two active fractions from Q-Sepharose eluted with 175 mM NaCl (FPP synthase) and 250 mM NaCl (GGPP synthase) were incubated with [^{14}C]IPP and GPP as allylic pyrophosphate primer. The reaction products obtained after enzymatic hydrolysis were analyzed by TLC followed by autoradiography as described in section 2. The positions of farnesol (FOH) and geranylgeraniol (GGOH) are indicated.

3.2. *Some properties of* Capsicum *FPP synthase*

A native molecular mass of 89 000 ± 5000 Da was estimated from the elution profile of FPP synthase on Sephacryl S-200 gel chromatography using protein markers. This native molecular mass and the above described results conclusively indicate that *Capsicum* FPP synthase is a dimer of identical subunits (43 000 Da). This data compares with values for the analogous enzyme from liver [13], yeast [13] and pea [8]. A slightly different value was reported for rubber prenyltransferase [9].

Capsicum FPP synthase is totally devoid of IPP isomerase activity and, in the presence of IPP, DMAPP and GPP are accepted as allylic pyrophosphate primers, while FPP is not accepted for further elongation. Several divalent cations were tested for their ability to activate *Capsicum* FPP synthase (Table II). Maximum activities were observed with Mg^{2+} or Mn^{2+}; however, at low concentrations, Mn^{2+} is preferred compared to Mg^{2+}, as already observed for the human liver prenyltransferase [14].

Table I

Purification of farnesyl pyrophosphate synthase from *Capsicum* fruits

Fraction	Total protein (mg)	Specific activity[a]	Purification factor
30 000 × *g* supernatant	1200	2.6	-
Q-Sepharose (batch)	500	6	2.3
Hydroxylapatite	63	30	11.5
Q-Sepharose	8	132	50.7
Phenyl Sepharose	1	480	184
Mono-Q	0.5	620	238

[a]Specific activity is expressed as nmol of IPP incorporated into FPP per mg of protein in 1 h

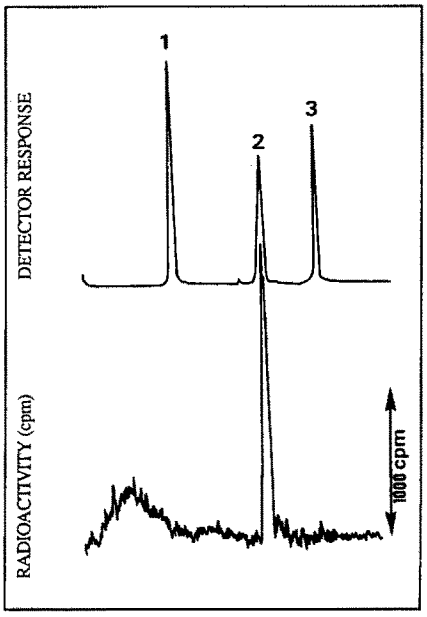

Fig. 2. Identification of enzymatically dephosphorylated product of FPP synthase by radio-gas chromatography. FPP synthase was incubated with [^{14}C]IPP and GPP as a primer. The products obtained after enzymatic hydrolysis, as described in section 2, were mixed with unlabelled standards before separation. The numbers above the trace refer to: (1) geraniol, (2) farnesol, (3) geranylgeraniol.

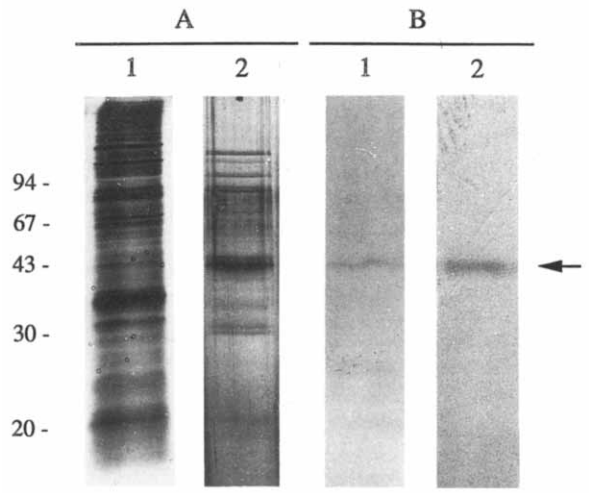

Fig. 3. SDS-PAGE and subsequent protein immunoblot analysis of *Capsicum* extract and purified FPP synthase. (A) SDS-PAGE of the crude 30 000 × *g* supernatant (lane 1) and purified FPP synthase from Mono-Q step (lane 2). (B) The polypeptides shown in lane 1 and 2 (panel A) were transferred on nitrocellulose paper. After incubation with the polyclonal anti-FPP synthase serum, the resulting immune complexes were detected with protein A-gold. The arrow indicates the position of FPP synthase. Protein standards on the left were (top to bottom): phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor.

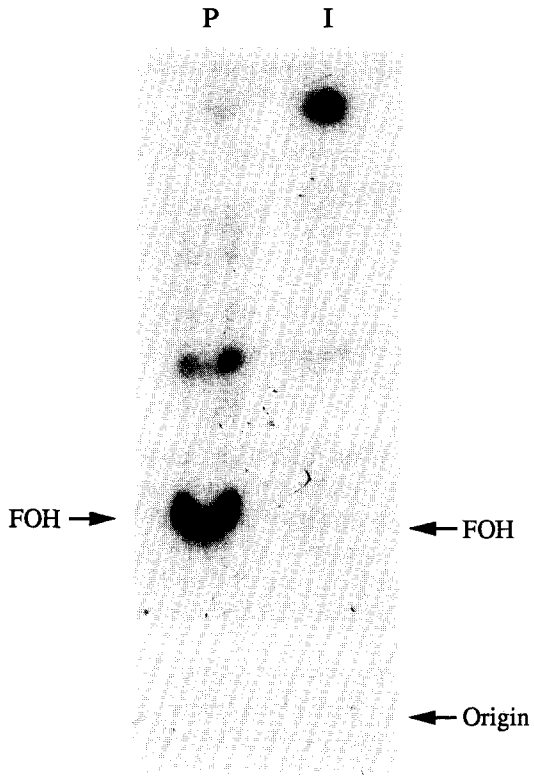

Fig. 4. Removal of FPP synthase activity in a cell free extract of *Capsicum* by polyclonal antiserum raised against purified FPP synthase. The crude 30 000 × *g* supernatant was incubated with preimmune serum (P) or antiserum (I) as described in section 2. Following centrifugation to remove the precipitate, the supernatant was used for enzymatic assay. The reaction products obtained after enzymatic hydrolysis were separed by TLC before detection and by autoradiography. Arrows indicate the position of farnesol (FOH).

3.3. *Comparative antigenicity of GGPP and FPP synthases from* Capsicum

Polyclonal antibodies directed against the plastid GGPP synthase previously purified [1] and polyclonal antibodies raised against FPP synthase were used to test whether or not these two enzymes are immunologically related. This hypothesis is based on the fact that both enzymes obey the same mechanism of catalysis [15] and accept the same allylic pyrophosphate partners DMAPP and GPP. To this purpose partially purified GGPP synthase and FPP synthase catalyzing the incorporation of 0.5 nmol of IPP/15 min were incubated either with 100 μl of anti-FPP or anti-GGPP antibodies for 1 h at 25°C. After centrifugation to remove the immunocomplexes, the supernatant was used for enzymatic assay. One of the most striking and unexpected insight into *Capsicum* prenyltransferases is that antibodies against FPP synthase did not inhibit plastidial GGPP synthase activity. Conversely, antibodies against plastidial GGPP synthase could not inhibit FPP synthase activity. Furthermore, we could not detect immunoreactive polypeptides when the total protein from *Capsicum* chromoplast stroma was separated by SDS-PAGE followed by electroblotting and immunodetection using FPP synthase antibodies (result not shown). These immunological differences based on the use of polyclonal antibodies, though not excluding possible conservation of the amino acid sequence of the active site of these enzymes, clearly indicate that FPP synthase and GGPP synthase have distinct antigenic determinants. From these results, we conclude and propose that FPP synthase in plant cell is probably strictly located in the extraplastidial compartment. In conclusion, the current data provide foundation for further characterization, precise study of the cellular localization and the regulation of plant FPP synthase underway in our laboratory.

Table II

Divalent cations requirement for purified FPP synthase

Cation	Activity (% of control)		
	10 mM	1 mM	0.1 mM
Mn^{2+}	10	42	100
Mg^{2+}	16	60	12
Co^{2+}	7	2	1
Ni^{2+}	1	1	1
Ca^{2+}	0	0	0
Cu^{2+}	0	0	0

The assay mixture was as described in section 2, except that Mn^{2+} was replaced by one of the indicated cations. Results are expressed as percent of control value determined in the presence of 0.1 mM Mn^{2+}.

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