INDUCTION OF PHYTOALEXIN SYNTHESIS IN SOYBEAN

Dimethylallylpyrophosphate:trihydroxypterocarpan dimethylallyl transferase from elicitor-induced cotyledons

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1. Introduction

Treatment of soybean (*Glycine max*) cotyledons with a glucan from mycelial walls of *Phytophtora megasperma* var. *sojae* (*Pms*-elicitor) leads to accumulation of glyceollin [1] and its isomers (*3–5*) [2,3] concomitant with large increases in the activities of enzymes involved in flavonoid biosynthesis [1]. [2-^{14}C]-Mevalonic acid was incorporated into glyceollin in elicitor-treated but not in untreated cotyledons. We now report for the first time on the presence of a dimethylallyl-pyrophosphate:trihydroxypterocarpan dimethylallyl transferase in a particulate fraction from elicitor-induced soybean cotyledons. This enzyme is very probably involved in the biosynthesis of the glyceollins.

2. Materials and methods

2.1. *Materials and synthesis of substrates*

[1-^{14}C]Isopentenylpyrophosphate (57 mCi/mmol) and L-[2,4,6-^{3}H]phenylalanine (60 Ci/mmol) were from Amersham Buchler. 3,6a,9-Trihydroxypterocarpan was isolated from induced soybean cotyledons [3]. [1-^{14}C]Dimethylallylpyrophosphate (DMAPP) was obtained from [1-^{14}C]isopentenylpyrophosphate (IPP) with a 50–100-fold purified isopentenylpyrophosphate isomerase (EC 5.3.3.2) from pig liver [4,5]. The radioactive DMAPP was further purified on Sephadex LH20 by elution with methanol/*n*-propanol/1 N NH_3 (6:2:2, v/v/v) containing 1 mM EDTA. Unlabelled DMAPP was synthesized from dimethylallyl alcohol (Aldrich) [7]. 3,6,9-[^{3}H]Trihydroxypterocarpan was isolated [3] from soybean cotyledons induced with *Pms*-elicitor [1] in the presence of L-[2,4,6-^{3}H]phenylalanine.

2.2. *Induction of soybean cotyledons*

The isolation of *Pms*-elicitor and induction of soybean (Harosoy 63) cotyledons was as in [1].

2.3. *Preparation of particulate fraction*

All preparations were at 4°C.

The wounded upper half of the cotyledons (2.5 g) exposed to the elicitor was ground with 0.5 g quartz sand and 7 ml 0.2 M Tris–HCl (pH 7.6) containing 2.8 mM mercaptoethanol and 1 mM EDTA in a chilled mortar. The extract was squeezed through 4 layers of cheese cloth and the filtrate centrifuged at 40 000 × *g* for 30 min. The pellet was resuspended in 1 ml Tris-buffer and homogenized in a Potter-Elvehjem homogenizer and then subjected twice for 15 s to ultrasonication at 0°C. This extract was used for the transferase assay.

2.4. *Transferase assay*

The transferase assay was carried out with (a) [1-^{14}C]DMAPP or (b) [^{3}H]trihydroxypterocarpan. (a) The assay system contained in 1.0 ml total vol. 100 μmol Tris–HCl (pH 7.6), 1–2 μmol [1-^{14}C]-DMAPP (4.6–9.1 × 10^4 dpm), 10–200 nmol trihydroxypterocarpan in ethylenglycolmonomethylether (5–25 μl), 30 μmol NaF, 4 μmol

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$MnCl_2$ and 50–100 μl of the homogenized pellet fraction. (b) The same incubation mixture as under (a) with [^{3}H]trihydroxypterocarpan (40–200 nmol; 3.3–8.2 × 10^3 cpm) and unlabelled DMAPP was used.

Incubation was carried out for 60 min at 30°C and was terminated by freezing the mixture with liquid nitrogen followed by lyophylization. The dried extract was resuspended in 200 μl water, saturated with NaCl and extracted 5 times each with 1 ml ethylacetate. The organic phase was dried over Na_2SO_4 and concentrated to ~100 μl at 20°C with a stream of N_2. The solution was applied to a silica gel plate (Merck F_{254}) and developed with toluene/chloroform/acetone (45:25:35, v/v/v). The plate was scanned for radioactivity. The radioactive zone (R_F 0.36) was scraped off and eluted with ethanol). The eluate was concentrated under N_2 to ~20 μl and injected into the high-pressure liquid chromatography (HPLC) apparatus. HPLC (Waters) was performed on a stainless steel column (0.9 × 25 cm) filled with LiChrosorb Si 60, 10 μm, Merck (Darmstadt), equipped with a 0.4 × 2 cm Corasil II precolumn. The system was operated at isocratic conditions with *n*-hexane/isopropanol (91:9, v/v) at a 2.5 ml/min flow rate.

2.5. *Mass and NMR spectra*

Mass spectra were taken on a Finnigan 4032 with a direct insertion probe. NMR spectra were measured in deutero-acetone on a Brucker WH-360 FT spectrometer.

3. Results and discussion

When a 40 000 × *g* particulate fraction from *Pms*-elicitor-induced soybean cotyledons [1] was incubated with [1-^{14}C]dimethylallylpyrophosphate a new radioactive product was formed which migrated on silica gel plates (toluene/chloroform/acetone) between glyceollin (fig.2,*3*) (R_F 0.45) and 3,6a,9-trihydroxypterocarpan (*1*) (R_F 0.30). The amount of this product was increased when *1*, isolated from copper-treated soybean cotyledons [3], was added to the incubation mixture. From a large scale incubation the radioactive product was isolated by preparative HPLC on LiChrosorb Si 60 using *n*-hexane/isopropanol. With either [1-^{14}C]DMAPP and *1* or unlabelled DMAPP and [^{3}H]trihydroxypterocarpan a single radioactive compound was obtained by HPLC with a 30 ml net retention vol. The radioactive profile corresponded exactly with the ultraviolet A_{280}. *1* had a higher retention volume at 47.5 ml. The radioactive product also gave one single peak in several thin-layer chromatography (TLC) systems and on a Sephadex LH-20 column with methanol as solvent.

The mass spectrum showed M^+ at 340 (17) and the following fragment ions (relative intensity in brackets): 322 (81), 267 (94), 266 (67), 152 (27), 137 (29), 115 (27), 97 (25), 83 (33), 69 (91), 57 (51), 55 (100). This spectrum is in agreement with the fragmentation pattern shown in fig.1 for a dimethylallylsubstituted pterocarpan. The compound showed the typical λ_{max} at 280 nm for pterocarpans.

The 360 MHz ^{1}H NMR spectrum of the prenylated product showed aromatic signals at δ 6.25 (d, J = 2 Hz), 6.42 (q, J = 8.2 Hz) and 7.20 (d, J = 8), coincident with those assigned to H_{10}, H_8 and H_7 of *5* [2] and *1* [3]. Together with singlets at δ 6.34 and 7.16, assigned to H_4 and H_1, this strongly suggests that the prenyl group is at C-2. The presence of a minor related compound was indicated by HPLC analysis showing a forerunning component appearing as a shoulder (~10%) on the main peak. The NMR spectrum also contained extra minor peaks, notably a doublet (J = 8 Hz) at δ 6.60 which was shown to be coupled to a doublet at δ 7.15. Integration confirmed that this compound constituted ~10% of the mixture. The observed signals are consistent with the formation of a minor isomer carrying the prenyl group at C-4.

These data confirm our earlier results demonstrating that prenylation can occur at both C-2 and C-4 in soybean leading to formation of the three glyceollin

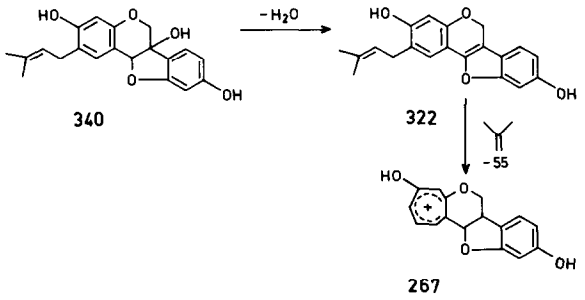

Fig.1. Mass spectrometric fragmentation of the prenylated product.

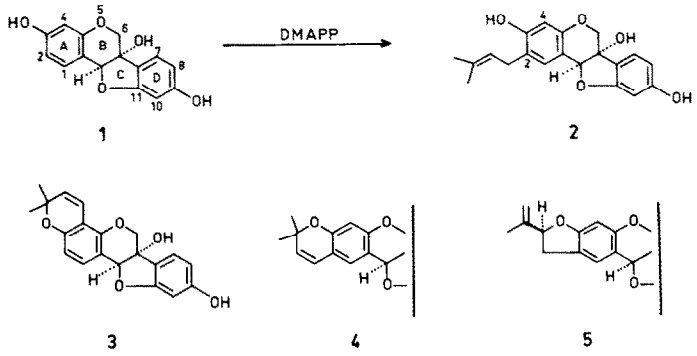

Fig.2. Structure of glyceollin isomers (*3–5*) and possible biosynthetic intermediates (*1,2*).

isomers (*3–5*) [2]. Also it is demonstrated that the prenylation occurs after formation of the pterocarpan moiety. The significance of the proportions of C-2 and C-4 prenylation observed here and the number of transferases involved is under investigation.

No transferase activity was detected in the supernatant of the 40 000 × *g* centrifugation and in preparations from wounded cotyledons which had not been induced with *Pms*-elicitor. Controls with the 40 000 × *g* pellet heated for 10 min in a boiling water bath were negative. Also, no transfer reaction was detected with [1-^{14}C]isopentenylpyrophosphate.

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